ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 13 Number 46
December 14, 2007
World J Gastroenterol
2007 December 14; 13(46): 6127-6290

Online Submissions
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www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

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National Journal Award
2005

World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 13 Number 46
December 14, 2007

Baishideng

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World Journal of Gastroenterology
Volume 13 **Number** 46 **December** 14, 2007

World Journal of Gastroenterology
Volume 13 **Number** 46 **December** 14, 2007

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NAME OF JOURNAL
World Journal of Gastroenterology

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Editorial Board of *World Journal of Gastroenterology,* 77 Shuangta Xijie, Taiyuan 030001,
Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com

PUBLISHING
Editorial Department of *World Journal of Gastroenterology,* 77 Shuangta Xijie, Taiyuan 030001,
Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of Newspapers and Journals
(Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE
December 14, 2007

CSSN
ISSN 1007-9327
CN 14-1219/R

Online Submissions: wjg.wjgnet.com
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2007 December 14; 13(46): 6127-6133
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Endoscopic treatment of chronic pancreatitis

Laurent Heyries, Jose Sahel

Laurent Heyries, Jose Sahel, Hôpital de la Conception, 147 Bd Baille, Marseille 13005, France
Correspondence to: Jose Sahel, Service de Gastroentérologie, Hôpital de la Conception, 147 Bd Baille, Marseille 13005, France. jose.sahel@ap-hm.fr
Telephone: +33-4-91384021 Fax: +33-4-91752304
Received: July 3, 2007 Revised: August 28, 2007

Abstract

Treatment of chronic pancreatitis has been exclusively surgical for a long time. Recently, endoscopic therapy has become widely used as a primary therapeutic option. Initially performed for drainage of pancreatic cysts and pseudocysts, endoscopic treatments were adapted to biliary and pancreatic ducts stenosis. Pancreatic sphincterotomy which allows access to pancreatic ducts was firstly reported. Secondly, endoscopic methods of stenting, dilatation, and stones extraction of the bile ducts were applied to pancreatic ducts. Nevertheless, new improvements were necessary: failures of pancreatic stone extraction justified the development of extra-corporeal shock wave lithotripsy; dilatation of pancreatic stenosis was improved by forage with a new device; moreover endosonography allowed guidance for celiac block, gastro-cystostomy, duodeno-cystostomy and pancreatico-gastrostomy. Although endoscopic treatments are more and more frequently accepted, indications are still debated.

Key words: Chronic pancreatitis; Endoscopic treatment

Heyries L, Sahel J. Endoscopic treatment of chronic pancreatitis. *World J Gastroenterol* 2007; 13(46): 6127-6133

http://www.wjgnet.com/1007-9327/13/6127.asp

METHODS

Endoscopic treatment needs a team (operator, anaesthesiologist) aware with Endoscopic Retrograde Cholangiopancreatography (ERCP) procedures. Specific material is necessary: good fluoroscopy with the possibility to magnify pictures, and a duodenoscope with a 4.2 channel allowing insertion of high calibre stent (10 Fr). Moreover, a wide variety of endoscopic ancillary instruments is essential: metallic and hydrophilic guide-wire, sphincterotomes, Dormia basket, balloon dilatators and bougie dilatators (5-11.5 Fr), but also very thin guide wire (0.025 inches), fine-tipped sphincterotomes, Soehendra extractors (cf infra)[1]. Impaction of stones in pancreatic ducts needs the use of extracorporeal shock wave lithotripsy before endoscopic stone extraction[2]. Endoscopic treatment also needs naso-pancreatic drains and pancreatic stents that are either polyethylene or Teflon. The choice of length, pattern or external calibre of the stent is decided according to the anatomy of pancreatic ducts. Usually, straight stents with proximal and distal external flaps (to avoid internal or external migration), are used. Other stents like single or double pig-tail stents can be used also.

Endoscopic pancreatic sphincterotomy (PS)

Firstly described by Fuji[3], PS is generally performed as the first step in order to improve access to the pancreatic duct. A short (5-6 mm) sphincterotomy is oriented at 13 h with a pure section cutting. Narrowness of pancreatic ducts has justified using special device (thin 0.025 guide-wire, fine-tipped sphincterotome). Biliary sphincterotomy, which was firstly recommended before PS, seems not to be systematically performed because, contrary to the firstly experience, PS alone is not associated with a secondary biliary stenosis[4].

Complications of PS occur in 4.2%-12.6% of cases[4,5]. The morbidity rate also depends on other endoscopic procedures done at the same time such as pancreatic stenting or stricture dilatation. It also depends on the inclusion of patients presenting with recurrent attacks of pancreatitis secondary to sphincter of Oddi dysfunction. In this case, morbidity rate is higher, reaching 12.5%[6]. PS is probably no more or no less harmful than a biliary sphincterotomy which is associated with a morbidity rate of 5.4%-9.8% of cases[7-9]. Morbidity of PS seems also lower in case of post-PS drainage with a naso-pancreatic drain or a pancreatic stent[6]. In case of complete obstruction of the main pancreatic duct in the head, it is sometimes possible to access to the body of the pancreatic duct through the accessory papilla.

Treatment of pancreatic duct strictures by dilatation and stenting

The procedure consists in setting a 3-4 m length, hydrophilic-top guide wire deep inside the main pancreatic duct to realise a stricture dilatation with balloon or dilatators in order to insert a stent the calibre (5-10 Fr) and length (3-12 cm) of which depends on pancreatic duct anatomy. The length of the stent is adapted to bridge the stenosis (duct stricture and/or stone); the calibre of the stent depends on the highest calibre of dilatator successfully inserted through the duct stricture.

In practice, chronic pancreatitis duct strictures are more difficult to pass than biliary stenosis. They are usually associated with an impacted stone which may prevent deep insertion of the guide-wire into the main pancreatic duct. Moreover, pancreatic duct strictures are often narrow and tight because of expanding pancreatic fibrosis. For these reasons, a high rate of failure of dilatation of pancreatic stricture has been reported. Recently, a new technique of dilatation has been reported by Brand *et al*[1]. who used a device (*Soehendra extractor*) previously designed for the extraction of migrated biliary stents. Forage is realised by screwing this instrument through pancreatic duct stenosis on the guidance of a guide-wire. The morbidity rate is low (0%-13%) because forage is realized in a fibrotic area. Finally, among patients with a stricture which could not be passed with a 7 Fr dilatator, this device (7 Fr or 10 Fr) allowed to pass over the stenosis in all cases[1,10].

Plastic stents are clogged by lithostatine precipates, carbonate of calcium and bio bacterial film in a mean time of four to six mo[11,12]. Therefore, stents have to be retrieved or exchanged every four mo during a variable stenting length of time according to the main series (2-12 mo)[13]. Some authors prefer to leave the pancreatic stent and to exchange it only in case of recurrence of symptoms or infection but this strategy is associated with a risk of complications[14]. Metallic stent has been proposed because of a longer time of patency but those stents could also be completely obstructed by intra-luminar inflammatory granuloma with a risk of septic complication[15]. The morbidity rate of pancreatic stenting is widely varied in series. Early complications (before d 30 after ERCP) are distinct of late complications (> 30 d). The main early complication is acute pancreatitis (5%-39%), most of cases are benign, oedematous, spontaneously resolutive forms. A few cases of pancreatic abscess or cholangitis have been reported in the preliminary publications[15,16]. Late complications are stent-related: although stent migration is rare (5%), stent occlusion is very frequently encountered. Ductal lesions after stenting (dilatation, irregularity, stenosis) have been reported in 21%-80% of cases[17]. These lesions are associated with endosonographic parenchymal signs in 68% of cases[18]. In fact, in more than half of the cases, stenting-ductal lesions will regress four mo after retrieval of the stent[17].

Pancreatic stones extraction and lithotripsy

Pancreatic stones may be retrieved only after a previously PS. Since pancreatic stones are often impacted in the pancreatic duct upstream a duct stenosis, extraction of pancreatic stones is more difficult than extraction of biliary stones. Many difficulties have to be solved before: stenosis above stones have to be dilated, stones have to be fragmented with extracorporeal shock wave lithotripsy (ESWL). After good results obtained for biliary and kidney stones, ESWL has been firstly proposed for pancreatic stones in 1987[2]. There are three kinds of lithotripter generators: electro magnetic, electro hydraulic and piezo-electric. Stones are visualised under fluoroscopic or ultrasonographic guidance or both, treatment is realized in pro-cubitus position, under general analgesia or sedation.

Results of the major series are reported on Table 1.

Table 1 Results of extracorporeal shock wave lithotripsy

Authors	Patients (*n*)	Sessions (*n*)	Symptom free (%)	Fragmentation (%)	Clearance (%)
Sauerbruch[19]	24	1.6	37	87	50
Ohara[20]	32	4.6	79	100	75
Costamagna[21]	35	1.9	17	100	80
Delhaye[22]	123	1.8	53	99	59
Schneider[23]	50	2.4	76	85	56
Total	264	1.6-4.6	17-79	85-100	50-75

Without ESWL, complete clearance of pancreatic stones was less than 40%. ESWL is successful in 85%-100% of cases and wash-out of stones was obtained in 50%-75%. Pain disappeared in 17%-79% of cases[19-23]. Morbidity of ESWL is difficult to distinguish from the morbidity related to other endoscopic procedures. Nevertheless, main adverse events of ESWL are abdominal pain and attack of acute pancreatitis. Success factors are more dependant of the site than the size of the stone: juxta-papillary and main pancreatic duct stones are easier to extract than stones located in the tail or in the side branches. Large stones seem easier to break because easier to localize. Duct stenosis is often associated with a pancreatic stone and is a factor of recurrence of pain despite a complete clearance. Fragments of stones are retrieved through PS during a new ERCP, using an extractor-balloon or a Dormia basket; a naso-pancreatic drain is sometimes left to wash the pancreatic ducts during 48 h[22].

Endoscopic drainage of pancreatic cysts and pseudo-cysts

Drainage of pancreatic cysts is realised through the stomach wall (gastro-cystostomy) or duodenum wall (duodeno-cystostomy) in case of trans-mural drainage or through the papilla in case of trans-papillary drainage. The trans-mural way is dedicated to bulging cysts into the stomach or duodenum. A diathermic puncture is realized perpendicularly on the site of maximal bulge. After insertion of the catheter deep inside the cyst cavity, a sample of cyst fluid is taken for bacteriologic, biochemical and cytological analysis. A guide-wire is inserted in the cavity in order to realise multiple loops. Then, a careful cystostomy of 5-8 mm with a papillotome or by balloon dilatation is realised. Large cystostomies appear to be associated with a higher risk of haemorrhage than balloon dilatation. Finally, one or two double-pig-tail stents are inserted. In case of infected cyst or large amount of necrotic tissues which could occlude the stent, a naso-cystic drain for washing seems more adapted than a stent.

Trans-papillary drainage is dedicated to communicating cysts. After a selective cannulation of the pancreatic duct, a guide-wire deeply inserted and a pancreatic sphincterotomy, a dilatation of the tract between the pancreatic ducts and the cyst (a down-stream duct stenosis usually being associated), is performed. A stent is inserted into the pancreatic duct in order to bridge the area of communication between the ductal system and the cyst. The mean time of drainage is usually two mo but depends on the persistence of the cyst on the morphologic explorations[24]. In case of co-existence of pancreatic duct

Table 2 Endoscopic treatment of pancreatic duct stenosis

Authors	Patients (*n*)	Technical Success (%)	Early improvement of pain (%)	Follow-up (mo)	Long-term improvement of pain (%)
Grimm[38]	70	58	82	2-36	57
Cremer[14]	76	94	94	18-72	94
Ponchon[39]	33	85	74	12	52
Sauerbruch[40]	24	87	83	24	50
Delhaye[11]	123	95	100	14	37
Schneider[23]	50	86	70	20	70
Binmoller[41]	93		74	58	64
Smits[42]	51	96	81	64	24
Dumonceau[43]	70		95	24	95
Adamek[44]	80			40	54
Heyries[13]	70	85	62	29	58
Rösch[45]	1018	70	-	59	65
Total	1758	85	81	30	61

lesions, a long-term pancreatic stenting is necessary to prevent a recurrence of the cyst.

Endo-ultrasonography

Interventional endo-ultrasonography (EUS) is particularly interesting in three issues: treatment of pain by coeliac neurolysis, drainage of pseudo cysts and trans-gastric pancreatic drainage. Coeliac block is associated with a low morbidity (diarrhoea in 3.5% of cases)[25,26]. Treatment of pancreatic cysts under EUS guidance is dedicated for pseudo cysts which are not bulging in the gut[27-32]. In case of complete obstruction of the pancreatic duct, a pancreatico-gastrostomy can be achieved under EUS guidance[33,34].

RESULTS AND INDICATIONS OF ENDOSCOPIC TREATMENT

The aim of endoscopic treatment is improvement of pain. Analysis of the literature is difficult because (a) the variability of pain during the time and between patients[35-37] (b) the pain during CP is multifactorial: ductal or interstitial pancreatic hyperpression, inflammatory infiltration of peri pancreatic nerves ("pancreatic neuritis") or pseudo-cysts. Other complications of CP could also be associated with pain: duodenal stenosis, duodenal cystic dystrophy, biliary stenosis or duodenal ulcer[35]. Moreover, endoscopic methods are different: biliary sphincterotomy is not always associated with a pancreatic sphincterotomy, time of pancreatic stenting (two months to undetermined), or number of stents. In fact, there are several methods of treatment which aim to obtain a satisfactory drainage of a pancreatic and/or biliary duct.

Treatment of pancreatic pain

Drainage of pancreatic duct is reported in numerous articles[11,13,14,23,38-45]. Technical success was obtained in 85% of cases (58%-96%). Stents are left during variable length of time, from two mo to endless. Short-term improvement of pain was obtained in 81% of cases (62%-100%). After a follow-up of 30 mo (14-60 mo), improvement of pain dropped to 61% of cases (24%-95%). There was no clinical predictive factor of success despite of an early stage of CP reported in three series[13,41,43]. Communicating cyst and juxta-ampullary stenosis were the two reported morphological predictive factor of success. Surprisingly, stop of alcohol intake did not seem to modify results of the endoscopic treatment. Nevertheless, alcohol intake has to be stopped because morbidity and mortality of CP are more attributed to toxic habits (alcohol or tobacco) than to CP itself[46]. In cases of complete obstruction of pancreatic duct preventing access *via* the papilla, EUS-guided pancreatico-gastrostomy can be done. Results of this method are preliminary: a short series of four cases has reported good results in one case, recurrence of pain in two cases managed with another endoscopic treatment (stenosis of the pancreatico-gastrostomy in one case, disruption and spontaneous migration of the stent in the other case), and failure in one case (12 mo of follow-up)[33]. An additional factor of pain is peri-pancreatic inflammatory infiltrate of nerves: a prospective randomized comparison of endoscopic ultrasound and computed tomography-guided celiac plexus block has reported better results under EUS whereby an immediate improvement of pain occurred in 50% of cases but this result dropped to 30% after six months of follow-up. Efficiency appeared significantly more prolonged in the EUS-group and the ratio cost-efficiency was also better in this group[47]. More recently, a prospective study including 90 patients reported an immediate improvement of pain in 55% and in 10% after six months of follow-up[25]. Young age or previously pancreatic surgery were factors of poor results. Indication of celiac plexus block is limited in CP because of a relative immediate efficiency and especially a frequently recurrence of pain after six mo of follow-up (Table 2).

Treatment of pancreatic cysts and pseudo cysts

Evaluation of patient and collection are the first step to decide strategic therapy. Ultrasonography, computed-tomography, MRCP and EUS make it possible for clinician to determine the two major risks of endoscopic treatment which are haemorrhage and perforation. Haemorrhage depends on the presence of pericystic or peridigestive vessels, segmental portal hypertension and the haemorrhagic content of the cyst. Perforation depends on the distance between the digestive-wall and

Table 3 Results of endoscopic cysto-enterostomy during chronic pancreatitis

Authors	Cysto-gastrost	Cysto-duodenost	Failure (*n*)	Recurrence (*n*)	Secondarysurgery	Morbidity (*n*)	Mortality (*n*)
Dohmoto[48]	5	1	0	-	1	0	0
Cremer[49]	11	22	1	3	5	3	0
Bejanin[50]	9	5	2	3	6	3	0
Barthet[51]	12	66	0	14	2	12	1
Smits[52]	16	10	3	3	8	5	0
Binmoeller[53]	24		4	6	5	5	0
Total	181		10 (5.5%)	29 (16%)	27 (15%)	28 (15.5%)	1 (0.5%)

Table 4 Results of endoscopic transpapillary drainage of pancreatic cysts

Authors	Patients (*n*)	Stent (Fr)	Healing (*n*)	Recurrence (*n*)	Morbidity (*n*)	Secondary surgery (*n*)
Kozarek[55]	8	-	7	0	3	4
Dohmoto[48]	6	7	6	1	2	0
[1]Barthet[56]	30	7-10	23	3	4	5
[2]Smits[52]	19	7-10	14	-	1	-
Binmoeller[53]	37	5-7	35	5	1	-
Catalano[57]	21	5-10	17	1	1	2
Total (*n*)	121	7 Fr	102 (85%)	10 (9.8%)	12 (10%)	11 (11%)

[1]transpapillary drainage + cysto-gastrostomy ($n = 5$); + cysto-duodenostomy ($n = 5$); [2]transpapillary drainage + cysto-gastrostomy ($n = 4$); + cysto-duodenostomy ($n = 2$); + cysto-gastrostomy and cysto-duodenostomy ($n = 1$).

the cyst which should not exceed 10 mm. Results of transmural drainage were reported in six series between 1989 and 1992 including 191 cysts (Table 3)[48-53]. Mean rate of healing, failure and recurrence were respectively 78% (51%-82%), 5.5% (0%-16%) and 6.5% (3%-13%). Secondary surgical procedure was necessary in 14.9% of patients (12%-30%). Morbidity was 15.5% including, according to increasing rates of frequency, haemorrhage, perforation, and infection. Haemorrhage seems more frequent in case of gastro-cystostomy than for duodeno-cystostomy[51]. The only death reported concerned a patient who presented cirrhosis complicated by portal hypertension and associated with haemorrhagic pancreatic ascitis[51]. Long-term results of transmural drainage are not well-known, follow-up not exceeding 31 mo. A recent study including 34 patients followed 46 mo, reported good results in 62% of cases (in intent to treat) with only 71% of initially technical success[54]. Three cases of recurrence were reported, of whom two cases were successfully managed endoscopically.

Results of trans-papillary drainage have been also reported in six series from 1991 to 1995 including 121 cysts (Table 4)[48,53,55-57]. Symptom-free rates were 87% (76%-87%) and healing rates of cysts were 84% (76%-94%). Recurrence of cyst was 9.2%, morbidity was 10% with essentially septic complications and post-ERCP acute pancreatitis. A secondary surgical procedure was necessary in 10.8% of cases (9%-50%). Endoscopic drainage is intended for symptomatic cysts. In the other cases, drainage is necessary if the size of the cyst is more than 4 cm, particularly if the cyst is localized out of pancreatic area because, in this case, it uncommonly collapses spontaneously[58]. Trans-papillary drainage appears a first choice treatment in case of CP because pancreatic stent treats also pancreatic ductal lesions down stream the cyst and because it is less invasive than transmural way. Transmural drainage is especially reserved to large cysts but must be avoided in presence of segmental portal hypertension. Results of EUS-guided pseudo-cysts drainage have been recently reported in six series including 69 cases[27-32]. The most important monocentric study included 35 patients of whom 20 pancreatic abscess: after 27 mo of follow-up, drainage was successful in 94% of cases, a pneuperitoneum occurred in a case and has been managed conservatively, recurrence of cyst occurred in three cases of whom two abscess, surgical drainage was necessary in four cases of whom were four abscess. This method seems satisfying but has to be more evaluated in larger series[31].

Endoscopic treatment of biliary stenosis

Long-term results of biliary stenting have been reported in three series including 102 patients[59-61]. Although initial improvement was reported in 100% of cases, the rate of symptom free patients decreased to 17.5% (10%-28%) after 10 mo (14-49 mo) of follow-up; moreover, 68% of patients underwent a surgical bili-digestive diversion or were still stenting. Plastic stents temporarily improve cholestasis but are not able to dilate adequately common bile duct. In contrast with the previous series, a recent study including 25 patients using balloon dilatation before biliary stenting has reported excellent results in 80% of cases after 13 mo of follow-up[62]. These recent optimistic results attributed to the balloon dilatation, have to be confirmed. Morbidity is low (8%-9%) except migration or obstruction of the stent observed in respectively 14/25 and 18/25 patients of series of Deviere *et al* and Vitale *et al*[59,62]. Draganov *et al* tried to improve results by using several stents: nine patients underwent a biliary stenting with 2-($n = 3$) or 3-($n = 6$) 10 French-stent; 48 mo after

Table 5 Results of endoscopic biliary stenting of biliary stenosis associated with chronic pancreatitis

Authors	Patients (*n*)	Clinical improvement (%)	Stent obstruction (%)	Stent migration (%)	Healing (%)	Length of time of stenting (mo)
Devière [59]	25	100	32	40	12	-
Barthet[60]	19	100	0	5	10.5	10
Smits[61]	58	100	62	7	27	10
Vitale[62]	25	100	56		80	13

retrieval of stents, biliary stenosis recurred in only 55% of cases, absence of cephalic pancreatic stones was a factor of success[63]. Because plastic stent are not adapted for a long-term drainage, Deviere *et al* tested metallic expansive stent with an excellent result in 18/20 patients, after 33 mo of follow-up[64]. Nevertheless, the two remaining patients presented an obstruction of the stent secondary to epithelial hyperplasia in contact with the stent. More recently, a study including 13 patients presenting a biliary stenosis and unfit for surgical procedure, reported good results in 9/13 (69%) patients after 50 mo of follow-up, mean time of stent patency was 60 mo[65]. A single stent was enough in five cases, obstruction of the stent was managed by insertion of a plastic stent inside the metallic stent (n = 3) or by an extractor balloon (n = 1). In four (21%) cases, biliary drainage was not effective because of occluded stent (n = 3) or duodenal migration of the stent (n = 1). Three patients died for a cause not related to the biliary stenosis. Nevertheless, those metallic stents being not extractable, after a long-term follow-up, there is a possibility of stent occlusion by granulation reaction against a foreign body (Table 5).

Management of pancreatic exocrine and endocrine functions

Although improvement of pancreatic exocrine and endocrine functions is frequently discussed in surgical series, this notion is seldom reported in endoscopic series[13,22]. This is probably due to the difficulties to correctly explore the pancreatic exocrine function and also to the relatively short-term follow-up of endoscopic series in compared with surgical series. A temporary improvement of diabetes mellitus has been reported in 10% of cases after endoscopic treatment, while aggravation was noted in 12%[22]. Although another series reported an improvement in 26% of cases[13], most of the series did not noted any improvement[42,44]. Therefore, diabetes mellitus alone should not be an indication of endoscopic treatment of CP.

Evaluation of the effects of endoscopic treatment on pancreatic exocrine function is also seldom precise. A few studies report a gain of weight but this gain probably reflects more improvement of pain than improvement of exocrine function[13]. Evaluation of pancreatic exocrine function with a C^{14} breath test reports a 50%-60% improvement after endoscopic treatment[22].

Endoscopic treatment of pancreatic fistulas

Three major series included 39 patients presenting with a pancreatic fistula after acute pancreatitis (n = 19) and associated with CP (n = 12)[55,66,67]. Treatment consisted in a trans-papillary drainage of pancreatic duct in 34 cases, associated with a transmural drainage of a cyst in four cases. Rate of success was 92 %, with complications occurring in seven cases (17%). Complications included mainly acute pancreatitis and sepsis. Seven (17%) patients underwent surgical procedures 11 to 16 mo after endoscopic treatment. Few isolated cases of pancreatico-pleural fistula successful treated by endoscopic drainage, have been reported[68]. Trans-papillary drainage is also reported as a successful treatment for pancreatico-peritoneal fistulas.

ENDOSCOPIC TREATMENT AND SURGICAL PROCEDURES

Up to now, few randomised series have covered this topic. A recent study concludes in favour of surgery[69]. This study randomized 72 patients and after a follow-up of five years, although incomplete improvement of pain was equivalent in the two groups (46% *versus* 52%), a significant difference appeared for the complete resolution of pain (37% in the surgical group *versus* 14% in the endoscopic group). Nevertheless this series presents a bias because 80% of patients in the surgical group underwent a resection procedure and only 20% underwent a derivation procedure. Therefore, results of endoscopic treatment have to be compared with surgical procedure of derivation. Moreover, half of patients accepted randomization between surgery and endoscopy, this high rate of refusal emphasizes the difficulties in comparing the two methods and to set-up this kind of study. Nevertheless, more recently, another prospective series reported that surgical drainage of the pancreatic duct was more effective than endoscopic treatment[70]. Thirty-nine symptomatic patients having CP with distal obstruction of the main pancreatic duct and without inflammatory mass were randomized: 19 underwent endoscopic trans-papillary drainage (16 of whom also underwent ESWL) and 20 had operative pancreatico-jejunostomy. After 24 mo of follow-up, patients who underwent surgery had a significant ($P < 0.001$) lower pain score compared to endoscopic drainage. Moreover, complete or partial pain relief was achieved in 75% of patients of "surgical group" and only 36% of patients of "endoscopic group" ($P = 0.007$). Morbidity rate and length of hospital stay were similar in the two groups but there were more procedures in the "endoscopic group" than in "surgical group" (a median of 8 *vs* 3). To conclude, strategy depends on the expertise of the local teams, endoscopic treatment could be proposed as a first line treatment, before surgical procedure.

CONCLUSION

Endoscopic treatment of CP has certainly improved during the last two decades. Although results are clearly accepted as excellent for pancreatic cysts and pancreatic fistulas, long-term improvement of biliary and pancreatic ducts stenosis remains controversial.

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S- Editor Liu Y **L- Editor** Roberts SE **E- Editor** Yin DH

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World J Gastroenterol 2007 December 14; 13(46): 6134-6139
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Smoking in inflammatory bowel diseases: Good, bad or ugly?

Peter Laszlo Lakatos, Tamas Szamosi, Laszlo Lakatos

Peter Laszlo Lakatos, Tamas Szamosi, 1st Department of Medicine, Semmelweis University, Budapest, Hungary
Laszlo Lakatos, 1st Department of Medicine, Csolnoky F. County Hospital, Veszprem, Hungary
Correspondence to: Peter Laszlo Lakatos, MD, PhD, 1st Department of Medicine, Semmelweis University, Koranyi str. 2/A, H-1083, Hungary. kislakpet@bel1.sote.hu
Telephone: +36-1-2100278-1500 Fax: +36-1-3130250
Received: August 27, 2007 Revised: September 20, 2007

Abstract

Smoking is an important environmental factor in inflammatory bowel disease (IBD), having different effects in ulcerative colitis (UC) and Crohn's disease (CD). A recent meta-analysis partially confirmed previous findings that smoking was found to be protective against ulcerative colitis and, after onset of the disease, might improve its course, decreasing the need for colectomy. However, smoking increases the risk of developing Crohn's disease and worsens its course, increasing the need for steroids, immunosuppressants and re-operations. Smoking cessation aggravates ulcerative colitis and improves Crohn's disease. Data are however, largely conflictive as well as the potential mechanisms involved in this dual relationship are still unknown. In this review article, the authors review the role of smoking in inflammatory bowel diseases.

Key words: Smoking; Crohn's disease; Ulcerative colitis; Phenotype

Lakatos PL, Szamosi T, Lakatos L. Smoking in inflammatory bowel diseases: Good, bad or ugly? *World J Gastroenterol* 2007; 13(46): 6134-6139

http://www.wjgnet.com/1007-9327/13/6134.asp

INTRODUCTION

The pathogenesis of ulcerative colitis (UC) and Crohn's disease (CD) has only been partly understood. Inflammatory bowel disease (IBD) is a multifactorial disease with probable genetic heterogeneity[1,2]. In addition, several environmental (eg, diet, smoking, measles or appendectomy) risk factors may contribute to its pathogenesis.

During the past decades, the incidence pattern of both diseases has changed significantly[3], showing some common but also quite distinct characteristics for the two disorders. Differences in geographic distribution, and particularly changes in incidence over time within one area, may provide insight into possible etiological factors[4]. It is very unlikely however, that these rapid changes attributed to variations in the genetic factors. On the contrary, environmental factors are likely to play an important role. Diet, as a luminal antigen, was thought to be an important factor in the pathogenesis of IBD[1,5]. In the last two decades, there has been a shift in the lifestyle in Eastern Europe, Asia, and Central America, as the lifestyle, including the diet, became more "Westernized". This possibility is further supported by the differences in incidence and prevalence found within one region.

A further important environmental factor studied extensively in both diseases is smoking. The link between smoking and (IBD) was first made in 1982 when Harries *et al*[6] noticed a low proportion of ulcerative colitis patients were smokers. Two years later a case-control study by Somerville *et al*[7] reported that the relative risk of developing Crohn's disease was 4.8 in those who smoked before disease onset, and 3.5 for those with a current smoking habit. In recent years, IBD has been classified into subtypes/phenotypes that are distinct and based on age at onset, disease location, and clinical behaviour. Knowledge of this heterogeneity has led to the re-examination of genetic and environmental influences on IBD. The relationship between smoking and IBD however, is far more complex than previously realized as clinical subtypes have become apparent. In this article, the authors give an updated review on the role of smoking in IBD.

EFFECT OF SMOKING CESSASION AND SMOKING ON THE RISK OF DEVELOPING IBD

Risk for developing ulcerative colitis

Ulcerative colitis affects predominantly non-smokers and former smokers. The percentage of current smokers (smoking more than seven cigarettes per week) in a group of patients with UC is about 10%-15%[8,9]. These percentages are significantly lower than those observed in a control population matched for sex and age (25%-40%). The meta-analysis by Calkins[10], conducted more than 15 years ago, yielded a pooled odds ratio of 0.41 (0.34-0.48) for current smokers compared with lifetime non-smokers. The effect of smoking seems to only postpone the event,

as the relative risk of UC was also higher in former smokers (OR: 1.64; 95% CI: 1.36-1.98). In a recent meta-analysis by Mahid *et al*[11] comparable values were reported, which also included new available data. Current smoking decreased the risk for UC (OR: 0.58; 95% CI: 0.45-0.75), while former smoking was associated with an increased risk (OR: 1.79; 95% CI: 1.37-2.34). Interestingly, in patients who stopped smoking, UC developed in 52% of patients, in the first three years after cessation, as reported by Motley *et al*[12] in concordance with other studies[13]. In contrast, active smoking in early childhood was associated with a gradually increased risk for developing UC (OR for smoking start < 10 years: 7.02 and < 15 years: 3.46)[14]. The same trend was observed for passive smoking by the mother (OR: 1.53, 95% CI: 0.93-2.49).

The relationship between smoking and ulcerative colitis has also been examined at a population level. The prevalence of UC was five-fold increased in patients from the Mormon Church in Britain and Ireland, where smoking is strongly discouraged, compared with that of the general population. In contrast, CD was equally as common[15]. In addition, a review of 56 epidemiological studies in Sweden over the time period from 1930 to 1990[16] demonstrated that the sex distribution of UC had changed from an earlier female predominance to a later male predominance. Over the same period, the proportions of smokers and ex-smokers among men and women have undergone reciprocal changes with an increase in women smokers relative to men, while the same change in predominance was not observed in contemporary pediatric studies. Somewhat contradictory, in a recent population-based case-control study[17], among others, ever smoking was also associated with increased risk (1.66; 95% CI: 1.17-2.35).

There are only very few data regarding the effect of smoking in indeterminate colitis[8,18]. The effect seems to be similar to that observed in UC, that is, a protective effect against the development of colitis and a possible beneficial effect on disease course.

Risk for developing Crohn's disease

The percentage of current smokers in a group of patients with CD is significantly higher than that observed in a control population matched for sex and age (45%-55% *vs* 30%-40%)[19]. In concordance, an increased life-time risk was reported in current smokers when compared with non-smokers by both Calkins *et al*[10] (OR: 2.0; 95% CI: 1.65-2.47) and in the more recent meta-analysis by Mahid *et al* (OR: 1.76; 95% CI: 1.40-2.22).

Compared with never-smokers, former smokers were reported to have an increased risk of developing CD[10]. This risk decreased only after four years of having quit smoking. In a recent population-based study by Bernstein *et al*[19], similar data were reported, both current smoking (OR: 1.96) and ever smoking (OR: 1.78) were associated with increased risk to develop CD. However, this later association could not be replicated in the recent meta-analysis by Mahid *et al* although a trend was observed ($P = 0.08$). In contrast, ever smoking was associated with increased risk (OR: 1.61; 95% CI: 1.27-2.03). The effect of passive smoking remains controversial[20]. In one recent prospective study[14] CD patients were more likely than controls to have prenatal smoke exposure (OR: 1.72; 95% CI: 1.1-2.71). In addition, the passive smoke exposure during childhood, with parents or other household members being smokers (OR: 2.04; 95% CI: 1.28-3.31) was also associated with increased risk, in concordance with previous data by Lashner *et al*[21].

EFFECT OF SMOKING AND ITS CESSATION ON DISEASE PHENOTYPE AND COURSE

Effect of smoking and its cessation on clinical course and extent of ulcerative colitis

Although the extent at diagnosis is not affected by smoking, UC usually runs a more benign disease course in smokers compared with non-smokers. Flare-up, hospitalization rates[13], the need for oral steroids[22] and colectomy rates[22,23], are reported to be lower, while age at onset is older in smokers compared with non-smokers, though not in all studies. Relapse rates are lower in patients who began smoking after the diagnosis of UC[24]. In concordance, in a recent Europe-wide population-based cohort[25] the relapse rate was lower (Hazard Ratio: 0.8; 95% CI: 0.6-0.9) in smokers compared with non-smokers, while it was higher in women. In a retrospective analysis of a large series of patients with UC, current smoking was found to decrease the 10-year cumulative colectomy risk from 0.42 to 0.32[22]. In concordance, a meta-analysis of several large series with a total of 1489 UC patients also found the risk for colectomy to be lower (OR: 0.57; 95% CI: 0.38-0.85) in current smokers compared with non-smokers[26].

In addition, in smokers with distal UC at diagnosis, the proximal extension of the disease is less frequent[22,27], while primary sclerosing cholangitis is observed almost exclusively in non-smokers[28]. Disease regression[29] was also more likely to occur in smokers compared with non-smokers or ex-smokers 5 years (30% *vs* 5% *vs* 8%), but not 10 years after the diagnosis. Also, those with extensive disease were the lightest smokers, whereas those with healthy colons were the heaviest smokers. Finally, current smokers have a lower incidence of pouchitis following coloproctectomy with ileal reservoir when compared with non-smokers[30,31].

In contrast, intriguing new data by Aldhous *et al*[29] showed that current and non-smokers had an almost identical age at onset (31.1 *vs* 29.4 years) and this was delayed only in ex-smokers (46.5 years). Colectomy rates were not different. This group however, had a greater exposure to smoking compared with the group of current smokers.

A link between smoking habits and the course of UC has also been reported. In intermittent smokers, many patients note symptom exacerbation when they stop smoking, followed by symptom relief when they smoke again[12]. In contrast, almost half of the intermittent smokers thought that their colitis symptoms improved while smoking at least 20 cigarettes per day[32]. Moreover, smokers with UC who quit, experience an increase in

disease activity, hospital admissions, and the need for major medical therapy (oral steroids, immunosuppressants), within the first years following the cessation of smoking[33]. However, the risk of colectomy in the short-term was not increased compared with matched non-smokers and continuing smokers.

Effect of smoking and its cessation on disease location, behaviour, and disease progression in Crohn's disease

Smoking is associated with disease location: most, but not all, studies report a higher prevalence of ileal disease and a lower prevalence of colonic involvement in smokers[34-36].

A recent review[36] and previous data have demonstrated that smoking, when measured up to the time point of disease behavior classification, was associated more frequently with complicated disease, penetrating intestinal complications[34,37,38], and greater likelihood to progress to complicated disease, as defined by development of strictures or fistulae[36], and a higher relapse rate[2,39]. Of note, previous severity of the disease, as assessed from the therapeutics needs, was found to be similar in young patients who started smoking and in their matched controls[10]. The need for steroids and immunosuppressants is increased in smokers compared with non-smokers[35]. Whether the daily dose (eg, more than 15 cigarettes per day) or the total pack years smoked is more important in the abovementioned associations remains questionable.

The risk of surgery as well as the risk for further resections during disease course is also higher in smokers, in most studies[34,41,42]. These findings were reinforced by Cottone *et al*[43] who have shown that macroscopic lesions on the ileal site of the anastomosis were observed 1 year after surgery in 70% of smokers, *versus* 35% of non-smokers and 27% of ex-smokers. The risk of symptomatic postoperative recurrence was more marked in heavy smokers than in mild smokers[43]. Noteworthy, immunosuppressive therapy was found to neutralize the effect of smoking on the need for surgery[40].

However, the harmful effect of smoking on the course of CD is not a universal finding. Studies in patients from Israel and Hungary have not found differences in the need for surgery or for immunosuppressants between smokers and non-smokers[2,44,45], and patients with only colonic involvement are less sensitive to the harmful effects of smoking[8]. Finally, the development and severity of perineal complications do not seem to be influenced by the smoking status[39].

In a recent paper by Aldhous *et al*[46] using the Montreal classification, the harmful effect of smoking was only partially confirmed. Although current smoking was associated with less colonic disease, the smoking habits at diagnosis were not associated with time to development of stricturing disease, internal penetrating disease, perianal penetrating disease, or time to first surgery. Age at diagnosis was also similar in current smokers and non-smokers (28.3 years and 28.9 years) and was only delayed in ex-smokers (43.2 years). However, the way in which they measured tobacco exposure was different from previous studies. "Current smokers" were defined as those who were smoking at the time of diagnosis or event (penetrating or stricturing complication) and "ex-smokers" had stopped for at least one year before the diagnosis or event (eg, a patient could have continued smoking up to 1 year before developing a complication but was considered an "ex-smoker").

The rationale for this may be that after surgery, the risk of endoscopic and clinical recurrence in former smokers who have not smoked for at least 1 year is similar to that of non-smokers[43]. Similarly, CD activity in ex-smokers is not different from that of non-smokers, and is less marked than in current smokers[39]. The beneficial effect of quitting smoking might be seen within the year following cessation. A large prospective intervention study by Cosnes *et al*[47] performed in a selected group of 59 patients who stopped smoking following a smoking cessation intervention, examined the disease course from 1 year following smoking cessation onwards. The flare-up rate, therapeutic needs, and disease severity were similar in patients who had never smoked and in those who stopped smoking, and both had a better course than current smokers. Quitters had a 65% lower risk of flare-up compared with continuing smokers. The need for corticosteroids, immunosuppressive therapy, or a dose increase of immunosuppressants was also lower. Interestingly, after quitting, some patients developed UC-like lesions of the distal colon, whereas previously they had typical CD.

Finally, in a prospective study during pregnancy, the improvement of disease activity was observed only in smokers, in parallel with a decrease in the daily cigarette smoking[48].

The role of gender, familial disease, and ethnicity

The effect of smoking is to some extent different between male and female patients. In CD, women are affected more drastically by smoking. The relative risk associated with smoking for women may be greater than for men: one study demonstrated a three-fold difference[20].

This was already demonstrated by Sutherland *et al*[42] in 1990, who reported that in a group of 174 patients who required surgery for Crohn's disease, smokers had a 29% greater risk than non-smokers, over 10 years. However, the increased risk was more marked in females than males (OR: 4.2; 95% CI 2.0-4.2 in females and 1.5; 95% CI 0.8-0.6 in males). In Crohn's colitis, smoking is clearly harmful for women, whereas colitis in men is not affected by smoking[8]. In the study by Cosnes *et al* current smoking hastened disease onset (from 35 to 29 years of age) and increased the need for immunosuppressants, only in women.

In UC, current and ex-smoking delayed disease onset in men (from 25 to 42 years of age), but not in women[50]. Similarly, when compared with non-smokers, male UC patients who smoked ran a more benign disease course as assessed by the decreased need for immunosuppressive therapy (8% *vs* 26%), whereas this difference was not observed in females as reported by Cosnes *et al*[8] and again smoking delayed the onset of disease, only in males.

Thus, the effect of smoking in both UC and CD seems to be modulated by gender, with women being affected more disadvantageously than men. This phenomenon deserves even more attention, since smoking habits are changing in the Western population, with a trend to a greater prevalence of smokers among young women[50].

Table 1 Smoking in IBD: Practice points

Ulcerative colitis (UC)	Crohn's disease (CD)
Current smoking decreases the risk for UC by app. 50%, in contrast former smoking is associated with an app. 2-fold increased risk	Both current and former smoking (presumable also passive smoke exposure during childhood) increases the risk of CD almost 2-fold
The protective effect is smaller in females	The risk is greater in females compared with males
Proximal extension of the disease is less likely in smokers as well as disease course is milder but the risk of lung cancer and vascular disease is higher	Smoking is associated with complicated (stricturing or penetrating) and ileal disease
Patients who stop smoking experience an increase in disease activity at least during the first year after cessation	Smokers with CD need more steroids, more immunosuppressants and more operations than non-smokers
The effect of smoking is similar in indeterminate colitis (less evidence is available)	Smoking cessation improves rapidly the course of CD
Nicotine-replacement therapies and antidepressants are useful in heavy smokers motivated to stop smoking	
Geographic differences exists (e.g. Israel, Korea)	

A gender difference in smoking habits may be a possible explanation, since women use more filtered cigarettes and lighter cigarettes, and might consequently have a higher relative exposure to smoke than to nicotine[51]. In addition, the negative effect of estrogens[52] on proinflammatory cytokine gene regulation and lymphocyte interactions might also play an important role.

In Israel, no association was reported between smoking and CD in Jewish patients, although the opposite association was found for UC[53]. Similarly, smoking was not associated to the risk for CD in some studies from Asia[54]. The reason why CD in Israeli Jews or Koreans is not as sensitive to smoking as in other populations is not clear. Explanations might include differences in genetic and/or environmental factors (eg, type of tobacco, way of smoking or other alimentary factors).

A further interesting question is whether the effect of smoking is similar in familial versus sporadic cases. Family studies have reported a high concordance rate within families, between smoking habits and the phenotype of IBD, CD developing in smokers, and UC in non-smokers[55]. The above association was refined in more recent studies. Tuvlin *et al*[56] reported that ex-smokers made up an increasing percentage of older patients diagnosed with UC, accounting for more than 35% of the attributable risk of late onset (> 45 years) UC and a large component of the second peak in diagnosis. In contrast, current smoking accounted for a large percentage of patients diagnosed at a younger age with familial CD but not with sporadic CD (Table 1).

MECHANISMS BEHIND THE EFFECT OF SMOKING ON IBD

The reason behind the opposite effects of smoking observed in CD and UC remain obscure. The effects of smoking and nicotine are numerous and since the pathogenesis of inflammatory bowel disease is only partially understood, any discussion on the possible mechanisms can only be speculative. In addition, although nicotine is thought to be the most important agent responsible for the effect of smoking, one should be careful, as some effects usually associated with smoking may not follow the use of therapeutic nicotine formulations. Of note in most studies, side-effects (nausea, headache, dermatitis) of nicotine therapies were frequent and tended to be greater than the clinical benefit[57].

Smoking has numerous specific and non-specific effects. It has been shown to affect the immune system, by influencing cellular and humoral immunity. Nicotine has been shown to decrease the synthesis of proinflammatory molecules, for example, interleukin (IL)-1β and TNFα by mouse colonic mucosa as well as the production of mucosal eicosanoids[58] and some proinflammatory cytokines by human mononuclear cells (eg, IL-2[59], IL-8, and TNFα[60]) partly by its action on the nicotinic acetylcholine receptor a7 subunit. Further evidence for anti-inflammatory properties comes from a surgically-induced ileus model, where carbon monoxide-treated mice were shown to have three times higher levels of IL-10. Macrophages from smokers express a selective functional deficiency in their ability to kill intracellular bacteria[61]. Finally, chronic exposure of rats to nicotine inhibits the antibody-forming cell response, impairs the antigen-mediated signaling in T-cells and induces T-cell anergy[62]. Other effects of nicotine or smoking on the intestine include the alteration of gut motility, the reduction of smooth muscle tone and contractility (modulated by nitric oxide)[63], decreased permeability[64], and alterations in the microcirculation[65], Furthermore, smoking also increases lipid peroxidation.

In UC, the colonic mucosal layer is thin or absent, in contrast to CD where it is significantly thicker[66], with nicotine having been shown to increase mucin synthesis[67,68]. Smokers with IBD have a significant reduction in mucosal cytokine levels, specifically, IL-1b and IL-8 in patients with UC, and IL-8 in patients with CD[69]. Beneficial effects of nicotine in active UC may be associated with a decrease in IL-8 expression. Hypoperfusion of the rectum and of acutely damaged colonic tissue may play an additional role[70]. On the contrary, in CD, several plasma antioxidant parameters are altered, the total radical-trapping antioxidant potential is decreased[71], and abnormalities are present in the microvasculature[65]. Smoking through increased carbon monoxide concentration might amplify the impairment in the vasodilation capacity of chronically inflamed microvessels, resulting in ischemia and perpetuating ulceration and fibrosis[70]. Furthermore, smoking is known to increase the thrombotic potential associated with vascular damage. A defect in bacterial clearance or macrophage deficiency might also play an important role.

CONCLUSION

In conclusion, smoking plays a dual role in IBD by increasing the risk for CD and decreasing that of UC. In an individual who is genetically at risk for IBD, smoking might be an important factor in determining disease phenotype. In addition, smoking also affects the disease course. It improves UC and worsens CD, more markedly in women, while smoking cessation is followed rapidly by a reversed effect. Since smoking is associated with several additional deleterious effects (eg, cardiovascular, lung cancer risk), gastroenterologists should encourage both UC and CD patients to quit smoking. Before stopping, UC patients should be informed about the potential risk of increase in disease activity, without a higher risk for surgery. In CD, the benefit of smoking cessation is well-proven, since patients who continue to smoke have a more severe course of disease with more complications, while ex-smokers run a similar course of disease to non-smokers.

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World J Gastroenterol 2007 December 14; 13(46): 6140-6149
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Small bowel capsule endoscopy in 2007: Indications, risks and limitations

Emanuele Rondonotti, Federica Villa, Chris JJ Mulder, Maarten AJM Jacobs, Roberto de Franchis

Emanuele Rondonotti, Federica Villa, Roberto de Franchis, Università degli Studi di Milano, IRCCS Fondazione Policlinico, Mangiagalli, Regina Elena.Unità Operativa di Gastroenterologia 3 via Pace 9, Milano 20122, Italy
Chris JJ Mulder, Maarten AJM Jacobs, VU University Medical Centre, Department of Gastroenterology and Hepatology, Boelelaan 1117, 1081 HV Amsterdam, The Nederlands
Correspondence to: Emanuele Rondonotti, MD, Universutà degli Studi di Milano, IRCCS Fondazione Policlinico, Mangiagalli, Regina Elena, Unità Operativa di Gastroenterologia 3, Via Pace 9, 20122 Milano,
Italy. emanuele.rondonotti@unimi.it
Telephone: +39-2-55035332 Fax: +39-2-50320747
Received: July 13, 2007 Revised: September 25, 2007

Abstract

Capsule endoscopy has revoluzionized the study of the small bowel by providing a reliable method to evaluate, endoscopically, the entire small bowel. In the last six years several papers have been published exploring the possible role of this examination in different clinical conditions. At the present time capsule endoscopy is generally recommended as a third examination, after negative bidirectional endoscopy, in patients with obscure gastrointestinal bleeding. A growing body of evidence suggests also an important role for this examination in other clinical conditions such as Crohn's disease, celiac disease, small bowel polyposis syndromes or small bowel tumors. The main complication of this examination is the retention of the device at the site of a previously unknown small bowel stricture. However there are also some other open issues mainly due to technical limitations of this tool (which is not driven from remote control, is unable to take biopsies, to insufflate air, to suck fluids or debris and sometimes to correctly size and locate lesions).The recently developed double balloon enteroscope, owing to its capability to explore a large part of the small bowel and to take targeted biopsies, although being invasive and time consuming, can overcome some limitations of capsule endoscopy. At the present time, in the majority of clinical conditions (i.e. obscure GI bleeding), the winning strategy seems to be to couple these two techniques to explore the small bowel in a painless, safe and complete way (with capsule endoscopy) and to define and treat the lesions identified (with double balloon enteroscopy).

Key words: Capsule endoscopy; Double balloon enteroscopy; Obscure glycemic index bleeding; Crohn's disease; Nonsteroidal anti-inflammatory drugs

Rondonotti E, Villa F, Mulder CJJ, Jacobs MAJM, de Franchis R. Small bowel capsule endoscopy in 2007: Indications, risks and limitations. *World J Gastroenterol* 2007; 13(46): 6140-6149

http://www.wjgnet.com/1007-9327/13/6140.asp

INTRODUCTION

The small bowel (SB) has been considered for a long time technically difficult to evaluate for many anatomical (i.e. distance from external orifices, length) and physiological (i.e. active peristalsis) reasons.

Until the introduction of Video Capsule Endoscopy (VCE) in clinical practice the small bowel was studied mostly with radiological or nuclear medicine techniques such as abdominal Computed Tomography (abdominal CT), abdominal Magnetic Resonance Imaging (abdominal MRI), small bowel follow through (SBFT), small bowel enteroclysis (SB enteroclysis) and 99_{m}TC scan. Although CT scan and abdominal MRI are highly sensitive in recognizing the presence of abdominal masses and allow an accurate evaluation of solid organs, lymph nodes and vessels, they are able to provide limited information about the small bowel wall. On the other hand, small bowel follow-through and small bowel enteroclysis, although specifically designed to evaluate the small bowel, have low sensitivity and specificity in recognizing small and flat lesions[1].

Additionally these two techniques are often poorly tolerated by patients and sometimes difficult to interpret.

The endoscopic evaluation of the small bowel represents the best possible approach to small intestinal diseases, allowing a direct visualization of small bowel mucosa, the collection of targeted biopsies and sometimes an effective treatment. Sonde enteroscopy, introduced because of its theoretical capability to visualize the entire small bowel (achievable in about 80% of examinations in clinical practice)[2,3], had been abandoned at the end of the 90's because of several technical limitations (angulation of the tip due to the presence of the balloon, duration of the examination, patient discomfort, inability to take biopsies)[4]. Push Enteroscopy (PE) is limited by the depth of insertion of the instrument to the proximal jejunum (about 90-150 cm from the oral route) and to

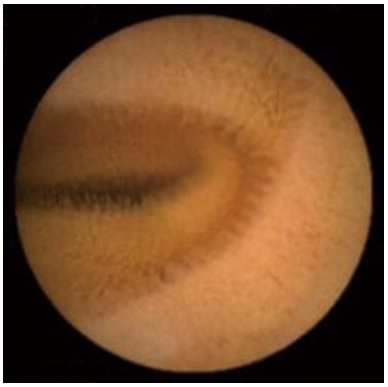

Figure 1 Normal small bowel.

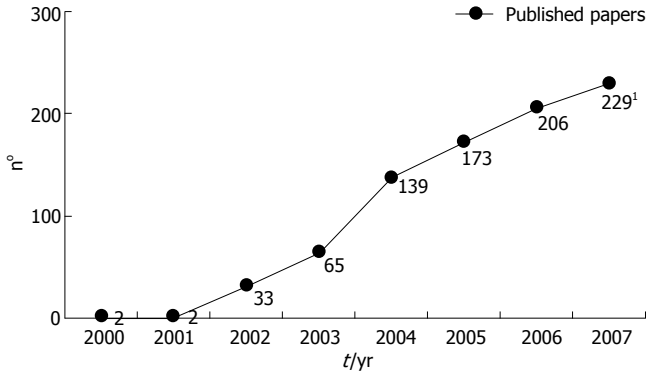

Figure 2 Published papers about capsule endoscopy between 2001 and 2007 (search engine: www.pubmed.org, key words: Capsule endoscopy OR capsule enteroscopy). [1]Estimated number of published articles in 2007 based on the number of papers published in the first seven months of the year.

the terminal ileum (50-80 cm in the retrograde way) and, despite sedation, is still poorly tolerated[5-7]. Intraoperative enteroscopy (IOE) is the most complete but also the most invasive means of examining the small bowel[7].

The introduction (in 2001)[8] and further continuous development of capsule endoscopy opened a new chapter in the study of small bowel diseases allowing, finally, to cross the frontier of the endoscopic examination of the small bowel. In fact this revolutionary technique made it possible, for the first time, to obtain high resolution endoscopic images of the entire small bowel (Figure 1) avoiding sedation, surgical intervention or radiation exposure. Capsule endoscopy showed, in everyday clinical practice, that the small bowel can be involved in several diseases (i.e. inflammatory, vascular, neoplastic, iatrogenic diseases). The knowledge of the large spectrum of lesions and diseases that can affect the small bowel stimulated the development and/or the implementation of other diagnostic and therapeutic techniques such as double balloon enteroscopy (DBE), MRI-enteroclysis and CT enteroclysis.

Performing a recursive search in the literature (by means of the most common search engine www.pubmed.org; using "capsule endoscopy OR capsule enteroscopy" as key words) we found a number of papers, increasing over the years, up to 754 (Figure 2). On the one hand this phenomenon certainly represents a proof of the revolutionary potential of this diagnostic tool in the field of small bowel endoscopy and, on the other hand, demonstrates the effort to establish the appropriate role of this device in different clinical conditions. Unfortunately about a quarter of published papers are case reports (187) or collections of small case series and 131 published papers are expert reviews. Following the rules of evidence based medicine[9] we can classify these papers at the lowest level of scientific evidence while, among the huge number of publications about capsule endoscopy, there are only 8 randomized controlled studies [five about bowel preparation, 2 about Nonsteroidal anti-inflammatory drugs (NSAIDs) induced damage and 1 about obscure GI bleeding (OGIB)][10-17] and 4 metanalyses (about OGIB or Crohn's disease)[18-21]; all these papers can be ranked as evidence grade 1c.

Mainly on the ground of the former 12 mentioned papers three Practice Guidelines have been published so far, two (in 2004 and 2006) on behalf of the European Society of Gastrointestinal Endoscopy (ESGE) and one on behalf of the American Society of Gastrointestinal Endoscopy (ASGE)[22-24] in 2006.

The aim of the present paper is to briefly review the evidence, available to date, about the use of capsule endoscopy for the study of the small bowel (starting from the studies providing the highest grade of evidence), to highlight the benefits of this technique but also to highlight risks and limitations which have emerged in these six years of use of the device in clinical practice.

INDICATIONS

Obscure GI bleeding

So far, OGIB is the main clinical indication for capsule endoscopy: about 70%-80%[25,26] of patients undergoing capsule endoscopy suffer from OGIB. The majority of studies published at the beginning of the experience with this new tool reported a high, although widely variable, diagnostic yield (ranging between 38% and 93%, about 75%-80% in most studies[27]). These studies, mainly performed in tertiary referral centres, collected highly selected patients with a long standing history of obscure GI bleeding, with low levels of haemoglobin at the time of the examination, who had undergone a huge number of prior examinations with negative results[28]. Subsequent studies performed on larger populations of patients, similar to those undergoing this examination in everyday clinical practice, showed a slightly lower diagnostic yield (about 50%)[29].

Although recent studies showed a decrease in the diagnostic yield of capsule endoscopy, two metanalyses[18,19] clearly demonstrated that capsule endoscopy in patients with obscure GI bleeding is superior to traditional radiological techniques (SBFT and SB enteroclysis) and PE. The latter comparison has been also recently confirmed in a specific prospective randomized controlled study[11]. The authors hypothesized that the high diagnostic yield of capsule endoscopy in this subgroup of patients may depend on the capability of the capsule to evaluate the mid-distal small bowel (particularly in comparison with PE) and/or to show small and flat lesions (vascular-

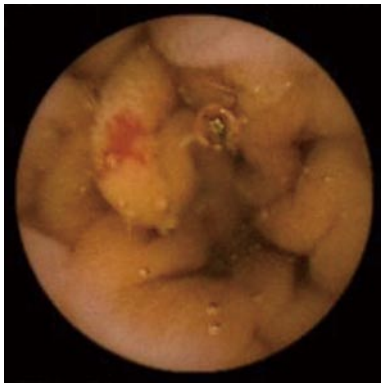

Figure 3 Artero-Venous Malformation (AVM) of the distal duodenum in a patient undergoing capsule endoscopy for obscure GI bleeding.

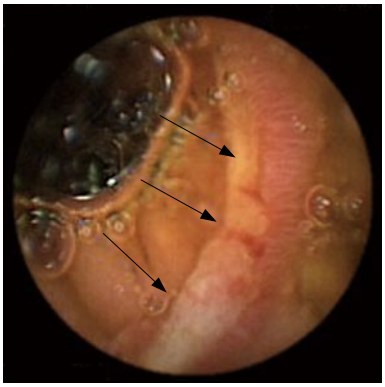

Figure 4 Jejunal ulcers in a patient with Crohn's disease.

Figure 3- or inflammatory-Figure 4) that are often missed by conventional radiological techniques.

As far as the possible factors potentially affecting the diagnostic yield of capsule endoscopy are concerned, the presence of active bleeding at the time of examination[30] or a short interval between the last episode of acute bleeding and capsule endoscopy[28-30], low levels of haemoglobin and high transfusion requirement have been found to be associated with a high diagnostic yield[31,32].

Since capsule endoscopy was introduced in clinical practice 6 years ago, some papers explored also the impact of this new technique on the outcome of patients with obscure GI bleeding. As expected, capsule endoscopy has been found to significantly modify the diagnostic and therapeutic work up immediately after the examination[33], decreasing the number of further examinations and reducing the length of hospital stay[10,34,35]. Nevertheless capsule endoscopy seems also to have a positive impact on long term follow up (mostly evaluated at 12-18 mo after the examination) in about 50%-66%[28,32] of patients and even patients with negative capsule endoscopy have a low probability of experiencing a new bleeding episode (the negative predictive value of capsule endoscopy ranges between 83% and 100%)[34,36].

On the ground of this large amount of published papers capsule endoscopy is now proposed by experts, in patients with obscure GI bleeding, as a third step after a negative bidirectional endoscopy[33], and scientific societies[22,23] define capsule endoscopy as a very valuable tool for investigating obscure gastrointestinal bleeding with the potential capability to improve outcomes.

Recently some Authors evaluated the possible role of capsule endoscopy in the diagnostic work up of patients with isolated iron deficiency anaemia. These studies[37,38], although small, reported that the diagnostic yield of capsule endoscopy in this clinical setting seems quite similar to that reported in patients with obscure GI bleeding (about 50%). These studies confirmed that capsule endoscopy is superior to conventional radiological techniques also in patients with iron deficiency anaemia.

Crohn's disease

We know that Crohn's disease can affect the small bowel: in approximately 45% of Crohn's disease patients the disease involves both the small bowel and the colon and in about 25% the disease is confined to the small bowel, primarily the ileum[39], that can be often difficult to evaluate with endoscopic (retrograde ileoscopy) or radiological methods. For these reasons and because of an increasing number of patients with ulcerative lesions suggesting Crohn's disease[28] has been discovered among subjects undergoing capsule endoscopy for other indications, capsule endoscopy has been also proposed to evaluate the small bowel mucosa of patients with Crohn's disease. However the possible presence of asymptomatic stenoses hampered, at least at the beginning of the experience, the use of this device in patients with a previously established Crohn's disease.

In fact, the first published papers[40,41] on this topic (in 2003) evaluated the diagnostic yield of capsule endoscopy in patients with suspected Crohn's disease (with negative traditional work up, including bidirectional endoscopy and mostly SBFT). The diagnostic yield of capsule endoscopy in this subset of patients (ranging between 33% and 70%)[18,19] has been found, in two independent metanalyses, to be higher when compared with other diagnostic techniques (such as SBFT, SB enteroclysis and retrograde ileoscopy). Marmo *et al*[18] comparing capsule endoscopy with radiological techniques also calculated that the number needed to diagnose (NND) for this subgroup is 2 (95% CI 2-3). Unfortunately the majority of studies aimed at evaluating the role of capsule endoscopy in patients with suspected Crohn's disease included a heterogeneous group of patients, seldom verified over time the final diagnosis by means of other independent diagnostic techniques (i.e. histology), and often used different criteria to classify the lesions identified. A recently published paper[42] tried to overcome possible confounding factors by clearly defining patients with suspected Crohn's disease and by verifying the diagnosis over time. In this paper Girelli *et al*[42] confirmed that capsule endoscopy is an effective tool to diagnose (positive likelihood ratio: 5.8) or to rule out (negative likelihood ratio: 0.08) small bowel Crohn's disease in this particular subset of patients. The authors also pointed out that, in patients with suspected Crohn's disease, assuming a 50% pre-test probability of disease, a positive capsule endoscopy gives a post-test probability of 85%.

The low frequency of capsule retention in patients undergoing capsule endoscopy for suspected Crohn's disease (approximately 1.5%, quite comparable with that reported in patients with obscure GI bleeding)[25]

encouraged the application of this new technique also in patients with established Crohn's disease[43-45]. As expected, in these patients capsule endoscopy showed a high diagnostic yield, significantly superior to that of retrograde ileoscopy and conventional radiological techniques[18,19]. Initial reports comparing capsule endoscopy with CT enteroclysis[46] in patients with established Crohn's disease, although small in size, seem to confirm that capsule endoscopy has a high capability of identifying small inflammatory lesions in the small bowel, and to significantly modify the subsequent management of the patients. However, Golder *et al*[47], using MRI enteroclysis to evaluate the small bowel in patient with Crohn's disease highlighted that, although capsule endoscopy is able to identify a larger number of lesions in the proximal -mid small bowel, in the distal small bowel, which is mostly affected by Crohn's disease, capsule endoscopy and MRI enteroclysis are closely comparable. The authors also pointed out that in these patients MRI enteroclysis identified significant extra intestinal findings in about 30% of cases.

When trying to compare different diagnostic tools for the study of the small bowel in patients with established Crohn's disease, we must keep in mind that capsule endoscopy has been performed exclusively in patients with non stricturing-non penetrating Crohn's disease. In fact all patients in whom a radiological technique showed a stenosis (or a fistula or an abscess) that must be considered as a positive finding of these examinations, were excluded from the comparative studies, leading to a significant and systematic underestimation of the true diagnostic yield of the radiological techniques. Nevertheless, although in the majority of cases, patients with strictures identified with radiological examinations were excluded from capsule endoscopy studies, capsule retention occurred in 5%-13% of cases[25,48]. Capsule retention in patients with established Crohn's disease can be managed, and sometimes partially solved, by giving steroids[49] or using DBE (both for capsule retrieval and stricture dilation)[50,51]. In this subset of patients, capsule endoscopy can be considered as a major complication because it often requires surgical intervention. The development of a dissolvable capsule (see below) may represent, in the near future, the best way to test intestinal patency before capsule endoscopy, in order to avoid capsule retention, especially in patients with Crohn's disease.

Practice guidelines from ESGE[22] suggested that capsule endoscopy, owing to its high diagnostic yield, should have a very important place in the diagnostic work up of patients with known or suspected Crohn's disease, but more large prospective studies are needed to evaluate the specificity of inflammatory lesions, the impact on long term outcome, the clinical significance of the assessment of the extent and severity of small bowel involvement and the risk of capsule retention.

Recent published studies showed also that VCE may have a role in assessing tissue healing after therapy with biologics, relapse after surgical intervention and small bowel evaluation in patients with ulcerative colitis undergoing total colectomy[52].

NSAIDs induced damage

Surprisingly two[13,15] of eight randomized controlled studies published on capsule endoscopy evaluated the role of this technique in assessing small bowel lesions due to NSAIDs consumption. This probably derived from the fact that these widely used drugs can induce small, spotty and superficial mucosal lesions (i.e. mucosal breaks) difficult to identify with other techniques.

Goldstein *et al*[15] clearly demonstrated that NSAIDs (i.e. naproxen), even if associated with proton pump inhibitors (omeprazole), caused more small bowel mucosal lesions than placebo, while Gomez *et al*[13], comparing different NSAIDs, showed that Ibuprofen seems to cause small bowel mucosal damage less frequently than other drugs (dexibuprofen and diclofenac). Another study by Goldstein *et al*[53] comparing COX2-inhibitors with naproxen plus omeprazole showed that among healthy subjects with no endoscopic lesions at baseline, celecoxib was significantly associated with fewer small bowel mucosal breaks than ibuprofen plus omeprazole.

Nevertheless the most important information in this field is the demonstration that small mucosal inflammatory lesions (such as mucosal breaks, small isolated erosion or superficial ulcers) have been detected in about 10%-13% of healthy subjects[15]. Although the clinical implications of these findings remain unclear, the occurrence of these lesions in young healthy subjects, define a new benchmark that must be considered in any further clinical study about capsule endoscopy.

Other indications

As far as celiac disease is concerned two studies[54,55] explored the performance of capsule endoscopy compared with histological evaluation of small bowel biopsies taken during gastroscopy in patients with suspected celiac disease.

Although both studies showed a high agreement between these two techniques (capsule endoscopy sensitivity 85%-87.5%, specificity 90.9%-100%, positive predictive value 96.5%-100% and a negative predictive value of 71.4%-88.9%) the authors underlined that, at present, traditional gastroscopy with duodenal biopsies remains the method of choice to assess mucosal atrophy in patients with suspected celiac disease. However, capsule endoscopy can be a suitable tool in patients, with high clinical suspicion of celiac disease, unable or unwilling to undergo traditional endoscopy.

At the present time, the main obstacle to the extensive use of capsule endoscopy in the diagnosis of celiac disease remains the high costs of the procedure, but also, as highlighted by Biagi *et al*[55], the difficulty in the graduation of mucosal atrophy (see below).

Two studies[57,58], published in 2005 and 2007 respectively, evaluated the role of capsule endoscopy in patients with complicated/refractory celiac disease. In this particular subset of patients capsule endoscopy has been performed to rule out malignant neoplasms [primarily enteropathy associated T-cell lymphoma (EATL)] or other complications (i.e. ulcerative jejunitis). The study of Culliford[57] depicted for the first time the spectrum

of findings (such as scalloping of folds, nodularity and villous atrophy, but also strictures, intussusceptions or submucosal masses), identified by capsule endoscopy in patients with complicated celiac disease, while Daum *et al*[58], demonstrated that capsule endoscopy adds significant clinical information affecting further management mostly in patients with refractory celiac disease type Ⅱ. Nevertheless, both studies included a small number of patients with refractory/complicated celiac disease undergoing a huge number of examinations to exclude strictures; in fact, as previously mentioned for Crohn' s disease, refractory celiac disease can also result in a structuring disease. For these reasons, and mainly because of its capability to take targeted biopsies, double balloon enteroscopy can represent, in this field, a reasonable alternative to capsule endoscopy[59].

Small bowel tumours are still considered, particularly when compared with gastric or colonic neoplasms, a rare disease accounting for 1% to 3 % of all primary gastrointestinal tumours[60], however, since the introduction of capsule endoscopy in clinical practice, some small studies have been published reporting a frequency of small bowel tumours ranging between 6% and 9%[61-65]. These studies, including a series of patients undergoing capsule endoscopy in which this tool was able to identify the presence of small bowel tumours, showed a higher than expected frequency of these tumours. However, two recently presented studies[66,67], published to date only in abstract form, showed a frequency of small bowel tumours ranging between 1.6% and 2.4%. There are no obvious explanations for this discrepancy between studies but the huge number of patients enrolled in the last two studies (more than 6000), the histological confirmation of all reported cases and the substantial concordance with data coming from surgical series, strongly decrease the reliability of earlier data.

All published series about capsule endoscopy in the diagnosis of small bowel tumours underlined that the main clinical indication for capsule endoscopy in these patients is obscure GI bleeding.

In agreement with previously published surgical series, small bowel tumours have been described at capsule endoscopy mostly as polyps (or masses) and stenoses, leading to capsule retention in about 10%[63] to 25%[67] of cases. The most frequent treatment in patients with small bowel tumours is surgical intervention, which, at the same time, allows the retrieval of capsules in case of retention of the device. Therefore, capsule retention in patients with small bowel tumours is considered nowadays as a minor complication.

Capsule endoscopy has also been proposed for the diagnosis and surveillance over time of patients with hereditary polyposis syndromes. The main advantage of this technique in this setting is the capability of this system to inspect the entire small bowel, avoiding radiation exposure and increasing patients' compliance, which is a key point in surveillance programs. Several studies evaluated the possible role of capsule endoscopy in patients with polyposis syndromes[68-70] confirming that, also in this field, capsule endoscopy is more accurate than conventional radiology (SBFT and SB enteroclysis)[71]. Nevertheless the same Authors underlined that the main limitation of this technique, particularly when compared with MRI-enteroclysis, is related to the estimation of size (see below) and location of polyps[68]. At the present time it is suggested that CE should be performed, instead of SBFT, at the time of diagnosis and, as a part of surveillance programs, every 2-3 years, but also as a first diagnostic step in patients with symptoms (i.e. abdominal pain or anaemia)[69,72]. Indeed, keeping in mind the limitations of capsule endoscopy in patients with polyposis syndromes, double balloon enteroscopy can become an important tool, to accurately size and locate lesions, but also to remove polyps identified by capsule endoscopy[73].

The role of CE is less established in patients affected by familial adenomatous polyposis. In fact the quick passage of the capsule through the proximal duodenum can hamper the accurate visualization of the periampullary area. For these reasons, at present, capsule endoscopy is not recommended when the diagnosis of FAP is already established, but may be considered as a part of surveillance for patients with severe duodenal polyposis[69,70]. In a recently published prospective study Wong *et al*[74] compared CE with push enteroscopy and lower endoscopy in 32 patients with FAP. They showed that, in a defined segment of the small bowel, CE diagnosed significantly fewer small-bowel polyps than standard endoscopy, showed only fair agreement with PE in determining polyp counts, and was fairly inaccurate in detecting large polyps and in sizing them.

Abdominal pain, as a possible indication for capsule endoscopy, is still largely debated. Although small bowel tumours have sometimes been identified in patients undergoing capsule endoscopy for unexplained abdominal pain[52], two studies[75,76] evaluating a group of 36 patients with chronic abdominal pain of unknown origin and previous negative diagnostic work-up, found that capsule endoscopy was negative or not clinically relevant in more than 85% of subjects. On the other hand May *et al*[77] clearly demonstrated that when chronic abdominal pain is associated with other signs or symptoms (weight loss > 10% of body weight, inflammation shown by laboratory tests, chronic anemia, or suspected mid-gastrointestinal bleeding) relevant, or potentially relevant, findings are diagnosed by capsule endoscopy in about 60% of cases.

Capsule endoscopy has also been used, with promising results, in other rare clinical conditions such as indeterminate colitis[78,79], small bowel transplantation[80], graft versus host disease[81,82], protein losing entheropathy[83], primitive lymphangectasia[84] (mostly in the pediatric population), Whipple disease[85] and irritable bowel syndrome (with clinical suspicion of celiac disease)[86].

RISKS AND LIMITATIONS

The majority of published papers we mentioned pointed out that the results obtained using capsule endoscopy in clinical practice mainly depend on the revolutionary technical characteristics of this device; however the same technical characteristics can represent, from a certain point of view, limitations of capsule enoscopy. These technical limitations can also explain, in the majority of cases, the clinical limitations of this examination.

Lewis *et al*[87] analyzing a master database, provided

by Given Imaging Ltd (Yoqneam, Israel), found that the global miss rate of capsule endoscopy is about 11% ranging between 0.5% for ulcerative disease and 18.9% for neoplastic disease. Despite the estimated miss rate, capsule endoscopy is significantly lower than that of conventional examinations (global miss rate: 73.3%, miss rate for ulcerative lesions and neoplastic disease: 78.7% and 63.2% respectively) these percentages, in some selected subgroups of patients (i.e. patients with small bowel tumour) are alarming.

Unfortunately there are no conclusive explanations for false negative capsule endoscopies but several factors such as the incompleteness of examination (that can occur in 15%-20% of cases), technical limitations (battery life duration, field of view) and the suboptimal cleanliness of the small bowel (mostly in distal segments) can play a role[88].

At present, although all published papers strongly underlined that small bowel cleanliness is a key point to ensure a complete and accurate examination, and several papers aimed at evaluating factors (dietary restrictions and/or laxatives and/or prokinetic and/or postural tricks) potentially affecting small bowel cleanliness[12,14,16,17,89-93] have been published, there are still no recommendations about small bowel preparation for capsule endoscopy.

This mainly depends on the fact that most studies are published in abstract form, the methodological quality of these studies is rather low, because randomized comparisons are only a small minority, different regimens (with different combinations of drugs) are compared in each study, and an accepted and validated scale to evaluate bowel cleanliness does not exist yet. Four[12,14,16,17] out of 8 controlled randomized studies published on the field of capsule endoscopy are aimed at identifying the best preparation regimen for VCE but, unfortunately, this is only another proof of the relevance of this point.

Despite the lack of any clinical study on this field, all Authors used an overnight fast. An agreement has been reached, basically on the ground of two studies[12,17], about the helpful role of simethicone, administered 20 min before the procedure, in reducing bubbles all along the small bowel, but, the main issue (the presence of liquid stools or fecal debris) which can affect the diagnostic yield of capsule endoscopy, remains to be solved.

In fact, although in 2004[24] the ESGE guidelines, on the ground of the study of Viazis *et al*[16], suggested 2-liters of a poly-ethylen-glycol (PEG) based solution the day before the examination, as small bowel preparation, the updated release of guidelines from the same scientific society (published in 2006)[22] does not recommend any particular schedule of preparation.

The absence of a remote control and of the capability of taking biopsies significantly decrease the specificity of capsule endoscopy findings, since the diagnosis can be based only on the endoscopic appearance. The low specificity of lesions observed at capsule endoscopy is an issue that affects all fields of application of this technique, especially regarding inflammatory lesions (i.e. erosions, ulcers-Figure 4) which can derive from acute and chronic inflammatory bowel diseases[94], ischemic[95], neoplastic[61-67], infectious[96,97] or iatrogenic[98] diseases. As previously mentioned, small and initial inflammatory lesions have also been described in healthy subjects[15].

Another clinical problem strictly dependent on the technical characteristics of the system is the problem of sizing and locating small bowel lesions. This problem, mainly highlighted in studies performed in patients with small bowel hereditary polyposis syndromes[68-70] has important clinical consequences. In fact the size and the location of the lesions are a key point to define, ultimately, the clinical significance of capsule endoscopy findings and to direct further management. In patients with obscure GI bleeding some Authors[99,100] suggested a possible three grade scale (from P0 to P3) to rank capsule endoscopy findings depending on the likelihood of these lesions to explain the reason for referral while, for patients with Crohn's disease two possible scores[43,101] have been proposed but not yet validated. In the field of celiac disease Biagi *et al*[56] clearly demonstrated a large inter- and intra-observer variation in the evaluation of the grade of mucosal atrophy (compared with traditional histology).

In patients with hereditary polyposis syndromes capsule endoscopy tends to overestimate the number of polyps while MRI-enteroclysis seems to be more reliable to correctly estimate the size of polypoid lesions, particularly for polyps of 1-2 centimetres, generally considered clinically relevant[71,74,102].

To improve the capability to estimate the size of polyps Racz *et al*[103] suggested the ingestion, 20' before the procedure, of mesalazine granules as "reference" while Greapler *et al*[104] demonstrated that training with a capsule with a graduated dome might be helpful.

Although awareness of this complication existed at the time of the introduction of this device in clinical practice, the risk of capsule retention at the site of a previously unknown small bowel stricture remains the main complication of capsule endoscopy. This complication seems to be seldom predictable by conventional radiology[105] but the development of a specific dissolvable capsule, even if its safety profile is still under discussion, seems to be a reliable test to screen patients at high risk for capsule retention[107,106-109]. As we know the frequency of capsule retention seems mostly dependent on the clinical indication for capsule endoscopy, ranging between 0% in healthy subjects and 21% in patients with intestinal obstruction[25]. Although capsule retention is the most feared complication of capsule endoscopy, in some selected patients (i.e. patients with small bowel tumor in which capsule retention has been described in 10%-25% of cases) it can be considered as "positive" or minor complication being a sort of "red flag" identifying the presence of the disease. On the contrary, capsule retention must be considered a serious and major complication in patients in whom surgical intervention must be avoided as long as possible (i.e. patients with Crohn's disease, in which capsule retention has been described in about 5%-13% of cases).

Although some case reports described a possible acute obstruction[110] or a possible perforation[111], due to capsule retention, these complications are nowadays considered exceptional. Retained capsules can be retrieved by means of surgical interventions (possibly in a laparoscopic

setting)[112] or by means of enteroscopy (PE or DBE, depending on the site of retention)[113].

Several studies demonstrated the safety of capsule endoscopy in patients with thoracic pace-makers or implanted defibrillators[114-116] and recently a capsule endoscopy has also been performed on a woman in her third trimester of pregnancy because of a life threatening haemorrhage showing a carcinoid tumor[117].

Last but not least, although recently published studies confirmed that this examination is cost-effective in patients with obscure GI bleeding[118], the cost of the procedure can prevent the use of this potentially helpful device in everyday clinical practice. To partially reduce costs of the procedure a possible "two steps" strategy (first step; revision of the video by the nurse and second validation of results by a physician) has been proposed[119,120].

CONCLUSION

Capsule endoscopy, introduced into clinical practice in 2001, revolutionized the study of the small bowel, providing for the first time, a reliable and painless method to evaluate this organ endoscopically. In this paper we critically evaluated the body of evidence produced in these last six years. Unfortunately the majority of published papers are case reports or expert reviews.

Capsule endoscopy has been proven to be significantly superior to conventional radiological techniques (SBFT or SB enteroclysis) for any clinical indication. However studies comparing capsule endoscopy with new imaging techniques (MRI-enteroclysis or CT-enteroclysis) are still few, small and mainly focused on some selected topic (i.e. polyposis syndromes). At the present time capsule endoscopy is recommended as the third examination, after negative bidirectional endoscopy, in patients with obscure GI bleeding. A growing body of evidence suggests also that capsule endoscopy can have a key role in other clinical conditions such as Crohn's disease, celiac disease, small bowel polyposis syndromes or small bowel tumours.

Although awareness of this complication existed at the time of the introduction of this device in clinical practice, the risk of capsule retention at the site of a previously unknown small bowel stricture remains the main complication of capsule endoscopy today. This complication seems to be seldom predictable by means of conventional radiology but the development of a specific dissolvable capsule might, in the near future, provide a safe and reliable test to identify patients at high risk for capsule retention.

