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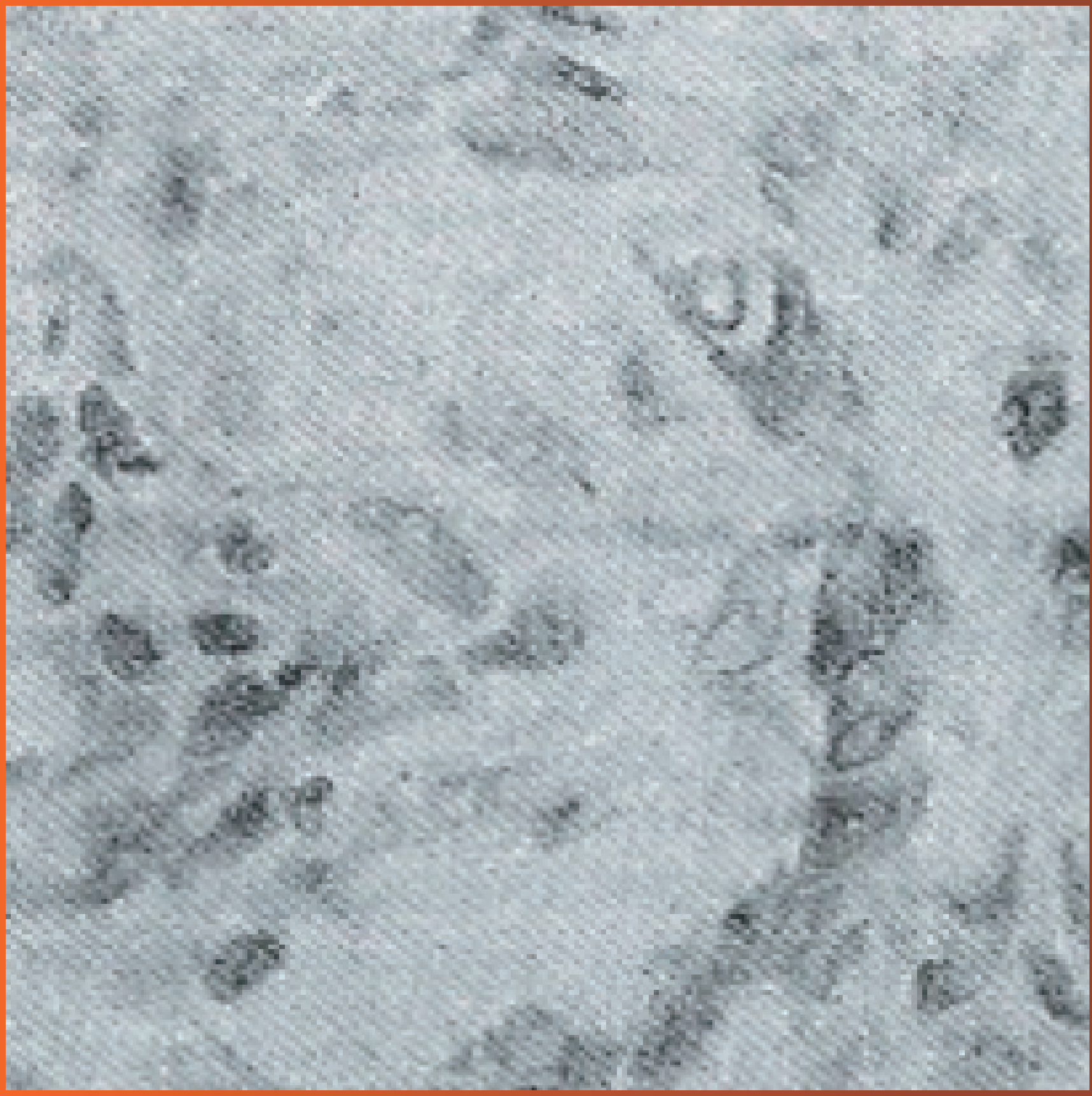

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COMMENTARY

Somatostatin: Likely the most widely effective gastrointestinal hormone in the human body

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Somatostatin (SS) was discovered from rat hypothalamus in 1968 and identified as a 14-amino acid peptide (SS-14) in 1973. Research achievements from studies of this fascinating hormone have drawn attention of scientists worldwide, yet it is not yet the time to make a complete conclusion on its broad distribution, physiologic functions, and roles in diagnosis and treatment of diseases. The results reported in the accumulated literature, however, do suggest its potential vast significant influence.

SS DISTRIBUTION[1]

SS-producing cells (D-cells) exist at high densities throughout the central and peripheral nervous systems, in the endocrine pancreas and the gut, as well as in small numbers in the thyroid, adrenals, submandibular glands, kidneys, prostate and placenta (Table 1). In rats, gut-localized SS accounts for about 65% of the total body distribution, followed by brain localization at 25% and the pancreas and the remaining organs at 5% each. The approximate relative amounts of SS in the central nervous system are mainly in the cortex (49%) and spinal cord (30%). The D-cells in pancreatic islets are proximal to the other hormone-producing islet cells (*i.e.* A (glucagon-producing), B (insulin-producing), and PP (pancreatic polypeptide-producing) cells) and function to mediate function and inhibit hypersecretion of glucagon, insulin and pancreatic polypeptide *via* paracrine signaling. The D-cells are also located in the mucosal glands located throughout the cardiac region of the stomach to the rectum, and their content of SS accounts for more than 90% of that in the entire gastrointestinal tract.

The SS in neurons populate both the submucosal and myenteric plexuses in all segments of the gastrointestinal tract, accounting for less than 10% of the total. Compared to SS concentration in plasma, that in cerebral spinal fluid is twice as much and in semen is 200-fold as high. Amniotic fluid is rich in SS. It is also present in some tumor tissues, including those of lung cancer, colon cancer and gastroenteropancreatic endocrine tumors. The extensive existence of SS in large amounts in the human body support the notion that it likely plays a significantly influential role in both physiologic and pathologic conditions.

SS BIOLOGIC/PHYSIOLOGIC ACTIONS[1,2]

SS exerts inhibitory effects on various organs. The broad array of SS actions includes regulation of neurotransmission, glandular secretion, smooth muscle contractility and cell proliferation. SS has been shown to inhibit virtually every known exocrine and endocrine secreted factor, including growth hormone, prolactin, thyrotropin, corticotropin, insulin, glucagon, vasoactive intestinal polypeptide, pancreatic polypeptide, gastrin, cholecystokin, motilin, neurotensin, thyroxin (T_4), triiodothyronine (T_3), calcitonin, aldosterone, catecholamine and renin. It has also been shown to inhibit splanchnic blood flow and movement functions (*i.e.* smooth muscle contractility), such as contractility of stomach, intestine and the biliary system, as well as gastric emptying, contractility of sphincter of Oddi, prolongation of gastrointestinal transit time, secretion such as for gastric acid and bicarbonate from the exocrine pancreas, release of pepsin, trypsin and the hemopoietic intrinsic factor as well as bile flow, absorption of glucose and other carbohydrates, amino acids and triglycerides, activity of the nervous system such as acetylcholine release from the gut mural myenteric plexuses, proliferation of cells and tumor growth, platelet aggregation, and function of activated immune cells. SS is also capable of stimulating water and electrolyte absorption, activating opioid receptors, and appears to excite the central neurons. The above-mentioned extensive inhibitory functions of SS suggest that, the SS is a widespread and powerful inhibitor capable of influencing a number of organs and tissues, and therefore of mediating homeostasis.

SOMATOSTATIN ANALOGUES[2-4]

To date, the clinical utility of SS has been limited due to its very short half-life (less than 3 min). Octreotide, the first synthetic SS analogue, first described by Bauer *et al* in 1982, possesses longer half life (up to 2 h) and could be injected intermittently, and even administered orally. This agent is also able to inhibit the release of growth hormone, glucagon, and insulin by 45-, 11-, and 1.3-times respectively, and more powerfully than SS-14 and without causing

Table 1 Localization of somatostatin

Organs	Type of Cells	Location
Major sites		
Nervous system	Neurons	Hypothalamus
		Cerebral cortex
		Limbic system
		Basal ganglia
		Major sensory systems
		Spinal cord
		Dorsal root ganglia
		Autonomic ganglia
Pancreas	D-cells	Islets
Gut	D-cells	Mucosal glands
	Neurons	Submucous and myenteric plexuses
Minor sites		
Adrenal		Scattered medullary cells
Placenta		Cytotrophoblasts in chorionic villi
Reproductive organs		Testis, epididymis, prostate
Submandibular Gland	D-cells	Scattered ductal cells
Thyroid	C-cells	Scattered parafollicular cells, coexisting with calcitonin
Urinary system		Scattered cells in renal glomerulus and collecting ducts
Miscellaneous		Thymus, heart, eyes, erythrocytes

Table 2 The 5 subtypes of human SS receptors

Feature	SS receptors				
	Subtype 1	Subtype 2	Subtype 3	Subtype 4	Subtype 5
Amino acid sequence	361	369 (A), 346 (B)	418	388	364
Chromosomal location	14	17	22 20	20 22	16
G protein coupling	Yes	Yes	Yes	Yes	Yes
Effector system					
Adenylate cyclase activity	Reduced	Reduced	Reduced	Reduced	Reduced
Tyrosine phosphatase activity	Increased	Increased	Not investigated	Not investigated	Not investigated
Possible binding affinity to receptors					
SS-14	++	++	++	++	++
SS-28	++	++	++	++	++
Octreotide	±	++	+	±	++
Vapreotide (RC-100)	±	++	+	+	+++
Lanreotide (BIM-23014)	±	++	+	+	+++
[1]Distribution in normal human tissue	Brain, lungs, stomach, jejunum, kidneys, liver, pancreas	Brain, kidneys	Brain, pancreas	Brain, lungs	Brain, heart, adrenal glands, placenta, pituitary, small intestine, skeletal muscle

[1]Not all human tissues were tested simultaneously. This table was modified from Steven *et al*.

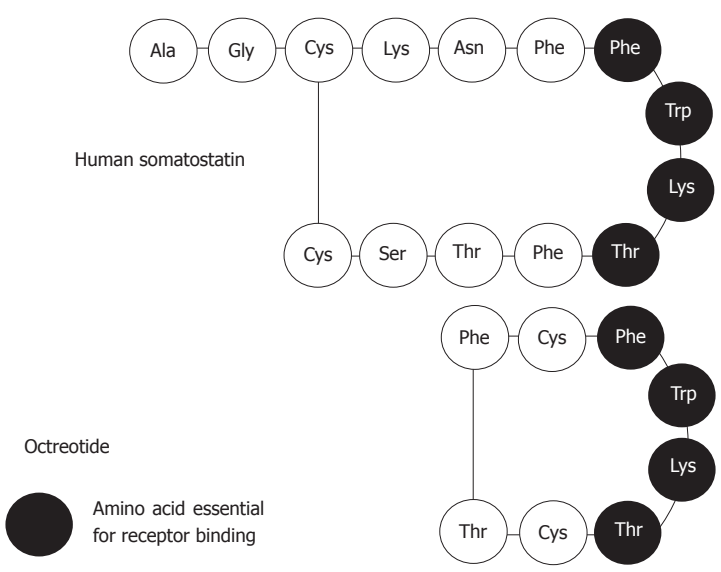

Figure 1 Amino acid composition of somatostatin and octreotide.

rebound hypersecretion of hormones. Octreotide, an 8-amino acid, was modified from SS-14 (Figure 1), with the 7th to 10th amino acids being maintained in common since they are the 4 amino acids essential for receptor binding. Octreotide has similar pharmacological effects as native SS, and its widespread clinical use had illuminated its manifold physiological functions and values in clinical application. The mechanism underlying SS analogues' clinical utility in diagnosis and treatment is the SS receptors that are present on tissue membranes and which specifically bind to the SS analogues, resulting in cell effects.

There are 5 subtypes of SS receptors (Table 2), which are identical in 42%-60% of their amino acid sequences and belong to a superfamily of receptors. The genes for these subtypes, located on different chromosomes, have suggested their different functions in different organs. As such, a variety of SS analogues might exert pharmacological actions by the specific binding with the various subtypes. The effects of treatment with SS analogues depend on both the ligands and the receptor subtypes.

OCTREOTIDE USE FOR DIAGNOSIS[5,6]

Radiolabelled SS analogue, administered intravenously and then binding to the SS receptors on tumor tissues is the resulting formation of radiolabelled aggregation. Scintigraphy using a γ camera allow for visualization of such SS receptor-positive tumors. ^{111}Octreotide is the most commonly used SS analogue because of the high density of SS receptors on neuroendocrine tumors of the gastroenteropancreatic system, with the total positivity rate ranging from 50% to 100%, and in most of the groups investigated it is above 80%. Metastatic liver tumors show lower rates of positivity. Pituitary adenoma may usually be visualized by scintigraphy. Adenocarcinomas originating from the breast, kidney, colon or ovary also express SS receptors. In ^{111}octreotide scintigraphy imaging, the diagnostic reliability and rate of positivity are better than the other current diagnostic imaging techniques. The diagnostic efficiency depends upon the amount of SS receptors expressed on the tumor cells. Octreotide injection therapy does not affect the diagnostic efficiency, but can increase the therapeutic effect.

OCTREOTIDE USE FOR TREATMENT[2,7,8]

Octreotide is the most widely used drug among the SS analogues. Its indications and clinical uses are as follows:

Pituitary adenomas and gastroenteropancreatic endocrine tumors

These tumors have a large number of SS receptors and are treated with octreotide effectively, especially the growth hormone-secreting pituitary tumors (acromegaly). The agent is usually delivered as a subcutaneous injection of 100 μg three times a day, and may result in rapid control of symptoms and hormone secretion. In about half of the patients treated with octreotide, the size of the tumor is reduced. Some cases require larger doses, up to 300 μg or 600 μg daily, to achieve the maximal benefit, but a dose above 600 μg per day rarely has additional effect. Because of the high sensitivity observed in elderly patients with growth hormone-secreting tumors, octreotide can be recommended as a primary treatment. There have been no reports of loss of the suppressive effect of octreotide on growth hormone secretion in patients with acromegaly, even after 10 years or more of uninterrupted treatment.

Pancreatic islet-cell tumors express SS receptors in more than 80% of cases, and daily injection of 150 μg to 300 μg may improve the clinical outcome in 50% to 85% of cases. This outcome may be mediated by both a direct inhibitory effect on hormone production by the tumor and indirect effects, such as on the resorption of intestinal fluid, the production of gastric acid, or intestinal contractility. Octreotide treatment is often effective initially, but after weeks to months of treatment the symptoms worsen and tumor hormone secretion increases in virtually all the patients; while this effect can be reversed by increasing the dose of octreotide, eventually the drug becomes ineffective in all patients. This resistance phenomenon may be explained by the growth of clones of tumor cells that lack SS receptors. Octreotide is especially valuable in controlling the symptoms associated with vipoma or glucagonoma, and the remaining islet cell tumors often express subtypes of the SS receptor that do not bind octreotide, decreasing the beneficial effect. Reportedly, 70%-90% of metastatic carcinoid tumors respond well to octreotide, with effective control of the symptoms and of the overproduction of serotonin or tachykinin. It may also temporarily inhibit tumor growth, and in about 20% of patients the enlarged lymph nodes and liver metastases shrink. The most important benefits of octreotide are improvements to patient quality of life and patient survival.

Probable indications

In accordance with its various functions, including suppression of endocrine and exocrine secretions of the gastroenteropancreatic system, reduction of splanchnic blood flow, prolongation of gastrointestinal transit time and stimulation of water and electrolytic absorption, octreotide can be used to treat the following diseases: variceal acute bleeding (by 48-h continuous intravenous administration, and which has a similar effect of emergency sclerotherapy); pancreatic fistula; post-elective pancreatic surgery complications when applied perioperatively; and some refractory secretory diarrheas (as in autoimmune disease syndrome, short bowel syndrome, radiation colitis, and chemotherapy conditions).

Potential role in oncologic treatment[2]

Octreotide has not been used formally as a clinical oncologic treatment, but several studies have showed its promise in this realm. Octreotide has been shown to inhibit tumor growth in animals and of tumor cell lines *in vitro*, exerting antiproliferative effects on tumor cells *via* it effects on the SS receptors. The efficiency of octreotide on tumors depends on both the presence and amount of SS receptors on tumor cells. Tumors of the nervous system have SS receptors, and these tumors account for 50% of solid tumors in children and adolescents, including the neuroblastoma, meningioma, low grade astrocytoma and medulloblastoma.

Octreotide in combination with anti-cancer drugs[9-11]

In Weckbecker's report, octreotide was applied in combination with the anti-cancer drugs mitomycin, doxorubicin, 5-fluorouracil, or Taxol to a pancreatic tumor cell line and to tumor-bearing nude mice. The results showed that the inhibitory effects of the anti-cancer drugs were enhanced markedly in a synergistic or additive manner, strongly suggesting the clinical utility of octreotide in combination with chemotheraputic agents for tumor therapy. Tamoxifen is an anti-oestrogenic agent used to treat breast cancer, but achievement of long-term effects in metastatic diseases is rare because developing resistance to the drug is common. Studies both *in vitro* and *in vivo* showed that the anti-proliferative effects of octreotide can enhance the anti-neoplastic effects of tamoxifen. Compared to tamoxifen mono-treatment, co-administration of octreotide plus tamoxifen yields better results with low toxicity and significantly lowered serum insulin-like growth factor I bioactivity.

Octreotide for SS-receptor-mediated radiotherapy

A β-emitter-labelled octreotide (90Y-SDZ 413) binds to SS receptors on rat pituitary cells and inhibits growth hormone release. Administration of 90Y-SDZ 413 to a nude mouse model with SS receptor-positive tumors led to SS receptor-specific accumulation of the labelled conjugate (tumor/muscle ratio: 52/1), and a single treatment of 90Y-SDZ 413 led to a decrease of 25% in tumor mass. These results suggest that receptor-targeted radiotherapy by means of a 90Y-labelled octreotide represents a new strategy for the treatment of SS receptor-positive tumors.

The mechanism by which octreotide exerts its therapeutic effects on cancers has not been fully elucidated, but studies have indicated that the effects occur as a result of direct anti-proliferative activities on the tumor cells and direct inhibition of angiogenesis in the tumor mass, and as a result of indirect inhibition of the secretion of growth hormone, insulin, and insulin-like factor I. A variety of tumors express different subtypes of the SS receptor, and each of the different kinds of SS analogues may not have high affinity with all subtypes of the SS-receptor. High affinity binding between an SS analogue and its specific subtypes is key for its optimal efficacy on tumors.

Miscellaneous experiences in octreotide treatment[13-15]

In patients with systemic sclerosis, octreotide can resolve the chaotic and non-propagative intestinal motility patterns so that they become well-coordinated-nearly to the extent as in healthy individuals-and can cause symptoms to subside. For patients with irritable bowel syndrome, octreotide can reduce the symptom of rectal pressure that underlies rectal urgency, thereby relieving a key component of the disease manifestation. In patients with dumping syndrome, octreotide can alleviate dumping by slowing gastric emptying, inhibiting insulin release, decreasing enteric peptide secretion, increasing intestinal absorption of water and sodium, slowing monosaccharide absorption, increasing gut transit time and preventing haemodynamic changes. For patients with psoriasis, a 12-wk course of octreotide treatment can ameliorate most symptoms. For patients wit pancreatitis, octreotide can also correct orthostatic hypotension and suppress the inflammatory reaction, the mechanism underlying its therapeutic effect involves inhibiting pancreatic exocrine and endocrine secretions and rectifying perioperative hypotension. Finally, patients with rheumatoid arthritis can benefit from the inflammation-resolving effects of octreotide and the therapeutic effect can be evaluated by scintigraphy of the involved joints.

Clinical usage and adverse effects of octreotide[2]

Octreotide can be delivered *via* intravenous and subcutaneous routes, but is mostly administered as a subcutaneous injection at present. Dosage ranges from 50 μg to 200 μg, 3 times a day, with the most common being 100 μg, 3 times a day. For advanced tumor cases, 500 μg to 1000 μg or 2000 μg, 3 times a day for 6 months has been used safely, but usage of the larger doses is more rare at present. The most common adverse effects include nausea, diarrhea, flatulence, abdominal cramps, and malabsorption of fat, all of which usually subside spontaneously in 10 to 12 d regardless of continuance of treatment.

Long-term treatment with octreotide (>1 mo) is associated with an increased incidence of cholesterol gallstones, with rates ranging from 20% to 30%. This effect seems to be related to dosage and duration of the octreotide treatment. In most cases, the gallstones remain asymptomatic and the management should be similar to that applied to patients with non-octreotide-induced gallstones. The process of gallstone formation during octreotide therapy probably

involves inhibition of gallbladder emptying and reduction of bile flow. Discontinuation of octreotide treatment tends towards gallstone resolution. A smaller dose, such as 100 μg, 3 times a day for less than one month, may present less to no risk of developing this problem. In the case of long-term octreotide treatment, however, intermittent usage may prevent gallbladder formation.

FUTURE PROSPECTIVES

Octreotide has widespread therapeutic effects on various diseases, and hundreds of articles on octreotide treatment are published annually. The reasons why this agent is capable of exerting such a broad range of effects remains unknown and further investigation is necessary. For patients with acromegaly, a long-acting preparation of 20 mg to 30 mg administered intramuscularly is capable of controlling growth hormone hypersecretion for 28 to 42 d as effectively as multiple daily subcutaneous injections. A long-acting intramuscular formulation of lanreotide (another SS analogue) can also maintain therapeutic effects for 2 wk.

Development of novel SS analogues and further development of their diverse preparations may speed up the progression of both basic and clinical research for the wide of array of disease conditions they are related to. Identification and characterization of the various subtypes of SS receptors in different organs and lesions, and of their specific ligands, will improve diagnosis and treatment as well. A current limitation of SS analogues is that they are expensive. When their costs are lowered in the future, they will be used more extensively and their theoretical basis and practical value will become more apparent as well. It can be predicted that octreotide will become a common drug in clinically use when it is able to be administered orally and for lower, more affordable cost.

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COMMENTARY

Advances and applications of enteroscopy for small bowel

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INTRODUCTION

Visual inspection of the entire small intestine is difficult and time-consuming, due to its anatomical location. While the usual techniques of endoscopy facilitate visualization of the most proximal and distal ends of the gut, only limited views of the terminal ileum (only about 5 cm to 25 cm) can be obtained by intubation of the ileocecal valve after total colonoscopy. The proximal jejunal mucosa (20 cm to 50 cm) can be inspected by standard colonoscopy or use of related modified instrumentation. Therefore, investigation of this region is dependent upon enteroclysis and angiography. The diagnostic efficacy of these procedures, however, is relatively low, and confirmative diagnosis has frequently been made only after laparotomy. Achievement of successful endoscopy for other parts of the gastrointestinal tract has allowed for further improvements to the enteroscopy technique. Since the 1970s, vigorous efforts have been made to produce three types of enteroscopes, namely the push type, sonde type, and ropeway type. The recent development of video endoscopes, including a new prototype video enteroscope for small bowel, has benefited clinical practice. In this paper, we describe the recent advances in enteroscopy, including its devices and applications.