Capsule endoscopy still suffers from some technical limitations (there is no remote control, it cannot take biopsies, insufflate air, suck fluids or debris) which can partially explain the clinical limitations/complications of this device (i.e. the difficulty in interpreting inflammatory lesions, in sizing and locating polyps, in grading mucosal atrophy).

The recently developed double balloon enteroscope, owing to its capability to explore a large part of the small bowel and to take targeted biopsies, although invasive and time consuming, can overcome some limitations of capsule endoscopy. At the present time, in the majority of clinical conditions (i.e. obscure GI bleeding), the winning strategy seems to be to couple these two techniques to explore in the most painless, safe and complete way the small bowel (with capsule endoscopy) and to define and treat the lesions identified (with double balloon enteroscopy).

ACKNOWLEDGMENTS

We would like to thank Erik Herdes for the tremendous, essential and continuous technical support.

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S- Editor Liu Y **L- Editor** Alpini GD **E- Editor** Lu W

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World J Gastroenterol 2007 December 14; 13(46): 6150-6155
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Telbivudine: A new treatment for chronic hepatitis B

Deepak N Amarapurkar

Deepak N Amarapurkar, Consultant Gastroenterologist, Department of Gastroenterology and Hepatology, Bombay Hospital & Medical Research Centre, Mumbai 400025, India
Correspondence to: Dr. Deepak N Amarapurkar, Department of Gastroenterology and Hepatology, Bombay Hospital & Medical Research Centre Mumbai, D-401.402 Ameya RBI Employees Co-op Hsg Society, Plot No. 947-950, New Prabhadevi Road, Prabhadevi, Mumbai 400025, India. amarapurkar@gmail.com
Telephone: +91-22-4222432 Fax: +91-22-4368623
Received: April 26, 2007 Revised: September 25, 2007

Abstract

Three hundred and fifty million people worldwide are estimated to be chronically infected with hepatitis B virus. 15%-40% of these subjects will develop cirrhosis, liver failure or hepatocellular carcinoma during their life. The treatment of chronic hepatitis B has improved dramatically over the last decade merits to the advent of nucleoside/nucleotide analogues and the use of pegylated interferons. Approved drugs for chronic hepatitis B treatment include: standard interferon-alpha 2b, pegylated interferon-alpha 2a, lamivudine, adefovir dipivoxil, and entecavir. Unfortunately, these agents are not effective in all patients and are associated with distinct side effects. Interferons have numerous side effects and nucleoside or nucleotide analogues, which are well tolerated, need to be used for prolonged periods, even indefinitely. However, prolonged treatment with nucleoside or nucleotide analogues is associated with a high rate of resistance. Telbivudine is a novel, orally administered nucleoside analogue for use in the treatment of chronic hepatitis B. In contrast to other nucleoside analogues, Telbivudine has not been associated with inhibition of mammalian DNA polymerase with mitochondrial toxicity. Telbivudine has demonstrated potent activity against hepatitis B with a significantly higher rate of response and superior viral suppression compared with lamivudine, the standard treatment. Telbivudine has been generally well tolerated, with a low adverse effect profile, and at its effective dose, no dose-limiting toxicity has been observed. Telbivudine is one of the most potent antiviral agents for chronic hepatitis B virus and was approved by the FDA in late 2006.

Key words: Telbivudine; Chronic hepatitis B; Hepatitis B virus; Nucleoside analogue; Antiviral agents; Pegylated interferons; Lamivudine; Adefovir dipivoxil; Entecavir

Amarapurkar DN. Telbivudine: A new treatment for chronic hepatitis B. *World J Gastroenterol* 2007; 13(46): 6150-6155

http://www.wjgnet.com/1007-9327/13/6150.asp

INTRODUCTION

Hepatitis B virus (HBV) infection is a significant health problem worldwide. Of the 6 billion worldwide populations, an estimated 2 billion have been infected by HBV[1]. It is estimated that 350-400 million people have chronic hepatitis B (CHB) infection[2]. There is clear epidemiologic evidence that chronic HBV infection can result in the development of hepatocellular carcinoma (HCC) and cirrhosis[3,4]. Approximately 15%-40% of HBV carriers develop cirrhosis, liver failure, and HCC; worldwide, more than 50% of primary HCC is related to chronic HBV infection[5]. Each year, 500 000 deaths are expected because of complications related to hepatitis B[6].

EPIDEMIOLOGY IN INDIA

In India nearly 3%-4% of the population is infected by the virus, and chronic hepatitis B constitutes more than 50% of the chronic hepatitis cases in the country[7]. The prevalence ranges from 1.1% to 12.2% with maximum incidence in Madhya Pradesh, Arunachal Pradesh and South India and least in Kashmir and Kerala[8]. There is a peak prevalence after the second decade of life. Most (90%) of these HBV infected subjects are HBeAg negative; the majority (80%) have normal ALT[9]. The prevalence of HBeAg among asymptomatic HBsAg positive persons varies from 9%-20%[9,10].

This, in the context of a large population and absence of a national immunization program would spell off a projected increasing burden of infection and liver disease due to HBV in this country in the years to come. In this perspective, the HBV epidemiology in India becomes relevant not only nationally, but also internationally, because of the possibility that India may soon have the largest HBV infection pool in the world. Economic burden is high and management is affected by cost and availability of diagnostic modalities and patients' awareness and compliance of various treatment options available. Though there have been several guidelines published by various organizations including the Indian association of Study of Liver, the management of HBV infection varies widely in the country. Many physicians in India still find it difficult to make satisfactory management decisions[11].

COST EFFECTIVENESS OF TREATMENT

The main obstacle to treatment in developing countries like India is the expenditure of drug therapy. Cost effective studies have shown savings in countries with intermediate or low endemicity[11]. In India cost of treatment with oral drugs like lamivudine and adefovir range from Rs. 3000 to Rs. 7000/year, a drastic reduction in cost compared to subcutaneous therapy. Additionally the orally used drugs are associated with a good response rate, excellent safety profile and making the overall treatment with oral drugs cost effective. However, the main limitation of these drugs is the emergence of drug-resistant viral strains during the course of treatment.

HBV INFECTION - NATURAL HISTORY

The natural history of CHB is complex and understanding it is important for the selection of patients for treatment. Infected individuals can go through four phases of infection: the immune tolerance phase, the immune reaction or clearance phase (HBeAg-positive, chronic HBV), the inactive carrier (low replication) phase, and the reactivation phase (HBeAg-negative chronic HBV)[1].

In the natural history of HBV infection, the most important event is HBeAg seroconversion characterized by loss of HBeAg and development of antibody to HBeAg (Anti HBe)[12]. The prognosis of chronic HBV infection is dependent upon the amount of inflammation, necrosis and fibrosis in the liver at this point of seroconversion. If significant liver damage is already present at this point, then the prognosis after seroconversion, spontaneous or treatment related is unlikely to be good, despite suppression of viral replication. On the other hand, if the seroconversion has occurred early and is maintained, then the long-term prognosis is excellent. In a subset of persons, this relationship between seroconversion and suppression of viral replication does not hold true. In them, despite anti-HBe positivity, active viral replication persists due to the emergence of mutants in the 'precore' and basal core promoter regions of HBV. This state, characterized by continuing viral replication despite anti HBe positivity has been termed as HBeAg negative CHB[13-15]. Compared with HBeAg-positive CHB, HBeAg negative CHB can follow an aggressive course, requires long or even infinite treatment, and leads to the rapid development of cirrhosis and HCC.

Goals of treatment

The ultimate goal of treatment is the eradication of HBV before it causes irreversible damage including cirrhosis and/or HCC. The eradication of HBV is impossible with currently approved drugs. This is because of extrahepatic reservoirs of HBV, integration of HBV DNA into host DNA, and the presence of covalently closed circular DNA (cccDNA) in the hepatocyte nucleus. Such cccDNA serves as a transcriptional template for HBV replication without the need for reinfection[16,17]. Current antiviral agents have little inhibitory effect on cccDNA, leading to high relapse rates after discontinuation of treatment.

The more realistic goals of therapy are early and prolonged viral suppression, remission of chronic liver disease, a decreased rate of cirrhosis, liver failure, HCC, and reduced morbidity and mortality.

TREATMENT OF CHB

The treatment of CHB has undergone tremendous change and continues to evolve with the advent of potent antiviral agents. The FDA currently approves interferon alpha-2b, pegylated interferon alpha-2a (PEG-IFN-α2a), and four oral agents, adefovir dipivoxil, entecavir, lamivudine and telbivudine as monotherapeutic agents[18].

IFN-α was the first FDA-approved medication for the treatment of CHB. Polyethylene glycol, an inert water soluble molecule when attached to standard interferon to form pegylated interferon decreases the clearance and antigenicity of interferon, thus extending and sustaining its activity *in vivo* allowing for once-weekly administration.

IFN-α and PEG-IFN-α2a have significant side effects, require injection therapy and are less efficacious in patients acquiring CHB during early childhood, for example Asian patients. Furthermore IFN treatment fails to decrease the chance of development of cirrhosis-related complications and HCC[19].

Lamivudine, an oral nucleoside analogue, was the second FDA-approved medication for the treatment of CHB. Viral breakthrough remains a problem with lamivudine with incidence of resistance ranging from 16%-32% after 1 year of therapy to as high as 58% with 2-3 years of therapy[20]. Furthermore, disease progression has been shown to resume following viral breakthrough.

Adefovir dipivoxil, an oral nucleotide analogue, was approved by the FDA in September 2003. Although resistance is less frequent with adefovir compared to lamivudine, this agent may be restricted by nephrotoxicity and a relatively modest potency[18].

Entecavir, a deoxyguanosine nucleoside analog, has recently been licensed by the FDA. Early trials have shown it to be more potent than lamivudine. However, care must be taken when using entecavir in individuals with renal dysfunction[18].

The newest antiviral nucleoside analogues like telbivudine was approved by the FDA based on the results of a Phase Ⅲ clinical trial for the treatment of CHB. The aim of this review article is to introduce and review current information on telbivudine for the treatment of CHB.

TELBIVUDINE

Telbivudine (LdT) is a novel agent for the treatment of CHB. It is an HBV-specific L-nucleoside analogue of thymidine. The chemical name of telbivudine is β-L-2-deoxythymidine (LdT). Telbivudine is an unsubstituted, unmodified β-L-2-nucleoside and the first compound of this series.

Mechanism of action

Telbivudine must be activated by phosphorylation and is efficiently metabolized to 5-triphosphate derivative.

5-triphosphate metabolite of β-L-2-deoxynucleosides interacts with the viral polymerase and inhibits viral replication and results in obligate chain termination of DNA synthesis[21]. This inhibition occurs mainly in the synthesis of the second strand of DNA (DNA-to-DNA transcription) for telbivudine (in contrast to lamivudine which strongly inhibited first strand DNA synthesis; RNA-to-DNA reverse transcription). Since transcription fidelity is higher in DNA-to-DNA synthesis than RNA-to-DNA synthesis, telbivudine treatment may have a slower rate of emergence of drug-resistant virus when compared to lamivudine[21].

Preclinical studies

There is no demonstrable toxic effect on human DNA polymerases α, β and γ by telbivudine after phosphorylation in the experiments using hepatoma cell lines, primary human peripheral blood monocyte cells and human foreskin fibroblasts and other cell types of mammalian origin[21].

In preclinical studies, telbivudine was investigated in rats and monkeys at concentrations substantially greater than the anticipated dose in humans. No significant toxic effects were observed in animal models, suggesting a minimal risk of cumulative, carcinogenic or reproductive toxicity in humans[22].

Pharmacokinetics

A phase Ⅰ/Ⅱ a dose-escalation study in HBV-infected patients showed that telbivudine is rapidly absorbed with the peak concentration reached within approximately 1-3 h. The plasma concentration of telbivudine increased proportionally with increasing doses in the 25-800 mg/d range studied. There were no serious adverse events in all the subjects either receiving telbivudine or placebo[23].

Systemic telbivudine is predominantly cleared unchanged by the kidneys with minimal metabolism and elimination via the hepatic route. Zhou *et al*[24] studied the pharmacokinetic profile of telbivudine administered to patients with moderate to severe renal impairment and hepatic impairment with regards to peak concentration and overall drug exposure [area under the curve (AUC)]. In patients with renal impairment, the AUC was two to three-fold higher in subjects with moderate renal impairment when dosing was normalized to 600 mg/d, suggesting that telbivudine dosage needs to be adjusted in renally impaired patients preferably by reducing the daily dose. Pharmacokinetic profiles were comparable in subjects with normal and impaired hepatic function. The pharmacokinetics are not altered by the food intake[24].

CLINICAL STUDIES

Dose ranging study

The excellent results achieved in the early human PK and safety studies led to the phase Ⅰ/Ⅱ clinical trial in patients with CHB[25]. In this first clinical study of telbivudine, safety, antiviral activity, and pharmacokinetics were assessed in 43 adults with hepatitis Be antigen-positive chronic hepatitis B. This placebo-controlled dose-escalation trial investigated 6 telbivudine daily dosing levels (25, 50, 100, 200, 400, and 800 mg/d); treatment was given for 4 wk, with a 12 wk follow-up. Serum HBV DNA levels were monitored *via* quantitative polymerase chain reaction (PCR). The results indicate that telbivudine was well tolerated at all dosing levels, with no dose-related or treatment-related clinical or laboratory adverse events. Telbivudine plasma pharmacokinetics was dose-proportional within the studied dose range. Marked dose-related antiviral activity was evident, with a maximum of telbivudine doses of 400 mg/d or more. In the 800 mg/d cohort, the mean HBV DNA reduction was 3.75 $\log_{10}$ copies/mL at wk 4, comprising a 99.98% reduction in serum viral load. Correspondingly, post treatment return of viral load was slowest in the high-dose groups[25].

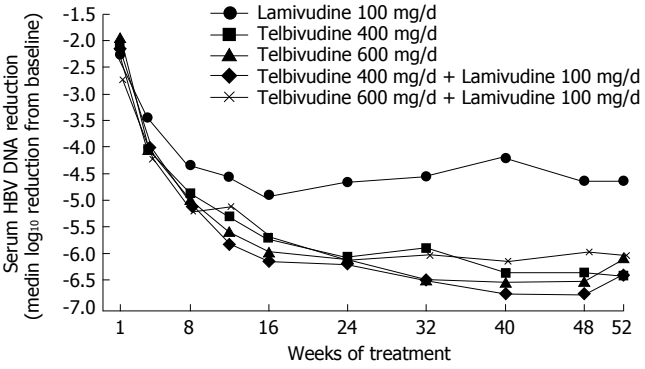

Figure 1 Median reductions in serum hepatitis B virus (HBV) DNA levels at wk 52 (log10 copies/mL) in all treatment groups.

Phase Ⅱb studies

Owing to the encouraging results in the phase Ⅰ/Ⅱ study, a multicenter, international phase Ⅱb trial was initiated. This randomized, double-blind trial evaluated the efficacy and safety of telbivudine 400 or 600 mg/d and telbivudine 400 or 600 mg/d plus lamivudine 100 mg/d (Comb400 and Comb600) compared with lamivudine 100 mg/d in hepatitis Be antigen (HBeAg)-positive adults with compensated chronic hepatitis B[26].

A total of 104 patients were randomized 1:1:1:1:1 among the 5 groups. Median reductions in serum hepatitis B virus (HBV) DNA levels at wk 52 ($\log_{10}$ copies/mL) are shown in Figure 1.

At wk 52, telbivudine monotherapy showed a significantly ($P < 0.05$ for each comparison) greater mean reduction in HBV DNA levels (Figure 1), clearance of polymerase chain reaction-detectable HBV DNA, and normalization of alanine aminotransferase (ALT) levels compared with lamivudine monotherapy, with proportionally greater HBeAg seroconversion and less viral breakthrough (Figure 2). Combination treatment was not better than telbivudine alone. All treatments were well tolerated[26]. This study also examined the prognostic significance of the magnitude of HBV DNA reduction at wk 24 during therapy. For patients with HBV DNA less than 3 log at wk 24 none developed viral breakthrough at wk 52. More importantly 100% of these patients had undetectable HBV DNA by PCR assay at wk 52. In

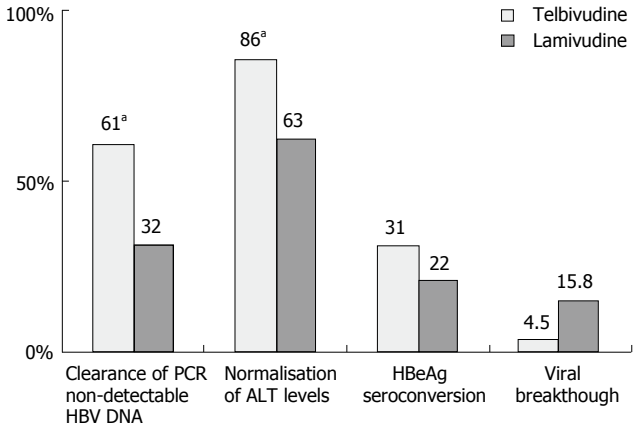

Figure 2 Comparison of telbivudine and lamivudine monotherapy. [a]$P < 0.05$.

addition, these patients had a higher rate of loss of HBeAg and higher chances of ALT normalization at wk 52. These important findings emphasize that treatment should target a rapid and maximal suppression of viral replication[26].

Phase Ⅲ studies

Efficacy *vs* lamivudine: Globe is a phase Ⅲ, double-blinded, randomized, international, multicenter clinical trial designed to compare telbivudine vs lamivudine in over 1369 individuals with CHB over a 2 years period[18].

Individuals entered into the study were screened for HBeAg positivity, and required HBV DNA more than 6 $\log_{10}$ copies/mL by Roche COBAS® Amplicor PCR assay, an ALT greater or equal to 1.3-10 times the upper limit of normal and compensated liver disease. They were stratified for HBeAg status (positive or negative) and ALT value (less or greater than 2.5 times the upper limit of normal). Individuals were randomized to receive 2 years of either: (1) Lamivudine 100 mg qd or (2) Telbivudine 600 mg qd.

The primary end-point of Globe was 'Therapeutic Response', a composite serological end-point comprising suppression of serum HBV DNA to below 5 $\log_{10}$ copies/mL.

Secondary end-points included reduction in serum HBV DNA, normalization of serum ALT, HBeAg loss, seroconversion, and safety[18].

At 1 year[18], a significant reduction in HBV DNA in the telbivudine exposed individuals compared with lamivudine was observed, there being a greater HBV clearance to PCR non-detectable levels in this group also (Table 1). Telbivudine was associated with fewer flares of serum ALT levels when compared to lamivudine. On reviewing histological findings, 65% of HBeAg positive individuals exposed to telbivudine had a significant improvement in histology *vs* 56% of those exposed to lamivudine ($P < 0.01$)[18].

For all clinical and virological efficacy parameters, efficacy at 1 year was proportional to HBV DNA level at wk 24 (Table 2). Achieving HBV DNA < 300 copies/mL at wk 24 with lamivudine or telbivudine was highly predictive of not developing resistance at wk 52. Overall significantly less resistance was seen in the telbivudine arm than the lamivudine arm (2%-3% *vs* 7%-8%). The ability to predict

Table 1 Efficacy at 1 yr in HBeAg positive and HBeAg negative individuals in the Globe study receiving telbivudine or lamivudine

	Telbivudine	Lamivudine	*P* value
HBeAg positive patients			
HBV DNA fall (mean $\log_{10}$)	-6.5	-5.5	< 0.01
HBV DNA non-detectable by PCR (%)	60	40	< 0.01
Treatment failure (%)[1]	5	13	< 0.01
HBeAg negative patients			
HBV DNA fall (mean $\log_{10}$)	-5.2	-4.4	< 0.01
HBV DNA non-detectable by PCR (%)	88	71	< 0.01
Treatment failure (%)[1]	< 1	3	NS

[1]Defined as HBV DNA > 5 $\log_{10}$ copies/mL.

Table 2 Efficacy at 1 yr against virological suppression at 24 wk in HBeAg positive and negative individuals in the Globe study

	Virological suppression at wk 24 (%)			
	< 300 copies/mL	300-1000 copies/mL	> 1000-10000 copies/mL	> 10000 copies/mL
HBeAg positive patients (wk 52)				
HBV DNA (< 300 copies/mL)	90	70	30	5
ALT normalization	90	89	80	54
Viral breakthrough	1	3	8	11
HBeAg negative patients (wk 52)				
HBV DNA (< 300 copies/mL)	93	66	38	10
ALT normalization	83	74	63	36
Viral breakthrough	0	9	18	32

subsequent outcomes at 24 wk enables clinicians to estimate a response and plan future therapeutic interventions.

The 2 years results of this study[27] were presented recently at the AASLD annual meeting at Boston. At 2 years telbivudine showed significantly greater therapeutic response, a greater reduction in HBV DNA from baseline and greater HBV DNA clearance to PCR negative. Telbivudine showed significantly less primary and secondary failure, breakthrough and resistance. Clinical adverse event profiles were similar between the two treatment groups (Table 3). Both study drugs were generally well-tolerated, with similar patterns of clinical adverse events. Clinical and virological efficacy at 2 years was also linked to magnitude of HBV suppression at wk 24 (Table 4)[28].

Another phase Ⅲ trial compared telbivudine with lamivudine in Chinese individuals with CHB[29], 87% of whom were HBeAg positive. At 52 wk, 70% of telbivudine exposed individuals had undetectable HBV DNA levels (defined as < 300 copies/mL by PCR) compared with only 43% of lamivudine treated individuals ($P < 0.001$). Telbivudine was superior to lamivudine in normalizing ALT levels (89% *vs* 76%, respectively, $P < 0.005$). In telbivudine exposed individuals who were HBeAg positive, on entering the study, 25% had seroconverted at 52 wk, compared with 18% of those treated with lamivudine. Both Telbivudine and lamivudine were equally well tolerated.

Table 3 Efficacy at 2 yr in HBeAg positive and HBeAg negative individuals in the Globe study receiving Telbivudine or lamivudine

	Telbivudine	Lamivudine	*P* value
HBeAg positive patients			
HBV DNA fall (mean $\log_{10}$)	-5.7	-4.4	< 0.05
HBV DNA non-detectable by PCR (%)	54	38	< 0.05
Treatment failure (%)[1]	4	12.3	< 0.05
HBeAg negative patients			
HBV DNA fall (mean $\log_{10}$)	-5	-4.2	< 0.05
HBV DNA non-detectable by PCR (%)	79	53	< 0.05
Treatment failure (%)[1]	0	3	< 0.05

[1]Defined as HBV DNA > 5 log10 copies/mL.

Efficacy *vs* adefovir: A third major phase Ⅲ study has compared the use of telbivudine and adefovir in HBeAg positive individuals with CHB for 24 wk[30]. At the end of the study, a significantly greater HBV DNA reduction was seen in those individuals exposed to telbivudine (6.37 *vs* 5.11 $\log_{10}$ copies/mL; $P < 0.01$). In individuals exposed to adefovir, 42% failed to reach a HBV DNA below 5 $\log_{10}$ copies/mL, compared with 5% in the telbivudine arm ($P < 0.01$). There was no significant difference in HBeAg loss or normalization of ALT levels between the two arms. There was no difference in adverse events between the two arms.

Resistance: Of the 1367 individuals included in the Globe ITT analysis, 81 patients experienced viral breakthrough[18]. Among these, genotypic resistance was confirmed in 69:17 telbivudine-treated patients and 52 lamivudine recipients. Following sequencing, all resistance was associated with M204 variants in the YMDD motif of the genome. Individuals failing lamivudine had acquired either M204I, M204V or a mixed picture of M204M/I/V. In those exposed to telbivudine, M204I was the only mutation detected in 16 of 17 telbivudine patients with resistance; the other patient carried a mixture of M204M/I/V.

The M204V lamivudine resistant mutation was associated with the 180M compensatory mutation, thus forming a double mutant, whereas the M204I telbivudine mutation was not. These results imply that telbivudine may suppress the emergence of fully resistant HBV *via* the M204V pathway that is dominant with lamivudine.

FUTURE TRENDS IN THE MANAGEMENT OF CHRONIC HEPATITIS B

Emerging data suggest that the magnitude of reduction in serum HBV DNA levels that are achieved early in the course of therapy with nucleos(t)ides predicts the likelihood of subsequent efficacy outcomes. Data from the GLOBE study suggest that patients who have rapid and profound viral response to treatment (serum HBV DNA < 300 copies/mL at 24 wk) tend to have a higher probability of maintaining the response and a lower probability of resistance over time. Conversely, patients who have a lower initial viral response tend to have a higher probability of low rates of response and resistance over time.

Table 4 Efficacy at 2 yr against virological suppression (PCR negative) at 24 wk in HBeAg positive and negative individuals in the Globe study

	Yr 2 outcome	Probability (%)
HBeAg positive	HBeAg seroconversion	45
	ALT normalization	79
	HBV DNA non-detectable by PCR	77
HBeAg negative	ALT normalization	77
	HBV DNA non-detectable by PCR	74

Understanding the factors that predict poor response and resistance can potentially permit treatment modification to optimize response to treatment. Therefore, the response to a single agent after 24 wk of therapy may be used effectively by the clinician to determine whether the patient is likely to benefit from continued monotherapy or if the addition of another agent may offer a better probability for improved long term outcomes.

A clinical trial to assess this approach with telbivudine will be conducted in India. The trial design will involve assessment of response, based on serum HBV DNA levels, at specific early time points starting at 24 wk. Based on the degree of viral suppression at these time points, telbivudine may be continued or another agent could be added.

CONCLUSION

Owing to its good safety profile and high antiviral potency, telbivudine is one of the most promising drugs for the treatment of CHB. Telbivudine is one of the new β-L-nucleoside analogues with potent antiviral activity against HBV. Telbivudine is an obligate chain terminator, incorporating into HBV DNA, and exerts a preferential effect on second strand DNA synthesis. The promising results of the early *in vitro* and animal studies paved the way for phase Ⅰ/Ⅱ human clinical trials. Phase ⅡB human clinical studies demonstrated superior antiviral efficacy of telbivudine, significantly better ALT normalization and better HBeAg loss as compared with lamivudine. Further large international multicenter phase Ⅲ studies have confirmed these results with telbivudine in comparison not only to lamivudine but also to adefovir.

Overall, telbivudine appears to be efficacious, easy to take with a good safety profile, proving to be a valuable therapeutic option in the management of hepatitis B.

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S- Editor Liu Y **L- Editor** Alpini GD **E- Editor** Lu W

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World J Gastroenterol 2007 December 14; 13(46): 6156-6165
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Jesús K Yamamoto-Furusho, *Series Editor*

Basic and clinical aspects of osteoporosis in inflammatory bowel disease

Lorena Rodríguez-Bores, Josué Barahona-Garrido, Jesús K Yamamoto-Furusho

Lorena Rodríguez-Bores, Josué Barahona Garrido, Jesús K Yamamoto-Furusho, IBD Clinic, Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Vasco de Quiroga 15 colonia Sección XVI, Tlalpan, CP 14000, México
Correspondence to: Jesús K Yamamoto-Furusho, MD, PhD, Head of IBD Clinic, Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Vasco de Quiroga 15 colonia Sección XVI, Tlalpan, CP 14000, México. kazuofurusho@hotmail.com
Telephone: +52-55-55733418-2705 Fax: +52-55-56550942
Received: August 26, 2007 Revised: September 25, 2007

Abstract

Low bone mineral density and the increased risk of fracture in gastrointestinal diseases have a multifactorial pathogenesis. Inflammatory bowel disease (IBD) has been associated with an increased risk of osteoporosis and osteopenia and epidemiologic studies have reported an increased prevalence of low bone mass in patients with IBD. Certainly, genetics play an important role, along with other factors such as systemic inflammation, malnutrition, hypogonadism, glucocorticoid therapy in IBD and other lifestyle factors. At a molecular level the proinflammatory cytokines that contribute to the intestinal immune response in IBD are known to enhance bone resorption. There are genes influencing osteoblast function and it is likely that LRP5 may be involved in the skeletal development. Also the identification of vitamin D receptors (VDRs) and some of its polymorphisms have led to consider the possible relationships between them and some autoimmune diseases and may be involved in the pathogenesis through the exertion of its immunomodulatory effects during inflammation. Trying to explain the physiopathology we have found that there is increasing evidence for the integration between systemic inflammation and bone loss likely mediated via receptor for activated nuclear factor kappa-B (RANK), RANK-ligand, and osteoprotegerin, proteins that can affect both osteoclastogenesis and T-cell activation. Although glucocorticoids can reduce mucosal and systemic inflammation, they have intrinsic qualities that negatively impact on bone mass. It is still controversial if all IBD patients should be screened, especially in patients with preexisting risk factors for bone disease. Available methods to measure BMD include single energy x-ray absorptiometry, DXA, quantitative computed tomography (QCT), radiographic absorptiometry, and ultrasound. DXA is the establish method to determine BMD, and routinely is measured in the hip and the lumbar spine. There are several treatments options that have proven their effectiveness, while new emergent therapies such as calcitonin and teriparatide among others remain to be assessed.

Key words: Inflammatory bowel disease; Osteoporosis

Rodríguez-Bores L, Barahona-Garrido J, Yamamoto-Furusho JK. Basic and clinical aspects of osteoporosis in inflammatory bowel disease. *World J Gastroenterol* 2007; 13(46): 6156-6165

http://www.wjgnet.com/1007-9327/13/6156.asp

INTRODUCTION

Patients with inflammatory bowel disease (IBD) are at increased risk of developing disorder in bone and mineral metabolism because of several factors, including the genetic influence, cytokine-mediated nature of the inflammatory bowel disease, the intestinal malabsorption resulting from disease activity or from extensive intestinal resection and the use of glucocorticoids to control disease activity. Apparently these disturbances may also be seen since childhood, and environmental factors such as malnutrition, immobilization, low body mass index (BMI), smoking and hypogonadism may also play a contributing role in the pathogenesis of bone loss. In IBD several studies demonstrate a negative correlation between bone mineral density (BMD) and glucocorticoid use, though there is evidence that may support the opposite. In order to answer the questions about the pathogenesis, we first have to determine the factors that are involved in this extraintestinal complication. The aim of this paper is to review the basic and molecular aspects with the clinical and therapeutic features and have an overview about the trends of the bone disease related to IBD.

EPIDEMIOLOGY

Bone mineral density is decreased in a proportion of subjects with IBD as shown by epidemiological studies. The current understanding about IBD and BMD is that

the overall risk of fracture may be slightly increased in IBD patients. IBD has been associated with an increased risk of osteoporosis and osteopenia and epidemiologic studies have reported an increased prevalence of low bone mass in patients with IBD. The prevalence rates from 2% to 30% for osteoporosis (OP), from 40% to 50% for osteopenia[1] and the overall prevalence of low bone mineral density is estimated in 15%. A population-based study compared IBD patients with the general population and reported similar increases in the fracture risk between Crohn's disease (CD) and Ulcerative colitis (UC)[2] and in comparison to control patients, similar to what other population-based studies have reported[3,4]. Some series have reported that in newly diagnosed IBD patients a reduced BMD has been found and this prevalence is slightly higher in patients with CD[3,5] whereas approximately 15% of patients with CD have osteoporosis[6]. There is contrasting data from a Danish case-control study where an increase in the risk of fracture among women with CD was seen, but not men with CD or patients with UC[7,8], also another study reported that the overall fracture rate in UC was similar to that of control subjects[9]. In regard to age and gender as risk factors, elderly have the highest risk of fracturing and this increased risk is evident across all age groups[3]. Some case control studies have demonstrated that gender, age, and body weight are the major determinants of bone mineral density in patients with CD. As in healthy individuals, the combined effect of these factors account for up to 50% of the variability in bone mineral density[10]. Male sex and increasing age were considered risk factors in predicting those with osteoporosis although most series report no significant difference between the genders.

Longitudinal studies show that the BMD changes are not excessive[11,12] and there is no exclusive pattern of low BMD that involves spine of the hip. The risk of hip fracture is increased by 86% in patients with CD and by 40% in patients with UC[2]. However the hip has been reported more frequently affected than the spine[13,14]. In a study of Stockbrugger *et al* significant number of fractures in IBD patients as in the general osteoporotic are asymptomatic, about 14.2% of the fractures seem to be underreported[15], though it is important to mention that osteoporosis occurrence is often underestimated[15].

GENETICS

Low bone mineral density and the increased risk of fracture in all gastrointestinal diseases including IBD have a multifactorial pathogenesis. There are a number of factors that can lead to enhanced bone loss, these also include genetic factors.

LRP5

Because of the central role of osteoblasts in bone formation, it is easy to think there are genes influencing osteoblast function and it is likely that LRP5 may be involved in the skeletal development. The protein encoded by *LRP5* is a member of the low-density lipoprotein-receptor (*LDLR*) gene superfamily[16] and is closely related to *LRP6*[17]. *LRP5* is transcribed in human bone tissue as well as in numerous other tissues. There is convincing findings that deleterious (loss of function) mutations in *LRP5* result in loss of function and cause bone defects such as the ones seen in pseudoglioma syndrome further supporting the critical role of this gene in skeletal integrity[18]. There is some data about the identification in normal healthy individuals of a gain of function mutation in the LDL receptor-related protein 5 (LPR5) gene resulting from a autosomal dominant high bone mass trait[19] and this gain of function mutation described in LRP5 produces increased bone mass with no adverse effect on skeletal structure, contrasting the loss of function mutation that maps to the same genomic region that contains LRP5 causes the osteoporosis pseudoglioma syndrome[20]. Polymorphisms rs491347 rs1784235 could be important to human osteoporosis phenotypes and may be considered as possible susceptibility factors for osteoporosis and fractures in humans[21]. A Japanese study found that the A1330 V polymorphism may contribute to osteoporosis susceptibility[22] and also was associated with reduced BMC and BMD values in healthy young Finnish men, providing evidence for the crucial role of LRP5 in peak bone mass acquisition[23].

VDR (Vitamin D receptor gene)

The identification of vitamin D receptors (VDRs) in peripheral blood mononuclear cells sparked the early interest in vitamin D as an immune system regulator[24]. Vitamin D deficiency has been linked to several different diseases, including the immune system-mediated OP such as IBD. The association of VDR gene BsmI polymorphism with OP has been studied by several investigators[24-28]. In addition, TaqI, FokI and ApaI polymorphisms of the VDR gene have also been described[25]. Regarding OP, most data concern to the BsmI polymorphism of the vitamin D receptor (VDR) gene.

Candidate genes

There are other candidate genes that seem involved with bone loss. Estrogen receptor alpha (ER alpha) play an important role in increasing BMD *via* mechanical strain and muscle mass[29]. The results of studies regarding the association between some common polymorphisms of the aromatase gene and bone mineral density and the risk of osteoporotic fractures are recognized[30]. Thus, aromatase is also an attractive osteoporosis candidate gene. The gene encoding TGFβ1 is a strong functional candidate for genetic susceptibility to osteoporosis. Several polymorphisms have been identified in TGFβ1, and previous work has suggested that allelic variants of TGFβ1 may regulate BMD and susceptibility to osteoporotic fracture[31]. During the last years, about 170 candidate genes have been published. There have been (e.g., VDR, ER-α, and COL1A1), as well as novel genes recently discovered to be important in bone and mineral metabolism. The newly studied genes include a big list CYP17 (17-hydroxylase)[32], CYP1B1 (cytochrome P450)[33], DBP (vitamin D-binding protein)[34], GH1 (growth hormone 1)[35], GnRH (gonadotropin-releasing

hormone 1[35]), IGF-Ⅱ (insulin-like growth factor Ⅱ)[37], LEPR (leptin receptor)[38], LRP5 (low-density lipoprotein receptor-related protein 5)[39], BMP2 (bone morphogenetic protein 2)[40], CCR2 (chemokine)[41], CLCN7 (chloride channel 7)[42], COMT (catechol-O-methyltransferase)[43], CTSK (cathepsin K)[44], DRD4 (dopamine receptor D4)[45], I-TRAF (TRAF family member-associated NF-κB activator)[46], LCT (lactase)[47], MIF (macrophage migration inhibitory factor)[48], MMP-1 (matrix metalloproteinase 1)[49], among many others, but their relationship with inflammation as a possible mechanism of osteoporosis still is not clear and the interaction with IBD bone disease has not been elucidated. The mechanisms involved and the potential usefulness of those genetic data in the prevention and management of osteoporosis need further investigation, also to determine the direct relation with IBD.

PATHOPHYSIOLOGY

Inflammation has now moved to the center of the physiopathologic mechanisms involved in the process of bone loss in IBD, there has been a considerable increase in knowledge surrounding the genetic determinants of osteoporosis. As well as genetic markers are potentially helpful in identifying high risk patients, the genetic variations of cytokines plays a key role in the regulation of the inflammatory response. Several studies are focused trying to identify genetic risk factors for rapid bone loss in IBD patients as a model of disease and inflammation-associated bone loss. Evidence accumulated in the past years support that interleukin 6 (IL-6) is a pathogenic factor in osteoporosis that results from the loss of either male or female sex steroids and have implicated IL-6 in the physiopathology of several other diseases caused by increased osteoclastic bone resorption including diseases such as Rheumatoid arthritis[50]. Genetic variations in the IL-6 and interleukin 1 receptor antagonist (IL-1ra) gene identify IBD patients at risk for increased bone loss. Allele status of the IL-1ra, IL-6, heat shock protein 70-2 and 70-hom (hsp 70-2, hsp hom) gene has been typed and correlated with clinical course of IBD and extent of bone loss[51]. These variations are independent determinants of bone loss in the setting of IBD, and have been identified as independent predictors of bone loss in the setting of postmenopausal osteoporosis, suggesting that IL-6 and IL-1ra determine the response of bone to different stressors such as the hypoestrogenic state or systemic inflammation[52,53]. Apparently, estrogen loss results in increased production of IL-6 by *ex vivo* bone marrow cell cultures and increased production of IL-6 follows the withdrawal of estradiol from primary culture[54,55]. It seems that IL-6 is responsible for increased bone resorption after loss of sex steroids and that gonadectomy prevents the increase in osteoclastogenesis in bone marrow and the increase in the number of osteoclasts in sections of trabecular bone[56]. The cytokines IL-1ra and IL-6 also have a central role in the paracrine stimulation of osteoclast development and regulation of the process of bone resorption[50,55]. Increasing evidence suggests that IL-6 type cytokines also promote the development of osteoblasts[50]. It has been observed that the carriage of the A2 allele of the IL-1ra gene is associated with reduced bone loss[52].

The interleukine-2 (IL-2) deficient mouse model of colitis is known to develop both osteopenia and colitis. Osteopenia was not evident in IL-2 deficient mouse cross-bred to be T-cell deficient, and osteopenia could be induced in T-cell-deficient mice by adoptive transfer of T cells from IL-2 deficient mice[57]. These data suggest that activated T cells are critical for mediating the osteopenia.

OPG-RANK-RANKL system

The receptor activator of nuclear factor κB ligand (RANKL) osteoprotegerin (OPG) system represents a potential link between inflammation and bone homeostasis and also an example of inflammation-mediated osteopenia such as IBD-associated osteopenia. The balance between RANKL and OPG (the soluble decoy receptor preventing ligation of RANKL) is of major importance to the regulation of osteoclastogenesis. The interaction of RANK on the surface of osteoclasts with its ligand RANKL induces osteoclastogenesis and conversely the interaction with the osteoblast derived soluble decoy receptor, osteoprotegerin (OPG)[58] blocks RANK-RANKL interaction inhibiting osteoclasts formation. Whether compounds stimulate RANK ligand or OPG will affect whether they induce or inhibit osteoclastogenesis. Pro-inflammatory cytokines induce RANKL and promote bone resorption with consecutive bone loss. Activated T cells can directly trigger osteoclastogenesis through RANKL leading to bone loss while OPG can block those effects[59-61]. Increased OPG levels may represent a continuing homeostatic response, attempting to reverse established osteopenia and RANKL driven osteoclastogenesis, thus maintaining normal bone mass. Inflammation seems to play an important role in the regulation of the OPG-RANK-RANKL system. To correlate it with chronic inflammatory states comparable to IBD, there have been some reports that show a direct correlation between serum OPG and erythrocyte sedimentation rate and a score of disease activity in patients with rheumatoid arthritis[62]. Soluble RANKL as well as OPG levels are elevated in rheumatoid arthritis, while high OPG and decreased RANKL levels have been reported in primary biliary cirrhosis[63,64]. Some of the osteoclastogenic factors released from the IBD mucosa (for example IL-1, IL-6 and TNFα) are thought to function indirectly via specific receptors on stromal osteoblastic cells to enhance RANKL expression[60,65,66]. Data suggests that OPG may be a protective host response that partially offsets the adverse skeletal effect created by the inflammation state. Moshen *et al*[67] described the alterations in the RANKL/OPG system in IBD and its relationship to decreased BMD. It has been demonstrated increased plasma levels of OPG as well as increased release from the inflamed colon in IBD, suggesting the macrophages and dendritic cells as colonic source of OPG in IBD. Apparently, no correlation was evident between corticosteroid and serum OPG[63] contrasting partially with other findings.

Corticosteroids

The controversial participation of glucocorticoid (GC) therapy in the pathogenesis of bone loss in IBD still has gaps to be fulfilled. It seems that there is an important relationship between dosage, duration and pattern of GC therapy and these factors are related to the incidence of pathological fractures[68]. Some studies indicate that fractures are present in 30%-50% of patients on GC therapy for chronic diseases[69] and several studies have demonstrated that dosage is associated with BMD[51,70-73]. On the other hand, several studies have reported the opposite[8,13].

The epidemiological data on fracture risk and bone loss in GC therapy do not distinguish the effects of drug and the effects of the underlying disease. It is known, for example, in rheumatoid arthritis, the risk of fracture is increased even in the absence of GC exposure, also it has been observed that osteoporosis is rapidly developed in recently diagnosed Crohn's disease without any effect of corticosteroids in the follow up. One study showed that the prevalence of osteoporosis in pediatric patients with IBD is approximately the same as in adult patients, showing that osteoporosis was already present before steroid treatment[74]. Contrasting data from other studies show that the extent of bone loss was no correlated to clinical severity of disease or application of corticosteroids[75-77]. The participation of GC in the pathophisiology of bone loss is complex. GCs influence the production and action of hormones that regulate bone and calcium metabolism and also have direct effects of GCs on bone. GCs increase the expression of receptor activator of nuclear factor κB ligand (RANK-L) and decrease the expression of its soluble decoy receptor osteoprotegerin (OPG) in stromal and osteoblastic cells[78] and also enhance the expression of macrophage colony-stimulating factor (M-CSF), which in the presence of RANK-L induces osteoclastogene sis[78-80]. GCs have direct effects on osteoclasts also by suppressing the expression of an autocrine cytokine, such as interferon I , that normally exerts inhibitory effects on osteoclastogenesis[80]. Also they inhibit the function of mature osteoblasts and suppress the synthesis of insulin-like growth factor- I , an agent that enhances bone formation[78,79].

The wingless-type (Wnt) signaling has emerged as a novel, key pathway for promoting osteoblastogenesis. The Wnt signal transduction comprises three intracellular pathways: the canonical pathway, the Wnt/planar-cell-polarity (PCP) pathway, and the Wnt/Ca^{2+} pathway[81,82]. Wnt signals are extracellularly regulated by several secreted antagonists including secreted frizzled-related protein (sFRP), Cerberus, Wnt inhibitory factor-1 (WIF-1), and dickkopf (Dkk)[83]. Some studies strongly suggest that the canonical pathway plays a central role in promoting bone formation[84-86]. Some groups have reported that glucocorticoid enhances the expression of dickkopf-1 (Dkk-1) in cultured human osteoblasts[87] by suppressesing the canonical Wnt signal[88].

DIAGNOSIS

Diagnosis of osteoporosis in IBD patients

Due to the low absolute risk of fracture remains controversial if all IBD patients should be screened, but it is suggested for avoiding the complications of osteoporosis, especially in patients with a preexisting bone disease, older than 65, and with risk factors for low bone mass as long-term steroid therapy (prednisone 5 mg daily for 6 mo or more)[88-91].

Both, the American College of Gastroenterology (ACG) and American Gastroenterological Association (AGA) issued position papers to offer guidance to the practicing clinician in the diagnosis and management of bone loss in IBD. These position papers recommended the selective screening of IBD patients with dual energy x-ray absorptiometry (DXA) scanning, and the criteria for DXA screening included: postmenopausal state , ongoing corticosteroid treatment, cumulative prior use of corticosteroids exceeding 3 mo, history of low trauma fractures, and age over 60. These criteria led to the detection of osteopenia or osteoporosis and initiation of specific therapies in the majority of patients[92].

Available methods to measure BMD include single energy x-ray absorptiometry, DXA, quantitative computed tomography (QCT), radiographic absorptiometry, and ultrasound. DXA is the establish method to determine BMD, and routinely is measured in the hip and the lumbar spine[93].

The T score was proposed by the World Health Organization (WHO) as the strongest determinant of fracture risk. T score is defined as the number of standard deviations (SD) by which a given BMD measurement exceeds or falls below the normal mean BMD of healthy 30-year-old individuals (peak bone mass). A BMD that is up to 1 SD below the peak bone mass is considered normal; between 1 to 2.49 SD below peak BMD is considered as osteopenic and to have mild to moderate bone deficiency; and $\geqslant$ 2.5 SD below the peak BMD are labeled osteoporotic and with marked bone deficiency. Individuals who have a fracture as a result of bone fragility are considered to have severe osteoporosis[93]. The z score is useful too, and is defined as the number of SDs by which a given BMD measurements exceeds or falls below the mean BMD of healthy individuals of the same age group. For the International Society for Clinical Densitometry (ISCD), z scores are preferred, and the WHO classification should not be applied in women before menopause and in men younger than 50[94].

TREATMENT OPTIONS

Calcium and vitamin D

It is known that calcium and vitamin D are essential in the metabolism of bone and so multiple trials have studied their benefit as treatment of osteoporosis. The use of calcium or/and vitamin D or its analogues have shown, in 2 meta-analysis, 1 Cochrane Review, and in a large placebo-controlled study, to have a small benefit in BMD and a controversial age-dependant trend, and not totally clear reduction of bone fractures, specially of the spine, in postmenopausal women[95-98]. In a randomized, placebo-controlled trial in glucocorticoid-using patients with IBD, the intake of vitamin D 250 IU and calcium 1000 mg/d had no significant benefit in bone density at 1 year of follow-up[99]. So, as described in a recent

consensus report, the supplementation with calcium and vitamin D is accepted as a cost-effective medication, and essential but insufficient, in the prevention and treatment of osteoporosis. The dosage that showed best is calcium 1200 mg/d and vitamin D 800 IU/d. The maximum benefit of calcium and vitamin D will generally be derived from combination therapy with an antiresorptive agent[100].

Bisphosphonates

The group of this antiresorptive analogue of pyrophosphate includes etidronate, pamidronate, tiludronate, alendronate, risedronate, and ibandonate.

Both, alendronate and risedronate, have shown to be effective in increasing BMD and reducing fractures in spine, hip and wrist for the treatment of osteoporosis in postmenopausal women. In a systematic review, meta-analysis and double blind and randomized study, they reduce vertebral fractures by 30% to 50%, with superiority for 70 mg once-weekly alendronate than daily 5 mg or once-weekly 35 mg of risedronate, and with similar tolerability profiles, at 1 or 2 years[101-105].

For the prevention and treatment of glucocorticoid-induced osteoporosis, in a randomized, double-blind, placebo-controlled, multicenter study, in patients receiving a minimum of 7.5 mg prednisone or its equivalent for diverse pathologies, all receiving 800-1000 mg elemental calcium and 250-500 IU of vitamin D, alendronate at a dosage of 5 or 10 mg/d significantly increased bone density compared to placebo at 1 year and reduced the incidence of bone fractures too, at 2 years[106,107].

In patients with moderate to high doses of corticoid therapy, a significant increase of BMD and a reduction of 70% in vertebral fracture risk was observed with risedronate 5 mg/d compared with the placebo group ($P = 0.01$). Risedronate was efficacious, irrespective of underlying disease and duration of corticosteroid therapy, and had a favorable safety profile, with a similar incidence of upper gastrointestinal adverse events to placebo[108,109].

Etidronate have shown to be superior to placebo for increasing BMD in lumbar spine and femoral neck, and reducing incidence of vertebral fractures with no effect in non-vertebral fractures in postmenopausal women[110].

A meta-analysis reported that intermittent cyclical etidronate (400 mg/d for 14 d, followed by 500 mg calcium daily for 76 d) in corticoid treated patients was effective in preventing bone loss, increasing bone mass but with no statistical significance on reduction of fractures[111].

Other bisphosphonate approved for the treatment of osteoporosis in postmenopausal women is the ibandonate in oral dosage of 2.5 mg/d, or intravenous dosage of 2 mg every 2 mo, or 3 mg every 3 mo, had shown to be better than placebo, increasing BMD and reducing bone fractures, with superiority of intravenous regimens[112].

For corticoid-induced osteoporosis, in an open-label, single-center, parallel-group, controlled study, participants received 500 mg/d calcium plus either 3-montly intravenous injections of 2 mg ibandronate or oral 1 mg/d alfacalcidol for 3 years, showing that the increase in BMD was much greater and the fractures were lower in the ibandronate than those in alfacalcidol group[113].

For the treatment of osteoporosis in IBD, bisphosphonates have been evaluated in few studies. In a 12-mo double-blind, randomized, placebo-controlled study of 10-mg daily dose of alendronate, that include 32 patients with CD in remission and without glucocorticoid treatment the BMD of the lumbar spine increased 4.6% ± 1.2% versus a decrease of 0.9% ± 1.0% in the placebo group ($P < 0.01$). BMD of the hip increased 3.3% ± 1.5% *vs* an increase of 0.7% ± 1.1% in the placebo group ($P < 0.08$)[114].

In 31 patients with CD and 30 with UC, in a double-blind placebo-controlled study, all taking 600 mg daily of calcium, after 1 year in the risedronate group the BMD of the spine and hip significantly increase in 2% and 1.9%, respectively[115]. After one year of monthly infusions of 30 mg iv pamidronate plus 500 mg calcium with 400 IU vitamin D in patients with CD, the BMD increased 2.6% (95% CI: 1.4-3.0) at the spine and 1.6% (95% CI: 0.6-2.5) at the hip versus 1.6% (95% CI: 0.1-3.2) at the spine and 0.9% (95% CI: 0.4-2.1) at the hip in the group with vitamin D and calcium supplements[116]. Stokker PC *et al*[117] reported a significant improve in T scores of lumbar spine and hip in 49 patients with IBD that received 30 mg iv pamidronate every 3 mo, plus 1000 mg of calcium and 400 IU of vitamin D daily.

Estrogens

Estrogens alone or with progestin stop progression of bone loss in postmenopausal women, increasing the BMD and reducing the incidence of spine and hip fractures by 34%[118]. Good response in preventing bone loss in patients under glucocorticoid treatment has been observed but the effect on prevention of bone fractures remains unclear, estrogens are not recommended for this purpose[119,120].

Raloxifene, a selective estrogen receptor modulator was approved for the prevention and treatment of postmenopausal spinal osteoporosis. In a meta-analysis of 7 clinical studies, raloxifene reduced the risk of vertebral fractures by 40% with a dose of 60 mg/d[121]. No studies with raloxifene have done yet in IBD patients.

EMERGENT THERAPIES

Calcitonin

Calcitonin intranasal spray, at doses of 200 IU/d plus 1000 mg calcium and 400 IU vitamin D, has been reported to reduce the risk of spine fractures by 33% in a 5-year follow-up time in postmenopausal women[122].

The efficacy of calcitonin for fracture prevention in steroid-induced osteoporosis remains to be established[123,124]. No studies have done for IBD-associated osteoporosis.

Teriparatide

The genetically engineered fragment of human parathyroid hormone, Teriparatide, stimulates new bone formation, leading to increased BMD. Teriparatide, at 20 and 40 micrograms daily subcutaneous injection, reduced the risk of vertebral and non-vertebral fractures in postmenopausal women[125]. It's also approved for FDA to increase bone mass in men with primary or hypogonadal osteoporosis[126].

The efficacy of teriparatide in preventing of treating glucocorticoid-induced or IBD-associated osteoporosis remains to be assessed. Hodsman AB[127] suggests that should be considered as treatment for patients with established glucocorticiod-induced osteoporosis who require long-term steroid treatment.

CONCLUSION

IBD has been associated with an increased risk of osteoporosis and osteopenia and epidemiologic studies have reported an increased prevalence of low bone mass in patients with IBD. While genetics play important role, there are other factors in the pathogenesis that play an important interaction and together with environmental influence lead to the an intriguing multifactorial pathogenesis that still has gaps to be fulfilled. Through the knowledge and understanding of basic aspects of bone disease in an autoimmune gastrointestinal scenario we can find leads to a better clinical performance and to bear new diagnostic techniques and breakthrough therapies for a better outcome in IBD patients.

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S- Editor Liu Y **L- Editor** Alpini GD **E- Editor** Lu W

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World J Gastroenterol 2007 December 14; 13(46): 6166-6171
World Journal of Gastroenterology ISSN 1007-9327

GASTRIC CANCER

Re-expression of methylation-induced tumor suppressor gene silencing is associated with the state of histone modification in gastric cancer cell lines

Chun-Feng Meng, Xin-Jiang Zhu, Guo Peng, Dong-Qiu Dai

Chun-Feng Meng, Xin-Jiang Zhu, Guo Peng, Dong-Qiu Dai, Department of Surgical Oncology, The First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China
Supported by National Natural Science Foundation of China, No. 30271477, No. 30572162
Correspondence to: Dr. Dong-Qiu Dai, Department of Surgical Oncology, The First Affiliated Hospital, China Medical University, No.155, Nanjing North Street, Heping District, Shenyang 110001, Liaoning Province, China. daidq63@163.com
Telephone: +86-24-83283560 Fax: +86-24-22703576
Received: July 18, 2007 Revised: September 15, 2007

Abstract

AIM: To identify the relationship between DNA hypermethylation and histone modification at a hypermethylated, silenced tumor suppressor gene promoter in human gastric cancer cell lines and to elucidate whether alteration of DNA methylation could affect histone modification.

METHODS: We used chromatin immunoprecipitation (ChIP) assay to assess the status of histone acetylation and methylation in promoter regions of the *p16* and *mutL homolog 1 (MLH1)* genes in 2 gastric cancer cell lines, SGC-7901 and MGC-803. We used methylation-specific PCR (MSP) to evaluate the effect of 5-Aza-2′-deoxycytidine (5-Aza-dC), trichostatin A (TSA) or their combination treatment on DNA methylation status. We used RT-PCR to determine whether alterations of histone modification status after 5-Aza-dC and TSA treatment are reflected in gene expression.

RESULTS: For the *p16* and *MLH1* genes in two cell lines, silenced loci associated with DNA hypermethylation were characterized by histone H3-K9 hypoacetylation and hypermethylation and histone H3-K4 hypomethylation. Treatment with TSA resulted in moderately increased histone H3-K9 acetylation at the silenced loci with no effect on histone H3-K9 methylation and minimal effects on gene expression. In contrast, treatment with 5-Aza-dC rapidly reduced histone H3-K9 methylation at the silenced loci and resulted in reactivation of the two genes. Combined treatment with 5-Aza-dC and TSA was synergistic in reactivating gene expression at the loci showing DNA hypermethylation. Similarly, histone H3-K4 methylation was not affected after TSA treatment, and increased moderately at the silenced loci after 5-Aza-dC treatment.

CONCLUSION: Hypermethylation of DNA in promoter CpG islands is related to transcriptional silencing of tumor suppressor genes. Histone H3-K9 methylation in different regions of the promoters studied correlates with DNA methylation status of each gene in gastric cancer cells. However, histone H3-K9 acetylation and H3-K4 methylation inversely correlate with DNA methylation status of each gene in gastric cancer cells. Alteration of DNA methylation affects histone modification.

Key words: Gastric cancer; DNA hypermethylation; Histone methylation; Histone acetylation; *p16*; *mutL homolog 1*; 5-Aza-2′-deoxycytidine; Trichostatin A

Meng CF, Zhu XJ, Peng G, Dai DQ. Re-expression of methylation-induced tumor suppressor gene silencing is associated with the state of histone modification in gastric cancer cell lines. *World J Gastroenterol* 2007; 13(46): 6166-6171

http://www.wjgnet.com/1007-9327/13/6166.asp

INTRODUCTION

Multiple recent reviews have shown that virtually all human cancer types have epigenetic abnormalities that collaborate with genetic changes to drive cancer development and progression[1-7]. Hypermethylation of DNA in promoter CpG islands of tumor suppressor genes (TSGs) is known to inhibit transcriptional initiation and cause permanent silencing of the genes, which play a crucial role in carcinogenesis[1,2]. It was reported that hypermethylation of DNA in promoter CpG islands and diminished expression are present in a number of tumor-related genes in gastric cancer, which is one of the major current causes for cancer death in Asian countries[8]. For example, silencing of the cyclin-dependent kinase inhibitor *p16* gene induced by hypermethylation can lead to disruption of cell cycle regulation and provide a growth advantage to affected cells[9]. A mismatch repair

gene, *MLH1*, is often silenced with aberrant CpG island hypermethylation in gastric cancers[10,11]. Except for DNA methylation, recent studies have demonstrated the importance of histone modification as another epigenetic mechanism in the organization of chromosomal domains and gene regulation[12-18]. Acetylation of H3-K9 and methylation of H3-K4 are associated with open chromatin configurations such as that found at transcriptionally active promoters. In contrast, methylation of H3-K9 is a marker of condensed, inactive chromatin of the sort associated with the inactive X-chromosome and pericentromeric heterochromatin[16,17,19,20].

It has also been shown that histone modification is crucial to the process of DNA methylation in some organisms and abrogation of H3-K9 methylation in Neurospora results in loss of DNA methylation[21]. It was reported that histone H3-K9 methylation directly correlates with DNA methylation of some tumor suppressor genes, while histone H3-K9 acetylation and histone H3-K4 methylation inversely correlate with DNA methylation of some tumor suppressor genes[22]. These data suggest a functional linkage between DNA methylation and histone modifications in gene repression. To better understand the relationship between DNA methylation and histone modification in cancer-associated gene silencing, we performed ChIP assay to assess the methylation and acetylation of H3-K9 and the methylation of H3-K4 at the *p16* and *MLH1* genes in two gastric cancer cell lines. We also treated the gastric cancer cell lines with the DNA methylation inhibitor, 5-Aza-dC, and the histone deacetylase inhibitor, TSA, to elucidate whether alteration of DNA methylation affects histone modification.

MATERIALS AND METHODS

Cell lines and culture conditions

Two cell lines derived from human gastric cancer, SGC-7901 and MGC-803, were cultured in RPMI 1640 supplemented with 10% fetal bovine serum(Gibco), penicillin (100 IU/mL) and streptomycin (100 μg/mL), and incubated in a humidified incubator containing 50 mL/L CO_2 at 37℃.

Treatment with 5-Aza-dC and TSA

TSA and 5-Aza-dC were purchased from Sigma. TSA was dissolved in absolute ethanol at a stock concentration of 3.3 mmol/L and stored at -80℃. 5-Aza-dC was dissolved in water at a stock concentration of 1 mmol/L and stored at -80℃. Cells were seeded at a low density in a 100 mm tissue culture dish and incubated for 24 h prior to treatment with 5-Aza-dC and TSA. 5-Aza-dC (5 μmol/L) was used for 72 h in the treatment. Culture medium containing 5-Aza-dC was exchanged every 24 h. TSA (300 nmol/L) was used for only 24 h in the treatment. 5-Aza-dC was used for 48 h followed by TSA for an additional 24 h in the combined treatment. Mock-treatment with an identical volume of absolute ethanol or water was used as a control.

Methylation-specific PCR

The genomic DNA was modified by bisulfite treatment, as described previously[23]. DNA was purified using a Wizard DNA clean-up system (Promega), precipitated with ethanol, and resuspended in 30 μL of Tris-EDTA buffer. Two microliters of the aliquot was used as a template. The primers used for MSP and additional PCR conditions are described elsewhere[22]. PCR products were separated by electrophoresis on 2% agarose gels and quantitated with the FluorChem 2.0 system. The experiment was repeated three times.

RT-PCR analysis of p16 and MLH1 expression

Total cellular RNA was extracted from each of the two cell lines with TriZOL (Invitrogen) according to the manufacturer's protocol. RNA was resuspended in nuclease-free water and quantitated with a spectrophotometer. Reverse transcription (RT) reactions were done on 2 μg of total RNA following the manufacturer's protocol (Promega). cDNA was amplified by PCR using primers as described previously. Reaction conditions for each PCR are described elsewhere[24]. PCR products were resolved on 2% agarose gels and quantitated using the Fluor Chem 2.0 system. The level was determined by quantifying the intensities of the PCR product versus *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). The experiment was repeated three times.

Chromatin immunoprecipitation assay

ChIP assays were performed as described previously with some modifications[24]. Briefly, proteins were cross-linked to DNA by adding formaldehyde directly into the culture medium to a final concentration of 4 g/L for 20 min at 37℃. After washing, the cell pellets were resuspended in 500 μL lysis buffer and sonicated thirty-five times, 2 s each. The average fragment size after sonication was 500 bp. The lysate (500 μL) was then divided into three fractions. The first and second fractions (200 μL each) were diluted in 1800 μL of lysis buffer, and the third fraction (100 μL) was used as an input control. The first lysate was incubated overnight at 4℃ with 5 μL anti-Lys-9 acetylated histone H3 antibody, 5 μL anti-Lys-9 dimethylated histone H3 antibody, or 5 μL anti-Lys-4 dimethylated histone H3 antibody (all antibodies from Upstate Biotechnology) overnight at 4℃. The second lysate was incubated with Tris-EDTA buffer (5 μL) as a negative control. Immune complexes were collected with 20 μL protein A-sepharose beads for 1 h at 4℃ with agitation. The cross-links were reversed by heating the sample at 65℃ for 5 h. After elution, the samples were digested with proteinase K. DNA was recovered by phenol extraction, precipitated with ethanol, and resuspended in Tris-EDTA buffer.

PCR analysis of immunoprecipitated DNA

Amplification was carried out with 2 μL of an immunoprecipitated DNA, a control without antibody or a 1:10 dilution of input DNA that was not immunoprecipitated. The primers used for ChIP and PCR conditions are described elsewhere[22]. We selected *P16-3, P16-6, MLH1-2* and *MLH1-3*. PCR products were electrophoresed on 2% agarose gels and quantitated with the FluorChem 2.0 system. The level of histone acetylation and methylation in each immunoprecipitation was measured by quantifying the intensities of the PCR product in

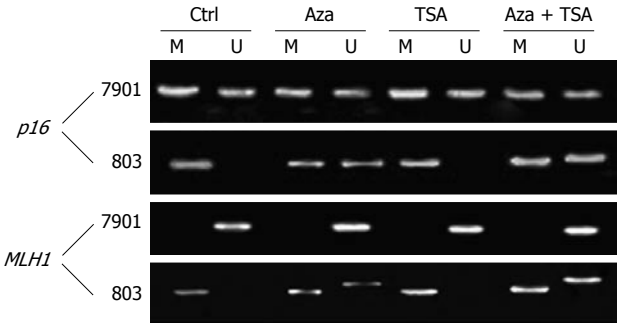

Figure 1 MSP analysis for promoter regions of gastric cancer cells after treatment with 5-Aza-dC, TSA or their combination. M: Methylated alleles; U: Unmethylated alleles; Ctrl: No treatment.

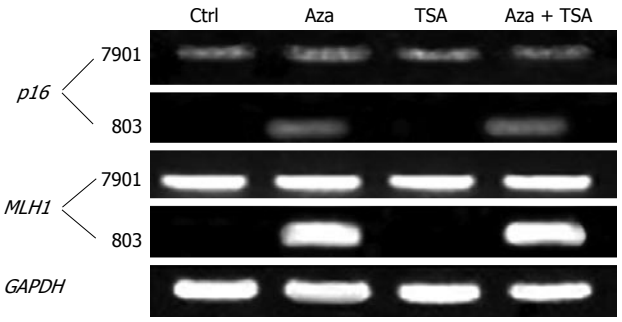

Figure 2 Expression and reactivation of *p16* and *MLH1* in gastric cancer cells after treatment with 5-Aza-dC, TSA or their combination. Ctrl: No treatment.

immunoprecipitated DNA versus input DNA diluted at 1:10 (total chromatin). The experiment was repeated three times.

RESULTS

MSP analysis for each promoter region after treatment with 5-Aza-dC, TSA or their combination

The two cell lines showed a characteristic DNA methylation status in each promoter region. As shown in Figure 1, *p16* was hypermethylated (both alleles methylated) in MGC-803 and partially methylated (only one allele methylated) in SGC-7901. *MLH1* was hypermethylated in MGC-803 but not methylated in SGC-7901.

5-Aza-dC and combined 5-Aza-dC and TSA resulted in demethylation of *p16* and *MLH1* in MGC-803, in which the silenced gene was associated with DNA hypermethylation. In contrast, TSA alone did not affect the DNA methylation status of *p16* and *MLH1*.

RT-PCR analysis for expression and reactivation of p16 and MLH1 after treatment with 5-Aza-dC, TSA or their combination

As shown in Figure 2, *p16* was expressed in SGC-7901 and minimally affected by TSA. *p16* was silenced in MGC-803 and TSA was not able to activate gene expression. In contrast, 5-Aza-dC alone reactivated expression of the *p16* in MGC-803. Similar results were obtained in *MLH1*, which was expressed in SGC-7901 but silenced in MGC-803. TSA had no effect on gene expression, while 5-Aza-dC reactivated the silenced gene. The combined

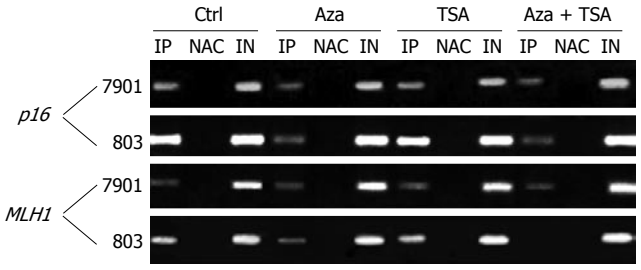

Figure 3 Status of histone H3-K9 methylation across TSGs and change in gastric cancer cells after treatment with 5-Aza-dC, TSA or their combination. Ctrl: No treatment; IP: Immunoprecipitated DNA; NAC: No-antibody control; IN: Input DNA from whole-cell lysate.

treatment with 5-Aza-dC and TSA increased gene expression.

ChIP assay for histone H3-K9 methylation across the promoter of TSG and change after treatment with 5-Aza-dC, TSA or their combination

The results of ChIP studies were almost identical in different regions of each promoter, and the values for each gene were averaged to present the data. In the promoter region of the *p16* gene, H3-K9 methylation was higher for MGC-803 than for SGC-7901. Similar results were seen at *MLH1*. SGC-7901 having no promoter DNA methylation at this locus, showed a low degree of H3-K9 methylation. MGC-803 had a higher degree of H3-K9 methylation across the promoter (Figures 3 and 4).

TSA alone had no effect on H3-K9 methylation, irrespective of DNA methylation status. In contrast, 5-Aza-dC had effects on H3-K9 methylation at the silenced loci, reducing histone H3-K9 methylation in the promoter showing partial methylation or hypermethylation (the promoter region of *p16* in both cell lines and the promoter region of *MLH1* in MGC-803). The combination of 5-Aza-dC and TSA had similar effects on histone H3-K9 methylation.

ChIP assay for histone H3-K9 acetylation across the promoter of TSG and change after treatment with 5-Aza-dC, TSA or their combination

The promoter region of the *p16* gene showed a higher degree of H3-K9 acetylation in SGC-7901 (partially methylated) than in MGC-803 (hypermethylated). Similar results were seen in the *MLH1* gene showing a low degree of H3-K9 acetylation in all parts of the promoter region in MGC-803. In contrast, a higher degree of H3-K9 acetylation was detected in SGC-7901 (both alleles non-methylated) at all *MLH1* regions studied (Figures 4 and 5A).

For the *p16* gene, treatment with TSA alone had no effect on H3-K9 acetylation in the SGC-7901 (partial DNA methylation) but slightly increased H3-K9 acetylation in the silenced MGC-803. Identical results were seen in *MLH1*. 5-Aza-dC increased H3-K9 acetylation at the loci with DNA hypermethylation (*p16* and *MLH1* in MGC-803) but had no effect on the loci with partial or no DNA methylation (*p16* and *MLH1* in BGC-7901). However, the combination of 5-Aza-dC and TSA increased H3-K9

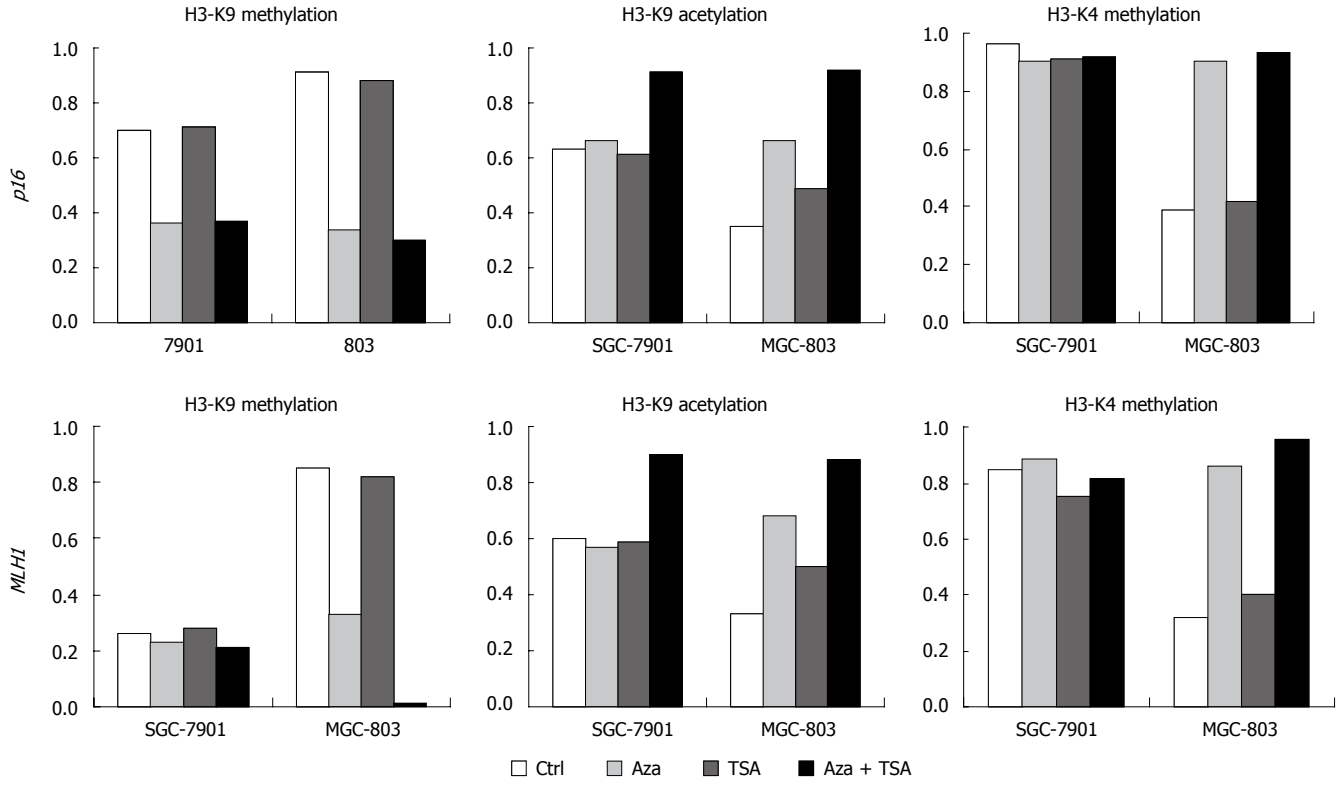

Figure 4 Summary of quantitative analysis for ChIP assays. Ratios of precipitated DNA over input DNA were used to calculate the relatively precipitated fold enrichment on the y axis.

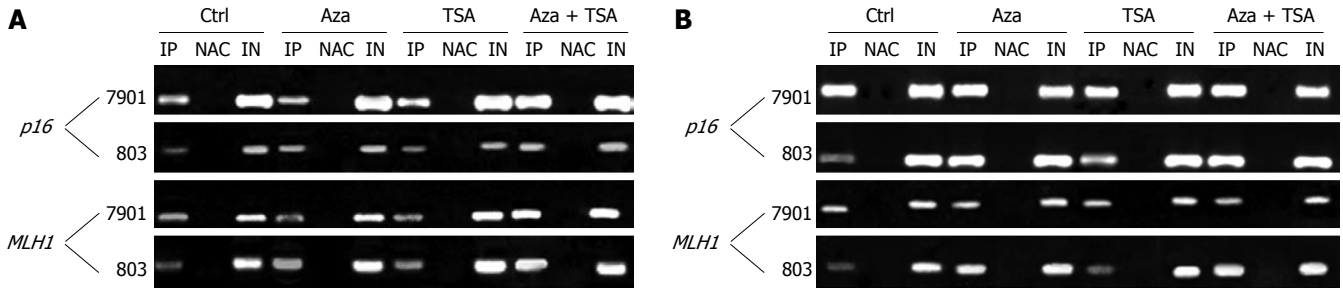

Figure 5 Status of histone H3-K9 acetylation (**A**) and histone H3-K4 methylation (**B**) across TSGs and change in gastric cancer cells after treatment with 5-Aza-dC, TSA or their combination. Ctrl: No treatment; IP: Immunoprecipitated DNA; NAC: No-antibody control; IN: Input DNA from whole-cell lysate.

acetylation effectively at all loci irrespective of DNA methylation status. The results of ChIP studies were almost identical in different regions of each promoter.

ChIP assay for histone H3-K4 methylation across the promoter of TSG and change after treatment with 5-Aza-dC, TSA or their combination

For the *p16* and *MLH1* genes, H3-K4 methylation was higher in SGC-7901 than in MGC-803. TSA did not affect H3-K4 methylation. However, 5-Aza-dC, or the combination of 5-Aza-dC and TSA, increased H3-K4 methylation at all silenced loci in MGC-803. Little change in H3-K4 methylation was observed in SGC-7901, where only partial or no methylation was observed (Figures 4 and 5B).

DISCUSSION

Silencing of tumor suppressor genes is related to epigenetic regulation of both DNA methylation and histone modification[25]. In the present study, hypermethylation in promoter CpG islands was significantly associated with *p16* and *MLH1* silencing. Furthermore, aberrantly silenced and DNA hypermethylated genes in gastric cancer cells were characterized by histone H3-K9 hypermethylation, H3-K4 hypomethylation and H3-K9 hypoacetylation.

DNA methylation and histone modification may act synergistically or antagonistically on gene expression[26,27]. We carried out ChIP assays to explore the relationship between DNA methylation and histone modifications. ChIP is a powerful technique to test for the presence of certain DNA-binding proteins that might modulate chromatin structure and/or transcriptional characteristics of the specific region of DNA with which they are associated. We made use of polyclonal antibodies generated against methylated and acetylated histone H3, all of which are proteins linked to chromatin modification

and regulation of transcription. In colorectal cancer, histone H3-K9 methylation directly correlates and histone H3-K4 methylation inversely correlates with DNA methylation of *p16, MLH1* and the O6-methylguanine-DNA methyltransferase gene, *MGMT*[22]. We demonstrated that histone H3-K9 methylation correlated, and histone H3-K9 acetylation and H3-K4 methylation inversely correlated very well with DNA methylation of *p16, MLH1* in SGC-7901 and MGC-803.

To further explore the relationship between DNA methylation and histone modification, we treated cancer cells with 5-Aza-dC and TSA. 5-Aza-dC, a DNA methyltransferase inhibitor, is sufficient to cause demethylation of the promoter region and reactivate expression of the hypermethylated, silenced gene[28,29]. TSA, a specific histone deacetylase (HDAC) inhibitor, has been permitted evaluation of the role of HDAC in silencing a variety of methylated genes[14]. It was reported that re-expression of DNA hypermethylated and silenced cancer genes can be induced through 5-Aza-dC-induced DNA demethylation, demethylated genes and the active marks, acetylated H3-K9 and methylated H3-K4 can be detected in HCT116 and DKO colon cancer[29-34]. However, one silencing mark, dimethylated H3-K9, is strikingly decreased[34]. In our study, when the CpG islands were hypermethylated, TSA increased histone acetylation, but had almost no effect on gene expression. In contrast, 5-Aza-dC reactivated expression of hypermethylation-induced silenced genes. Our findings on histone acetylation are consistent with previous reports linking the effect of DNA methylation and histone deacetylation on transcriptional silencing, demonstrating that DNA methylation is dominant over histone deacetylation in maintaining a silent state at hypermethylated promoters[22]. Furthermore, TSA and 5-Aza-dC play a different role in histone methylation. In the present study, 5-Aza-dC, but not TSA, could reactivate expression of the silenced genes and completely reverse key histone methylations surrounding the gene promoter, indicating that reactivation of silenced genes correlates much better with decreased histone H3-K9 methylation and increased H3-K4 methylation than with increased H3-K9 acetylation. We speculate that histone methylation plays a critical role in the maintenance of promoter DNA methylation-associated gene silencing in gastric cancer.

After 5-Aza-dC treatment, we observed a complete reversal of histone modification at the *p16* and *MLH1* promoter in MGC-803 cells. Acetylated H3-K9 and methyl-H3-K4 levels were increased, whereas methyl-H3-K9 levels decreased, suggesting that DNA hypermethylation may be essential for maintaining histone modification at gene promoters silenced due to aberrant DNA hypermethylation. DNA methylation plays a direct role in both genes silencing and maintaining a repressive histone modification at a hypermethylated gene promoter in cancer. Data show that DNMT1 interacts with HDAC activity in complexes bound to DNA, suggesting that it can recruit histone modifiers to DNA[33-35]. It was reported that DNA modification itself, or components of the DNA methylating machinery such as DNMTs or methyl-CpG binding proteins, can directly interact with histone methyltransferases or proteins in regions containing DNA methylation and allow them to set up an alterative histone modification[2], showing that histone methylation depends on DNA methylation.

COMMENTS

Background

Gastric cancer is a malignant tumor threatening human health worldwide. It has been shown that epigenetic mechanism plays an important role in the occurrence and development of gastric cancer. However, the role of histone modification and the relationship between DNA hypermethylation and histone modification at a hypermethylated, silenced tumor suppressor gene promoter in human gastric cancer remain unclear.

Research frontiers

Histone modification plays a prominent role in the epigenetic regulation of gene transcription. There is evidence that dysregulation of epigenetic process causes transcriptional repression of a subset of genes, contributing to the pathogenesis of many cancers. The relationship between aberrant epigenetic changes and tumorigenesis is still to be identified.