ENTEROSCOPE INSERTION TECHNIQUE FOR THE DIFFERENT DEVICE TYPES

Push type enteroscope[1-4]

Push type enteroscopes, such as the SIF-RP, SIF-B and SIF-10, are long forward-viewing fiberscope devices. At present, SIF-10 is the most widely used for examination of the upper small intestine[1-5]. The insertion technique for the push type enteroscopes can be described as follows: The patient should fast for at least for 12 h prior to the procedure. On the procedure day, a local anesthesia (such as lidocaine) is sprayed over the patient's pharynx. The patient is placed on the fluoroscopic table in the left lateral decubitus position. The enteroscope is swiftly introduced into the duodenum, with minimal distention of the stomach. As the instrument is advanced, the patient is moved into various positions to assist in endoscope passage; meanwhile, abdominal pressure helps to minimize or prevent loop formation in the stomach and, simultaneously, withdrawal of the enteroscope can also straighten the formed loop and allow for paradoxical advancement of the instrument tip. When the entire length of the insertion tube is passed, glucagon (1.0 mg) should be administered to inhibit peristalsis. Full insufflation of the bowel with air reduces the height of the intestinal folds. The endoscope may be withdrawn slowly as the bowel is carefully inspected. Usually, endoscopists are able to advance the instrument to an average depth of 35 cm to 60 cm beyond the ligament of Treitz with a mean 50 min procedure time; biopsy is also possible at this step. However, the extent of insertion is limited to the proximal jejunum at 20 cm to 60 cm due to the crooked nature of the duodenum, which is embedded in the retroperitoneum and which makes the transmission of propelling force difficult. As a result, a large loop tends to form in the stomach, consuming the effective length of the scope. In order to overcome this problem, the new push type enteroscope SIF-10L was developed. This type of scope is equipped with a stiffening tube that is specially made as an auxiliary device. Shimizu *et al*[6] described the insertion technique for the SIF-10L. When the scope is advanced to the duodenojejunal junction and its position has been confirmed by fluoroscopy, the distal tip is maximally flexed for fixation at the duodenojejunal junction and the scope can be pulled until it is straight from the descending portion of the duodenum. After that, the stiffening tube, which was lubricated prior to the procedure, is gently advanced over the shaft into the descending portion of the duodenum. When the insertion of the sliding tube is completed, it is fixed on the mouthpiece by the stopper to protect against its being pulled out. The scope is then pushed into the deeper part of the jejunum with concurrent observation of the intestinal lumen. When the maximal working length is achieved, the distal tip is again fixed and the sliding tube is gently advanced beyond the duodenojejunal junction to prevent loop formation in the horizontal portion of the duodenum. The passage of the stiffening tube is observed by fluoroscopy. At this point, the scope is again advanced to its full working length. Insertion extent is determined by measuring the length of the scope from the distal

end of the sliding tube, with measurements made on an X-ray film. It has been reported that the time for insertion can range from 7 min to 30 min, with a mean of 11.7 min and the range of observable length being 60 cm to 120 cm from the distal end of the stiffening tube which could be inserted beyond the duodenojejunal junction, at a mean insertion length of 105.8 ± 17.5 cm. By introducing the stiffening tube as an auxiliary device, the insertion extent can be increased remarkably.

Sonde type enteroscope

Several types of sonde scopes are currently available and in clinical use[7-11]. The sonde type small intestinal fiberscope (SSIF-VII) is one among the series of prototype fiberscopes; its features include a 5 mm diameter, 2790 mm length, forward-viewing capacity and 90-degree angle view. It can be passed transnasally and migrates distally, responding to peristaltic activity. The essential step of the procedure is a technique called "piggy backing" in which a long endoscope is introduced into the stomach carrying the enteroscope through the pylorus and beyond the ligament of Treitz, which is facilitated by grasping with forceps and pulling into place. The balloon at the tip of the instrument advances the enteroscope in the small bowel when the endoscope is removed. Metoclopramide is then administered to stimulate small bowel motility. Mucosal inspection is performed when the scope has reached the distal ileum. Glucagon is administered to arrest small bowel activity. The whole procedure lasts 3-8 h, with an average duration of 4.3-6 h[7-9]. However, it is estimated that only approximately 50% of the lumen view is visible during the controlled withdrawal due to the absence of tip deflection, the relatively small angle of view, and occasional rapid withdrawal of the instrument through loops of small intestine or migration to the colon. The another disadvantage of this scope and its application technique is that biopsy is impossible. The newest sonde type enteroscope (SSIF-type 10) that has been developed features a 2 mm biopsy channel, which makes target biopsy possible even in distal parts of the small intestine[9-10]. Finally, a new prototype video enteroscope (ESI-2000, Pentax) has been developed and successfully applied in clinic[11-13].

Ropeway type enteroscope[15-19]

Several types of ropeway enteroscopes, such as FIS-II, SIE-RP and SIE-2C, are currently available. The key step for application of the ropeway enteroscopes, during the examination, is insertion of a long intestinal teflon string that is advanced perorally and discharged from the anus. Completion of this step, however, may require at least 24 h. Once this step is finished, the ropeway enteroscope can be pulled through the gastrointestinal tract with the aid of the Teflon string relatively quickly (within 10 min). Observation and biopsy throughout the entire intestine are possible. Use of this method causes the intestine to shorten, like an accordion, which precludes complete observation; moreover, stenotic and diffuse lesions in the small bowel disallow free passage of the string and limit the effectiveness of this device. Finally, excessive traction on the string augments the risk of instrument-induced perforation. From a physical standpoint, this procedure may cause discomfort to the patient and requires general anesthesia. Thus, application of ropeway enteroscope is limited in clinical practice.

APPLICATIONS IN SMALL BOWEL DISEASES

Obscure gastrointestinal bleeding

Obscure gastrointestinal bleeding is a troublesome medical problem. Although a wide range of investigational tools are available to aid clinicians in localizing sites of gastrointestinal blood loss, approximately 5% of the patients with GI bleeding continue to bleed from an unidentified source. In recent years, some authors have reported their experiences in use of small bowel enteroscope for detecting the source and cause of obscure bleeding. For nearly 26%-77% of those patients, correct diagnosis or identification of the site of occult bleeding was achieved[2,20-22]. Moreover, these results indicated that the following specific lesions could cause obscure bleeding: arteriovenous malformations (AVMs), hemangiomas, Meckel's diverticulum, jejunal diverticulum, jejunal leiomyoma, leiomyosarcoma, ulcerative jejunitis, Peutz-Jeghers syndrome, and Crohn's disease. Of the known causes of obscure gastrointestinal bleeding, AVMs appear to be the most common source. Lewis and Waye[7] employed the SSIF-VII sonde type enteroscope to investigate the cause of chronic gastrointestinal obscure bleeding in 60 patients and found that 33% (n = 20) had blood loss within the small bowel, with 80% (n = 16) of those being caused by AVMs and 15% (n = 3) by ulcers; for only 1 of the 20 patients with small bowel blood loss was the bleeding foci not found, despite the presence of fresh blood. AVMs were limited to the proximal jejunum in 7 patients, distal small intestine in 3 patients, and diffuse small bowel in 6 patients. Foutch *et al*[2] described a group of patients with gastrointestinal bleeding of obscure origin and reported finding a definite cause of bleeding for 15 cases by using the push type enteroscope, 12 of which were diagnosed as AVMs. Similar results reported by another group support the general finding that AVMs are the main cause of obscure gastrointestinal bleeding[20].

Detection of small bowel tumor

Small bowel tumors are difficult to detect using conventional diagnostic techniques. Imaging examinations, such as barium X-ray, ultrasonography and angiography, often produce false negative test results, which can reportedly delay diagnosis for 12 mo or more in nearly one-quarter of patients with primary small bowel neoplasms[23]. At present, enteroscopy is considered to play a precise role in the diagnosis of small bowel disease. Kawai and colleagues published their 11-year experience of detecting and identifying lesions using three types of enteroscope[16], in which a total of 19 lesions were detected and identified as cancer (n = 3), malignant lymphoma (n = 5), leiomyosarcoma (n = 1), leiomyoma (n = 5), hemangiopericytoma (n = 1) and Peutz-Jeghers polyp (n = 5). The authors described the endoscopic appearances of the tumors, which were classified among three types: type Ⅰ, sessile tumor; type Ⅱ, semipedunculated or pedunculated tumor; type Ⅲ, large tumor with central depression. Type Ⅲ was further classified into two subgroups: type Ⅲa, tumors partially encirculating the bowel; type Ⅲb, tumors completely encirculating the bowel. Malignant tumors showed various appearance ranging from type Ⅱ to type Ⅲb, but benign tumors never showed type Ⅲb. Shinya and Mc Sherry[19] also presented their experience with push type and sonde type enteroscopes, and reported that among the 3 cases total 2 were diagnosed as duodenal adenocarcinoma and one as a jejunal hemangiolymphangioma.

Application in familial adenomatosis coli (FAC)

Upper gastrointestinal lesions have been considered rare in patients with FAC or Gardner's syndrome, although recent studies have indicated that FAC and Gardner's syndrome are the same disease and affect the upper as well as the lower gastrointestinal tract[24-26]. Iida *et al*[25] used the push type enteroscope and found that jejunal polypoid lesions were detected in 9 of 10 patients (90%) with FAC or Gardner's syndrome. The lesions were located in the upper jejunum, particularly within about 20 cm below the duodenojejunal junction. The number of polyps varied from 1 to 20, and their sizes were 3 mm or less in diameter. Endoscopically, the polypoid lesions appeared sessile and whitish. Histologic findings of the lesion biopsy specimens revealed a tubular adenoma with mild to moderate epithelial atypia in all 9 cases. Other authors reported 3 cases of jejunal carcinoma in patients with FAC/Gardner's syndrome[28,29]. However, such a situation is quite rare, since the jejunal lesions are usually very small, asymptomatic, and adenomatous in histology. Therefore, as treating physicians we should pay attention to related changes and careful follow-up of patients is needed with endoscopic removal of larger polyps, whenever feasible.

Application in other small bowel diseases

Correct diagnosis of small intestine diseases requires not only intestinal visualization but also biopsy of lesions. Barkin *et al*[4] examined 31 patients with clinically suspected small bowel disease using a PCF-135 flexible pediatric colonoscope perorally and 14 patients were confirmed by biopsy, including celiac disease (n = 5), Whipple's disease (n = 2), lymphoma (n = 1), adenocarcinoma (n

= 1), Crohn's disease (*n* = 1), tropical sprue (*n* = 1), amyloidosis (*n* = 1), enteritis (*n* = 1) and cytomegalovirus infiltration (*n* = 1). Such diseases as Whipple's disease, tropical sprue and amyloidosis could not be diagnosed if biopsies were not available. Tada *et al* examined 15 cases of Crohn's disease using the sonde type enteroscope, and the presence of small intestinal lesions was confirmed in 13 cases, while the small intestine was not involved in the remaining 2. The characteristic findings of Crohn's disease were observed, including longitudinal ulcers, circular ulcers, irregular ulcers, aphthoid ulcers, cobble-stone appearance, inflammatory polyps, pseudo-diverticular formation, and stenosis. The author considered that the enteroscope device was superior to X-ray instrumentation for detecting tiny lesions, and that enteroscopy plays an important role in diagnosis of small bowel disease; further developments and improvements in enteroscopy will support its more widespread use clinically. Different types of enteroscopes have different characteristics, for instance, the push type enteroscope is suitable to detect a lesion on the upper jejunum or obtain a biopsy in the case of a diffusely spreading lesion. The ropeway type enteroscope is helpful for observation of the entire intestine, whereas the sonde type is useful for the examination of emergency cases of gastrointestinal bleeding, in cases of poor general condition, or when a stenosing lesion is located at the distal parts of the small intestine where neither the push type scope nor the ropeway type scope can be introduced. Thus, we can observe small intestinal diseases sufficiently when the suitable enteroscope is employed. However, the questions remain as to how to best utilize the various enteroscopes for pathophysiological research of the small intestine and how to improve the insertion techniques so as to make it easier in technical operation and for patient tolerance.

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ORIGINAL RESEARCH

Clinical significance of CD44v mRNA detection by PCR in peripheral blood of patients with hepatocellular carcinoma

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Abstract

AIM: To study the clinical significance of detecting the expression of CD44v mRNA in the blood of patients with hepatocellular carcinoma (HCC).

METHODS: The expression of CD44v mRNA was detected in blood with RT and diploid PCR, and the clinical significance was discussed based on the result of pathological examination and follow-up.