Innovations and breakthroughs

In some cancers, histone H3-K9 methylation directly correlates and histone H3-K4 methylation inversely correlates with DNA methylation of some TSGs. Our findings may reflect the mechanism underlying inactivation of tumor suppressor genes by DNA hypermethylation and histone modification during gastric tumorigenesis. DNA methylation may affect histone modification in human gastric cancer.

Applications

Reactivation of the *p16* and *MLH1* gene by DNA methyltransferase inhibitor alone or in combination with histone deacetylase inhibitor suggests that the two agents can be used in treatment of gastric cancer.

Terminology

Histone is the major component of chromatin functioning as a DNA packaging unit and as a transcriptional regulator. The amino-terminal tails of histones protrude from the nucleosome and are subjected to chemical modifications including phosphorylation, acetylation and methylation. These modifications of histones affect the access of regulatory factors and complexes to chromatin and influence gene expression. These different combinations of histone modifications at different residues may act synergistically or antagonistically on gene expression.

Peer review

This is an important and interesting study, and the manuscript is well-written. The major finding in this paper is that treatment with 5-Aza-dC, a DNA methylation inhibitor and TSA, a histone deacetylase inhibitor, affects the expression of tumor suppressor genes, including *p16* and *MLH1*, by reversing the hypermethylation and inhibiting histone deacetylation in human gastric cancer cells.

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S- Editor Zhu LH **L- Editor** Wang XL **E- Editor** Ma WH

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World J Gastroenterol 2007 December 14; 13(46): 6172-6182
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Protective effects of erythropoietin against acute lung injury in a rat model of acute necrotizing pancreatitis

Oge Tascilar, Güldeniz Karadeniz Cakmak, Ishak Ozel Tekin, Ali Ugur Emre, Bulent Hamdi Ucan, Burak Bahadir, Serefden Acikgoz, Oktay Irkorucu, Kemal Karakaya, Hakan Balbaloglu, Gürkan Kertis, Handan Ankarali, Mustafa Comert

Oge Tascilar, Güldeniz Karadeniz Cakmak, Ali Ugur Emre, Bulent Hamdi Ucan, Oktay Irkorucu, Kemal Karakaya, Hakan Balbaloglu, Mustafa Comert, Department of Surgery, Zonguldak Karaelmas University, School of Medicine, Kozlu-Zonguldak 67600, Turkey
Ishak Ozel Tekin, Department of Immunology, Zonguldak Karaelmas University, School of Medicine, Kozlu-Zonguldak 67600, Turkey
Burak Bahadir, Gürkan Kertis, Department of Pathology, Zonguldak Karaelmas University, The School of Medicine, Kozlu-Zonguldak 67600, Turkey
Serefden Acikgoz, Department of Biochemistry, Zonguldak Karaelmas University, School of Medicine, Kozlu-Zonguldak 67600, Turkey
Handan Ankarali, Department of Biostatistics, Zonguldak Karaelmas University, School of Medicine, Kozlu-Zonguldak 67600, Turkey
Correspondence to: Dr. Güldeniz Karadeniz Cakmak, Zonguldak Karaelmas Universitesi, Arastirma ve Uygulama Hastanesi Bashekimligi, Kozlu-Zonguldak 67600, Turkey. gkkaradeniz@yahoo.com
Telephone: +90-372-2610169 Fax: +90-372-2610155
Received: August 1, 2007 Revised: September 16, 2007

Abstract

AIM: To investigate the effect of exogenous erythropoietin (EPO) administration on acute lung injury (ALI) in an experimental model of sodium taurodeoxycholate-induced acute necrotizing pancreatitis (ANP).

METHODS: Forty-seven male Wistar albino rats were randomly divided into 7 groups: sham group (n = 5), 3 ANP groups (n = 7 each) and 3 EPO groups (n = 7 each). ANP was induced by retrograde infusion of 5% sodium taurodeoxycholate into the common bile duct. Rats in EPO groups received 1000 U/kg intramuscular EPO immediately after induction of ANP. Rats in ANP groups were given 1 mL normal saline instead. All animals were sacrificed at postoperative 24 h, 48 h and 72 h. Serum amilase, IL-2, IL-6 and lung tissue malondialdehyde (MDA) were measured. Pleural effusion volume and lung/body weight (LW/BW) ratios were calculated. Tissue levels of TNF-α, IL-2 and IL-6 were screened immunohistochemically. Additionally, ox-LDL accumulation was assessed with immune-fluorescent staining. Histopathological alterations in the lungs were also scored.

RESULTS: The mean pleural effusion volume, calculated LW/BW ratio, serum IL-6 and lung tissue MDA levels were significantly lower in EPO groups than in ANP groups. No statistically significant difference was observed in either serum or tissue values of IL-2 among the groups. The level of tumor necrosis factor-α (TNF-α) and IL-6 and accumulation of ox-LDL were evident in the lung tissues of ANP groups when compared to EPO groups, particularly at 72 h. Histopathological evaluation confirmed the improvement in lung injury parameters after exogenous EPO administration, particularly at 48 h and 72 h.

CONCLUSION: EPO administration leads to a significant decrease in ALI parameters by inhibiting polymorphonuclear leukocyte (PMNL) accumulation, decreasing the levels of proinflammatory cytokines in circulation, preserving microvascular endothelial cell integrity and reducing oxidative stress-associated lipid peroxidation and therefore, can be regarded as a cytoprotective agent in ANP-induced ALI.

Key words: Erythropoietin; Acute pancreatitis; Acute lung injury; Acute respiratory distress syndrome; Cytokine

Tascilar O, Cakmak GK, Tekin IO, Emre AU, Ucan BH, Bahadır B, Acikgoz S, Irkorucu O, Karakaya K, Balbaloglu H, Kertis G, Ankarali H, Comert M. Protective effects of erythropoietin against acute lung injury in a rat model of acute necrotizing pancreatitis. *World J Gastroenterol* 2007; 13(46): 6172-6182

http://www.wjgnet.com/1007-9327/13/6172.asp

INTRODUCTION

Acute pancreatitis (AP) is a life-threatening necro-inflammatory disease with significant morbidity and mortality rates, especially when complicated by systemic inflammatory response syndrome (SIRS) and multiple organ failure (MODS)[1,2]. Death occurs in 60% of the patients within the first 6 d of disease onset and pulmonary complications including acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)

account for a significant number of these deaths[3]. The exact mechanisms by which diverse etiological factors induce an attack are indefinite, but once the disease process is initiated, common inflammatory and repair pathways are invoked. Within the first few days following the onset of AP, lung injury occurs as a consequence of AP, whereas sepsis is a dominant cause for lung injury and mortality in the later phase of the disease process[4]. Despite improved understanding of the pathogenesis of ARDS, pharmacological modalities are ineffective in decreasing its mortality. None of the randomized clinical trials using novel therapeutic agents has demonstrated an improvement in patient outcome. Consequently, effective therapeutic interventions are thus called for.

Erythropoietin (EPO), a 30.4-kDa glycoprotein and a member of the type I cytokine superfamily, was first introduced as a hormone that regulates erythroid progenitors within the bone marrow to mature into erythrocytes, through binding to its specific cell surface receptors[5]. Hence, EPO is approved for the treatment of anemia as a consequence of a variety of disorders. In the current era, the premise that EPO is essential only for erythropoiesis has been changed according to the researches demonstrating the existence of EPO and its receptor in other organs and tissues outside of liver and kidney, such as brain, heart, pancreas, as well as vascular, gastrointestinal and reproductive systems[6,7]. Beyond its hematopoietic properties, EPO modulates a broad array of vital cellular processes including progenitor stem cell development, cellular integrity, and angiogenesis[8,9]. Additionally, in various tissues, EPO inhibits the apoptotic mechanisms of injury, including preservation of cellular membrane asymmetry to prevent inflammation[10-12]. Experimental evidence supports a vigorous cytoprotective effect and EPO is now considered to have applicability in a variety of disorders, such as cerebral ischemia, myocardial infarction, and chronic congestive heart failure[12-15]. Wu *et al*[16] demonstrated that pretreatment with EPO appears to attenuate ischemia-reperfusion-induced lung injury. This function is partly related with the ability of EPO to inhibit the accumulation of polymorphonuclear leukocytes (PMNL) in lung tissue and decrease the systematic expression of tumor necrosis factor-α (TNF-α). In addition to these studies, it has been reported that EPO can attenuate different kinds of lung injuries, showing that rats exposed to hyperoxia exhibit well-maintained alveolar structure and enhanced vascularity when treated with EPO[17]. Importantly, EPO can protect the ultrastructure of tracheobronchial epithelia and pulmonary type Ⅱ epithelia of rats during traumatic brain injury[18,19].

AP associated lung injury is a multifactorial phenomenon with various phases. In the light of the above-mentioned findings, the present study was to evaluate the hypothesis that EPO administration offers pulmonary protective effect against pancreatitis induced lung injury in rats.

MATERIALS AND METHODS

Animals

Forty-seven male Wistar albino rats weighing 250-300 g were housed under constant temperature (22℃) and humidity in a 12-h dark/light cycle.

Experimental design

The experiments were conducted following the Ethic Committee Faculty of Medicine, University of Zonguldak Karaelmas guiding principles for the care and use of laboratory animals. The animals were randomized into seven experimental groups as follows: sham group in which rats received sham operation (n = 5), 3 ANP groups in which acute necrotizing pancreatitis (ANP) was induced by retrograde infusion of sodium taurodeoxycholate and 1 mL normal saline (0.9% NaCl) was given intramuscularly immediately after induction of AP (n = 7 each), 3 EPO groups in which AP was induced by the same way and 1000 U/kg EPO (Eprex, Epoetin alfa, Janssen-Cilag AG, Sweden) was injected intramuscularly immediately after induction of AP. All animals in the ANP and EPO groups were sacrificed at postoperative 24 h, 48 h and 72 h, respectively. Histopathological, biochemical and immunohistochemical evaluations were performed.

Induction of acute pancreatitis

Anesthesia was induce by injecting ketamine HCL at 100 mg/kg im and laparotomy was performed under strict sterile conditions. An upper midline abdominal incision was made to identify the common pancreaticobiliary duct. The duodenal wall was punctured at its antimesenteric aspect with a 24-gauge Ⅳ catheter (Novacath, Medipro A.Ş., Istanbul, Turkey). The catheter was advanced 5 mm into the common duct through the papilla of Vater. ANP was induced by retrograde infusion of 0.2 mL 5% sodium taurodeoxycholate (Sigma, St.Louis, MO, USA) over 3 min using an infusion pump as previously described[20] and the pacreaticobiliary duct was clamped near the liver hilum throughout the inraductal infusion in all groups, except for sham group. Animals in sham group were subjected to anesthesia, laparotomy and duodenal manipulation, but not to biliopancreatic duct cannulation. The midline incision was closed in two layers with 4/0 silk suture (Ethicon, Edinburg, UK). Rats were allowed to recover from anesthetic and returned to their cages with free access to water and food after surgery.

Sampling procedures

All the rats were sacrificed by aortic puncture method. The abdominal and thoracic cavities were entered to obtain blood and lung samples. Blood samples were centrifuged at 1800 × g for 15 min at 4℃ to obtain plasma and stored at -80℃ for biochemical analysis. Then, the rats were killed with the lung removed immediately. Random cross-sections of the lung tissue were fixed in 10% neutral phosphate-buffered formalin and embedded in paraffin wax for histopathological examination. Samples of lung tissue were weighed and stored at -85℃ for subsequent biochemical and immunohistochemical measurements.

Assessment of pulmonary effusion

The thorax was opened to collect pleural effusion (PE) by suction which was measured volumetrically. Care was also taken to eliminate blood contamination with PE. The

lungs were then removed and all surrounding tissues were dissected and weighed with an analytical balance. The volume of PE (mL) and the lung weight/body weight (LW/BW) ratios were calculated and considered as an index of pulmonary edema.

Biochemical analysis

Serum amylase, IL-2 and IL-6 assay: Serum amylase levels were measured by a Beckman Coulter LX-20® system analyzer (Fullerton, CA, USA) using Beckman kits (Fullerton, CA, USA), following the manufacturer's instructions. IL-2 and IL-6 levels in the serum were measured with commercially available kits (Biosource International, Commercial ELISA Kit, California, USA).

Lung tissue malondialdehyde (MDA) assay: MDA levels in the lung tissue were measured in tissue homogenate. In brief, tissue was homogenized with cold 1.15% KCl to make a 10% homogenates, and 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 with NaOH and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid were added to 0.2 mL of 10% tissue homogenates. The mixture was made up to 4.0 mL with distilled water and heated in an oil bath at 95℃ for 60 min. After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1, v/v) were added and the solution was shaken vigorously. After centrifugation at 4000 r/min for 10 min, the organic layer was taken with its absorbance measured at 532 nm on a Shimadzu UV 1601 spectrophotometer. As a standard, 1.1.3.3 tetraetoxypropane was used. MDA concentration per gram tissue was calculated (nmol/gr tissue).

Immunohistochemical method for screening IL-2, IL-6 and TNF-α in the lung tissue: Cryostat sections of lung tissue (7 μm) were fixed with absolute ethanol and stained with avidin biotin complex based immunohistochemical method. Immunohistochemistry was performed to observe peroxidase diaminobenzidine reaction. Cytokine staining was performed with biotinylated mouse anti-rat IL-2, IL-6, TNF-α antibodies (Biosource International, California, USA). Streptavidin-peroxidase (HRP) and diaminobenzidine (DAB) were purchased from DAKOCytomation (Denmark). Ethanol-fixed tissue sections were treated with biotinylated mouse anti-rat IL-2, IL-6 and TNF-α for 30 min, washed three times with PBS, incubated for an additional 30 min with streptavidin-HRP and washed three times with PBS. The sections were then treated with 0.03% 3, 3-diamino benzidine tetrahydrochloride plus 0.01% hydrogen peroxide in 50 mmol/L Tris-HCl buffer (pH 7.4) for 10 min. All incubations were performed at room temperature. The sections were examined under a light microscope by an independent observer, blind to the study.

Immune-fluorescent staining method for screening ox-LDL in the pancreas and lung tissues: Rat lung and pancreas were obtained and stored at -85℃. Slides were prepared from the 7 μm-thick frozen lung biopsy sections. Slides were further divided into two pieces: one for the test and the other for the negative control. Thirty μL human polyclonal anti-ox-LDL IgG solution as primary antibody was added only on the test slides and the control slides were manipulated with phosphate- buffered solution (PBS) as the same amount of primary antibody. After a 30-min incubation in a humid chamber at room temperature, both the control and test slides were washed with phosphate-buffered saline and 30 μL fluorescent isothiocyanate (FITC)-labeled anti human IgG was administered as a conjugate substance. The slides were incubated for a further 30 min at room temperature and washed with the standard PBS solution. After drying, the slides were covered with a mounting medium and examined under a fluorescent microscope (LEICA DMRX, Germany).

Histopathologic analysis

The lung tissue samples were fixed in 10% formalin immediately after removal, embedded in paraffin, sectioned at 5 μm intervals, stained with hematoxylin and eosin, and examined under a light microscope. Histopathological evaluation and scoring of the parameters were performed by a single pathologist unaware of the treatment groups. Morphometric analysis of histological sections was accomplished with the point counting technique. For this purpose, we used an optical microscope provided with an integrating eyepiece containing 100 points and 50 lines. The following parameters were evaluated as previously described[21,22].

Alveolar distension and collapse index: At a magnification of × 100, we analyzed 10 randomly selected fields of the proximal and 10 fields of the distal sections. We designated grades 0, 1, 2, and 3 to microscopic fields respectively as 0%, 25%, 50%, and over 50% of the area with either alveolar distension or alveolar collapse.

Alveolar edema index: At a magnification of × 400, we analyzed 10 randomly selected fields of the proximal and 10 fields of the distal sections. The relationship between the number of points of the eyepiece falling on alveolar edema and the number of points falling on the whole alveolar lumen was determined.

Alveolar cellularity index: We analyzed 10 microscopic fields from each lung slide at a magnification of × 1000. The alveolar cellularity index was obtained by the relationship between the lines of the integrating eyepiece crossing a nucleus and the lines crossing alveolar septa.

Polymorphonuclear cell (PMNL) index: We analyzed 10 microscopic fields from each lung slide at a magnification of × 1000. PMNL index was obtained by the relationship between the lines of the integrating eyepiece crossing a nucleus and the lines crossing alveolar septa.

Statistical analysis

Statistical analysis was performed using SPSS version 11.5 for Windows XP. The results were expressed as mean + standard deviation (SD). The differences in serum amylase, IL-2 and IL-6 were assessed by Welch test and *post hoc* Games-Howell test or one way ANOVA and *post hoc* Tukey

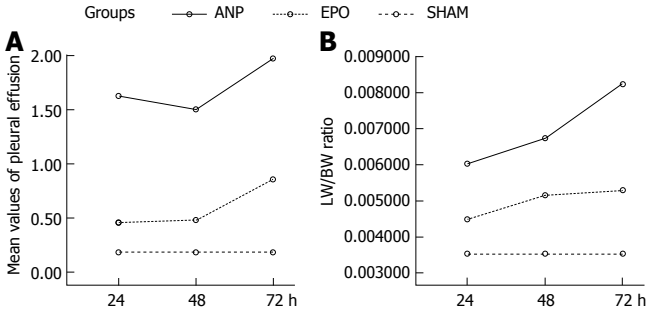

Figure 1 Values of pleural effusion volumes (**A**) and calculated LW/BW ratios (**B**) (mean ± SD).

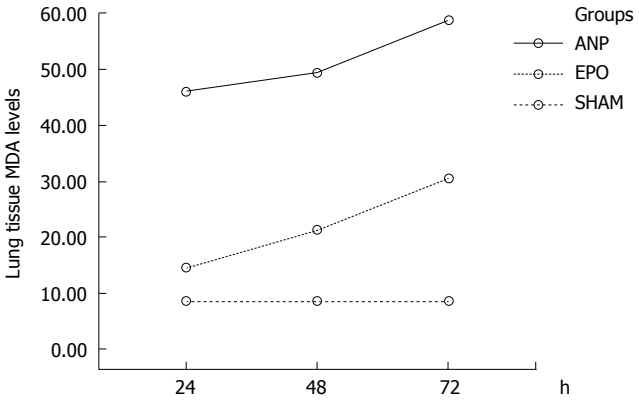

Figure 2 Values of MDA in lung tissue (mean ± SD).

HSD test where appropriate. The differences betweeen groups (ANP, EPO, sham), time course (three different hours) and its interaction in terms of tissue MDA levels, pulmonary effusion volume, calculated LW/BW ratio, and mean histopathological scores, were analyzed by factorial analysis of variance with a single control. $P < 0.05$ was considered statistically significant.

RESULTS

Pleural effusion and LW/BW ratio

The mean pleural effusion volume (mL) and the calculated LW/BW ratio were significantly increased in ANP groups when compared to EPO groups ($P < 0.0001$). The mean ± SD volume of pleural effusion measured was 1.62 ± 1.08 mL, 1.5 ± 0.33 mL and 1.97 ± 0.39 mL in 3 ANP groups and 0.45 ± 0.37 mL, 0.48 ± 0.38 mL and 0.85 ± 0.13 mL in 3 EPO groups, respectively. No statistically significant difference was detected between sham and EPO groups (0.18 ± 0.08 mL *vs* 0.45 ± 0.37 mL, 0.48 ± 0.38 mL and 0.85 ± 0.13 mL, $P > 0.05$ for each). The volume of pleural effusion was statistically significant higher in ANP groups than in sham group (0.18 ± 0.08 mL *vs* 1.62 ± 1.08 mL, 1.5 ± 0.33 mL and 1.97 ± 0.39 mL, $P < 0.001$ for each).The time course of pleural effusion volume in 3 ANP groups is shown in Figure 1A. In terms of LW/BW ratio, a statistically significant difference was seen from 24 h to 72 both in 3 ANP groups (0.006 ± 0.0022 *vs* 0.008 ± 0.0019, $P < 0.05$) and in 3 EPO groups (0.004 ± 0.0008 *vs* 0.005 ± 0.0011, $P < 0.05$) and the mean calculated ratio was higher at 72 h for each. In comparison to sham group, no statistically significant difference was found in EPO groups (0.003 ± 0.0004 *vs* 0.004 ± 0.0008, 0.005 ± 0.0009 and 0.005 ± 0.0011, $P > 0.05$), where as ANP resulted in a significant increase in calculated LW/BW ratio at 24 h, 48 h, and 72 h (0.003 ± 0.0004 *vs* 0.006 ± 0.0022, 0.007 ± 0.0016, and 0.008 ± 0.0019, $P < 0.05$ for each) (Figure 1B). The pleural effusion values and LW/BW ratio are listed in Table 1.

Biochemical analysis

Serum amylase, IL-2 and IL-6 assay: A statistically significant increase was detected in the mean ± SD serum levels of amylase in 3 ANP groups when compared to sham group (534 ± 124 u/L *vs* 3502 ± 1830 u/L, 3759 ± 1505 u/L and 5056 ± 1872 u/L, $P < 0.05$ for each). The mean ± SD value of serum amylase was 3502 ± 1830 u/L, 3759 ± 1505 u/L and 5056 ±1872 u/L in 3 ANP groups and 1523 ± 514 u/L, 2317 ± 311 u/L and 735 ± 454 u/L in 3 EPO groups, respectively. On the other hand, no statistically significant difference was found between the ANP and EPO groups with respect to the time intervals ($P > 0.05$). The serum levels of IL-6 were significantly lower in 3 EPO groups than in 3 ANP groups ($P = 0.001$ for each). The IL-6 value (mean ± SD) was 24.4 ± 3.26 pg/mL, 27.7 ± 3.74 pg/mL and 33.2 ± 2.1 pg/mL respectively in 3 ANP groups, and 12.2 ± 2.15 pg/mL, 12.8 ± 1.89 pg/mL and 13.8 ± 3.24 pg/mL respectively in 3 EPO groups. We did not observe any statistically significant difference in IL-2 values among these groups (7.4 ± 2.88 pg/mL *vs* 7.7 ± 3.17 pg/mL, 9.3 ± 2.74 pg/mL, 7.2 ± 3.1 pg/mL, 6.9 ± 2.04 pg/mL, 7.7 ± 2.93 g/mL and 10.6 ± 3.9 pg/mL, $P > 0.005$ for each). The mean ± SD values of serum amylase, IL-2, IL-6 and tissue MDA are listed in Table 2.

Lung tissue MDA assay: Pulmonary injury in ANP groups was characterized by an increase in lung tissue MDA levels, an indicator of lipid peroxidation. The lung tissue MDA levels were significantly reduced in EPO groups at 24 h, 48 h, and 72 h, when compared to ANP groups ($P < 0.0001$). The MDA value (mean ± SD) was 45.9 ± 6.8 nmol/gr tissue, 49.3 ± 9.5 nmol/gr tissue, and 58.8 ± 9 nmol/gr tissue respectively in 3 ANP groups and 14.7 ± 2.1 nmol/gr tissue, 21.2 ± 2.7 nmol/gr tissue, and 30.4 ± 2.1 nmol/gr tissue respectively in 3 EPO groups. A statistically significant increase in MDA values was noted at 24 h-72 h (45.9 ± 6.8 nmol/gr tissue *vs* 58.8 ± 9 nmol/gr tissue, and 14.7 ± 2.1 nmol/gr tissue *vs* 30.4 ± 2.1 nmol/gr tissue, $P < 0.0001$) and 48 h to 72 h (49.3 ± 9.5 nmol/gr tissue *vs* 58.8 ± 9 nmol/gr tissue and 21.2 ± 2.7 nmol/gr tissue *vs* 30.4 ± 2.1 nmol/gr tissue, $P = 0.001$) in either ANP or EPO groups. The mean MDA value was higher at 72 h. In comparison with sham group, the MDA levels were significantly higher in all the other groups (8.5 ± 3.1 nmol/gr tissue *vs* 45.9 ± 6.8 nmol/gr tissue, 49.3 ± 9.5 nmol/gr tissue, 58.8 ± 9 nmol/gr tissue, 21.2 ± 2.7 nmol/gr tissue, and 30.4 ± 2.1 nmol/gr tissue, $P < 0.001$ for each) except for EPO groups at 24 h (8.5 ± 3.1 nmol/gr tissue *vs* 14.7 ± 2.1 nmol/gr tissue, $P = 0.224$) (Figure 2).

Table 1 Pleural effusion volume and LW/BW ratios in different groups (mean ± SD)

Groups	Sham	ANP1	ANP2	ANP3	EPO1	EPO2	EPO3
Pleural effusion (mL)	0.18 ± 0.08	1.62 ± 1.08	1.5 ± 0.33	1.97 ± 0.39	0.45 ± 0.37	0.48 ± 0.38	0.85 ± 0.13
LW/BW ratio	0.003 ± 0.0004	0.006 ± 0.0022	0.007 ± 0.0016	0.008 ± 0.0019	0.004 ± 0.0008	0.005 ± 0.0009	0.005 ± 0.0011

Table 2 Serum amylase, IL-6, IL-2 and tissue MDA levels in different groups (mean ± SD)

Groups	Sham	ANP1	ANP2	ANP3	EPO1	EPO2	EPO3
Amylase (U/L)	534 ± 124	3502 ± 1830	3759 ± 1505	5056 ± 1872	1523 ± 514	2317 ± 311	735 ± 454
IL-6 (pg/mL)	4.7 ± 2.01	24.4 ± 3.26	27.7 ± 3.74	33.2 ± 2.1	12.2 ± 2.15	12.8 ± 1.89	13.8 ± 3.24
IL-2 (pg/mL)	7.4 ± 2.88	7.7 ± 3.17	9.3 ± 2.74	7.2 ± 3.1	6.9 ± 2.04	7.7 ± 2.93	10.6 ± 3.9
MDA (nmol/gr tissue)	8.5 ± 3.1	45.9 ± 6.8	49.3 ± 9.5	58.8 ± 9	14.7 ± 2.1	21.2 ± 2.7	30.4 ± 2.1

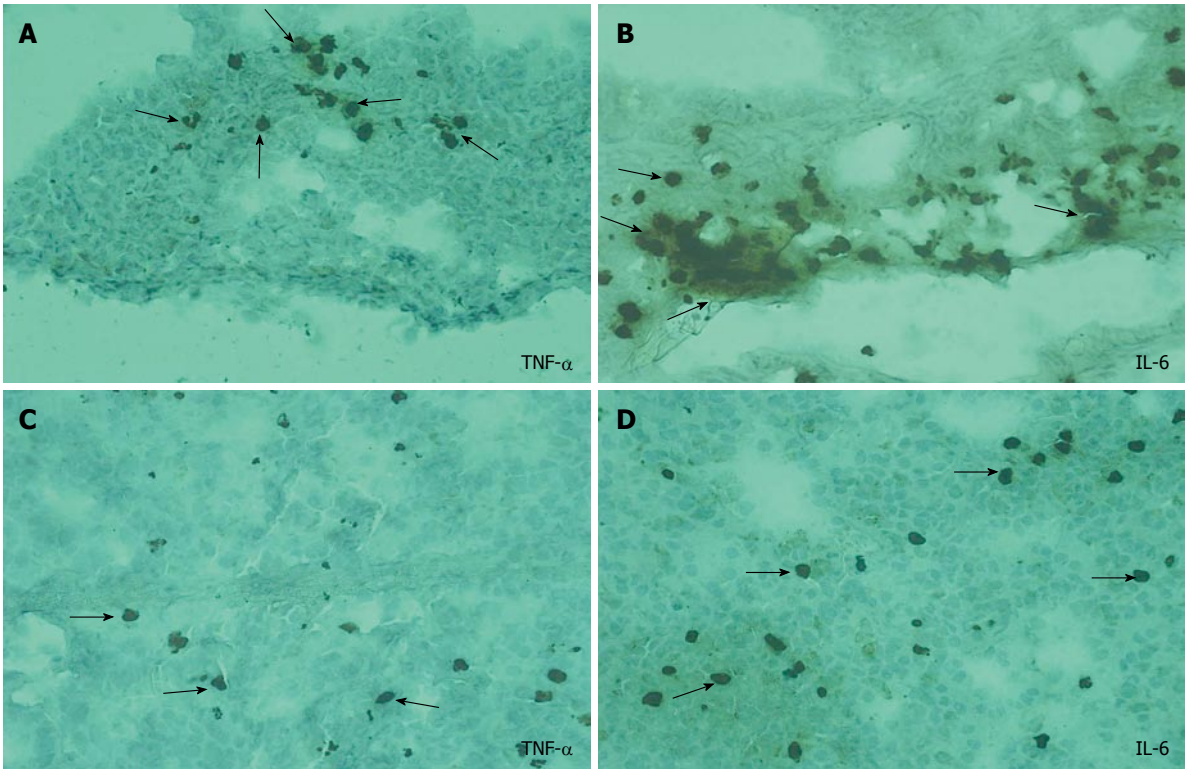

Figure 3 Light microscopic view of immunohistochemical staining for intracellular accumulations of TNF-α and IL-6 in the lung sections of ANP groups (**A** and **B**) and EPO groups (**C** and **D**) at 72 h. Arrows indicate the significantly positive staining in ANP groups (**A** and **B**) and less intensive immnunohistochemical staining in EPO groups (**C** and **D**).

Immunohistochemical screening: The intracellular accumulation of TNF-α and IL-6 was evident in the lung tissues of ANP groups (Figure 3A and B) when compared to EPO groups, particularly at 72 h (Figure 3C and D). No significant difference in IL-2 accumulation was detected among the groups.

Immune-fluorescent screening of ox-LDL: As we did not observe any positive immunofluorescent staining either in pancreas or in lung tissue of sham and EPO groups, a significant positive staining for ox-LDL was determined in ANP groups, which became much evident at 72 h (Figure 4A and B).

Histopathologic analysis

Alveolar distention and collapse: Alveolar distention and collapse were significantly intense in ANP groups at 24 h, 48 h and 72 h when compared to EPO groups ($P < 0.0001$). The alveolar distention and collapse scores (mean ± SD) for ANP and EPO groups calculated at 24 h, 48 h and 72 h were 0.85 ± 0.69, 1.57 ± 0.78, and 1.71 ± 0.75 *vs* 0.71 ± 0.75, 1 ± 0.57 and 0.42 ± 0.53 respectively. Only ANP groups demonstrated a significant difference at 72 h in comparison with sham (1.71 ± 0.75 *vs* 0.4 ± 0.54, $P = 0.03$) (Figure 5A).

Alveolar edema index: Alveolar edema index was

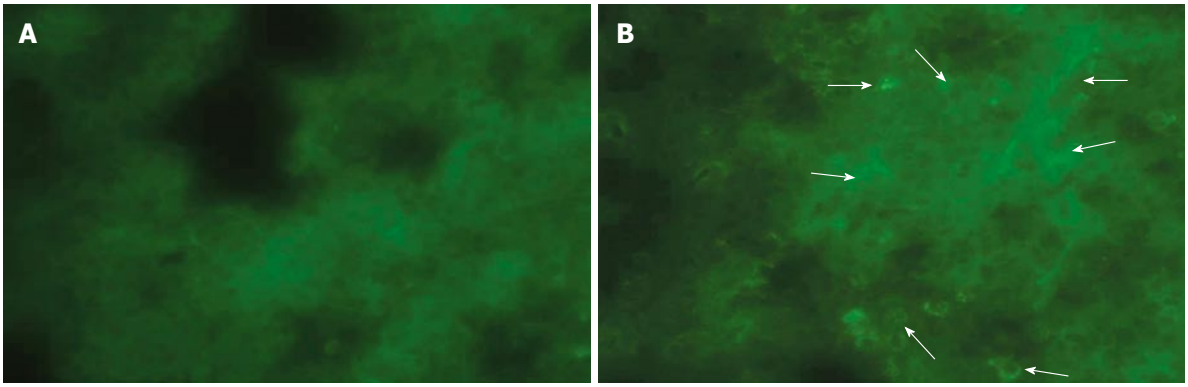

Figure 4 Lung tissue sections from EPO groups and ANP groups showing no fluorescent staining (**A**) and positive fluorescent staining (**B**) at 72 h. Arrows indicate the accumulation areas of ox-LDL in the lung.

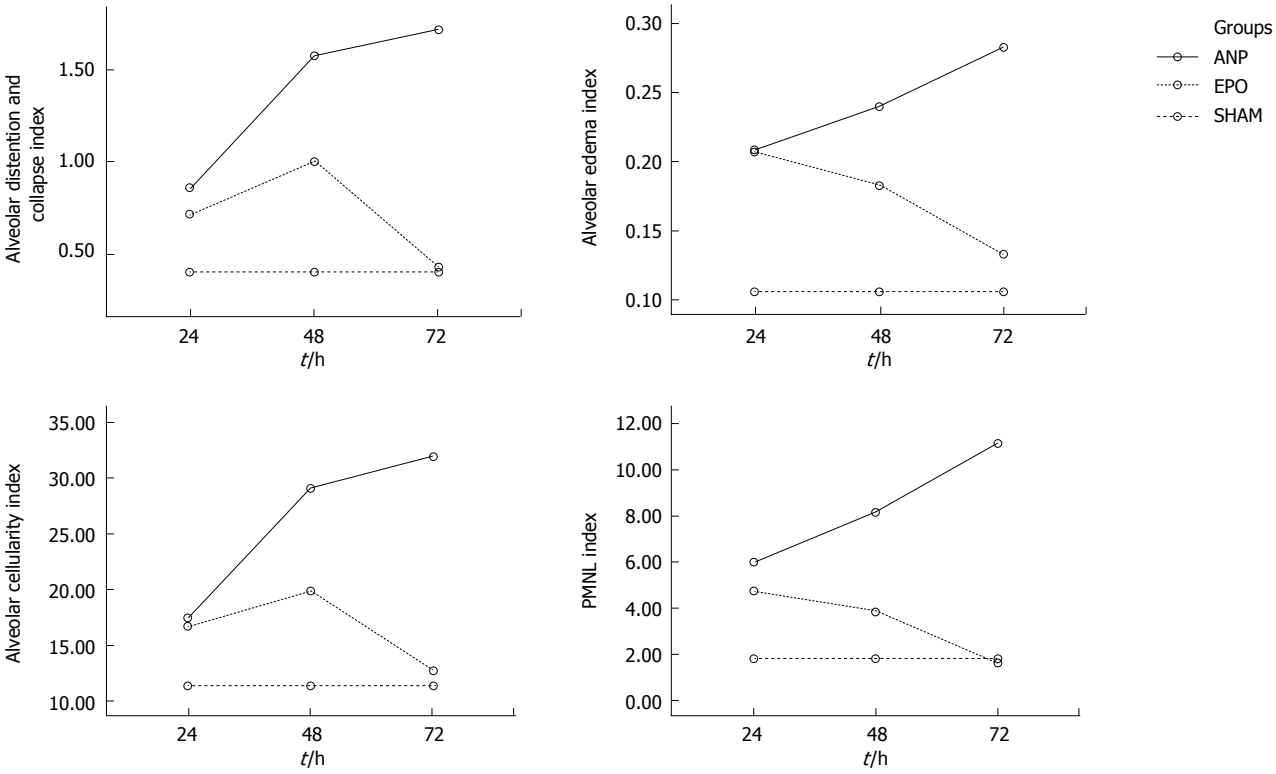

Figure 5 Mean values of alveolar distension and collapse index (**A**), alveolar edema index (percentage of the alveolar lumen filled with edema) (**B**), alveolar cellularity index (**C**) and polymorphonuclear cell index (**D**) obtained in the seven groups.

significantly different both in ANP groups and in EPO groups depending on the time course ($P = 0.002$). Alveolar edema was more intense in ANP groups at 48 h and 72 h, when compared to EPO groups (0.24 ± 0.05 *vs* 0.18 ± 0.03, $P < 0.05$ and 0.28 ± 0.03 *vs* 0.13 ± 0.04, $P < 0.01$). Moreover, at 72 h the mean alveolar edema index determined was the highest in ANP groups and the lowest in EPO groups (0.28 ± 0.03 *vs* 0.13 ± 0.04). In comparison with sham group, ANP groups had a significantly increased mean alveolar edema index at 24 h, 48 h and 72 h (0.1 ± 0.02 *vs* 0.2 ± 0.08, $P = 0.019$; 0.1 ± 0.02 *vs* 0.24 ± 0.05, $P = 0.001$; and 0.1 ± 0.02 *vs* 0.28 ± 0.03, $P = 0.0001$; respectively). On the other hand, no statistically significant difference was detected between sham group and EPO groups at 48 h and 72 h (0.1 ± 0.02 *vs* 0.18 ± 0.03, $P = 0.149$ and 0.1 ± 0.02 *vs* 0.13 ± 0.04, $P = 0.968$), which might propose that EPO treatment could decrease alveolar edema index at 48 h and 72 h (Figure 5B).

Alveolar cellularity index: Alveolar cellularity index was significantly different in either ANP groups or in EPO groups depending on the time course ($P = 0.011$). There was no significant difference in alveolar cellularity index between ANP and EPO groups at 24 h (17.42 ± 9.16 *vs* 16.71 ± 8.61, $P > 0.05$), whereas the mean value for ANP groups was significantly increased at 48 h and 72 h (29.14 ± 8.39 *vs* 19.85 ± 5.89, $P < 0.05$ and 32 ± 6.42 *vs* 12.71 ± 7.11, $P < 0.01$). Additionally, the mean alveolar cellularity index at 72 h was the highest in ANP groups and the lowest in EPO groups (32 ± 6.42 *vs* 12.71 ± 7.11).

Table 3 Histopathological index scores of lung injury (mean ± SD)

Groups	Sham	ANP1	ANP2	ANP3	EPO1	EPO2	EPO3
Alveolar distention collapse	0.4 ± 0.54	0.85 ± 0.69	1.57 ± 0.78	1.71 ± 0.75	0.71 ± 0.75	1 ± 0.57	0.42 ± 0.53
Alveolar edema index	0.1 ± 0.02	0.2 ± 0.08	0.24 ± 0.05	0.28 ± 0.03	0.2 ± 0.04	0.18 ± 0.03	0.13 ± 0.04
Alveolar cellularity index	11.4 ± 8.14	17.42 ± 9.16	29.14 ± 8.39	32 ± 6.42	16.71 ± 8.61	19.85 ± 5.89	12.71 ± 7.11
PMNL cell index	1.8 ± 1.78	6 ± 3.51	8.14 ± 3.48	11.14 ± 5.55	4.71 ± 2.36	3.85 ± 2.19	1.57 ± 1.61

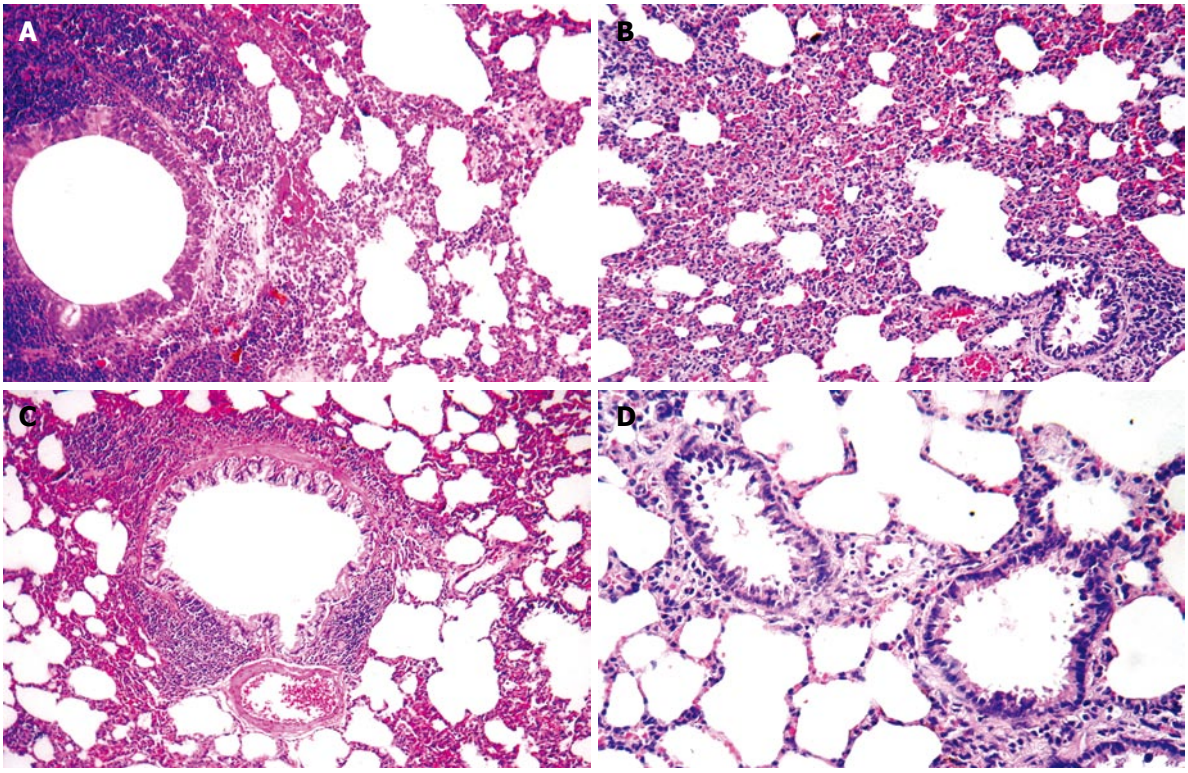

Figure 6 Light microscopy revealing significant lung injury-associated alveolar septal thickening, interstitial edema, infiltration of inflammatory cells, destruction of alveolar wall (emphysema), and microabscess formation in ANP groups at 48 h (**A**) and 72 h (**B**), and attenuation of inflammatory reaction, edema and emphysema in EPO groups at 48 h (**C**) and 72 h (**D**).

Compared with sham group, alveolar cellularity index was significantly increased in only ANP groups at 48 h and 72 h (11.4 ± 8.14 *vs* 29.14 ± 8.39, $P = 0.006$ and 11.4 ± 8.14 *vs* 32 ± 6.42, $P = 0.001$). This might suggest that EPO administration following ANP could decrease alveolar cellularity index at 48 h and 72 h (Figure 5C).

PMNL index: A statistically significant difference was observed in PMNL index between ANP and EPO groups with respect to the time intervals ($P = 0.009$). PMNL index was similar either in ANP groups or in EPO groups at 24 h (6 ± 3.51 *vs* 4.71 ± 2.36, $P > 0.05$). However, EPO treatment significantly decreased the mean PMNL index at 48 h and 72 h (8.14 ± 3.48 *vs* 3.85 ± 2.19, $P < 0.05$ and 11.14 ± 5.55 *vs* 1.57 ± 1.61, $P < 0.01$). The mean ± SD value at 72 h was the greatest in ANP groups and the lowest in EPO groups (11.14 ± 5.55 *vs* 1.57 ± 1.61). There was no statistically significant difference in PMNL index at 24 h, 48 h and 72 h between ANP and EPO groups (1.8 ± 1.78 *vs* 4.71 ± 2.36, $P = 0.315$; 1.8 ± 1.78 *vs* 3.85 ± 2.19, $P = 0.930$; and 1.8 ±1.78 *vs* 1.57 ± 1.61, $P = 1.00$, respectively), whereas ANP induction resulted in an increased PMNL index at 48 h and 72 h (1.8 ± 1.78 *vs* 8.14 ± 3.48, $P = 0.028$ and 1.8 ± 1.78 *vs* 11.14 ± 5.55, $P = 0.0001$, respectively) but not at 24 h (1.8 ± 1.78 *vs* 6 ± 3.51, $P = 0.725$). This might be explained as EPO administration could decrease PMNL index at 48 h and 72 h (Figure 5D). The histopathological indexes of lung injury (mean ± SD) are listed in Table 3. The representative light microscopic views of lung injury at 48 h and 72 h, and inecrotizing pancreatitis with severe fatty necrosis in ANP and EPO groups are shown in Figures 6 and 7. According to the above-mentioned criteria, it might be speculated that EPO administration could alleviate pulmonary injury by decreasing alveolar edema, alveolar cellularity and PMNL indexes at 48 h and 72 h following taurocolic acid-induced pancreatitis. The effect of EPO on alveolar distention and collapse was restricted at 72 h.

DISCUSSION

ANP is an inflammatory disorder with various systemic

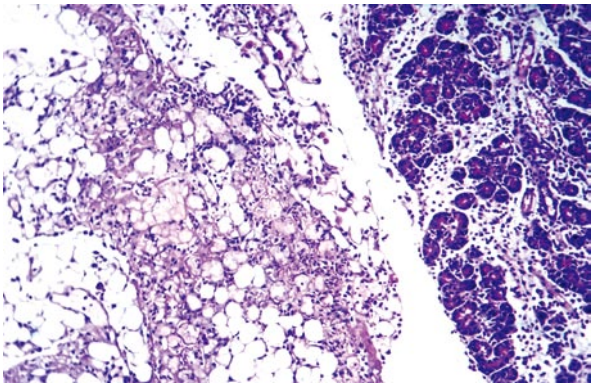

Figure 7 Light microscopic view of pancreatitis with severe fatty necrosis.

complications. ALI and ARDS are the most dreadful complications of ANP and impending catastrophe which is difficult to deal with clinically. Various medications directed at key stages of the pathophysiology are not clinically efficacious as indicated in the preceding experimental trials[23]. Therefore, therapies for preventing or reversing lung injury would be ideal for the treatment of AP[1,2,21,24-26].

Randomized studies of AP in the clinical setting do have limitations. In this regard, reliable AP animal models are of paramount importance. Taurocholate infusion model is a well-established ANP rat model that induces multiple organ failure involving the lung[27]. Moreover, Milani *et al*[28] found that mechanical and morphologic alterations in pancreatitis-associated pulmonary injury in rats are similar to those observed in humans.

The pathophysiology of ALI/ARDS and most of other pulmonary complications is multifactorial in ANP. The major pathway is the induction of a strong inflammatory response both in experimental models and in patients[29]. Regardless of the priming process, the disease progression can be viewed as three phases in continuum: local inflammation of the pancreas (a generalized inflammation stage and SIRS), and the final stage of multiorgan dysfunction[24,25]. The first sign of MODS is often the impaired lung function that manifests itself clinically as ARDS[1,2,24,25,30]. SIRS is one of the crucial reasons for pancreatitis-associated lung injury and PMNL plays a central role with various inflammatory cytokines and reactive oxygen species (ROS)[2,29,31]. Many researchers have focused their efforts on preventing AP-induced lung injury by pharmacologic interventions. Attractively, recent works have discovered the potential role of EPO as a multifunctional endogenous mediator offering cytoprotective effect against injury in various tissues including lung[16-19]. In multiple species including humans, many tissues injured by ischemia, mechanical trauma, excitotoxins, and other stressors are significantly improved by administration of EPO following injury[32]. The presence of a therapeutic window dictates specific time constraints for efficacious administration of exogenous EPO as a cytoprotectant[5]. According to this hypothesis we administered EPO immediately following the induction of pancreatitis and evaluated its effect in three different time courses.

The principle mechanism by which EPO confers tissue protection involves the modulation of cellular apoptosis. EPO inhibits the apoptotic mechanisms of injury, including preservation of cellular membrane asymmetry to prevent inflammation, can therefore be regarded as a general tissue-protective cytokine[11,12,14]. Agents that can prevent apoptosis can be effective long after the occurrence of injury[5]. This phenomenon might describe the long protective effect of EPO on lung injury in our study, particularly at 48 h and 72 h. We would also like to emphasize that, in patients with a severe attack, the effects of distant organ damage including lung injury, are often not fully established and become apparent only over the following 48 h. There is thus a therapeutic window between hospital presentation and development of distant organ dysfunction. As an obvious time window existed in this process, therapeutic approach should focus on it during this period. From this assumption, the animals were sacrificed on postoperative hours 24, 48 and 72 for histopathological and biochemical evaluations in our study.

Another crucial determinant for observing the cytoprotective effect of EPO is the serum concentration. The serum concentration of EPO required for tissue protection is higher than that required for erythropoiesis. Preclinical data suggest that the minimum therapeutic level needed for protection against tissue injury appears to be 300-500 mIU/kg body weight (intravenously or intraperitoneally) for the organs to be adequately investigated. EPO administration (100-1000 U/kg body weight) achieves possible systemic protective effects whereas high doses of EPO (3000-5000 U/kg body weight) are necessary for cardioprotection and neuroprotectin[33]. According to this, we administered EPO at the dose of 1000 mIU/kg body weight to observe the cytoprotective effects.

EPO plays a dual role in vascular protection by preserving endothelial cell integrity[6,8,9], thus playing a role in maintaining the integrity of microvasculature[11]. One of the major factors for the development of alveolar edema in ANP is the increased microvascular permeability. The experimental protocol we performed let us to measure the amount of edema within alveoli. We used alveolar edema index and alveolar distension and collapse index as markers of ALI according to the previous observations suggesting that histologic evidence of pulmonary tissue injury can appear before the development of clinically relevant respiratory mechanical changes[22]. We prefer three different time courses, since pulmonary injury indexes are quite intense in taurocolic acid induced acute pancreatitis on d 1 and 3, some of which persist through d 8[22]. In the present study, pulmonary edema, alveolar cellularity index and PMNL index (pulmonary injury index) were significantly reduced in EPO groups at 48 h and 72 h, suggesting that EPO can preserve endothelial cell integrity.

Oxidative stress has been implicated as a crucial landmark by increasing endothelial permeability in ARDS[1]. ROS scavengers possess protective effect against local acute pancreatitis-associated with lung injury[21,34,35]. In addition to other effects, EPO has been demonstrated in various tissues to be an antioxidant as it can decrease

the plasma iron concentration and increase the ability of plasma to inhibit lipid peroxidation[36,37]. In the present study, we determined the tissue levels of thiobarbituric acid reactant MDA, which is considered a good indicator of lipid peroxidation, and found a significant decrease in EPO group when compared to ANP groups in all three time courses. This might be attributed to the antioxidant effect of EPO. Furthermore, the tissue damage induced by ANP was associated with a significant ox-LDL accumulation either in pancreas samples or in lung tissue specimens. Ox-LDL is an early product of lipid peroxidation and ox-LDL accumulation in pancreatitis is associated with lung injury.

At present, the role of inflammatory mediators in the pathogenesis of ARDS has become a hot issue in the research field. EPO has been demonstrated to prevent cellular inflammation by inhibiting several proinflammatory cytokines, such as IL-6, TNF-α, and monocyte chemoattractant protein[7,38]. Attractively, these effects of EPO can be mediated by both hormonal and paracrine modalities[38]. There is mounting evidence that proinflammatory cytokines are the agents behind the systemic complications of AP[1,2]. It was reported that systemic inflammation plays a role in development of ALI triggered by pancreatitis[30]. The critical players of this process include proinflammatory cytokines including IL-1β, TNF-α, IL-6, IL-8, and platelet activating factor (PAF)[29]. Among these, the serum and/or tissue levels of TNF-α, IL-2 and IL-6 were analyzed in this study. Regardless of the model of acute pancreatitis, inhibition of the potent cytokine TNF-α might decrease organ injury and improve survival[39]. The tissue levels of TNF-α in the lungs were analyzed with immunohistochemical staining. Since no quantitative analysis was possible unavailable techniques, we evaluated this parameter not statistically but morphologically. IL-6 is another proinflammatory cytokine, and its high circulating level has been shown to be an excellent predictor of the severity of ARDS with different etiologies, including AP[40]. Moreover, IL-6 has been proposed to be one of the best prognostic parameters for pulmonary failure in human AP[30,41]. Mayer *et al*[41] have confirmed the important role of soluble IL-2 receptors (a lymphocyte activation marker), as a marker for severe AP, especially when complicated by lung or kidney failure or sepsis during lethal course of the disease[41]. In the present study, pulmonary injury in ANP groups was characterized by the increased serum or tissue IL-6 and TNF-α level. EPO treatment significantly decreased IL-6 and TNF-α level which might be due to the antiinflammatory properties of its molecule. However, we did not determine a statistically significant difference in the IL-2 level among the groups. This result might reflect the ineffectiveness of EPO on lymphocyte activity.

In conclusion, EPO administration plays a crucial role in preventing histological changes of ALI induced by experimental ANP. Moreover, it can significantly reduce the circulating and tissue levels of proinflammatory cytokines which have been considered the key factors for ALI. Additionally, oxidative stress markers are decreased particularly at 72 h following the induction of pancreatitis that might be attributed to the long-lasting antioxidant effect of EPO. All these findings show that EPO can attenuate ANP-induced lung injury by inhibiting PMNL accumulation, decreasing the circulating levels of proinflammatory cytokines, preserving microvascular endothelial cell integrity and reducing oxidative stress-associated lipid peroxidation. Years of clinical application in patients with anemia and chronic renal disease indicate that EPO is safe and well tolerated and can act as an ideal cytoprotective agent[7,42,43]. Nevertheless, the issue which should also be taken into consideration is that EPO is not an absolutely innocent agent with subsequent clinical toxic effects. Therefore, it would be of value to investigate its pharmacodynamics, pharmacokinetics, side-effects, administration routes and doses before used as a potential candidate for the treatment of ANP-associated ALI in routine clinical practice. In other words, this is a preliminary study and more experiments are necessary for the efficacy and potentially cytoprotective mechanisms of EPO action.

COMMENTS

Background

Pulmonary complication is the major cause for mortality in acute necrotizing pancreatitis (ANP). Since no absolutely effective treatment is available at present, therapies for preventing or reversing lung injury would be ideal for the treatment of AP.

Research frontiers

Erythropoietin (EPO) has long been known as a glycoprotein hormone that regulates erythropoiesis in mammals. Beyond its hematopoietic properties, EPO modulates a broad array of vital cellular processes including progenitor stem cell development, cellular integrity, and angiogenesis. EPO has recently been demonstrated to play a role in prolonging cell survival by acting as an antiapopitotic agent. EPO inhibits the apoptotic mechanisms of injury including preservation of cellular membrane asymmetry to prevent inflammation, and can therefore be regarded as a general tissue-protective cytokine. Additionally, experimental evidence supports a vigorous cytoprotective effect of EPO, which is now considered to have applicability in a variety of disorders, such as cerebral ischemia, myocardial infarction, and chronic congestive heart failure.

Related publications

The present study was an experimental study addressing the beneficial effects of EPO on lung injury. We cited several articles from other investigators reporting researches of EPO action on various tissues including lungs.

Innovations and breakthroughs

Recent works have discovered the potential role of EPO as a multifunctional endogenous mediator offering cytoprotective effect against injury in various tissues including the lungs. Pretreatment with EPO appears to attenuate ischemia-reperfusion-induced lung injury and hyperoxic lung injury in neonatal rats. From this point of view we evaluated the potantial protecting effects of EPO against acute lung injury in a rat model of ANP. Our data show that EPO administration can alleviate pulmonary injury parameters in experimental pancreatitis.

Applications

The impending catastrophe in ANP is generally preceded by acute lung injury. Despite improved understanding of the pathogenesis of ARDS, pharmacological modalities are ineffective in decreasing its mortality. None of the randomized clinical trials using novel therapeutic agents has demonstrated an improvement in patient outcome. The verification of cytoprotective effects of EPO on acute lung injury in a model of experimental pancreatitis might shed some valuable light on the novel effective therapeutic interventions.

Terminology

Erythropoietin (EPO), a 30.4-kDa glycoprotein and a member of the type I

cytokine superfamily, was first introduced as a hormone that regulates erythroid progenitors within the bone marrow to mature into erythrocytes, through binding to its specific cell surface receptors. Acute necrotizing pancreatitis (ANP) is a life-threatening necroinflammatory disease of pancreas with significant morbidity and mortality rates. Acute lung injury (ALI) is one of the most dreadful complications of AP which might be described as the continuum of pathological responses to pulmonary parenchymal injury. Acute respiratory distress syndrome (ARDS) is a severe form of ALI and acute pulmonary inflammation syndrome and resultant increased capillary endothelial permeability with clinical features of severe dyspnea and extreme hypoxemia refractory to a high inspired oxygen concentration.

Peer review

This is a well-designed and interesting study about the beneficial effects of EPO on lung injury in an experimental model of ANP. Since this is a preliminary study as discussed by the authors, more comprehensive experiments should be carried out to reveal the underlying cellular mechanisms of EPO's cytoprotective action against lung injury.

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S- Editor Liu Y **L- Editor** Wang XL **E- Editor** Ma WH

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World J Gastroenterol 2007 December 14; 13(46): 6183-6190
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Carbon liberated from CO-releasing molecules attenuates leukocyte infiltration in the small intestine of thermally injured mice

Bing-Wei Sun, Qin Jin, Yan Sun, Zhi-Wei Sun, Xi Chen, Zhao-Yong Chen, Gediminas Cepinskas

Bing-Wei Sun, Qin Jin, Yan Sun, Zhi-Wei Sun, Xi Chen, Zhao-Yong Chen, Department of Burns and Plastic Surgery, Affiliated Hospital, Jiangsu University, Zhenjiang 212001, Jiangsu Province, China
Gediminas Cepinskas, Centre for Critical Illness Research, Lawson Health Research Institute, 800 Commissioners Rd. E., London, Ontario, N6A 4G4, Canada
Correspondence to: Dr. Bing-Wei Sun, Department of Burns and Plastic Surgery, Affiliated Hospital, Jiangsu University, 438 Jiefang Rd, Zhenjiang 212001, Jiangsu Province, China. sunbinwe@hotmail.com
Telephone: +86-511-85026183 Fax: +86-511-5029089
Received: July 12, 2007 Revised: September 8, 2007

Abstract

AIM: To determine whether Carbon (CO) liberated from CO-releasing molecules attenuates leukocyte infiltration in the small intestine of thermally injured mice.

METHODS: Thirty-six mice were assigned to four groups. Mice in the sham group (n = 9) were underwent to sham thermal injury; mice in the burn group (n = 9) received 15% total body surface area full-thickness thermal injury; mice in the burn + CORM-2 group (n = 9) were underwent to the same thermal injury with immediate administration of tricarbonyldichlororut henium (Ⅱ) dimer CORM-2 (8 mg/kg, i.v.); and mice in the burn+DMSO group (n = 9) were underwent to the same thermal injury with immediate administration of 160 μL bolus injection of 0.5% DMSO/saline. Histological alterations and granulocyte infiltration of the small intestine were assessed. Polymorphonuclear neutrophil (PMN) accumulation (myeloperoxidase assay) was assessed in mice mid-ileum. Activation of nuclear factor (NF)-κB, expression levels of intercellular adhesion molecule-1 (ICAM-1) and inducible heme oxygenase in mid-ileum were assessed.

RESULTS: Treatment of thermally injured mice with CORM-2 attenuated PMN accumulation and prevented activation of NF-κB in the small intestine. This was accompanied by a decrease in the expression of ICAM-1. In parallel, burn-induced granulocyte infiltration in mid-ileum was markedly decreased in the burn mice treated with CORM-2.

CONCLUSION: CORM-released CO attenuates leukocyte infiltration in the small intestine of thermally injured mice by interfering with NF-κB activation and protein expression of ICAM-1, and therefore suppressing the pro-adhesive phenotype of endothelial cells.

Key words: Leukocyte infiltration; Carbon monoxide; Thermal injury; Small intestine

Sun BW, Jin Q, Sun Y, Sun ZW, Chen X, Chen ZY, Cepinskas G. CO liberated from CO-releasing molecules attenuates leukocyte infiltration in the small intestine of thermally injured mice. *World J Gastroenterol* 2007; 13(46): 6183-6190

http://www.wjgnet.com/1007-9327/13/6183.asp

INTRODUCTION

Systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF) still continue to be leading causes of morbidity and mortality in severe burn patients[1,2]. The intestine is considered to be the critical organ in the development of organ dysfunction in trauma, burns, and intensive care unit patients[3]. Thermal injury is accompanied by complex events that exert deleterious effects on various organs, such as the small intestine, distant from the original burn wound. Following thermal injury, the small intestine is subjected to ischemia, and consequently, especially during burn resuscitation, reperfusion injury occurs[4]. Intestinal ischemia-reperfusion results in organ injury through both tissue hypoxia and reperfusion phenomena mediated by neutrophils[5,6]. A variety of cytokines are released into the microcirculation by neutrophils, endothelial cells and monocytes during phases of hypoxia and reperfusion[7,8]. Although the pathophysiological basis of organ damage remains unclear, there is increasing evidence that leukocyte infiltration into intestinal tissue plays an important role in bacterial or endotoxin translocation and development of SIRS after thermal injury[9-12].

A lot of evidence indicates that endogenous Carbon (CO), a by-product of inducible heme oxygenase (HO-1), modulates inflammation. In addition, some experiments have determined that the administration of exogenous CO inhibits lipopolysaccharide (LPS)-induced production

of cytokines both *in vivo* and *in vitro*, and consequently exhibits an important cytoprotective function and anti-inflammatory properties that are beneficial for the resolution of acute inflammation[13-15].

Recently, transitional metal carbonyls have been identified as potential CO-releasing molecules (CORMs), with the potential to facilitate the pharmaceutical use of CO by delivering it to tissues and organs[16]. CORMs have been shown to act pharmacologically in rat aorta and cardiac tissue in which liberation of CO induced vasorelaxant effects[17-20] and decreased myocardial ischemia-reperfusion injury[21,22], respectively. Our previous studies[23,24] have shown that burn-induced overexpression of adhesion molecules [such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1] on endothelial cells and leukocytes may contribute to liver and lung tissue injury, subsequently leading to multiple organ dysfunction syndrome (MODS). We also confirmed that CORM-released CO attenuated leukocyte sequestration in the liver and lungs of burned mice by interfering with NF-κB activation and protein expression of ICAM-1, therefore suppressing the pro-adhesive phenotype of endothelial cells. However, it is still unknown if CORM-released CO can exert its anti-inflammatory and protective effects in the small intestine. Based on these preliminary observations, in this study we employed tricarbonyldichlororuthenium (Ⅱ) dimer (CORM-2), one of the novel group of CORMs, to determine whether it attenuated leukocyte infiltration to the intestinal tissue of thermally injured mice.

MATERIALS AND METHODS

Materials

CORM-2 was obtained from Sigma-Aldrich and was solubilized in DMSO to obtain a 10 mmol/L stock solution. Polyclonal or monoclonal antibodies against ICAM-1 and HO-1 were purchased from Santa Cruz Biotechnology. All other chemicals were reagent grade and obtained from Sigma unless otherwise stated.

Animals and burn protocol

C57BL/6 mice (36 male; body weight 20 ± 2 g) were fed a standard laboratory diet and water *ad libitum*. Mice were assigned to four groups. Mice in the sham group (n = 9) were underwent to sham thermal injury; mice in the burn group (n = 9) received 15% total body surface area (TBSA) full-thickness thermal injury; mice in the burn + DMSO group (n = 9) were underwent to the same thermal injury with immediate administration of 160 μL bolus injection of 0.5% DMSO/saline; and mice in the burn + CORM-2 group (n = 9) were underwent to the same thermal injury with immediate administration of CORM-2 (8 mg/kg, i.v.). The experimental protocol was approved by The Council on Animal Care at Jiangsu University for the protection and welfare of animals. Under anesthesia with spontaneous inhalation of isoflurane/N_2O (Abbott Laboratories, Mississauga, ON, Canada) in a 60% oxygen/40% nitrogen mixture, the dorsum of each mouse was shaved and the animal was subjected to 15% TBSA full-thickness thermal injury as previously described[25,26]. Sham animals were immersed in a water bath at room temperature. All animals were resuscitated with 1.5 mL saline immediately after thermal (or sham) injury. No wound care was required for the burn wounds. This burn method achieves a histologically proven, full-thickness scald injury[27,28]. The animals were sacrificed at 24 h after experimental manipulation.

Table 1 Histological scoring system for ileum and jejunum sections stained with hematoxylin/eosin

	Granulocyte infiltration			Hydropic degeneration		
	0	1	2	0	1	2
Ileum	No	Moderate	Intense	No	Moderate	Intense
Jejunum	No	Moderate	Intense	No	Moderate	Intense

Ileum histologic studies

The mid-ileum specimens harvested from different groups of animals were immersed in 4% formaldehyde solution at 24 h after thermal injury. The tissue was embedded in paraffin wax, serially sectioned, and stained with hematoxylin-eosin. Ileal morphologic characteristics were evaluated by light microscopy. Ileum tissue was evaluated for density of granulocytes and degree of hydropic degeneration. Tissues were evaluated in a semi-quantitative manner by two experienced independent examiners that were blinded to the experimental groups (Table 1). A scoring system was used for each item using 0 up to 2 points for the different states of organ damage (with 2 being most granulocytes, edema and degeneration; Table 1). Afterwards, the mean ± SEM of each item was calculated.

Preparation of intestinal homogenates

Immediately after withdrawing blood, the intestine was exposed. Leaving approximately the first 5-cm-long proximal segment of the intestine, 3-cm-long segments of jejunum and ileum were removed, cleaned, and snap-frozen in liquid nitrogen. The samples were stored at -70℃. Equal weights (100 mg wet weight) of intestine from various groups were suspended in 1 mL PBS and sonicated (30 cycles, twice, for 30 s) on ice[29]. Homogenates were cleared by centrifuging at 12000 r/min at 4℃, and the supernatants were stored at -70℃. Protein levels in the homogenates were determined using the Bio-Rad (Hercules, CA, USA) assay kit.

Myeloperoxidase (MPO) activity

MPO activity was measured in ileum tissue using a procedure similar to that documented by Hillegas *et al*[30,31]. Tissue samples were homogenized in 50 mmol/L potassium phosphate buffer (PB) (pH 6.0), and centrifuged at 10000 × g (10 min); pellets were suspended in 50 mmol/L PB containing 0.5% hexadecyltrimethylammonium bromide. After sonication, the samples were centrifuged at 10000 × g for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mmol/L PB, o-dianisidine, and 20 mmol/L H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO

Table 2 Histological scoring (score in Table 1) of ileum and jejunum tissue stained with hematoxylin/eosin 24 h after thermal injury

	Granulocyte infiltration			Hydropic degeneration		
	Sham	Burn	Burn + CORM	Sham	Burn	Burn + CORM
Ileum	0.60 ± 0.1	1.92 ± 0.4[a]	0.88 ± 0.2[c]	0	1.8 ± 0.2	1.7 ± 0.1
Jejunum	0.54 ± 0.1	1.5 ± 0.2[a]	0.64 ± 0.2[c]	0	1.6 ± 0.1	1.6 ± 0.2

0, no injury; 1, moderate injury; 2, severe injury. Values are presented as mean ± SEM. [a]$P < 0.05$ *vs* sham group; [c]$P < 0.05$ *vs* burn group.

present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Measurement of ICAM-1

ICAM-1 levels in ileum tissue homogenates were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Western blot analysis

Tissues were homogenized for extraction in ice-cold mild lysis buffer, containing 1% Nonidet P-40, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate (pH 7.2), 2 mmol/L EDTA, 50 mmol/L sodium fluoride, 0.2 mmol/L sodium vanadate, and 1 μg/mL aprotinin. The tissue homogenates were centrifuged at 20000 × *g* for 15 min and supernatants were collected. SDS-PAGE was performed on equivalent amounts of protein samples using precast 7% resolving/4% stacking Tris/HCl gels (Bio-Rad, Hercules, CA, USA). Separated proteins were then transferred to PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked in 5% non-fat milk in TBS buffer containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Blocked membranes were incubated with primary antibodies specific for mouse ICAM-1 and HO-1 at a concentration of 1:1000 and 1:5000, respectively, in TBST overnight at 4℃. Then, the membranes were washed and probed with horseradish-peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at room temperature. Chemiluminescence detection was performed with the Amersham enhanced chemiluminescence detection kit according to the manufacturer's instructions. To ensure a similar amount of protein in each sample, the membranes were "stripped off", reprobed with actin, developed with horseradish-peroxidase-conjugated secondary antibody, and visualized by enhanced chemiluminescence.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear protein from ileum tissue was extracted using our previously described method[32,33]. Briefly, frozen tissues were weighed, transferred to Corex tubes and homogenized in four volumes (w/v) of PBS containing 2 mmol/L phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 3000 × *g* for 10 min, and the pellet was then resuspended in 2 mL buffer A [0.3 mol/L sucrose, 5 mmol/L dithiothreitol (DTT), 5 mmol/L $MgCl_2$ 10 mmol/L Tris/HCl, 0.1% Triton X-405], and further homogenized using a Dounce homogenizer. After filtration through a 100-μm nylon mesh, the suspension was centrifuged at 1000 × *g* for 5 min at 4℃. The pellet (nuclei) was washed in buffer A (without 0.1% Triton X-405) and centrifuged (1000 × *g* for 5 min at 4℃), and then the nuclei were extracted on ice for 30 min in 60 μL buffer B containing 20 mmol/L HEPES, 0.75 mmol/L spermidine, 0.15 mmol/L spermine, 0.2 mmol/L EDTA, 2 mmol/L ethylene glycol-bis (b-aminoethyl ether)-*N, N, N', N'*-tetraacetic acid, 2 mmol/L DTT, 20% glycerol, and 1 mmol/L PMSF (4℃) in the presence of 0.4 mol/L NaCl. Finally, the samples were centrifuged for 10 min at 21000 × *g* (4℃), and the supernatants were collected and stored at -80℃ as the nuclear protein fraction.

For EMSA, 5 μg total nuclear proteins was incubated with 1.0 pmol double-stranded γ[^{32}P] ATP end-labeled oligonucleotides containing consensus binding sequences for NF-κB (sense strand 5'-AGGGACTTTCCGCTG GGGACTTTCC-3') in a binding buffer (10 mmol/L HEPES, pH 7.9, 80 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, and 10% glycerol), as described previously[34]. Samples were incubated for 30 min at room temperature and then run through a 4% non-denaturing polyacrylamide gel (0.5 × TBE buffer) at 280 V for 1 h. The gel was dried and then exposed to X-ray film (Kodak) for 4-6 h in cassettes at -80℃. Signal detection and quantification were performed by computer-assisted densitometry.

Statistical analysis

All the values are presented as mean ± SE. Statistical analysis was performed by ANOVA and Student's *t* test for the comparisons. $P < 0.05$ was considered to be statistically significant.

RESULTS

Histology

Histological analysis showed that the ileum from sham mice had the normal architecture of the intestinal epithelium and wall, while thermal injury induced severe edema and sloughing of the villous tips, as well as infiltration of inflammatory cells into the mucosa (Figure 1). Semi-quantitative analysis of histological samples of ileum and jejunum showed that granulocyte infiltration in the burned mice was significantly increased compared to that in the sham group. Administration of CORM-2 (8 mg/kg, i.v.), significantly decreased granulocyte infiltration. However, CORM-2 did not improve the hydropic degeneration induced by thermal injury in either the ileum or jejunum (Table 2).

Effect of CORM-2 on MPO activity in small intestine of thermally injured mice

To determine whether the burn-induced increase in polymorphonuclear neutrophil (PMN) accumulation in the small intestine was effectively prevented by CORM-2,

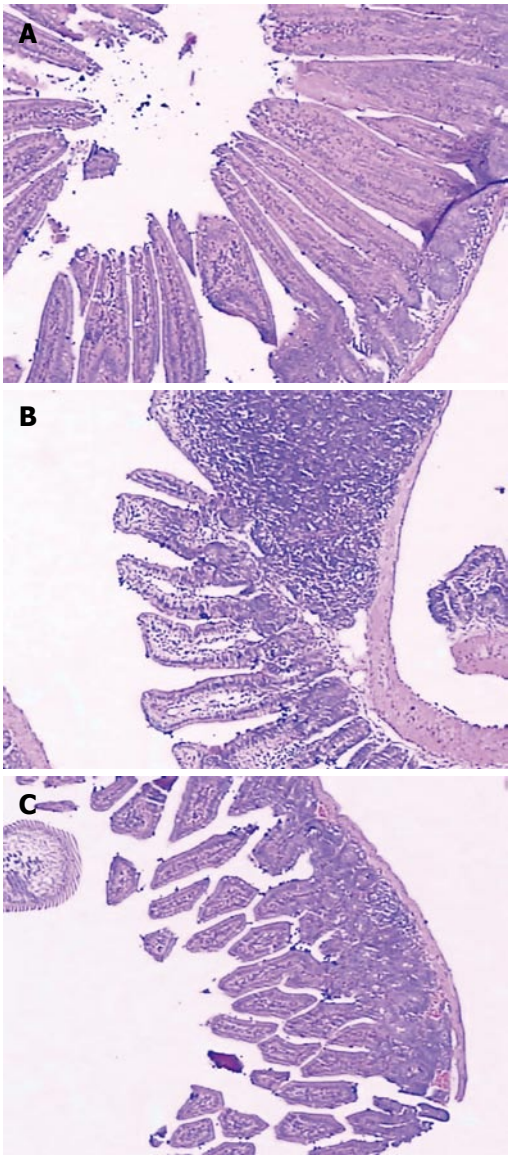

Figure 1 Effects of CORM-2 on small intestine injury in thermally injured mice. Mice were injected i.v. with CORM-2 (8 mg/kg) immediately after thermal injury. Mice in the DMSO group received a 160-μL bolus injection of 0.5% DMSO/saline. Mid-ileum sections from sham-treated mice had normal architecture of the intestinal epithelium and wall (**A**); Mid-ileum sections from thermally injured mice showed inflammatory cell infiltration through the wall, concentrated below the epithelial layer, edema of the distal portion of the villi, and necrosis of the epithelium at the villous tips (**B**); Ileum section from burned mice treated with CORM-2 (**C**) showed a significant decrease in granulocyte infiltration, while no marked improvement of hydropic degeneration. The figure is representative of at least three experiments performed on different days.

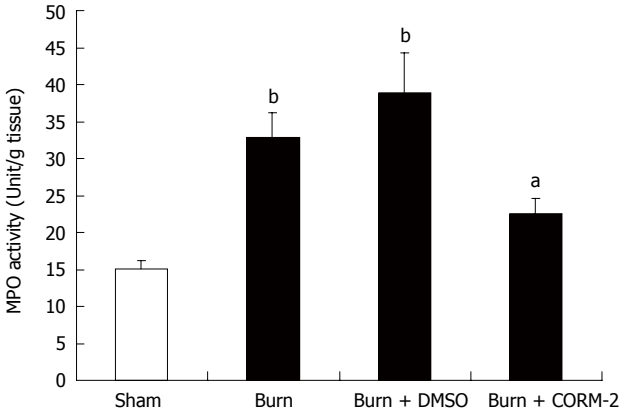

Figure 2 Effects of CORM-2 on MPO activity in the small intestine of thermally injured mice. Mice were challenged with thermal injury and treated with CORM-2 as described in Figure 1. MPO activity in the mid-ileum was assessed 24 h following thermal injury. Results are mean ± SE, $^{b}P < 0.01$ *vs* sham mice. $^{a}P < 0.05$ *vs* burned mice.

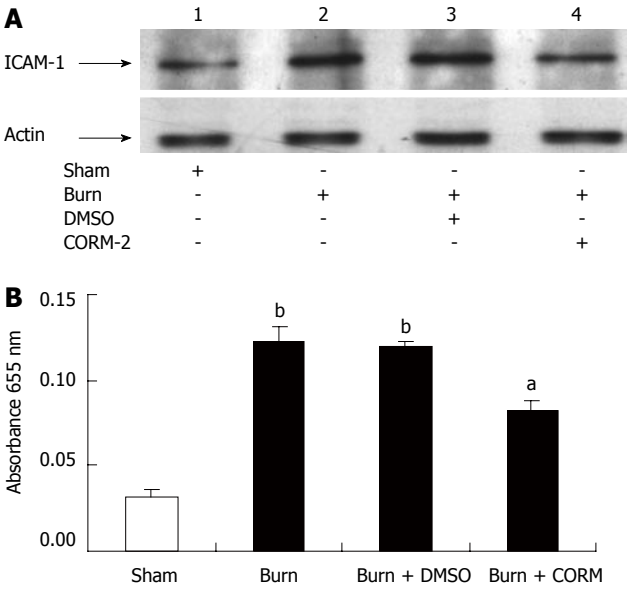

Figure 3 Effects of CORM-2 on protein expression of ICAM-1 in the ileum tissue of thermally injured mice. Mice were challenged with thermal injury and treated with CORM-2 as described in Figure 1. Protein expression of ICAM-1 was analyzed by Western blotting (**A**) and ELISA (**B**) 24 h after thermal injury. A representative experiment is shown in A. $^{b}P < 0.01$ *vs* sham-treated; $^{a}P < 0.05$ *vs* burned mice.

the activity of MPO, an enzyme in azurophilic granules of neutrophils, was assessed. Extracts of the ileum samples were examined for content of MPO 24 h after thermal injury. The mean MPO levels are shown in Figure 2. MPO activity in organs obtained from burned mice was markedly increased compared to that in the sham group ($P < 0.01$), while it was significantly decreased by treatment with CORM-2 ($P < 0.05$).

Effect of CORM-2 on expression of ICAM-1 in the small intestine of thermally injured mice

At 24 h after a 15% TBSA full-thickness thermal injury, the expression of ICAM-1 in the ileum was significantly increased compared to that in the sham-treated animals. Administration of CORM-2 (8 mg/kg, i.v.) significantly decreased expression of ICAM-1 (Figure 3).

Effect of CORM-2 on expression of HO-1 in the small intestine of thermally injured mice

At 24 h after 15% TBSA full-thickness thermal injury, the expression of HO-1 in the small intestine significantly increased compared to that in the sham-treated animals. *In vivo* administration of CORM-2 (8 mg/kg, i.v.), expression of HO-1 in the ileum tissue of burn mice was more significantly increased compared to burn group (Figure 4).

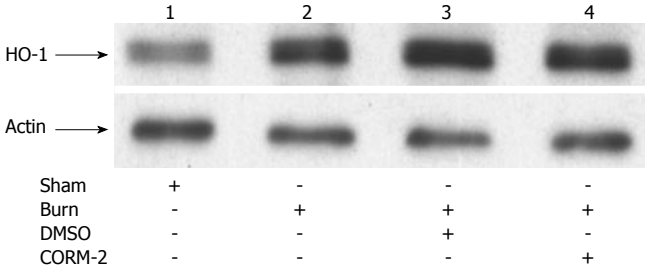

Figure 4 Effects of CORM-2 on protein expression of HO-1 in the ileum tissue of thermally injured mice. Mice were challenged with thermal injury and treated with CORM-2 as described in Figure 1. Protein expression of HO-1 was performed by Western blotting 24 h after thermal injury. A representative experiment showed that HO-1 was significantly up-regulated by thermal injury (lane 2). Expression of HO-1 in the small intestine of thermally injured mice treated with CORM-2 was more significantly increased compared to burned mice without CORM-2 (lane 4).