RESULTS: CD44v mRNA was detected in the blood of 10/15 patients, giving a positive rate of 66.67%. In the 13 patients who showed response in the follow up period, the CD44v mRNA expression was positive in 9 and negative in 4. Recurrence rate was higher in the patients with positive CD44v mRNA expression than in those with negative CD44v mRNA expression, and the clinical pathological indices were also higher in the former than in the latter.

CONCLUSION: Detection of the expression level of CD44v mRNA in blood of the patients with HCC can be used as an adjuvant means for differential diagnosis, prediction and monitoring of HCC recurrence.

Key words: CD44; Liver neoplasms; mRNA; Hepatocellular carcinoma; Polymerase chain reaction

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INTRODUCTION

CD44 is an adhesive molecule located on the cell surface, where it participates in cell-cell and cell-matrix interactions[1,2]. "Standard" CD44 can be modified by the insertion of transcripts from at least five extra exons[3], after which splice variants of CD44 (CD44v) can be produced. A single cell may contain two or more variant mRNAs from the same gene simultaneously. CD44v may play an important role in tumor growth and metastasis. The human homologue of rat metastasis-associated CD44v has been isolated from the cell lines of human non-small cell lung carcinoma, colon carcinoma, and breast carcinoma[4]. Furthermore, CD44v expression has been detected in fresh human tumor tissues of the breast, colon[7], and gastrointestinal tract[8], as well as in melanoma[9] and metastases in brain[10].

Gene expression of a single tumor cell in 10^7 white blood cells can be detected by sensitive RT-PCR[7]. Thus, it is possible to detect the gene expression of a free tumor cell in the blood of patients with malignant diseases. In the study described herein, we explored an auxiliary method for the diagnosis and monitoring of recurrence of malignant tumors by detecting CC44v mRNA expression in the peripheral blood of patients with hepatocellular carcinoma (HCC).

MATERIALS AND METHODS

Experimental specimens

On a Ficoll-Hypaque gradient, free tumor cells were collected from blood samples obtained from the peripheral vein of 15 patients with HCC prior to surgical intervention. Meanwhile, tumor tissue samples were obtained from the resected HCC specimens and control blood samples were obtained from 16 patients with benign diseases (including chronic cholecystitis, chronic abscess of liver, chronic hepatitis B, cirrhosis, inflammatory pseudotumor of liver, and cavernous hemangioma of liver). All samples were stored at -196 ℃ and kept frozen until use.

RNA isolation and RT-PCR

RNA was extracted from the free tumor cells and the tumor tissue samples according to the method of Chomzynski and Sacchi[11]. RT was carried out on the total RNA sample using an AMV reverse transcriptase kit (Promega Co., USA). First-strand cDNA was synthesized at 42 ℃ for 60 min in 20 μL reaction solution. The first round of PCR was carried out with the synthesized cDNA in 50 μL reaction solution (upstream primer: 5'-GACAGACACCTCAGTTTTTCTGGA-3'; downstream primer: 5'- TTCCTTCGTGTGTGGGTAATGAGA-3') for 30 cycles. The conditions of PCR were 94 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 1.5 min. The second round of PCR was carried out under the same conditions as the first round (upstream primer: 5'-GACAGACACCTCAGTTTTTCTGGA-3'; downstream primer: 5'-TTCCTTCGTGTGTGGGTAATGAGA-3') fro 30 cycles. PCR products

Table 1 Follow-up data of 15 detected patients with primary liver cancer

CD44vmRNA in blood	Detected, *n*	Positive AFP	Followed up, *n*	Responders, *n*	Recurrence, *n*	Recurrence rate, %	Survivors, *n*	Survival rate, %
+	10	8	10	9	9	100.00	1	11.11
-	5	3	5	4	1	25.00	3	75.00
Total	15	11	15	13	10	76.92	4	30.77

Table 2 Clinicopathological data of the patients who responded during follow-up

CD44v MRNA in blood	Followed up, *n*	Responders, *n*	DTC	PTC	TEPV	DTN
+	10	9	8	2	9	7
-	5	4	1	1	2	1
Total	15	13	9	3	11	8

Note: DTC: deficiency of tumor capsule; PTC: penetration of tumor capsule; TEPV: tumor embolus in portal vein observed by microscopy; DTN: daughter tumor nodules observed by microscopy. $\chi^2_{DTC} = 5.7778$, $P < 0.025$; $\chi^2_{PTC} = 0.0164$, $P > 0.9$; $\chi^2_{TEPV} = 5.3182$, $P < 0.025$; $\chi^2_{DTN} = 4.9661$, $P < 0.05$.

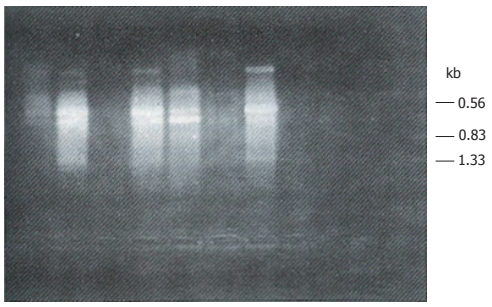

Figure 1 Electropherogram of PCR products.

(20 μL) were separated on an ethidium bromide-stained 1.5% agarose gel and analyzed.

Pathological indices for metastasis

All tumor samples were observed under microscopy to determine whether the tumor capsule was intact or deficient, whether it had been penetrated by tumor cells, whether tumor cells had migrated into the portal vein of normal liver tissues, and whether daughter tumor nodules appeared in normal liver tissues near the main mass. Follow-up after tumor resection was carried out for 20-22 mo and was conducted by questionnaire.

Statistical analysis

Statistical analysis was carried out using the chi-square test.

RESULTS

All the tumor tissue samples and 10 of the 15 blood samples obtained from the patients with HCC expressed CD44v mRNA. No CD44v mRNA expression was detected in the control blood samples (Tables 1 and 2, Figure 1).

In the patients with positive CD44v mRNA expression in blood, the recurrence rate was higher and the survival rate was lower than in the patients with negative CD44v mRNA expression ($\chi^2 = 8.775$, $P < 0.005$ and $\chi^2 = 5.7778$, $P < 0.025$).

DISCUSSION

Free tumor cells can appear in the peripheral vein of patients with malignant tumors of the digestive system[12]. Tumor cells can enter into the vein through the short path involving the microartery vein and elastic blood capillaries[13]. Our study has shown that liver cancer cells may enter into the peripheral vein *via* 1) collateral circulation of the portal caval vein, or 2) liver vein → inferior caval vein → capillary of lung → artery → blood capillary of tissue → peripheral vein of body.

It is possible to detect the CD44v mRNA expression of free tumor cells in blood by RT-PCR. This method may be an auxiliary indicator for diagnosis of malignant tumors. CD44v can confer metastatic potential on tumor cells, which was supported by the findings of our study[14]. In this study, the metastatic data were more robust in patients with positive CD44v mRNA expression in blood than in patients with negative CD44v mRNA expression; additionally, the recurrence rate was higher and the survival rate was lower in the former than in the latter. Thus, CD44v expression in blood may indicate metastatic potential of cancer and become an auxiliary means for monitoring recurrence.

Although detection of alpha-fetoprotein (AFP) is considered the most effective method for HCC diagnosis, there are still about 10% to 30% of patients with HCC who present with negativity for AFP. In the current study, we found that the CD44v mRNA expression in blood of patients with HCC was not related to AFP, suggesting that this method can be a complementary indicator for HCC diagnosis.

However, CD44v mRNA expression in blood cannot be a specific diagnostic index for primary hepatic carcinoma because the specificity of CD44v in different tumors was not clear. Yet, it may be an auxiliary method for differential diagnosis of malignant and benign diseases (*i.e.*, HCC and non-typical abscess of liver, inflammatory pseudotumor of liver, *etc.*).

The reasons for CD44v mRNA expression being positive in only 10 of 15 blood samples of HCC in the current study, but being positive in all of the 15 tumor tissue samples, may be as follows: (1) none of tumor cells were collected from blood or there may have been no free tumor cells in the blood; (2) the experimental method used was not optimal or sufficient; and (3) the expression level of CD44v mRNA in tumors was low. Therefore, the detection method for CD44v mRNA expression in blood should be further improved.

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ORIGINAL RESEARCH

Analysis of two constitutive forms of microsomal heme oxygenase in different rat tissues

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Abstract

AIM: To isolate and purify the heme oxygenase (HO) isoform in microsomal fractions of Sprague-Dawley rat liver and brain in order to understand the characteristics of the two constitutive forms and the mechanism of the occurrence of hyperbilirubinemia.

METHODS: After induction by hematin and phenylhydrazine, the rat liver and brain microsomal fractions were isolated and purified by DEAE-Sephacel and hydroxyapatite. Activity and the apparent molecular weight of the two isoforms [heme oxygenase 1 (HO-1) and heme oxygenase-2 (HO-2)] were measured. Kunming mice were used to prepare antiserum against purified liver HO-2. Rat liver HO-1 and brain HO-2 preparations were analyzed by the western immunoblotting technique.

RESULTS: Two isoforms were purified and identified in the treated rat liver, and HO-1 was the predominant form with a ratio of 2:1. In the native state, HO-2 activity was detectable but HO-1 activity was increased in response to hematin and phenylhydrazine, while HO-2 activity was fully refractory to these agents. The apparent molecular weights of HO-1 and HO-2 were about Mr 30000 and Mr 36000 under reducing conditions, respectively. In the untreated liver and treated brain, only one peak of HO activity exhibiting an elution profile similar to that of HO-2 of the treated liver was detected. The presence of an activity peak was not found in the elution profile at the region where the inducible isoform of HO (HO-1) was expected. The apparent molecular weight in treated brain preparation was identical to that of the purified liver HO-2. Cross-reactivity of HO-2 in the brain microsomal preparation was established, but a reactivity of HO-1 in the liver was not observed by western immunoblotting analysis when antiserum to liver HO-2 was applied.

CONCLUSION: Two constitutive forms of HO, designated as HO-1 and HO-2, exist in the treated rat liver. HO-1 is an inducible enzyme. In the treated rat brain only HO-2 exists and is a molecular entity similar to that found in liver. The two constitutive forms were different in molecular weight and in inducibility and immunochemical properties.

Key words: Heme oxygenase; Liver; Brain; Hyperbilirubinemia

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INTRODUCTION

Oxidative degradation of heme (protoheme, Fe protoporphyrin IX, heme b) to the open tetrapyrrole, biliverdin, is catalyzed by the microsomal enzymes heme oxygenase (HO) and NADPH-cytochrome C (P-450) reductase. Although the exact mechanism of heme degradation is not yet fully understood, it is generally accepted that the role of HO is to bind the substrate in a specific orientation, such that upon degradation the predominant end product is the IX-α isomer of biliverdin; the dual function of the reductase is to activate molecular oxygen and to promote the reduction of heme iron to the ferrous state. In mammals, the cytosolic enzyme, biliverdin reductase, catalyzes the conversion of biliverdin to bilirubin[1-6].The purpose of the present study was, therefore, to verify the possibility of the existence of multiple forms of HO in the rat liver and brain, and if existing to compare the properties of these enzymatic isoforms in order to understand their mechanisms of action.

MATERIALS AND METHODS

Materials and animals

Hematin was purchased from Sigma. Sodium cholate and DTT were purchased from Serva. Sprague-Dawley (S-D) rats (weight range: 180-220 g) and Kunming mice were purchased from the Shanghai Institute of Cell Biology, Academia Sinica.