Effect of CORM-2 on activity of NF-κB in the small intestine of thermally injured mice

Binding of nuclear protein to the radiolabeled consensus binding sequences of NF-κB was assessed by EMSA. At 24 h after 15% TBSA full-thickness thermal injury, NF-κB activation in the ileum was markedly increased, and this was markedly inhibited by administration of CORM-2 (8 mg/kg i.v.) (Figure 5).

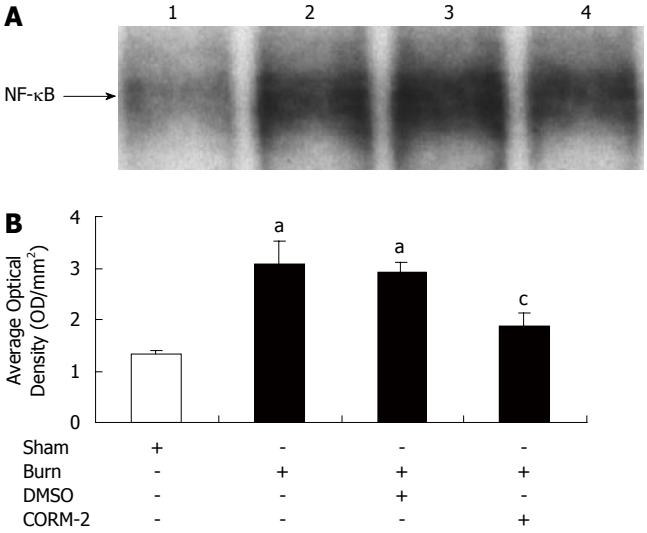

Figure 5 Effects of CORM-2 on NF-κB activation in the ileum tissue of thermally injured mice. Mice were challenged with thermal injury and treated with CORM-2 as described in Figure 1. Measurement of NF-κB activity was performed by EMSA with ^{32}P-labeled NF-κB probe and 5 μg nuclear extract from the ileum of sham, burn, burn + DMSO and burn+CORM-2 mice at 24 h after thermal injury. NF-κB activation in the ileum of thermally injured mice was markedly increased (lane 2), and this activity was inhibited by CORM-2 (lane 4). A representative experiment is shown in **A**, and quantitative results (average optical density) of three experiments are shown in **B**. $^{a}P < 0.05$ *vs* sham-treated; $^{c}P < 0.05$ *vs* burned.

DISCUSSION

Major burns alter immune function, which produces an imbalance between pro- and anti-inflammatory cytokine synthesis, and increases susceptibility to post-burn infection and sepsis[35-37]. Also, severe burns cause damage to multiple organs distant from the original burn wound, leading to MOF, a serious clinical problem. The intestine is one of the most sensitive tissues to ischemia and reperfusion induced by thermal injury. PMNs may play an important role in ischemic injury, and reperfusion of intestine is associated with accumulation of PMNs in the intestinal tissue. It has been suggested that tissue accumulation of PMNs is a key event that determines the severity of ischemia-reperfusion injury[8].

We report here that CORM-released CO exerts a protective effect against the pathological changes caused by thermal injury of the small intestine. Importantly, this exogenous CO showed effective inhibition of activation of NF-κB and expression of ICAM-1. Thus, we propose that CORM-2 contributes to the attenuation of leukocyte infiltration to the intestinal tissue after burn challenge. What is, then, the mechanism by which attenuation of PMN infiltration to the intestine is caused by thermal injury?

Many experimental studies have highlighted the specific and independent role of exogenous CO (i.e. CO inhalation) in the modulation of inflammation[38,39]. Recently some new metal carbonyl-based compounds (CORMs) that have the ability to release CO in biological systems have been identified and synthesized. The vasoactive, antihypertensive and anti-rejection effects of CORMs have been demonstrated to be due to the CO liberated by the compounds. CORM-2, a DMSO-soluble CORM, also has exhibited anti-inflammatory actions in an *in vitro* model of LPS-stimulated murine macrophages[40].

MPO is an enzyme that is found predominantly in the azurophilic granules of PMNs. Tissue MPO activity is frequently utilized to estimate tissue PMN accumulation in inflamed tissues, and correlates significantly with the number of PMNs determined histochemically in tissues[41]. In the present study, we found that intestinal MPO activity was markedly elevated after thermal injury, and administration of CORM-2 led to significant down-regulation of MPO activity. This indicates that CORM-2 effectively prevents PMN chemotaxis and infiltration in the small intestine after thermal injury, which consequently decreases the production of oxidants and reduces tissue oxidative injury, which contributes to MODS. In parallel, histological analysis in this study indicated that mid-ileum sections from thermally injured mice showed inflammatory cell infiltration through the wall, concentrated below the epithelial layer, edema of the distal portion of the villi, and necrosis of the epithelium at the villous tips. On the contrary, ileum sections from mice treated with CORM-2 showed a significant decrease in leukocyte infiltration.

PMN-endothelial cell interactions are supposed to play a central role in the pathogenesis of intestinal barrier failure following thermal injury and ischemia-reperfusion[42]. The presence of ICAM-1, which mediates leukocyte adhesion, correlates with infiltration of leukocytes into inflammatory lesions[43,44]. It seems to be the initial marker of inflammatory reactions and is involved in the acute inflammatory reaction following burns[45]. ICAM-1 activates leukocytes and endothelial cells, which in turn, prompt the release of various inflammatory mediators. This may result in SIRS, acute respiratory distress syndrome, and MODS, which may develop further into progressive MOF and death[46-48]. The present

results showed that at 24 h post-burn, the expression of ICAM-1 in intestinal tissue was markedly up-regulated. CORM-2 was able to inhibit the up-regulation of ICAM-1 induced by thermal injury. Our findings strongly indicate that CORM-2 appears to inhibit leukocyte activation and adhesion, and consequently, might effectively decrease the inflammatory response in the small intestine induced by burns.

HO is a rate-limiting enzyme that is responsible for the catabolism of heme into bilirubin, free iron, and CO. Three HO isoforms have been identified: HO-2 and HO-3 isoforms are believed to be constitutive and physiologically expressed, whereas HO-1 isoform is a stress-responsive protein that is induced by various stimuli. The adaptive response of HO-1 to various stimuli suggests that it may play an important role in protection against the inflammatory response and oxidative injury[49]. Other studies have shown that up-regulation of endogenous HO-1 ameliorates inflammatory responses and/or tissue damage[50]. In this study, we found that HO-1 was significantly up-regulated by thermal injury. Interestingly, the expression of HO-1 in the small intestine of thermally injured mice treated with CORM-2 was more significantly increased compared to burned mice without CORM-2 (Figure 4). This result indicates that not only major burn injury might significantly induce the expression of HO-1, but also the increase in HO-1 expression can be further enhanced by the administration of CORM-2. Through the by-product (CO and/or biliverdin), the potent cytoprotective and anti-inflammatory functions were ultimately led to exert.

NF-κB family members control transcriptional activity of various promoters of proinflammatory cytokines, cell-surface receptors, transcription factors, and adhesion molecules that are involved in intestinal inflammation[51,52]. Stimuli like oxidative stress, cytokines (interleukin-1, interleukin-6, tumor necrosis factor-α), bacteria and viruses can release NF-κB from its inactive cytoplasmic form to the nucleus[53,54]. Thermal injury has been known to induce hepatic NF-κB expression associated with hepatic cell apoptosis and proliferation[55], but its effect on NF-κB activation in the intestine has never been clarified. Previously, using a thermal injury model in mice, we have shown that CORM-2 plays a pivotal role in inhibition of NF-κB activity in the liver, which subsequently decreases hepatocellular secretion of inflammatory cytokines and burn-related hepatic dysfunction. In this study, NF-κB activity in mid-ileum was elevated by thermal injury, while it was markedly inhibited by administration of CORM-2. These results show that CORM-2 plays, at least partly, an important role in inhibition of NF-κB activity in the small intestine. Therefore, the role of NF-κB activation and the regulation of CORM-2 in thermal-injury-induced intestinal damage requires further study.

In conclusion, the present study serves to clarify the role of CORM-2, one of the novel CORMs, on the mechanisms of anti-inflammation and cytoprotection. Application of CORM-2 to thermally injured mice attenuated PMN accumulation, and prevented activation of NF-κB in the small intestine. This was accompanied by a decrease in expression of ICAM-1, and an increase in expression of HO-1. Taken together, these findings indicate that CORM-released CO modulates gut inflammation in burned mice by interfering with NF-κB activation, and protein expression of ICAM-1 and HO-1, and therefore suppresses the pro-adhesive phenotype of endothelial cells. Further studies are now required to understand the detailed mechanisms of the anti-inflammatory effects mediated by CORMs, and to contribute to the development of a therapeutic approach to protect against gut damage during severe burn injury.

COMMENTS

Background

SIRS and MOF still continue to be leading causes of morbidity and mortality in severe burn patients. The intestine is considered to be the critical organ in the development of organ dysfunction in trauma, burn and intensive care unit patients. Thermal injury is accompanied by complex events that exert deleterious effects on various organs, such as the small intestine, distant from the original burn wound. Following thermal injury, the small intestine is subjected to ischemia, and consequently, especially during burn resuscitation, reperfusion injury occurs. Intestinal ischemia-reperfusion results in organ injury through both tissue hypoxia and reperfusion phenomena mediated by neutrophils. A variety of cytokines are released into the microcirculation by neutrophils, endothelial cells and monocytes during hypoxia and reperfusion. Although the pathophysiological basis of organ damage remains unclear, there is increasing evidence that leukocyte infiltration into intestinal tissue plays an important role in bacterial or endotoxin translocation, and development of SIRS after thermal injury.

Research frontiers

Major burns alter immune function, which produces an imbalance between pro- and anti-inflammatory cytokine synthesis, and increases susceptibility to post-burn infection and sepsis. Also, severe burns cause damage to several organs distant from the original burn wound, which leads to MOF, a serious clinical problem. The intestine is one of the most sensitive tissues to ischemia and reperfusion induced by thermal injury. PMNs may play an important role in ischemic injury, and reperfusion of the intestine is associated with accumulation of PMNs in the intestinal tissue. It has been suggested that tissue accumulation of PMNs is a key event that determines the severity of ischemia-reperfusion injury.

Innovations and breakthroughs

Our study is believed to be the first to observe that CORM-released CO attenuates leukocyte infiltration in the small intestine of thermally injured mice, and the possible mechanisms involved.

Applications

Our research observed that CORM-released CO attenuates leukocyte infiltration in the small intestine of thermally injured mice by interfering with NF-κB activation and protein expression of ICAM-1, and therefore suppresses the pro-adhesive phenotype of endothelial cells. This may have a significant clinical impact in the future.

Terminology

CORMs: transitional metal carbonyls that have been identified as potential CO-releasing molecules with the potential to facilitate the pharmaceutical use of CO by delivering it to tissues and organs.

Peer review

This is a well-written paper that suggests the benefit of CORMs after burn injury. Although the mechanism remains to be determined, I think it may be suitable for publication in WJG. It will be nice to detail other experiments using other doses of CORMs.

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World J Gastroenterol 2007 December 14; 13(46): 6191-6196
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Heterozygous nucleotide-binding oligomerization domain-2 mutations affect monocyte maturation in Crohn's disease

Marilena Granzotto, Elisa Fabbro, Massimo Maschio, Stefano Martelossi, Sara Quaglia, Alberto Tommasini, Gianni Presani, Alessandro Ventura

Marilena Granzotto, Stefano Martelossi, Alberto Tommasini, Gianni Presani, Alessandro Ventura, Institute for Child Health Burlo Garofolo, Trieste 34137, Italy
Elisa Fabbro, Massimo Maschio, Sara Quaglia, Alberto Tommasini, Alessandro Ventura, Department of Sciences of Reproduction and Development, University of Trieste, Trieste, Italy
Supported by the Italian Ministry of Health, No. 150/03
Correspondence to: Dr. Marilena Granzotto, Laboratory of Immunology, Institute of Child Health IRCCS Burlo Garofolo, Via dell'Istria 65/1, Trieste 34137, Italy. granzotto@burlo.trieste.it
Telephone: +39-40-3785216 Fax: +39-40-3785210
Received: March 21, 2007 Revised: August 25, 2007

Key words: Crohn's disease; Dendritic cells; Immunophenotype; Muramyldipeptide; Nucleotide-binding oligomerization domain-2

Granzotto M, Fabbro E, Maschio M, Martelossi S, Quaglia S, Tommasini A, Presani G, Ventura A. Heterozygous nucleotide-binding oligomerization domain-2 mutations affect monocyte maturation in Crohn's disease. *World J Gastroenterol* 2007; 13(46): 6191-6196

http://www.wjgnet.com/1007-9327/13/6191.asp

Abstract

AIM: To investigate the function of monocytes in Crohn's disease (CD) patients and to correlate this with disease-associated nucleotide-binding oligomerization domain-2 (*NOD2*) gene variants.

METHODS: Monocytes from 47 consecutively referred CD patients and 9 healthy blood donors were cultured with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), and stimulated with lipopolysaccharide (LPS) or muramyldipeptide (MDP), the putative ligand of *NOD2*.

RESULTS: We found that monocytes from CD patients differentiated *in vitro* to mature dendritic cells (DCs), as determined by immunophenotype and morphology. *NOD2* genotype was assessed in all subjects, and we observed high CD86 expression on immature and LPS-stimulated DCs in *NOD2* mutated CD patients, as compared with *wtNOD2* CD patients and controls. By contrast, CD86 expression levels of DCs induced to maturity with MDP derived from *NOD2*-mutated subjects were comparable to those of normal subjects. The amount of IL-12p70 in patient-cell cultures was larger than in controls after LPS treatment, but not after treatment with MDP.

CONCLUSION: Our results suggest that DCs obtained from patients with mutations in the *NOD2* gene display an activated phenotype characterized by high CD86 expression, but have a diminished response to MDP when compared to the terminal differentiation phase. We speculate that the altered differentiation of monocytes might lead to an imbalance between inflammation and the killing ability of monocytes, and may be relevant to the pathogenesis of CD.

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract, influenced by both environmental factors and genetic predisposition[1-3]. A significant advance in the understanding of its pathogenesis was achieved by the identification of nucleotide-binding oligomerization domain-2 (*NOD2*) as the first susceptibility gene for CD in Caucasian populations[4,5]. This was the demonstration that a frameshift mutation (1007fs) and two nucleotide polymorphisms (R702W and G908R) in the coding region of *NOD2* predispose people to the disease. Since the identification of *NOD2* mutations associated with CD, much attention has been given to the function of monocytes in which this gene is constitutively expressed[6-8].

The idea that the NOD2 protein is involved in the induction of nuclear factor-kappa B (NF-κB) pro-inflammatory signaling pathways in response to bacterial infections may be relevant for understanding the role of intestinal bacteria in CD[9-12]. Indeed, functional assays on CD-associated isoforms of NOD2 have yielded controversial results concerning the production of inflammatory cytokines and responses to bacteria. NOD2 has been shown to act as both an inducer and a regulator of NF-κB and cytokine production[13,14]; more precisely, muramyldipeptide (MDP)-induced activation of NF-κB lacks mononuclear cells in CD patients homozygous for the 1007fs mutation[15]. Conversely, interleukin (IL)-12 production induced by toll-like receptor 2 (TLR2) is negatively regulated in mice by MDP co-stimulation of *NOD2*; although this effect is absent in *NOD2*-/- mice[14]. Moreover, cells obtained from knock-in mice for the 3020insC *NOD2* mutation show enhanced NF-κB activity,

as well as increased production of IL-1β after MDP stimulation[10]. It is noteworthy that this pro-inflammatory phenotype is also associated with an impaired response to *Listeria monocytogenes* challenge[13]. These data show that CD patients' lymphomonocytes have a defect in the stress-induced production of IL-8, which may be responsible for the impaired response to bacteria[16,17].

Taken together, these data suggest a complex pathogenic model of CD, in which genetic factors favor an imbalance between the inflammatory response and the killing of mucosal bacteria. This is similar to the picture observed in some primary immunodeficiencies. Indeed, a histological lesion typical of CD, chronic granuloma, is common also in some deficiencies of the phagocytic immune system such as chronic granulomatous disease, congenital neutropenia and Wiskott-Aldrich syndrome[18,19]. Based on this, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been beneficially used in patients affected by CD, probably through strengthening their natural immunity[20-22], although it shows no direct anti-inflammatory activity.

We thus hypothesized that, besides the inflammatory response itself, the function of the monocyte-derived immune system may be impaired in CD patients because of mutations in the *NOD2* gene. Therefore, we looked for possible defects in monocyte differentiation in CD patients and the relationship with the *NOD2* genotype.

MATERIALS AND METHODS

Patients

The subjects in this study were 47 patients consecutively referred to our institute for CD, 28 males and 19 females, with a mean age of 16.6 (range 4-33) year, and a mean age at diagnosis of 12.8 year (range 1 mo-18 year). All 47 CD patients were sporadic cases. Thirty had active disease and 17 had clinical, echographic and endoscopic remission at the time of analysis. For *NOD2* genotyping, a control group of 69 blood donors was analyzed. For the functional study of monocytes, the control group was 9 healthy adult blood donors (5 males and 4 females, mean age 24.3 years, range 21-38) who tested negative for CD-associated *NOD2* variants. The study was approved by our local independent ethics committee, and informed consent was obtained from all patients (or their parents) and blood donors.

Genetic analysis

Patients were genotyped for R702W and G908R mutations (identified by PCR amplification and enzymatic digestion) and for the 1007fs mutation (analyzed by amplification and sequencing). DNA was extracted from peripheral blood of patients and controls using a Genomix kit (Talent, Italy). PCR reactions were performed using specific primers for the three mutations (sequences are shown in Table 1), Taq polymerase (Amplitac Gold, Applied Biosystems, Foster City, CA, USA) and a thermal cycler Gene Amp 9700 (PE Applied Biosystems). After denaturing at 95℃ for 10 min, amplification was obtained after 35 cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s. For just the 1007fs mutation, 10 amplification cycles at 95℃ for 30 s, 53℃ for 30 s, and 72℃ for 30 s, followed by another 35 cycles at 95℃ for 30 s, 53℃ for 30 s (touch down step: decrease of 0.5℃/cycle), and 72℃ for 30 s. A final step at 72℃ for 7 min was used to stop the reactions. A restriction enzyme digestion assay was performed to detect both R702W and G908R using Msp1 and HhaI, respectively. After digestion, the presence of a wild-type allele resulted in an intact fragment, whereas the variant was characterized by two bands. PCR reaction products underwent electrophoresis on 1.5% agarose gels and were visualized by ethidium bromide staining. Sequencing was carried out for 1007fs detection. Reactions were performed with a Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and on an ABI PRISM 3100 Sequence Detector. Subjects with at least one heterozygous CD-associated variant were categorized as *mtNOD2*, while patients with a homozygous wild type *NOD2* sequence were categorized as *wtNOD2*.

Table 1 Sequence primers used for detection of *NOD2* mutations

Primers	Sequence
Arg702W (forward)	5′-GGCGCCCCTGGAATTC-3′
Arg702W (reverse)	5′-CCTCACCCGGTGCAGC-3′
Gly908Arg (forward)	5′-CCCAGCTCCTCCCTCTTTC-3′
Gly908Arg (reverse)	5′-AAGTCTGTAATGTAAACGCCAC-3′
Leu1007fsinsC (forward)	5′-GAATGTCAGAATCAGAAGGG-3′
Leu1007fsinsC (reverse)	5′-GTCTCACCATTGTATCTTCTTTTC-3′

Cell isolation and dendritic cell generation

To generate *ex vivo* dendritic cells (DCs) from patients and controls, mononuclear cells were isolated by Ficoll separation density-gradient centrifugation, resuspended at a concentration of 2-5 × 10^6 cells/mL in complete RPMI-1640 medium containing 0.1% fetal calf serum (FCS), and allowed to adhere to the well surface of 24-well flat bottom plates (Corning, New York, USA) for 30 min at 37℃ in a 5% CO_2 incubator[23]. After washing twice with PBS to remove non-adherent cells, monolayer cells were cultured for DC differentiation in 0.5 mL RPMI-1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 500 ng/mL GM-CSF (Strathmann Biotec AG, Germany), and 500 ng/mL IL-4 (Strathmann Biotec). After a 3-d culture, 100 μL medium was replaced with a fresh one containing the above-mentioned cytokines. Cell morphology was monitored by light microscopy. Analysis of cell surface marker expression was performed on a suspension of cells harvested on d 8.

Stimulation of DCs

On d 6 of culture, immature DCs were further maturated by adding either 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, Italy) plus 500 ng/mL interferon-γ (INF-γ; Strathmann Biotec) or 500 ng/mL MDP (Sigma-Aldrich) plus 500 ng/mL INF-γ for two more days. After 48 h stimulation, cells were harvested and analyzed by flow cytometry.

Flow cytometry analysis

Cell surface marker expression was evaluated by triple

Table 2 Genotype and allelic frequencies for *NOD2* variants in CD patients and controls

	CD (n = 47)	Healthy controls (n = 69)
Genotype		
Hz	17/47 (36.1%)	4/69 (5.8%)
Double Hz	3/47 (6.4%)	0
mtNOD2	20/47	4/69
wtNOD2	27/47	0/69
Allelic frequencies		
R702W	5.30%	1.45%
G908R	7.45%	0.75%
1007fs	8.50%	0.75%

Table 3 CDAI and drug therapy in *mtNOD2* and *wtNOD2* patients

	mtNOD2 (n = 20)	*wtNOD2* (n = 27)
CDAI	24.5	25.2
Active disease	14	16
Steroids	7	9
Methotrexate	1	1
Azathioprine	4	5
Aminosalicylic acid	6	8
Salazopyrine	4	4
Infliximab	1	1
Thalidomide	4	5

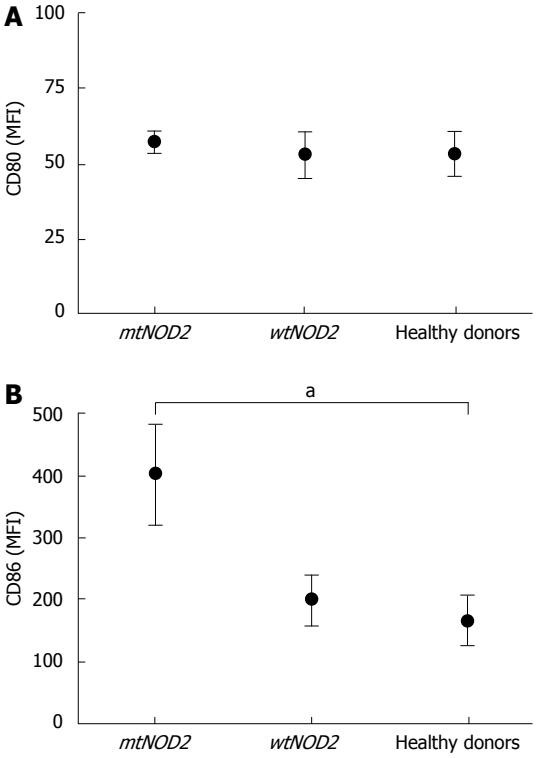

Figure 1 Expression of CD80 (**A**) and CD86 (**B**) in immature DCs, derived from healthy donors, and *mtNOD2* and *wtNOD2* patients. $^{a}P < 0.05$, Student's t test.

immunofluorescence staining with the following monoclonal antibodies: anti-CD80-FITC, anti-CD86-PE, anti-CD83-PE, anti-CD1a-PE, anti-CD33-TC (Caltag Laboratories, Burlingame, CA, USA), anti-CD14-FITC (clone TUK4; Dako Cytomation, Denmark) and anti-HLADR FITC (Becton Dickinson, San Jose, CA, USA). Samples were acquired using a FACScan flow cytometer (Becton Dickinson) and data analysis was performed using CellQuest software (Beckton Dickinson, San Jose, CA, USA). A total of 5000 events were analyzed for each sample.

Cytokine quantification

Supernatants of cell cultures were harvested and stored at -80℃ until measurement of cytokines. Production of IL-12p70 was quantified using an ELISA (Bender MedSystems, Burlingame, CA, USA) according to the manufacturer's instructions.

Statistical analysis

The t test was used to evaluate significant differences. Statistical analysis was performed with the GraphPad Prism program (San Diego, CA, USA), and $P < 0.05$ was considered significant.

RESULTS

NOD2 allelic variants in CD

Seventeen of the 47 CD patients were heterozygous for *NOD2* allelic variants associated with CD, and 3 were double heterozygous for two mutations. The allelic frequencies of R702W, G908R and 1007fs *NOD2* variants in CD patients and healthy donors are shown in Table 2. There was no correlation in our series between disease activity, pharmacological treatment, inflammatory localization and *NOD2* genotype. The mean Crohn's Disease Activity Index (CDAI) and pharmacological treatments are summarized in Table 3.

High CD86 expression in immature DCs derived from NOD2-mutated CD patients

In order to obtain immature DCs, monocytes were first cultured with IL-4 and GM-CSF for 6 d and analyzed for the expression of the two co-stimulatory molecules CD80 and CD86. Under microscopy, the cells from CD patients and controls showed a typical dendritic morphology. Immunocytometry showed no significant differences in CD80 expression in either *mtNOD2* or *wtNOD2* patients as compared with controls. However, CD86 expression was higher in CD patients than in controls, although the difference was not significant; however, it tended to be much higher when *mtNOD2* patients were compared to *wtNOD2* patients and controls ($P = 0.04$) (Figure 1).

Greater effect of LPS as compared to MDP on CD86 upregulation in NOD2-mutated CD patients

After LPS stimulation, terminal differentiation was obtained in DCs in patients and controls. Cells expressed high levels of activation/maturation markers, such as CD83, HLADR and CD1a, without any statistically significant differences among groups (Figure 2). Indeed, DCs from *mtNOD2* patients tended to show higher CD83 expression levels, while those from *wtNOD2* patients presented lower CD1a expression levels. MDP stimulation did not alter these results. Different behavior was shown

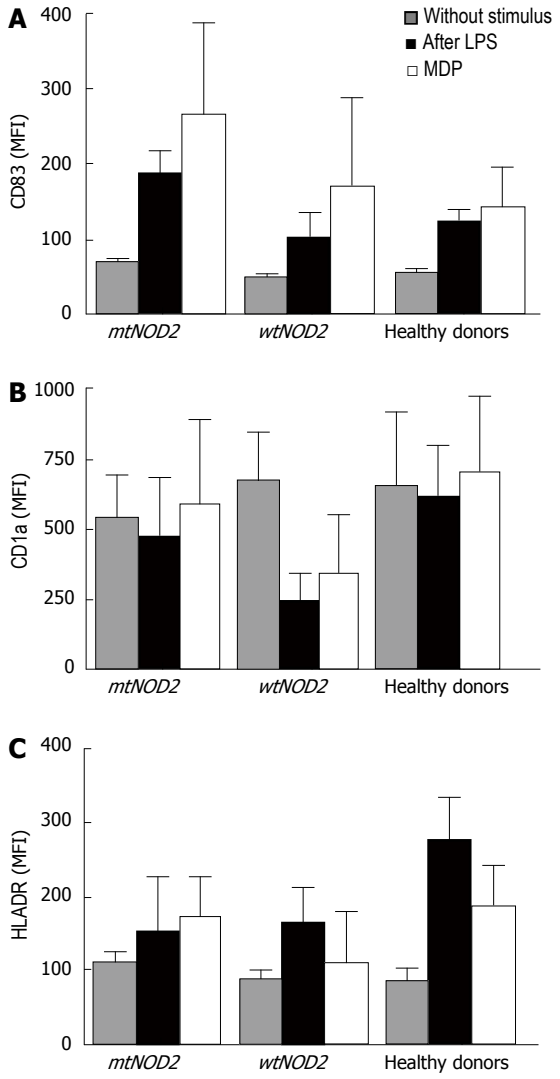

Figure 2 Expression of CD83 (**A**), CD1a (**B**) and HLADR (**C**) in DCs, derived from healthy donors, and *mtNOD2* and *wtNOD2* patients, without stimulus , after LPS or MDP stimulation.

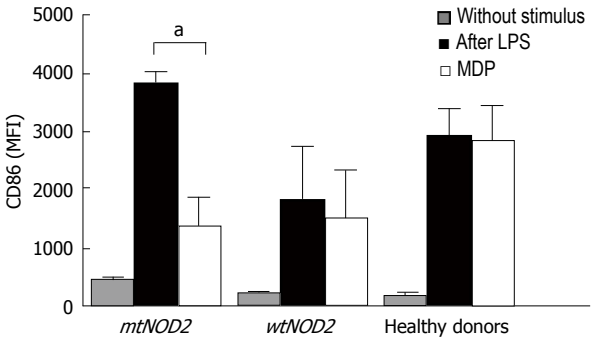

Figure 3 Expression of CD86 in DCs derived from *mtNOD2* and *wtNOD2* patients and healthy donors without stimulus, after LPS or MDP stimulation. [a]$P < 0.05$ LPS- *vs* MDP-stimulated *mtNOD2* patients, Student's *t* test.

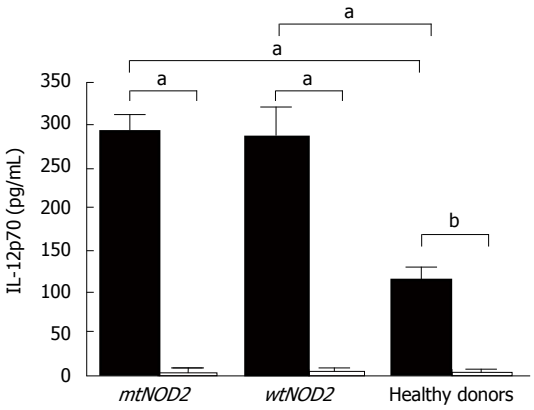

Figure 4 IL-12p70 levels (pg/mL) in monocyte-culture supernatants after stimulation with LPS (■) or MDP (□) in *mtNOD2* and *wtNOD2* patients and healthy controls. [a]$P < 0.05$ LPS- *vs* MDP-stimulated *mtNOD2* patients, LPS- *vs* MDP-stimulated *wtNOD2* patients, LPS-stimulated *mtNOD2* patients *vs* LPS-stimulated healthy donors, LPS-stimulated *wtNOD2* patients *vs* LPS-stimulated healthy donors; [b]$P < 0.01$ LPS- *vs* MDP-stimulated healthy donors; Student's *t* test.

by CD86 in *mtNOD2* patients compared to *wtNOD2* patients and healthy donors (Figure 3). A significantly greater up-regulation of CD86 was shown in *mtNOD2* after LPS stimulation compared to after MDP stimulation ($P = 0.016$). This difference was completely absent in *wtNOD2* and controls.

High levels of IL-12p70 expression in LPS-matured DCs from CD patients

MDP and LPS stimulation of monocyte cultures from patients and healthy donors was evaluated by measuring the production of the bioactive form of IL-12p70 (Figure 4), a major regulatory cytokine of the adaptive immune response. LPS induced higher levels of cytokines in CD patients (whether *mtNOD2* or *wtNOD2*) (mean values ± SD: 291 ± 42 pg/mL in *mtNOD2* and 285 ± 61 pg/mL in *wtNOD2*) in comparison to controls (112 ± 38.2 pg/mL), which showed a statistically significant difference ($P < 0.05$). MDP, on the other hand, was inactive, thus preventing cytokine production in DCs from CD patients (mean values ± SD: 3.2 ± 7.15 pg/mL in *mtNOD2* and 2.8 ± 6.2 pg/mL in *wtNOD2*) and healthy donors (1.94 ± 3.46 pg/mL, $P = 0.0079$).

DISCUSSION

Although several genes involved in CD have been described to date, the pathogenesis of the disease remains largely unknown[24,25]. It is thought that the disease arises from abnormal crosstalk between a changing intestinal flora and the host in the presence of a genetic background able to influence the integrity of the intestinal barrier and/or the functioning of the innate immune response[1,26]. Since the identification of *NOD2* mutations is associated with CD, much attention has been placed on the functions of monocytes in which this gene is constitutively expressed[6]. It has been hypothesized that some slight innate immunity defects may underlie the pathogenesis of CD. Kramer *et al* have recently identified a defect in response to MDP stimulation of DCs obtained from patients with homozygous 3020insC *NOD2* mutations, which suggests that a defect in the production of cytokines like IL-10 plays a role in the pathogenesis of the disease, by diminishing immune tolerance to intestinal bacteria.

In this work, we tested the ability of monocytes from CD patients to differentiate *in vitro* into DCs. The results indicated that monocytes differentiated into DCs using conventional stimuli. However, DCs obtained from CD patients with *mtNOD2* showed some differences in their expression of activation antigens before and after stimulation. These differences are not likely to depend on disease activity or drugs, as the *NOD2* genotype did not influence such aspects in our study (as in other published series). The most striking difference is the elevated expression of CD86 on immature *mtNOD2* DCs. Moreover, the *mtNOD2* group displayed a greater difference in CD86 up-regulation after LPS as compared to MDP stimulation. This was partially in agreement with the observations of Kramer *et al*[27] in patients with a homozygous 3020insC *NOD2* mutation, whose DCs failed to up-regulate CD80 and CD86 upon MDP stimulation. In our experiment, it was of particular interest that the expression of these activation markers was higher in immature cells, thus suggesting a continuous stimulation of these cells *in vivo*. Indeed, a high expression of activation markers on peripheral blood monocytes from CD patients has been reported[28]. Moreover, it is noteworthy that we could see these differences in heterozygous *mtNOD2* subjects when compared to *wtNOD2* CD patients and controls. This suggests some interference of mutated and normal proteins, perhaps in the process of homodimerization[29].

In conclusion, we showed that DCs obtained from patients with mutations in *NOD2* tended to be more activated than those obtained from *wtNOD2* and controls. However, during terminal differentiation, these DCs were less responsive to MDP compared to LPS. This may be the cause of an imbalance between inflammation and the killing ability of monocytes that may be relevant to the pathogenesis of CD.

COMMENTS

Background

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. The incidence of the disease is rising in countries with improving socio-economic conditions. Although several hypotheses have been raised about the environmental factors involved in the risk of CD, the issue remains unresolved. Genetic data have recently identified genes responsible for susceptibility to CD and provided new tools for studying interactions between the immune system and the environment in the pathogenesis of CD.

Research frontiers

Several lines of evidence suggest that the disease may arise from an altered sensing of the microbial environment in the gut by the innate immune system. (1) CD patients produce antibodies against common intestinal commensal microbes. (2) Elemental diet is an effective treatment for CD. (3) Variants of the *NOD2* gene, which is involved in control of inflammatory responses to bacteria in monocytes, confer susceptibility to CD. (4) An inflammatory disease of the gut is typical of most primary immunodeficiencies involving the innate system. (5) Granulocyte monocyte-colony stimulating factor (GM-CSF) has been shown to ameliorate CD. However, the study of *NOD2* mutants has brought controversial results regarding the interpretation of CD pathogenesis. The study of the behavior of CD monocytes can help clarify this issue.

Innovations and breakthroughs

Most previous studies have analyzed *NOD2* mutations in cellular models, and have concluded that the consequences of *NOD2* mutation are either pro-inflammatory (activation of nuclear factor-κB) or a deficiency in response to bacteria. However, the situation *in vitro* is more complex. We demonstrated abnormal behavior of monocytes from CD, with an easier capacity to become activated but with only minor ability to complete differentiation. While other studies have shown a defective differentiation only for *NOD2* homozygous patients, we demonstrated that some differences may be present also in heterozygous patients. We hypothesized that the altered differentiation of monocytes might lead to an imbalance between inflammation and the killing ability of monocytes, and therefore is probably relevant to the pathogenesis of CD.

Applications

This study can help in the understanding of the therapeutic paradox of a disease that can be treated both with anti-inflammatory drugs and with cytokines able to strengthen the innate immune response. Further studies will be needed to determine those CD patients who are more likely to receive benefits from these two different treatment options.

Terminology

wtNOD2 represents the form of the gene without mutations. DCs are the most effective cells in presenting antigens to and stimulating T cells. They can develop from monocytes and histiocytes. Muramyldipeptide (MDP) is the minimal bioactive peptidoglycan motif common to all bacteria, and it is the essential structure required for adjuvant activity in vaccines.

Peer review

Generally this is a well written paper and provides further evidence concerning the effects of *NOD2* mutants on immune responses to bacterial stimuli.

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S- Editor Zhu LH **L- Editor** Kerr C **E- Editor** Liu Y

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World J Gastroenterol 2007 December 14; 13(46): 6197-6202
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Accurate positioning of the 24-hour pH monitoring catheter: Agreement between manometry and pH step-up method in two patient positions

Mehmet Fatih Can, Gokhan Yagci, Sadettin Cetiner, Mustafa Gulsen, Taner Yigit, Erkan Ozturk, Semih Gorgulu, Turgut Tufan

Mehmet Fatih Can, Gokhan Yagci, Sadettin Cetiner, Taner Yigit, Erkan Ozturk, Turgut Tufan, Departments of General Surgery, Gulhane Military Medical Academy, Etlik, Ankara, Turkey
Mustafa Gulsen, Semih Gorgulu, Gastoenterology and Hepatology, Gulhane Military Medical Academy, Etlik, Ankara, Turkey
Mehmet Fatih Can, Gokhan Yagci, Sadettin Cetiner, Mustafa Gulsen, Taner Yigit, Erkan Ozturk, Semih Gorgulu, "Reflux Research Group", Gulhane Military Medical Academy, Etlik, Ankara, Turkey
Correspondence to: Mehmet Fatih Can, MD, Departments of General Surgery, Gulhane Military Medical Academy, Kent Koop Mh. 11.Cd. Guneyce Merkez Sit. A/10, 06370, Batikent Ankara, Turkey. mfcan@gata.edu.tr
Telephone: +90-505-4568779 Fax: +90-312-3045100
Received: July 2, 2007 Revised: September 14, 2007

Abstract

AIM: To investigate the agreement between esophageal manometry and pH step-up method in two different patient positions.

METHODS: Eighteen subjects were included in the study. First, the distance from the nose to the proximal border of the lower esophageal sphincter (LES) was measured manometrically. Then a different investigator, who was blinded to the results of the first study, measured the same distance using the pH step-up method, with the patient in both upright and supine positions. An assessment of agreement between the two techniques was performed.

RESULTS: In the supine position, the measurement of only one subject was outside the range accepted for correct positioning (≤ 3 cm distal or proximal to the LES). In the upright position, errors in measurement were recognized in five subjects. Bland-Altman plots revealed good agreement between measurements obtained manometrically and by the pH-step up method with the patient in the supine position.

CONCLUSION: In the case of nonavailability of manometric detection device, the pH step-up method can facilitate the positioning of the 24 h pH monitoring catheter with the patient in the supine position. This should increase the use of pH-metry in clinical practice for subjects with suspected gastroesophageal reflux disease if our results are supported by further studies.

Key words: pH monitoring; Esophageal manometry; pH step-up method; Gastroesophageal reflux

Can MF, Yagci G, Cetiner S, Gulsen M, Yigit T, Ozturk E, Gorgulu S, Tufan T. Accurate positioning of the 24-hour pH monitoring catheter: Agreement between manometry and pH step-up method in two patient positions. *World J Gastroenterol* 2007; 13(46): 6197-6202

http://www.wjgnet.com/1007-9327/13/6197.asp

INTRODUCTION

Although the number of patients with a confirmed diagnosis with gastroesophageal reflux disease (GERD) is low, the condition is thought to affect many more people worldwide[1]. The evaluation of patients with reflux symptoms often requires using an advisable diagnostic tool both for distinguishing between physiologic and pathologic reflux, and for the purpose of treatment. New diagnostic tests (multichannel impedance monitoring, wireless pH monitoring capsules, *etc.*) have been developed and have become popular over the last decade, especially in leading industrial countries[2]. However, the conventional 24 h ambulatory pH monitoring continues to be the most common diagnostic test for determining pathologic acid reflux in developing countries. Traditionally, the distal pH sensor of the monitoring catheter is positioned 5 cm above the proximal border of the lower esophageal sphincter (LES), since the threshold used to discriminate the diagnostic cut-off level of acid exposure has been validated at this point[3]. The most popular method used for detecting this location is prior esophageal manometry. However, several measures have been employed to accurately determine the location of the LES, since manometric measurement (despite being essential to recognize motility disorders) is time consuming, is an invasive procedure[4-10], is uncomfortable for patients, and

leads to an increase in the cost of the diagnostic work-up.

In the past, the pH step-up method has been used for this purpose, but the results obtained were conflicting[5,11]. It is well known that gravity and body position have an effect on organ position, and in particular abdominal contents[12]. In the present study, we investigated the influence of body position on the location of the pH monitoring catheter during the pH step-up process. We hypothesized that localizing the pH probe with the patient in a supine position was more accurate since it is the same position as is used in manometric measurement.

MATERIALS AND METHODS

Preparation for the study

The study was conducted between May 2004 and July 2005, in a Military Medical Academic Hospital, in the GI Endoscopy and Manometry Laboratory of the General Surgery Department. Eighteen patients with reflux symptoms, but without hiatus hernia, (ten male and eight female), ranging in age from 22 to 68 (median age 46) years, and referred for esophageal manometry and esophageal pH monitoring constituted the study group. All esophageal tests were performed by two physicians, each of whom was blind to the results obtained by the other, however, all therapeutic decisions were made by a multidisciplinary gastroesophageal reflux team.

The tests were explained in detail to each patient, and written consent was obtained. All patients had been investigated endoscopically prior to the study, and only two had esophagitis. Patients with esophagitis had grade B disease according to the Los Angeles Classification[13] and there was no evidence of any serious disease (Barrett's esophagus, carcinoma *etc*.). Esophageal pH monitoring determined the presence of GERD in eleven cases (GERD group) while physiologic acid reflux was observed in the remaining subjects (Non-GERD group). All medications which could potentially impact esophageal motility, LES pressure and acid reflux were discontinued fifteen days prior to the tests.

Study protocol

An eight-lumen polyvinyl catheter with an external diameter of 4.5 mm [four lumens arranged 90º from each other for the most distal openings on the same circle, and the remaining four lumens with openings 5, 10, 15 and 20 cm proximal to the distal one respectively (Solar, MMS B.V. Enschede, The Netherlands)] was used for esophageal manometry. Once perfused and filled with distilled water, each lumen was connected to a pressure transducer-recorder system. Perfusion was maintained by fluid flow of 1 mL/min. After a six hour fast, patients were admitted to the laboratory. Topical anesthesia was applied, and with the patient in the supine position the catheter was introduced through the nose and into the stomach. The distance from the proximal margin of the LES to the nostril, the mean resting pressure and length of the LES, receptive relaxation of the LES, waves of esophageal peristalsis stimulated by wet swallow, and upper esophageal sphincter location were detected by using the stationary pull-through method. Each of the pulling steps was at intervals of 1 cm. The actual location of the proximal margin of the LES was based on the mean location of four radially oriented openings. The catheter was removed after measurements of the upper esophageal sphincter resting pressure and the location site was recorded.

After obtaining the manometric values, a different investigator who was blind to these findings performed the pH study. For this purpose, we used a double electrode ambulatory pH monitoring catheter [first sensor on the tip, and another 15 cm above the first (pH probe meter, MMS B.V. Enschede, The Netherlands)], calibrated with buffer fluids at pH 7.0 and 1.0, immediately prior to the test. The catheter was introduced into the stomach through the nose, with the patient in the upright position and breathing shallow. After confirmation of acidic pH, the catheter was withdrawn gradually until an abrupt rise in pH to > 4.0 (pH step-up) was detected. The pH readings were used to indicate the proximal margin of the LES at the esophagogastric junction. To confirm this point, the catheter was withdrawn at least a further 10 cm and then re-inserted into the stomach. The process was repeated two more times. The mean value of the three readings was used when there was a difference in the three measurements.

All tests after esophageal manometry were repeated with patients in the supine position. Following this stage, the first investigator rejoined the study and positioned the distal electrode of the catheter 5 cm above the upper margin of the LES, based on the actual LES location determined by manometric study. A chest x-ray was obtained to ensure against any bends or rolls. If any bend, roll, or dislocation was noted, the catheter location was re-confirmed after making the necessary corrections. Finally, ambulatory 24 h pH monitoring was initiated, with comments made on the usage rules of the recording machine.

Statistical analysis

The results of statistical analysis are noted in the text as mean ± standard deviation (SD) except for age. To analyze the significance of the results, the SPSS version 11.0 software package program (SPSS Inc, Chicago, IL, USA) was used. All data obtained from manometric measurements were compared between patients with pathologic and physiologic reflux using the Mann-Whitney-*U* test. The results of measurement and analysis with respect to esophageal motility above the LES and upper esophageal sphincter functions were not included in this report. An agreement between methods was assessed using the Bland-Altman analysis. This method tests agreement between two measurements, one of which is generally accepted as the gold standard. In addition, correlation between distances measured manometrically and by pH step-up method was calculated using Spearman's rho test. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Patients referred for esophageal manometry and 24 h ambulatory pH monitoring were identified. None of

Table 1 Comparative results of GERD and non-GERD groups

Variables (mean ± SD)	**GERD group**	**Non-GERD group**
n (male/female)	11 (6/5)	7 (4/3)
Age (yr)	48 (26-68)	43 (22-61)
LES length (cm)	3.27 ± 0.9	2.86 ± 0.7
LES resting pressure, mmHg	10.82 ± 2.6	18.43 ± 4.0[a]
Distance: nose to the PB-LES, cm (by manometry)	42 ± 3.8	41.7 ± 3.5
Distance: nose to the PB-LES, cm (by pH step-up in upright position)	44.7 ± 2.6	43.8 ± 3.5
Distance: nose to the PB-LES, cm (by pH step-up in supine position)	43.4 ± 3.0	42.1 ± 3.3
Clear detection of receptive relaxation of the LES (*n*)	9/11	7/7

[a]$P < 0.05$ *vs* GERD group (Mann-Whitney-*U* test). LES: Lower esophageal sphincter; PB-LES: Proximal border of the LES.

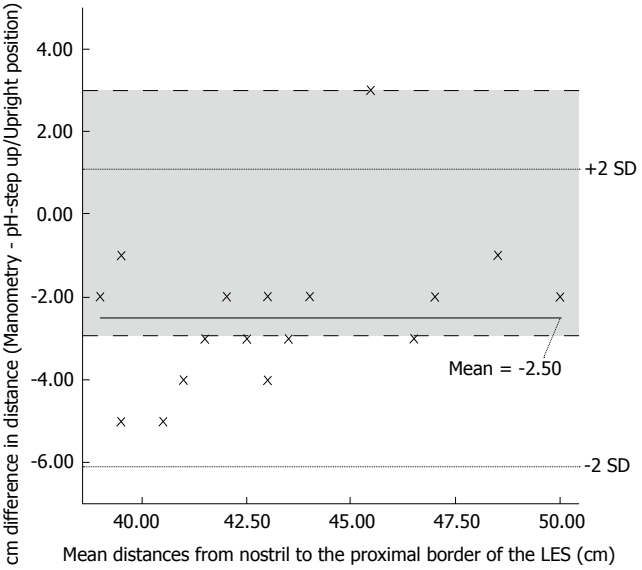

Figure 1 Bland-Altman scatter graph plotted to assess agreement between measurements obtained manometrically and by the pH step-up method with patients in the upright position. Grey area surrounded by longer dashed line indicates clinically acceptable limits.

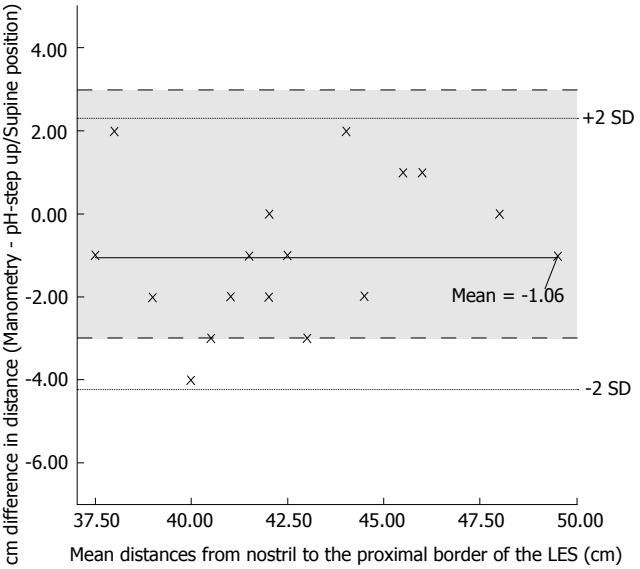

Figure 2 Bland-Altman scatter graph plotted to assess agreement between measurements obtained manometrically and by the pH step-up method with patients in the supine position. Grey area surrounded by longer dashed line indicates clinically acceptable limits.

the subjects had a hiatus hernia, an esophageal motility disorder, or dysfunction of the upper esophageal sphincter. According to the DeMeester reflux scoring system[14], eleven patients had acid reflux above the pathologic level; while the remaining subjects experienced reflux within the physiologic range (data not provided).

The results of comparison between the LES length and the mean resting pressure of the LES are given in Table 1. The LES length was 3.27 ± 0.9 in the GERD group and 2.86 ± 0.7 in the non-GERD group ($P = 0.268$). There was a significant difference between the mean resting LES pressures (10.82 ± 2.6 for GERD group and 18.43 ± 4.0 for non-GERD group, $P = 0.02$). In addition, a receptive relaxation at the level of LES was clearly observed in all but two subjects whose mean resting LES pressures were comparatively lower (7 and 8 mmHg, respectively).

In both study groups, the distance from the nose to the proximal border of the LES, detected manometrically was in the range of 37 to 49 (average 41.89 ± 3.58) cm. The same distance measured by the pH step-up method ranged between 40 and 51 (average 44.39 ± 2.95) cm in the upright position, and between 37 and 50 (average 42.94 ± 3.09) cm in the supine position. Bland-Altman (bias) statistics showed a good agreement between the distances measured manometrically and by the pH step-up method. This agreement was found within clinically acceptable limits (difference ± 3 cm) in measurements obtained with patients in the supine position [difference (mean ± SD): -1.06 ± 1.76, CI 95% for supine position and -2.5 ± 1.82, CI 95% for upright position] (Figures 1 and 2). When the differences are analyzed based on the individual results, a difference of > 3 cm between measurements obtained manometrically and by the pH step-up method in a supine position was observed in only one subject, where as there were five subjects who had a difference > 3 cm in the measurements performed in the upright position. A strong correlation was also noted between the two methods (correlation coefficient: 0.842, $P < 0.0001$ for manometry/pH step-up in the upright position, and correlation coefficient: 0.891, $P < 0.0001$ for manometry/pH step-up in the supine position, respectively, using the Spearman's rho test). A comparison of the GERD *vs* non-GERD patients with respect to the measurement obtained with each method-manometry, pH step-up in an upright position, and pH step-up in a supine position-showed no statistically significant difference between the two groups.

DISCUSSION

Our comparative study of LES function in patients with pathologic *vs* physiologic reflux are in agreement with established data regarding the pathogenesis of GERD. We observed a significant difference in the LES resting pressures of both groups, but not in the LES lengths.

Similar results were reported in a previous study[15]. Another report demonstrated that patients with reflux esophagitis had a lower minimum LES pressure compared with healthy subjects[16]. However, we could not determine the receptive relaxation of the LES during wet swallow in two patients with GERD. This was perhaps due to the presence of very low resting pressures, which made measurement of the relaxation pressure difficult. The results of pH monitoring and the management of patients with pathologic reflux have not been provided in this report.

For an accurate positioning of a pH electrode, esophageal manometry is widely accepted as the gold standard. Almost all of the other methods recommended for precise pH electrode placement (endoscopy, fluoroscopy, transnasal fiberoptic laryngoscopy, *etc.*), require interventions which increase the cost and are more invasive. It is for this reason that we recommend the pH step-up method, which aims at identifying the proximal border of the LES and only requires the use of pH monitoring catheter. Our results are based on the detection of an abrupt rise in the pH as the catheter is withdrawn from the acidic pH of the stomach into the neutral (pH > 4) environment of the esophagus. In contrast to other studies[5,17,18], some reports have stressed that the pH step-up method cannot be used for positioning of the pH electrode in the esophagus[11,19,20]. One of these studies by Marples and colleagues[20], suggested that false positioning of the pH electrode may occur (they detected incorrect positioning of 10 cm above and 16 cm below the LES) if the pH step-up method is used. Pehl and co-workers interpreted this inter-individual variance as a methodological bias, and have explained this on the basis of a lower position of the electrode, presumably because of the inability to detect acidic content secondary to the location of the electrode in the fundic air, when the patient is in the upright position[18]. This explanation supports our results, as it suggests that in the supine position, the withdrawal process may help in the correct positioning of the pH electrode. In fact, it should be recognized that a significant number of physicians interested in GERD and related illnesses have to manage such patients without esophageal manometry, since this test is still not commonly available, especially in community hospitals and small medical centers in developing countries[21]. For example, in the city of Ankara (the second largest city in Turkey), there are ten medical departments using esophageal pH monitoring in adult patients. However, adult participants are investigated by manometry in only five of these centers. This rate is similar to that in pediatric centers; therefore, a correct guide to the use of the pH step-up method is required.

We proposed to answer the following three questions with this study: (1) Could manometric determination of the LES level be safely replaced by the pH step-up method? (2) Does the degree of reliability change with the position of patient during pull-through?, and (3) Is there any difference in the measurements of GERD *vs* non-GERD patients? Esophageal manometry is generally performed when the patient is in a supine position and motionless; this provides the most accurate results from level-oriented pressure sensors. These sensors are properly calibrated prior to each measurement. When an individual reclines from the sitting to the supine position, the fundic air is dispersed throughout the stomach[22], and thus most of the acidic fluid content of the stomach comes into contact with inferior surface of the LES. Therefore, we hypothesized that the distal electrode of the pH monitoring catheter more precisely registers the pH change during the withdrawal process when the patient is in the supine position. Furthermore, as previously reported, LES pressure is higher in the supine position compared to the sitting position, particularly in patients with reflux esophagitis[22]. This may facilitate determining the pH change by preventing the escape of acid fluid into the esophagus, especially in patients whose LES pressure is very low. Despite this, reflux can occur when the patient is in a supine position. Therefore, to avoid a flawed measurement during a possible reflux episode, we repeated the withdrawal process two more times. In another report supporting the reliability of our measurements, the supine position was shown to have no greater influence on the amount of possible acid exposure than that of a 20 degree head-up position in healthy individuals, even if the stomach was full[23]. A previous study by Decktor *et al* showed that the placement of the esophageal pH monitoring catheter across the gastroesophageal junction did not increase gastroesophageal reflux[24]. These findings support our hypothesis with respect to the trustworthiness of the method.

Another possible reason for the difference in the measurements between the upright and supine positions is the effect of gravity on organ placement. It is well known that gravity, plays a significant role in posture and the proprioceptive location of body parts[12]. This effect may change an organ's location if it is sufficiently free from adjacent structures. There is a continuous movement of internal organs, caused by factors such as breathing, postural changes, and muscle contractions, which affects the abdominal contents as well as the diaphragm[25,26]. Although less than that compared to intra abdominal organs, there is measurable movement with changes in posture of organs within the ribcage. The pericardium, a structure that is adherent to adjacent tissues, has been shown to be mobile in the sagittal plane[27]. In light of these observations, it is possible that the LES may have a similar posture-related movement. This would help explain the difference in the measurement between the nostril and the proximal border of LES in the two positions. The present study showed no difference in the distance between nose and the proximal border of the LES between patients with and without GERD. These results were obtained both by the pH step-up technique as well as by the manometric method (Table 1). This finding is of critical importance as it indicates that the pH step-up method can be used in both GERD and non-GERD patients.

Measuring the exact location of the proximal border of the LES is presumably impossible because the LES is a ring that is localized in a diagonal plane in association with complex vector volumes[28]. Therefore, a difference in distance of up to 2 cm may be determined by different radially-oriented openings during manometric measurement. For this reason, an electrode positioned

by any method, at 3 cm above or below the manometric position, is commonly accepted as accurate[11,18]. However, if the electrode is positioned with a difference of 5 cm or more, a significant error in acid detection can occur. Anggiansah and co-workers reported that in nine out of twenty GERD patients, the clinical diagnoses according to the DeMeester reflux scoring system, was altered if the pH electrode was placed 10 cm above the LES[29]. Another study showed that there was a two-fold greater measurement of reflux events if an electrode was positioned at 1 cm compared with 5 cm above the gastroesophageal junction[30]. According to our results, all but one pH step-up measurement (successful in 17 of 18) made in the supine position was in the acceptable range (± 3 cm), whereas five measurements failed to determine the acceptable pH-step up location (successful in 13 of 18) in the upright position. Bland-Altman agreement plots demonstrated the superiority of measurements obtained in the supine position.

These findings clearly indicate the need for accurate placement of the ambulatory pH monitoring catheter with the patient in a supine position rather than in an upright position. However, the relatively small number of patients that were investigated may restrict the power of this conclusion. The total number of individuals tested was low because the study was designed to include subjects without hiatus hernia. Therefore, additional prospective, double-blind trials with larger number of subjects are needed for better understanding of this issue. Although patient position during diagnostic work-up may seem to be of little significance, the overall number of individuals who suffer from suspected reflux symptoms makes this issue quite important. In our opinion, physicians who manage patients with GERD should use this technique in clinical practice, especially in centers where manometric devices are not available.

In summary, we consider 24-h pH monitoring as the most valuable diagnostic tool in GERD; until such time as new methods now under development become widely available. Esophageal manometry is still the most reliable method for determining the proximal border of the LES. However, it has several limitations to its routine use. Hence, we recommend an easy method of placement of the ambulatory pH monitoring catheter. To our knowledge, this is the first study designed to assess the effect of patient's position on pH monitoring catheter location by an evidence-based clinical trial. Further studies with larger number of subjects are needed to confirm these results. We conclude that if the catheter is positioned when the patient is in a supine position rather than an upright position, the results obtained are more accurate. This may increase both the use of pH measurements without manometry and improve the diagnosis rate of GERD in developing regions of the world.

ACKNOWLEDGMENTS

The authors thank Sezgin Ulusahin, Mahsuni Bahar and Nuh Gumus for their support in collecting the data.

COMMENTS

Background

24-h pH monitoring remains the most crucial test to determine pathologic acid reflux in gastroesophageal reflux disease. Accurate placement of 24-h pH monitoring catheter requires prior esophageal manometry that enables physicians to precisely detect the upper border of the lower esophageal sphincter (LES). Manometric measurement is a relatively invasive procedure, is uncomfortable to patients, and leads to increase in the cost as well as the time spent on investigating each patient. The pH step-up method has been recommended for this purpose; however, published reports provide conflicting results on its usefulness. In the present study, we investigated the influence of patient posture on the pH step-up method, which resulted in improvement in the accuracy of the test.

Research frontiers

In an attempt to find an alternative method to esophageal manometry, different workers have recommended other techniques such as endoscopy, fluoroscopy and transnasal fiberoptic laryngoscopy; however, all of these add new difficulties and additional cost to the diagnostic workup. By contrast, the pH step-up method only requires the use of a monitoring catheter, however any alteration in the location of the pH sensor results in false measurement. This fundamental aspect of the study led us to the finding that the location and level of the pH sensor in the esophagus can alter with posture. To date, this finding has not been reported; and therefore, we decided to prospectively investigate the effect of patient posture on the position of pH monitoring catheter.

Innovations and breakthroughs

The present study revealed that the pH monitoring catheter could be more accurately positioned when the examination is performed with patient in a supine position. Bland-Altman (bias) statistics showed a good agreement between the distances measured manometrically and by the pH step-up method; however, this agreement was found within clinically acceptable limits (difference ± 3 cm) only with the measurements obtained with patients in the supine position. The possible factors and effects leading to this result are discussed in the present article.

Applications

The main debate about the use of the pH step-up method is focused on the ability to accurately position the monitoring catheter. The results of the present study suggest that examination carried out with the patient in the supine position increases the likelihood of accurate placement. Despite these encouraging findings, because of the small number of patients included in the present study, further trials with larger study population will be required to confirm our findings. If confirmed, this technical step will help make 24-hour pH monitoring a routine procedure. We believe that catheter placement for ambulatory pH monitoring can be more easily managed by using the pH step-up method.

Terminology

pH step-up: defines a sudden increase in the pH as the pH monitoring catheter is withdrawn from the acidic content of the stomach into the neutral (pH > 4) environment of the esophagus. It indicates that the pH sensor has crossed the gastroesophageal junction. Blant-Altman analysis: This test is used to compare the bias (the mean of the differences) and limits of agreement (bias ± 2 SD of bias) between two methods, one of which is accepted as the gold standard.

Peer review

In this manuscript, the authors ascertained the accuracy of the step-up method in intra-esophageal pH monitoring. The study was well performed and the conclusion is clear and very interesting.

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S- Editor Zhu LH **L- Editor** Anand BS **E- Editor** Wang HF

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World J Gastroenterol 2007 December 14; 13(46): 6203-6207
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Combination of allopurinol and hyperbaric oxygen therapy: A new treatment in experimental acute necrotizing pancreatitis?

Bilgin Comert, Ahmet Turan Isik, Sezai Aydin, Ergun Bozoglu, Bulent Unal, Salih Deveci, Nuket Mas, Esref Cinar, Mehmet Refik Mas

Bilgin Comert, Division of Intensive Care, Departments of Internal Medicine, Gulhane School of Medicine, Ankara 06018, Turkey
Ahmet Turan Isik, Ergun Bozoglu, Division of Geriatric Medicine, Department of Internal Medicine, Gulhane School of Medicine, Ankara 06018, Turkey
Sezai Aydin, Department of Surgery, Guven Hospital, A. Ayranci, Ankara 06018, Turkey
Bulent Unal, Department of Surgery, Numune Training Hospital, Sihhiye, Ankara 06018, Turkey
Salih Deveci, Department of Pathology, Gulhane School of Medicine, Ankara 06018, Turkey
Nuket Mas, Department of Anatomy, Medical Faculty, Baskent University, Ankara 06018, Turkey
Esref Cinar, Department of Infection Disease, Gulhane School of Medicine, Ankara 06018, Turkey
Mehmet Refik Mas, Department of Internal Medicine, Gulhane School of Medicine, Ankara 06018, Turkey
Correspondence to: Dr. Ahmet Turan Isik, Associate Professor, Division of Geriatric Medicine, Departments of Internal Medicine, Gulhane School of Medicine, GATA Geriatri BD, Etlik, Ankara 06018, Turkey. ahmetturanisik@yahoo.com
Telephone: +90-312-3043121 Fax: +90-312-3044000
Received: July 24, 2007 Revised: September 16, 2007

Abstract

AIM: To investigate the individual and combined effects of allopurinol and hyperbaric oxygen (HBO) therapy on biochemical and histopathological changes, oxidative stress, and bacterial translocation (BT) in the experimental rat acute pancreatitis (AP).

METHODS: Eighty-five Sprague-Dawley rats were included in the study. Fifteen of the eighty-five rats were used as controls (sham, Group Ⅰ). AP was induced via intraductal taurocholate infusion in the remaining seventy rats. Rats that survived to induction of acute necrotizing pancreatitis were randomized into four groups. Group Ⅱ received saline, Group Ⅲ allopurinol, Group Ⅳ allopurinol plus HBO and Group Ⅴ HBO alone. Serum amylase levels, oxidative stress parameters, BT and histopathologic scores were determined.

RESULTS: Serum amylase levels were lower in Groups Ⅲ, Ⅳ and Ⅴ compared to Group Ⅱ (974 ± 110, 384 ± 40, 851 ± 56, and 1664 ± 234 U/L, respectively, $P < 0.05$, for all). Combining the two treatment options revealed significantly lower median [25-75 percentiles] histopathological scores when compared to individual administrations (13 [12.5-15] in allopurinol group, 9.5 [7-11.75] in HBO group, and 6 [4.5-7.5] in combined group, $P < 0.01$). Oxidative stress markers were significantly better in all treatment groups compared to the controls. Bacterial translocation into the pancreas and mesenteric lymph nodes was lower in Groups Ⅲ, Ⅳ and Ⅴ compared to Group Ⅱ (54%, 23%, 50% *vs* 100% for translocation to pancreas, and 62%, 46%, 58% *vs* 100% for translocation to mesenteric lymph nodes, respectively, $P < 0.05$ for all).

CONCLUSION: The present study confirms the benefit of HBO and allopurinol treatment when administered separately in experimental rat AP. Combination of these treatment options appears to prevent progression of pancreatic injury parameters more effectively.

Key words: Experimental pancreatitis; Allopurinol; Hyperbaric oxygen

Comert B, Isik AT, Aydın S, Bozoglu E, Unal B, Deveci S, Mas N, Cinar E, Mas MR. Combination of allopurinol and hyperbaric oxygen therapy: A new treatment in experimental acute necrotizing pancreatitis? *World J Gastroenterol* 2007; 13(46): 6203-6207

http://www.wjgnet.com/1007-9327/13/6203.asp

INTRODUCTION

Acute pancreatitis (AP) is an untreatable condition with a wide clinical spectrum ranging from a mild, self-limited disease to severe organ failure[1]. Translocation of enteric bacteria is the most important cause of infection in the pancreatic tissue, and subsequent events such as sepsis and related complications[2,3]. Currently, several treatment options have been proposed for the septic complications of AP.

Hyperbaric oxygen (HBO) therapy has been investigated in several experimental and clinical conditions which cannot be treated with currently available medical

or surgical options[4,5]. HBO has been shown to have bactericidal activity against anaerobic bacteria[6]. In addition, HBO reduces the incidence of bacterial translocation[7]. It also lowers nitric oxide production and enhances several activities including bactericidal action of neutrophils, angiogenesis and wound healing[8]. Lin *et al* showed that repeated HBO therapy in endotoxic rats reduced inflammatory mediators and free radicals, as well as mortality[9]. In a rat model of tourniquet-induced ischemia-reperfusion skeletal muscle injury, HBO attenuated the reperfusion-induced increase in catalase activity and malondialdehyde (MDA)[10]. These studies demonstrated that HBO treatment, especially when administered in repeated doses has antioxidant rather than oxidative effect.

Xanthine oxidase plays an important role in the migration of microorganisms from the intestinal lumen to intra-abdominal spaces in pathological conditions, an event termed bacterial translocation (BT)[11,12]. Allopurinol (ALPL) has antioxidant properties, and previous studies have shown that antioxidant therapy reduces tissue injury and bacterial translocation in experimental pancreatitis[13,14].

The present study was carried out to investigate the individual and combined effects of ALPL and HBO on biochemical and histopathological changes, oxidative stress, and BT during the course of experimental rat pancreatitis.

MATERIALS AND METHODS

The study was approved by the Institutional Animal Use and Care Committee of the Gulhane Medical Academy and performed in accordance with the National Institutes of Health guidelines for the care and handling of animals.

Animals

Eighty-five male Sprague-Dawley rats weighing from 280 to 350 g were obtained from Gulhane School of Medicine Research Center (Ankara, Turkey). Before the experiment, the animals were fed standard rat chow and water ad libitum and housed in metabolic cages with controlled temperature and 12 h light/dark cycles for at least 1 wk.

Induction of pancreatitis

Anesthesia was induced with Sevoflurane (Sevorane® Liquid 250 mL, Abbott, Istanbul, Turkey) inhalation. Laparotomy was performed through a midline incision. The common biliopancreatic duct was cannulated with a 28 gauge 1/2 inch, micro-fine catheter. One microaneurysm clip was placed on the bile duct below the liver and another around the common biliopancreatic duct at its entry into the duodenum to avoid reflux of enteric contents into the duct. One mL/kg of 3% sodium taurocholate (Sigma, St. Louis, MO, USA) was slowly infused into the common biliopancreatic duct, with the infusion pressure maintained below 30 mmHg, as measured with a mercury manometer[15]. When the infusion was finished, the microclips were removed, and the abdomen was closed in two layers. All procedures were performed using sterile techniques.

Study protocol

Fifteen rats had a sham operation and served as the controls (Group Ⅰ). AP was induced *via* intraductal taurocholate infusion in the remaining seventy rats. Five of seventy rats died during the 6 h induction period. All surviving animals were randomization into four groups, six hours after the induction of pancreatitis. Group Ⅱ (n = 17) received saline (1 mL/kg, bid, sc), Group Ⅲ (n = 16) ALPL (200 mg/kg per day, bid, sc)[16], Group Ⅳ (n = 16) ALPL plus HBO (2.8 atmospheric pressure, bid, 90 min each, total 4 sessions)[17] and Group Ⅴ (n = 16) HBO alone. Five rats in Group Ⅱ, three rats each in Groups Ⅲ and Group Ⅳ, and four rats in Group Ⅴ died during the treatment period. Fifty-four hours after induction, all the surviving animals were killed with an intracardiac injection of pentobarbital (200 mg/kg). Data was collected on serum amylase levels, oxidative stress parameters [MDA, superoxide dismutase (SOD) and glutathione peroxidase (GSHPx)], bacterial translocation and histopathologic scores.

Laboratory tests

Blood samples were taken from the heart before the animals were sacrificed for serum amylase levels. A Hitachi 917 auto analyzer (Roche Diagnostics, Germany) was used for the amylase assay. Amylase level was expressed as U/L.

Histopathologic analysis

A portion of the pancreatic tissue from each rat was fixed in 10% neutral buffered formalin and embedded in paraffin. One paraffin section, stained with hematoxylin and eosin, was examined from each animal. Two pathologists who were blinded to the treatment protocol scored the tissues for edema, acinar necrosis, inflammatory infiltrate, hemorrhage, fat necrosis, and perivascular inflammation, in 20 different fields. The scores for each of the histologic abnormalities were added up, with a maximum score of 24, as defined by Schmidt *et al*[18].

Quantitative cultures and bacterial identification

The areas of the pancreas showing macroscopic necrosis and visible mesenteric lymph nodes were excised, weighed, and homogenized. The homogenates were diluted serially, quantitatively plated in duplicate on phenylethyl alcohol and MacConkey Ⅱ agar, and incubated aerobically at 37℃ for 24 h. The bacterial counts were expressed as colony-forming units (cfu/g tissue), and counts of 1000 cfu/g or higher were considered to be indicative of a positive culture. Gram-negative bacteria were identified with the API-20E system (BioMerieux Vitek, Hazelwood, MO, USA). Gram-positive bacteria were identified to the genus level by means of standard microbiologic methods[19,20].

Evaluation of oxidative stress

Pancreatic tissue samples were homogenized in cold KCl solution (1.5%) in a glass homogenizer on ice. The samples were centrifuged and the supernatant was used for the assays described below.

Tissue MDA concentration was estimated by the method of Ohkawa *et al*[21]. The supernatant was

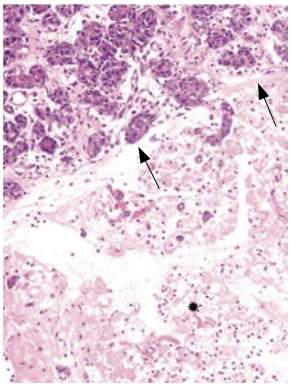

Figure 1 Light microscopy showing extensive necrosis (star) and relatively normal acinar structure (arrows) between the necrotic areas in the control group (HE, × 200).

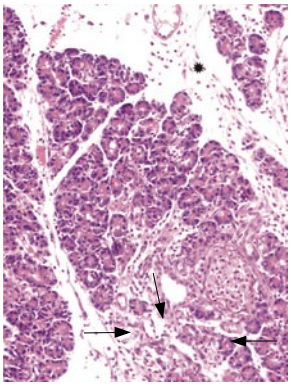

Figure 2 Light microscopy showing interlobular edema (star) and necrotic area (arrows) in the combined treatment group (HE, × 200).

resuspended in 4 mL water, 0.5 mL glacial acetic acid and 0.5 mL 0.33% aqueous thiobarbituric acid solution. The mixture was heated for 60 min in a boiling water bath. After cooling, the complex formed by thiobarbituric acid reactant substances was extracted into an n-butanol phase, and the formed chromogen was measured at 532 nm by spectrophotometer. A standard absorption curve for MDA was prepared using tetramethoxy propane solution. MDA levels were expressed as nmol/g tissue.

For the measurement of SOD activity, the supernatant was diluted 1:400 with 10 mmol/L phosphate buffer, pH 7.00. Twenty five μL of diluted supernatant was mixed with 850 μL of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) in a buffer solution containing 50mmol/L 3-cyclohexylaminol-1-propanesulfonic acid (CAPS) and 0.94 mmol/L ethylenediamine tetraacetic acid (EDTA) at pH 10.2. At this stage, 125 μL of xanthine oxidase (80 U/L) was added to the mixture and the absorbance increase was followed at 505 nm for 3 min against air. Twenty five microlitres of phosphate buffer or 25 μL of various standard concentrations were used as blank or standard determinations. SOD activity was expressed as U/g tissue[22].

For GSHPx measurement, the reaction mixture consisted of 50 mmol/L tris buffer, pH 7.6 containing 1mmol/L of Na_2EDTA, 2 mmol/L of reduced glutathione (GSH), 0.2 mmol/L of reduced nicotinamide adenine dinucleotide (NADPH), 4 mmol/L of sodium azide and 1000 U of glutathione reductase (GR), Fifty microlitres of supernatant and 950 μL of reaction mixture, or 20 μL of supernatant and 980 μL of reaction mixture were mixed and incubated for 5 min at 37℃. The reaction was initiated with 8 mmol/L H_2O_2, and the decrease in NADPH absorbance was followed at 340 nm for 3 min. The enzyme activity was expressed as U/g tissue[23].

Table 1 Serum amylase levels, oxidative stress parameters, bacterial translocation, histopathologic scores, and mortality rates in the different study groups

	Group Ⅰ (n = 15)	Group Ⅱ (n = 12)	Group Ⅲ (n = 13)	Group Ⅳ (n = 13)	Group Ⅴ (n = 12)
Amylase (U/L)	278 ± 44	1664 ± 234	974 ± 110	384 ± 40	851 ± 56
Oxidative stress					
MDA (nmol/g)	12.3 ± 0.4	28.3 ± 0.7	18.2 ± 0.4	16.4 ± 0.4	20.1 ± 0.5
SOD (U/g)	395 ± 7	254 ± 6	345 ± 16	282 ± 8	300 ± 9
GSHPx (U/g)	51.6 ± 2.0	30.8 ± 0.9	35.8 ± 1.5	48.4 ± 0.7	45.6 ± 1.4
Bacterial translocation					
MLNs	2 (13%)	12 (100%)	8 (62%)	6 (46%)	7 (58%)
Pancreas	2 (13%)	12 (100%)	7 (54%)	3 (23%)	6 (50%)
Histopathologic score	2 (1-3)	18 (14.5-19)	13 (12.5-15)	6 (4.5-7.5)	9.5(7-11.75)
Mortality	0/15 (0%)	5/17 (29%)	3/16 (19%)	3/16 (19%)	4/16 (25%)

MDA: Malondialdehyde; SOD: Superoxide dismutase; GSHPx: Glutathione peroxidase; MLNs: Mesenteric lymph nodes.

Statistical analysis

The results of parametric tests were expressed as mean ± SE. Nonparametric values were expressed as median (25-75 percentiles). The significance of differences in the histopathologic scores and serum amylase levels was assessed by the Kruskal-Wallis test. Subgroup analyses were performed by the Mann-Whitney *U* test or *t*-test as appropriate. The significance of differences in oxidative stress parameters was determined by Oneway ANOVA test and Tukey HSD procedure as post hoc test. Probabilities less than 0.05 were considered significant. All statistical measurements were made using SPSS PC ver. 11.05 (SPSS Inc. USA).

RESULTS

All rats except those in Group I developed acute pancreatitis, demonstrated by macroscopic parenchymal necrosis, and abundant turbid peritoneal fluid (Figure 1). Histopathological scores were significantly lower in all treatment groups (Group Ⅲ, Group Ⅳ and Group Ⅴ) compared to Group Ⅱ (13 [12.5-15], 6 [4.5-7.5], 9.5 [7-11.75], 18 [14.5-19]; $P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively). The most favorable results were seen in the combination treatment group (Figure 2, Table 1).

Serum amylase levels were lower in Groups Ⅲ, Ⅳ and Ⅴ compared to Group Ⅱ (974 ± 110, 384 ± 40, 851 ± 56, 1664 ± 234 U/L; $P < 0.05$, $P < 0.001$, and $P < 0.02$, respectively, Table 1). Oxidative stress markers showed significantly lower levels in all treatment groups compared to the controls. Tissue MDA levels in Groups Ⅲ, Ⅳ, and Ⅴ were significantly lower than in Group Ⅱ (18.2 ± 0.4, 16.4 ± 0.4, 20.1 ± 0.5 nmol/g, *vs* 28.3 ± 0.7 nmol/g, respectively,

$P < 0.01$ for all, Table 1). Tissue SOD activity in Groups Ⅲ, Ⅳ, and Ⅴ was significantly higher compared to Group Ⅱ (345 ± 16 U/g, 282 ± 8 U/g, 300 ± 9 U/g, *vs* 254 ± 6 U/g; respectively, $P < 0.01$ for all, Table 1). In addition, GSHPx activity in Groups Ⅲ, Ⅳ and Ⅴ was significantly higher than in Group Ⅱ (35.8 ± 1.5 U/g, 48.4 ± 0.7 U/g, 45.6 ± 1.4 U/g, *vs* 30.8 ± 0.9 U/g; respectively, $P < 0.01$ for all, Table 1). BT to pancreas and mesenteric lymph nodes was reduced significantly in the three treatment groups (Group Ⅲ, Ⅳ and Ⅴ) compared to the control group (pancreatic tissue: 54%, 23%, 50%, *vs* 100%; $P < 0.02$, $P < 0.001$, and $P < 0.002$, respectively; MLNs: 62%, 46%, 58%, *vs* 100%; $P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively, Table 1). Bacterial growth was seen in all tissue specimens obtained from the pancreas and MLNs in the control groups. Of the three treatment groups, combination treatment (Group Ⅳ) was most effective in preventing BT (3/13 [23%] to pancreatic tissue, and 6/13 [46%] in MLNs). The best results in terms of amylase levels, histopathological score, oxidative stress markers and BT were seen in rats receiving the combination treatment, compared to animals receiving a single treatment and the control group. Five rats in Group Ⅱ, three rats each in Groups Ⅲ and Ⅳ, and four rats in Group Ⅴ died before the 54th h of induction of pancreatitis. Mortality rates between groups, except the sham group, were statistically not significant.

DISCUSSION

Pancreatic infection is a serious complication of acute necrotizing pancreatitis. The failure of gut barrier results in bacterial translocation and subsequently septic complication of pancreatitis[2,3,24]. For this reason, prevention of contamination of the necrotic pancreatic tissue is very important, and the new generation antibiotics are of significant advantage in this respect. Although in experimental and clinical studies, the use of antibiotic has been shown to be beneficial[2]; in a randomized, controlled study prophylactic antibiotic therapy was found to have no effect on the mortality[25].