Preparation of tissue

The S-D rats were given ad libitum access to food and water. Hypoxia was induced in the rats by exposure to a gas mixture of 5%-7% oxygen and 93%-97% nitrogen for 60 min to increase the

Table 1 Levels of sIL-2R, ALT, and HBV DNA in the sera of patients with chronic HBV infection (mean ± SD)

Purified fractions	Total protein, mg	Total activity, $\times 10^3$ U	Specific activity, U/mg	Recovery, %	Purification, -fold
HO-1					
Solubilized microsomes	82	68.6 (64.0-73.2)	837.5 (781.4-893.6)	100	1.0
DEAE-Sephacel	8.7	18.3 (16.6-20.0)	2097.3 (1902.9-2291.7)	10.7	2.5
Hydroxyapatite	1.2	5.1 (5.0-5.2)	4301.1 (3692.8-4909.3)	1.5	5.1
HO.2					
Solubilized microsomes	82	68.6 (64.0-73.2)	837.5 (781.4-893.6)	100	1.0
DEAE-Sephacel	4.9	7.7 (7.3-8.0)	1574 (1504.5-1643.6)	6.0	1.9
Hydroxyapatite	3.1	6.7 (5.3-8.0)	2146.3 (1708.5-2584.0)	3.8	2.6

blood brain barrier permeability, after which injections of 40 μmol/kg hematin (pH 7.4, intraperitoneally) and 100 mg/kg phenylhydrazine (intravenously) were given. The control animals (untreated, hypoxic) received saline injections. The animals were euthanized 20 h after treatment for analysis.

Resolution and isolation of HO isoforms

All operations were carried out below 4 ℃. The rat tissues were homogenized in 2.0 volumes of 20.0 mmol/L potassium phosphate buffer (pH 7.4) containing 0.1 mmol/L EDTA and 135.0 mmol/L KCl; the resulting homogenate was centrifuged at 10000 × *g* for 20 min. The supernatant fractions were collected and subsequently centrifuged at 150000 × *g* for 1 h. Washing, resuspension, and solubilization of the microsomes were performed according to Maines' method[1,4]. The solubilized microsomes were diluted with 1.0 volume of 0.05 mmol/L DTT solution (pH adjusted to 8.0) and loaded onto a DEAE-Sephacel column (2.5 cm × 13.6 cm) that was previously equilibrated with 20.0 mmol/L Tris HCl buffer (pH 7.5) containing 0.05 mmol/L EDTA, 0.5% Triton X-100, 0.1% sodium cholate and 0.05 mmol/L DTT. The column was eluted with concurrent linear gradients of KCl (0-0.4 mol/L) and Triton X-100 (0.5%-0.9%) prepared with equilibration buffer (75 mL). Fractions (3.0-4.0 mL) were collected and analyzed for HO activity.

Purification of HO-1 and HO-2

The pooled DEAE-Sephacel fractions containing the first and second peaks of HO activity were dialyzed against distilled water for 24 h at temperature below 4 ℃, and then concentrated and respectively loaded onto a hydroxyapatite column equilibrated with 10.0 mmol/L potassium phosphate buffer (pH 7.5). The column was washed with 10.0 mmol/L potassium phosphate buffer (pH 7.2) containing 0.05 mmol/L EDTA, 0.4% Triton X-100 and 0.1% sodium cholate, followed by the same solution supplemented with 20% glycerol. The column was eluted with a linear gradient of 10.0-260.0 mmol/L potassium phosphate in the above buffer, and the fractions containing HO activity were collected.

Preparation of biliverdin reductase from rat liver

Each rat liver sample was homogenized in 2.0 volumes of 0.1 mol/L potassium phosphate buffer (pH 7.4). The resulting homogenate was centrifuged at 9000 × *g* for 20 min. The supernatant containing biliverdin reductase was prepared by additional centrifugation of that supernatant fraction at 105000 × *g* for 1 h. The protein concentration of the supernatant fraction was adjusted to 10.0 mg/mL. Biliverdin reductase activity was determined by measuring the increase in spectra absorption due to the production of bilirubin.

Assay of HO isoforms activities

HO isoform activities were routinely determined according to the previously described methods in[1] and[4]. One unit of HO activity was defined as the amount of enzyme catalyzing the formation of 1 nmol of bilirubin in 1 h.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of HO isoforms

Electrophoretic procedures were performed according to Laemmli's method in order to identify HO isoforms.

Production of antiserum

The purified rat liver HO-2 fraction (69 μg) was mixed with 100 μL of 0.9% NaCl solution and injected intraperitoneally into Kunming mice (10-15 g). These mice were then boosted with antigen (69 μg) every second week, for a total of three times. The mice were bled at 10 days after the final booster and sera were collected and stored at -20 ℃.

Western blotting

Western blotting was performed as follows. Protein samples, rat liver HO-1 and rat brain HO-2 fractions, were subjected to SDS-PAGE in 1.5 mm slab gels according to the procedure of Laemmli. The separated polypeptides were then transferred electrophoretically from the gel to a nitrocellulose membrane using a transfer buffer consisting of 48 mmol/L Tris base, 39 mmol/L glycine, 20% (v/v) methanol and 0.037% (w/v) SDS (pH 8.3). Following electroblotting, the nitrocellulose blot was stained with Ponceau S for protein detection. Destaining was performed with distilled water. For immunostaining, the nitrocellulose blot was incubated at 37 ℃ for 1 h with bovine serum albumin in 20.0 mmol/L Tris-HCl buffer (pH 7.5) containing 0.5 mol/L sodium chloride, in order to saturate all protein binding sites. The nitrocellulose blot was then incubated with antiserum against rat liver HO-2 at 37 ℃ for 1 h, washed several times with PBS-Tween-20 solution and then incubated for 1 h at 37 ℃ with peroxidase anti-mouse IgG diluted with PBS solution. The blot was stained with diaminobenzidine (DAB) solution, washed with distilled water, and air-dried.

RESULTS

Purification and identification of the two forms of HO in two tissues

Rat liver microsomal fractions were loaded for DEAE-Sephacel chromatography, and 3.0-4.0 mL fractions were collected and analyzed for HO activity. Two peaks exhibiting HO activity were eluted (Figure 1). These peaks were designated as HO-1 and HO-2 according to the sequence of their elution from the column. The pooled DEAE-Sephacel fractions containing HO-1 and HO-2 activities were loaded onto hydroxyapatite column, and the final purified HO-1 and HO-2 preparations exhibited only single bands, respectively, when visualized by staining with Coomassie blue following SDS-PAGE. The final HO-1 and HO-2 preparations showed specific activities of up to 4300 nmol of bilirubin/mg of protein·h and 2150 nmol of bilirubin/mg of protein·h, respectively (Table 1).

The treated rat brain and untreated rat liver microsomal fractions were also solubilized and respectively subjected to ion exchange chromatography of DEAE-Sephacel. The chromatographic elution patterns of HO activity were compared with those of treated rat liver. In the treated rat brain and untreated rat liver, only one peak of HO activity was detected. An activity peak was not present in the eluate corresponding to the region where the inducible isoform of HO, HO-1, was detected (Figure 1). Tables 2 and 3 show the procedures and the activity for purification of treated rat brain HO-2

Table 2 Purification of HO-2 from treated rat brain microsomal fractions

Purified fractions	Total protein, mg	Total activity, $\times 10^3$ U	Specific activity, U/mg	Recovery, %	Purification, -fold
HO-2					
Solubilized microsomes	56.0	24 (16.9-31.1)	428.7 (302.7-554.7)	100	1.0
DEAE-Sephacel	20.1	12.2 (10.3-14.1)	607.7 (512.0-703.4)	35.9	1.4
Hydroxyapatite	1.6	10.6 (10.0-11.2)	6478.1 (6102.4-6853.8)	2.9	15.1

Table 3 Purification of HO-2 from intact rat liver microsomal fractions

Purified fractions	Total protein, mg	Total activity, $\times 10^3$ U	Specific activity, U/mg	Recovery, %	Purification, -fold
HO-2					
Solubilized microsomes	662.7	394.7 (351.8-437.6)	595.6 (530.9-660.3)	100	1
DEAE-Sephacel	10.2	16 (13.8-18.2)	1574.4 (1358.7-1789.5)	1.5	2.6
Hydroxyapatite	2.4	8.2 (7.3-9.0)	3381.6 (3020.1-3743.2)	0.4	5.7

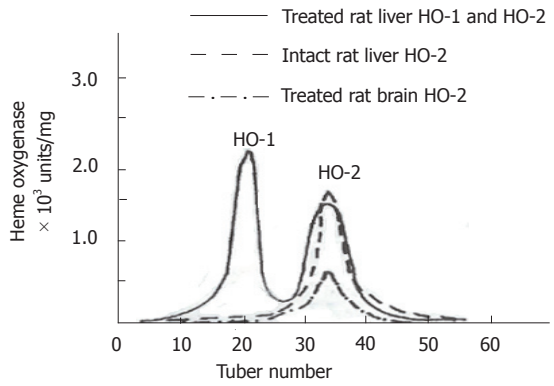

Figure 1 DEAE-Sephacel chromatography of rat liver and brain solubilized microsomes. Solubilized microsomal fractions were loaded onto a DEAE-Sephacel column (2.5 cm × 13.6 cm) equilibrated with 20.0 mmol/L Tris-HCl buffer (pH 7.5) containing 0.05 mmol/L EDTA, 0.5% Triton X-100, 0.1% sodium cholate, and 0.05 mmol/L DTT. The column was eluted with concurrent linear gradients of KCl (0-0.4 mol/L) and Triton X-100 (0.5%-0.9%) in the same buffer. The fractions (3.0-4.0 mL) were collected and analyzed for enzyme activities.

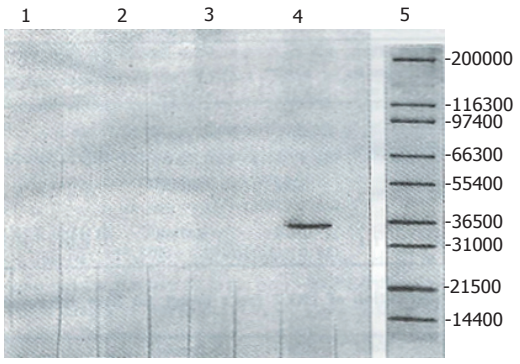

Figure 2 Western immunoblot of rat liver HO-1 and brain HO-2 preparations. Preparations of HO-1 and HO-2 were subjected to SDS-polyacrylamide slab gel electrophoresis, electroblotted onto nitrocellulose membranes and visualized as described in the "experimental procedures". 1 and 3, controls; 2, liver HO-1; 4, brain HO-2; 5, molecular weight markers.

and untreated liver HO-2, respectively.

Characteristics of HO-1 and HO-2

Two forms of HO from the treated rat liver were subjected to electrophoresis and two distinct bands exhibiting HO activity were detected, with the migration patterns of HO-1 and HO-2 being different. This differential separation pattern suggested the presence of distinct molecular properties associated with HO-1 and HO-2. The purified HO-2 after SDS-PAGE treatment displayed a higher monomeric molecular weight. The apparent molecular weights for HO-1 and HO-2 were Mr 30000 and Mr 36000, respectively. The apparent molecular weight in treated brain microsomal preparation was identical to the purified liver HO-2 (Mr 36000). The HO-1 activity was increased in response to hematin and phenylhydrazine, while that of HO-2 was fully refractory to these agents. The blot was treated with anti-rat liver HO-2 serum followed by anti-mouse IgG-peroxidase conjugate and then stained for peroxidase activity. As expected, rat brain HO-2 preparation gave a reddish brown band in the region of molecular weight Mr 36000. However, the rat HO-1 preparation failed to show a stained band (Figure 2).

DISCUSSION

We have purified and identified two constitutive forms of HO from rat liver and brain, and provided evidence for decidedly different characteristics of the two forms. These two forms are named HO-1 and HO-2. The results indicate that in the liver of treated rats, there exist two different molecular types of HO. It appears that HO-1 and HO-2 substantially differ in their molecular composition and structure as indicated by their chromatographic behavior and electrophoretic migration pattern. Differences in amino acid composition or sequence usually result in such observations. It is rather curious that in the untreated rat liver only the HO-2 isoform exists, and the HO-1 form of the enzyme could not be detected. This finding suggests that the amount of the HO-1 form is too low to be detected. The present findings also show that the activity of HO-1 in the treated rat liver apparently surpasses that of HO-2, and the HO-2 activity is almost refractory, indicating that only the activity of HO-1 is inducible.