Although the potential role of xanthine oxidase in the presence of barrier failure and translocation of bacteria across the gut lumen has been shown in a previous study[14], the degree to which such a mechanism is involved in the pathogenesis of pancreatic infection is not known, and whether an inhibitor of this enzyme has a preventive effect is not clear[11,12]. The role of HBO therapy in the prevention of infectious complications, mainly through the reduction of oxidative stress and bacterial translocation in experimental acute pancreatitis has been reported previously[7,26]. Our group had previously investigated the efficacy of individual administration of allopurinol and HBO in preventing bacterial contamination of pancreatic tissue. In the present study, we examined the impact of combining allopurinol and HBO therapy[7,14].

We observed that both allopurinol and HBO had beneficial effects on the biochemical and histological abnormalities, oxidative stress and bacterial translocation. The present report represents the first study examining the effects of a xanthine oxidase inhibitor plus HBO therapy in acute pancreatitis. The individual effects of the two treatments on amylase levels were nearly the same. However, HBO treatment resulted in greater reduction in the histopathological scores, while allopurinol alone did not produce satisfactory histological recovery. The histological abnormalities in the combined treatment group were significantly less compared with the use of allopurinol and HBO alone, indicating a potentiation of effect. Allopurinol also decreased the oxidative stress parameters, as it has been reported previously[13,14,27,28], although allopurinol was found to have no effect on the incidence and severity of endoscopic retrograde cholangiopancreatography (ERCP)-induced pancreatitis in studies on human subjects by Budzynska *et al*[29]. When allopurinol was co-administered with HBO at the same doses, the overall antioxidant effect did not increase. These results correlate well with the histological recovery seen in animals treated with individual drugs and combination therapy. However, when the data regarding oxidative stress was examined, it was interesting to note that combination therapy was more effective in increasing the anti-oxidant system. These findings suggest that the improvement in pancreatic morphology was related to the increase in the anti-oxidant system.

Bacterial translocation was very similar in the individual treatment groups. Again, bacterial contamination of the pancreatic tissue was significantly less in the combined treatment group, indicating a potentiating effect. Although xanthine oxidase, an important source of endothelial cell-derived superoxide and hydrogen peroxide, plays a primary role in ischemia-reperfusion injury, which contributes to the failure of the intestinal barrier[11], it can be postulated that even with the addition of antioxidant activity of allopurinol, inhibition of xanthine oxidase was not superior compared to the use of HBO alone. However, a combination of these two agents may produce remarkable inhibition of bacterial translocation, perhaps through different mechanisms including not only xanthine oxidase inhibition and antioxidant activity but also direct antibacterial, immunological, angiogenic and cellular-subcellular effects.

Finally, the present study confirmed our previous observations on the efficacy of HBO and allopurinol in experimental acute necrotizing pancreatitis and also demonstrated that a combination of these treatment options prevented more effectively the progression of pancreatic injury. Nevertheless, the activity and potency of xanthine oxidase, the importance of blocking its activity, and the detailed effects of HBO on this enzyme in the intestines and in the pancreas in acute pancreatitis need further examination.

COMMENTS

Background

The severity of acute pancreatitis may range from a mild, self-limited illness to a catastrophic disease with multiple potentially severe complications and risk of death. Translocation of bacteria from the intestines is one of the most important factors in the development of septic complications and mortality in acute pancreatitis

Research frontiers

Most of the experimental and clinical studies designed to reduce morbidity and mortality in acute pancreatitis are focused on minimizing the extent of necrosis and the prevention of bacterial contamination of necrotic pancreatic tissue.

Innovations and breakthroughs

Several studies have assessed the effect of allopurinol and hyperbaric oxygen on bacterial translocation, oxidative stress, and histology in experimental acute necrotizing pancreatitis. The present study was carried out a rat model to evaluate the effect of combined allopurinol and hyperbaric oxygen treatment on bacterial translocation, oxidative stress and the course of acute necrotizing pancreatitis.

Applications

If these results are confirmed on further studies, combination treatment with allopurinol and hyperbaric oxygen can be applied clinically in patients with acute necrotizing pancreatitis to prevent oxidative stress and bacterial translocation.

Peer review

This paper examines the effects of allopurinol and hyperbaric oxygen on taurocholate infusion-induced acute necrotic pancreatitis in rats. It was observed that both treatments improved the pathological abnormalities, and combination of the two modalities provided further improvement.

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S- Editor Zhu LH **L- Editor** Anand BS **E- Editor** Wang HF

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World J Gastroenterol 2007 December 14; 13(46): 6208-6212
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Early diagnosis and prediction of severity in acute pancreatitis using the urine trypsinogen-2 dipstick test: A prospective study

Erdinc Kamer, Haluk Recai Unalp, Hayrullah Derici, Tugrul Tansug, Mehmet Ali Onal

Erdinc Kamer, Haluk Recai Unalp, Hayrullah Derici, Tugrul Tansug, Mehmet Ali Onal, Department of Surgery, Izmir Ataturk Training and Research Hospital, Izmir 35820, Turkey
Correspondence to: Dr. Erdinc Kamer, Department of Surgery, Izmir Ataturk Training and Research Hospital, 1834 sk. No. 9/4 35530 Karşıyaka- İzmir, Turkey. erdinc.kamer@gmail.com
Telephone: +90-232-2444444 Fax: +90-232-2434848
Received: May 11, 2007 Revised: September 4, 2007

Kamer E, Unalp HR, Derici H, Tansug T, Onal MA. Early diagnosis and prediction of severity in acute pancreatitis using the urine trypsinogen-2 dipstick test: A prospective study. *World J Gastroenterol* 2007; 13(46): 6208-6212

http://www.wjgnet.com/1007-9327/13/6208.asp

Abstract

AIM: To evaluate the use of the trypsinogen-2 dipstick (Actim Pancreatitis) test for early diagnosis and prediction of severity in acute pancreatitis (AP).

METHODS: Ninety-two patients with AP were included in this study. The control group was 25 patients who had acute abdominal pain from non-pancreatic causes. Urine trypsinogen-2 dipstick test (UTDT) and conventional diagnostic tests were performed in all patients. Patients were divided by the Atlanta classification into two groups as having mild or severe pancreatitis.

RESULTS: UTDT was positive in 87 (94.6%) of the AP patients and in two (8%) controls ($P < 0.05$). Positive UTDT was found in 61 (92.4%) of 66 (71.7%) patients with mild pancreatitis and in all (100%) of the 26 (28.3%) with severe pancreatitis ($P > 0.05$). UTDT positivity lasted longer in severe pancreatitis compared with that in mild pancreatitis (6.2 ± 2.5 d *vs* 2.0 ± 1.43 d, $P < 0.05$). The sensitivity, specificity, positive predictive value, negative predictive value (NPV), positive likelihood ratio (PLR) and negative likelihood ratio (NLR) of UTDT were 91%, 72%, 96.6%, 70.4%, 3.4 and 0.1, respectively.

CONCLUSION: UTDT is a simple, rapid and reliable method for use on admission. It has high specificity and low NLR for early diagnosis and prediction of severity in AP. However, its relatively low NPV does not allow trypsinogen-2 dipstick test to be a stand-alone tool for diagnosis of acute pancreatitis; the use of other conventional diagnostic tools remains a requirement.

Key words: Acute pancreatitis; Urine trypsinogen-2 dipstick test; Early diagnosis; Disease severity

INTRODUCTION

Most patients with acute pancreatitis (AP) have a mild and self-limited form of the disease that resolves spontaneously, but approximately 20% of attacks are severe and represented by pancreatic necrosis, sepsis, and fulminant multiorgan/system failure with a life-threatening morbidity and a mortality rate of 20%-30%. Hence, early diagnosis and prediction of severity in AP has particular significance[1-6].

Early prediction of the severity of AP is difficult[3,7]. Multifactorial scoring systems like the Ranson prognostic signs and the Glasgow score can only be evaluated 48 h after admission. The acute physiology and chronic health evaluation Ⅱ (APACHE Ⅱ) score has the invaluable advantage of being useful within a few hours after admission, and it can be assessed serially. However, it is cumbersome, which limits its use in clinical practice[7]. The current gold standard for staging AP combines clinical criteria with computed tomography (CT), but this has limited availability, high costs, exposes the patient to ionizing radiation, and lacks sensitivity and specificity in the early stage of the disease. Several laboratory markers have been evaluated as replacements for the multifactorial scoring systems, with CT being the most widely used[8,9]. Alternatively, magnetic resonance imaging can be used, e.g. in case of contraindications to intravenous CT contrast agents[10]. Various biochemical tests such as the urine trypsinogen-2 dipstick test (UTDT) have been developed over the past ten years for early diagnosis and prediction of severity in AP. Trypsinogen occurs as two major isoenzymes, trypsinogen-1 (cationic) and trypsinogen-2 (anionic), which are secreted at high concentrations into pancreatic fluid with a small proportion escaping into the circulation. Trypsinogen-1 and trypsinogen-2 are eliminated from the blood circulation by the kidneys. In AP, concentrations of trypsinogen-2 in serum and urine are higher than those of trypsinogen-1[11-13]. Although

UTDT has been evaluated in many studies, details of the clinical use of this test for early diagnosis and prediction of severity in AP remain obscure[3,13-15]. The aim of our prospective study was to evaluate the use of the trypsinogen-2 dipstick (Actim Pancreatitis) test in early diagnosis and prediction of severity in AP, and to compare the sensitivity, specificity and prognostic value of the this test with those of serum amylase, serum lipase and APACHE Ⅱ score.

MATERIALS AND METHODS

Materials

The prospective study population consisted of 92 consecutive patients with AP (study group: 69 males, 23 females; median age 58.9 year, range 36-80) and 25 consecutive patients with acute abdominal disease of extrapancreatic origin (control group: 15 males, 10 females; median age 59.2 year, range 34-78) admitted to the emergency unit at Izmir Ataturk Education and Research Hospital between January 2003 and July 2005. The study was approved by the Committee on Research Ethics at our hospital, and all patients gave their informed consent for inclusion in the study.

Study design

Diagnosis of AP was based on a history of prolonged upper abdominal pain, serum amylase at least three times the upper limit of normal and the presence of edema or necrosis on abdominal ultrasonography and/or contrast-enhanced CT. Patients who were admitted after the first 24 h after the onset of abdominal pain were not included in the study. APACHE Ⅱ score values were calculated on admission and at 48 h. Body mass index (BMI) of all patients was calculated. CT was performed selectively in patients with severe pancreatitis as predicted by either one of the two scoring systems (Ranson criteria > 2 or APACHE Ⅱ score > 7). Under the Atlanta classification, AP is predicted as severe if it is accompanied by single or multiorgan failure, local complications, 3 or more on the Ranson criteria, or an APACHE Ⅱ score of ⩾ 8 points[1].

Methods

Serum amylase and lipase concentrations were measured using enzymatic assay (Architect C 8000; Abbott, Abbott Park, IL, USA; reference interval, 26-100 U/L and 13-60 U/L, respectively). The Actim Pancreatitis test strip (Medix Biochemica, Kauniainen, Finland), an immunochromatographic test, was used for urine trypsinogen-2 determination (detection limit 50 μg/L). The tip of the strip was immersed into a urine-containing vial and was held for 20 s before being completely taken out of the vial. The strip was then kept at room temperature for 5 min to observe whether urine reacted with blue latex particles covered by monoclonal antitrypsinogen-2 antibodies. Excess (> 50 μg/L) urinary trypsinogen-2 caused the occurence of 2 blue stripes, while only one stripe (referred to as the control stripe) was observed when urinary trypsinogen-2 concentration was within the normal range. The appearance of the control stripe confirmed the accuracy of the assay, while no blue stripes on the test strip suggested an erroneous test, in which case the test was repeated[16].

Table 1 Diagnosis and/or etiology of pancreatitis in the control and study groups

Control group	*n* (%)	Study group	*n* (%)
Familial Mediterranean fever	5 (20)	Gallstone	58 (63.0)
Acute appendicitis	5 (20)	Idiopatic	18 (19.6)
Acute cholecystitis	4 (16)	Post-ERCP	8 (8.7)
Perforated peptic ulcer	3 (12)	Alcohol	5 (5.4)
Acute cholangitis	2 (8)	Hypertriglyceridemia	3 (3.3)
Pelvic inflammatory disease	2 (8)		
Intestinal obstruction	2 (8)		
Gastric cancer	1 (4)		
Meckel diverticulitis	1 (4)		

ERCP: Endoscopic retrograde cholangiopancreaticography.

Statistical analysis

Data were expressed as the mean ± SE. The Mann-Whitney *U* and McNemar tests were used where appropriate for statistical analysis. All *P* values were two-tailed, and those with $P < 0.05$ were defined as statistically significant. For serum concentrations of amylase and lipase, a threefold increase in the reference values recommended by our laboratory were selected as cut-off values. Using these cut-off points, the sensitivity, specificity, positive (PPV) and negative predictive value (NPV), and positive (PLR) and negative likelihood ratio (NLR) in establishing the diagnosis of AP were calculated. The SPSS/PC 10.0 (SPSS, Chicago, IL, USA) statistical package was used on a personal computer for the analysis of data.

RESULTS

The mean ages of patients in the study and control groups were 58.9 ± 14.2 year ($n = 92$, range, 36-80) and 59.2 ± 13.5 year ($n = 25$, range, 34-78), respectively ($P = 0.928$). No significant difference was found between the two groups in terms of gender (study group, M/F = 69/23; control group, M/F = 15/10; $P = 0.113$). Gallstones were the most common causes of AP ($n = 58$, 63%), while familial Mediterranean fever and acute appendicitis were the most common causes of acute abdominal pain in patients in the control group ($n = 5$, 20% and $n = 5$, 20%, respectively) (Table 1). Mean BMI of patients in the study and control groups were 24.0 ± 3.8 kg/m^2 (range, 16-33) and 23.5 ± 3.4 kg/m^2 (range, 16-30), respectively ($P = 0.902$). Both mean serum amylase concentrations and mean serum lipase concentrations were significantly higher in patients in the study group compared with those in the control [amylase, 600.8 ± 189.7 U/L (range, 376-1250) *vs* 67 ± 32.5 U/L (range, 18-176), and lipase, 96.2 ± 42.4 U/L (range, 58-230) *vs* 33.8 ± 15 U/L (range, 13-65); $P = 0.000$)].

UTDT on admission was positive in 87 (93.5%) of the 92 patients in the study group, and in two (8%) of the 25 patients in the control group ($P = 0.000$). In the control

Table 2 Demographics and results for control and study groups

	Control group (n = 25)	Study group (n = 92)	P
Age (yr)[1]	59.2 ± 13.5 (34-78)	58.9 ± 14.2 (36-80)	0.928
Gender (F/M)	15/10	69/23	0.113
BMI (kg/m^2)[1]	23.5 ± 3.4 (16-30)	24.0 ± 3.8 (16-33)	0.902
Amylase (> 100 U/L)[1]	67 ± 32.5 (18-176)	600.8 ± 189.7 (376-1250)	0.000
Lipase (> 60 U/L)[1]	33.8 ± 15 (13-65)	96.2 ± 42.4 (58-230)	0.000
UTDT +/- (on admission)	2/23	87/5	0.000
UTDT +/- (48 h later)	1/23	87/5	0.000
Duration of UTDT positivity (d)[1]	2.0 ± 1.4 (1-3)	3.6 ± 2.1 (2-13)	0.000
APACHE Ⅱ score (on admission)[1]	4.6 ± 1.5 (3-8)	6.1 ± 2.7 (3-13)	0.006

[1]Values expressed in mean ± SE (inter-quartile range).

Table 3 Demographics and results for control and study groups (Classified as mild and severe AP)

	Control (n = 25)	Mild AP (n = 66)	Severe AP (n = 26)	P^1	P^2	P^3
Age (yr)[4]	59.2 ± 13.5	58.7 ± 14.3	60.2 ± 14.9	0.883	0.796	0.655
Gender (F/M)	15/10	51/15	17/9	0.118	0.776	0.365
BMI (kg/m^2)[4]	23.5 ± 3.4	23.5 ± 3.9	24.2 ± 4.1	0.627	0.507	0.217
Amylase (> 100 U/L)[4]	67 ± 32.5	578.6 ± 171	658.2 ± 222	0.000	0.000	0.068
Lipase (> 60 U/L)[4]	33.8 ± 1	76.0 ± 23.9	110.4 ± 54.2	0.000	0.000	0.000
Positive UTDT (on admission)	2/23	61/5	26/0	0.000	0.000	0.351
Positive UTDT (48 h later)	1/24	61/5	26/0	0.000	0.003	0.351
Duration of UTDT positivity (d)[4]	2.0 ± 1.4	2.6 ± 0.6	6.2 ± 2.5	0.000	0.000	0.000
APACHE-Ⅱ score (on admission)[4]	4.6 ± 1.5	4.7 ± 1.4	9.7 ± 1.8	0.807	0.000	0.000
APACHE-Ⅱ score (after 48 h)[4]	-	4.24 ± 1.4	11.5 ± 2.9	-	-	0.000
Ranson score (on admission) > 2[4]	-	1.03 ± 0.8	3.73 ± 1	-	-	0.000

[1]Control group *vs* mild AP; [2]Control group *vs* severe AP; [3]Mild AP *vs* severe AP; [4]Values expressed as means ± SD.

group, false-positive UTDTs were normalized 1 d later in a patient with acute cholecystitis and 3 d later in a patient with gastric cancer. On the other hand, UTDT positivity lasted for an average of 3.6 ± 2.1 d (range, 2-13) in patients in the study group. APACHE Ⅱ score (cut-off > 8) was 6.1 ± 2.7 (range, 3-13) and 4.6 ± 1.5 (range, 3-8) in the study and control groups, respectively (P = 0.006) (Table 2).

Of the patients with AP, 66 (71.7%) had mild disease and 26 (28.3%) had severe disease according to the Atlanta classification[1]. Gallstones were the most common cause of both mild and severe AP (n = 45, 68.2% and n = 13, 50%, respectively). No significant difference was found between patients with mild and severe AP in terms of BMI (P = 0.217). However, both Ranson and APACHE Ⅱ scores on admission were significantly higher in patients with severe AP than in those with mild AP [Ranson, 3.73 ± 1.04 (range, 2-5) *vs* 1.03 ± 0.82 (range, 0-3), and APACHE Ⅱ, 9.73 ± 1.75 (range, 8-13) *vs* 4.68 ± 1.38 (range, 3-8), respectively; P = 0.000]. Likewise, APACHE Ⅱ score determined at 48 h later was significantly higher in patients with severe pancreatitis than in those with mild pancreatitis (4.24 ± 1.44 *vs* 11.5 ± 2.88, P = 0.000). Sensitivity and specificity of APACHE Ⅱ score on admission were 56.0% and 89.4%, respectively.

Table 4 Sensitivity, specificity, PPV, NPV, PLR and NLR of serum amylase, serum lipase, UTDT and APACHE Ⅱ scoring systems in AP

	Sensitivity %	Specificity %	PPV %	NPV %	PLR	NLR
On admission						
Serum amylase (> 100 U/L)	78.0	87.3	94.8	61.5	6.1	0.3
Serum lipase (> 60 U/L)	86.2	89.4	96.6	76.0	8.1	0.2
UTDT, positive	91.0	72.0	96.6	70.4	3.4	0.1
APACHE Ⅱ > 8	56.0	89.4	61.0	84.2	5.3	0.5

Table 5 Comparisons of sensitivity and specificity of UTDT in AP in present study with those reported previously

Reference	Sensitivity (%)	Specificity (%)
Kemppainen *et al* 1997[29]	94	95
Kylanpaa-Back *et al* 2000[28]	96	92
Lempinen *et al* 2001[5]	62	87
Pezzilli *et al* 2001[27]	53.3	-
Lempinen *et al* 2003[7]	72	81
Chen *et al* 2005[16]	89.6	85.7
Saes *et al* 2005[30]	68	86.4
Present study	91	72

Mean serum amylase concentrations were not significantly different in patients with severe and mild AP (658.2 ± 222.5 U/L *vs* 578.6 ± 171.3 U/L, respectively, P = 0.068), while serum lipase concentrations in patients with severe AP were significantly higher than those in patients with mild AP (76.0 ± 23.9 U/L *vs* 110.4 ± 54.2 U/L, respectively, P = 0.000). UTDT was positive in 61 of the 66 (92.4%) patients with mild AP and in all 26 (100%) patients with severe AP (P = 0.351). No significant difference was found between patients with severe AP and those with mild AP in terms of UTDT positivity at 48 h after admission (P = 0.351). Positive UTDT continuation averaged 2.6 ± 0.6 d (range, 2-4) and 6.2 ± 2.5 d (range, 3-13) in patients with mild and severe AP, respectively (P = 0.000) (Table 3).

In AP, the sensitivity, specificity, PPV, NPV and PLR of serum amylase and lipase were 78%, 87.3%, 94.8%, 61.5% and 6.1, and 86.2%, 89.4%, 96.6%, 76.0% and 8.1 respectively, while UTDT sensitivity was 91%; specificity was 72%; PPV was 96.6%; NPV was 70.4% and PLR was 3.4 (Table 4). Sensitivity and specificity rates of UTDT obtained in our study were compared with those reported in the literature and are listed in Table 5.

DISCUSSION

AP presents in various clinical forms ranging from

mild abdominal discomfort to multiple organ failure. After a mild pancreatitis attack, 80% of patients recover completely, while the disease worsens in 20% and has a mortality rate of 30%[1,17-19]. Thus, early diagnosis and determination of severity of AP are of great importance in terms of mortality and morbidity.

Several methods have been used to diagnose AP and determine its prognosis and severity; these include scoring systems (such as Ranson, Glasgow, and APACHE), biochemical parameters [e.g. serum amylase, lipase, C-reactive protein (CRP), trypsinogen-activation peptide (TAP), interleukins 6 and 8, carboxypeptidase B activation peptide, tumor necrosis factor-α, platelet-activating factor, polymorphonuclear elastase, and serum procalcitonin], and imaging techniques (such as CT)[5,20-22]. The methods that can be used to compare the predictive value of different tests have been summarized in a paper by Jaeschke[23]. What is needed is an immediate test with high specificity and low NLR[24].

In the mid 1990s, urine trypsinogen concentration and TAP were reported to be of high sensitivity and specificity in diagnosing and predicting severity of AP. Since then, determinations of urine trypsinogen concentration and TAP have been considered as good alternative biochemical tests[25,26]. Lempinen et al have compared urinary trypsinogen-2 with urinary TAP and serum CRP for early differentiation between severe and mild AP and concluded that urinary trypsinogen-2 is superior to serum CRP, and is as good as or even better than urinary TAP for the early prediction of disease severity in the first 24 h of admission for AP[7].They have also noted that the result of a trypsinogen-2 dipstick test is available within 5 min, whereas TAP requires a laborious ELISA method, which takes several hours and requires skilled laboratory personnel; the rapid urinary trypsinogen-2 test does not require the use of laboratory equipment[7].

Sensitivity and specificity of UTDT in AP has been reported in the literature as 53.3%-96% and 85.7%-95%, respectively[22,25-31]. We calculated a sensitivity of 91% and specificity of 72% for UTDT. Consistent with a previous report, we found higher sensitivity for UTDT compared with that for serum amylase and lipase concentrations (91% *vs* 78% and 86%, respectively)[4]. However, Pezzilli *et al* reported a low sensitivity for UTDT in their study in which 30 patients with AP were investigated, 11 of whom were included at 2-3 d after onset of the attack[27]. We believe that this late inclusion of a considerable number of patients in the aforementioned study might have affected urinary trypsinogen-2 concentrations and, thus might have decreased the sensitivity and specificity of UTDT for AP diagnosis. In agreement with this view, Chen et al have recently reported a gradually decreasing sensitivity for UTDT in diagnosis of AP from the first to the fourth day of admission (i.e. 90.6%, 81.2%, 59.4% and 50% on the first, second, third and fourth days of admission, respectively)[16]. Considering the effect of late admission (which resulted in delayed UTDT), we did not include patients who were admitted 24 h after the onset of abdominal pain. Thus, we obtained a homogeneous study group in terms of timing of UTDT. We found a positive UTDT in 93.5% and 8% of patients with AP and those with acute abdominal pain due to non-pancreatic causes, respectively. This statistically significant difference supports the use of UTDT in AP diagnosis within the first 24 h of acute abdominal pain.

Chen *et al* have concluded that UTDT can be used in the differential diagnosis of AP due to its high NPV[16]. Lempinen *et al* and Kylanpaa-Back *et al* have reported NPV rates for UTDT of 85% and 99%, respectively[5,28]. We found that the NPV for UTDT was 70.4%, which is higher than the NPV of serum amylase and close to that of serum lipase. We believe that AP diagnosis must be confirmed by other biochemical tests and imaging techniques in patients with a positive UTDT. This is because urinary trypsinogen-2 concentrations may also be increased in other diseases such as hepatobiliary and pancreatic malignancies, colon cancers, and chronic pancreatitis[13]. On the other hand, we found that the PPV (96.6%) for UTDT was higher than that of serum amylase and was equal to that of serum lipase. Thus, we believe that the use of UTDT is advantageous for an early diagnosis of AP because of its rapid action, high sensitivity and high PPV.

UTDT has been reported to have a direct correlation with the severity of AP, as its sensitivity increases with increased severity of AP[4,16]. We have been unable to confirm this conclusion, because in our study we found the sensitivity of UTDT was 87.2% in severe AP and 82.8% in mild AP. However, these rates were not significantly different. On the other hand, a longer duration of UTDT positivity in severe AP compared with that in mild AP was detected. These data suggest that repeating UTDT every day may allow a clinician to predict the severity of AP that varies with time. Thus, such patients will probably benefit from admission to a medical center, prophylactic antibiotic administration, early enteral nutrition, and early endoscopic retrograde cholangiopancreatography in pancreatitis of suspected biliary origin[25].

In conclusion, while there are a few studies on the value of the trypsinogen-2 dipstick test as a predictive test for the severity of AP, its relatively low NPV does not allow UTDT to be a stand-alone tool for diagnosis of AP. Thus, the use of other conventional diagnostic tools becomes an additional requirement. However, UTDT is a simple, rapid and a reliable method that can be used on admission with high specificity and low NLR for early diagnosis and prediction of severity in AP.

COMMENTS

Background

Urine trypsinogen-2 dipstick test (UTDT) is simple, rapid and a reliable method that can be used on admission with high specificity and low negative likelihood ratio (NLR) for early diagnosis and prediction of severity in acute pancreatitis (AP).

Research frontiers

Early diagnosis and prediction of severity in AP is of particular significance.

Innovations and breakthroughs

Although UTDT has been evaluated in many studies, clinical use of this test for early diagnosis and prediction of severity in AP is obscure. The aim of our

prospective study was to evaluate the use of a trypsinogen-2 dipstick (Actim Pancreatitis) test in early diagnosis and prediction of severity in AP and to compare the sensitivity, specificity and prognostic value of this test with those of serum amylase, serum lipase and APACHE Ⅱ score.

Applications

UTDT is a simple, rapid and reliable method that can be used on admission for early diagnosis and prediction of severity in AP.

Terminology

Various biochemical tests, one of which is the UTDT, have been developed over the past ten years for early diagnosis and prediction of severity in AP. Trypsinogen occurs as two major isoenzymes, trypsinogen-1 (cationic) and trypsinogen-2 (anionic), which are secreted at high concentrations into pancreatic fluid with a small proportion escaping into the circulation. Trypsinogen-1 and trypsinogen-2 are eliminated from the blood circulation by the kidneys. In AP, concentrations of trypsinogen-2 in serum and urine are higher than those of trypsinogen-1.

Peer review

The authors evaluated the use of a trypsinogen-2 dipstick (Actim Pancreatitis) test in early diagnosis and prediction of severity in AP. UTDT is a simple, rapid and a reliable method that can be used on admission with high specificity and low NLR for early diagnosis and prediction of severity in AP.

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S- Editor Zhu LH **L- Editor** Kerr C **E- Editor** Liu Y

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World J Gastroenterol 2007 December 14; 13(46): 6213-6218
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effect of Breathwalk on body composition, metabolic and mood state in chronic hepatitis C patients with insulin resistance syndrome

M Vázquez-Vandyck, S Roman, JL Vázquez, L Huacuja, G Khalsa, R Troyo-Sanromán, A Panduro

M Vázquez-Vandyck, S Roman, A Panduro, Molecular Biology in Medicine, Civil Hospital of Guadalajara, Fray Antonio Alcalde, Guadalajara Jalisco, México
M Vázquez-Vandyck, S Roman, A Panduro, Health Sciences University Center, University of Guadalajara, Guadalajara Jalisco, México
JL Vázquez, Epidemiology Department, Regional General Hospital No.110, Mexican Institute of Social Security and Public Health Department, University of Guadalajara, Guadalajara Jalisco, México
L Huacuja, Chronic Degenerative Diseases Institute, Health Sciences University Center, University of Guadalajara, Guadalajara Jalisco, México
G Khalsa, Kundalini Research Institute, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States
R Troyo-Sanromán, Research Unit, Health Sciences University Center, University of Guadalajara, Guadalajara Jalisco, México
Supported by Grant Salud-Conacyt-2004-C01-025 to AP
Correspondence to: Arturo Panduro, MD, PhD, Molecular Biology in Medicine, Civil Hospital of Guadalajara Fray Antonio Alcalde, P.O. Box 2-500, Guadalajara, Jalisco 44280, Mexico. apanduro@prodigy.net.mx
Telephone: +52-33-36147743 Fax: +52-33-36147743
Received: August 8, 2007 Revised: September 15, 2007

Abstract

AIM: To identify the anthropometric, metabolic and mood state in hepatitis C virus (HCV)-infected patients from the west of Mexico and to evaluate the effect of Breathwalk (BW), a combination of walking, synchronized breathing and focussed attention, on those patients.

METHODS: In an experimental study, 17 patients with serological and molecular diagnosis of HCV, not receiving pharmacological treatment, were studied. One hour sessions of BW were practiced 3 times at week for six months. Body composition was assessed by electric impedance. Biochemical profiles and insulin resistance (IR) risk was assessed by conventional methods. Mood state was evaluated with specific and open questions at the beginning and at the end of the program.

RESULTS: Seventy percent of patients were overweight or obese, and 77% of the patients presented with IR at the beginning of the study. Improvements were observed at the 3rd mo, and statistically significant differences were recorded at the 6th mo using the fitness score (76 *vs* 83, $P < 0.01$), in alanine aminotransferase (ALT) (106 ± 93 U/L *vs* 59 ± 32 U/L, $P < 0.01$), total bilirubin (0.09 ± 1 mg/dL *vs* 0.62 ± 0.2 mg/dL, $P < 0.01$), ALT/AST ratio (1.04 *vs* 0.70, $P < 0.01$), triglycerides (165 ± 86 mg/dL *vs* 124 ± 49 mg/dL, $P < 0.01$) and the IR risk (4.0 *vs* 2.7). Most patients (88%) indicated to feel better at the end of BW ($P < 0.01$).

CONCLUSION: Breathwalk has an important effect on body composition, lipid profile and liver enzymes. It is also easy, inexpensive and has a beneficial effect on metabolic and mood state in HCV patients.

Key words: Breathwalk; Chronic hepatitis C; Insulin resistance; Obesity; Quality of life

Vázquez-Vandyck M, Roman S, Vázquez JL, Huacuja L, Khalsa G, Troyo-Sanromán R, Panduro A. Effect of Breathwalk on body composition, metabolic and mood state in chronic hepatitis C patients with insulin resistance syndrome. *World J Gastroenterol* 2007; 13(46): 6213-6218

http://www.wjgnet.com/1007-9327/13/6213.asp

INTRODUCTION

It has been estimated that hepatitis C virus (HCV) has infected more than 170 million people globally and is responsible for up to 70% of all viral chronic infections[1]. Furthermore, obesity, type 2 diabetes (DM2) and insulin resistance (IR) are current health problems that especially affect chronic HCV patients[2-6]. The occurrence of obesity is an additional risk factor that can further deteriorate the health condition of chronic hepatitis C patients[7], with increased insulin resistance[8] and potential development of diabetes and progression to liver fibrosis[9,10]. Also, poor quality of life related to changes in mood, emotional state, and depressions are common findings among chronic HCV patients[11,12]. This is mainly attributed to the impact of the diagnosis and subsequent anxiety over long-term health[12]. This situation may limit the tolerability of antiviral treatment and reduce compliance[11], together with the fact that HCV patients with excess body weight are known to be poor responders to antiviral therapy in comparison to normal-weight individuals[13,14]. Therefore, it is important to search for additional therapeutic strategies

focused on lifestyle habits in chronic HCV patients that can contribute to improve metabolic and liver function as well as better quality of life.

Conventional therapeutic interventions for chronic HCV patients are primarily focused on antiviral regimens with minor attention on lifestyle modifications[15]. For instance, in the past and up to date, it has been stated that patients diagnosed with liver cirrhosis regardless of the stage must remain in a resting state or maintain minimal physical activity. Even when exercise and/or physical activity is a common therapeutic modality recommended for obese, DM2 and IR patients[16,17], studies related to the modality and strategy of exercise that could be beneficial for the patients with chronic liver disease are limited[18]. Breathwalk (BW) is a novel exercise strategy that is different from conventional walking, in fact it synchronizes walking steps with specific breath patterns and mental sustained attention[19].

Since obesity and IR are dependent on genetic and environmental factors of each population, the identification of such variables and implementation of specific strategies are necessary in order to achieve a better quality of life and response to antiviral treatments. The aim of the present study was to measure anthropometric and metabolic parameters as well as the mood state in HCV-infected patients from the west of Mexico and to evaluate the effect of BW on those parameters.

MATERIALS AND METHODS

Patients

In an experimental study, 22 patients with chronic hepatitis C infection attending the Gastroenterology and Molecular Biology in Medicine Departments at the Civil Hospital of Guadalajara were included. Molecular and serological diagnosis of HCV was performed as described before[20]. All patients had serologic and molecular diagnosis (PCR) of chronic hepatitis C virus and were not receiving interferon or any other specific treatment. The Civil Hospital of Guadalajara is one of the biggest public hospitals in Mexico that mainly attends low income patients that have no other social health security. Due to the cost of interferon, the low income level and the large number of patients with HCV attending the hospital, most of them do not receive specific pharmacological treatment. Patients with HCV infection without interferon treatment were invited to participate in the program. Only those volunteers who signed a written consent were included. Clinical evaluations involved identification of the stage of the disease through Child-Pugh score. Patients with decompensated hepatic disease and/or Child C were not included. Twenty two patients remained until after the third month of the program. At this time period, an important improvement was registered, however 5 patients did not continue and dropped out because of the following reasons: two patients were from out of the city and returned to their villages, one patient felt better and decided not to further attend and 2 more patients did not show up. The protocol was conducted in accordance with the Helsinki declaration and with approval by the ethical committee of the hospital.

Body composition

Body composition was measured by electric impedance technique (In Body 3.0, Biospace, Inc). Patients were indicated to fast at least 8 h, to not carry out any type of exercise 5 d before evaluation and to empty the bladder and bowels before testing[21,22]. The parameters evaluated in body composition were body mass index BMI (kg/m^2), percentage of fat and muscle, waist circumference (WC) and fitness score. Patients were classified for risk of metabolic complications associated with obesity, according to WC. High risk of cardiovascular disease was marked at 94 cm of WC for men and 80 cm for women according to WHO[23]. Also the Official Mexican Norm for the classification of BMI[24] was used.

Biochemical profile

Blood samples for measurement of the biochemical profile were obtained after an overnight fast (12 h minimum). The biochemical profile included lipid profile [total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL)], liver function tests aspartate amine transferase (AST), alanine amine transferase (ALT), total bilirubin, glucose and insulin. Routine biochemical test were performed by the use of manual enzymatic assays (Human Gessellschaft für Biochemica und Diagnostica mbH, Germany). Insulin levels were detected by microparticle enzyme immunoassay (Abbott Laboratories, North Chicago, IL). Insulin resistance was measured by the homeostasis model of assessment (HOMA)[25,26] and/or the triglycerides/high-density lipoprotein (TG/HDL) ratio[27].

Evaluation of mood state

Initially and at the end of the BW program, an interview was carried out with specific questions in order to assess the patients' mood regarding their illness during this intervention as described elsewhere[28,29]. Also, an interview was performed with two questionnaires. The first had two types of questions: open and with multiple option answers related to negative and positive mood states. One open question was: How is your mood state actually? Options for positive mood state were: Happy, calm, good in general and negative mood options were: angry, nervous, anguished, fear, bad in general.

The second questionnaire asked specific questions like: "Have you reduced the time spent in your job or other activities?" "Have you been done less of what you like to do?" "Have you done your job or others activities carelessly?" These specific questions had a transformed score after evaluation. Patients were scored in four categories: excellent (100 points), good (66.66 points), regular (33.33) and bad (0 points).

Breathwalk protocol

Breathwalk is an exercise strategy that consists basically of walking with different synchronized breath patterns enhanced with a meditative episode[19]. Physical movements in BW are combined aerobic and resistance exercises. The first and basic tool is a conscious complete deep breathing. While inhaling in a rhythmic and flowing way, air is taken from the stomach and directed smoothly towards the

Table 1 Demographic and clinic characteristics of patients with HCV infection at the beginning of the breathwalking (BW) program

Parameter	HCV group *n* = 17	Reference range
Age (yr)	51 ± 10.2	-
Sex F/M (%)	11/6 (65/35)	-
DM2 (%)	3 (18)	-
BMI (kg/m^2)	27.6 ± 3.2	< 25
Waist circumference (cm)	88.0 ± 10.4	< 80 cm F, < 94 cm M
Fat percentage (mean)	32.3 ± 7.1	< 28% F, < 20% M
Soft lean mass (kg)	44.2 ± 7.9	-
Glucose (mg/dL)	94.0 ± 16.5	< 110
ALT (U/L)	106.7 ± 93.1	< 55
AST (U/L)	101.4 ± 66.6	< 40
Cholesterol (mg/dL)	157.1 ± 35.0	< 200
Triglycerides (mg/dL)	165.0 ± 86.5	< 160
HOMA ratio	3.7 ± 1.8	< 2.5
Metabolic syndrome (%)	10 (59)	[1]

DM2: Type 2 diabetes mellitus; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; F: Female; M: Male; HOMA: Homeostasis model of assessment. [1]NCEP (National cholesterol education program) ATP Ⅲ (adult treatment panel Ⅲ) 2001/2 criteria from metabolic syndrome.

thoracic chest and it finishes in a lightly upward movement of the clavicle and the superior part of the chest. While exhaling, all movements are inverted. This breathing pattern is continually practiced throughout the BW session.

The one hour session is composed of five steps. During the first 10 min, the participant began with a series of specific movements to warm up both arm and leg muscles and to relax the entire body, complete deep breathing as described above, in a synchronized pattern with body movements and was mentally focus on breathing. The following 5 min were dedicated to mentally scan the body posture while walking consciously and breathing patterns were continually synchronized. In the next 25 min, the participant was engaged in a quicker walking rhythm in which inhaling and exhaling patterns are performed at different intervals and combined with silently short-repeated phrases. The walking episode was concluded in the next 5 min by gradually reducing the walking pace. In the final remaining time, a new series of resistance exercises were initiated together with stretching and concluded with an episode of meditative visualization. The physical activity of BW was imparted by a qualified doctor trained in this program. Patients practiced a 1 h session of BW, three times a week for 6 mo.

Statistical analysis

Data and statistical analysis only of the 17 patients that remained until the end of the program were included. Numeric data was grouped by mean ± SD and minimum and maximum as indicated. Results at 3 and 6 mo of the program were compared against the basal using the non-parametric Wilcoxon rank test. A P value less than < 0.05 was considered statistically significant.

RESULTS

Demographic and clinical characteristics of HCV patients that participated in the BW program are shown in Table 1. A higher proportions of women than men is in agreement with the frequency of HCV in the west of Mexico as reported before[30,31]. Normal weight was present only in 29.4% of the patients, overweight in 17.6% and obesity in 53%. Five patients had cirrhosis and the rest had fibrosis and liver steatosis. Waist circumference was 84.9 ± 10.0 cm in women and 97.6 ± 11.6 cm in men. Waist circumference above 80 cm for women and more than 90 cm for men is considered a risk factor for the development of type 2 diabetes mellitus and/or insulin resistance in Mexico[24].

Type 2 diabetes was present in 18% of the patients studied, whereas dyslipidemia was in 41.1%. Two patients had isolated hypercholesterolemia and 5 presented with isolated hypertriglyceridemia. Insulin resistance as determined by the HOMA index was present in 77% of the participants.

Anthropometric measurement in HCV patients at baseline, 3rd and 6th mo of the program are shown in Table 2. In spite of minimal changes in body weight, a reduction in BMI and waist circumference was observed during the program period, as well as a statistically significant increase in the fitness score ($P < 0.001$) at the 3rd and 6th mo.

An improvement in the lipid profile and liver function tests was observed during the BW program (Table 3). A statistically significant reduction in triglycerides was detected at 6th mo ($P < 0.01$). This reduction was

Table 2 Anthropometric measurements in chronic HCV patients at start, 3 and 6 mo of breathwalking (BW) intervention

Parameter	Unit of parameter	Basal	3 mo	6 mo
Weight	kg	69.1 ± 11.0	68.2 ± 10.8	68.4 ± 10.4
BMI	kg/m^2	27.6 ± 3.3	26.7 ± 3.6	26.5 ± 3.0[a]
Fat	%	32.3 ± 7.1	31.6 ± 7.1[b]	31.5 ± 6.6[a]
Muscle	kg	44.2 ± 7.9	45.3 ± 9.4	45.2 ± 9.4
WC	cm	88.0 ± 10.4	87.2 ± 10.0	86.0 ± 9.4[b]
Fitness	Score	76.0 ± 13.4	81.0 ± 3.0[b]	83.0 ± 2.6[d]

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ between baseline and 3 or 6 mo.

Table 3 Biochemical profile in chronic HCV patients at start, 3 and 6 mo of breathwalking (BW) (mean ± SD)

Biochemical parameter	Basal	3 mo	6 mo
Glucose (mg/dL)	94.0 ± 16.5 (77-128)	93.5 ± 21.8 (71-142)	87.2 ± 12.3 (75-121)
ALT (U/L)	106.6 ± 93.1 (18-349)	82.8 ± 63.4 (20-241)	59.4 ± 32.7[b] (17-112)
AST (U/L)	101.4 ± 66.6 (32-265)	87.8 ± 61.8 (20-226)	84.7 ± 61.8 (24-241)
ALT/AST ratio	1.04	0.94	0.70[b]
Total bilirubin (mg/dL)	1.38 ± 1.2 (0.30-5.2)	1.22 ± 1.1 (0.29-4.9)	0.62 ± 0.36[b] (0.0-1.1)
Cholesterol (mg/dL)	157.1 ± 86.5 (103-231)	148.0 ± 38.4 (82-217)	146.0 ± 39.8 (67-205)
Triglycerides (mg/dL)	165.0 ± 86.5 (86-378)	141.9 ± 46.4 (85-210)	124.6 ± 49.2[b] (64-197)
TG/HDL ratio	4.0	3.9	2.7[a]

Data are expressed in mean ± SD. TG/HDL: Triglycerides/high density lipoprotein. [a]$P < 0.05$, [b]$P < 0.01$ between baseline and 3 or 6 mo.

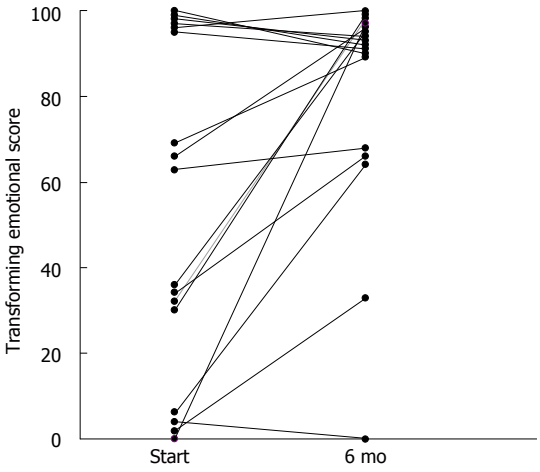

Figure 1 Patients' mood state with respect to disease at the beginning (start) and at the end of breathwalk (BW) after 6 mo. Wilcoxon rank test. $P < 0.01$ between start and 6 mo.

associated with a statistically significant decrease of the TG/HDL ratio. Improvement in liver function tests was documented by a statistically significant decrease in bilirubin ($P < 0.01$) and ALT serum levels after six months ($P < 0.01$), as well as in the ratio ALT/AST ($P < 0.01$).

Viral load was documented only in 7 patients at the beginning and at the end of the BW program. A reduction of the viral load was detected in 4 patients; from 78667 ± 50000 copies baseline levels to 600 ± 0 at the end of BW intervention. Patients that showed an improvement in the viral load had HCV genotype 1b, 2a, 2c and 3. In the other three patients no changes in viral load were detected.

At the beginning of the program, 47% of the HCV patients expressed negative mood states (depression, anger, anguish, fear). At mo 6 of BW, 94% of the patients presented with positive mood states (Figure 1). At the end of BW, the patients report improved emotional state ($P < 0.001$) compared with the beginning in all categories (Table 4). In general, the majority of the patients were motivated towards physical activity, optimistic towards life, and they referred being happy.

Table 4 Mood state based on transformed score evaluation at start, 3 and 6 mo of breathwalking (BW)

Mood state category	Transformed score	Baseline *n* (%)	3 mo *n* (%)[b]	6 mo *n* (%)[b]
Excellent	100	6 (35)	11 (65)	15 (88)
Good	66.66	3 (18)	5 (29)	1 (6)
Regular	33.33	4 (23.5)	0 (0)	1 (6)
Bad	0	4 (23.5)	1 (6)	0 (0)

Wilcoxon rank test; [b]$P < 0.001$ between start and 3 or 6 mo.

DISCUSSION

Obesity and DM2 are an epidemiological problem worldwide, associated with changes in lifestyle. Traditional homeland dietary and physical activity habits have shifted to a mixture of occidentalized and traditional diets combined with more sedentary life. Therefore, the extent of obesity and DM2 in HCV infected patients could be expected to vary in each continent or country mainly due to the diversity of race and environmental factors present in the population. More than fifty percent of the adults are overweight or obese in Mexico[32], and the association of HCV infection with obesity, which by itself is associated with insulin resistance[33], feasibly explains why 77% of the studied patients presented with IR at the time of the study. Hypercholesterolemia and hypertriglyceridemia were the two main dyslipidemia present in 41% of the participants. Indeed, hypertriglyceridemia has been associated with insulin resistance and development of DM2[34]. Therefore, a further rise in DM2 incidence could be expected in these HCV-infected patients in a near future. This reinforces the need to establish specific strategies based on lifestyle changes in order to prevent further metabolic abnormalities or deterioration of liver function.

In order to analyze the effect of BW on lipid profile, only patients that did not vary their dietetic habits during the program were evaluated. A statistically significant decrease in triglycerides at mo 6 of the program was observed as well as the decrease of the TG/HDL ratio. Therefore, BW had an important effect on both types of dyslipidemia and IR without a dietary intervention as seen in other studies[16,17,35]. However, the inclusion of an individualized dietetic program considering their own culture and specific habits could exert a further decrease on dyslipidemia and BMI.

The BW intervention achieved an important effect on the fitness score with a statistically significant increase at 3 mo of the program. The fitness score is an impedance measurement that reflects the muscle/fat ratio, which explains the lack of significant changes in weight, especially during the initial weeks or month of the exercise.

It has been shown that walking requires a longer time period to exert its beneficial effect on lipid metabolism in obese and patients with DM2[36,37]. An advantage that BW has over conventional walking is that it is an aerobic exercise where the patients maintain up to 70% VO_{2max} through rhythmic walking, and during the same session resistance exercise is included and associated with mental attention. Furthermore, it has been shown that anaerobic exercise (exercise load), which could be harmful for the patient with chronic liver disease, does not have significant effect on lipid metabolism at shorter periods of time[38].

An important effect of BW on liver function was also observed in HCV-infected patients. A statistically significant decrease of ALT, the ratio ALT/AST and total bilirubin was detected at mo 6 of the program as well as a decrease of the viral load in four of seven patients, where RNA-HCV was quantified at the beginning and at the end of the program. Little is known about the physiopathological mechanism related to the effect of exercise on the improvement of liver function tests in patients with chronic liver disease. However, even when it is known that exercise can improve insulin resistance at the cellular level[39], it can be speculated that a decrease in fatty liver would cause less liver injury, although further studies are required in order to determine any proposed

mechanism. Also, the small number of analyzed patients with changes in viral load may only indicate the importance of performing further studies with this approach, primarily in those patients that have not responded to antiviral treatment[14] either because of the virus genotype[40] or as a secondary effect of the interferon by itself[41].

At the beginning of the program, only 12% of the patients reported a positive mood state, however, negative mood state shifted towards a positive mood score in 88% of the patients at the end. Most studies focus on analyzing the effect of exercise on metabolic parameters, yet it is unusual to evaluate the emotional state of the patients[42]. The observed significant change in mood state and therefore in quality of life may be attributed to the synchronized breathing patterns combined with walking throughout the BW routine. A meditative state of mind is provoked that allows disconnection from the external environment and liberates negative thought and feeling[43]. Another important factor that may have contributed to the results in this study is the fact that, throughout the six-month program, a close relationship was established between the instructor and the patients. This could have enhanced compliance and adherence of the patients to exercise and therefore to the changes in habits.

The fact that in the past and even in current days exercise has been controindicated in patients with chronic liver disease appears to be proven wrong, since in this study, chronic HCV patients with fibrosis and compensated cirrhosis improved in their metabolic and hepatic profiles at the end of the program. This approach is important because it can be incorporated as a complementary treatment in patients who are candidates for interferon treatment including non-responder patients. However, further studies should be continued to evaluate the effect of an integrative management of the patient that includes antiviral treatment, specific types of exercise, diet and mood state.

In conclusion, this study identifies a specific effect of BW on anthropometric, metabolic and liver functional tests, and demonstrates the importance of the patient's mood state to enhance compliance to the program. Furthermore, this approach could be used in patients living in developing countries, where the cost of the antiviral treatment is expensive and in the mean time government authorities and/or international institutions can aid with financial funding to treat these patients.

ACKNOWLEDGMENTS

We thank Daniel Quezada for his laboratorial and technical support and kind attention to this work and Teresa Salcedo for the typing of the manuscript.

COMMENTS

Background

In the last few years, two different issues have been on top of the table in the hepatology field: the association of obesity and type 2 diabetes in patients infected with hepatitis C virus versus the traditional medical indication of resting in patients with chronic liver disease and, on the other hand, the current problem of the nonresponder patient to antiviral treatments, especially in those who are overweight or obese.

Research frontiers

Whereas it is clear that an increase in physical activity and/or specific exercises are indicated in overweight, obese and type 2 diabetes patients, there are few studies related to the effect of exercise on metabolic and hepatic function parameters in patients with liver disease and even to further approach patient's emotions and well-being. Such strategy intends to involve different research areas so to achieve an integrative evaluation of patients that includes the study of metabolic parameters, liver function tests, emotional state, diet and exercise that are otherwise accessed in an individual manner.

Innovations and breakthroughs

This study represents an initial attempt to use Breathwalk as a specific technique in chronic liver disease patients, and to test in the near future the effect of different physical activity and exercise strategies on metabolic, liver function and anthropometric parameters, as well as emotional states in these patients.

Applications

Breathwalk is an innovative exercise technique that is easy to perform which could be implemented as a tool for patients with chronic liver diseases, especially at early stages of disease and in other chronic pathological conditions such as obesity, metabolic syndrome and type 2 diabetes.

Terminology

Breathwalk: An exercise technique that synchronizes walking steps, specific breathing patterns and focused attention. No-responders: Patients who do not achieve to lower viral loads after administration of the standard doses of antiviral monotherapy or combination therapy after (months) treatment.

Peer review

The manuscript studies the impact of breathwalking exercise on metabolic and fitness paramters in patients with chronic hepatitis C infection. It is interesting and of possible relevance to the field.

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S- Editor Zhu LH **L- Editor** Kremer M **E- Editor** Liu Y

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World J Gastroenterol 2007 December 14; 13(46): 6219-6225
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Inflammatory cytokines suppress arylamine *N*-acetyltransferase 1 in cholangiocarcinoma cells

Benjaporn Buranrat, Auemduan Prawan, Banchob Sripa, Veerapol Kukongviriyapan

Benjaporn Buranrat, Auemduan Prawan, Veerapol Kukongviriyapan, Department of Pharmacology, Faculty of Medicine, Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen 40002, Thailand
Banchob Sripa, Department of Pathology, Faculty of Medicine, Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen 40002, Thailand
Supported by Khon Kaen University Research Fund, Grant from National Science and Technology Development Agency through the Research-Team-Strenghtening Grant Scheme 2006
Correspondence to: Veerapol Kukongviriyapan, Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. veerapol@kku.ac.th
Telephone: +66-43-348397 Fax: +66-43-348397
Received: July 25, 2007 Revised: September 14, 2007

Abstract

AIM: To evaluate the effect of inflammatory cytokines on arylamine *N*-acetyltransferase 1 (NAT1), which is a phase-Ⅱ enzyme involved in the biotransformation of aromatic and heterocyclic amines found in food, drugs and the environment.

METHODS: Human cholangiocarcinoma KKU-100 cells were treated with a mixture of proinflammatory cytokines (interferon-γ, interleukin-1β, and tumor necrosis factor-α) for 48 h, and the effect on NAT1 activity was assessed by high performance liquid chromatography, while *NAT1* expression was determined by reverse-transcription polymerase chain reaction. The oxidative stress on the cells was examined by the formation of nitric oxide, superoxide anion and glutathione (GSH) levels. The cells were also treated with *S*-nitroso-glutathione (GSNO), a nitric oxide donor, to see if the responses were similar to those obtained with the inflammatory cytokines.

RESULTS: Cytokines suppressed NAT1 activity, reducing the V_{max} without affecting the K_m. Cytokines also had a significant impact on the induction of nitric oxide production and in reducing the redox ratios of glutathione (GSH) and GSH disulfide. Treatment with GSNO for 2-48 h reduced NAT1 activity without affecting the GSH ratio. Moreover, inflammatory cytokines and GSNO suppressed NAT1 mRNA expression.

CONCLUSION: These findings indicate an association between inflammation and suppression of NAT1, which perhaps contributes to chemical-mediated toxicity and carcinogenesis.

Key words: Arylamine *N*-acetyltransferase 1; Phase Ⅱ drug-metabolizing enzyme; Inflammatory cytokine; Oxidative stress; Cholangiocarcinoma

Buranrat B, Prawan A, Sripa B, Kukongviriyapan V. Inflammatory cytokines suppress arylamine *N*-acetyltransferase 1 in cholangiocarcinoma cells. *World J Gastroenterol* 2007; 13(46): 6219-6225

http://www.wjgnet.com/1007-9327/13/6219.asp

INTRODUCTION

Arylamine *N*-acetyltransferases (NATs) are well known polymorphic phase-Ⅱ drug-metabolizing enzymes. Human NAT1 and NAT2 are encoded by two closely related *NAT1* and *NAT2* genes[1,2]. NAT1 mRNA and protein are expressed in a wide range of tissues, whereas NAT2 mRNA and protein are present mainly in the liver and the gastrointestinal tract[3]. Biliary epithelial cells do not express NAT2 but retain NAT1 activity[4].

NATs are important enzymes capable of acetylation reactions, which are involved in the detoxification and metabolic activation of various chemicals in drugs, food and the environment[5]. *N*-acetylation is usually considered a detoxification process, because it renders the nitrogen atom less susceptible to oxidation, a process primarily mediated by cytochrome P450 1A2[6]. In contrast, *O*-acetylation is an activation step for heterocyclic and aromatic amines.

Genetic polymorphisms of NAT1 have been identified, but the relationship between genotypes and phenotypes is not clear[7]. Several studies have suggested that NAT1 and NAT2 acetylation polymorphism plays a role in carcinogenesis in humans exposed to certain carcinogenic chemicals[5]. Our previous studies suggested that *NAT1* and *NAT2* polymorphisms may be modifiers of individual risk for cholangiocarcinoma, a cancer of the biliary epithelium[8]. Overexpression of NAT1 in breast tumors is associated with growth properties, as well as chemotherapeutic drug resistance[9] and drug allergy, in particular, cutaneous drug reactions associated with sulfonamides, whereas inactivation of the enzyme may contribute to drug toxicity and cancer risk[10,11]. More recently, NAT1 activity was shown to

be suppressed by oxidant species such as hydrogen peroxide (H_2O_2) and peroxynitrite[12,13]. Peroxynitrite and H_2O_2 irreversibly inactivate NAT1 by oxidation of its conserved catalytic cysteine residue to sulfinic or sulfonic forms[12,13].

Chronic infection and inflammation are important risks factors for several cancers, including cholangiocarcinoma, a highly malignant adenocarcinoma originating from the cholangiocytes. The highest incidence of cholangiocarcinoma worldwide is seen in Northeast Thailand[14]. Infection of the biliary system with liver fluke (*Opisthorchis viverrini*) and possibly exposure to carcinogenic chemicals are believed to be causally related to cholangiocarcinoma[15,16]. The association of cholangiocarcinoma and liver fluke was observed in hospital case series which showed an excessively increased risk in patients with liver fluke infestation. Inflammation of the biliary tract caused by mechanical injury and the release of metabolic products from the flukes, together with the damaging effects of reactive metabolites from endogenous and environmental chemicals have been proposed as the responsible factors[17], which induce alterations in gene expression resulting to cellular hyperproliferation and development of neoplasia[15,16]. In an animal model of cholangiocarcinogenesis, hamster livers infected with liver flukes showed inflammation of the bile duct epithelium, and contained 8-oxo-deoxyguanosine and 8-nitroguanine adducts, which are biomarkers of DNA attack by reactive oxygen and nitrogen species[18]. Thus, inflammatory processes can cause oxidative stress and thereby affect NAT1 activity.

In the present study, we examined the effect of a combination of proinflammatory cytokines on KKU-100 cholangiocarcinoma cells. We also assessed the effect of cytokines on NAT1 activity and the development of oxidative stress; some of these effects were reproduced by a nitric oxide donor.

MATERIALS AND METHODS

Human CCA cell line

The human biliary epithelial cell line KKU-100, derived from intrahepatic cholangiocarcinoma, was established in our institute[19]. The cells were cultured in Ham's F12 containing 4 mmol/L L-glutamine, 1 mmol/L Na-pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin and 10% fetal bovine serum and maintained under an atmosphere of 5% CO_2 at 37℃. The media was renewed every 3 d. The cells were trypsinized with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) and subcultured in the same media. Twenty four hours after subculture, cells at approximately 70% confluence were exposed to a combination of inflammatory cytokines consisting of human interleikin-1β (IL-1β) (1 ng/mL), interferon-γ (IFN-γ) (400 U/mL), and tumor necrosis factor-α (TNF-α) (500 U/mL) (Biosource International Camarillo, CA) for 48 h or S-nitroso glutathione (GSNO, 100 μmol/L) for 2 or 48 h. In experiments determining nitrite production, KKU-100 cells were cultured in non-phenol red medium.

Biochemical assays

Nitrite assay: After treatment of the cell cultures, the accumulation of nitrite in the culture medium was assessed by mixing an equal volume of the medium with Griess reagent (containing 0.1% N-1-napthylethylenediamine in water and 1% sulfanilamide in 5% H_3PO_4). Absorbance was read at 540 nm with an ELISA plate reader.

Superoxide production: KKU-100 cells were cultured in 35-mm dishes with cytokines for 48 h. Cell cultures were washed with Tris-buffered saline (TBS) (10 mmol/L Tris HCl and 150 mmol/L NaCl, pH 7.3) and incubated for 30 min with phorbol-12-myristate-13-acetate (PMA) (0.68 μg/mL) or with N^G-nitro-L-arginine methylester (L-NAME) (100 μmol/L) or for 5 min with NADPH (200 μmol/L). Lucigenin (100 μmol/L) was added to the culture dishes, and chemiluminescence was recorded using a luminometer (Luminometer model 20/20^n, Turner Biosystems, CA).

Assay of GSH and glutathione disulfide: After treatment with cytokines, the cells were trypsinized and washed with cold tris buffer saline (TBS) by centrifugation at 1500 × *g* at 4℃ for 10 min and resuspended in TBS buffer. One hundred microliters of cell suspensions were reacted with 10 μL of 1-methyl-2 vinylpyridinium triflate (M2VP) (3.3 mmol/L) as a GSH scavenger for assay of GSH disulfide (GSSG)[20] or with distilled water for assay of total GSH, and cell suspensions were stored frozen at -20℃ until analysis. Total GSH and GSSG were assayed according to the Tietze method[21]. The amount of reduced GSH was calculated from total GSH and GSSG. Another aliquot of cell suspensions was used to determine protein content in a Bradford dye binding assay with bovine serum albumin as the standard.

Assay of NAT1 activity: NAT1 enzyme activity was assayed using high performance liquid chromatography according to a previously described method[4], with some modifications. Briefly, the cell cultures were washed with TBS and scraped into a microcentrifuge tube with lysis buffer (1X cell lysis buffer containing 1 mmol/L dithiothreitol [DTT] and 0.1 mmol/L phenylmethylsulfonyl fluoride [PMSF]). The cells were vortexed and centrifuged at 12000 × *g* at 4℃ for 30 min. The supernatant (cytosol) was stored in 10% (v/v) glycerol. The cytosol protein was used for assays of protein concentration and NAT1 activity. The reaction mixture consisted of 40 μL cytosol (final concentration 50 μg/mL), 20 μL acetyl CoA-regenerating system (DL-acetylcarnitine, 5.4 mg/mL, carnitine acetyltransferase (1 U/mL) in NAT assay buffer (225 mmol/L triethanolamine HCl, 4.5 mmol/L EDTA, and 4.5 mmol/L DTT, pH 7.5), and 20 μL acetylCoA (final concentration 100 μmol/L). The reaction was initiated by addition of 10 μL *p*-aminobenzoic acid (PABA) in 2.5% dimethylsulfoxide (final concentration 1.25-100 μmol/L) and incubated for 30 min. The reaction was stopped by addition of 10 μL of 15% perchloric acid and centrifuged at 12000 × *g* at 4℃ for 10 min. The supernatant was injected directly onto a high performance liquid chromatography column (YMC-PACK Pro-C_{18}, 5 μm, 150 mm × 4.6 mm; YMC Co., Japan) and

A

Treatment	K_m (μmol/L)	V_{max} (pmol/min per mg protein)
Control	2.8 ± 0.3	211.2 ± 2.9
Cytokine mixture	3.3 ± 0.2	135.7 ± 3.3[a]

Values are shown as mean ± SE.

B

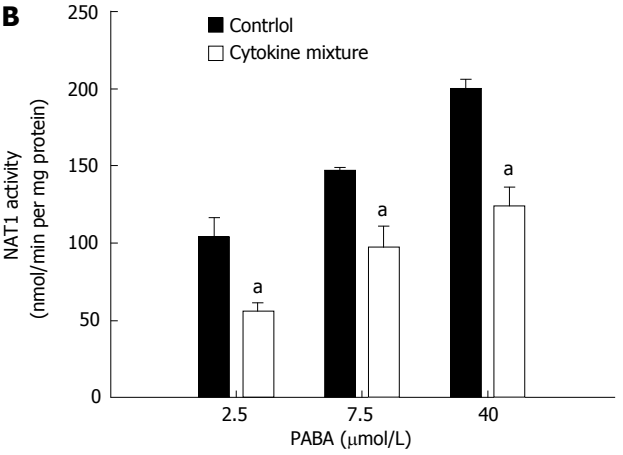

Figure 1 The effect of a mixture of inflammatory cytokines on NAT1 activity in KKU-100 cells. Assays were performed with the cytosol fraction extracted from KKU-100 cells and treated with a combination of cytokines for 48 h. **A**: Kinetic analysis of NAT1 activity; **B**: Activity of NAT1 at various substrate concentrations (PABA) of KKU-100 cells with or without treatment with cytokines. [a]$P < 0.05$ between cytokine treated and control groups.

eluted with a mobile phase consisting of water:acetonitrile: acetic acid:triethylamine:tetrahydrofuran (90.2:8.5:1:0.05:0.25 v/v) at a flow rate of 1.0 mL/min. *N*-acetyl-aminobenzoic acid (Ac-PABA) was detected using a fluorescence detector (Waters 740 scanning fluorescence detector; Waters Corp, Milford) set for excitation at 270 nm and emission at 340 nm. Analytical precision was evaluated by intra- and inter-day assay validation. The coefficients of variation were less than 5% and 10%, respectively. The detection limit of Ac-PABA was less than 1 pmol.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was extracted from KKU-100 cells using Trizol®LS reagent according to the manufacturer's instructions. Total RNA (3 μg) was reverse-transcribed in a 20 μL volume containing 0.5 μg oligo(dT)$_{15}$ primer, 20 units RNasin® ribonuclease inhibitor and ImProm-Ⅱ™ reverse trancriptase (Promega, Madison, WI) in 10x polymerase chain reaction (PCR) buffer, 3 mmol/L $MgCl_2$, and 1 mmol/L dNTPs. The first-strand cDNA was synthesized at 42℃ for 60 min. Reverse transcription products were used as a template for PCR. PCR amplification was performed using specific primers for *NAT1* and farnesyl-diphosphate farnesyltransferase1 *(FDFT1)* as an internal control. The PCR primer sequences were: *NAT1* forward primers, 5'-CCTAGAAGACAGCAAATACCG-3'; *NAT1* reverse primers, 5'-AGCCCACCAAACAGTGA-3' (PCR product: 170 bp); *FDFT1* forward primers, 5' -TTTAACTTC TGTGCTATTCCAC-3'; *FDFT1* reverse primers, 5'-TCTCCAGTCTGAACATAGTC-3' (size of PCR product: 325 bp).

PCR was performed in a final volume of 25 μL containing cDNA template, 1.5 μmol/L of each *NAT1* primer or 0.4 μmol/L of each *FDFT1* primer, 1 U Platinum® Tag DNA polymerase (Invitrogen, Carlsbad, CA), 3 mmol/L $MgCl_2$, and 0.8 mmol/L dNTPs using a Px2 Thermal Cycle (Thermo Electron, Milford, MA). After an initial denaturating step at 94℃ for 5 min, 32 PCR cycles were performed for NAT1 and 28 cycles for FDFT1, as follows: denaturating for 1 min at 94℃, annealing for 1 min at 55℃, and extension for 1 min at 72℃. The final extension was performed at 72℃ for 10 min. The PCR products were separated by electrophoresis on a 3% agarose gel containing ethidium bromide. Gels were visualized and photographed. Band density was analyzed with Gel-Pro3 software. The relative amount of NAT1 mRNA was expressed as a ratio of FDFT1 mRNA.

Statistical analysis

Data are expressed as mean ± SE of duplicate assays from three independent experiments. Student's *t*-test was used to determine significant differences between each experimental group. The level of significance was set at $P < 0.05$.

RESULTS

Effect of the cytokine mixture on the kinetics of NAT1 acetylation

NAT1 activity of KKU-100 cells and Michaelis-Menten constants for PABA *N*-acetylation are shown in Figure 1A. Treatment with the mixture of inflammatory cytokines for 48 h did not affect cell viability (data not shown) but resulted in a significant decrease in $V_{max(apparent)}$ without affecting $K_{m(apparent)}$ (Figure 1A). This implies a change in the amount of enzyme but not in its affinity. The initial velocities of PABA *N*-acetylation by the cytosolic enzyme from KKU-100 cells are shown in Figure 1B.

Effect of the cytokine mixture on production of nitric oxide and superoxide

Since direct application of oxidant species has been reported recently to suppress NAT1 activity, our experiment showed that treatment with a mixture of cytokines could induce oxidative stress by overproduction of nitric oxide, detected by nitrite assay. Whereas basal production of nitric oxide by KKU-100 cells was nearly absent, production was greatly stimulated by inflammatory cytokines (Figure 2A).

To determine if KKU-100 cells were capable of releasing superoxide anion, aggravating oxidative stress, the cells were cultured with a mixture of cytokines. The cells exhibited low basal release of superoxide; the levels increased slightly after cytokine treatment (Figure 2B). To determine whether the low levels of superoxide were due to scavenging subsequent to overproduction of nitric oxide, the cells were treated with a nitric oxide synthase inhibitor. Inhibition of nitric oxide synthases by L-NAME did not increase superoxide levels relative to the control or baseline values (Figure 2B). When the cells were incubated with NADPH, the substrate of NADPH oxidases, superoxide production in the control and cytokine-treated groups was greatly increased and reached similar levels. Furthermore, treatment with PMA, a protein kinase C activator, resulted in a marked increase in superoxide production in the cytokine-treated group but not in the controls (Figure 2B).

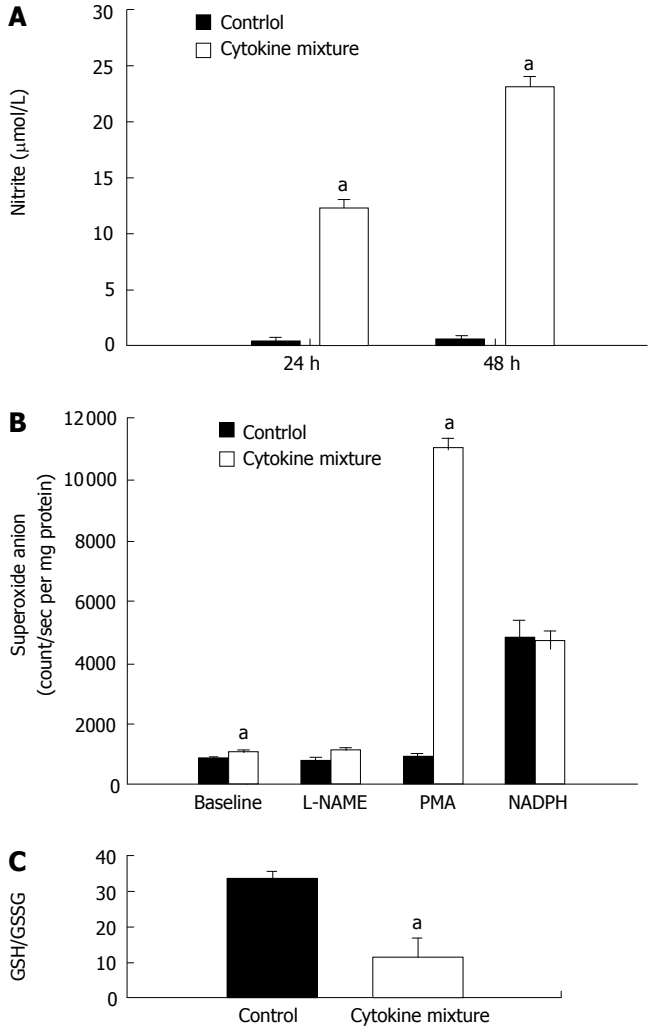

Figure 2 Oxidant status of KKU-100 cells after exposure to a mixture of inflammatory cytokines. **A**: Stimulation of nitric oxide production, assayed as nitrite levels. Cultured media was collected at 24 and 48 h after exposure; **B**: Superoxide formation. Cell cultures were washed and incubated with PMA (0.68 μg/mL), L-NAME (100 μmol/L), or NADPH (200 μmol/L), and superoxide production was measured using the chemiluminescence method; **C**: Redox status was assessed as the GSH and GSSG ratio. Results are presented as mean ± SE from 3 separate experiments. $^{a}P < 0.05$ between cytokine treated and the respective control groups.

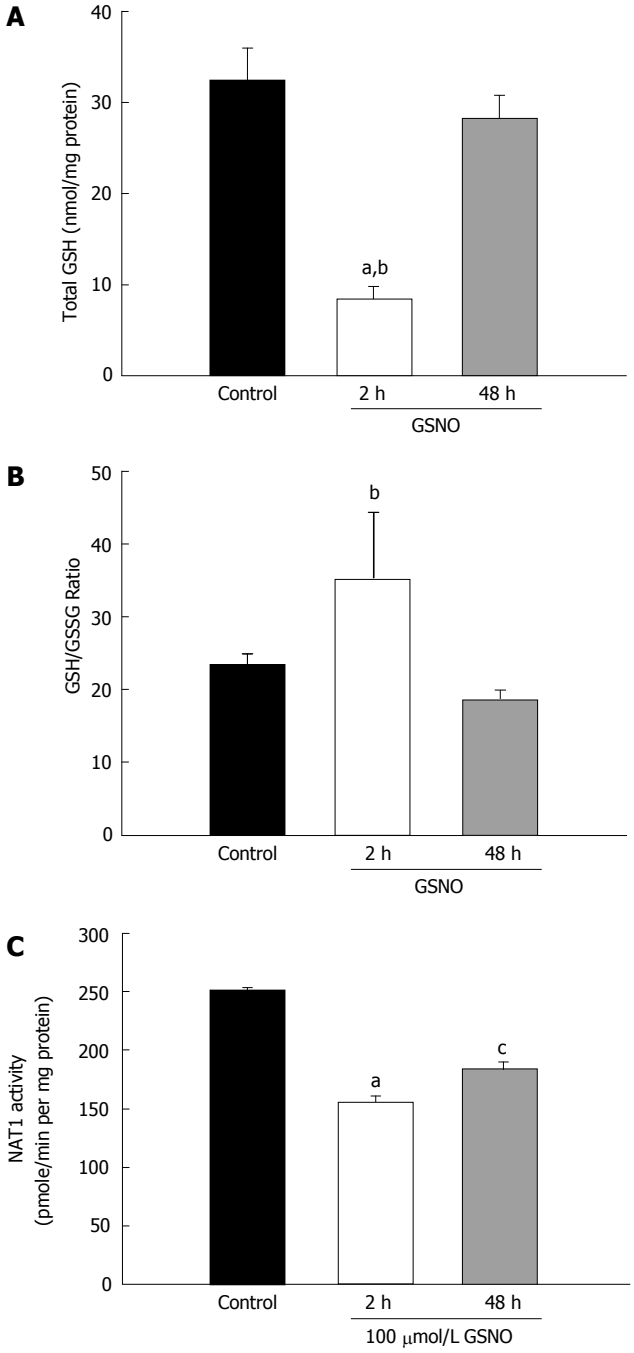

Figure 3 Effect of treatment with the nitric oxide donor GSNO on KKU-100 cells. Cell cultures were treated with 100 μmol/L GSNO for 2 and 48 h. **A**: Total GSH in the cells; **B**: GSH/GSSG ratio; **C**: Activity of NAT1 PABA-acetylation. Bars represent the mean ± SEM from 3 separate experiments. $^{a}P < 0.05$ *vs* control groups, $^{c}P < 0.05$ *vs* the 48-h treatment group.

Effect of the cytokine mixture on GSH levels

Since treatment with inflammatory cytokines may result in the formation of free radicals, further experiments were performed to determine if treatment will alter GSH levels and the oxidative status. KKU-100 cells exposed to the cytokine combination for 48 h showed no significant change in the total GSH levels (control: 36.7 ± 2.8 nmol/mg protein, cytokine-treated: 31.9 ± 14.5 nmol/mg protein). However, there was a marked reduction in redox status (GSH/GSSG ratio; Figure 2C) of the treated cells.

Effect of GSNO on NAT1 activity and redox status

The above noted experiments demonstrated that the mixture of cytokines induced nitric oxide production and oxidative stress. The next experiment investigated whether a nitric oxide donor would produce similar results. Treatment with GSNO elicited a very large decrease in total GSH within 2 h; however, the GSH levels normalized to the control levels at 48 h (Figure 3A). There was no significant change in the GSH/GSSG ratio after GSNO treatment (Figure 3B). However, treatment with GSNO reduced NAT1 activity as early as 2 h, and the suppression persisted at 48 h (Figure 3C).

Effect of the cytokine mixture and GSNO on expression of NAT1

Reverse transcription PCR was used to assess the effects of proinflammatory cytokines and nitric oxide donors on the expression of NAT1 mRNA. The results are shown in

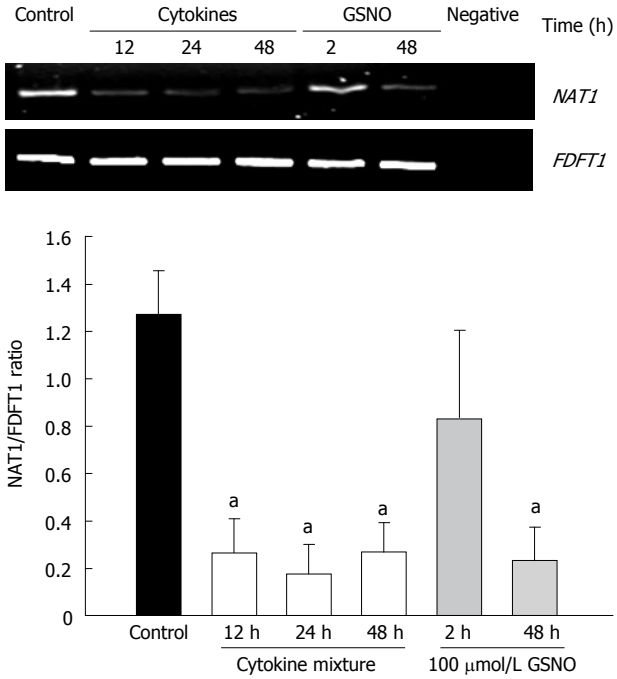

Figure 4 Effect of the cytokine mixture and a nitric oxide donor on the expression of NAT1 in KKU-100 cells. KKU100 cells were incubated with a mixture of cytokines for 48 h or with 100 μmol/L GSNO for 2-48 h. Cells were harvested, and RNA was extracted and analyzed by reverse transcription PCR using FDFT1 as an internal control. $^{a}P < 0.05$ *vs* controls.

Figure 4. Cytokine treatment elicited a decrease in NAT1 mRNA levels. Similarly, GSNO resulted in reduction of the NAT1 mRNA at 48 h, but not at 2 h.

DISCUSSION

The catalytic activity of NAT enzymes is dependent on a reactive cysteine residue, since its activity is inhibited when this residue is modified by *N*-hydroxy-arylamine compounds, hydroxamic acids[22], enzyme substrates[10], as well as oxidant species[12,13,23]. Oxidative stress and chronic inflammation are inseparable such that inflammation inevitably produces oxidant species, resulting in tissue damage[24]. The present study shows that proinflammatory cytokines suppress NAT1 activity similar to treatment with oxidant species[12,13]. Kinetic analysis of NAT1 showed a significant decrease in V_{max}, suggesting that the reduction of NAT1 activity was due to a decrease in the enzyme level. This alteration was probably due to enzyme inactivation or suppression of *NAT1* expression.