With the same procedure, only HO-2 could be clearly detected in the brain, and the HO-1 form was absent, after the brain barrier permeability had been increased by hypoxia in order to induce the brain microsomal HO isoforms with hematin and phenylhydrazine. This finding, however, does not suggest that this isoform is absent in the organ; rather, it may reflect inability of the presently used procedures to detect the exceedingly low level of the isoform and expression of the HO-1 isoform in the brain being suppressed under various conditions. Surprisingly, the brain displayed a higher level of HO-2 activity than the liver, possibly reflecting a major biological adaptation[2]. HO-1 and HO-2 preparations were analyzed by the western immunoblotting technique. As expected, the rat brain HO-2 preparation exhibited immunological reactivity with antibody to rat liver HO-2, but the rat HO-1 preparation did not, indicating that these two HO preparations are antigenically different.

The present studies further suggested that the liver and brain HO-2 are similar protein entities, according to the following criteria: (1) similarity in molecular weight; (2) cross-reactivity with antiserum to rat liver HO-2; and (3) similarity in chromatographic behavior on a DEAE-Sephacel column.

Collectively, this study showed that the two constitutive forms differ in molecular weight and in inducibility and immunochemical properties and that these differences are highly important for understanding the occurrence of hyperbilirubinemia, particularly in the premature newborn.

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ORIGINAL RESEARCH

Effects of endotoxin on expression of *ras*, *p53* and *bcl-2* oncoprotein in hepatocarcinogenesis induced by thioacetamide in rats

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Abstract

AIM: To evaluate the relationship between expression of *ras*, *p53* and *bcl-2* gene products and hepatocarcinogenesis since the endotoxemia produced from lipopolysaccharide administration and/or the hypophagocytic state of splenectomy significantly accelerated hepatocarcinogenesis induced by thioacetamide.

METHODS: The hepatocarcinoma model was induced by 6-mo oral intake of 0.03% thioacetamide. During the hepatocarcinoma modeling process, rats were additionally treated with splenectomy and/or lipopolysaccharide administration. The techniques of flow cytometry, immunohistochemistry and immunoelectronmicroscopy were applied for quantitative analysis of the expression of oncogene proteins.

RESULTS: In this model system, overexpression of ras p21 protein mainly occurred in the precancerous cell population or in cells in the early stage of hepatocyte transformation. The levels of ras p21 declined when nuclear DNA aneuploidy increased. Expression of bcl-2 protein slowly and steadily rose, with more hepatocytes staying in S+G2M phases, as the hepatocarcinoma became more malignant. p53 was moderately expressed during hepatocarcinogenesis. There was no statistical correlation between endotoxemia levels and the changes in levels of *ras*, *p53* and *bcl-2* gene products.

CONCLUSION: Overexpression of oncogene ras p21 was considered likely to be a precursor of premalignant hepatocytes and possibly as responsible for the initiation of hepatocarcinogenesis. Bcl-2 protein expression is proportional to the severity of malignancy in hepatocarcinogenesis. p53 may be involved in a key pathway underlying the transformation and development processes of hepatocarcinoma. This study confirmed the hypothesis that there are multiple genes and multiple steps involved in hepatocarcinogenesis. Expression of oncogene proteins reflects the properties of the premalignant and malignant cells, but is not directly related to endotoxemia statistically.

Key words: Genes, ras; Genes, p53; Oncogene proteins; Gene expression; Liver neoplasms; Thioacetamide

Yang JM, Han DW, Liang QC, Zhao JL, Hao SY, Ma XH, Zhao YC. Effects of endotoxin on expression of *ras, p53* and *bcl-2* oncoprotein in hepatocarcinogenesis induced by thioacetamide in rats. *World J Gastroenterol* 1997; 3(4): 213-217 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/213.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.213

INTRODUCTION

Chemically-induced hepatocarcinogenesis in rats is widely used to assess the carcinogenic risk of chemicals to humans and to study the molecular pathogenesis in order to improve hepatoma prevention and treatment strategies for humans. Based on our previous observations that endotoxin can enhance hepatocarcinogenesis in rats induced by oral intake of thioacetamide (TAA), we hypothesized that endotoxin is responsible for, at least some of, the changes in levels of oncogenes and tumor suppressor genes that occur during the development of TAA-induced hepatocarcinoma.

A multitude of studies have provided evidence that members of the ras oncogene family (Ha-ras, Ki-ras and N-ras, activated by point mutation in codons 12, 13 and 61 respectively) are responsible for cell transformation[1]. NIH-373 cell transfection and immunohistochemical analysis, in particular, have yielded data that demonstrates the presence of a dominant activated N-ras gene in premalignant and malignant cells, which also appears to play a key role in the initiation of hepatocarcinogenesis[2-4].

Mutations in the p53 tumor suppressor gene are frequently detected in many human and animal cancers (approximately 50%), and studies of its consequent misexpression have provided clues to the etiology and molecular pathogenesis of neoplasia[5,6]. In addition to its role in neoplastic transformation, the p53 protein plays an important role in normal cell function and its encoding gene has been found to be highly conserved among vertebrates, allowing extrapolation of data from animal models[7].

The *bcl-2* gene was first discovered in non-Hodgkin's B-cell lymphomas and its presence in tumors is due to a *bcl-2* gene

Table 1 Oncogene ras p21 expression and DNA content in the various study groups

Group	*n*	FI, x ± s	% cells labeled, x ± s	Cases with positive ras p21 expression	DNA index, x ± s
Sham	5	1	17 ± 4	1	1
TAA control	6	1.29 ± 0.085	18 ± 5	6	1.15 ± 0.21
TAA + LPS	6	1.60 ± 0.071[a]	24 ± 7[a]	6	1.24 ± 0.25[a]
TAA + ST	6	1.52 ± 00105[a]	15 ± 3[b]	6	1.44 ± 0015[a, b]
TAA + ST + LPS	6	1.32 ± 0.061[b, c]	49 ± 9[a, b, c]	6	1.56 ± 0.07[a, b, c]
CBRH-7919 cells	1	1.54	42	1	1.34

[a]$P < 0.02$ *vs* TAA control group; [b]$P < 0.02$ *vs* TAA + LPS group; FI: fluorescence index; [c]$P < 0.02$ *vs* TAA + ST group.
CBRH-7919: rat hepatocarcinoma cell line; DNA index: ratio of mean nuclear DNA content of the treated rat to mean DNA content of normal rats.

translocation from 18q21 to cis-configuration. However, high levels of bcl-2 protein production in a variety of human solid tumors have been observed in the without translocation or alterations in the structure of the *bcl-2* gene[8]. It is generally accepted that the bcl-2 protein contributes to the process of neoplastic cell expansion by blocking normal physiological cell death[9]; in addition, gene transfer-mediated elevation in bcl-2 protein levels have been shown to render tumor cells relatively more resistant to induction of apoptosis by chemotherapeutic drugs[10]. Therefore, bcl-2 has been theorized to play a significant role in the origins of cancer and in its therapy.

Considering the important contributions of *ras*, *p53* and *bcl-2* genes to carcinogenesis, the aim of this study was to explore the relations between expression of oncoproteins and malignity or incidence of hepatocarcinoma using a TAA-treated model system coupled with splenectomy (ST) and/or endotoxin (lipopolysaccharide, LPS) treatment.

MATERIALS AND METHODS

Hepatoma models and treatment

The protocol course of TAA-induced modeling encompassed 4 mo for cirrhosis and 6 mo for liver tumor. Female Wistar rats (provided by the Experimental Animals Center of Shanxi Medical University), weighing 125 ± 9 g, were housed in wire-bottom cages under a 12 h light/dark cycle and fed with a balanced pet diet ad libitum. Animals were randomly assigned to the following five groups: sham group (n = 5), which was the untreated control group that received tap water ad libitum; TAA control group (n = 6), which was given 0.03% w/v TAA (purity > 99%; Shanghai Central Chemical Factory) in drinking water; TAA + ST group (n = 6), which was splenectomized 1 week before commencement of the experiment; for the final two groups, mice first treated as the TAA + LPS group (n = 6) or the TAA + ST + LPS group (n = 6) were given 0.8 mg LPS (*Escherichia coli* sterotype 055:B5; Sigma) in drinking water containing 0.03% TAA for the last 2 months of the modeling course. The sham group, without splenectomy, underwent midline laparotomy and spleen manipulation as the sham surgery procedure. All surgeries were carried out under light ether anesthesia and all procedures were performed under sterile conditions. At the end of the 6 mo modeling course, all rats were euthanized by over-anesthetization with ethyl ether and the liver was excised for the following preparations.

Immunofluorescence flow cytometry

Expression of oncogene proteins was quantitatively determined as described by Zuo *et al*[11]. Briefly, ~2 g of excised liver tissue was immediately minced in 0.05% collagenase (type IV; Sigma) and filtered through a 200 mesh stainless steel filter. A single-cell suspension was prepared and fixed in 70% ethanol. After washing with PBS, the cells were resuspended in a solution containing 0.1% Triton X-100 and 5% goat serum, in order to increase the permeability of the cell membrane and to block the non-specific binding sites of IgG. Antibodies, including pan ras (F132, a mouse monoclonal IgG_2b antibody), p53 (CM1, a rabbit polyclonal antibody) and bcl-2 (N19, a rabbit polyclonal antibody) were purchased from Santa Cruz Biotechnology Inc., United Kingdom. They were diluted at 1:100 and incubated with 10^5 cells respectively at 37 ℃ for 30 min. After washing twice, the cells were incubated with 100 μL of species-specific secondary-FITC-IgG (Lot 9609; Military Medical Academy) at 37 ℃ for 30 min. The negative control (omission of the primary antibody) and the positive control (the rat hepatocarcinoma cell line CBRH-7919, provided by Shanghai Institute of Cell Biology) were generated using the same procedure described above. For flow cytometric analysis, 10000 cells from each sample were passed through a 400 mesh filter and then quantitatively examined on the Fluorescence Activated Cell Sorter FACS-420 (Becton Dickinson, United States). Data were analyzed on an IBM PC-compatible computer with HP-300 Consort 30 software. The quantitative expression of oncogene product was calculated according to Morker *et al*[12], as follows:

$$\text{Fluorescence index (FI)} = \frac{\text{Mean channels of the treated rat - Mean channels of negative controls}}{\text{Mean channels of the sham group}}$$

The criteria for oncoprotein expression was as follows: FI value > 1, positive; FI ≤ 1, negative.

In addition, liver cells were stained with propidium iodide reagents (50 mg/L propidium iodide, 20 mg/L RNase, and 1% Triton X-100) at 4 ℃ for 30 min, and the nuclear DNA content was determined by flow cytometry.

Immunohistochemistry

Immunohistochemical staining was conducted using the streptavidin/peroxidase kit (SP-TM; Zymed, United States) and the biotin-streptavidin method[13] with slight modification. Briefly, all liver sections were fixed with neutral buffered 10% formalin and embedded in paraffin. The paraffin-embedded sections were deparaffinized through an alcohol series graded with distilled water. The resultant hydrated sections were then incubated in 0.3% hydrogen peroxide for 10 min to quench the endogenous peroxidase activity. Liver sections were then incubated in a salt buffer (pH 6.0) at 92-98 ℃ for 10 min to restore antigenicity, followed by incubation in 5% normal blocking serum for 20 min to suppress non-specific binding of IgG. The preparations were then incubated sequentially with primary antibodies (diluted 1:50 in PBS) at 4 ℃ for overnight, followed by sequential incubation with the biotinylated secondary antibodies (diluted 1:100 in 1% BSA-PBS) at 37 ℃ for 10 min and streptavidin horseradish peroxidase (diluted 1:100) at 37 ℃ for 20 min. The colored reaction product was developed with diaminobenzidine (DAB). The sections were lightly counterstained with hematoxylin. Immunostaining by replacing primary antibody with PBS was also conducted as a negative control.

Immunoelectronmicroscopy

Anti-mouse IgG/colloidal gold (10 nm) was obtained from Beijing Zhongshan Biotechnology Co. LTD. The liver ultrathin sections were sequentially incubated with pan ras p21 antibody and the secondary immunogold antibody using the procedures described by Yin *et al*[14].