Inflammatory cytokines induce the expression of inducible nitric oxide synthase (iNOS) and increase nitric oxide production in cholangiocytes[25]. Our results are consistent with this finding, in fact the basal nitric oxide production was very low but the production increased markedly after treatment with inflammatory cytokines. It is plausible that induction of nitric oxide formation is partly responsible for the suppression of NAT1 activity. Treatment with a nitric oxide donor inhibited NAT1 in a manner similar to cytokine treatment, supporting the possibility that nitric oxide and perhaps peroxynitrite play an important role in modulation of NAT1 activity.

A previous study showed that treatment of breast cancer cells with peroxynitrite irreversibly inactivated NAT1[12]. Peroxynitrite is formed by a reaction between nitric oxide and superoxide anion. In the present study, inflammatory cytokines did not induce a large increase in superoxide, even when nitric oxide formation was inhibited by L-NAME. However, NADPH oxidases, which are membrane bound enzymes that require assembly of subunits from cytosol to become fully functional, are responsible for the formation of superoxide in phagocytic and nonphagocytic cells[26]. They may be upregulated by inflammatory cytokines, as has been shown in smooth muscles and kidney cells[27]. The NADPH oxidases in KKU-100 cells may also be upregulated, however stimulation by PMA, a protein kinase C activator that mediates phosphorylation and recruitment of oxidase subunits[26] may be required to render the NAT1 enzyme fully functional. Together, these findings suggest that superoxide and perhaps peroxynitrite did not play a major role in the cytokine-induced suppression of NAT1 activity in KKU-100 cells. Nevertheless, the role of superoxide and peroxynitrite *in vivo*, a situation where infiltrating macrophages are present, has not been investigated.

The ability of inflammatory cytokines to induce oxidative stress was clearly evident in the present study, as there was a marked increase in pro-oxidant status, evidenced by a decrease in the redox ratio of GSH/GSSG in cultured cells exposed to the mixture of cytokines. In contrast, treatment with GSNO did not affect the redox ratio at any time point. However, total GSH, representing the major intracellular antioxidant pool, decreased substantially after GSNO treatment, especially at the 2-h time point. Altogether, the differences in the effects of cytokines and GSNO suggest that the effects of the cytokine mixture were mediated by nitric oxide and redox-sensitive pathways.

A recent report has shown that intrahepatic cholangiocarcinoma seen in liver fluke endemic areas is characterized by altered expression of drug metabolizing genes, whereas that from non-endemic areas such as Japan shows alteration in the growth factor signaling genes[17]. This may indicate that drug metabolizing genes are involved in the metabolism of potential carcinogenic chemicals. However, information regarding NAT1 expression is currently not available. Regulation of *NAT1* expression has been under study for a long time[23,28]. Previous studies have shown that suppression of NAT1 activity by substrates or oxidant species is due to direct inhibition of the enzyme molecules[13,29], since the expression of NAT1 mRNA does not show any alteration[29]. Our study is the first to demonstrate that treatment with a mixture of cytokines suppresses NAT1 mRNA expression. Using deletion mutant constructs, a promoter site for basal *NAT1* expression was identified[30] comprising of an activator protein 1 (AP-1) binding site. Transcription factor AP-1 is upregulated or downregulated by oxidative stress and inflammatory cytokines, depending upon the oxidant levels and the type of inflammatory cytokines[31,32]. Suppression of *NAT1* expression in KKU-100 cells may involve down regulation or inactivation of AP-1, although specific evidence in this regard remains

to be established. Direct inhibition of NAT1 by oxidant species (nitric oxide) cannot be ruled out, particularly in *in vivo* conditions; this may occur concurrently with suppression of expression.

In summary, treatment with inflammatory cytokines suppresses NAT1 activity and mRNA expression in cholangiocarcinoma KKU-100 cells. This suppression was associated with oxidative stress and nitric oxide production. These findings show that inflammation can suppress NAT1, a key cellular defense enzyme. Moreover, such a suppression may be implicated in drug toxicity and cancer risk.

ACKNOWLEDGMENTS

Auemduan Prawan was a research fellow of Thailand Research Fund through the Basic Research Grant scheme.

COMMENTS

Background

Arylamine *N*-acetyltransferases-1 (NAT1) is an important phase Ⅱ drug metabolizing enzyme that is constitutively expressed in most tissues. Its activity is inactivated by oxidant species.

Research frontiers

NAT1 expression has been demonstrated in cholangiocarcinoma cells (CCA), and polymorphism of NAT genes has been implicated as a risk factor for cancer. Inflammation of the bile duct resulting from opisthorchiasis may alter the activity of the drug metabolizing enzymes including cytochrome P450 and NAT1. Modulation of NAT1 activity may be implicated in drug induced-toxicity and carcinogenesis.

Innovations and breakthroughs

Suppression of NAT1 activity and gene expression is associated with cytokine-induced oxidative stress. A combination of proinflammatory cytokines induces changes in cellular redox and nitric oxide production in CCA and these may be involved in down-regulation of NAT1.

Applications

NAT1 activity could be modulated by inflammation and this may be associated with drug induced toxicity and carcinogenesis.

Terminology

Opischorchiasis: liver fluke (Opisthorchis viverrini) infection of the bile duct.

Peer review

This manuscript describes the intriguing idea that inflammatory cytokines, which are known to be upregulated in cholangiocarcinoma, may function in part by suppressing the activity and/or expression of NAT1, an enzyme thought to be involved in the detoxification of xenobiotics. The authors also show a parallel increase in oxidative stress and nitric oxide after treatment with inflammatory cytokines. Treatment of the cell line with nitric oxide donors also shows a similar suppression of NAT1 activity and expression.

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World J Gastroenterol 2007 December 14; 13(46): 6226-6230
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Predictors of premature delivery in patients with intrahepatic cholestasis of pregnancy

Jurate Kondrackiene, Ulrich Beuers, Rimantas Zalinkevicius, Horst-Dietmar Tauschel, Vladas Gintautas, Limas Kupcinskas

Jurate Kondrackiene, Limas Kupcinskas, Department of Gastroenterology, Kaunas University of Medicine, Kaunas, Lithuania
Ulrich Beuers, Department of Gastroenterology and Hepatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
Rimantas Zalinkevicius, Institute of Endocrinology, Kaunas University of Medicine, Kaunas, Lithuania
Horst-Dietmar Tauschel, Dr. Falk Pharma GmbH, Freiburg, Germany
Vladas Gintautas, Department of Obstetrics and Gynecology, Kaunas University of Medicine, Kaunas, Lithuania
Supported in part by a Grant from the Science Foundation of Kaunas University of Medicine and by Dr. Falk Pharma GmbH, Freiburg, Germany
Correspondence to: Jurate Kondrackiene, MD, PhD, Department of Gastroenterology, Kaunas University of Medicine, Eiveniu Street 2, Kaunas 50009, Lithuania. jukond@takas.lt
Telephone: +370-37-326896 Fax: +370-37-326508
Received: June 20, 2007 Revised: August 29, 2007

Abstract

AIM: To evaluate the predictive value of clinical symptoms and biochemical parameters for prematurity in intrahepatic cholestasis of pregnancy (ICP).

METHODS: Sixty symptomatic patients with ICP were included in this retrospective analysis. Preterm delivery was defined as delivery before 37 wk gestation. Predictors of preterm delivery were disclosed by binary multivariate logistic regression analysis.

RESULTS: Mean time of delivery was 38.1 ± 1.7 wk. No stillbirths occurred. Premature delivery was observed in eight (13.3%) patients. Total fasting serum bile acids were higher (47.8 ± 15.2 *vs* 41.0 ± 10.0 μmol/L, $P < 0.05$), and pruritus tended to start earlier (29.0 ± 3.9 *vs* 31.6 ± 3.3 wk, $P = 0.057$) in patients with premature delivery when compared to those with term delivery. Binary multivariate logistic regression analysis revealed that early onset of pruritus (OR 1.70, 95% CI 1.23-2.95, $P = 0.038$) and serum bile acid (OR 2.13, 95% CI 1.13-3.25, $P = 0.013$) were independent predictors of preterm delivery.

CONCLUSION: Early onset of pruritus and high levels of serum bile acids predict preterm delivery in ICP, and define a subgroup of patients at risk for poor neonatal outcome.

Key words: Intrahepatic cholestasis; Delivery; Pregnancy

Kondrackiene J, Beuers U, Zalinkevicius R, Tauschel HD, Gintautas V, Kupcinskas L. Predictors of premature delivery in patients with intrahepatic cholestasis of pregnancy. *World J Gastroenterol* 2007; 13(46): 6226-6230

http://www.wjgnet.com/6226-6230/13/6226.asp

INTRODUCTION

Liver disorders during pregnancy range from benign nuisance to progressive and potentially lethal disorders for mothers and/or children. This is exemplified by intrahepatic cholestasis of pregnancy (ICP), which starts with modest itching and can end in intrauterine fetal demise[1]. ICP is a liver disorder unique to pregnancy and disappears after delivery. However, it frequently recurs in subsequent pregnancies or when women begin taking oral contraceptives.

The condition is very common in Chile and Bolivia (6%-27%), and in Sweden (1%-1.5%). The incidence of ICP is lower elsewhere in Europe (0.1%-1.5%) and the United States (0.7%)[2,3]. Genetic predisposition and hormonal factors have crucial roles in the pathogenesis[2]. There is increasing evidence that genetically determined dysfunction of canalicular transporters may be a risk factor for development of ICP[4-9]. ICP has been linked to adverse maternal and fetal outcomes. The main symptom is pruritus without evidence of skin lesions, which appears most typically in the third trimester of pregnancy. Laboratory tests demonstrate an increase in serum bile acids and aminotransferases[2,10,11]. ICP is essentially benign in mothers. The major consequences of this disease are premature delivery in 19%-60% of cases[12,13], stillbirths in 1%-2%[13,14] and fetal distress in 22%-33%[15,16]. The mechanisms by which ICP leads to poor fetal outcome are unclear. Recent clinical and biochemical studies have provided evidence for altered metabolism of bile acids and progesterone in ICP, although it remains unclear whether these changes are specific for ICP or are rather the consequence of cholestatic injury[17-20]. In a study from Sweden, a correlation between fetal complications and serum bile acids levels was demonstrated[3].

Various strategies have been proposed to improve obstetric outcome. Nevertheless, in several studies, the investigators have concluded that fetal death in ICP may not be predictable by traditional antepartum surveillance, and that delivery after establishment of fetal lung maturity may reduce fetal mortality rate[13-15]. Obstetric management consists of weighing the risk of premature delivery against the risk of sudden death *in utero*. As well, it has to be considered that induction of labor is associated with a higher frequency of complications such as surgical delivery compared to spontaneous labor[2]. To allow term delivery (≥ 37 wk) in patients with ICP, it appears essential to ascertain early prognostic markers for poor fetal outcome.

In our previous prospective therapeutic trial[21], we observed a significant effect of ursodeoxycholic acid (UDCA) in comparison to cholestyramine on pruritus, serum liver tests, and the duration of pregnancy in patients with ICP. However, the treatment groups did not differ significantly in the number of premature deliveries (< 37 wk gestation). Therefore, the aim of the current study was to re-evaluate clinical symptoms and biochemical parameters as potential predictors of spontaneous preterm births in patients with ICP.

MATERIALS AND METHODS

Sixty patients with ICP defined by (1) development of pruritus during the second or third trimester of pregnancy, and (2) total fasting serum bile acids (TBA) ≥ 11 μmol/L, were included in this retrospective analysis. All patients were seen at the Kaunas Medical University Hospital, Lithuania between October 1999 and September 2002. Patients with chronic liver diseases, skin diseases, allergic disorders, symptomatic cholelithiasis, and ongoing viral infections affecting the liver (hepatitis A, B and C virus, cytomegalovirus, herpes simplex virus, and Epstein-Barr virus) were excluded. All patients participated in a randomized parallel-group study as reported previously[21]. In contrast to the previous prospective trial, the actual retrospective analysis included patients with elevated TBA ≥ 11 μmol/L only.

Pruritus intensity was assessed daily by patients using a subjective score: 0, no pruritus; 1, mild pruritus, occasional; 2, moderate pruritus, intermittent during the day with asymptomatic periods prevailing; 3, severe pruritus every day with symptomatic periods prevailing; 4, severe, constant pruritus day and night. Serum liver tests and fasting serum bile acids were evaluated at the time of the first presentation. Serum liver tests were determined using routine laboratory techniques. Bile acids were analyzed by gas-liquid chromatography as described previously[21,22]. Fasting serum samples were stored at -20℃ until analyzed. Ultrasonography of the abdomen and serology of viral hepatitis were performed to exclude other causes of liver disease in every patient before enrollment.

The Obstetric and Gynecology Clinic of Kaunas Medical University Hospital is a tertiary care maternity center that provides all obstetric services for women with complicated pregnancies, for a stable and ethnically uniform population of 2 million inhabitants. Most of the high-risk deliveries in the area took place in this clinic. Fetal status was monitored in the same hospital every week. Pregnancy outcome and newborn status (term and mode of delivery, Apgar score at 1 and 5 min, asphyxial events, and newborn weight) were assessed by obstetricians and neonatologists, who were not given any specific instructions concerning date and form of delivery. Spontaneous preterm birth was defined as delivery before 37 wk gestation after the spontaneous onset of labor.

Statistical analysis

The results are expressed as means ± SD. Comparison of parametric, normally distributed data was performed by Student's *t* test. The difference between two samples was calculated using the Mann-Whitney test. Correlation analysis was assessed by Spearman's rank correlation. Multivariate analysis of significant prognostic factors of delivery before 37 wk gestation was based on binary multivariate logistic regression analysis. Factors found to be significant or having a trend towards significance (TBA concentrations, onset of pruritus) were selected for this model. Statistical analysis was conducted with SPSS 12.0. All reported *P* values were two-sided, and $P < 0.05$ was considered statistically significant.

RESULTS

Sixty patients who met the inclusion criteria were included in the study. Age ranged between 18 and 40 year (median 27.0), median gestational age was 35.0 wk (range, 22-39), median time of onset of pruritus was 32.0 wk (range, 20-37). Twenty-eight (46.6%) women were primiparous and 32 (53.4%) were multiparous. Recurrence of ICP was reported by 19 (31.6%) patients, 14 of these had a history of preterm delivery, and two of intrauterine fetal death. Ten (16.6%) patients had been users of oral contraceptives, of whom three women had experienced pruritus during use. Gallstone disease was diagnosed in seven (11.6%) cases. One (1.6%) patient had a urinary tract infection. No stillbirths were observed. The Apgar score at 1 min was 8.5 ± 0.7, and at 5 min, 9.0 ± 0.6. Delivery was after 38.1 ± 1.7 wk. Postnatal development was normal in all babies. Pregnancy ended prematurely in eight (13.3%) patients: in three receiving UDCA and in five treated with cholestyramine. Table 1 compares the clinical characteristics of patients who had deliveries before 37 wk and those who had delivery after 37 wk gestation. Significantly higher levels of TBA (47.8 ± 15.2 *vs* 41.0 ± 10.0 μmol/L, $P < 0.05$), and a tendency towards earlier onset of pruritus (29.0 ± 3.9 *vs* 31.6 ± 3.3 wk, $P = 0.057$) were found in cases of premature delivery when compared with term delivery. The correlation coefficient between TBA levels and pruritus scores tended to be higher in patients with preterm delivery (0.733) when compared to those with term delivery (0.523). In cases of preterm delivery (< 37 wk gestation), TBA concentration correlated positively with onset of pruritus ($r = 0.678$), bilirubin ($r = 0.538$), alanine aminotransferase ($r = 0.343$), and aspartate aminotransferase ($r = 0.308$), whereas correlation was weaker in term delivery.

To unravel potential factors that may affect the time of delivery, we instituted a binary multivariate logistic

Table 1 Clinical characteristics at the time of first presentation of patients with ICP who had delivery before and after 37 wk gestation

Characteristics	Delivery before 37 wk *n* = 8	Delivery after 37 wk *n* = 52	*P*
Age (yr)	26.6 ± 7.2	28.3 ± 5.4	0.433
Onset of pruritus (wk)	29.0 ± 3.9	31.6 ± 3.3	0.057
Intensity of pruritus (score)	3.1 ± 0.4	2.9 ± 0.6	0.361
ALT (U/L)	187.3 ± 87.2	210.4 ± 149.3	0.721
AST (U/L)	126.6 ± 78.8	140.1 ± 101.4	0.855
AP (U/L)	386.9 ± 132.7	372.1 ± 136.2	0.707
γGT (U/L)	35.6 ± 23.2	24.4 ± 14.1	0.072
Bilirubin (μmol/L)	10.3 ± 4.0	15.5 ± 13.1	0.202
TBA before treatment (μmol/L)	47.8 ± 15.2	41.0 ± 10.0	0.041

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AP, alkaline phosphotase; γGT, γ-glutamyltransferase; TBA, total bile acids.

Table 2 Potential risk factors for preterm delivery: Predictive value as evaluated by binary multivariate logistic regression analysis

Factor	OR	95.0% CI	*P*
Onset of pruritus (wk)	1.703	1.227-2.947	0.038
Total bile acids before treatment (μmol/L)	2.128	1.126-3.252	0.013

regression model. The factors that were found to be significant or those that had a trend towards significance (TBA concentrations, onset of pruritus) were selected for this model. Binary multivariate logistic regression analysis demonstrated that serum bile acid concentration (OR 2.13, 95% CI 1.13-3.25, $P = 0.013$) and onset of pruritus (OR 1.70, 95% CI 1.23-2.95, $P = 0.038$) were the most important independent variables predicting preterm delivery (Table 2).

DISCUSSION

Although recent studies have improved our understanding of the underlying pathophysiological disturbances and their association with specific symptoms during ICP, the pathogenesis and prognosis of pregnancy have remained obscure. The current study aimed to unravel potential risk factors for fetal prematurity. We found that earlier onset of pruritus and higher TBA concentrations were associated with preterm delivery in our cohort of patients with ICP. The correlation between premature delivery and onset of pruritus is a new and interesting finding; although, high TBA levels have already been described as predictors of fetal outcome in other cohorts[3,23].

ICP is the most common liver disorder unique to pregnancy. In Lithuania, a retrospective analysis disclosed a rate of 0.4% of ICP in 16252 pregnant women over a period of 5 year (1996-2000; J. Kondrackiene, unpublished data). Although essentially benign in the mother, ICP may adversely affect the prognosis of the fetus. ICP has been reported to be associated with increased rates of spontaneous premature delivery[12,13]. According to the Lithuanian Medical Birth Register (2004), the total premature birth rate was 5.3%. Our study showed a 13.3% incidence of preterm delivery in patients with ICP.

The mechanism of preterm delivery remains unclear. Germain *et al*[24] have shown that during ICP, activation of the oxytocin receptor pathway is possibly caused by a cholic-acid-mediated increase in oxytocin-receptor expression. The placenta plays a crucial role in protecting the fetus from the adverse effects of potentially toxic endogenous substances, including TBA[25]. High levels of maternal TBA affect placental transport, placental hormone production, and chorionic vessel constriction[17]. In animal models, maternal hypercholanemia may affect the vectorial transfer of bile acids through the creation of inversely directed gradients, as compared with the physiological situation[26], and by impairing the ability of the trophoblast to transport bile acids[18]. A study from Argentina has shown that asymptomatic hypercholanemia of pregnancy, defined as TBA > 11 μmol/L in healthy pregnant women, does not necessarily lead to ICP[27]. Glantz *et al*[3] have demonstrated that no increase in fetal risk is detected in ICP patients with TBA levels < 40 μmol/L, and have proposed that these women can be managed expectantly. However, a recent case of fetal death at 39 wk and 3 d in a patient with ICP, who had low TBA concentrations at the time of diagnosis, has been reported[28]. This raises a crucial question: is the fasting TBA level sufficient to predict fetal outcome? Should testing be repeated on a weekly basis or discontinued once a less-than-critical level has been determined? What if concentrations increase dramatically over the following weeks, although levels are comparably low at the time of diagnosis for which the prognostic value has been evaluated[29]? Therefore, it is important to evaluate other clinical factors that are possibly associated with prematurity. We found significantly higher levels of TBA, and a tendency towards earlier onset of pruritus, although non-significant, in patients who had premature delivery, when compared with cases of term delivery. The binary multivariate regression analysis revealed that the TBA levels and early onset of pruritus were the most important independent factors predicting premature delivery.

In the current retrospective study, we did not analyze the effect of treatment on preterm delivery. Indeed, among the women who had births before 37 wk gestation, three of eight patients had received UDCA, and five were treated with cholestyramine. The size of the cohort may have been too small to detect any difference in the rate of preterm delivery between patients treated with UDCA and those treated with cholestyramine, although the timepoint of delivery was significantly earlier in patients treated with cholestyramine than in those treated with UDCA in our previous analysis. As well, the rate of preterm delivery in the present cohort was lower than that reported in other studies. This could in part be due to increased attention devoted to ICP during the study[3].

The current analysis indicated that early onset of pruritus, along with markedly elevation of TBA levels, may predict premature delivery, which represents a potential risk factor for the fetus in women with ICP. Because the

prognosis remains unpredictable in some cases[28], our current strategy is to begin pharmacological treatment after confirmation of diagnosis in all ICP patients. The treatment of choice is UDCA, which has improved maternal and fetal morbidity in several clinical trials and observational studies[21,31-34]. When lung maturity is achieved for those patients with risk factors of prematurity, delivery should be considered.

In conclusion, the present study indicated that early onset of pruritus and high levels of TBA were the most important factors associated with preterm delivery in a well-defined cohort of patients with ICP; thereby, defining a group at risk of poor neonatal outcome and so requiring active management.

COMMENTS

Background

Intrahepatic cholestasis of pregnancy (ICP) is characterized by pruritus and an elevation in serum bile acid concentrations. The major consequences of this disease are premature delivery, stillbirth and fetal distress. The mechanisms by which ICP leads to poor fetal outcome are unclear, although a role for bile acids or toxic metabolites of bile acids has been suggested. Currently, the hydrophilic bile acid ursodeoxycholic acid (UDCA) is the most effective treatment for ICP. Various strategies have been proposed to improve obstetric outcome. In several studies, the investigators have concluded that fetal death in ICP may not be predictable by traditional antepartum surveillance, and that delivery after establishment of fetal lung maturity may reduce fetal mortality rate. To allow for term delivery ($\geqslant$ 37 wk) in patients with ICP, it appears essential to disclose early prognostic markers for a poor fetal prognosis.

Research frontiers

There is increasing evidence that genetically determined dysfunction in the canalicular ABC transporters might be risk factors for development of ICP. Heterozygous mutations in the MDR3 gene (encoding for a canalicular phospholipid translocator involved in the biliary secretion of phospholipids) have been found. Recent clinical and biochemical studies provided evidence of abnormal metabolites impairing hepatobiliary carriers for an altered metabolism of bile acids and progesterone in ICP although it remains unclear whether these changes are specific for ICP or are rather the consequence of cholestatic injury in ICP.

Innovations and breakthroughs

We found that earlier onset of pruritus and higher fasting serum bile acid concentrations were associated with preterm delivery in our cohort of patients with ICP. The correlation between premature delivery and onset of pruritus is a new and interesting finding; although, high serum bile acid levels have been described as predictors of fetal outcome in other cohorts.

Applications

The present study indicates that early onset of pruritus and high levels of serum bile acid are the most important factors associated with preterm delivery in patients with ICP; thereby, defining a group at risk of poor neonatal outcome and so requiring active management.

Peer review

This is a well-written manuscript reporting on a cohort of 60 patients with symptomatic ICP. Early onset of pruritus and high levels of serum bile acids predict preterm delivery in intrahepatic cholestasis of pregnancy and define a subgroup of patients at risk of poor neonatal outcome.

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S- Editor Zhu LH **L- Editor** Kerr C **E- Editor** Ma WH

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World J Gastroenterol 2007 December 14; 13(46): 6231-6235
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Bevacizumab plus infusional 5-fluorouracil, leucovorin and irinotecan for advanced colorectal cancer that progressed after oxaliplatin and irinotecan chemotherapy: A pilot study

Hyuk-Chan Kwon, Sung Yong Oh, Suee Lee, Sung-Hyun Kim, Hyo-Jin Kim

Hyuk-Chan Kwon, Sung Yong Oh, Suee Lee, Sung-Hyun Kim, Hyo-Jin Kim, Departments of Internal Medicine, Dong-A University College of Medicine, Busan 602-715, Korea
Correspondence to: Hyo-Jin Kim, MD, PhD, Department of Internal Medicine, Dong-A University College of Medicine, 3-1 Dongdaeshin-dong, Seo-gu, Busan 602-715, Korea. kimhj@dau.ac.kr
Telephone: +82-51-2402951 Fax: +82-51-2402088
Received: June 17, 2007 Revised: September 6, 2007

Kwon HC, Oh SY, Lee S, Kim SH, Kim HJ. Bevacizumab plus infusional 5-fluorouracil, leucovorin and irinotecan for advanced colorectal cancer that progressed after oxaliplatin and irinotecan chemotherapy: A pilot study. *World J Gastroenterol* 2007; 13(46): 6231-6235

http://www.wjgnet.com/1007-9327/13/6231.asp

Abstract

AIM: To evaluate the combination of bevacizumab with infusional 5-fluorouracil (5-FU), leucovorin (LV) and irinotecan (FOLFIRI) in patients with advanced colorectal cancer (CRC) pretreated with combination regimens including irinotecan and oxaliplatin.

METHODS: Fourteen patients (median age 56 years) with advanced CRC, all having progressed after oxaliplatin- and irinotecan-based combination chemotherapy, were enrolled in this study. Patients were treated with 2 h infusion of irinotecan 150 mg/m^2 on d 1, plus bevacizumab 5 mg/kg iv infusion for 90 min on d 2, and iv injection of LV 20 mg/m^2 followed by a bolus of 5-FU 400 mg/m^2 and then 22 h continuous infusion of 600 mg/m^2 given on two consecutive days every 14 d.

RESULTS: The median number of cycles of chemotherapy was six (range 3-12). The response rate was 28.5%, one patient had a complete response, and three patients had a partial response. Eight patients had stable disease. The median time to progression was 3.9 mo (95% CI 2.0-8.7), and the median overall survival was 10.9 mo (95% CI 9.6-12.1). Grade 3/4 neutropenia occurred in five patients, and two of these developed neutropenic fever. Grade 3 hematuria and hematochezia occurred in one. Grade 2 proteinuria occurred in two patients. However, hypertension, bowel perforation or thromboembolic events did not occur in a total of 90 cycles.

CONCLUSION: Bevacizumab with FOLFIRI is well tolerated and a feasible treatment in patients with heavily treated advanced CRC.

Key words: Bevacizumab; Irinotecan; Leucovorin; 5-fluorouracil; Colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer deaths worldwide, with 945 000 new cases and 492 000 CRC-related deaths in 2000[1,2]. Up to 30% of patients present with metastatic disease, and approximately 50%-60% eventually develop metastatic or advanced disease[1]. The management of patients with metastatic CRC has changed dramatically over the last 5 years, with increased chances of prolonged survival. In particular, combination chemotherapy regimens including irinotecan and oxaliplatin have markedly improved response rates and prolonged median survival over fluorouracil (FU) with leucovorin (LV)[3,4]. However, there is a paucity of data about third-line chemotherapy in patients who are resistant to irinotecan- or oxaliplatin-based chemotherapy[5-7].

Angiogenesis is required for tumor growth and metastasis, which makes it an attractive target for biologically based cancer therapy[8-10]. Vascular endothelial growth factor (VEGF) is the most potent and specific target for cancer therapy, and has been identified as a crucial regulator of both normal and pathological angiogenesis, with increased expression being observed in many human tumor types[11-13]. In CRC, increased VEGF expression correlates with invasiveness, vascular density, metastasis, recurrence and prognosis[14,15]. In preclinical studies, a murine anti-human monoclonal antibody directed against VEGF has been shown to inhibit the growth of human tumor xenografts[16-18]. As well, the combination of anti-VEGF antibody and chemotherapy in nude mice injected with human cancer xenografts has demonstrated an increased antitumor effect compared with antibody or chemotherapy treatment alone[19].

Bevacizumab, a recombinant humanized monoclonal antibody targeting VEGF, has been evaluated in various solid tumors[20]. In phase I trials, bevacizumab was generally well tolerated and did not demonstrate dose-limiting toxicity or interactions with commonly used chemotherapy

regimens[21]. In a phase 2 trial of treatment of CRC, the addition of bevacizumab to FU/LV increased the response rate, the median time to disease progression, and the median duration of survival[22]. Recently, it has been shown in randomized phase Ⅲ trials that bevacizumab, when combined with irinotecan plus bolus FU/LV in the first-line treatment of metastatic CRC, and with oxaliplatin plus continuous FU/LV (FOLFOX) in second-line treatment leads to an increased median survival, progression-free survival (PFS), and response rate compared with cytotoxic chemotherapy alone[23,24].

The goal of this trial was to evaluate the safety and activity of bevacizumab plus LV, 5-FU and irinotecan (FOLFIRI) in patients with advanced CRC that had progressed after treatment with both irinotecan- and oxaliplatin-based chemotherapy regimens.

MATERIALS AND METHODS

Eligibility criteria

The eligibility criteria were as follows: histologically confirmed CRC (adenocarcinoma), bidimensionally measurable disease, no secondary malignancy, age > 18 years, Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0-2, and a life expectancy of > 3 mo. Adequate hematological, hepatic and renal function (including urinary excretion of no more than 500 mg protein/d) were also required. Patients should have had and failed both oxaliplatin- and irinotecan-based treatment prior to enrolment. Failure due to significant intolerance to either drug was allowed.

Exclusion criteria included thromboembolism that required therapeutic anticoagulation, central nervous system metastasis, and major surgery within 6 wk, non-healing wounds, uncontrolled hypertension, pregnant or lactating women, bleeding diathesis, active or recent cardiovascular disease or cerebrovascular accident, or prior bevacizumab therapy. The pretreatment characteristics of the patients are presented in Table 1. Written informed consent was required before chemotherapy.

Table 1 Patient characteristics

Characteristics	No. of patients
Median age (range)	56 yr (29-69)
Sex	
Male	9
Female	5
Performance (ECOG)	
0-1	12
2	2
CEA (ng/mL)	
< 5	3
⩾ 5	11
Primary site	
Colon	8
Rectum	6
Sites of metastasis	
Liver	7
Lung	8
Lymph nodes	7
Peritoneum	3
Others	2
Number of metastasis	
1	3
⩾ 2	11
Adjuvant chemotherapy	
Yes	9
No	5

CEA: Carcinoembryonic antigen.

Treatment protocols and dose modification

On d 1, irinotecan (150 mg/m^2) was administered in 500 mL normal saline or dextrose as a 2-h iv infusion. On d 1 and 2, LV (20 mg/m^2) was administered as a iv bolus, immediately followed by 5-FU (400 mg/m^2) given as a 10-min iv bolus, followed by 5-FU (600 mg/m^2) as a continuous 22-h infusion. Bevacizumab administration always followed chemotherapy. Bevacizumab was given at 5 mg/kg as an iv infusion every 2 wk. The first infusion was given over 90 min, the second over 60 min, and if both were well tolerated, subsequent infusions were given over 30 min. No premedication was given.

Dose modifications of irinotecan or 5-FU were made for hematological or non-hematological toxicity, on the basis of the most severe grade of toxicity that occurred during the previous cycle. Treatment was delayed until the absolute number of neutrophils was > 1500/μL, platelets were > 100000/μL, and recovery occurred from mucositis, diarrhea, or skin toxicity to grade 1 or less. The 5-FU dose was reduced after the occurrence of National Cancer Institute Common Toxicity Criteria (NCI-CTC) grade 3 diarrhea, stomatitis or dermatitis. For toxicity of grade 3 or higher, a dose reduction of irinotecan by 20% was prescribed by the protocol. Bevacizumab was retained for uncontrolled hypertension or proteinuria of > 2 g in 24 h. Bevacizumab was discontinued for grade 3 or 4 hemorrhage, thromboembolic events that required full-dose anticoagulation, or any grade 4 toxicity.

Treatment was administered until the disease progressed, unacceptable toxic effects developed, or the patient refused further treatment.

Pretreatment and follow-up evaluation

Pretreatment evaluation included physical examination, complete blood cell counts, blood chemistry, tumor marker level, and radiological examination [chest posterior-anterior (PA) view radiography, computed tomography (CT) and other imaging techniques as clinically indicated] within 1 mo of starting chemotherapy. Tumor responses were determined by WHO criteria[25]. Complete blood cell counts, serum chemistry, including liver and renal function, and chest PA radiography were performed at least every 2 wk, and tumor assessment by CT was performed every three cycles.

Statistical analysis

Efficacy analysis was performed according to the intention-to-treat principle. Patients were considered assessable for response if they were eligible, had measurable disease, and had received at least one dose of study therapy. In the analysis of survival and subsequent treatment, all patients

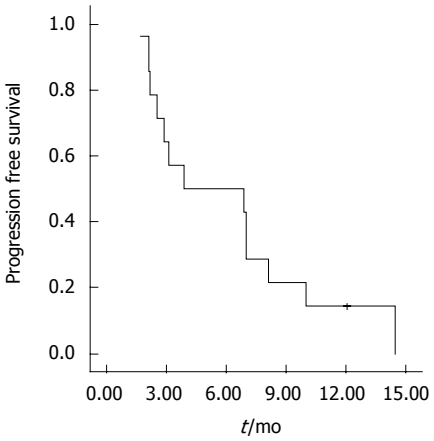

Figure 1 PFS curve. Median PFS was 3.9 mo (95% CI: 2.0-8.7).

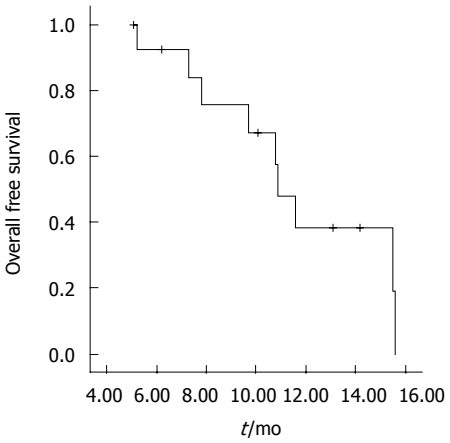

Figure 2 OS curve. Median OS was 10.9 mo (95% CI: 9.6-12.1).

were followed until death, loss to follow-up, or termination of the study.

PFS and overall survival (OS) were calculated using the Kaplan-Meier method. PFS was calculated from the date therapy started to the date of disease progression, and OS was calculated from the date therapy started to the date of death. All data were analyzed using SPSS software (version 12.0, Chicago, IL, USA).

RESULTS

Patient characteristics

Between June 2005 and June 2006, a total of 14 patients were assigned for treatment at the Department of Internal Medicine, Dong-A University Medical Center, Busan, Korea. Demographic details of the patients included in the study are shown in Table 1. There were nine male and five female patients, median age 59 years (range 29-69). All patients had progressed after prior irinotecan- and oxaliplatin-based regimens. All patients used bevacizumab/FOLFIRI treatment within 6 mo after both irinotecan and oxaliplatin treatment failures. All 14 patients were assessable for response and for toxicity and survival.

Objective tumor responses and survival

There were a median six cycles of chemotherapy (range 3-12). Chemotherapy was stopped due to disease progression in 12 patients, one discontinued because of toxicity and another because of unrelated events with no progression. Median follow-up duration was 10.1 mo. Response rate was 28.5% (95% confidence interval (CI) 15.0%-36.5%), one patient had a complete response, and three had a partial response. The median duration of response was 7.7 mo. Eight patients had stable disease, and two had disease progression. PFS was 3.9 mo (95% CI; 2.0-8.7), and median OS was 10.9 mo (95% CI; 9.6-12.1). Figures 1 and 2 show PFS and OS curves, respectively. Oral 5-FU was administered to the 12 patients with disease progression. No objective responses were documented after salvage therapy.

Table 2 Toxicity (n = 14)

	NCI-CTC grade			
	1	2	3	4
Hematological				
Anemia	7	3		
Leukopenia	2	2	2	
Neutropenia		3	4	1
Thrombocytopenia	1	3	1	
Non-hematological				
Nausea/vomiting	2	1		
Mucositis	1			
Diarrhea		2		
Proteinuria	1	2		
Hematuria	1		1	
Asthenia		4		

Toxicity

A total of 90 cycles of chemotherapy were administered to the patients. All patients who received at least one dose of bevacizumab and chemotherapy were assessable for adverse events. Dose modifications or interruptions were required in 30% of patients. The incidence of hematological and non-hematological toxicity is summarized in Table 2. The major grade 3/4 hematological toxicity included neutropenia (35.7%) and thrombocytopenia (7.1%). There were two cycles of neutropenic fever. Grade 1/2 nausea, vomiting, diarrhea and mucositis developed in 6 patients; however, this toxicity was mild and manageable. In one patient who had rectal cancer with bladder invasion, massive hematuria and hematochezia occurred; he was taken off the study because of toxicity. However, hypertension, bowel perforation or thromboembolic events did not occur. There was no treatment-related death. There have been nine reported deaths, of which eight were because of disease progression, and one was because of pneumonia in the present study.

DISCUSSION

Patients with advanced CRC treated with 5-FU, irinotecan and oxaliplatin in combination or sequentially may survive for 18-21 mo[3,4,26,27]. However, if these three standard drugs fail, there are no accepted treatment options. There have been few clinical trials in a third-line setting that can

provide historical estimates of PFS and OS[5-7,28,29]. A recent study has shown that patients treated with cetuximab in combination with irinotecan achieved significant activity[6]. The response rate was 22.9% and time to progression and OS were 4.1 and 8.6 mo, respectively. Promising data from a small randomized phase Ⅱ trial have recently shown that bevacizumab when added to cetuximab or to cetuximab plus irinotecan has a high activity in chemotherapy-refractory CRC[28]. Panitumumab, a human monoclonal antibody against epidermal growth factor receptor (EGFR), has also been shown to be active in irinotecan- and oxaliplatin-refractory metastatic CRC[23]. However, other reports have shown no clinical benefits[5,7].

The improvement in the clinical outcome afforded by the addition of bevacizumab to 5-FU suggests that blocking VEGF may be a broadly applicable approach to the treatment of CRC[22]. Adding bevacizumab to both first- and second-line combination chemotherapy improves response, time to progression, and OS, but not without toxicity[23,24]. The addition of bevacizumab 5 mg/kg biweekly significantly improved the primary outcome of median survival from 15.6 mo with irinotecan/5-FU bolus infusion/LV (IFL) alone to 20.3 mo with IFL/bevacizumab. Bevacizumab also significantly increased response rate from 34.8% to 44.8%, and prolonged time to progression from 6.2 to 10.6 mo[23]. Compliance was also excellent in this study. As well, results from a phase Ⅲ study in patients with previously treated metastatic colon cancer have revealed improved OS in patients who receive bevacizumab (10 mg/kg) with FOLFOX, as compared with those treated with FOLFOX alone, 12.5 versus 10.7 mo[24].

However, a recent large non-randomized study has shown that the combination of bevacizumab and a bolus regimen of 5-FU/LV is not sufficiently active in heavily pretreated, bevacizumab-naive patients to support the use of bevacizumab with bolus 5-FU/LV in chemotherapy-refractory metastatic CRC. The combination of bevacizumab and 5-FU/LV was associated with a low response rate: 4% based on investigator assessment and 1% based on independent review. Median PFS and OS were 3.7 and 9.1 mo, respectively[7]. This study demonstrated that for patients with advanced CRC that had progressed after treatment with both oxaliplatin- and irinotecan-based chemotherapy regimens, response rate was 28.5%, with approximately 58% of the patients showing stable disease. Median PFS was 3.9 mo and median OS was 10.9 mo. We used irinotecan instead of bolus 5-FU/LV; therefore, the response rate and survival were increased compared with those in the earlier study. Further studies will be needed to confirm these results.

Previous phase 1 and 2 clinical trials have suggested that treatment with bevacizumab alone or with chemotherapy results in an increased incidence of thrombosis, bleeding, proteinuria and hypertension[21,22]. In two phase Ⅲ investigations, the risk of venous thromboembolism was not increased by bevacizumab, but there was a small increased risk of both bleeding and bowel perforations, as well as a consistent increase in hypertension[23,24]. Hemorrhage has also been seen more frequently with bevacizumab treatment as compared with chemotherapy alone[22]. The majority of patients had minor hemorrhage, but 10% of patients had gastrointestinal hemorrhage, and 43% were grade 3/4. However, a larger phase Ⅲ trial did not demonstrate an increased incidence of grade 3/4 bleeding[23]. We did not find any excess of such side effects, compared with previous studies, except for one case of massive bleeding. The reason why there was no thrombosis, hypertension and bowel perforation may have been due to the small number of patients and their relatively young age.

An analysis of predictive markers has shown indeed that bevacizumab increases the activity of irinotecan plus FU/LV, regardless of the level of VEGF expression, thrombospondin expression, and microvessel density[30]. In this study, we evaluated the correlation between expression of VEGF and microvascular density and clinical outcome, and we found no significant results (data not shown).

COMMENTS

Background

There is a paucity of data about third-line chemotherapy in patients who are resistant to irinotecan- or oxaliplatin-based chemotherapy. Bevacizumab, a recombinant humanized monoclonal antibody targeting vascular endothelial growth factor (VEGF), has been evaluated in various solid tumors.

Research frontiers

The goal of this trial was to evaluate the safety and activity of bevacizumab plus irinotecan (FOLFIRI) in patients with advanced colorectal cancer (CRC) that had progressed after treatment with both irinotecan- and oxaliplatin-based chemotherapy regimens.

Innovations and breakthroughs

Bevacizumab with FOLFIRI is well tolerated and feasible in heavily treated patients with advanced CRC.

Applications

We will evaluate correlations between expression of VEGF and microvascular density and clinical outcomes.

Terminology

Bevacizumab: monoclonal antibody against VEGF, which aids growth and metastasis of several cancers. Irinotecan, oxaliplatin: chemotherapeutic agents that are useful in CRC.

Peer review

The manuscript evaluates the safety and activity of bevacizumab plus FOLFIRI in patients with advanced CRC that had progressed after treatment with both irinotecan- and oxaliplatin-based chemotherapy regimens. The method is simple and correct. The manuscript is written in correct English with minor language polishing.

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S- Editor Zhu LH **L- Editor** Kerr C **E- Editor** Ma WH

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www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2007 December 14; 13(46): 6236-6242
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Predictive factors for interferon and ribavirin combination therapy in patients with chronic hepatitis C

Joonho Yoon, Jong In Lee, Soon Koo Baik, Kwang Ho Lee, Joon Hyung Sohn, Hyean-Woo Lee, Jun Namkung, Sei Jin Chang, Jong-Whan Choi, Hyun-Won Kim, Byung-Il Yeh

Joonho Yoon, Joon Hyung Sohn, Hyean-Woo Lee, Jun Namkung, Jong-Whan Choi, Hyun-Won Kim, Byung-Il Yeh, Department of Biochemistry and Institute of Basic Medical Sciences, Wonju College of Medicine, Yonsei University, Wonju 220-701, Republic of Korea
Jong In Lee, Soon Koo Baik, Department of Internal Medicine, Wonju College of Medicine, Yonsei University, Wonju 220-701, Republic of Korea
Kwang Ho Lee, Department of Anesthesiology, Wonju College of Medicine, Yonsei University, Wonju 220-701, Republic of Korea
Sei Jin Chang, Department of Preventive Medicine and Institute of Occupational Medicine, Wonju College of Medicine, Yonsei University, Wonju 220-701, Republic of Korea
Soon Koo Baik, Jun Namkung, Sei Jin Chang, Byung-Il Yeh, Institute of Lifelong Health, Wonju College of Medicine, Yonsei University, Wonju 220-701, Republic of Korea
Supported by a Research Grant from Yonsei University, Wonju College of Medicine, YUWCM 2004-03
Correspondence to: Byung-Il Yeh, Department of Biochemistry, Wonju College of Medicine, Yonsei University, 162 Ilsandong, Wonju, Gangwondo 220-701,
Republic of Korea. biyeh64@hotmail.com
Telephone: +82-33-7410282 Fax: +82-33-7430411
Received: August 9, 2007 Revised: September 20, 2007

Abstract

AIM: To confirm the predictive factors for interferon (IFN)-α and ribavirin combination therapy for chronic hepatitis patients with hepatitis C virus (HCV) genotype 1b.

METHODS: HCV RNA from 50 patients infected with HCV genotype 1b was studied by cloning and sequencing of interferon sensitivity determining region (ISDR), PKR-eIF2α phosphorylation homology domain (PePHD). Patients were treated with IFN-α and ribavirin for 6 mo and grouped by effectiveness of the therapy. A variety of factors were analyzed.

RESULTS: Our data showed that age, HCV RNA titer, and ISDR type could be used as the predictive factors for combined IFN-α and ribavirin efficacy. Characteristically, mutations in PePHD appeared only when the combination therapy was effective. Other factors, such as sex and alanine aminotransferase (ALT) level, were not related to its efficacy. Adjusting for age and HCV RNA titer indicated that the ISDR type was the most potent predictive factor.

CONCLUSION: HCV RNA ISDR type is an important factor for predicting efficacy of IFN-α and ribavirin combination therapy in Korean patients.

Key words: Hepatitis C virus -1b; Interferon-α; Ribavirin; Interferon sensitivity determining region; PKR-eIF2α phosphorylation homology domain

Yoon J, Lee JI, Baik SK, Lee KH, Sohn JH, Lee HW, Namkung J, Chang SJ, Choi JW, Kim HW, Yeh BI. Predictive factors for interferon and ribavirin combination therapy in patients with chronic hepatitis C. *World J Gastroenterol* 2007; 13(46): 6236-6242

http://www.wjgnet.com/1007-9327/13/6236.asp

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped RNA virus with a positive single-stranded RNA genome about 9600 nucleotides in length. This RNA encodes a single polypeptide approximately 3000 amino acids in length. The polypeptide is post-translationally cleaved into structural and non-structural proteins[1-3]. HCV infection is a major cause for chronic liver disease worldwide[4]. Eighty percent or more of acutely infected patients develop chronic hepatitis, which progresses to liver cirrhosis in about 20% of cases and hepatocellular carcinoma in 5%. These complications arise even if the disease remains asymptomatic[5].

Alpha-interferon (IFN-α) treatment effectively reduces viral load, but complete eradication of the virus is achieved in less than 20% of patients treated with IFN-α alone and in 40%-47% of patients treated with combined IFN-α and ribavirin[6-8]. The treatment outcome largely depends on the sensitivity of HCV genotype and IFN-α[9,10]. About 10 years before, IFN-α and ribavirin combination therapy has begun to be used instead of IFN monotherapy. Ribavirin is an oral nucleoside analogue acting on a broad spectrum of DNA and RNA viruses. It has been proposed to have both direct antiviral and immunomodulatory effects[11], but the detailed mechanism remains unclear. Ribavirin used as monotherapy is known to have little or no activity against HCV.

The HCV genotype appears to be a major determinant for IFN efficacy, because patients infected with HCV genotypes 2 and 3 respond better to IFN monotherapy than patients with genotype 1[9]. The IFN-based therapy effectiveness is not satisfactory, especially in patients with HCV genotype 1b[12-14], the most common genotype in Korea[15,16], Japan[17], southern and eastern Europe[18,19]. IFN is also inconvenient to use, costly, and has a variety

of possible complications. Thus, many studies have been carried out to determine the predictive factors for the efficacy IFN therapy[12-19].

Response to interferon monotherapy is associated with several host and viral factors. HCV genotype 1b, low viral load, and rapid HCV RNA clearance from the serum have been identified as favorable predictors for a sustained response to IFN therapy[20-22]. Since Enomoto *et al*[23] reported that genetic variability in a 40 amino acid stretch (amino acids 2209-2248) and mutations in the NS5A region of HCV and in designated interferon sensitivity determining region (ISDR), has become a predictive factor for IFN therapy. Studies from Japan[23], Sweden[24] and Spain[25] have shown that ISDR is an effective predictor. However, studies from Western countries displayed that ISDR is not a good predictor[26-28]. Studies on the relationship between IFN-α and ribavirin combination therapy and ISDR have controversial results[29-32].

Taylor *et al*[31] reported that a HCV envelope protein (E2) contains a sequence similar to the phosphorylation site on eIF2-α for the interferon-inducible cellular protein kinase PKR. The PKR-eIF2-α phosphorylation homology domain (PePHD) on E2 may serve as a pseudosubstrate for PKR and inhibit its function, reducing the antiviral effect of interferon. Thus, the PePHD region might also be involved in IFN resistance of chronic hepatitis C to IFN therapy. However, the role of this region is also controversial so far[31].

In Korea, the HCV prevalence is about 1%-2%, but studies to analyze the predictive factors for combined IFN-α and ribavirin therapy have not been performed. To identify these factors, we investigated the relationship between combined IFN-α and ribavirin efficacy and a variety of factors such as ISDR sequence, PePHD sequence, age, ALT level, and HCV RNA titer in Korean patients with HCV genotype 1b.

MATERIALS AND METHODS

Patents and treatment

Serum was collected from HCV genotype 1b-infected patients admitted to Wonju Christian Hospital. Only HCV genotype 1b was used in this study because it is the most common HCV genotype in the Republic of Korea. Sera were screened by a third generation ELISA method with an anti-HCV antibody. The patients were treated with IFN-α and ribavirin for 6 mo. Three million units of IFN-α was injected every two days, and 9 mg of ribavirin was orally administrated during the same period. The patients who did not receive the treatment were excluded. After the 6-mo combination therapy, the patients were classified into complete response group and no-response group. In the complete response group, HCV RNA titer was less than 50 IU/mL and ALT levels were within the normal range. In the no-response group, HCV RNA titer was over 50 IU/mL even if the ALT levels were normal.

cDNA preparation

HCV RNA was extracted from sera as previously described[33]. After ethanol precipitation, each RNA pellet was dissolved in 10 μL of diethylpyrocarbonate (DEPC)-treated distilled water for cDNA preparation. cDNA synthesis was performed as previously described[34] with certain modifications. For the synthesis of cDNA of HCV, an aliquot of RNA (10 μL) isolated from the sera of patients was mixed with 1 μL of random hexamer (1 μmol/L), 2 μL of reaction buffer (250 mmol/L Tris-HCl pH 8.3, 250 mmol/L potassium chloride, 50 mmol/L magnesium chloride, 50 mmol/L dithiothreitol and 2.5 mmol/L spermidine) and 5.5 μL of DEPC-treated water was added. After the contents were heat-treated for 5 min at 65℃, 20 units (0.5 μL) of RNase inhibitor and 10 units (1 μL) of AMV reverse transcriptase were added. The mixture was incubated at 37℃ for 30 min, followed by at 99℃ for 1 min to inactivate the enzyme. PCR was performed as described previously[16]. The ISDR and PePHD primer sequences are listed in Table 1. PCR products were subjected to agarose gel electrophoresis in Tris-acetate-EDTA buffer and visualized with ethidium bromide staining under an ultraviolet transilluminator.

ISDR and PePHD sequencing

RT-PCR amplified products, including the ISDR and/or PePHD regions, were purified from agarose gel and glass milk (Gene Clean II kit, Bio 101, USA), and then subcloned by inserting the cDNA into a pGEM-T TA-cloning vector (Promega). The clones from each of the individual patient's plates were randomly selected and plasmid prepared from each clone was used as a template for DNA sequencing which was performed as previously described[35].

HCV RNA quantitation

In order to determine the HCV RNA titer, a quantitative and competitive polymerase chain reaction (QCPCR) assay was carried out as previously described[36]. As a first step, cDNA encoding the 5'-untranslated region of HCV was subcloned into a pGEM vector (pGEM5'UTR). Using PCR, the internal control plasmid, pGEM5'UTRDel, was constructed by deletion of nucleotides between the 87 and 165 nucleotides in the 5'-UTR of the HCV genome. The internal control RNA was synthesized *in vitro* by T7 RNA polymerase from a linearized template derived from the pGEM5'UTRDel plasmid. The amount of RNA synthesized *in vitro* was determined by measurement of the absorbance at 260 nm. A known copy number of the RNA was included as an internal control in order to quantify the viral RNA. The data were analyzed by Quantity One® 1-D analysis software (Bio-Rad).

Statistical analysis

Comparisons between groups were made by the Student's *t*-test. The *P* values were determined between the two groups with regard to age, ALT, amino acid mutations in PePHD, and HCV RNA titer. $P < 0.05$ was considered statistically significant. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for ISDR to test its predictive value for combination therapy by logistic regression analysis.

RESULTS

Patient characteristics

We collected serum from 50 HCV genotype 1b-infected

Table 1 Primer sequences used to amplify ISDR and PePHD

Region	Primer direction	Sequence (5′ to 3′)	Nucleotide No
ISDR	Outer sense	TGGATGGAGTGCGGTTGCACAGGTA	6703-6727
	Outer antisense	TGTAAAACGACGGCCAG	7296-7320
	Inner sense	TCTTTCTCCGTGGAGGTGGTATTGC	6722-6741
	Inner antisense	CAGGAAACAGCTATGACC	7275-7294
PePHD	Outer sense	TGACTACCCATACAGGCTCT	2180-2199
	Outer antisense	AAGGAAGGAGAGATTGCCAT	2725-2744
	Inner sense	AAGGTTAGGATGTATGTGGG	2238-2257
	Inner antisense	ATTGAGGACCACCGAGTTCT	2689-2708

ISDR: Interferon sensitivity determining region; PePHD: PKR-eIF2α phosphorylation homology domain; PKR: RNA-activated protein kinase.

Table 2 Characteristics of the complete response group patients before IFN-α and ribavirin combination therapy

	Age (yr)	Sex	ALT	Transfusion history[1]	HCV RNA titer[2]	ISDR Type[3]	No. of Amino Acid Mutations of PePHD
A-1	50	M	178	×	3.92	1	0
A-2	49	F	135	○	3.27	1	1
A-3	40	M	106	×	4.16	1	1
A-4	45	M	218	○	5.48	1	0
A-5	51	F	92	×	4.04	1	0
A-6	41	M	143	×	3.75	2	0
A-7	56	F	167	×	4.24	2	0
A-8	58	M	86	×	5.26	2	0
A-9	49	M	99	○	5.12	2	1
A-10	40	F	129	○	4.41	2	0
A-11	54	F	201	×	3.73	2	1
A-12	53	M	179	×	4.21	2	1
A-13	52	M	234	×	4.25	2	0
A-14	47	M	311	×	5.11	2	0
A-15	49	M	86	×	6.12	3	1
A-16	53	F	220	×	5.91	3	0
A-17	47	F	194	×	4.82	3	0
A-18	49	M	246	×	5.44	3	1
A-19	51	M	441	○	5.22	3	2
A-20	47	F	305	○	4.85	3	0
A-21	55	M	119	×	3.73	3	0

ISDR: Interferon sensitivity determining region; PePHD: PKR-eIF2α phosphorylation homology domain. [1]○ indicates history of transfusion and × indicates no history of transfusion. [2]The unit of HCV RNA titer before treatment (log copies/mL). [3]ISDR type: 1 (wild type, no amino acid substitution), 2 (intermediate type, 1-3 amino acid substitutions), 3 (mutant type, ⩾ 4 amino acid substitutions).

Table 3 Characteristics of the no-response group patients before IFN-α and ribavirin combination therapy

	Age (yr)	Sex	ALT	Transfusion history[1]	HCV RNA titer[2]	ISDR type[3]	No. of Amino Acid Mutations of PePHD
B-1	53	M	185	×	5.25	1	0
B-2	48	M	320	×	6.11	1	0
B-3	44	F	125	○	4.88	1	0
B-4	49	F	175	×	5.72	1	0
B-5	54	M	151	○	6.21	1	0
B-6	58	F	190	×	5.14	1	0
B-7	62	F	212	×	6.35	1	0
B-8	64	M	252	○	7.11	1	0
B-9	45	M	145	×	6.82	1	0
B-10	49	M	138	×	4.95	1	0
B-11	42	F	120	○	5.54	1	0
B-12	55	M	95	×	6.25	1	0
B-13	53	M	142	×	6.73	1	0
B-14	55	F	185	×	6.76	1	0
B-15	57	M	258	×	4.85	1	0
B-16	53	F	175	○	6.33	2	0
B-17	49	M	241	×	5.81	2	0
B-18	44	M	183	×	6.32	2	0
B-19	59	F	167	×	5.89	2	0
B-20	54	M	171	○	6.14	2	0
B-21	52	F	217	×	6.23	2	0
B-22	55	M	222	×	5.88	2	0
B-23	63	F	235	×	6.32	2	0
B-24	47	F	161	×	6.47	2	0
B-25	51	M	96	○	5.31	2	0
B-26	54	F	80	×	5.85	2	0
B-27	55	F	192	○	6.42	3	0
B-28	58	M	234	×	5.93	3	0
B-29	48	M	341	×	4.98	3	0

ALT: Alanine aminotransferase; ISDR: Interferon sensitivity determining region. [1]○ indicates history of transfusion and × indicates no history of transfusion. [2]The unit of HCV RNA titer before treatment (log copies/mL). [3]ISDR type: 1 (wild type, no amino acid substitution), 2 (intermediate type, 1-3 amino acid substitutions), 3 (mutant type, ⩾ 4 amino acid substitutions).

patients. Among them, 21 patients completely responded to the 6-mo combination therapy, while 29 patients showed no response. Group A was designated as the complete response group and group B as the no-response group. The characteristics of each patient prior to combination therapy are shown in Tables 2 and 3.

HCV RNA quantitation

As shown in Tables 2 and 3, the HCV RNA titer had a wide distribution. In the complete response group the HCV RNA titer was between $10^{3.27}$-$10^{6.12}$ copies per mL and $10^{4.85}$-$10^{7.11}$ copies per mL, respectively, in the no-response group. The average RNA titer of the response and no-response groups was 4.62 ± 0.80 and 5.59 ± 0.61, respectively. These values were statistically significant ($P < 0.05$, Table 4).

ISDR and PePHD amino acid sequences

The ISDR and PePHD amino acid sequences and the HCV genotype 1b prototype sequence (HCV-J) are shown in Figure 1. The complete response group had 1-10 amino acid substitutions while the no-response group had 1-8 amino acid substitutions in the ISDR (Figure 1A and B). The PePHD region had 1-2 amino acid substitutions in several cases of complete response group and no amino acid substitutions in the no-response group (Figure 2).

A

2209								2248
	PSLKA	TCTTH	HDSPD	ADLIE	ANLLW	RQEMG	GNITR	VESEN
1	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
2	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
3	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
4	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
5	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
6	- - - R -	- - - - -	- - N - -	P - - - -	- - - - -	- - - - -	- - - - -	- - - - -
7	- - - - -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
8	- - - - -	- - - - -	- - - - -	V - - - -	- - - - -	- - - - -	- - - - -	- - - - -
9	- - - - -	- - - - -	- - - - -	P - - - -	- - - - -	- - - - -	- - - - -	- - - - -
10	- - - - -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
11	- - - - -	- - - - C	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
12	- - - - -	- - - - R	- - - - -	V - - - -	- - - - -	- - - - -	- - - - -	- - - - -
13	L - - - -	- - - - -	- - - - -	I - - - D	- - - - -	- - - - -	- - - - -	- - - - -
14	- - - - -	- - - - -	- - - - -	L - - - -	- - - - -	- - - - -	- - - - -	- - - - -
15	V - - - -	A Y I - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
16	- - S - T	- Y I - -	R G - - -	- - - - -	- - - - -	- - - - -	- D - - -	- - - - -
17	L - - - -	A - - - N	- - - - -	V - - - -	- - - - -	- - - - -	- S - - -	- - - - -
18	V - - - -	A - - - -	- - - - -	- - - - -	- - - - -	- - - K -	- T - - -	- - - - K
19	L - - - -	- - R R -	- - - - -	- - D - -	- - - - -	W - - K -	- - - - -	- - - - -
20	L - - - -	- - - R -	N - - V -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
21	O - S - T	- Y I - Q	Y - G - -	V - - - -	- - - - -	- - - - -	- D - - -	- - - - -

B

2209								2248
	PSLKA	TCTTH	HDSPD	ADLIE	ANLLW	RQEMG	GNITR	VESEN
1	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
2	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
3	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
4	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
5	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
6	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
7	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
8	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
9	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
10	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
11	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
12	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
13	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
14	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
15	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
16	- - - - -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
17	- - - - -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
18	- - - - -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
19	- - - - -	- - - - C	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
20	- - M - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
21	- - - - -	- - - - C	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
22	- - - - -	- - - - R	- V - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
23	- - - R -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
24	L - - - -	- - - P -	- - - - -	- - - - -	- - - - -	W - - - -	- - - - -	- - - - -
25	- - - - -	- - - - R	- V - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
26	- - - - -	- - - - -	- - - - -	P - - - -	- - - - -	- - - - -	- - - - -	- - - - -
27	L - - - -	A - - A R	- G F I -	- - - - -	- - - - -	- - - - -	- E - - -	- - - - -
28	- - - - -	- G - - -	- - - - -	- - - - D	- - - - -	P - - - -	- D - - -	- - - - -
29	L - - - -	- - R R -	- - - - -	V - - - -	- - - - -	- - - - -	- - - - -	- - - - -

Figure 1 ISDR sequences (2209-2248) of HCV in complete response group (**A**) and in no-response group (**B**). Dashes indicate the amino acid residues identical to the sequences on the top in each panel.

Response to combination therapy in different groups

As shown in Table 4, patients were younger in the complete response group than in the no-response group. The HCV RNA titer was also significantly different. No differences were found in gender or ALT levels. We classified ISDR sequences into three groups based on the number of amino acid mutations, as previously described[23]. These three groups were analyzed by an odds ratio to define the responsiveness to combination therapy. The ISDR group responses to combination therapy are shown in Table 5. Intermediate (one to three amino acid changes) and mutant (four or more amino acid changes) ISDRs showed an increased responsiveness to the combination therapy. The odds ratio was 2.46 and 7.00, respectively, assuming the wild type had 1.00. The age, PePHD mutations, and HCV RNA titer were significantly different between the two groups (Table 4). Considering these factors, ISDR type might be a better predictive factor for combination therapy responsiveness. After adjusting for age and HCV RNA titer, the odds ratio for intermediate and mutant ISDRs was 3.57

	Complete response group			No-response group		
	RS	ELSPL	LLSTT	RS	ELSPL	LLSTT
1	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
2	- A	- - - - -	- - - - -	- -	- - - - -	- - - - -
3	- Q	- - - - -	- - - - -	- -	- - - - -	- - - - -
4	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
5	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
6	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
7	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
8	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
9	- A	- - - - -	- - - - -	- -	- - - - -	- - - - -
10	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
11	- E	- - - - -	- - - - -	- -	- - - - -	- - - - -
12	- Q	- - - - -	- - - - -	- -	- - - - -	- - - - -
13	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
14	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
15	- -	A- - - -	- - - - -	- -	- - - - -	- - - - -
16	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
17	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
18	- -	Q- - - -	- - - - -	- -	- - - - -	- - - - -
19	- A	Q- - - -	- - - - -	- -	- - - - -	- - - - -
20	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
21	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
22				- -	- - - - -	- - - - -
23				- -	- - - - -	- - - - -
24				- -	- - - - -	- - - - -
25				- -	- - - - -	- - - - -
26				- -	- - - - -	- - - - -
27				- -	- - - - -	- - - - -
28				- -	- - - - -	- - - - -
29				- -	- - - - -	- - - - -

Figure 2 PePHD sequences (659-670) of HCV in complete response group (n = 21) and no-response group (n = 29). Dashes indicate the amino acid residues identical to the sequences on the top in each panel.

Table 4 Statistical analysis of age, ALT and HCV RNA titer before treatment

Group	Male:Female	Age (yr)	ALT	HCV RNA titer before treatment
A	13:08	49.3 ± 5.0[a]	185.2 ± 89.1	4.62 ± 0.80[b]
B	16:13	52.8 ± 5.7[a]	186.5 ± 61.7	5.95 ± 0.61[b]

ALT: Alanine aminotransferase. The data of age, ALT and HCV RNA titer before treatment are shown in mean ± SD. A: Complete response group. B: No-response group. [a]P = 0.032 between A and B. [b]P = 0.001 between A and B.

Table 5 Logistic regression analysis of ISDR

ISDR type[1]	Crude OR	95% CI	Adjusted OR[2]	95% CI[2]
Wild	1.00		1.00	
Intermediate	2.46	0.64-9.39	3.57	0.66-19.36
Mutant	7.00	1.29-37.91	9.67	1.16-80.65

[1]ISDR wild type had no mutation. Intermediate type had 1-3 mutations and mutant type had more than 4 mutations. [2]After adjustment for age and HCV RNA titer before treatment. OR: Odds ratio; 95% CI: 95% confidence interval.

and 9.67, respectively. According to these results, patients with mutant ISDR strains would likely respond better to combination therapy than those with wild type ISDR strains.

DISCUSSION

Identifying host and viral factors can predict the response of HCV-infected patients to IFN-α and ribavirin combination therapy. Studies showed that factors such as the HCV genotype 1b and viral load are associated with resistance of HCV-infected patients to INF therapy[37,38]. It was reported that resistance of HCV genotype 1b-infected patients to INF therapy is influenced by a region of the NS5A viral phenotype[23,39]. Mutations in this region, known as ISDR, are beneficial for patients receiving IFN therapy, whereas the wild type virus is resistant to IFN treatment[23,39]. Other studies fail to confirm the association between ISDR genotypes and IFN responsiveness[26,27,40-44], thus it remains a controversial issue[45,46].

Combined IFN and ribavirin therapy has replaced IFN monotherapy for HCV-infected patients about 10 years before. In the present study, to identify the predictive factors for effective combination therapy, we investigated the relationship between the response to combination therapy and a variety of factors. Only patients with HCV genotype 1b were studied because this genotype is known to be more resistant to interferon treatment than the other genotypes and is the most prevalent genotype in Korea[15,16].

In this study, age, PePHD mutations, HCV RNA titer, and ISDR subtype were found to be the predictive factors for combined IFN-α and ribavirin therapy for HCV genotype 1b infection. On the other hand, gender and ALT level were not associated with the combination therapy efficacy. These results are consistent with many previous studies, but contrary to others[26,27,31]. Such a difference indicates that these factors are not always accurate predictors for IFN response. This effect may be due to the pleiotropic nature of IFN activity, in addition to other cellular and viral

genes that also modulate the effectiveness of INF therapy for chronic hepatitis C. This is the first study to determine the factors that predict the effectiveness of combination therapy in Korean patients. Therefore, this study may reflect the Korean genetic characteristics.

HCV seems to have a defense strategy against the host cellular responses induced by IFN[47]. The E2 protein appears to play a major role as a potential immune response target, and may interfere with cellular effectors induced by IFN[48]. Information about the clinical implications of E2 containing PePHD, is still limited. Analysis of a small series of HCV genotype 1-infected patients showed that amino acid sequence variability in the PePHD region was similar in responders and no-responders, indicating that the PePHD region is very stable over time[49-51]. In our study, a sequence analysis of the PePHD region in 50 patients found mutations in eight cases, all in the complete response group, suggesting that mutations in the PePHD region are associated with the response to combination therapy. In other studies, a few cases showed some PePHD mutations in the no-response group, though more mutations appeared in the complete response group[31,52]. Therefore, further study is needed to determine why mutations only occur in the complete response group of HCV-infected patients in Korea.

Some studies showed that the association of ISDR mutation rate with treatment response, but the other studies did not[31,45]. One of the Korean studies reported that the effect of INF monotherapy is not associated with the ISDR mutation rate[53]. It is not sure, but the different result may be due to the treatment methods and the sample size.

In conclusion, response of HCV genotype 1b-infected patients to combination therapy is influenced, at least in part, by HCV RNA titer, age, PePHD mutations, and ISDR subtype, but not by gender and ALT level. After adjusting for age and HCV RNA titer, ISDR subtype may be the most potent predictive factor for combination therapy efficacy in Korean chronic hepatitis patients with HCV genotype 1b.

COMMENTS

Background

The effectiveness of IFN-α and ribavirin combination therapy in Hepatitis C virus (HCV) infected patients is not satisfactory, especially in patients with HCV genotype 1b. IFN is also inconvenient to use, costly, and has a variety of possible complications. Therefore, it is necessary to determine the predictive factors for IFN therapy.

Research frontiers

It is very important to know the pathogenesis of HCV-infected patients to block the progression of hepatocellular carcinoma.

Innovations and breakthroughs

This is the first study to determine the factors that predict the effectiveness of IFN-α and ribavirin combination therapy in Korean patients. In this study we used a large number of samples to determine the ISDR subtype, HCV RNA titer, age, and PePHD mutations.

Applications

The predictive factors for IFN-α and ribavirin combination therapy in patients with HCV genotype 1b can be used to select its candidates.

Peer review

This article is of theoretical and practical importance. The results show that HCV RNA ISDR type may be an important factor for predicting the efficacy of IFN-α and ribavirin combination therapy in Korean patients.

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S- Editor Zhu LH **L- Editor** Wang XL **E- Editor** Li HY

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World J Gastroenterol 2007 December 14; 13(46): 6243-6248
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effect and mechanism of β-L-D4A on DNA polymerase α

Yan Li, Ju-Sheng Lin, Ying-Hui Zhang, Xiao-Yan Wang, Ying Chang, Xing-Xing He

Yan Li, Ju-Sheng Lin, Ying-Hui Zhang, Xiao-Yan Wang, Ying Chang, Xing-Xing He, Institute of Liver Diseases, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Street, Wuhan 430030, Hubei Province, China
Supported by National Natural Science Foundation of China, No. 30330680
Correspondence to: Dr. Ju-Sheng Lin, Professor, Institute of Liver Diseases, Tongji Medical College, 1095 Jiefang Street, Wuhan 430030, Hubei Province, China. jslin@tjh.tjmu.edu.cn
Telephone: + 86-27-83663661 Fax: +86-27-83663661
Received: July 28, 2007 Revised: August 27, 2007

Abstract

AIM: To investigate the safety of β-L-D4A on DNA polymerase α.

METHODS: Ion exchange chromatography was used to separate DNA polymerase α from crude extract of human Hela cells. Detailed kinetic parameters were determined for β-L-D4A against DNA polymerase α.

RESULTS: DNA polymerase α was purified with 4% yield and 31000 units/mg specific activity. The Michaelis constant (Km = 3.22 μmol/L), 50% inhibition concentration (IC50 = 178.49 μmol/L) and inhibition constant (Ki = 126 μmol/L) of β-L-D4A were determined by kinetic analysis.

CONCLUSION: β-L-D4A is a more safe nucleoside for hepatitis B virus (HBV) infection with a lower host toxicity.

Key words: Nucleoside; β-L-D4A; DNA polymerase α; Kinetic study; Side effect; Hepatitis B virus

Li Y, Lin JS, Zhang YH, Wang XY, Chang Y, He XX. Effect and mechanism of β-L-D4A on DNA polymerase α. *World J Gastroenterol* 2007; 13(46): 6243-6248

http://www.wjgnet.com/1007-9327/13/6243.asp

INTRODUCTION

Infection with hepatitis B virus (HBV) is a global medical problem[1]. With the support of National Natural Science Foundation of China[2], we have found a novel nucleoside analog (β-L-D4A), a potent and selective inhibitor of HBV replication, which is stronger than lamivudine (3TC)[3,4]. Because the nucleoside and nucleoside analog could serve as substrates for normal DNA polymerases, resulting in inhibition of cellular DNA synthesis, we decided to study the side effect of β-L-D4A on DNA polymerases[5].

It has been found that many kinds of DNA polymerase have a crucial role in DNA replication, among which, the high molecular weight class of eukaryotic DNA polymerase α (EC2.7.7.7), first identified in calf thymus extract and found to be ubiquitous among eukaryotes, constitutes the predominant polymerase species in actively dividing cells[6,7]. So ion exchange chromatography was used to separate DNA polymerase α from crude extract of human Hela cells, which could be used as a substrate to evaluate the safety of nucleoside-β-L-D4A.

MATERIALS AND METHODS

Reagents

β-L-D4 triphosphate was chemically synthesized in our institute with the help of Pharmaceutic College of Wuhan University and identified by infrared mass spectra, nuclear-magnetic resonance. 3TC 5' triphosphate was provided by Professor Cheng YC (School of Medicine, Yale University, New Haven, CT). Hela S3 cells were purchased from Institute of Immunity, Tongji Medical College, DE52 and P11 cellulose resin were from Whatman while HTP was from Bio-Rad. DNA-cellulose and activator calf thymus DNA were obtained from SIGMA. α-^{32}P-dTTP was from Amersham. All other tissue culture reagents were from Gibco.

All operations were performed at 0℃-4℃. Unless otherwise noted, all buffers contained 1 mmol/L β-mercaptoethanol and 1 mmol/L EDTA.

Preparation of cytoplasmic crude extract

Frozen cells (approximately 80 g) were suspended in 300 mL of 2 mmol/L $MgCl_2$, 1 mmol/L p-toluenesulfonyl fluoride, 10 mL/L isopropyl alconol, and 5 mmol/L KPO_4 (pH7.5), and broken in a Dounce homogenizer after 20 min at 0℃. Nuclei were spun down at 800 r/min for 10 min, and mitochondria were pelleted at 13000 r/min for 20 min. The mitochondrial pellet was washed with 80 mL extraction buffer and re-sedimentated with the two supernatants combined (a). Fraction a was dialyzed for 8-12 h against 10 volumes twice with 0.2 mol/L sodium acetate (pH5.5) each time, after which a flocculent precipitate was removed by centrifugation at 13000 r/min for 20 min (b). Fraction b was over-layered with 117 mL/L sucrose in 0.2 mol/L KPO_4 (pH8.2) and spun for 90 min at 40000 r/min in a Beckman rotor. The supernatant was saved (c).

First DEAE-cellulose chromatography

Fraction c was diluted to a final concentration of 0.1 mol/L KPO_4 at 1:1 with 1 mmol/L β-mercaptoethanol, 1 mmol/L EDTA and loaded onto a DEAE-cellulose column (5 cm × 20 cm) equilibrated with the same buffer. The column was washed with 3 volumes of equilibration buffer, and the adsorbed activity was then eluted in a single step with 0.2 mol/L KPO_4 (pH8.2). All the active fractions from the step were pooled (d).

Second DEAE-cellulose chromatography

Fraction d was diluted to a final concentration of 0.1 mol/L KPO_4 with 1 mmol/L β-mercaptoethanol, 1 mmol/L EDTA and loaded onto a second DEAE-cellulose column (2.5 cm × 25 cm). The column was washed with 3 volumes of equilibration buffer and then developed with a 5-6 column volume gradient from 0.1 mol/L to 0.2 mol/L KPO_4 (pH8.2).

Phosphocellulose chromatography

The peak fractions of protein obtained from DE52 were pooled and dialyzed against 300 g/L sucrose, 200 mL/L ethylene glycol, 0.1 mol/L KPO_4 (pH7.2) (e). Fraction e was loaded onto a phosphocellulose column (2.5 cm × 20 cm). The column was developed with a 6-volume gradient from 0.1 mol/L to 0.3 mol/L KPO_4 (pH7.2).

Hydroxylapatite chromatography

The peak fractions of DNA polymerase α from P11 were pooled and dialyzed against 300 g/L sucrose, 0.05 mol/L KPO_4 (pH 6.8) (f). Fraction f was diluted at 1:1 with 1.0 mol/L KCl, 4 mL/L Triton X-100 and loaded onto a hydroxylapatite column (1 cm × 13 cm). The column was eluted with a 15-volume gradient from 0.025 mol/L to 0.2 mol/L KPO_4 (pH7.2).

DNA-cellulose chromatography

The peak fractions were pooled and concentrated by dialysis against 300 g/L sucrose, 0.1 mol/L KPO_4 (pH7.5) (g). Fraction g was diluted to a final concentration of 0.02 mol/L KPO_4 (pH7.5) at 1:4 with 1 mmol/L β-mercaptoethanol, 1 mmol/L EDTA, and loaded onto a DNA cellulose column (1 cm × 10 cm). The column was developed with a 20-volume gradient from 0.02 mol/L to 0.2 mol/L KPO_4 (pH7.5).

Protein concentration

Bradford method was used to measure the protein concentration, which was operated by directions of the reagent box.

Assay of DNA polymerase α

The final volume of reaction mixture was 50 μL containing 2 μL DNA polymerase α, 50 μmol/L d CTP, 50 μmol/L d GTP, 50 μmol/L d ATP, 50 μmol/L α-^{32}Pd TTP (100 cpm/pmol), 100 μg/mL activated calf thymus DNA, 50 mmol/L Tris-HCl (pH7.5), 0.5 mmol/L $MnCl_2$, 100 mmol/L KCl and 2.5 mmol/L DTT. The mixture was Incubated at 37℃ for 15 min, and spotted onto DE81 filter paper. The paper was washed three times with 5% Na_2HPO_4 (10 min each time), twice with water (5 min each time), dried and assayed for the acid-insoluble radioactivity.

Sodium dodecyl sulfatd polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to detect the purity of DNA polymerase α.

Western blot

Western blot was performed to confirm the protein bands.

Detailed kinetic parameters were determined using DNA polymerase α which was prepared, pooled and stored at -80℃ until use in experiments. Potential inhibitors at various concentrations were added to 5 μL reaction mixture. Control samples (without inhibitor) included 5 μL solution in PBS, but no inhibitors. DNA polymerase α activity was measured.

Michaelis constant (Km)

To find the inhibition type, α-^{32}P-dTTP(100 cpm/pmol) was performed at the concentration of 1, 3, 5, 7, 10 μmol/L while inhibitors were added at the concentration of 10 μmol/L of each compound. DNA polymerase α was determined. The data were analyzed with GraphPad Prism 4 Demo, which could finish Line weaver-Burk Plot and L-B linear secondary regression with the enzyme kinetics template.

Fifty percent inhibition concentration (IC50)

To determine the concentration required to inhibit DNA polymerase α activity by IC50, initial experiments were designed with the gradient (1 μmol/L-400 μmol/L) of each compound. The concentration of α-^{32}P-dTTP (100 cpm/pmol) was 3 μmol/L. All experiments were performed twice. Inhibition rate = (1 - count of drug class/blank control) × 100%. Half logarithm plot was used to process the data. DNA polymerase α activity in the control sample was set at 100%.

Inhibition constant (Ki)

To find the inhibition constant (Ki), each compound was studied at five different inhibitor concentrations (10, 40, 100, 160, 200 μmol/L), and at various substrate concentrations (2, 5 μmol/L) in duplicate. Dixon plot was used to deal with the data.