Statistical analysis

All results are expressed as the x ± s. Data were analyzed by Student's *t*-test and multiple regression. P values < 0.05 were considered statistically significant.

RESULTS

Expression of oncogene ras p21 was quantitatively detected by flow cytometry. The fluorescence index indicated the potentiality of gene product expression. Table 1 shows that the TAA-treated group with 2-mo administration of LPS presented the highest expression of ras p21 despite their relatively low DNA content, reflecting the malignity of hepatocarcinoma to a great extent. However, the group with the

Table 2 p53 expression and hepatocarcinoma rate in the various study groups

Group	*n*	FI, x ± s	% cells labeled, x ± s	Cases with positive ras p21 expression	DNA index, x ± s
Sham	5	1	32	1	0
TAA control	6	1.15 ± 0.067	22 ± 12	6	17
TAA + LPS	6	1.32 ± 0.024[a]	20 ± 8.2	6	33
TAA + ST	6	1.22 ± 0.088[a]	25 ± 12	6	50
TAA + ST + LPS	6	1.29 ± 0.083[a]	30 ± 14	6	67
CBRH-7919 cells	1	1.35	9	1	

[a]$P < 0.01$ *vs* TAA control group; [b]$P < 0.05$ *vs* TAA + LPS group; [c]$P < 0.01$ *vs* TAA + ST group. CBRH-7919: rat hepatocarcinoma cell line.

Table 3 Expression of bcl-2 protein and percentage of cells in S and G_2M phases

Group	N	FI, x ± s	% cells labeled, x ± s	Cases with positive ras p21expression	DNA index, x ± s
Sham	5	1	23	1	25 ± 3.2
TAA control	6	0.96 ± 0.14	23 ± 6	4	33 ± 4.7
TAA + LPS	6	1.06 ± 0.07	37 ± 14	6	35 ± 4.5
TAA + ST	6	1.12 ± 0.11	28 ± 14	6	35 ± 7.7
TAA + ST + LPS	6	1.30 ± 0.09[a, b, c]	25 ± 6	6	45 ± 3.9[a, b, c]
CBRH-7919 cells	1	1.56	15	1	30

[a]$P < 0.05$ *vs* TAA control group; [b]$P < 0.05$ *vs* TAA + LPS group; [c]$P < 0.05$ *vs* TAA + ST group. CBRH-7919: rat hepatocarcinoma cell line.

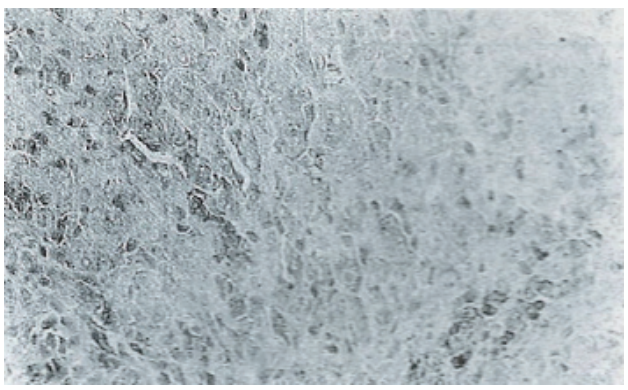

Figure 1 Immunostaining of a rat malignant nodule with anti-p21 monoclonal IgG2b antibody detected by the biotin-streptavidin method. Magnification × 10.

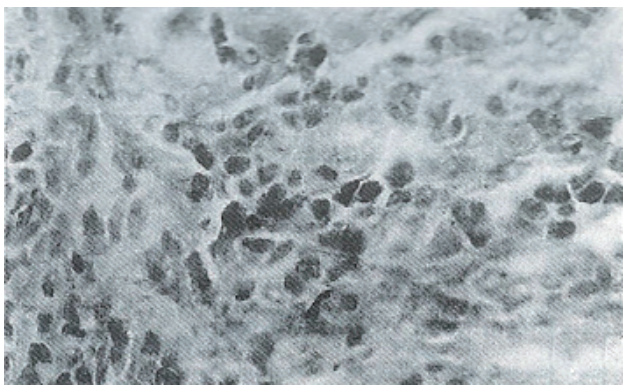

Figure 2 Immunostaining of a rat malignant foci with anti-p53 polyclonal antibody detected by the biotin-streptavidin method. Magnification × 40.

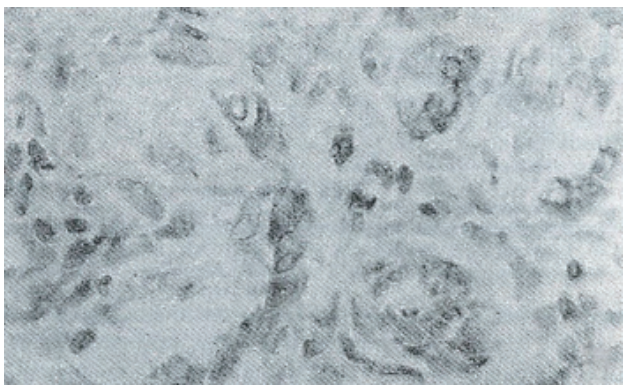

Figure 3 Immunostaining of a rat malignant foci with anti-bcl-2 polyclonal antibody detected by the biotin-streptavidin method. Magnification × 40.

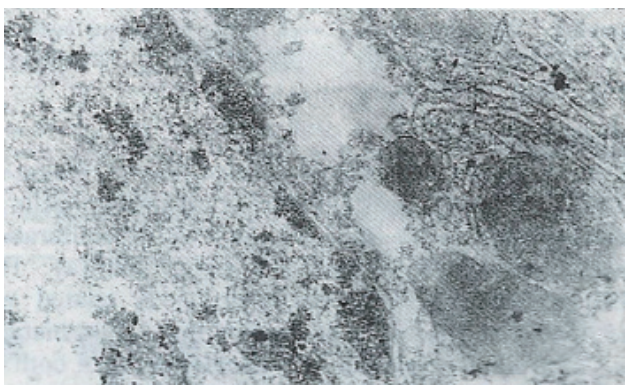

Figure 4 Immunostaining of ultrastructure of a rat normal hepatocyte with anti-p21 IgG/ colloidal gold (φ 10 nm) antibody. Magnification × 2000

highest DNA content had an average value of fluorescence index that was significantly decreased ($P < 0.01$) but a percentage of labeled cells that was obviously increased ($P < 0.01$).

Similar to the results of ras p21 expression, the flourescence index of the p53 tumor suppressor gene product was highest in the TAA + LPS group ($P < 0.01$) and relatively higher in the TAA + ST + LPS group ($P < 0.05$). However, the alteration in expression did not correlate with the hepatocarcinoma rate (Table 2).

Unlike the results of p53 and ras p21, the expression of the bcl-2 protein was consistently correlated with DNA index ($r = 0.93$, $P < 0.01$) and the percentage of cells in S plus G-2M phases ($r = 0.86$, $P < 0.05$). The rat hepatocarcinoma cell line CBRH-7919 was used as a positive control of expression of oncogene product when the samples of hepatocytes were analyzed by flow cytometry (Table 3).

Immunohistochemical analyses showed that immunostaining for the ras p21 oncogene was mainly localized to the cytoplasm in malignant cells and in dysplastic hepatocytes in the hyperplastic nodules. It was noticed that expression of ras p21 was more apparent in hepatocytes adjacent to neoplastic lesions than in hepatic tumor tissues (Figure 1). The hyperexpression of p53 was present in the nuclei of malignant hepatocytes (Figure 2), and bcl-2 protein was moderately expressed in some cytoplasm of neoplastic foci (Figure 3).

Immunoelectronmicroscopy showed that in contrast to the data presented in Figure 4, overexpression of ras p21 dominantly occurred on the increased nuclear heterochromatin of malignant hepatoma and secondarily on the endoplasmic reticulum adjacent to the nuclear membrane (Figure 5).

DISCUSSION

The proto-oncogene *ras* gene family (Ha-ras, Ki-ras and N-ras) commonly exists in normal mammalian cells and its products are involved in cell proliferation, metabolism, differentiation and various signaling pathways. Here, the mutation-activated *ras* gene

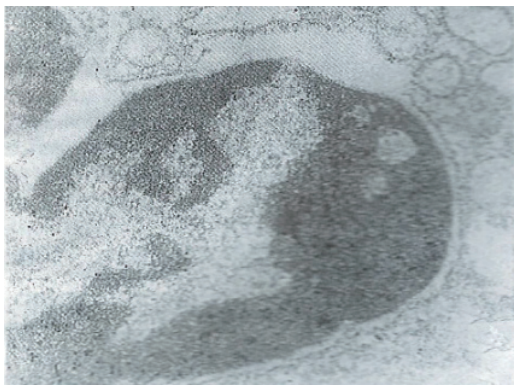

Figure 5 Immunostaining of ultrastructure of a rat malignant hepatocyte with anti-p21 IgG/ colloidal gold (φ 10 nm) antibody. Magnification × 2000.

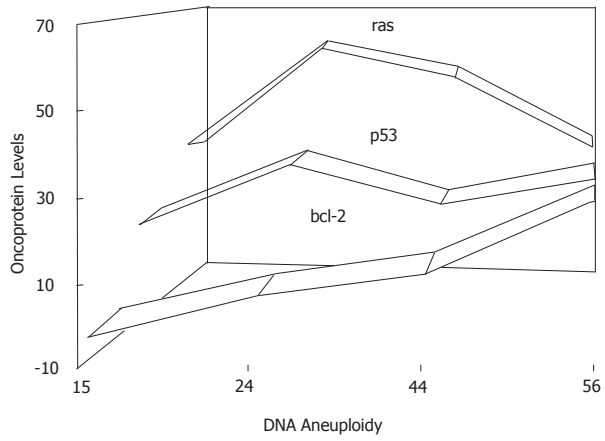

Figure 6 Relationship between hepatocarcinoma malignancy and expression of oncogene proteins detected by immunofluorencence flow cytometry.

was found to be associated with hepatocarcinogenesis. It has been reported that mutation of Ki-ras in codon 13 is present in liver angiosarcoma of humans as well as animals exposed to vinyl chloride[4]. Activated Ha-ras has also been detected in spontaneous hepatoma of the B6C3F1 mouse[15] and 2 amino 3 methyl-imidazo quinolone-induced hepatocarcinoma in rat[16]. However, the mutation-activated N-ras oncogene has been demonstrated frequently in hepatocarcinomas.

The p21 *ras* gene product plays a key role in cell transformation, as indicated by its strong expression in preneoplastic cells[17]; thus, ras p21 may be a biomarker for tumor diagnosis and prognosis. Our study, along with others[17,18], demonstrate oncogene ras p21 expression as a early event of hepatocarcinogenesis, since its expression levels do not correspond with nuclear DNA content. In contrast, we found that p21 expression declines when the DNA content is increased in the premalignant and malignant liver. Therefore, the inverse correlation between the levels of ras p21 production and the severity of liver cancerous lesions indicates that ras p21 is a potent trigger for transformation of the hepatocyte phenotype and it is not crucial for maintenance of the transformed cell phenotype. It is generally accepted that members of the *ras* gene family, especially N-ras, are "transforming genes" involved in initiation of carcinogenesis[1,2,17,18]. Therefore, although we observed a high content of p21 protein in the model system that occurred in response to additional administration of LPS purified from *E. coli*, it is questionable whether LPS itself induces overexpression of ras p21 or the property of preneoplastic hepatocytes. The increased heterochromatin in the malignant hepatocytes showed overexpression of ras p21, which may imply some mechanism of hepatocarcinogenesis.

The 17-y history of research into the p53 tumor suppressor gene has indicated that p53 protein is involved in gene transcription, DNA synthesis and repair, genomic plasticity, and programmed cell death. So it is not surprising that p53 is a functional component of signaling pathways central to human carcinogenesis. Deactivation of p53 causes loss of tumor suppressor function and gain of oncogenic activity, and this dynamic represents one of the explanations for pathogenesis of cell transformation. The genetic mutation of p53 and of some viral oncoproteins binding to the p53 protein can lead to deactivation of p53 functions; for example, aflatoxin B1 induces hepatocellular carcinoma by mutation at codon 249 of p53[19] and HBV X protein binding to the p53 protein mediates p53 inactivation and remains an important possible mechanism of primary hepatocellular carcinoma lacking a p53 mutation[20]. In addition, some small carcinogen-DNA adducts, such as O6 methylguanin, may cause DNA polymerase to misread the base pairing, and bulky DNA adducts may render the bases unreadable, thereby increasing errors during DNA replication[21].