RESULTS

DEAE-cellulose chromatography

The first DE52 chromatography was to remove nucleic acid. The curve of A280 showed a broad peak including 74 tubes from the 16th tube (0.115 mol/L) to the 90th tube (0.185 mol/L) in the second DE52 chromatography (Figure 1A). The protein in 74 tubes was collected and the ingredient was confirmed by assay of DNA polymerase α activity. The total protein was 0.36 mg measured by Bradford method.

Phosphocellulose chromatography

Protein determined by A280 was eluted sharply as a

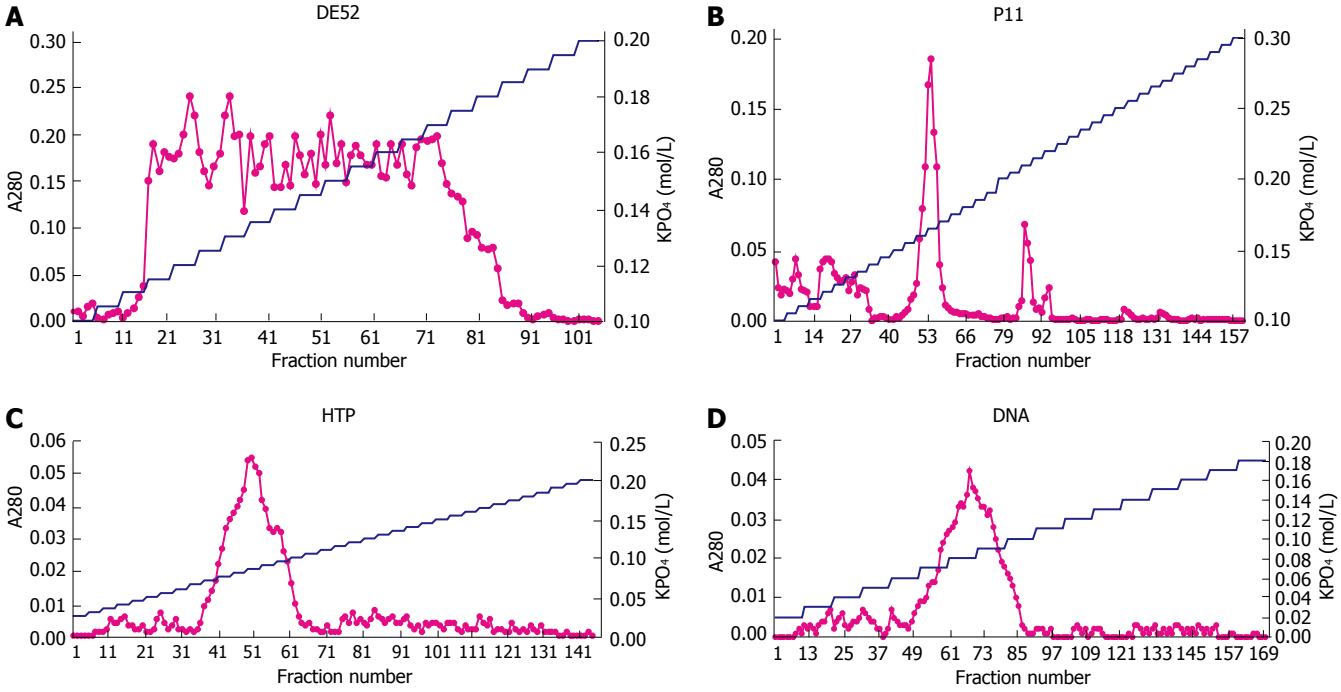

Figure 1 A280 and gradient concentration of KPO_4 in each chromatography. The curve with spots is for A280, the other is for KPO_4. **A**: A broad peak of protein from the 16th tube (0.115 mol/L) to the 90th tube (0.185 mol/L), clearly observed in the second DE52 chromatography; **B**: Phosphocellulose chromatography displaying a major peak of protein identified at 0.165 mol/L KPO_4 and a minor trailing shoulder at 0.21 mol/L; **C**: Hydroxylapatite chromatography showing a single sharp peak of protein at 0.085 mol/L KPO_4; **D**: DNA-cellulose chromatography revealing a single sharp peak at 0.085 mol/L KPO_4.

Table 1 Protein, activity, specific activity, purification times and yield of each step

Step	Protein/ mg	Activity/ units	Specific activity/ (units/mg)	Purification times	Yield(%)
Crude extract	9.000	800	89		
First DEAE	0.880	480	545	6	60
Second DEAE	0.360	260	722	8	33
P11	0.039	240	6154	69	30
HTP	0.008	120	15000	169	15
DNA	0.002	32	31000	348	4

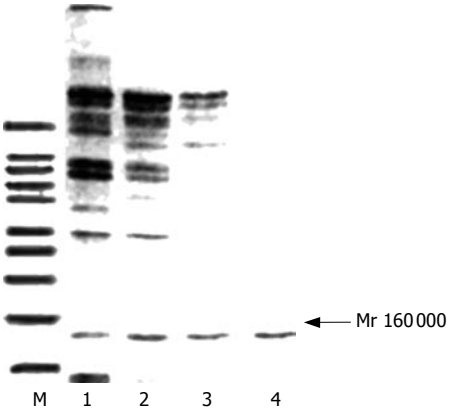

Figure 2 SDS-PAGE displaying decreased bands after purification steps-DE52 (lane 1), P11 (lane 2), HTP (lane 3) and DNA (lane4), and M (marker), While DNA-cellulose chromatography demonstrating only one band (M_r 160 000).

major peak at 0.165 mol/L, with a minor trailing shoulder occasionally present at 0.21 mol/L (Figure 1B). Fractions from 0.15 mol/L to 0.18 mol/L KPO_4 were pooled because the minor peak was not the protein as expected. The total protein was 0.039 mg.

Figure 3 Western blot of the fractions of DNA-cellulose chromatography. When mouse anti-human DNA polymerase α monocloned antibody was used as the primary antibody, the fraction was found to be DNA polymerase α.

Hydroxylapatite chromatography

Fractions from the 37th to the 64th tube were collected because a single sharp peak appeared at 0.085 mol/L KPO_4 (Figure 1C). The characterization was certified by its activity and the protein was 0.008 mg.

DNA-cellulose chromatography

Protein was eluted in a single sharp peak at 0.085 mol/L KPO_4 (Figure 1D). As the activity was measured, the region from 0.07 mol/L to 0.10 mol/L KPO_4 was pooled and concentrated by dialysis against 300 g/L sucrose, 0.1 mol/L KPO_4 (pH7.5).

Analysis of protein and activity

When the specific activity became higher, the total protein, total activity and yield became lower in each stage, illustrating that DNA polymerase α was purified step by step while the other components were removed (Table 1).

SDS-PAGE and Western blot

SDS-PAGE showed that the purification of protein was effective because the bands decreased with the step of purification and finally there was only one band (Figure 2). Western blot revealed that the band was DNA polymerase α (Figure 3).

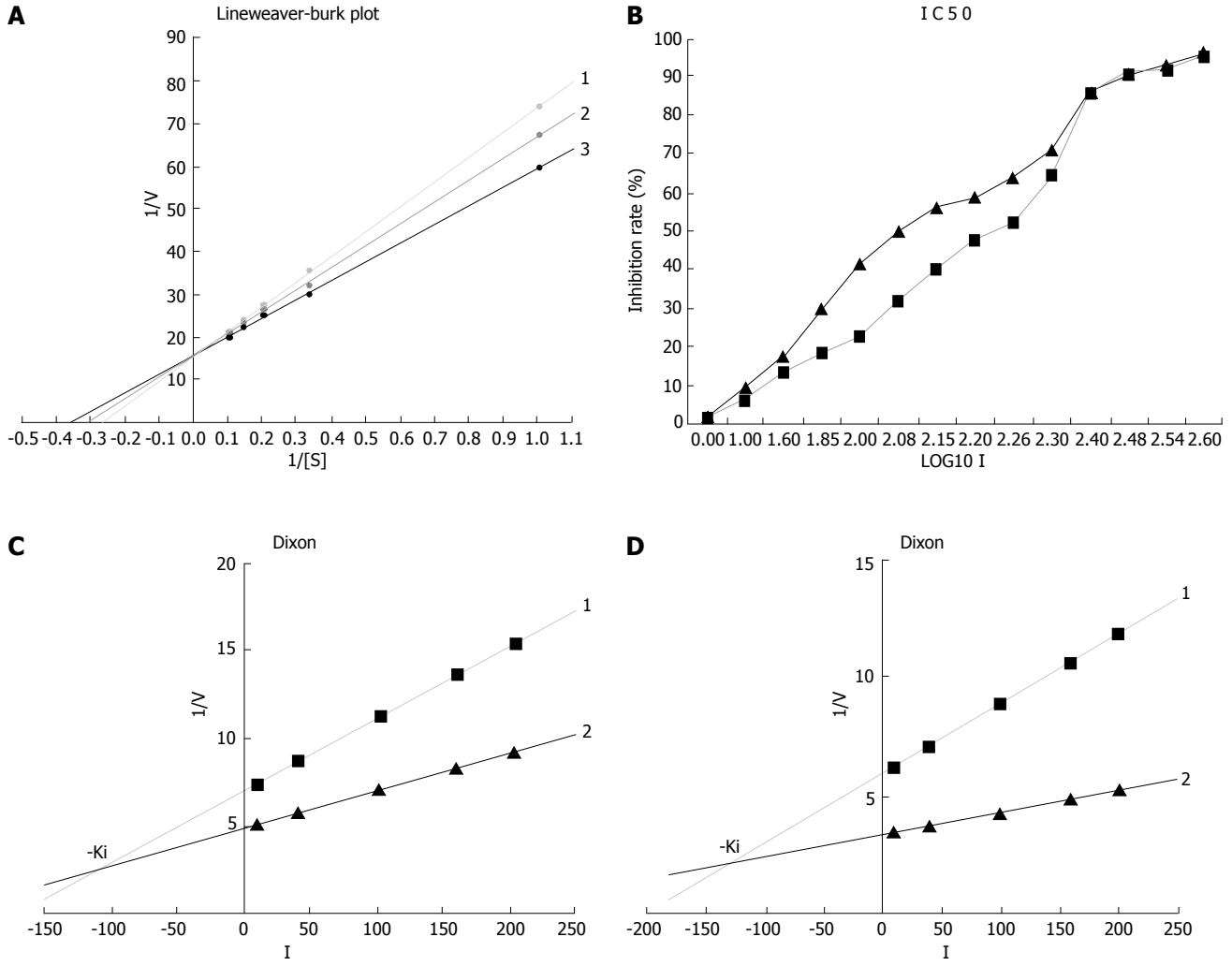

Figure 4 Determination of detailed kinetic parameters Km (**A**), IC50 (**B**), and Ki (**C** for 3TC, **D** for β-L-D4A).

Michaelis constant (Km)

The inhibition mechanism was observed by visual inspection of graphical plots after data linearization (Line weaver-Burk plot) using the GraphPad Prism 4 Demo software. The L-B linear regression equations in Figure 4A showed 3TC5'-triphosphate, y = 15.922 + 57.978x (r^2 = 0.9999), Km = 3.64 μmol/L; β-L-D4A, y = 15.879 + 51.163x (r^2 = 0.9993), Km = 3.22 μmol/L; blank without inhibitor, y = 15.860 + 43.493x (r^2 = 0.9995), Km = 2.74 μmol/L.

Fifty percent inhibition concentration (IC50)

IC50 was calculated using the half logarithm plot, demonstrating that β-L-D4A (178.49 μmol/L) was 1.5 fold more potent than 3TC (122.41 μmol/L) (Figure 4B).

Inhibition constant (Ki)

The Ki was also estimated using the GraphPad Prism 4 Demo software. The two linear regression equations in Figure 4C were 3TC, y = 0.0408x + 6.9594 (r^2 = 0.9998) and y = 0.0212x + 4.8957 (r^2 = 0.9991) while the equations in Figure 4D were β-L-D4A, y = 0.0297x + 6.0389 (r^2 = 0.9965) and y = 0.0095x + 3.4904 (r^2 = 0.9996). The Ki of 3TC was 105 μmol/L which was lower than β-L-D4A (126 μmol/L).

DISCUSSION

HBV infection, associated with the risk of developing liver cirrhosis and hepatocellular carcinoma, is a worldwide public health problem. Two billion people worldwide show evidence of having been infected with HBV, and more than 350 million of them are chronically infected. Persons with chronic hepatitis B are at a high risk of developing hepatic cirrhosis and primary hepatocellular carcinoma, leading to 500000 to 1.2 million deaths annually worldwide. Antiviral chemotherapy remains the only choice of treatment for controlling HBV infection in these individuals, for whom available HBV vaccines provide no benefit. At present, approved therapeutics for chronic HBV infection are α-interferon or nucleoside and nucleoside analogs[8]. Drawbacks of treatment with α interferon include a low sustained response rate, undesirable side effects, the need for parenteral administration, and high cost[9,10]. Treatment with nucleosides such as 3TC is less costly and more convenient[11,12]. The fundamental concern is that while initial treatment of patients with 3TC results in a rapid decrease in HBV DNA blood levels, its efficacy is severely compromised in most patients by the development of antiviral resistance after prolonged therapy[13,14]. Although the use of nucleoside and nucleoside analogs as anti-

hepadnaviral agents is similarly disappointing, prospects for their future use are bright, as several of recently developed analogs have been found to be potent and can be used as selective inhibitors of HBV replication. In our previous work, the novel nucleoside (β-L-D4A) was synthesized and its inhibitiory actions against HBV were studied and found to be much better than 3TC[15,16]. The inhibition of HBV replication by nucleoside analogs results from the recognition of nucleoside analog triphosphates (TPs) by the RNA-dependent DNA polymerase of HBV (HBV Pol). The triphosphate derivatives could serve as substrates for human DNA polymerases, inhibiting cellular DNA synthesis. For the systemic evaluation of the safety of the new drug, kinetic analysis of DNA polymerases must be performed.

Of the multiple DNA polymerases occurring in eukaryotes, only DNA polymerase α is able to initiate the synthesis of new strands[6], because it can initiate the replication of DNA by cooperating with RNA polymerase[17-19].

In the present study, DNA polymerase α was purified step by step because the total protein and activity decreased while the specific activity raised and the number of protein bands was cut down till only one band was left in SDS-PAGE (Mr 160 000). Western blot and the specific activity (31 000 units/mg) confirmed that the scheme was effective. The enzyme obtained lays a foundation for the next research.

Nucleoside analogs are chemically synthesized drugs that are able to mimic natural nucleosides[20,21]. They exert their antiviral effect, after anabolism to the triphosphate form, by acting as alternate substrates for the virally encoded reverse transcriptase[22,23]. Incorporation of the nucleoside analog monophosphate into the viral DNA, results in premature termination of viral DNA synthesis. Nucleoside analogs competitively inhibit DNA-dependent reverse transcriptase activity of the viral polymerase[24,25]. As DNA polymerase α has the similar substrate and mechanism to HBV viral polymerase, the drugs could serve as substrates for human DNA polymerase α, inhibiting cellular DNA synthesis[26,27]. Because DNA polymerase α exhibits neither exonuclease nor endonuclease activity, mistake cannot be repaired[28,29]. To determine the safety of nucleosides on cells in the present study, DNA polymerase α was purified and kinetic analysis was performed under the identical conditions of ionic strength, pH, divalent metal ion concentration, and DNA substrate.

The Km increased and Wmax unchanged (Figure 4A), confirming that both of β-L-D4A and 3TC are the competitive inhibitors of DNA polymerase α. In our study, the drugs inhibited DNA polymerase α activity by acting as competitive alternate substrates. 3TC was more effective than β-L-D4A as inhibitors of DNA polymerase α because IC50 of β-L-D4A was 1.5 fold more potent than 3TC and the Ki of 3TC was 105 μmol/L, much lower than β-L-D4A (126 μmol/L), demonstrating that β-L-D4A is significantly more safe than 3TC.

In conclusion, β-L-D4A is a safe drug for HBV infection because it is endowed with lower host toxicity in comparison to 3TC. Furthermore, combined therapy of β-L-D4A and lamivudine for HBV infection can be explored.

ACKNOWLEDGMENTS

The authors thank Xiao-Dan Jiang, Institute of Immunity, Tongji Medical College.

COMMENTS

Background

Since hepatitis B virus (HBV) replication involves a virally encoded reverse transcriptase (RT), a number of L-configuration nucleoside analogs with an inhibitory effect on RT have emerged as potent antiviral agents against HBV infection. However, most anti-HBV nucleoside analogs tested to date have only transient and limited effects on a small number of HBV-infected individuals with moderate to severe side effects. In light of the fact, development of novel antiviral agents is an extremely important undertaking.

Research frontiers

In recent years, a considerable interest has been focused on the use of 2', 3'-dideoxynucleosides (DDNs) in the treatment of chronic HBV infection. β-L-D4A, a novel L-nucleoside, can effectively block the production of HBV in 2.2.15 cells *in vitro*, but no information is available on the safety evaluation of this new compound.

Innovations and breakthroughs

In this research, kinetic analysis of DNA polymerase α cytotoxicity, was investigated to evaluate the safety of this new compound.

Applications

β-L-D4A is a safe drug for HBV infection because it is endowed with a low host toxicity.

Terminology

β-L-D4A, [5-(6-amino-9H-purin-9-yl)-2,5-dihydrofuran-2-yl] methanol, is a novel L-nucleoside.

Peer review

This is an interesting paper describing the effect of β-L-D4A (a novel nucleoside analog) on DNA polymerase α. The authors separated DNA polymerase α from crude extract of human Hela cells and studied its enzyme kinetics, showing that the enzyme is less inhibited than lamivudine, thus β-L-D4A may be an effective nucleoside for HBV infection with a lower host toxicity.

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World J Gastroenterol 2007 December 14; 13(46): 6249-6253
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effects of endogenous nitric oxide induced by 5-fluorouracil and L-Arg on liver carcinoma in nude mice

Xiao-Yan Yin, Jun-Mei Jiang, Ji-Yong Liu, Ju-Ren Zhu

Xiao-Yan Yin, Endoscopy Center, Affilated Hospital of Medical College, Qingdao University, Qingdao 266003, Shandong Province, China
Jun-Mei Jiang, Ji-Yong Liu, Ju-Ren Zhu, Department of Gastroenterology, Shandong Provincial Hospital, Jinan 250021, Shandong Province, China
Supported by the Grants for the Excellent Youth Scientists of Shandong Provincial Science Commission. No. 2000BB2DBA1
Correspondence to: Dr. Jun-Mei Jiang, Department of Gastroenterology, Shandong Provincial Hospital, Jinan 250021, Shandong Province, China. jiangjunm@hotmail.com
Telephone: +86-532-82911525 Fax: +86-532-82911999
Received: April 11, 2007 Revised: September 1, 2007

Abstract

AIM: To study the effects of endogeous nitric oxide induced by 5-fluorouracil (5-FU) and L-arginine (L-Arg) on the human liver carcinoma model in nude mice.

METHODS: The human liver carcinoma model in nude mice was established with BEL-7402 cells and normal saline (NS), 5-FU and 5-FU + L-Arg injected intraperitoneally. The tumor size was measured. The necrotic degree and range were observed under microscope. The apoptosis of cancer cell was detected by turmina deoxynucleotidyl transferanse mediated dUTP nick end labeling (TUNEL) method. Immunohistochemical method was performed to determine the expression of iNOS, P16, BAX. The chemical colorimetry was used to test the activity and nitrate reductase method was adopted to test the concentration of nitric oxide (NO) in the tumor tissue. The BI2000 pathological image analyzer was used to analyze the result of immunohistochemistry.

RESULTS: 5-FU combined with L-Arg could inhibit the tumor growth apparently. In NS, 5-FU and 5-FU+L-Arg groups, the changes of tumor volumes were 257.978 ± 59.0, 172.232 ± 66.0 and 91.523 ± 26.7 mm^3, respectively ($P < 0.05$ 5-FU *vs* 5-FU + L-Arg group; $P < 0.05$ NS *vs* 5-FU + L-Arg group; $P < 0.05$, NS *vs* 5-FU group). The necrotic range and apoptosis index were significantly increased after the drug injection. The necrotic range was biggest in 5-FU + L-Arg group ($\chi^2 = 15.963$, $P < 0.05$). The apoptosis indexes were as follows: NS, 17.4% ± 6.19%; 5-FU, 31.3% ± 12.3%; and 5-FU + L-Arg, 46% ± 15.24% ($P < 0.05$, 5-FU *vs* 5-FU + L-Arg; $P < 0.05$, NS *vs* 5-FU + L-Arg; $P < 0.05$, NS *vs* 5-FU). The expression and activity of iNOS were increased in the tumor tissue. The concentration of NO was also increased. *F* of optical density of iNOS, iNOS activity and NO concentration are 31.693, 21.949, and 33.909, respectively, $P < 0.05$. The concentration of NO was related to the expression of P16 and BAX. The correlation coefficient was 0.764 and 0.554.

CONCLUSION: 5-FU combined with L-Arg can inhibit the growth of tumor in nude mice. The effect may be related to inducing the synthesis and increasing the activity of iNOS. The production of NO is increased, and it can enhance the expression of apoptosis-related gene and antioncogene.

Key words: 5-Fluorouracil; L-Arginine; Animal model; Nitric oxide synthase; Nitric oxide

Yin XY, Jiang JM, Liu JY, Zhu JR. Effects of endogenous nitric oxide induced by 5-fluorouracil and L-Arg on liver carcinoma in nude mice. *World J Gastroenterol* 2007; 13(46): 6249-6253

http://www.wjgnet.com/1007-9327/13/6249.asp

INTRODUCTION

Over the past decade or so, it has become evident that free radical gas nitric oxide acts as a novel transcellular messenger molecule in many key physiological and pathological processes. It appears that high levels of nitric oxide (NO) may be cytostatic or cytotoxic for tumor cells[1,2]. NO is a small gaseous molecule generated in a wide variety of cells as a product of the conversion of L-arginine into L-citrulline by the enzyme nitric oxide synthase (NOS). There have been few reports about the antitumor effects of endogenous nitric oxide *in vivo*[3,4]. In this study, we observed the inductive effects of 5-fluorouracil (5-FU) and L-arginine (L-Arg) on the endogenous nitic oxide in the human live carcinoma model in nude mice and proved the antitumor effects of the endogenous nitric oxide in the primary hepatic carcinoma and found effective adjuvant for 5-FU.

MATERIALS AND METHODS

Materials

Male Balb/c nude mice were provided by the animal center of Shandong University. BEL-7402 cells were obtained from Shandong Academy of Medical Sciences, China.

Immunochemical kit and *in situ* cell apoptosis detection kit were purchased from Wuhan Boster Technology Company, China. Nitric oxide synthase and NO detection kits were purchased from Nanjing Jiancheng Bioengineering Institute, China. 5-FU was produced by Shanghai Xudong Haipu Pharmacentical Company. L-Arg was produced by Shanghai Xinyi Jinzhu Pharmacentical Company.

Cell lines and culture conditions

Human primary hepatocellular cancer cell line BEL-7402 was maintained in a humidified, 5% CO_2 atmosphere and cultured in DMEM, supplemented with 10% fetal calf serum and penicillin-streptomycin mixture.

Grouping and animal model

BEL-7402 cells were cultured *in vitro* to the log growth phase, centrifuged and washed with PBS to a concentration of 1×10^7/mL, and 0.2 mL cell solution was subcutaneously xenografted in the neck or the flank of 4-6 wk old male Balb/c nude mice. The mice were housed and maintained in lamina flow cabinets under specific pathogen-free conditions. Ten day after establishment of the model when tumors reached a mean diameter of 0.5 cm, mice were randomly divided into three groups (10 mice per group) and treated as follows: group A was injected intraperitoneally with 0.2 mL normal saline, group B was injected with 5-FU at a dose of 20 mg/kg, and group C was injected with 5-FU at 20 mg/kg and L-Arg at 100 mg/kg. The drugs were injected every other day from d 11 to d 15. The mice were killed at 17d after the experiment finished.

Tumor inhibition rate in vivo

The largest diameter (a) and its vertical diameter (b) of the tumors were measured with caplipers every two days after the tumor appeared. The volume of tumor was equal to $1/2ab^2$. The tumor inhibition rate (TIR) was calculated with the following formula:

TIR = control group (v_1 - v_0) - treatment group (v_1 - v_0)/control group (v_1 - v_0)

(v_0: the volume before injection of drugs, v_1:the volume after injection of drugs).

Histology

The sections were stained with hematoxylin-eosin to evaluate the degree and range of the necrosis. It was divided into 4 degrees according to the percentage of the necrotic area: ≤ 25% (+), 25%-50% (++), 50%-75% (+++), > 75% (++++)[5].

TUNEL

TUNEL method was used to detect the apoptosis of tumor cells according to the instructions of the manufacturer. Cells with brown or yellow nuclei were assumed as apoptotic cells. The number of apoptotic cells and total cancer cells was counted under light microscope at 400 × magnification in 5 fields of version and the average values were used for the calculation of apoptosis index (AI) according to the formula: AI = (apoptotic cells/total cancer cells) × 100%.

Immunohistochemical staining

Tumor specimens were dissected from mice and fixed in 10% buffered formalin solution overnight. They were then embedded in paraffin and sectioned in 4 μm thicknesses. SABC immunohistochemistry was performed according to the manufacturer's instructions to detect the gene expression of iNOS, P16 and BAX. Briefly, the tissue sections were deparaffinized in xylene at 37℃ for 20 min. Endogenous peroxide was blocked by incubating the slides with 30 mL/L H_2O_2 for 10 min at 37℃. Sections were incubated with primary antibodies of iNOS, P16 and BAX at 4℃ overnight respectively. Staining was visualized with DAB for 10 min at room temperature. Finally, the sections were counterstained for nuclei by hematoxylin solution. The results were analyzed with a BI2000 pathological image analyzer and expressed as optical density.

iNOS activity and NO concentration assay

NOS catalyzed the formation of NO and L-citrulline from L-arginine and molecular oxygen, and NO reacted with a nucleophile to generate color compounds. The absorbance of NOS at 530 nm was calculated and expressed as U/mg protein. One unit of NOS activity was defined as the production of 1 nmol nitric oxide per second per mg tissue protein. Total NOS activity was measured as follows: 10% tissue homogenate (100 μL) was incubated with 200 μL substrate buffer, 10 μL reaction accelerator and 100 μL color development reagent at 37℃ for 15 min after mixing. Then 100 μL clearing reagent and 2 mL stop solution were added, mixed and absorbances were read at 530 nm. For measuring iNOS activity, an inhibitor was added before incubation according to the manufacturer's instructions. Total protein concentration was determined using the Coomassie blue method with bovine serum albumin as standard.

Tumor samples were thawed, weighed and homogenized 1:9 w:v in 0.9% saline. The homogenates were then centrifuged at 1000 r/min for 5 min at 4℃, the supernatant was taken for NO assay and total protein determination.

NO was assayed spectrophotometrically by measuring total nitrate plus nitrite (NO_3^- plus NO_2^-) and the stable end products of NO metabolism. The procedure was performed by the manufacturer's instructions. Results were expressed as μmol/g protein.

Statistical analysis

All data were analyzed with SPSS11.0 statistical software. One-way ANOVA, Bonferroni, Kruskal-Wallis H test and correlated analysis were performed.

RESULTS

Tumor inhibition rate in vivo

5-FU could inhibit the growth of the liver carcinoma in nude mice. When the substrate L-Arg was supplemented, tumor inhibition rate increased significantly (Table 1).

Histology

There was different necrosis in the tumor tissues. The

Table 1 Changes of tumor volume and tumor inhibition rate

	n	Changes of tumor volume (mm^3)	Tumor inhibition rate (%)
A: NS group	10	257.978 ± 59.0[a]	
B: 5-FU group	10	172.232 ± 66.0[c]	33.24
C: 5-FU+L-Arg group	10	91.523 ± 26.7[e]	64.52

[a]$P < 0.05$, group B *vs* C; [c]$P < 0.05$, A *vs* C; [e]$P < 0.05$, A *vs* B.

Table 2 Necrotic range in tumor tissues

	+	+ +	+ + +	+ + + +
A: NS group	6	3	1	
B: 5-FU group		5	3	2
C: 5-FU+L-Arg group		1	4	5

$\chi^2 = 15.963$, $P < 0.05$.

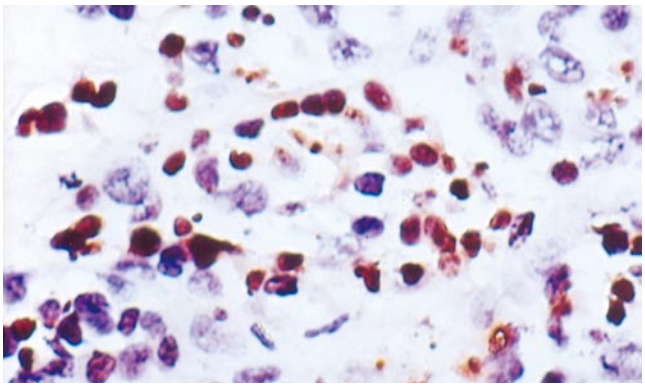

Figure 1 The apoptosis cells in tumor tissues (× 400).

necrotic range enlarged greatly after treatment with 5-FU and L-Arg (Table 2).

Apoptosis index

There were a lot of apoptosis cells in 5-FU and 5-FU+L-Arg groups, while only scattered apoptosis cells in NS group. The apoptosis cells are shown in Figure 1. The apoptosis indexes were as follows: NS group 17.4% ± 6.19%; 5-FU group 31.3% ± 12.3%; and 5-FU+L-Arg group 46% ± 15.24%. [a]$P < 0.05$ group B *vs* group C; [c]$P < 0.05$ A *vs* C; [e]$P < 0.05$ A *vs* B. There was significantdifference between the two groups.

iNOS activity, NO concentration and immunohistochemical results of iNOS

The optical density of iNOS, iNOS activity and NO concentration were increased after treatment with 5-FU. When the substrate L-arg was supplemented, they were increased more significantly (Table 3). The expression of iNOS is shown in Figure 2.

Correlation analysis between concentration of NO and expression of P16, BAX

The expression of P16 and BAX was increased after

Table 3 Optical density of iNOS ,iNOS activity and NO concentration in tumor tissues

	Optical density of iNOS (μmol/L)	Activity of iNOS (U/mg protein)	Concentration of NO
A: NS group	0.4621 ± 0.0115[a]	4.87 ± 2.5[a]	6.58 ± 3.2[a]
B: 5-FU group	0.4783 ± 0.0107[c]	9.83 ± 2.31[c]	17.97 ± 6.16[c]
C: 5-FU+L-Arg group	0.5269 ± 0.0034[e]	15.1 ± 4.91[e]	30.41 ± 8.81[e]

F of optical density of iNOS , iNOS activity and NO concentration are 31.693, 21.949 and 33.909, respectively, [a]$P < 0.05$, B *vs* C; [c]$P < 0.05$, A *vs* C; [e]$P < 0.05$, A *vs* B.

Table 4 The optical density of P16 and BAX in tumor tissues

	Optical density of BAX	Optical density of P16
A: NS group	0.4501 ± 0.0114[a]	0.4565 ± 0.0139[a]
B: 5-FU group	0.4788 ± 0.0068[c]	0.4939 ± 0.041[c]
C: 5-FU+L-Arg group	0.5045 ± 0.0199[e]	0.5451 ± 0.027[e]

F of optical density of BAX andP16 is 23.51 and 16.13. [a]$P < 0.05$, B *vs* C; [c]$P < 0.05$, A *vs* C; [e]$P < 0.05$, A *vs* B.

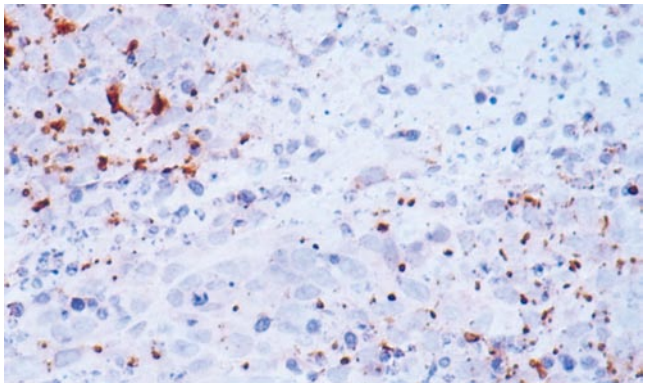

Figure 2 The expression of iNOS in tumor tissues (× 200).

treatment with 5-FU and L-Arg. $P < 0.05$ (Table 4). The result of Pearson analysis showed that the concentration of NO was correlated with the expression of P16 and BAX. The correlation coefficient was 0.764 and 0.554.

DISCUSSION

Hepatocellar carcinoma is one of the most common malignant tumors in China. Surgical resection is the best choice. But many patients could not be treated surgically when diagnosis was made too late. Chemotherapy becomes the important treatment for liver cancer[6,7]. 5-FU is used extensively in the chemotherapy of hepatocellular carcinoma[8,9]. It is well known that mechanism of the anti-tumor effects is to interfere with DNA or protein synthesis[10]. 5-FU can also induce the apoptosis of different tumor cells, but the mechanism is unclear[11,12]. Oshima T *et al* reported that 5-FU induced iNOS expression and NO produced by gastric cancer cells, and NO participated in antitumor activity in gastric cancer cells. These effects may be mediated by TNF-alpha

production[13]. 5-FU could induce the cytokines such as interleukin-1beta (IL-1beta) and TNF-alpha[14]. Other studies showed that the iNOS expression was stimulated by interleukin-1beta (IL-1beta) and TNF-alpha[15]. So we speculated that the 5-FU might induce the iNOS by induction of cytokines. However, this need to be proved by further studies. We found that 5-FU could induce the expression of iNOS of BEL-7402 cells *in vitro*. When the L-Arg was sufficient, the production of NO increased obviously. It caused the increases of apoptosis and necrosis of BEL-7402 cells. NO increased the chemotherapy sensitivity of BEL-7402 cells to 5-FU[16]. In this study, the expression and activity of iNOS increased after the treatment with 5-FU and L-Arg, and the concentration of NO in the tumor tissue was increased at the same time. This suggests that the endogenous NO plays an important role in the anti-tumor effects of 5-FU and L-Arg.

The present study showed that the high concentration of NO could be cytotoxic for tumor cells or inhibit the tumor growth[17,18]. The high concentration of NO could interfere with the citric acid cycle, and arrest the cell in the S stage[19,20]. More importantly, NO has been shown to bind rapidly with high affinity to ferrous iron (Fe^{2+}). As a consequence, NO can bind easily to free iron, iron within iron-sulphur centres, and iron within hemoproteins[21,22]. NO can cause DNA damage via the generation of peroxynitrite (ONOO-) and N_2O_3[23,24]. One of the consequences of the NO-mediated DNA damage is to trigger p53 accumulation, which can induce apoptosis[25,26]. NO can induce the apoptosis of tumor cell by the regulation of the apoptosis and cell cycle related protein, such as P21, BAX, cyclin D *etc*[27-29]. Nicola *et al* reported that NO mediates chemosensitivity in tumor cells, and Hypoxia-induced drug resistance appears to result, in part, from down-stream suppression of endogenous NO production. These results raise the possibility that administration of small doses of NO mimetics could be used as an adjuvant in chemotherapy[30]. In this study, the expression of P16 and BAX was found correlated with the concentration of NO, suggesting that NO can increase the expression of apoptosis-related gene and antioncogene. The necrotic range and apoptosis index increased more significantly in the 5-FU and L-Arg group than other two groups. We think that the high concentration of NO may kill the tumor cells or induce apoptosis of the tumor cells directly. On the other hand, the production of NO also improved the chemosensitivity of tumor cells to 5-FU.

L-Arg is not an essential amino acid, and it can be synthesized in the liver. However, liver function of liver cancer patients is poor, and the concentration of L-Arg in the body is likely low. When patients are undergoing chemotherapy with 5-FU, added with L-Arg to improve the production of endogenous NO in liver cancer cells may enhance the therapeutic effects and improve the antitumor ability of the patients. L-Arg may be an effective adjuvant for 5-FU.

COMMENTS

Background

Hepatocellar carcinoma is one of the most common malignant tumors in China. 5-FU is used extensively for the treatment of liver cancer. The effects of 5-FU alone is not satisfied. It is necessary to find the effective adjuvant for 5-FU.

Research frontiers

The expression of iNOS in the human liver carcinoma and the effects of endogenous nitric oxide on the BEL-7402 cells have been examined.

Innovations and breakthroughs

In the present study, the effects of endogenous nitric oxide on the liver carcinoma in nude mice have been studied.

Applications

The L-Arg can be used as the adjuvant for 5-FU in the treatment of liver cancer.

Terminology

L-Arginine (L-Arg) is a semi-essential amino acid that possesses numerous useful physiologic properties. NOS can catalyzethe L-Arg to form the nitric oxide. When the L-Arg is sufficient, the production of nitric oxide can be increased.

Peer review

This manuscript is very interesting. The title accurately reflects the major contents of the article. The results provide sufficient experimental evidences from which conclusions are drawn. The conclusions are scientifically reliable and valuable.

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S- Editor Liu Y **L- Editor** Ma JY **E- Editor** Ma WH

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World J Gastroenterol 2007 December 14; 13(46): 6254-6258
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Two novel germline mutations of MLH1 and investigation of their pathobiology in hereditary non-polyposis colorectal cancer families in China

Chao-Fu Wang, Xiao-Yan Zhou, Tai-Ming Zhang, Ye Xu, San-Jun Cai, Da-Ren Shi

Chao-Fu Wang, Xiao-Yan Zhou, Tai-Ming Zhang, Da-Ren Shi, Department of Pathology, Cancer Hospital of Fudan University, Shanghai 200032; Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China
Ye Xu, San-Jun Cai, Department of Abdominal Surgery, Cancer Hospital of Fudan University, Shanghai 200032, China
Supported by the Key Project of Shanghai Medical Subjects, No.05 Ⅲ 004 and Shanghai Pujiang Program, No. 06PJ14019
Correspondence to: Dr. Xiao-Yan Zhou, Laboratory of Molecular Pathology, Cancer Hospital of Fudan University, 270 Dongan Road, Shanghai 200032, China. xyzhou100@yahoo.com
Telephone: +86-21-64175590-3646
Received: May 16, 2007 Revised: August 20, 2007

Abstract

AIM: To detect germline mutations of MLH1, and investigate microsatellite instability and expression of MLH1 in tumor tissues of hereditary non-polyposis colorectal cancer (HNPCC) with two novel germline mutations, and further investigate the pathobiology of the two novel mutations of MLH1.

METHODS: RNA was extracted from the peripheral blood of 12 patients from 12 different families that fulfilled the Amsterdam Ⅱ Criteria for HNPCC. Germline mutations of MLH1 were determined by RT-PCR, followed by cDNA sequencing analysis. PCR-GeneScan analysis was used to investigate microsatellite instability with a panel of five microsatellite markers (BAT26, BAT25, D5S346, D2S123 and mfd15), along with immunohistochemical staining to detect the expression of MLH1 protein in two patients' tumor tissues with novel mutations.

RESULTS: Three germline mutations were found in four patients, one of the mutations has previously been reported, but the other two, CGC→TGC at codon 217 of exon 8 and CCG→CTG at codon 581 of exon 16, have not been reported. The two patients' tumor tissues with novel mutations had high-frequency microsatellite instability that showed more than two unstable loci, and both tumors lost their MLH1 protein expression.

CONCLUSION: The two novel germline mutations of MLH1 in HNPCC families i.e. CGC→TGC at codon 217 of exon 8 and CCG→CTG at codon 581 of exon 16, are very likely to have pathological significance.

Key words: Colorectal cancer; Hereditary non-polyposis colorectal cancer; MLH1 gene; Germline mutation; Microsatellite instability; Gene sequencing

Wang CF, Zhou XY, Zhang TM, Xu Y, Cai SJ, Shi DR. Two novel germline mutations of MLH1 and investigation of their pathobiology in hereditary non-polyposis colorectal cancer families in China. *World J Gastroenterol* 2007; 13(46): 6254-6258

http://www.wjgnet.com/1007-9327/13/6254.asp

INTRODUCTION

Colorectal cancer remains a serious public health challenge worldwide. According to the different molecular mechanisms, colorectal cancer is divided into two groups: sporadic and genetic. Hereditary non-polyposis colorectal cancer (HNPCC), the most common hereditary colon cancer syndrome[1], is a predominantly inherited disease associated with increased lifetime risk of a range of cancers, including colorectal and endometrial cancers, as well as extracolonic gastrointestinal, genitourinary, ovarian and brain cancers[2-9]. HNPCC development is associated with the functional deficiency of germline MMR genes. Up to now, seven MMR genes have been found, and among these, MLH1 and MSH2 are very closely associated with HNPCC[10-12]. Carriers of germline MMR mutations have a > 80% risk of cancer by the age of 75[13-15]. Twelve patients fulfilling Amsterdam Criteria Ⅱ from China were explored in this study. Two novel MLH1 mutations were detected, and the pathobiology of the novel mutations was investigated.

MATERIALS AND METHODS

Subjects and samples

Twelve patients of Chinese descent from families fulfilling Amsterdam Ⅱ Criteria were selected through the clinic at the Cancer Hospital of Fudan University, Shanghai, China. Personal and family cancer histories were obtained from the patients and participating relatives, and cancer diagnosis was confirmed by reviewing records and pathology reports. Informed consent was obtained from each participant. Three microliters of peripheral blood of each participant was taken. Total RNA of the peripheral blood was extracted using Trizol (Sigma), according to the

Table 1 Sequences and localization of primers used for Amplification of cDNA of MLH1

Sense	Antisense
MLH1-1F (1-18)	MLH1-5R (2198-2175)
CTTGGCTCTTCTGGCGCC	GAGCGCAAGGCTTTATAGACAATG
MLH1-4F (1333-1353)	MLH1-6R (2484-2459)
GCTGAAGTGGCTGCCAAAAAT	TATGTTAAGACACATCTATTTATTTA

manufacturer's instructions. Twenty unrelated volunteers from families without HNPCC were used as controls.

RT-PCR

cDNA was synthesized with RT (Roche Diagnostics), using 0.5 μg total RNA and specific primers complementary to the 3' end of the MLH1 (2484-TATGTTAAGACACAT CTATTTATTTA-2459). cDNA of MLH1 was amplified in two overlapping fragments using primers (Table 1) that generated products of approximately 2000 bp. PCR was performed using Expand Long Template PCR (Roche Diagnostics): 94℃ for 5 min; 10 cycles at 94℃ for 30 s, 59℃ for 30 s, and 68℃ for 3 min; 32 cycles at 94℃ for 30 s, 57℃ for 30 s, 68℃ for 3 min; and a final extension at 68℃ for 7 min[16].

Sequencing

The purified PCR fragments were sequenced directly using a DNA sequencing kit with BigDye Terminators on an ABI3700 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer' s instructions. The cDNA of MLH1 (2484 bp) was sequenced in six overlapping fragments using primers described in Table 2.

Microdissection and minimal amount of DNA extraction

One 5-μm and four 7-μm paraffin-embedded sections of tumor tissues were deparaffinized. The 5-μm sections were stained with hematoxylin and eosin and served as controls. The 7-μm sections were lightly stained with hematoxylin for microdissection. The microdissection was performed under a dissection microscope with a scalpel. Tumor cells should account for at least 80% of the total cells isolated. The microdissected tissues were transferred directly into an Eppendorf tube with 150 μL cell lysis buffer (0.5 mol/L Tris, 20 mmol/L EDTA, 10 mmol/L NaCl, 10 g/L SDS, 0.5 g/L Proteinase K). The subsequent DNA extraction was performed according to the protocol of the DNA extraction kit (Daxia Biotech, Shanghai, China). Genomic DNA was also extracted from peripheral white blood cells.

Microsatellite instability (MSI) analysis

Matched normal and tumor DNA was investigated with a panel of microsatellite markers (mononucleotide repeats BAT26 and BAT25, dinucleotide repeats D5S346, D2S123 and mfd15)[17]. The primer pairs were synthesized by Shenyou Biotech (Shanghai, China). Each forward primer was labeled with a fluorescent dye at the 5' end to enable the PCR products to be detected by an ABI 310 automated DNA sequencer. After successful amplification, the 2-μL PCR products were mixed with 12.5 μL deionized formamide and 2 μL 350 Rox Sizer. The mixture was denatured, snap-cooled and electrophoresed on an ABI 310 automated DNA sequencer according to the manufacturer's recommendation. The electrophoresis results were analyzed by GeneScan Software (Applied Biosystems). MSI was determined according to the method of Gebert *et al*[18]. Additional peaks (bands) at microsatellite loci in the tumor compared with normal tissue from the same patient were interpreted as MSIs. Cases with MSIs in more than two loci were interpreted as exhibiting high MSI.

Immunostaining for MLH1

A monoclonal antibody against MLH1 (Pharmingen, San Diego, CA, USA) was prepared at a 1:40 dilution. The antibody was detected by the EnVision method. Diminished expression of MLH1 in cancer tissues was demonstrated when there was complete absence of detectable nuclear staining of neoplastic cells. Infiltrating lymphocytes, as well as normal colonic crypt epithelium next to the tumor area, served as internal positive controls[19,20].

RESULTS

Germline mutations of MLH1

Four germline mutations were detected at three different sites of MLH1, involving four patients, which were at 649 codon 217 exon 8: CGC→TGC in family H2, at 1742 codon 581 exon 16: CCG→CTG in family H31, and at 1151 codon 384 exon 12: GTT→GAT in family H109 and H114. All three were missense mutations (Table 3, Figures 1 and 2). Their polymorphism possibilities were excluded by visiting the mutation database of MMR genes (www.INSIGHT-group.org). The mutation in families H109 and H114 has been reported to be pathogenic, while the mutations in H2 and H31 are not. The three abnormalities in MLH1 were not found in the control group.

MSI analysis

Four loci in BAT25, BAT26, D2S123 and D5S346 showed MSI in the tumor tissue of the patient from the H2 family, and four loci in BAT25, BAT26, D2S123 and Mfd15 showed MSI in the tumor tissue of the patient from the H31 family. According to the criteria above for MSI, the tumor tissues of the two patients had high MSI (Figure 3).

Immunohistochemistry of MLH1

There was no expression of MLH1 protein in the tissues of the two patients. As a control, MLH1 protein was detected in infiltrating lymphocytes and the normal colonic crypt epithelium next to the tumor area in the patient from the H2 family, and in the stromal cells in the patient from the H31 family (Figures 4 and 5).

DISCUSSION

Colorectal cancer is one of the most common malignant tumors; furthermore, its incidence is increasing continuously. HNPCC, genetic colorectal cancer, accounts for about 10% of all colorectal cancer. Compared with sporadic colorectal cancer, HNPCC shows special characteristics associated with its molecular mechanisms

Table 2 MLH1 primers used for sequencing of cDNA

	Sense		Antisense
MLH1-1F	CCTGGCTCTTCTGGCGCC	MLH1-1R	CTTTTCTCCTCGTGGCTATGTTGT
MLH1-2F	ATGTGCTGGCAATCAAGGGA	MLH1-2R	GGTGCACATTAACATCCACATTCT
MLH1-3F	CCAAAAACACACACCCATTCCT	MLH1-3R	CCTTTGTTGTATCCCCCTCCA
MLH1-4F	GCTGAAGTGGCTGCCAAAAAT	MLH1-4R	CATCTTCCTCTGTCCAGCCACTC
MLH1-5F	TTGCCATGCTTGCCTTAGATAGTC	MLH5R	GAGCGCAAGGCTTTATAGACAATG
MLH1-6F	GCTCCATTCCAAACTCCT	MLH1-6R	TATGTTAAGACACATCTATTTATTTA

Table 3 Germline mutations of MLH1

Families	Genes	Exon	Codons affected	DNA change	Amino acid change	Mutation types
H2	MLH1	8	217	C→T, at 649	Arg→Cys	Missense
H31	MLH1	16	581	C→T, at 1742	Pro→Leu	Missense
H109	MLH1	12	384	T→A, at 1151	Val→Asp	Missense
H114	MLH1	12	384	T→A, at 1151	Val→Asp	Missense

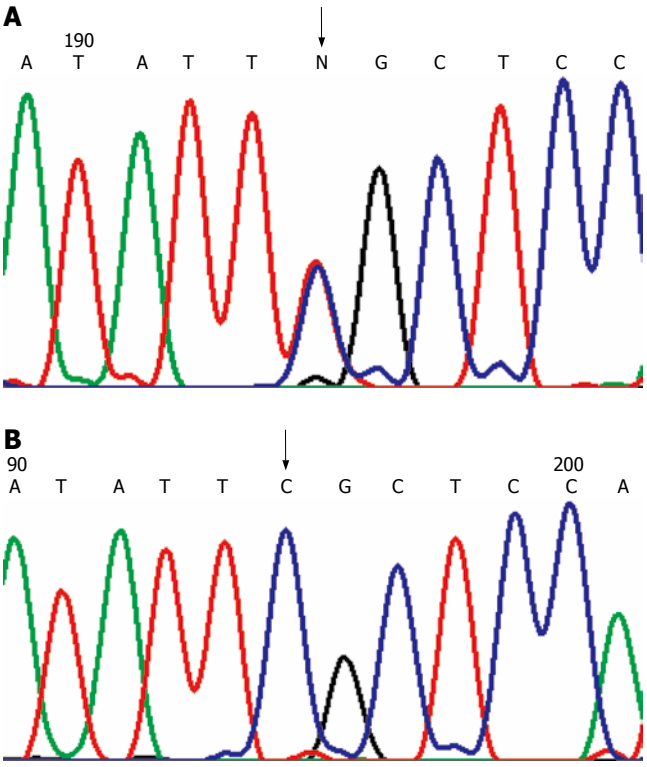

Figure 1 Missense mutations. (**A**): MLH1 mutation in H2 family at 649 codon 217 exon 8: CGC→TGC. The arrow shows the site of the mutation; (**B**): Wild-type sequence. The arrow shows the corresponding site.

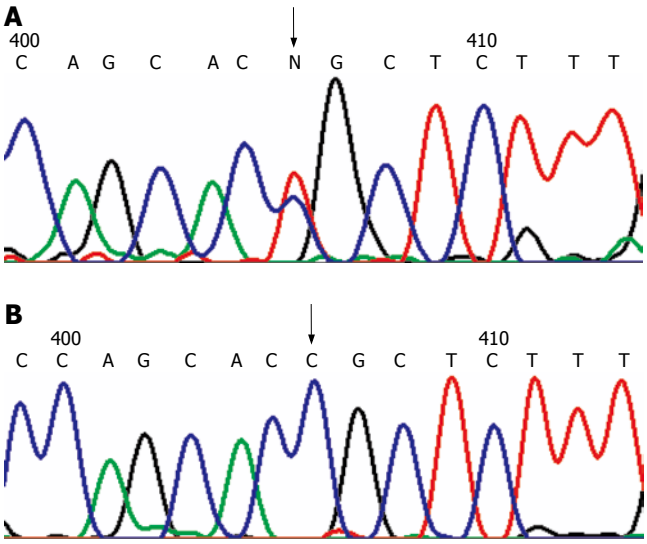

Figure 2 Missense mutation. **A**: MLH1 mutation in H31 family at 1742 odon 581 exon 16: CCG→CTG. The arrow shows the site of the mutation; **B**: Wild-type sequence. The arrow shows the corresponding site.

and clinic features. HNPCC, as the most common hereditary colon cancer syndrome, is characterized by early onset of colorectal cancer, location of tumors in the proximal colon, and an increased risk of neoplasms of extracolonic organs, including endometrium, stomach, urothelium, small intestine, and ovary, multiple metachronous colorectal cancer[21-24], and better prognosis than that in sporadic cases[25-30]. Development of HNPCC is closely associated with deficiency or loss of MMR gene function. Identification of MMR gene germline mutations can have direct clinical implications in counseling and management of HNPCC families[31].

HNPCC has gained worldwide recognition, and several developed countries, such as the United States, Germany, Finland and the Netherlands, have established HNPCC genetic institutions, and several clinical criteria for the diagnosis of concerned families have been suggested. However, at present, there are only are a few institutions in China that are engaged in research on HNPCC. Our hospital collaborative group on HNPCC has been involved in the field for a few years, and has set up an HNPCC database. We detected the 12 random samples from our database that fulfilled Amsterdam Criteria Ⅱ, using an mRNA-based sequencing technique, and three germline mutations of MLH1 were found. All three were missense mutations, two of which have not been reported previously. Diagnosis of HNPCC was based on finding the pathological germline mutation in MMR gene. In the present series, difficulties in the assessment of pathogenicity were mostly associated with missense mutations. Peltomaki *et al* thought the missense mutation meeting the following criteria was pathogenic (1)

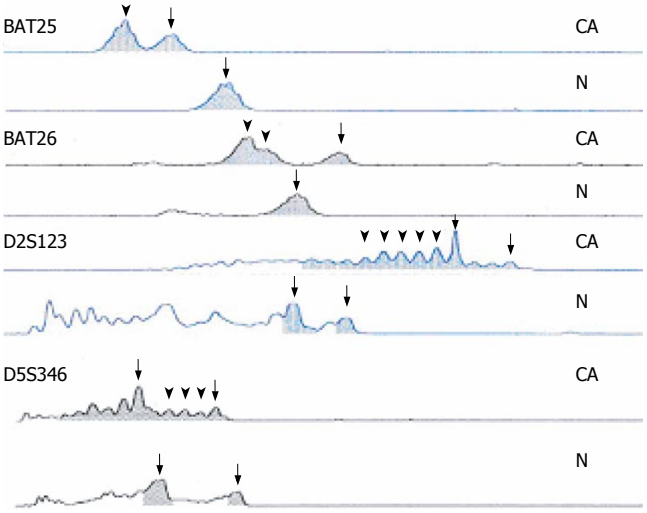

Figure 3 Four sites of MSI in H2 family. CA represents the tumor tissue, N represents the control tissue, the arrow-heads show new waves, and the arrows show wild ones.

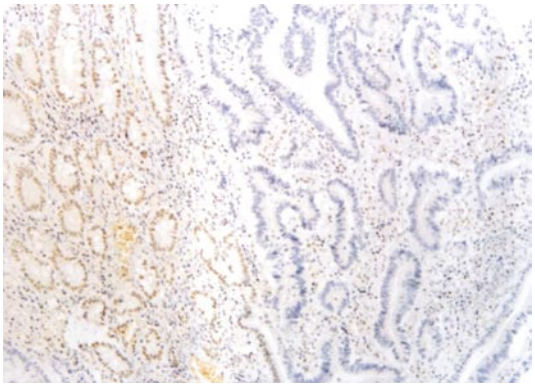

Figure 4 MLH1 protein in H2 was negative in the tumor glands (right), and positive in the mucous glands next to the tumor tissue (EnVison, × 200).

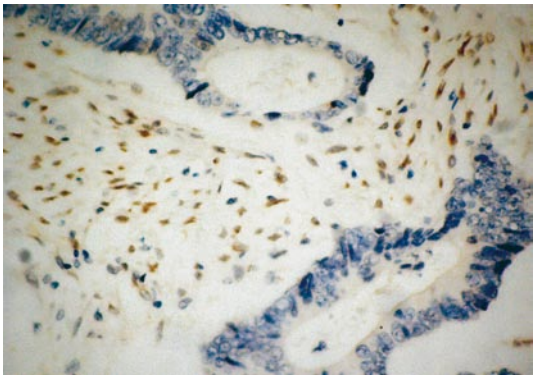

Figure 5 MLH1 protein in H31 was negative in the tumor glands, and positive in the stroma cells (EnVison, × 400).

that it led to a nonconservative aminoacid change, (2) that the involved codon was evolutionarily conserved, (3) that the alteration did not occur in the normal population, and (4) that it cosegregated with the disease phenotype[11]. The patient in the H2 family was affected by colorectal cancer at 36 years of age, his mother was affected by endometrial cancer at 54 years of age, and one of his aunts was affected by colorectal cancer at 48 years of age. The patient in the H31 family was affected by colorectal cancer at 39 years of age, her father was affected by colorectal cancer at 50 years of age, and her uncle was affected by gastric carcinoma at 57 years of age. Their family histories suggested co-segregation of the mutations with the disease. Both of the novel mutations lead to amino acid changes, and the changed amino acids belong to the non-conserved ones. According to the criteria above, we estimated that the two novel mutations were pathogenic. In order to further evaluate the mutations, DNA were isolated from the two patients' tumor tissues, and GeneScan was employed for MSI analysis, and immunohistochemistry was used to detect the expression of MLH1 protein in tumor tissues[32,33]. The tumor tissues of the two patients showed high MSI and lack of MLH1 protein expression. Based on the above results, we concluded that the two novel mutations were very likely pathogenic.

Differentiating HNPCC from sporadic colorectal cancer has practical clinical value, and the identification of HNPCC depends on the detection of germline mutations of the MMR gene. More MMR genes should be investigated besides MLH1, and when a novel mutation is found, its pathogenic evaluation should be carried out so that more HNPCC can be identified.

COMMENTS

Background

Hereditary non-polyposis colorectal cancer (HNPCC) is one of the most common autosomal dominantly inherited cancer syndromes and accounts for 10% of all colorectal cancer. HNPCC shows its own characteristics associated with its molecular mechanisms, clinical features, method of treatment, and management of HNPCC families. It has gained worldwide recognition, and the International Collaborative Group on Hereditary Non-polyposis Colorectal Cancer (ICG-HNPCC) was founded in 1990. At present, there are a few ways to screen HNPCC families; however, only the pathological germline mutation in the mismatch repair (MMR) gene can be used for identifying HNPCC families.

Research frontiers

Development of HNPCC is closely associated with deficiency or loss of function of MMR genes. At least 5 MMR genes, MLH1, MSH2, MSH6, PMS1, and PMS2, have been implicated in HNPCC. Genetic linkage analysis showed that germline mutations of MLH1 and MSH2 account for nearly 90% of all the germline mutations found in HNPCC. Germline mutations of MLH1 were detected in this study.

Innovations and breakthroughs

There are a few ways to screen HNPCC families, however, the most specific method is to detect germline mutations of MMR genes. In present study, a new method, RNA-based sequencing analysis, was used. Two novel germline mutations in MLH1 were found, and their possible pathobiology was investigated by PCR-GeneScan analysis and immunohistochemical staining.

Applications

Identification of pathological germline mutations in MMR genes is the gold standard for HNPCC. Differentiating HNPCC from sporadic colorectal cancer has direct clinical implications for counseling and management of HNPCC family members.

Peer review

HNPCC is different from sporadic colorectal cancer(SCRC), and to differentiate HNPCC from SCRC owns practical clinical implications. The study detected germline mutations of MLH1 with a new method, and investigated the pathobiology of the detected novel mutations in MLH1. The study is a valuable contribution with potential clinical importance.

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S- Editor Liu Y **L- Editor** Kerr C **E- Editor** Li HY

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World J Gastroenterol 2007 December 14; 13(46): 6259-6263
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effects of short-term application of low-dose growth hormone on trace element metabolism and blood glucose in surgical patients

Kun Qian, Zhi Wan, Lang-Song Hao, Ming-Ming Zhang, Yong Zhou, Xiao-Ting Wu

Kun Qian, Lang-Song Hao, Ming-Ming Zhang, Yong Zhou, Xiao-Ting Wu, Department of General Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Zhi Wan, Pediatrics, West China Second Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Correspondence to: Professor Xiao-Ting Wu, Department of General Surgery, West China Hospital, Sichuan University, 37 Guo Xue Road, Chengdu 610041, Sichuan Province, China. hxjsqk@163.com
Telephone: +86-13980523523 Fax: +86-288-5422483
Received: June 14, 2007 Revised: October 8, 2007

Abstract

AIM: To investigate the effects of short-term application of low-dose growth hormone on trace element metabolism and blood glucose in surgical patients

METHODS: A total of 48 consecutive patients undergoing abdominal operations were randomized to receive either subcutaneous rhGH (0.15 IU/kg) or placebo (menstruum) injections daily for 7 d after surgery. The two groups had similar nutrition intake. Blood, feces, urine and drain samples were collected to measure zincum, cuprum and ferrum as well as glucose levels. Accumulative intake, excretion and balance of zincum, cuprum and ferrum, apparent absorption (AA) and apparent utilization (AU) of zincum, cuprum and ferrum, blood glucose levels and adverse events were estimated.

RESULTS: There were no differences in accumulative intake and drain excretion between the two groups. The feces excretion and accumulative excretion of cuprum were lower in the rhGH group ($P < 0.05$). The urinary excretion of zincum, cuprum and ferrum was all significantly decreased in the rhGH group ($P < 0.05$) and the accumulative balance of zincum, cuprum and ferrum was improved compared with the placebo group ($P < 0.05$). AA of cuprum in the rhGH group was almost twice as much as the placebo group ($P < 0.05$), and AU of zincum, cuprum and ferrum was all improved in the rhGH group ($P < 0.05$). The mean blood glucose level was significantly higher in the rhGH group than in the placebo group from d 3 to d 6 after operation ($P < 0.05$).

CONCLUSION: Postoperative low-dose rhGH treatment improves the retention of zincum, cuprum and ferrum and decreases the excretion of zincum, cuprum and ferrum, improves the balance of zincum, cuprum and ferrum, and promotes the AA and AU of zincum, cuprum and ferrum. rhGH can be well tolerated without significant adverse effects and the blood glucose level can be well controlled.

Key words: Growth hormone; Metabolism; Trace elements; Zincum; Cuprum; Ferrum

Qian K, Wan Z, Hao LS, Zhang MM, Zhou Y, Wu XT. Effects of short-term application of low-dose growth hormone on trace element metabolism and blood glucose in surgical patients. *World J Gastroenterol* 2007; 13(46): 6259-6263

http://www.wjgnet.com/1007-9327/13/6259.asp

INTRODUCTION

Patients undergoing abdominal surgery often suffer from severe trauma or infection caused by catabolic responses[1], which cannot be prevented by conventional parenteral or enteral nutrition formulas[2,3]. Administration of recombinant human growth hormone (rhGH) has been shown to significantly maintain the nitrogen balance and increase the protein synthesis in surgical patients receiving either parenteral or enteral nutrition[4-7]. Most of such studies paid more attention to nitrogen balance and protein metabolism changing associated with rhGH treatment. However, there are few studies focusing on the effects of rhGH on trace element metabolism in patients. The present study was to evaluate the effects of rhGH on trace element metabolism and blood glucose levels in selective abdominal surgical patients.

MATERIALS AND METHODS

Patients

The study was conducted in accordance with the guidelines for Good Clinical Practice and the provisions of the Declaration of Helsinki in 1995 as revised in Edinburgh 2000, and approved by the Ethical Review Committee of West China Hospital. Only those who consented to

participate in the study after explanation of the objectives and protocol were included in the study. Signed, informed consent was obtained from all patients and their close relatives.

Forty-eight adult patients were enrolled in the study and all met the following criteria: undergoing a selective abdominal operation, aged 18-75 years, willing and being able to comprehend the protocol and give written informed consent. Exclusion criteria were as follows: severe bacterial infection, liver and renal dysfunction, previous or current treatment with corticosteroids, diabetis mellitus or fasting glucose levels greater than or equal to 7.0 mmol/L, metabolic diseases, gestation, severe malnutrition (serum albumin < 21 g/L), tumor recrudescence or metastasis.

Study design

The study was a randomized prospective double-blind, placebo-controlled clinical trial. Eligible patients were randomly assigned to rhGH group or placebo group (24 each group). The randomization codes were prepared with the random number table according to the design of a computer. Patients, surgeons and nursing staff members remained blind to the allocation status of the study drugs throughout the experiment.

After operation, all patients received continuous combined intravenous or/and enteral nutrition. The daily total caloric requirement was 20 kcal/kg and total nitrogen requirement was 140 mg of nitrogen/kg. Parenteral nutrition (PN) solution was prepared aseptically using commercially available products, including vitamins, trace elements and electrolytes (Addamel, Vitlipid, Soluvit and Glycophos; Fresenius Kabi Deutschland Gmbh, Bad Homburg, Germany). Amino acid injections were provided as 8.5% and 11.4% Novamin (Fresenius Kabi Deutschland Gmbh). Energy calories were provided with glucose and fat emulsion injections (50% glucose and 20% Lipovenos® MCT; Fresenius Kabi Deutschland Gmbh). All the nutrients were given in all-in-one bag. Enteral nutrition (EN) emulsion (Fresenius Kabi Deutschland Gmbh) was provided orally or via a nasogastric tube with a continuous perfusion pump.

Postoperatively, patients received general intravenous infusion with only glucose on d 1; PN provided only half of total caloric and nitrogen requirement on d 2 and all of total requirement on d 3; on d 4, PN provided 2/3 of total requirement and EN provided another 1/3; on d 5, PN provided 1/3 of total requirement and EN provided 2/3; only EN emulsion was given from d 6 to d 9.

From d 3 to d 10 post operation, patients were randomly assigned to receive identical-looking treatments consisting of either rhGH (JINTROPIN®, 0.15 mg/kg) or menstruum injection (1 mL, consisting of glycin, mannitol, lactose and sodium bicarbonate) subcutaneously once daily. rhGH and placebo were provided by GeneScience Pharmaceutical Co. Ltd, Changchun, China.

Laboratory tests

Blood samples were drawn from each patient before operation to measure baseline values and on d 3 and 10 after operation to study the rhGH effect. Complete blood cell count was estimated by the XE-2100 (Sysmex, Kobe, Japan). Plasma glucose, serum urea nitrogen, creatinine, bilirubin, alanine aminotransferase, alkaline phosphatase, total protein, albumin and electrolytes were estimated using an Olympus AU5400 autoanalyser (Olympus, Tokyo, Japan).

Table 1 Baseline characteristics of patients (mean ± SD)

Variable	Placebo (n = 24)	rhGH (n = 24)	P
Age (yr)	58.50 ± 9.35	59.08 ± 10.93	0.789
	39-75	35-74	
Sex (femal/male)	11/13	9/15	0.558
Weight (kg)	57.90 ± 8.42	56.19 ± 11.83	0.567
Height (cm)	162.42 ± 6.92	162.88 ± 7.16	0.823
Sepsis score	0.79 ± 0.98	0.67 ± 0.87	0.742
Operation position, n (%)			
Resection of stomach	6 (25)	5 (20.8)	0.297
Resection of colon	5 (20.8)	7 (29.2)	
Resection of rectum	12 (50)	9 (37.5)	
Others	1 (4.2)	3 (12.5)	
Acumulative intakes of energy (10^3 kcal)	7.98 ± 0.67	7.76 ± 0.76	0.292
Acumulative intakes of nitrogen (g)	54.84 ± 5.23	53.91 ± 7.05	0.605

Trace element balance

Daily trace element input was assumed to be the trace element contents (zincum, cuprum, ferrum) in PN/or EN solution given. Daily trace element loss was assessed by collecting 24-h output and measuring the trace element contents in feces, urine and drains. Accumulated trace element balance was calculated by subtracting 7 d trace element output from 7 d trace element input. Trace element contents in samples were determined by the inductively coupled plasma atomic emission spectrometry (ICPAES) and estimated by the IRIS ADVANTAGE 1000 (Thermo Elemental, USA).

Statistical analysis

All data were assessed for normality of distribution and equality of variance. Student's t-test and multiple correlation analysis were used to compare normal distribution of data. Data are presented throughout as mean ± SD. All data analyses were performed using the program SPSS 11.5 for Windows. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

There was no difference in baseline characteristics between the two groups (Table 1).

Accumulative intake, excretion and balance of zincum, cuprum and ferrum

As shown in Table 2, there were no differences in accumulative intake and drain excretion between the two groups. The feces excretion and accumulative excretion of cuprum were lower in the rhGH group. The urinary excretion of zincum, cuprum and ferrum was

Table 2 Accumulative intake, excretion and balance of zincum, cuprum and ferrum

Test	Zincum		Cuprum		Ferrum	
	Placebo (n = 24)	rhGH (n = 24)	Placebo (n = 24)	rhGH (n = 24)	Placebo (n = 24)	rhGH (n = 24)
Accumulative intake (mg)	65.79 ± 6.38	71.92 ± 6.96	10.01 ± 0.85	10.82 ± 0.92	65.08 ± 8.50	73.25 ± 9.29
Urinary excretion (mg)	36.74 ± 5.76	32.51 ± 5.55[a]	0.37 ± 0.08	0.28 ± 0.05[a]	18.65 ± 5.99	12.10 ± 3.92[a]
Feces excretion (mg)	19.14 ± 6.49	18.07 ± 7.50	4.21 ± 1.00	1.74 ± 0.95[a]	22.38 ± 9.82	19.65 ± 3.71
Drain excretion (mg)	2.74 ± 0.48	2.48 ± 0.62	0.84 ± 1.00	0.61 ± 0.05	14.83 ± 4.05	16.69 ± 3.32
Accumulative excretion (mg)	58.62 ± 8.69	53.06 ± 9.35	5.42 ± 1.42	2.63 ± 0.95[a]	55.86 ± 12.19	48.44 ± 6.34
Accumulative balance (mg)	7.17 ± 5.90	18.86 ± 6.24[a]	4.59 ± 1.33	8.19 ± 0.28[a]	9.22 ± 8.74	24.81 ± 6.79[a]

[a]P < 0.05 *vs* placebo group.

Table 3 Comparison between apparent absorption (AA) and apparent utilization (AU) of zincum, cuprum and ferrum (mean ± SD)

Test	Zincum		Cuprum		Ferrum	
	Placebo (n = 24)	rhGH (n = 24)	Placebo (n =24)	rhGH (n = 24)	Placebo (n = 24)	rhGH (n = 24)
Accumulative intake (mg)	65.79 ± 6.38	71.92 ± 6.96	10.01 ± 0.85	10.82 ± 0.92	65.08 ± 8.50	73.25 ± 9.29
Accumulative intake of EN (mg)	46.29 ± 6.38	52.42 ± 6.96	6.17 ± 0.85	6.98 ± 0.92	61.72 ± 8.50	69.89 ± 9.29
Urinary excretion (mg)	36.74 ± 5.76	32.51 ± 5.55	0.37 ± 0.08	0.28 ± 0.05	18.65 ± 5.99	12.10 ± 3.92
Feces excretion (mg)	19.14 ± 6.49	18.07 ± 7.50	4.21 ± 1.00	1.74 ± 0.95[a]	22.38 ± 9.82	19.65 ± 3.71
Apparent absorption (%)	59.09 ± 10.56	66.25 ± 11.07	31.80 ± 13.78	75.21 ± 13.69[a]	64.94 ± 11.72	71.87 ± 4.00
Apparent utilization (%)	15.21 ± 5.43	30.02 ± 6.23[a]	54.35 ± 8.87	81.36 ± 8.57[a]	37.31 ± 6.85	56.34 ± 6.99[a]

[a]P < 0.05 *vs* placebo group.

Table 4 Comparison of blood glucose levels

Test	D1	D3	D4	D5	D6	D7	D8	D9
Glucose (mmol/L)								
Placebo	5.26 ± 1.09	5.68 ± 1.33	5.81 ± 1.56	5.84 ± 1.48	5.95 ± 2.34	6.01 ± 2.64	5.66 ± 2.03	5.70 ± 1.89
rhGH	5.14 ± 0.64	6.71 ± 1.93[a]	7.17 ± 1.86[a]	8.28 ± 2.30[a]	7.68 ± 2.15[a]	7.29 ± 2.93	6.40 ± 2.00	6.20 ± 2.13

[a]P < 0.05 *vs* placebo group.

Table 5 Main adverse events

Event	Placebo	rhGH
Hyperglycemia	4	23[a]
Tetter	1	0
Sepsis	0	0
Infection	2	3
Death	0	0

[a]P < 0.05 *vs* placebo group.

all significantly decreased in the rhGH group and the accumulative balance of zincum, cuprum and ferrum was significantly improved compared with the placebo group.

Apparent absorption (AA) and apparent utilization (AU) of zincum, cuprum and ferrum

The cuprum was mostly excreted *via* feces. AA of cuprum in the rhGH group was almost twice as much as that in the placebo group, and AU of zincum, cuprum and ferrum was improved in the rhGH group (Table 3).

Blood glucose levels and adverse events

The main adverse effects seen during the study are summarized in Tables 4 and 5. The mean blood glucose level was significantly higher in the rhGH group than in the control group from d 3 to d 6 after operation (Table 4). Twenty-there patients in the rhGH group experienced hyperglycemia and 5 of them required insulin treatment (Table 5). Furthermore, 3 patients had other mild adverse events (1 with edema, 1 with tetter and 1 with fever). In the placebo group, 3 of 4 patients presenting hyperglycemia required insulin treatment. Five placebo-treated patients experienced mild electrolyte imbalance, which was not related the trial drug used. There was no significant difference in complete blood cell count, liver and renal function, body weight and daily clinical parameters such as temperature, blood pressure, and pulse, between the two groups.

DISCUSSION

Many attempts have been made to reverse the catabolic changes that occur in postoperative patients. Conventional nutrition support is unable to provide adequate nutritional supplements to increase or even maintain body proteins and trace elements in hypercatabolic response conditions[8-10]. Recent studies indicate that rhGH can stimulate body protein synthesis and produce nitrogen-spacing effects[11-13]. However, the impact of rhGH on body trace elements and blood glucose has not been

investigated in patients receiving PN or EN following selective gastrointestinal surgery[14-16]. In the present experiments, we studied the effects of rhGH on trace element metabolism and blood glucose. The number of patients enrolled in the study was based on previous experiments and the dosage of rhGH used[7,17,18].

Massive trace elements are lost after selective operation because of decreased intake, loss from wound surface, redistribution in the body and increased urinary excretion[19,20]. Even supplying adequate nutritional support cannot prevent such a massive loss of trace elements. Zincum, cuprum and ferrum are very important trace elements in the human body and can sensitively reflect changes in gastric diseases[21]. In this study, low-dose rhGH treatment reduced the urinary excretion of zincum, cuprum and ferrum, thus improving their accumulative balance compared with the placebo group. Meanwhile, the apparent absorption and utilization of zincum, cuprum and ferrum in the rhGH group were also increased. However, the AU of zincum in the rhGH group (30.02%) was almost two times higher than that in the placebo group. The AU of cuprum and ferrum in the rhGH group was also about 1.5 times higher than that in the placebo group. These data indicate that low-dose rhGH treatment can reduce the excretion of zincum, cuprum and ferrum, increase their utilization, and maintain the retention and balance of zincum, cuprum and ferrum.

Changes in zincum, cuprum and ferrum metabolism are mainly associated with protein synthesis and breakdown. Since proteins are carriers of many trace elements, rhGH may also improve protein synthesis, reduce protein breakdown, promote recovery of intestinal mucosa, increase mucosa thickness, improve intestinal barrier function, and increase absorption of trace elements[22-24]. In our study, the apparent absorption and utilization of zincum, cuprum and ferrum were improved in the rhGH group.

It was reported that GH given during sepsis can impair immune function and result in hyperglycemia, which may explain why acute critically ill patients do not benefit from GH treatment[25,26]. However, selective surgical patients can safely administer GH after the acute inflammatory response stage. rhGH treatment was generally well tolerated with no serious adverse events occurred in our trial. No death occurred in the GH-treated group, confirming its safety. These results are contrary to the increased mortality among critically ill patients treated with GH[25]. We hypothesize that this discrepancy might be due to the difference in study patients. In our study, the patients were selective surgery subjects. rhGH given during the response to stress leads to uncontrolled systemic inflammation in Takala's study[25].

The main adverse event of rhGH treatment is hyperglycemia. Insulin resistance caused by rhGH plays an important role in the elevation of blood glucose. Other reasons include nutrition support and systemic inflammation syndrome[27,28]. In our study, hyperglycemia caused by rhGH administration was mild and controlled by insulin. Considering the difference between critically ill patients and selective surgery patients, rhGH seems to be well tolerated after operation.

Since our study included 14 cancer patients in the rhGH group, the potential tumor-promoting effect of GH should be addressed. In animal models, the role of rhGH administration in promoting tumor recurrence is controversial[29-31]. It was reported that GH could promote host growth selectively and inhibit tumor metastasis[32,33]. Only two trials have assessed the impact of GH on tumor recurrence in humans. Based on 2632 adverse events, the National Cooperative Growth Study analyzed the recurrence of brain tumors in patients receiving long-term GH replacement, showing that there is no evidence that GH increases tumor recurrence[34]. Only one study has investigated the impact of short-term treatment with three different doses of GH on long-term tumor recurrence in postoperative cancer patients[35], finding that 35% rhGH-treated patients have tumor recurrence in comparison to 44% placebo-treated patients. Based on the above two studies, we believe that when complete resection and appropriate antineoplastic treatment are administered, cancer patients can safely receive short-term GH treatment.

In conclusion, postoperative low-dose rhGH treatment improves the retention and decreases the excretion of zincum, cuprum and ferrum, increases the balance and promotes their apparent absorption and utilization. rhGH is also well tolerated with no significant adverse effects and can control the blood glucose level. A larger trial is required to determine the clinical endpoints such as infection, morbidity, mortality and tumor recurrence.

COMMENTS

Background

Patients undergoing abdominal surgery often suffer from severe trauma or infection caused by catabolic responses, which cannot be prevented by conventional parenteral or enteral nutrition formulas. Administration of recombinant human growth hormone (rhGH) has been shown to significantly maintain the nitrogen balance and increase the protein synthesis in surgery patients receiving either parenteral or enteral nutrition.

Research frontiers

Many studies paid attention to nitrogen balance and protein metabolism associated with rhGH treatment. However, there are few studies focusing on the effects of GH on trace element metabolism in patients. This study was to evaluate the effects of rhGH on trace element metabolism and blood glucose levels in selective abdominal surgical patients.

Innovations and breakthroughs

This study evaluated the effects of rhGH on trace element metabolism and blood glucose levels in selective abdominal surgical patients. Postoperative low-dose rhGH treatment improves the retention of zincum, cuprum and ferrum, and decreases their excretion, increases their balance and promotes their apparent absorption and utilization. rhGH is well tolerated with no significant adverse effects and can control the blood glucose level.

Applications

The results of this study will promote the short-term low-dose rhGH application in clinical practice. Hyperglycemia is the main adverse event of short-term low–dose rhGH treatment.

Terminology

Biosynthetic human growth hormone, also referred to as recombinant human growth hormone, is also called somatropin and abbreviated as rhGH.

Peer review

This is the first study analyzing the effects of growth hormone on trace element metabolism and significantly adds our knowledge on the beneficial effect of short-term GH application.

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S- Editor Langmann T **L- Editor** Wang XL **E- Editor** Yin DH

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World J Gastroenterol 2007 December 14; 13(46): 6264-6268
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Relationship between survivin expression and recurrence, and prognosis in hepatocellular carcinoma

Chao-Ping Ye, Cheng-Zhi Qiu, Zhong-Xin Huang, Qi-Chen Su, Wei Zhuang, Rui-Lan Wu, Xin-Feng Li

Chao-Ping Ye, Cheng-Zhi Qiu, Wei Zhuang, Xin-Feng Li, Department of Hepatobiliary Surgery, Second Affiliated Hospital of Fujian Medical University, Quanzhou 362000, Fujian Province, China
Zhong-Xin Huang, Qi-Chen Su, Rui-Lan Wu, Department of Pathology, Second Affiliated Hospital of Fujian Medical University, Quanzhou 362000, Fujian Province, China
Correspondence to: Chao-Ping Ye, Department of Hepatobiliary Surgery, Second Affiliated Hospital of Fujian Medical University, Quanzhou 362000, Fujian Province, China. xulina9995@yahoo.com.cn
Telephone: +86-595-22780153 Fax: +86-595-22793591
Received: June 15, 2007 Revised: September 2, 2007

Abstract

AIM: To study the expression of the inhibitor of apoptosis protein survivin in hepatocellular carcinoma (HCC), and its correlation with clinicopathological factors, cell proliferation, recurrence and prognosis after hepatectomy.

METHODS: Immunohistochemical staining of survivin and Ki-67 was performed by the standard streptavidin-peroxidase technique on paraffin sections of 55 cases of HCC.

RESULTS: The positive rate of survivin in HCC was 52.7% (29/55). Significant correlation was found between survivin expression with portal vein thrombi and intrahepatic matastasistic nodes ($P < 0.05$). The recurrent rate in survivin-positive HCC was significantly higher than that in survivin-negative HCC after hepatectomy, the 1- and 3-year survival rate in patients with survivin-positive tumors was significantly lower than that in patients with survivin-negative tumors (58.62 and 10.34% *vs* 76.92 and 30.77%, $P < 0.05$, log-rank test). The proliferation index (Ki-67) in survivin-positive HCC (33.83% ± 18.90%) was significantly higher than that in survivin-negative HCC (19.60% ± 19.35%) ($P < 0.05$).

CONCLUSION: Survivin may play an important role in progression of HCC by promoting cell proliferation, and may be positively correlated with high risk of disease recurrence and poor prognosis in HCC. Its expression may serve as a prognostic factor for patients with HCC after hepatectomy.

Key words: Hepatocelluar carcinoma; Survivin; Proliferation index; Prognosis; Immunohistochemistry

Ye CP, Qiu CZ, Huang ZX, Su QC, Zhuang W, Wu RL, Li XF. Relationship between survivin expression and recurrence, and prognosis in hepatocellular carcinoma. *World J Gastroenterol* 2007; 13(46): 6264-6268

http://www.wjgnet.com/1007-9327/13/6264.asp

INTRODUCTION

Although surgical resection is the most important method for hepatocellular carcinoma (HCC), the recurrent rates may be as high as 50% at 2 years after hepatectomy[1]. The recurrence of HCC may be related to a variety of factors, including biological markers. Molecular prognostic markers are likely to be of greatest benefit in the effective management of patients with HCC, however, these factors have not yet been sufficiently defined in patients with a high risk of cancer recurrence.

Survivin is a recently described member of the family of inhibitor of apoptosis proteins (IAPs). Recently, it has been shown that survivin is strongly associated with apoptosis, cell proliferation and cell-cycle control[2-5]. Survivin plays a crucial role in the genesis and progression of malignancy and is an important prognostic parameter in tumors[6-10]. This study investigated the expression of survivin in HCC and its correlation with clinicopathological factors, cell proliferation and prognosis.

MATERIALS AND METHODS

Materials

Tissue samples were obtained between December 2000 and December 2003 from 55 patients with HCC (41 men, 14 women; 24-74 years old, mean age, 48.65 years). There were 27 patients with stage Ⅰ-Ⅱ, and 28 with stage Ⅲ-Ⅳ cancer. None of the patients received radiotherapy, chemotherapy or immunotherapy before surgery.

Reagents

Rabbit anti-human survivin polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-human Ki-67 monoclonal antibody

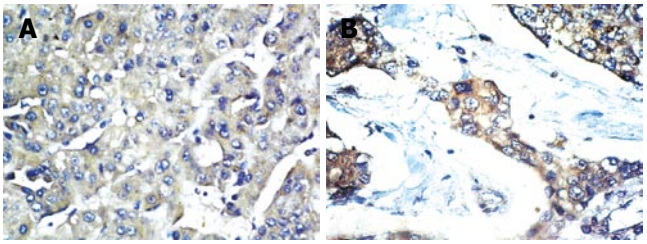

Figure 1 **A:** Positive expression of survivin in HCC (SP, × 200); **B**: Positive expression of survivin in HCC (SP, × 400).

(MBI.1), streptavidin-peroxidase (SP) staining kit and diaminobenzidine (DAB) kit were supplied by Maixin-Bio, Fuzhou, China.

Methods

Expression of survivin and Ki-67 was detected using SP immunohistochemistry. Briefly, after deparaffinization and rehydration, antigen retrieval was accomplished by incubation in 0.01 mol/L citric acid buffer (pH 6.0), boiling for 1 min in a pressure cooker, and cooling and washing in tap water. The sections were incubated with hydrogen peroxide for 10 min and washed in PBS. Non-specific reactions were blocked by incubation in a solution containing normal serum. The sections were incubated with a primary antibody (anti-survivin or Ki-67 antibody) overnight at 4℃. The working dilution of anti-survivn antibody was 1:100. The sections were rinsed with PBS, and then incubated for 10 min at room temperature with biotinylated secondary antibody. After washing, streptavidin-biotin complex conjugated to horseradish peroxidase was applied for 10 min at room temperature. After three rinses with PBS, the sections were incubated with DAB, rinsed with distilled water, counterstained with hematoxylin, and dehydrated and coverslipped. The sections were prepared for microscopy. Colorectal cancer tissues were used as a positive control. As a negative control, PBS was used to replace primary antibody.

Scoring criteria for survivin expression

Intensity and percentage of positive cells were used to evaluate each tissue section. The mean percentage of positive tumor cells and normal epithelial cells in at least five areas at × 400 magnification was determined and assigned to one of five categories: 0, < 5%; 1, 5%-24%; 2, 25%-49%; 3, 50%-74%; and 4, ≥ 75%. The intensity of survivin immunostaining was scored as 0 (achromatic), 1 (light yellow), 2 (yellow), and 3 (brown). The percentage of positive cells and staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores < 1 were defined as negative; all others were defined as positive.

Determination of the Ki-67 proliferation index

At least five high-power fields were chosen randomly in each section, and 500 cells were counted for each field. The Ki-67 proliferation index was defined as the number of Ki-67-positive nuclei divided by the total number of colorectal cancer cells counted, and was expressed as a percentage.

Table 1 Correlation between survivin expression and clinicopathology in HCC *n* (%)

Clinicopathological factor		*n*	Survivin expression		*P* value
			positive	negative	
Sex	Male	41	23	6	0.392
	Female	14	6	8	
Age (yr)	≤ 55	40	22	18	0.581
	> 55	15	7	8	
Tumor site	Right lobe	30	14	16	
	Left lobe	20	12	8	0.615
	Whole liver	5	3	2	
HBsAg	Positive	42	21	21	0.467
	Negative	13	8	5	
Differentiation	Moderate to well	40	23	17	0.247
	Poor	15	6	9	
Intrahepatic metastatic nodes	(+)	21	16	5	0.006
	(–)	34	13	21	
Portal vein thrombi	(+)	14	12	2	0.004
	(–)	41	17	24	
Tumor capsule	(+)	22	12	10	0.825
	(–)	33	17	16	
Tumor size (cm)	≤ 5	16	8	8	0.795
	> 5	39	21	18	
AF P(μg/L)	< 400	19	7	12	0.086
	≥ 400	36	22	14	
Hepatocirrhosis	(+)	37	21	16	0.391
	(–)	18	8	10	
Tumor stage	Ⅰ-Ⅱ	27	14	13	0.898
	Ⅲ-Ⅳ	28	15	13	

Statistical analysis

The survival curves were assessed by the Kaplan-Meier method and compared by a log-rank test. The χ^2 test was performed for enumeration data comparison, and the *t* test was used for comparison of measurement data. $P < 0.05$ was considered statistically significant. All data analysis was performed with commercially available statistical analysis software packages (SSPS 11.5, SSPS, Chicago, IL, USA).

RESULTS

Relationship between expression of survivin and clinical pathology

Survivin protein expressed as brown-yellow particles in the cytoplasm after staining, and only one expressed both in the cytoplasm and nucleus after staining. The positive staining rate for survivin in the cytoplasm and nuclei was 29/55 (52.7%) (Figure 1). There was a significant correlation between survivin expression and portal vein thrombi and intrahepatic metastatic nodes ($P < 0.05$). However, it was not related to the following factors: age and sex of the patient, tumor location, tumor differentiation, tumor size, presence of tumor capsule, clinical stage, complicating liver cirrhosis, preoperative alpha fetoprotein (AFP) level, and hepatitis B surface antigen (HBsAg) (Table 1). These findings suggest that the expression of survivin may be significantly associated with metastasis.

Relationship between expression of survivin and proliferation index

Ki-67 showed as brown-yellow particles in the nuclei after

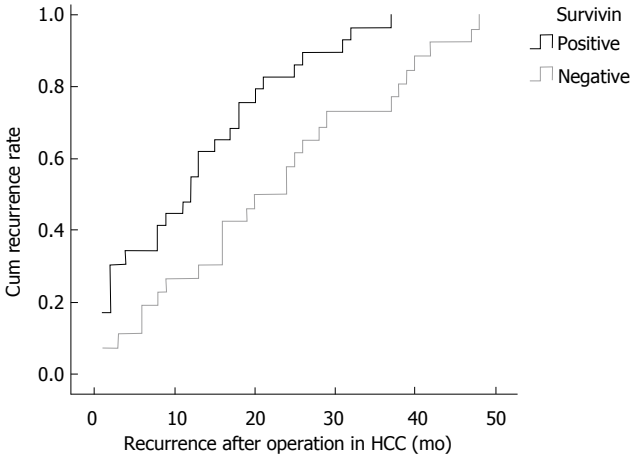

Figure 2 Correlation between survivin expression and recurrence rate of HCC after hepatectomy.

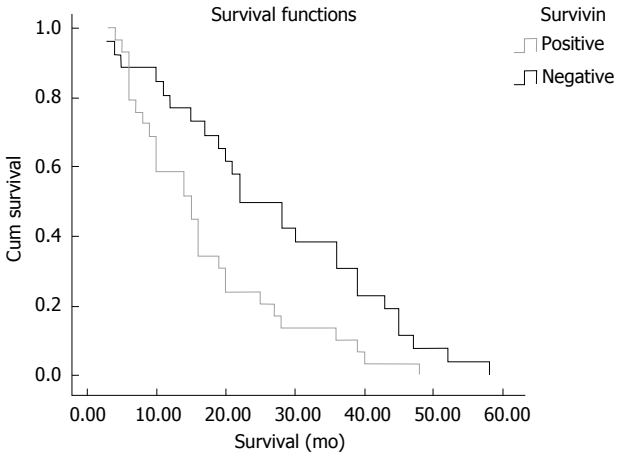

Figure 3 Kaplan-Meier curves for overall survival rates of patients with HCC according to survivin expression.

staining. Ki-67 labeling index in survivin-positive cancer was 33.83% ± 18.90%, while it was 19.60% ± 19.35% in negative tumor. The difference was significantly different ($P < 0.05$). This suggests that the expression of survivin may promote the proliferation of HCC.

Relationship between expression of survivin and recurrence and prognosis of HCC

The 1- and 3-year recurrence rates in survivin-positive HCC were 55.17% and 96.55%, respectively, while the rates were 26.91% and 73.08%, respectively, in survivin-negative HCC after hepatectomy. The recurrent time of survivin-positive HCC was significantly advanced ($P < 0.05$, Figure 2). Furthermore, the 1- and 3-year survival rates in survivin-positive HCC were 58.62% and 10.34% after hepatectomy, respectively, but for survivin-negative HCC, the rates were 76.92% and 30.71%, respectively. The 1- and 3-year survival rates were significantly lower in patients with survivin-positive HCC than those in survivin-negative HCC ($P < 0.05$, Figure 3). The expression of survivin may be used as an indicator for prognosis of HCC.

DISCUSSION

Among the recently described IAP family, survivin is characterized by a unique structure with a single BIR and no zinc-binding domain[11], and is undetectable in terminally differentiated adult tissues, but becomes notably expressed in the most common human cancers, including esophageal, stomach, colorectal, breast and pancreatic carcinoma[12-16]. Survivin has also been implicated in the control of cell-cycle kinetics and inhibition of apoptosis[17-19].

In our current study, we demonstrated that the expression of survivin was detected in 52.7% of patients with HCC, mainly localized in the cytoplasm of the carcinoma cells, with rare appearance in the nucleus. On the other hand, there have also been reports of a nuclear presence of survivin in HCC[20,21]. In previous studies, immunohistochemical analysis or RT-PCR of surgically resected tissues has revealed that approximately 30%-90% of HCC are positive for survivin expression[22-26]. The reasons for the difference may be the following: during the cell-division cycle, mRNA expression for survivin is extremely low in the G1 phase, and in the S phase is six times higher, while in the G2/M phase, the expression level of survivin increases suddenly to be 40 times higher than that in G1[27]. Therefore, the tumor cells in the G1/S phase may represent negative expression, which would lead to different expression rate of survivin in different study. On the other hand, different criteria for positive expression of survivin or experimental methods may explain the different expression rates of survivin.

Ki-67 is considered to be more useful for the determination of the proliferative activity of HCC, and is known as a risk factor for HCC. In our study, we surprisingly found that expression of the proliferation index in survivin-positive HCC was higher than that in survivin-negative HCC. The results suggest that survivin may promote cell proliferation, and contribute to the development of HCC. Survivin may interact with the cell-cycle regulator Cdk4, which leads to Cdk2/cyclin E activation and Rb phosphorylation. As a result of survivin/Cdk4 complex formation, p21 is released from its complex with Cdk4 and interacts with mitochondrial procaspase-3 to suppress Fas-mediated cell death. Survivin can also inhibit the activity of caspase-3/7 directly or indirectly, and this results in the generation and development of HCC[28-30]. The up-regulated expression of the proliferation index in survivin-positive HCC also suggests that survivin plays an important role in tumor progression.

The survivin expression in HCC was significantly correlated with portal vein thrombi and intrahepatic metastatic nodes. Therefore, survivin may play an important role in the development of HCC. Compared to survivin-negative HCC, survivin-positive HCC had a higher recurrent rate and lower 1- and 3-year survival rates. Survivin expression may play a role in the tumor biological characteristics of HCC, and may be a prognostic factor after hepatectomy. The study by Ikeguchi *et al* has also shown that high expression of survivin is associated with high recurrence and low survival rates[23]. Normal shedding of cells initiates the apoptosis process, but the over-expression of survivin exerts an anti-apoptotic effect,

which leads to a high rate of cell proliferation. Therefore, survivin may play an important role in the progression of HCC and may facilitate metastatic spread *via* the blood stream.

In conclusion, survivin expression in HCC was significantly correlated with portal vein thrombi and intrahepatic metastatic nodes. There was a significant positive correlation between survivin expression and proliferation index. Survivin plays an important role in HCC progression through promoting cell proliferation, and may be a prognostic marker for HCC.

COMMENTS

Background

Survivin is a recently described member of the family of inhibitor of apoptosis proteins (IAPs). It has been shown that survivin is strongly associated with apoptosis, cell proliferation and cell-cycle control, and becomes markedly expressed in the most common human cancers.

Research frontiers

Immunohistochemical staining of survivin and Ki-67 was performed by the standard streptavidin-peroxidase (SP) technique for paraffin sections of hepatocellular carcinoma (HCC) tissues.

Innovations and breakthroughs

We demonstrated that the positive rate of survivin in HCC was 52.7%, and a significant correlation was found between survivin expression and portal vein thrombi and intrahepatic metastatic nodes. The recurrence rate in survivin-positive HCC was significantly higher than that in survivin-negative HCC after hepatectomy. The 1- and 3-year survival rates of patients with survivin- positive tumors were significantly lower than those in patients with survivin-negative tumors. The proliferation index (Ki-67) in survivin-positive HCC was significantly higher than that in survivin-negative HCC.

Applications

Survivin may play an important role in progression of HCC by promoting cell proliferation, and may be positively correlated with a high risk of disease recurrence and poor prognosis in HCC. Its expression can serve as a prognostic factor for patients with HCC after hepatectomy.

Peer review

This is an interesting correlative study of survivin expression and survival in a cohort of 55 patients. A significantly worse survival was observed in surviving-positive tumors. The data are of high quality.

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World J Gastroenterol 2007 December 14; 13(46): 6269-6273
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Relationship between vascular invasion and microvessel density and micrometastasis

Yan-Dong Wang, Pei Wu, Jia-Ding Mao, He Huang, Fan Zhang

Yan-Dong Wang, Department of Surgery, Wannan Medical College, Wuhu 241001, Anhui Province, China
Pei Wu, Jia-Ding Mao, He Huang, Department of Surgery, Affiliated Yijishan Hospital, Wannan Medical College, Wuhu 241001, Anhui Province, China
Fan Zhang, Department of Pathology, Affiliated Yijishan Hospital, Wannan Medical College, Wuhu 241001, Anhui Province, China
Correspondence to: Pei Wu, Department of Surgery, Affiliated Yijishan Hospital, Wannan Medical College, Wuhu 241001, Anhui Province, China. wp5708@sohu.com
Telephone: +86-13637134296
Received: April 17, 2007 Revised: September 12, 2007

Key words: Vascular invasion; Reverse transcriptase-polymerase chain reaction; Microvessel density; Micrometastasis

Wang YD, Wu P, Mao JD, Huang H, Zhang F. Relationship between vascular invasion and microvessel density and micrometastasis. *World J Gastroenterol* 2007; 13(46): 6269-6273

http://www.wjgnet.com/1007-9327/13/6269.asp

Abstract

AIM: To evaluate the relationship between vascular invasion and microvessel density (MVD) of tissue and micrometastasis in blood.

METHODS: Vascular invasion was detected by both hematoxylin and eosin staining and immunohistochemiscal staining. Blood samples were collected from 17 patients with vascular invasion and 29 patients without vascular invasion and examined for cytokeratin20 (CK20) expression by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Microvessel density of tissue samples was also determined by immunohistochemistry using antibodies to CD105.

RESULTS: CK20 was detected in 12 of the 17 patients with vascular invasion and in 9 of the 29 patients without vascular invasion. Positive RT-PCR was significantly correlated with vascular invasion (70.6% *vs* 30.0%, $P < 0.05$). The average MVD was significantly higher in patients with positive vascular invasion than in patients with negative vascular invasion (29.2 ± 3.3 *vs* 25.4 ± 4.7, $P < 0.05$). The vascular invasion detected with hematoxylin-eosin staining was less than that with immunohistochemical staining. There was a significant difference between the two staining methods (19.6% *vs* 36.9%, $P < 0.05$).

CONCLUSION: Positive CK20 RT-PCR, depth of tumor invasion, lymph node status, metastasis and MVD are significantly correlated with vascular invasion. Immunohistochemical staining is more sensitive than hematoxylin-eosin staining for detecting vascular invasion.

INTRODUCTION

Vascular invasion is one of the most important clinicopathologic characteristics of malignant tumor. Since the initial report by Brown and Warren in 1938 demonstrating an increased visceral metastasis in rectal cancer patients with vascular invasion, a number of investigators have examined the influence of vascular invasion by colorectal cancer[1].

The presence of vascular invasion which is not a consistent finding is associated with an increased incidence of lymph node and distant metastasis and a corresponding decrease in survival[1]. Since polymerase chain reaction (PCR) invented by Mullis in 1989, it has become a standard and mature laboratory technique to detect micrometastasis in patients with malignant tumor[2]. In this study, we detected cytokeratin20 (CK20) mRNA expression in portal system blood[3-5] and microvessel density (MVD) of tissue to evaluate the relationship between vascular invasion and MVD[6] of tissue and metastasis in blood.

MATERIALS AND METHODS

Blood and tissue samples

Portal system blood was obtained before operation from 27 gastric cancer patients and 19 colorectal cancer patients. A venous catheter was inserted into the right gastric omental veins of gastric cancer patients and corresponding veins of colorectal cancer patients and blood samples were collected. The initial 5 mL blood was discarded to reduce possible contamination and the following 5 mL of blood drawn using a new syringe, was used for RNA extraction[7].

Tissue samples from 27 gastric cancer patients and

Table 1 Oligonucleotide primers

cDNA	Primer	Sequence	Product length (bp)
CK20	Outer sense	5'-GAGGTTCAAC TAACGGAGCT-3'	253
	Outer antisense	5'-TCTCTCTTCCA GGGTGCTTA-3'	
	Inner sense	5'-GCCTTGAGATA GAACTCCAG-3'	
	Inner antisense	5'-ACGTCTTCTCC TTCCAGAAG-3'	
GAPDH	Sense	5'-CAGGGCTGCTT TTAACTCTG-3'	385
	Antisense	5'-CTGTTGTCGGAG TTCTAGTAG-3'	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

19 colorectal cancer patients were formalin-fixed and parafin-embedded. The tissue samples were cut into 1 μm-thick sections, mounted onto slides coated with polylysin and examined with hematoxylin-eosin and immunohistochemical staining.

Detecting vascular invasion

Vascular invasion examined with hematoxylin-eosin staining was defined either by the presence of neoplastic cells with fibrin clots, erythrocytes, or both in endothelial cell-lined spaces without erythrocyte extravasation in the surrounding tissues or by the presence of neoplastic cells within the smooth muscle cell-lined spaces[8]. Vascular invasion examined with immunohistochemical staining was defined by the presence of at least one tumor cell cluster which was clearly visible in decorated vascular spaces where endothelial cells were stained brown[9]. According to the immunohistochemical staining, the fibrin clots or erythrocytes surrounding neoplastic cells should be considered. Vascular invasion was confirmed by at least one staning method.

Detecting CK20 mRNA in portal system blood

Isolation of mononuclear cells: Blood mononuclear cells (MNCs) were isolated by density gradient centrifugation through Ficoll-Hypaque, and washed twice with phosphate-buffered saline (PBS). Cell pellets were snap frozen in liquid nitrogen and stored at -80℃ until use.

RNA extraction: Total RNA was extracted from the MNC pellets with TRIzol reagent (Invitrogen Biotech, USA) according to the manufacturer's instructions.

Reverse transcriptase: An aliquot of 2 μg MNC RNA was pre-incubated with 0.5 μg of oligo(dT)$_{15}$ primer in 14 μL solution for 5 min at 70℃. After chilling on ice, 6 μL of 5-fold synthesis buffer, 25 U of RNase inhibitor, 1.5 μL of dNTPs (final concentration of 0.5 mmol/L) and 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase were added. The reaction mixture was then incubated for 60 min at 42℃. The reaction was terminated by heating at 95℃ for 5 min.

Table 2 Comparison between HE and immunohistochemical staining

	Vascular invasion		χ^2	*P*
	(+)	(-)		
HE staining	9	37	19.087	< 0.05
Immunohistochemical staining	17	29		

McNemar's test for correlated proportions, $\chi^2 = 8.003$, $P < 0.05$ *vs* immunohistochemical staining.

Polymerase chain reaction (PCR): PCR was carried out as described previously[10]. The sequences of primers used are shown in Table 1. To distinguish from contaminating genomic DNA, we selected both upstream and downstream primers at different exons. Integrity of the isolated RNA was demonstrated by RT-PCR analysis of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR products were visualized after electrophoresis with ethidium bromide staining under a UV transilluminator.

Detecting microvessel density of tissue

CD105 antigen was detected by immunohistochemistry on a separate slide using a monoclonal mouse antibody following a standard protocol. Microvessel density was assessed as previously described[11].

Statistical analysis

Statistical analysis was performed using the likelihood chi-squared analysis, Fisher's exact test or Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of vascular invasion

Vascular invasion was detected in 9 patients with hematoxylin-eosin staining and in 17 patients with immunohistochemical staining. There was a significant difference in vascular invasion detected by the two methods (Table 2, Figure 1A and B).

Relationship between vascular invasion, MVD and micrometastasis

CK20 was detected in 12 of the 17 patients with vascular invasion and in 9 of the 29 patients without vascular invasion. Positive RT-PCR was significantly correlated with vascular invasion. The average MVD was significantly higher in patients with positive vascular invasion (29.2± 3.31) than in those with no vascular invasion (Tables 3 and 4, Figure 2).

Comparison of clinicopathologic features

Clinicopathologic features such as depth of invasion, lymph node status and metastasis were associated with the presence of vascular invasion (Table 3).

DISCUSSION

Since vascular invasion first reported by Brown and

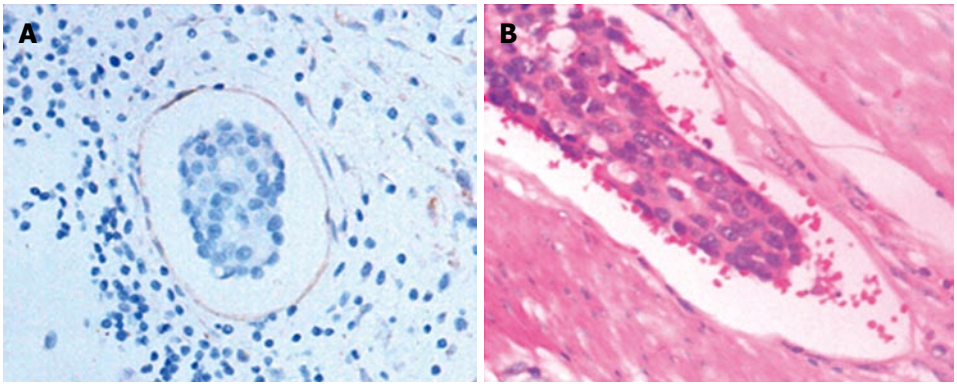

Figure 1 Immunohistochemical staining (**A**) and hematoxylin-eosin staining (**B**) of tumor cells (× 400) showing a tumor cell cluster in vascular spaces with brown-stained endothelial cells and tumor cells in blood vessel spaces with erythrocytes surrounded.

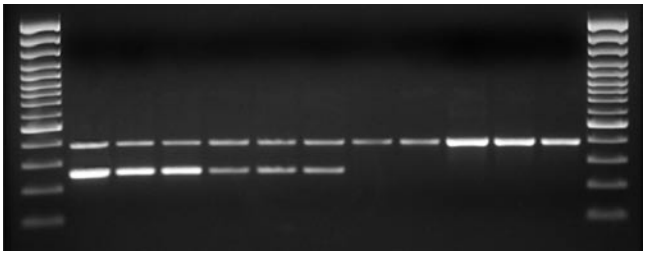

Figure 2 Expression of both CK20 mRNA and GAPDH detected in six patients and expression of only GAPDH detected in five patients.

Table 3 Comparative data on vascular invasion

	n	VI(+)	VI(-)	χ^2	*P*
CK20 mRNA					
Positive	21	12	9	6.758	< 0.05
Negative	25	5	20		
Age (yr)					
< 50	14	4	10	0.607	> 0.05
⩾ 50	32	13	19		
Size (cm)					
< 5	31	12	19	0.125	> 0.05
⩾ 5	15	5	10		
Differentiated					
Well	11	2	9	2.351	> 0.05
Moderately	20	8	12		
Poorly	15	7	8		
Serosa invasion					
Negative	14	2	12	4.440	< 0.05
Positive	32	15	17		
Lymph node metastasis					
Negative	18	3	15	5.225	< 0.05
Positive	28	14	14		
Distant metastasis					
Negative	38	9	29	16.520	< 0.05
Positive	8	8			

Statistical analysis by chi-square test. VI: Vascular invasion.

Table 4 Average number of microvessels of tissue in VI positive and negative patients

	n	MVD	*t*	*P*
VI				
Positive	17	29.2 ± 3.3	2.987	< 0.05
Negative	29	25.4 ± 4.7		

Statistical analysis of independent samples by *t* test. VI: Vascular invasion; MVD: Microvessel density.

Warren in 1938, a lot of studies have examined the influence of vascular invasion on survival[1]. Horn and colleagues found that vascular invasion is an independent prognostic factor for distant metastasis but not for survival[12]. However, Chapuis and colleagues found that vascular invasion is an independent prognostic factor for survival[13], but this was not confirmed by Wiggers *et al*[14] or Minsky *et al*[15]. In this study, we examined CK20 mRNA expression in patients with or without vascular invasion to evaluate the relationship between vascular invasion and microvessel density of tissue and micrometastasis in blood.

Vascular invasion and micrometastasis

Tumor metastasis is an orchestrated multistep process that may involve direct, hematogenous or lymphatic spread[16,17]. Tumor metastasis requires an exodus of cancer cells from the primaty site, endurance outside the hormonal and nutritional milieu of the primary site, evasion of the body' s immune surveillance, as well as adhesion, invasion, and penetration at a distant site, and organization of metastatic tissue in the secondary site with neovascularization[18]. Primary tumor invades blood and/or lymphatic vessels departing from the primary site[19]. In this study, CK20 mRNA was detected in 12 of 17 patients with positive vascular invasion, and in 9 of 29 patients with no vascular invasion, suggesting that vascular invasion is closely related to micrometastasis in blood, depth of tumor invasion, lymph node status and distant metastasis. Therefore, CK20 mRNA can be considered an indirect prognostic factor for survival. There is evidence that distant metastases are associated with the neoplastic invasion of relatively large veins at the tumor's periphery[20-22].

Vascular invasion and angiogenesis

Angiogenesis is the propelling force for tumor growth and metastasis[23-25]. To progress to a larger size, incipient neoplasms must have an angiogenic ability, which involves the sprouting of new blood vessels from preexisting capillaries, and requires the multiplication and migration of endothelial cells, remodeling of extracellular matrix, tube formation, and recruitment of surrounding structures to maintain the newly formed vessels[26]. In

this study, the average MVD was significantly higher in patients with vascular invasion than in patients with no vascular invasion, suggesting that angiogenesis is closely related with microvessel density of tissue[27] and clinical aggressiveness of tumor[28].

Detection of vascular invasion

Vascular invasion was detected with hematoxylin-eosin staining and immunohistochemical staining, respectively. The heterogeneous positive rate suggests immunohistochemical staining is more sensitive than hematoxylin and eosin staining for the detection of vascular invasion. Fibrin clots, erythrocytes, or both in endothelia-lined spaces without erythrocyte extravasation in the surrounding tissues must be concerned if detected with HE staining. However, we had to decide whether a tumor cell cluster is clearly visible in decorated vascular spaces where endothelial cells are stained brown when detected with immunohistochemical staining. Our results are consistent with the reported data[29,30].

COMMENTS

Background

Since the initial report by Brown and Warren in 1938 demonstrating an increased visceral metastasis in rectal cancer patients with vascular invasion, a number of investigators have examined the influence of vascular invasion by colorectal cancer. The presence of vascular invasion is associated with an increased incidence of lymph node and distant metastasis and a corresponding decrease in survival. However, this is not a consistent finding.

Research frontiers

Horn and colleagues found that vascular invasion is an independent prognostic factor for distant metastasis but not for survival. By multivariate analysis, Chapuis and colleagues found vascular invasion to be an independent prognostic factor for survival, but this was not confirmed by Wiggers *et al* or Minsky *et al*.

Innovations and breakthrough

Though several articles have reported the prognostic value of vascular invasion, the results are not consistent, and no study has focused on micrometastasis in patients with vascular invasion. In this study, we detected cytokeratin20 (CK20) mRNA expression in portal system blood and microvessel density of tissue to evaluate the relationship between vascular invasion and microvessel density of tissue and metastasis in blood.

Applications

We recommend vascular invasion as a method of choice for predicting prognosis of gastric and colorectal cancer patients. Patients with vascular invasion are more likely to need adjuvant therapies.

Terminology

CK20: It belongs to the epithelial subgroup of the intermediate filament family. Because of its restricted range of expression in humans, it has become an important tool for detecting and identifying metastatic cancer cells by immunohistochemistry and PCR analysis. Factor Ⅵ: Vascular invasion is usually defined by the presence of neoplastic cells with fibrin clots, erythrocytes, or both in endothelia-lined spaces without erythrocyte extravasation in the surrounding tissues or by the presence of neoplastic cells within the smooth muscle cell-lined space

Peer review

This subject is valuable for understanding the importance of vascular invasion of cancer in predicting the prognosis of such patients. It also provides a better way to increase the detection rate of vascular invasion with immunohistochemical staining.

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S- Editor Zhu LH **L- Editor** Wang XL **E- Editor** Wang HF

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World J Gastroenterol 2007 December 14; 13(46): 6274-6276
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

A phantom gallbladder on endoscopic retrograde cholangiopancreatography

Jinhong Xing, Jeremy Rochester, Caroline K Messer, Bruce P Reiter, Mark A Korsten

Jinhong Xing, Jeremy Rochester, Caroline K Messer, Bruce P Reiter, Mark A Korsten, Gastroenterology and Radiology Programs, James J. Peters VA Medical Center, Bronx, NY 10468, Montefiore Medical Center, Bronx, NY, Mount Sinai School of Medicine, New York, NY and Albert Einstein College of Medicine, Bronx, New York 10468, United States
Correspondence to: Mark A Korsten, MD, James J Peters VA Medical Center, 130 W. Kingsbridge Road, Bronx, New York 10468, United States. korsten.mark@med.va.gov
Telephone: +1-718-5849000-6753 Fax: +1-718-5791635
Received: April 20, 2007 Revised: September 6, 2007

Abstract

Various complications have been related to laparoscopic cholecystectomy but most occur shortly after the procedure. In this report, we present a case with very late complications in which an abscess developed within the gallbladder fossa 6 years after laparoscopic cholecystectomy. The abscess resolved after treatment with CT-guided extrahepatic aspiration. However, 4 years later, an endoscopic retrograde cholangiopancreatography (ERCP) performed for choledocholithiasis demonstrated a "gallbladder" which communicated with the common bile duct via a patent cystic duct. This unique case indicates that a cystic duct stump may communicate with the gallbladder fossa many years following cholecystectomy.

Key words: Laparoscopic cholecystectomy; Complication; Abscess; Gallbladder; Endoscopic retrograde cholangiopancreatography

Xing J, Rochester J, Messer CK, Reiter BP, Korsten MA. A phantom gallbladder on endoscopic retrograde cholangiopancreatography. *World J Gastroenterol* 2007; 13(46): 6274-6276

http://www.wjgnet.com/1007-9327/13/6274.asp

INTRODUCTION

Laparoscopic cholecystectomy has been widely accepted as a preferred option for the management of patients with cholelithiasis. Complications such as biliary injuries[1,2] intestinal ischemia[3] and biliary-colonic fistula[4] have been reported, but most occur shortly after surgery. In this report, we present a case with very late complications in which an abscess developed within the gallbladder fossa 6 years after laparoscopic cholecystectomy. Four years later, despite the clinical certainty that it had been removed 10 years previously, a "gallbladder" communicating with the common bile duct *via* a cystic duct was demonstrated by endoscopic retrograde cholangiopancreatography (ERCP).

CASE REPORT

A 73-year-old male presented with complaints of right upper quadrant discomfort, fatty food intolerance and bloating. He eventually underwent an elective laparoscopic cholecystectomy in early October 1996. A J-P drainage tube was placed during the operation. The drainage was initially dark bloody, then became bilious, but gradually became clear over the next several days. The patient was discharged home after 1 wk. A follow-up abdominal CT scan was performed in May 1997, six months after the procedure. There were no fluid collections in the gallbladder fossa or peritoneal cavity.

The patient did well until early October 2002, six years after cholecystectomy, when he presented with complaints of fever, chills and mild pain in the right upper quadrant. Sonographic examination of the abdomen showed a normal sized liver with non-dilated intrahepatic ducts and common bile duct. Of note, a gall bladder-like structure was seen in the gallbladder fossa. An abdominal CT confirmed the presence of a 4 cm encapsulated fluid collection in the gallbladder fossa (Figure 1). A subsequent HIDA scan showed prompt excretion of the radiotracer into the small bowel with no evidence of biliary leak. A CT-guided transhepatic intra-abdominal aspiration was then performed (Figure 2). Turbid-appearing fluid was readily aspirated from the extrahepatic fluid collection. Gram stain of the aspirated fluids showed the presence of many white blood cells. Cultures were positive for moderate Enterobacter SP in the aspirates. The persistent low-grade fever subsided shortly after the CT-guided aspiration, and the patient was discharged home with no abdominal complaints. In August 2004 (22 mo after aspiration), an abdominal CT scan was repeated and no gallbladder fossa fluid collection was observed.

In May 2006, the patient was again admitted with the sudden onset of abdominal pain, icterus and fever. A CT scan showed a dilated common bile duct. MRCP showed intra- and extrahepatic biliary

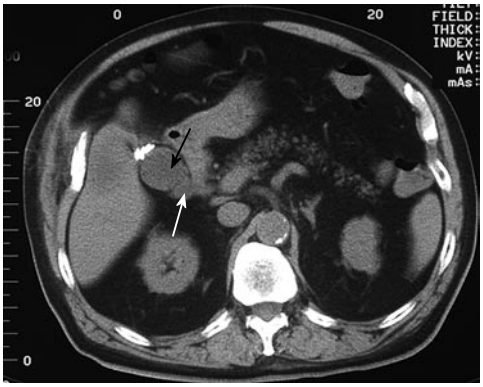

Figure 1 Abdominal CT scan six years after laparoscopic cholecystectomy. A 4 cm encapsulated fluid collection in the gallbladder fossa (black arrow) with adjacent "cystic duct-like" collections was observed (white arrow).

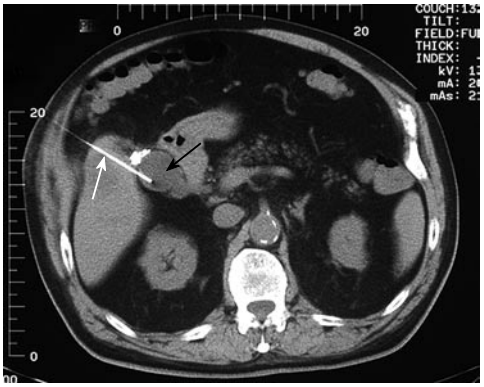

Figure 2 A CT-guided transhepatic percutaneous aspiration needle (white arrow) was seen entering the extrahepatic fluid collection (black arrow).

dilatation as well as a round, low signal filling defect within the distal common bile duct. During ERCP, the cholangiogram revealed a mildly dilated proximal common bile duct with 2 mobile stones (Figure 3A). A gallbladder-like structure that communicated with the common bile duct was also visualized in the gallbladder fossa (Figure 3B). Sphincterotomy was performed, and numerous stone fragments were removed from the CBD. A plastic biliary endoprosthesis (10F) was placed in the distal common bile duct. The patient was discharged home without any complication or discomfort two days after the ERCP. One month later, the stent was removed and the common bile duct appeared normal. A "phantom" gallbladder image reappeared on the cholangiogram.

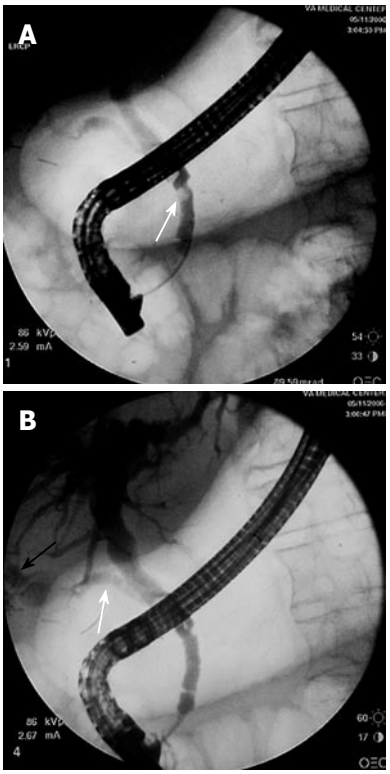

Figure 3 Calculi (white arrow) in the common bile duct were noted during the ERCP (**A**). In addition, a gallbladder-like structure (black arrow) communicated with the common bile duct *via* the cystic duct (white arrow) was also observed (**B**).

DISCUSSION

It is not uncommon to develop a fluid collection in the gallbladder fossa shortly after laparoscopic cholecystectomy. Kang *et al*[5] studied 106 consecutive patients 24 h after laparoscopic cholecystectomy using ultrasound. They identified small fluid collections in the gallbladder fossa in 56 (53.0%) patients. In another study[6], the gallbladder fossa of 70 asymptomatic patients was sonographically examined within two weeks of laparoscopic cholecystectomy. The authors reported the presence of homogeneous echogenic structures in the gallbladder bed in 35 (50%) patients, inhomogeneous structures in 25 (35.7%) cases, and, cystic structures, resembling the normal gallbladder, in 6 (8.5%) cases. The nature of the fluid collection varied, but included seromas, hematomas, abscesses and biloma.

In the present case, the fluid collection inside the gallbladder fossa observed 6 years after the procedure proved to be an abscess on the basis of the turbid, non-bilious, culture positive fluid retrieved during CT-guided aspiration. The abscess resolved after percutaneous transhepatic aspiration and antibiotic treatment. The most recent finding in this patient, a gallbladder-like structure inside the gallbladder fossa that directly communicated with the common bile duct, is likely related in some manner to this prior abscess. One possibility is that the previously documented abscess may have resolved by fistualizing into the cystic duct. Alternatively, increased pressure within the cystic duct as a result of biliary obstruction from choledocholithiasis may have caused rupture of the stump and its subsequent breakthrough into a residual cavity in the gallbladder fossa.

By whatever mechanism, this case demonstrates that, post-cholecystectomy, a cystic duct stump may potentially communicate with extrabiliary structures in and around the gallbladder fossa. In this respect, we believe that this is the first reported case in which a cystic structure resembling a radiologically typical "gallbladder" was seen on ERCP many years after this organ was surgical removed.

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S- Editor Liu Y **L- Editor** Ma JY **E- Editor** Ma WH

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World J Gastroenterol 2007 December 14; 13(46): 6277-6280
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Prolonged cholestasis following successful removal of common bile duct stones: Beware patients on estrogen therapy

JM Dunn, A McNair

JM Dunn, A McNair, The Queen Elizabeth Hospital, Woolwich, London, United Kingdom
Correspondence to: A McNair, Gastroenterology Department, The Queen Elizabeth Hospital, Stadium Road, Woolwich, London, SE18 4QH, United Kingdom. a.mcnair@nhs.net
Telephone: +44-8-8365579 Fax: +44-8-8365581
Received: May 8, 2007 Revised: August 24, 2007

Abstract

There are various well described forms of chronic cholestatic jaundice in adults, such as autoimmune cholangitis, drug-induced cholangitis and intrahepatic cholestasis of pregnancy. We present two cases of prolonged cholestasis following removal of gallstones at endoscopic retrograde cholangiopancreatography (ERCP) and subsequent clear cholangiography. Both patients were taking oral estrogens at the time of presentation, which were subsequently withdrawn. The first case responded rapidly to corticosteroid treatment, and the second case had a much slower resolution with ursodeoxycholic acid. Both cases highlighted the significance of estrogen-induced cholestasis in female patients with protracted jaundice following ERCP and removal of intra-ductal stones. After oral estrogens are discontinued, a short course of steroids needs to be considered.

Key words: Estrogen; Cholestasis; Gallstones; Steroids; Ursodeoxycholic acid

Dunn JM, McNair A. Prolonged cholestasis following successful removal of common bile duct stones: Beware patients on estrogen therapy. *World J Gastroenterol* 2007; 13(46): 6277-6280

http://www.wjgnet.com/1007-9327/13/6277.asp

INTRODUCTION

Estrogens have been a well-recognized cause of cholestatic jaundice since 1962[1], and are commonly used in an experimental model of hepatocellular cholestasis. The similarity between such drug reactions and the syndrome of intrahepatic cholestasis of pregnancy has been reported[2]. Withdrawal of the estrogenic effect by delivery of the fetus or drug withdrawal leads to improvement of liver function. This tends to occur over several weeks or months[3]. It has been shown in observational studies that estrogen therapy is an important risk factor for gallbladder disease[4]. A recent randomized double-blind placebo-controlled trial of otherwise healthy postmenopausal women has demonstrated that the risk of adverse biliary tract outcomes, such as cholecystitis, is substantially increased by exogenous estrogen therapy[5]. Estrogens are thought to promote gallstone formation by significantly elevating the biliary cholesterol saturation index and a reduction of the nucleation time[6]. We present two cases of prolonged cholestasis after removal of obstructing common bile duct (CBD) stones at endoscopic retrograde cholangiopancreatography (ERCP).

CASE REPORTS

Case 1

A 25-year-old Caucasian woman presented at 6 wk post partum with pruritus and right upper quadrant pain. There was no jaundice evident, and she had been asymptomatic throughout her pregnancy. She had stopped taking Microgynon (ethinlyestradiol 30 μg and levonorgestrel 150 μg) 1 year previously. Her mother had undergone cholecystectomy aged 50 years. Physical examination was unremarkable, and liver function tests showed a total serum bilirubin level of 43 μmol/L, alanine aminotransferase (ALT) 187 IU/L, alkaline phosphatase (ALP) 81 IU/L and gamma-glutamyltranspeptidase (γ-GT) 88 IU/L (Figure 1). Liver ultrasound scan showed multiple calculi in the gall bladder and a CBD of 6 mm, with no evidence of intraductal stones.

She presented again 6 wk later with worsening pruritus, biliary colic and jaundice. The patient had restarted Microgynon in the interim period. She denied exposure to alcohol or illegal drugs. There were no risk factors for viral hepatitis. Her weight had dropped by 8 kg and she had icterus. Abdominal palpation revealed a tender right upper quadrant but no hepatosplenomegaly. Retesting of liver biochemistry showed a total serum bilirubin level of 163 μmol/L and a twofold increase in serum ALP. ALT remained elevated at 163 IU/L. Abdominal ultrasound scanning showed a thick-walled edematous gallbladder with multiple small calculi, one impacted in the neck of the gallbladder. The CBD measured 3.5 mm.

Endoscopic retrograde cholangiopancreatography (ERCP) was performed and revealed two calculi in the CBD, the largest measuring 5 mm. A 12-mm

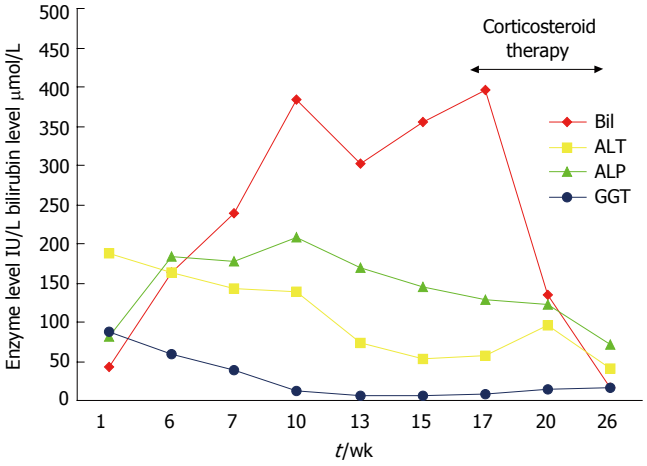

Figure 1 Case 1: Liver biochemistry and response to corticosteroids.

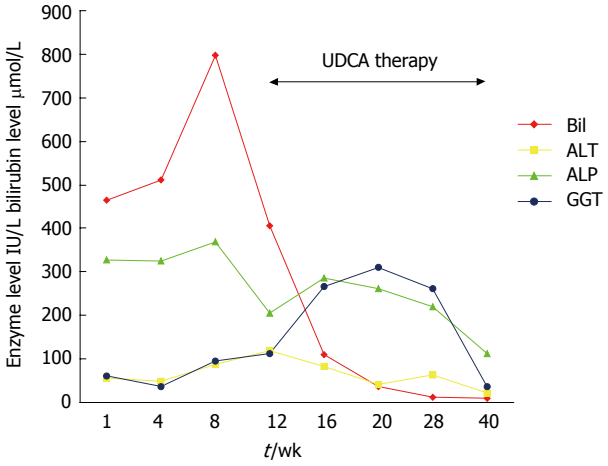

Figure 2 Case 2: Liver biochemistry and response to UDCA.

sphincterotomy was made and the CBD dredged with a balloon. Both stones were removed and an occlusion cholangiogram confirmed clearance of the duct. Her total serum bilirubin level continued to rise over the next 10 wk. A second ERCP performed 3 wk after the first showed a dilated CBD, but no obvious filling defects. The sphincterotomy was extended. A balloon dredge and occlusion cholangiogram confirmed that the duct was clear. Microgynon was stopped at this point. Autoantibody profile and hepatitis A, B and C serology were negative. Ceruloplasmin and ferritin levels were normal. The hemoglobin level was stable and the haptoglobin level normal. Because of persisting jaundice, a third ERCP was performed 3 wk later and revealed a normal condition.

Seventeen weeks following the initial presentation (7 wk after stopping the oral contraceptive pill), total serum bilirubin level continued to rise and reached 386 μmol/L. The patient was treated with prednisolone 40 mg/d, which was reduced by 5 mg/d at weekly intervals over a period of 8 wk. Response to treatment is shown in Figure 1. Her symptoms improved dramatically during treatment with the corticosteroid, with a concomitant restoration of normal liver biochemistry. She has now been followed up for more than 12 mo and is asymptomatic with weight gain of 10 kg.

Case 2

A 66-year-old Caucasian woman presented with a 4-wk history of jaundice, right upper quadrant pain and pruritus. She had a past history of hysterectomy and bilateral salpingo-oopherectomy, carried out when she was 40 years old, complicated by post-operative deep vein thrombosis. She started Premarin (conjugated estrogens) 625 μg for menopausal symptoms when she was 50 years old, and had been taking this continuously up to the time of her presentation. The patient had no history of jaundice, hepatitis, blood transfusion or travel outside Western Europe. She had no risk factors for viral hepatitis and did not drink alcohol. On examination she was jaundiced, with no signs of chronic liver disease, and was afebrile. Tenderness on palpation of the right hypochondrium was evident, and the liver was palpable 2 cm below the costal margin.

Laboratory investigation revealed a total serum bilirubin of 464 μmol/L, ALT 56 IU/L, ALP 328 IU/L and γ-GT 60 IU/L. Serological tests for hepatitis A, B and C were negative. Anti-nuclear factor, and anti-mitochondrial and smooth muscle antibodies were also negative. Ultrasound examination of the liver revealed a dilatated CBD measuring 10 mm and multiple small radio-opaque calculi in the duct.

The patient underwent ERCP and sphincterotomy; all intraductal stones were removed with a balloon. However, 8 wk after her initial presentation, the patient remained markedly jaundiced with worsening pruritus, malaise, anorexia and weight loss of 6 kg. Liver function tests had worsened: total serum bilirubin 798 μmol/L, ALT 87 IU/L, ALP 370 IU/L and γ-GT 96 IU/L (Figure 2). Premarin was stopped.

A CT scan showed a normal gallbladder, with no evidence of malignancy and no biliary tree dilatation. A repeat ERCP showed a normal biliary system with no evidence of retained CBD stones. Ursodeoxycholic acid (UDCA) 1200 mg/d was commenced following ERCP. Four weeks later, her symptoms had begun to improve, and her bilirubin level had fallen to around half its peak value. After a further month, her symptoms had resolved completely, although her liver biochemistry did not completely return to normal until 40 wk after her initial presentation.

DISCUSSION

In both cases, prolonged cholestasis after removal of CBD stones at ERCP was thought to have been caused by oral estrogen therapy. The association between oral estrogen-containing preparations and several cholestatic syndromes has been well recognised since 1962[1]; and of the canalicular type with little portal inflammation. It is thought that these reactions are because of the estrogen component; hence it is widely used as an experimental model of hepatocellular cholestasis. This effect is mediated by its glucuronidated metabolites, which inhibit canalicular bile salt and glutathione excretion, which results in inhibition of bile salt transport[7].

Clinical features include malaise, pruritus, jaundice and

anorexia with subsequent weight loss, were seen in both our patients. These features are also seen in the clinical syndrome of intrahepatic cholestais of pregnancy (ICP). Indeed a Chilean group has found that, in 42 patients with cholestatic jaundice following use of oral estrogen compounds, 27 had previously jaundice and pruritus during pregnancy, which suggests a link between the two disorders[2]. This must be considered in the context of the strong geographical predilection of this disease to Chile. Crucially, withdrawal of the estrogenic effect by delivery of the fetus or drug withdrawal leads to improvement in liver function. This tends to take place over several weeks or months. Neither of the patients described here had a history of ICP, although liver function tests had not previously been performed.

Case 1 had proven gallstones at 6 wk post partum and liver function tests were consistent with stones in the CBD. The rapid deterioration in liver function both clinically and biochemically after commencing oral estrogens, and the resolution on discontinuation of the treatment support the hypothesis that the cholestasis was a drug-induced liver injury. Other potential causes were excluded. In particular, the absence of antimitochondrial antibodies, normal intrahepatic bile ducts on ERCP, and the spontaneous normalization of liver biochemistry excluded both primary biliary cirrhosis and primary sclerosing cholangitis.

Case 2 had been on estrogen replacement therapy for 16 year at the time of her presentation with obstructive jaundice. However, the only factor that could account for the prolonged cholestasis was the estrogen therapy, and the temporal relationship between stopping estrogens and improvement in liver function further supports the conjecture that hormone replacement therapy was responsible (although UDCA therapy may have played a role in the resolution of the cholestasis).

Since estrogen therapy alone did not cause jaundice in this patient, there must have been an interaction between the effects of mechanical obstruction to bile flow and the cholestatic effects of estrogens. The patient has remained off estrogens since the procedure.

The cause of the differences in time for resolution of jaundice in cases 1 and 2 is unclear. The jaundice in both cases was likely to have resolved following the withdrawal of estrogens, and it is possible that the longer duration of jaundice in case 2 was simply a reflection of the variable length of time for estrogen-induced cholestasis to resolve following treatment withdrawal. Typically, this takes several weeks to months, but it has been reported to take up to 10 years[3]. It is also possible that the difference in time for the jaundice to resolve reflected differences in the modes of action of the drugs used to treat the cholestasis. The anti-inflammatory effects of corticosteroids may be of benefit for individuals with a pre-existing mechanical obstruction. It is known that when biliary obstruction occurs, an inflammatory response is mounted with the release of pro-inflammatory cytokines (e.g. tumor necrosis factor-α and interleukin-1), a reduction in the expression of nuclear bile acid receptor, and infiltration of neutrophils, all of which are thought to aggravate cholestatic injury[8].

Corticosteroid use has previously been described in two cases reports of prolonged cholestasis following ERCP and successful removal of gallstones[9]. The patients were not taking estrogens (both were male) and the authors hypothesized that the canalicular function had been directly compromised by a mechanical obstruction. They also postulated that the radiocontrast medium infused under high pressure during ERCP may have had a toxic effect, with disruption of the canalicular membrane, but no evidence was given to support this theory. The two cases presented differed from both our patients, who were taking oral estrogens. Whilst the onset of jaundice appears to have been precipitated by the presence of obstructing intraductal calculi, the estrogen therapy appears to have been responsible for the persistence of cholestasis. Presumably, the biliary obstruction in some way sensitized the biliary canaliculi to the cholestatic effects of estrogens, perhaps through an associated inflammatory response (discussed above) that led to the prolonged cholestasis. The interaction between these two factors may in part be idiosyncratic and/or dependent on genetic factors, since this has not previously been reported.

UDCA was used in the second case because of concerns about side effects, particularly steroid-induced osteoporosis in this postmenopausal woman. Its use resulted in a less impressive response, but it may not have influenced the course of the cholestasis. However, evidence from a number of large studies has confirmed the efficacy of UDCA in ICP, and the drug is now used routinely for treating this condition[10]. The use of UDCA in treating cholestasis secondary to oral estrogen therapy in humans has not been reported, although ethinylestradiol-induced cholestasis in rats has been shown to respond to UDCA[11]. The authors of that study concluded that UDCA increased bile flow by increasing bile acid secretion, through the normalization of the expression of the canalicular bile salt export pump. UDCA has also been noted to decrease the glucuronidation of estrogens, thereby decreasing the production of cholestatic metabolites[12].

Although corticosteroid treatment appeared more effective than UDCA, the two agents have been compared in a randomized study in ICP. Treatment with UDCA led to a significant reduction in ALT and bilirubin and an improvement in pruritus, while dexamethasone had no such effects[13].

Endoscopists who carry out ERCPs should be aware of the potential causes of persistent jaundice following the removal of intraductal stones. In female patients with protracted jaundice, the possibility of estrogen-induced cholestasis should always be considered, and oral estrogens discontinued. In such cases, a short course of corticosteroid treatment should be considered.

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World J Gastroenterol 2007 December 14; 13(46): 6281-6283
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Penetrating ectopic peptic ulcer in the absence of Meckel's diverticulum ultimately presenting as small bowel obstruction

Hilary Hurley, Ronan A Cahill, Paul Ryan, Ashraf I Morcos, Henry P Redmond, Helen M Kiely

Hilary Hurley, Ronan A Cahill, Henry P Redmond, Department of Surgery, Cork University Hospital, Cork, Ireland
Paul Ryan, Helen M Kiely, Department of Pathology, Cork University Hospital, Cork, Ireland
Ashraf I Morcos, Department of Gastroenterology, Mercy University Hospital, Cork, Ireland
Correspondence to: HP Redmond, Professor, Department of Surgery, Cork University Hospital, Wilton, Cork, Ireland. rcahill@rcsi.ie
Telephone: +353-21-4922371
Received: July 16, 2007 Revised: September 6, 2007

Abstract

We report here how a heterotopic penetrating peptic ulcer progressed to cause small bowel obstruction in a patient with multiple previous negative investigations. The clinical presentation, radiographic features and pathological findings of this case are described, along with the salient lessons learnt. The added value of wireless capsule endoscopy (WCE) in such circumstances is debated.

Key words: Non-Meckelean ectopic peptic ulcer; Bowel obstruction

Hurley H, Cahill RA, Ryan P, Morcos AI, Redmond HP, Kiely HM. Penetrating ectopic peptic ulcer in the absence of Meckel's diverticulum ultimately presenting as small bowel obstruction. *World J Gastroenterol* 2007; 13(46): 6281-6283

http://www.wjgnet.com/1007-9327/13/6281.asp

INTRODUCTION

Heterotopic gastric mucosa resulting in peptic ulceration in the absence of a Meckel's diverticulum is a most unusual cause of small bowel symptomatology[1]. Although wireless capsule endoscopy (WCE) enables painless visualization of the small bowel in a non-invasive manner, its added value in cases not presenting with occult gastrointestinal blood loss is unclear. In this report, we consider what extra value this technology added to the care of a patient who had had multiple negative standard investigations, and ultimately required a laparotomy and ileal resection for definitive management.

CASE REPORT

A 77-year-old man presented to the Accident and Emergency Department of our hospital with subacute small bowel obstruction, having complained for many years of intermittent severe central abdominal discomfort associated with episodic distension and constipation. Prior to this admission, these symptoms had been extensively investigated by means of plain radiology, computerized tomography (CT) and barium contrast studies, as well as by both upper and lower gastrointestinal endoscopy, but to no avail. Furthermore, laparotomy at the time of one such presentation had been performed but without therapeutic benefit, as no evidence of overt intestinal pathology or abnormality was found (in particular, there was no evidence of Meckel's diverticulum). On this latest presentation, the clinical examination was consistent with subacute small bowel obstruction (distended but soft, moderately tender abdomen, with hyperactive bowel sounds and signs of extracellular fluid depletion). Plain abdominal radiography revealed the presence of dilated loops of the small bowel, while subsequent abdominal CT confirmed this finding but did not identify a transition point or any mass lesion. Initial treatment involved correction of fluid and electrolyte abnormalities and nasogastric aspiration for symptomatic relief. Mindful of his past history, when the symptoms persisted for more than 48 h, small bowel barium follow-through was performed. This also failed to define any specific pathology, although the barium failed to progress beyond the proximal jejunum.

As the patients symptoms had somewhat relented by this stage and he resumed passing flatus again, he was therefore permitted some oral diet which he tolerated despite some intermittent, colicky pain. Although a second laparotomy was advocated because of the ongoing persistence of low-grade symptoms, the patient was reluctant, on empiric reasons alone, to consider this given the lack of impact from his previous operation. WCE was therefore performed on a somewhat speculative basis. This test again demonstrated a prolonged transit time through the small bowel (the capsule had not progressed through the jejunum after 7 h), but the last frame of the study showed a raised mucosal jejunal lesion adjacent to an area of severe luminal stenosis (Figure 1). Along with the non-resolving symptoms, identification of this suspicious mucosal lesion, compounded by capsule retention, strengthened the case for exploratory laparotomy. The patient consented. At operation, two

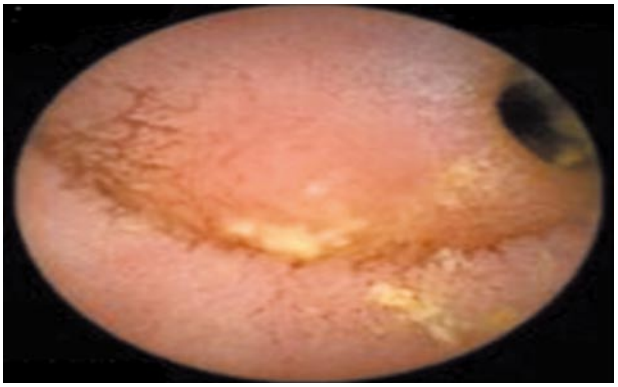

Figure 1 Image of jejeunal luminal stenosis from WCE.

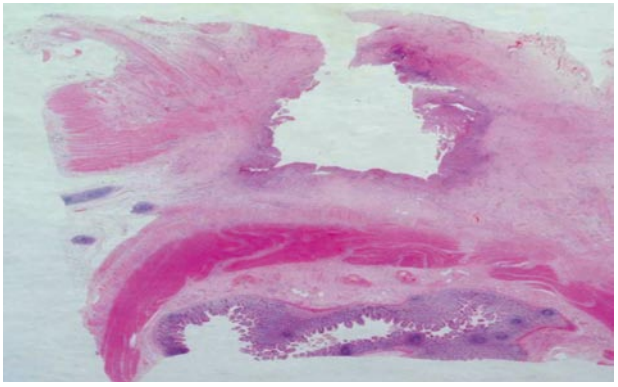

Figure 2 Photomicrograph of a deep penetrating ulcer with underlying fibrosis replacing muscularis propria, extending through the serosa adherent to an underlying small bowel loop (lower field).

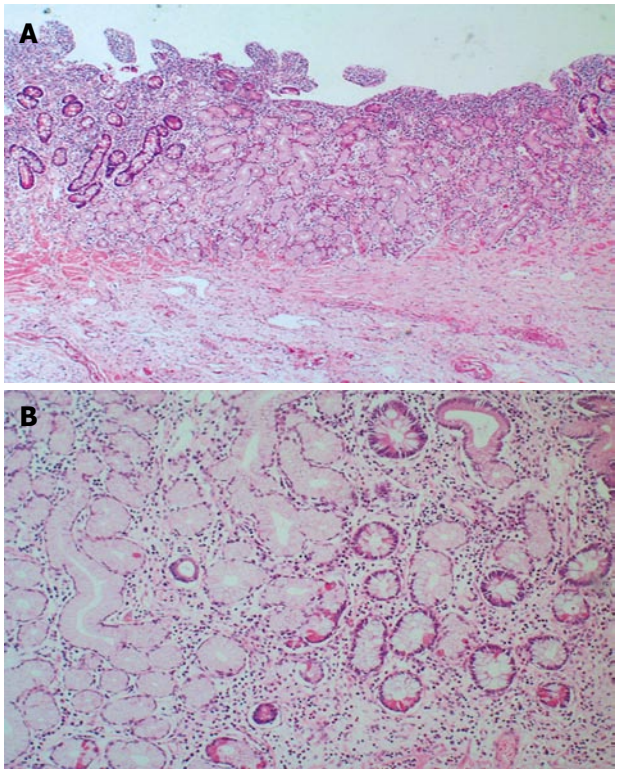

Figure 3 Photomicrographs showing (**A**) heterotopic gastric foveolar mucosa with a few normal small intestinal glands; and (**B**) scattered cells with eosinophilic cytoplasm compatible with acid-secreting cells.

adjacent loops of densely adherent jejenum were obvious among other areas of peritoneal adhesions. There were also features of chronic dilatation proximal to an area of luminal stricturing within one of these loops of bowel. Careful adhesiolysis permitted resection of a segment of chronically obstructed aperistaltic small bowel, as well as allowing for the retrieval of the capsule. A primary hand-sewn end-to-end jejunojejunal anastomosis was performed. Opening of the specimen subsequently revealed an underlying oval mucosal ulcer with punched out edges extending through the wall, which caused fibrosis and adhesion to the adjacent loops of bowel (Figure 2). Histological examination of the resected specimen identified foci of heterotopic gastric mucosa adjacent to this deep penetrating chronic peptic ulcer (Figure 3). The patient thereafter made an uncomplicated recovery and remains symptom-free 6 mo later.

DISCUSSION

Heterotopic gastric mucosa in the small bowel, other than in Meckel's diverticulum or other congenitally anomalous bowel, is rare[2]. Such lesions usually cause small bowel obstruction, either secondary to a mechanical lesion in the lumen[1], or intermittently due to intussusception of the ectopic mass[2-5]. Acute presentation with a perforating ulcer is rarely seen; indeed, there have been only five cases reported in the literature[1-4], with a further five being reported as causing hemorrhage due to ulcer penetration[1]. In our patient, an ulcerating ectopic mucosal lesion presented unusually as small bowel obstruction secondary to an extensive fibrotic reaction. In this case, there was no macroscopic evidence of diverticulum, luminal duplication, or congenital anomaly. Due to the dense adhesional mass and desmoplastic reaction, it was not possible to identify whether the ulcer was on the mesenteric or antimesenteric border of the small bowel, but the penetrating jejunal ulcer was clearly present at the site of the transition point between the dilated and normal caliber small bowel. Microscopic examination of the lesion yielded evidence of ectopic gastric mucosa adjacent to the deep penetrating ulcer, associated with features of chronic inflammation and fibrosis. A diagnosis of chronic peptic ulcer, secondary to heterotopic gastric mucosa with reactive adjacent fibrosis and adhesions was therefore concluded.

The development of WCE technology has changed investigative endoscopy of the small bowel into a much less invasive and more complete examination. A now-proven reliable tool for verifying the state of the small bowel[2], it has been particularly successful in finding the cause of obscure gastrointestinal bleeding[3] and chronic diarrhea, and in evaluating the extent of Crohn's disease. It is not generally used in cases of small bowel obstruction, due to the risk of capsule retention that should precipitate surgical intervention. In patients with undiagnosed abdominal pain, the yield from the use of WCE appears low, whereas in this case, it proved useful in securing a pathological diagnosis and furthering management[4], as there was an

understandable reluctance by the patient to proceed to a second laparotomy without a strong positive indication and likelihood of therapeutic benefit. This case therefore adds some weight to the argument that judicicious use of WCE in small bowel obstruction may identify the site of obstruction and guide surgical intervention[5,6]. In particular, this could be of great significance for a surgeon attempting to localize otherwise non-apparent pathology intraoperatively. In this case, WCE was considered only in the setting of multiple negative investigations and a high probability of operative intervention, given the high risk of capsule retention.

While WCE added little objective evidence to the findings at the latter operation, it is intriguing to speculate how this test may have guided intervention at the time of first surgery. Although small bowel ulcers, ileal tuberculosis and even worm infestation have previously been demonstrated by WCE, most small bowel pathology found to date has been Crohn's disease or NSAID-related lesions of the distal small bowel[5]. There is only one previous case in the literature of heterotopic gastric mucosa of the small bowel identified by capsule endoscopy[6]. Although the captured images in this case did not diagnose the specific lesion, they did suggest a mucosal anomaly and secondary stenosis, which was subsequently histologically identified as ulcerating heterotopic gastric mucosa. If knowledge of such an abnormality had been available prior to the first operation, perhaps that intervention could have been tailored with therapeutic benefit.

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World J Gastroenterol 2007 December 14; 13(46): 6284-6285
World Journal of Gastroenterology ISSN 1007-9327

LETTERS TO THE EDITOR

Hepatic hydrothorax occurring rapidly after manual abdominal compression

Kazufumi Dohmen, Hirofumi Tanaka, Masatora Haruno, Yoshiyuki Niho

Kazufumi Dohmen, Hirofumi Tanaka, Masatora Haruno, Yoshiyuki Niho, Internal Medicine, Chihaya Hospital, Fukuoka 813-8501, Japan
Correspondence to: Dr. Kazufumi Dohmen, Internal Medicine, Chihaya Hospital, 2-30-1 Chihaya Higashi-ku, Fukuoka 813-8501, Japan. dohmen-kzfm@cool.odn.ne.jp
Telephone: +81-92-6612211 Fax: +81-92-6830411
Received: August 22, 2007 Revised: September 10, 2007

Abstract

Hepatic hydrothorax is a relatively infrequent but potentially serious complication of liver cirrhosis that often causes respiratory dysfunction. Several hypotheses for the development of hepatic hydrothorax have been suggested to explain a transdiaphragmatic shift of ascitic fluid through small defects between the peritoneal cavity and the pleural space. However, the rapid development of hydrothorax within several hours is seldom encountered. In addition, the causal factors for rapid passage of ascitic fluid into the pleural cavity are unknown. This report describes a patient with liver cirrhosis who suffered rapid development of a hydrothorax after manual compression of the abdomen.

Key words: Hydrothorax; Liver cirrhosis; Abdominal compression

Dohmen K, Tanaka H, Haruno M, Niho Y. Hepatic hydrothorax occurring rapidly after manual abdominal compression. *World J Gastroenterol* 2007; 13(46): 6284-6285

http://www.wjgnet.com/1007-9327/13/6284.asp

TO THE EDITOR

A 63-year-old female with Child-Pugh grade C liver cirrhosis was admitted to Chihaya Hospital, Fukuoka, Japan because of ascitic fluid and hepatic encephalopathy. Physical examination revealed a markedly distended abdomen and peripheral edema. Her laboratory blood tests showed a white blood cell count of 9550/L, hemoglobin of 7.6 mg/dL, platelet count of 54000/L, aspartate aminotransferase 39 IU/L (normal 7-38), alanine aminotransferase 16 IU/L (normal 4-43), gamma glutamyltransferase 45 IU/L (normal 16-73), alkaline phosphatase 600 IU/L (normal 104-338), serum albumin 2.0 mg/dL (normal 3.8-5.2), total bilirubin 1.5 mg/dL (normal 0.2-1.1), and serum ammonia 255 μg/dL (normal 30-86). Chest radiography showed no pleural effusion (Figure 1), while abdominal computed tomography imaging was characteristic of cirrhosis with a massive amount of ascitic fluid (Figure 2).

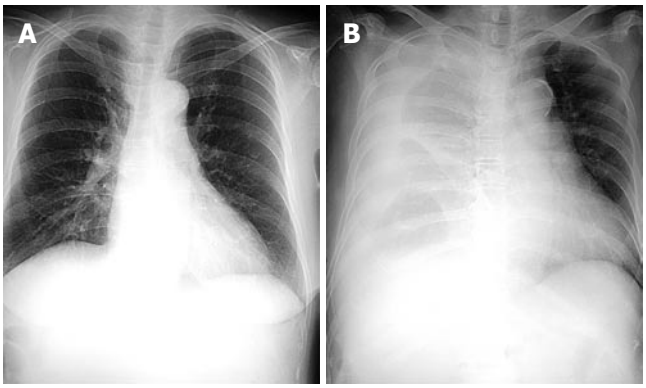

Figure 1 Chest X-ray. **A**: Before manual compression of the abdomen; **B**: on the next day, after manual compression of the abdomen.

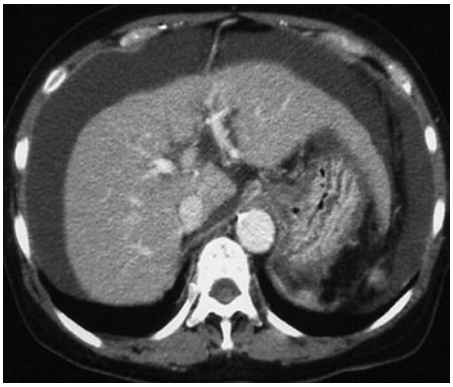

Figure 2 Abdominal computed tomography before manual compression of the abdomen shows liver cirrhosis with a massive volume of ascitic fluid.

After admission, the patient was treated with branched-chain amino acid supplementation, diuretics and lactulose enema, in combination with antibiotics for ascites and hepatic encephalopathy. Hepatic encephalopathy had been well controlled; however, it manifested in association with constipation on fourteenth day after admission. Constipation was not relieved after the administration of laxative drugs and a lactulose enema. Therefore,

manual abdominal compression was performed gently for approximately 1 h by a nurse to accelerate bowel movement. After that, a large amount of stool was evacuated and hepatic encephalopathy improved. However, during the following night, progressive dyspnea occurred and analysis of blood oxygen showed hypoxemia. Chest radiography on the next day revealed a massive right-sided pleural effusion (Figure 1), while ultrasonography showed that the ascitic fluid had disappeared. Pleural effusion obtained by thoracenteses showed a transudate, which indicated the rapid migration of ascitic fluid into the right hemithorax.

The transfer of large volumes of fluid from the abdomen to the pleural space through defects in the diaphragm can easily be understood. Once the diaphragmatic communications begin to leak fluid in response to abdominal pressure such as a cough or muscle strain, the combination of a hydrostatic gradient of negative intrathoracic pressure and positive intra-abdominal pressure passively produces a unidirectional flow of ascitic fluid into the pleural space[1,2]. The occurrence of hydrothorax induced by tense and prolonged abdominal compression has not previously been reported, although there have been a few reports demonstrating the actual condition of the rapid occurrence of hepatic hydrothorax[3].

Interestingly, in this case with cirrhosis and a massive volume of ascitic fluid, the imaging data and clinical course strongly suggested that the rapid migration of ascitic fluid into the pleural space occurred due to the manual abdominal compression. Therefore, clinicians need to be aware of the possibility that massaging the abdomen can cause a hepatic hydrothorax in patients with a massive volume of ascitic fluid.

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World J Gastroenterol 2007 December 14; 13(46): 6286
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Liver Diseases Branch - NIDDK, National Institutes of Health, 10 Center Drive, Bldg. 10, Room 9B11,Bethesda, MD 20892, United States

Joseph Daoud Boujaoude
Assistant Professor, Department of Gastroenterology, Hotel-Dieu de FranceHospital, aint-Joseph University, Beirut 961, Lebanon

Frank J Burczynski
Faculty of Pharmacy, University of Manitoba, 50 Sifton Road, Winnipeg, Manitoba, R3T 2N2, Canada

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Division of Research and Education, Scott and White Hospital and The Texas A&M University System, Health Science Center College of Medicine, Temple, Texas 76504, United States

Nikolaus Gassler, Professor
Institute of Pathology, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

Shannon S Glaser, Dr
Department of Internal Medicine, Scott & White Hospital, 702 SW HK Dodgen Loop, Medcial Research Building, Temple 76504, United States

Kazuhiro Hanazaki, MD
Professor and Chairman, Department of Surgery, Kochi Medical School, Kochi University, Kohasu,Okohcho, Nankoku, Kochi 783-8505, Japan

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Institute of Immunology, Rikshospitalet University Hospital, N-0027 Oslo, Norway

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Leonidas G Koniaris, Professor
Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave., Miami, FL 33136, United States

Shiu-Ming Kuo, MD
University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo 14214, United States

Stephen J Lanspa, Dr
Medical Dean's Office, Creighton University, 2500 California Plaza, Criss II, Room 103, Omaha, Nebraska 68178, United States

Chang Hong Lee, MD
Department of Internal Medicine, Konkuk University Hospital, Konkuk University School of Medicine, 4-12 Hwayang-dong, Gwangjin-gu, Seoul, 143-729, Korea

Stephan Menne, Assistant Professor
Department of Clinical Sciences/GI Unit, College of Veterinary Medicine, Cornell University , C2-005 Veterinary Medical Center, Ithaca, NY 14853, United States

Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman
Department of Medical Oncology, Kanazawa Medical University,1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Yuji Naito, Professor
Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

Yaron Niv, Professor
Department of Gastroenterology, Rabin Medical Center, Beilinson Campus, Tel Aviv University, 2 Hadekel St., Pardesia 42815, Israel

Thierry Piche , MD, PhD
Department of Gastroenterology, Archet 2 Hospital, 151 RTE ST Antoine de Ginestiere 06202, Nice CEDEX 3, France

Paolo Del Poggio, Dr
Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy

Bernardino Rampone, Dr
Department of General Surgery and Surgical Oncology, University of Siena, viale Bracci, Siena 53100, Italy

Vasiliy I Reshetnyak, MD, PhD, Professor
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Damian Casadesus Rodriguez, MD, PhD
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Tomohiko Shimatani, Assistant Professor
Department of General Medicine, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan

Ross C Smith, Professor
Department of Surgery, University of Sydney, Royal North Shore Hospital, St Leonards, New South Wales 2065, Australia

Nick Paul Thompson, MD
Department of Medicine, Freeman Hospital, Newcastle Upon Tyne, NE7 7DN, United Kingdom

Siegfried Wagner, Professor
Medizinische Klinik II, Klinikum Deggendorf, Perlasberger Str. 41, Deggendorf 94469, Germany

Scott A Waldman, MD, PhD, FCP
Division of Clinical Pharmacology, Departments of Medicine and Biochemistry and Molecular Pharmacology, Jefferson Medical College, Thomas Jefferson University, 1100 Walnut Street, MOB Suite 813, Pennsylvania 19107, Philadelphia, United States

Fritz von Weizsäcker, Professor
Department of Medicine Schlosspark-Klinik, Humboldt University, Heubnerweg 2, Berlin D-14059, Germany

Yoshio Yamaoka, MD, PhD, Associate Professor
Department of Medicine/Gastroenterology, Baylor College of Medicine and VA Medical Center (111D), 2002 Holcombe Blvd, Houston, Texas 77030, United States

Online Submissions: wjg.wjgnet.com
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World J Gastroenterol 2007 December 14; 13(46): 6287
World Journal of Gastroenterology ISSN 1007-9327

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www.come-innsbruck.at/events/ibd2007/default.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
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Meeting BSG Annual Meeting
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Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
11-15 April 2007
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www.easl.ch/liver-meeting

Meeting SAGES 2007 Annual Meeting -part of Surgical Spring Week
18-22 April 2007
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www.sages.org/07program/index.php

Meeting Falk Symposium 159: IBD 2007-Achievements in Research and Clinical Practice
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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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