The wild-type p53 protein exists in a very small quantity and has a very short half-life in the cell nucleus, and these two features complicate its detection by routine immunological methods. Missense mutations often increase the half-life and quantity of the p53 protein by 20-fold, so p53 overexpression is a surrogate marker for missense mutation. Previous studies have noted that alteration of the function of the p53 pathway (*via* mutation or epigenetic inactivation) are associated with aneuploidy and increased proliferative rates in ovarian, colorectal and gastric cancers, features which are also considered precursors for tumor progression and poor prognosis[22]; however, some authors have reported that changes in the levels of p53 protein are not related to tumor differentiation[23]. Our study showed that a high content of p53 protein, as detected by flow cytometry and immunohistochemistry, was present in the model system with LPS treatment or in the hyperplastic nodules and malignant liver lesions, but neither specifically related to endotoxemia levels nor to malignancy. These findings suggested that the overexpression of p53 protein was relatively associated with LPS challenge and to both the early and late events of hepatocarcinogenesis, but the precise mechanism remains unclear.

The bcl-2 proto-oncogene on chromosome 18 may normally exert a regulatory function towards avoidance of cell apoptosis (programmed cell death), mainly in tissues where apoptosis may represent a specific control mechanism of cell turnover. Bcl-2 protein localized in mitochondria, endoplasmic reticulum and nuclear membrane has been shown to contribute to neoplastic cell expansion by blocking programmed cell death[24]. Bcl-2 expression has been detected in hepatocarcinoma and other solid tumors but appears to be absent in normal and dysplastic hepatocytes[25]. In normal liver, the majority of hepatocytes exist in a state of proliferative quiescence (G0 phase), but can enter a cell renewal compartment upon appropriate stimulation[26]. Dysplastic hepatocytes and regenerating hepatocytes in cirrhotic nodules have failed to show bcl-2 protein expression, but some types of dysplasia represent a precancerous lesion[27]. Thus, bcl-2 expression seems to be a relatively late event in hepatocarcinogenesis. In our study, the data from flow cytometry analysis with an antibody specific for bcl-2 protein indicated that expression of bcl-2 protein is significantly correlated with malignity of hepatocarcinoma (r = 0.93, $P < 0.01$), which is in agreement with findings from other studies[25-27]. On the other hand, the bcl-2 protein was also found to be related to the percentage of hepatocytes in S and G2M phases (r = 0.86, $P < 0.05$). Therefore, inhibition of apoptosis may be one of the mechanisms of carcinogenesis as little is known about the intracellular mechanisms underlying programmed cell death. Unlike other oncogene proteins described in the literature so far, bcl-2 acts in both S and G2M transitions and the potential impact of bcl-2 functions seem to, in addition to protecting cells from apoptosis, play a more complex role in morphogenesis, differentiation and homeostasis.

It is generally accepted that there are multiple genes and steps involved in carcinogenesis. The quantitatively analyzed data obtained in the current study showing elevations of ras, p53, and bcl-2 proteins in a mass of normal, premalignant and malignant cells in rat liver, together with the immunohistochemstry data, confirm the hypothesis that ras mainly contributes to neoplastic transformation and bcl-2 to malignant progression, and with p53 contributing to several pathways during hepatocarcinogenesis. Although endotoxemia was found to be significantly correlated with the malignity or incidence of rat hepatocarcinoma, it was not directly related to the overexpression of ras, p53 or bcl-2 proteins (maximum: r = 0.78, $P > 0.05$). Thus, it is suggested

that expression of oncogene proteins may represent some of the properties of the hepatocarcinoma itself (Figure 6).

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ORIGINAL RESEARCH

Effects of somatostatin analog on splanchnic hemodynamics and plasma glucagon level in portal hypertensive rats

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Abstract

AIM: To investigate the effects of somatostatin analog on splanchnic hemodynamics and plasma glucagon level in portal hypertensive rats.

METHODS: Twenty-eight male Sprague-Dawley rats were equally divided into a intrahepatic portal hypertension (IHPH) model group (n = 14, established by injection of CCl_4) and a prehepatic portal hypertension (PHPH) model group (n = 14, established by stenosis of the portal vein). Animals in each group were subdivided into an octreotide treatment (injection) group and a control (normal saline injection) group. Seven age-matched unmodeled/untreated normal rats served as controls. The mean systemic arterial pressure (MSAP) and free portal venous pressure (FPP) were measured. The splanchnic blood flow was detected by injection of toad blood red cell labelled with ^{51}Cr and $^{125}I{\cdot}T_3$. The concentration of plasma glucagon was determined by radioimmunoassay.

RESULTS: All rats with portal hypertension showed significantly decreased splanchnic blood flow and FPP in response to octreotide treatment, as well as markedly increased splanchnic vascular and portal venous resistance. The octreotide treatment did not appear to significantly lower the plasma glucagon levels in either the peripheral or the portal veins.

CONCLUSION: Octreotide induces a decrease in splanchnic blood flow in rats with portal hypertension, and this effect results primarily from direct vasoconstriction and to a lesser extent from decreased plasma glucagon level.

Key words: Portal hypertension; Octreotide; Glucagon; Splanchnic hemodynamics; Somatostatin analog

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INTRODUCTION

Somatostatin has been widely used as treatment for variceal bleeding in patients with portal hypertension. However, the collective research on the mechanisms of somatostatin therapeutic action have not definitely determined whether the decreased splanchnic blood flow and free portal venous pressure (FPP) result from splanchnic vasoconstriction or decreased concentration of plasma glucagon or both[1]. The present study was designed to observe the effects of a commonly used somatostatin analog, octreotide, on splanchnic hemodynamics and concentration of plasma glucagon using rat model systems of both intrahepatic portal hypertension (IHPH) and prehepatic portal hypertension (PHPH) in order to investigate the underlying mechanism.

MATERIALS AND METHODS

Thirty-five male Sprague-Dawley rats, weighing 31.7 ± 15.8 g, were used in the study. All animals were housed in an environmentally controlled vivarium and allowed free access to a standard pellet diet and water.

IHPH modeling

Rats were given a subcutaneous injection of CCl_4 (60% vol/vol in mineral oil, at a dose of 0.3 mL/100 g body weight) every 4 days for a total of 20 times. During the full modeling course, the rats were allowed to drink 10% alcohol.

PHPH modeling

Rats were put under ether anesthesia and after surgically isolating the portal vein a 7-gauge needle was placed alongside it. A ligature was then tied snugly to the needle and the vein at a location between the portal hepatic vein and the coronary vein. The needle was then removed to yield a calibrated stenosis of the portal vein.

Experimental and control treatment groups

Fourteen of the rats used in the IHPH and PHPH modeling (n = 7 each) were divided into two groups: octreotide (injection) treatment and untreated (normal saline injection) control. In addition, 7 age-matched unmodeled/untreated rats served as normal controls.

Hemodynamics study

According to the method described by Zang *et al*[2], toad red

Table 1 Effects of octreotide on splanchnic hemodynamics

	Normal Cont	IHPH Cont	IHPH Thera	PHPH Cont	PHPH Thera
THBF, mL/min/100 g BW	6.30 ± 0.5	5.86 ± 0.9	4.53 ± 0.9[c]	2.80 ± 0.3[a]	2.12 ± 0.4[e]
THBF, mL/min/g LW	2.50 ± 0.3	1.61 ± 0.3[a]	1.37 ± 0.3[a]	1.17 ± 0.2[ae]	0.09 ± 0.2[ae]
PVI, mL/min/100 g BW	4.85 ± 0.4	7.03 ± 0.7[a]	5.85 ± 0.6[c]	7.86 ± 0.4[ae]	5.38 ± 0.6[ca]
SVR, mmHg·min/mL·100 g BW	26.68 ± 2.1	15.86 ± 2.3[a]	20.64 ± 2.8[c]	12.84 ± 1.0[a]	19.88 ± 2.4[ca]
PVR, mmHg·min/mL·100 g BW	1.72 ± 0.2	2.01 ± 0.3[a]	1.95 ± 0.2[a]	1.76 ± 0.1[e]	2.20 ± 0.3[c]
PSS, %	1.46 ± 0.3	34.29 ± 11.7[a]	38.77 ± 11.2[a]	94.4 ± 1.3[ae]	87.7 ± 4.5

[a]$P <$ 0.05 *vs* unmodeled/untreated normal controls; [c]$P <$ 0.05 *vs* control group; [e]$P <$ 0.05 *vs* the IHPH control group.
Cont: Control; Thera: Therapy; THBF: Total hepatic blood flow; PVI: Portal venous inflow; SVR: Splanchnic vascular resistance; PVR: Portal venous resistance; PSS: Portosystemic shunt.

blood cells labelled with ^{51}Cr and ^{125}I·T$_3$ were prepared for use in hemodynamics study. The study was carried out at 2 wk after creation of the portal hypertension. Briefly, the modeled rats were fasted, but allowed access to water, for 18 h prior to experimental use. Following anesthetization with pentobarbital sodium (30 mg/kg, intra-abdominal injection), the rats were placed on a heated surgical table in a supine position with rectal temperature maintained at 37 ± 0.5 ℃. The left femoral vein was dissected and cannulated with a PE-50 catheter for octreotide and normal saline injection. The right femoral artery was dissected and cannulated with a PE-50 catheter for mean systemic arterial pressure (MSAP) measurement and blood collection. A PE-50 catheter was also introduced into the right carotid artery and then into the left ventricle for injecting toad red blood cell labelled with radioisotope. Ten minutes after all procedures were completed, MSAP was measured. The zero reference point was placed 1 cm above the operating table, and the PE-50 catheter was connected to a transducer for continuous monitoring of MSAP. The octreotide treatment group of rats received an injection of 25 μg/kg body weight in 0.2 mL normal saline into the femoral vein followed by a 30-min infusion delivered at a rate of 0.02 mL/min. The untreated control group received an injection of normal saline that was administered intravenously. MSAP was measured at 1 and 30 min after the octreotide or normal saline administration. Subsequently, 0.3 mL of toad red blood cells labelled with 1-Cr was injected into the left ventricle over a duration of 20 s. A reference blood sample was drawn from the femoral artery at a rate of 1 mL/min for a duration of 10 s prior to and at 60 s after the injection of the radioactive toad red blood cells. A midline incision was then made and the spleen was gently taken out of the abdomen and injected with 0.5 mL of toad red blood cells labelled with ^{125}I·T$_3$ over a duration of 10 sec. After the puncture point bleeding was controlled by local pressure, the spleen was placed back into the abdominal cavity. Five minutes later, the main trunk of the portal vein was dissected. FPP was measured by inserting a heparin solution-filled 4-gauge needle connected to a catheter going directly into the portal vein and which was connected to an IVAC560 transducer for venous pressure measurement. Blood samples (1.5 mL) were drawn from both the portal vein and the carotid artery to determine the concentrations of plasma glucagon. Animals were then sacrificed by an intra-arterial bolus of saturated 10% KCl solution. All abdominal organs including the liver, spleen, pancreas, stomach, small and large intestine, and mesentery, as well as the lung, were removed and weighed. These organs were cut into small fractions for measurement of radioactivity.

Determination of plasma glucagon concentrations

The blood samples were placed into chilled collection tubes containing EDTA (25 g/L) and aprotinin (5 × 10^5 U/L) and immediately centrifuged (2400 × *g* for 20 min) at 4 ℃; the separated plasma was stored at -40 ℃ until use for determination of glucagon concentration by radioimmunoassay with a specific antibody for glucagon (DPC, United States).

Statistical analysis

All values are expressed as x ± s. Variance analysis and Student's *t*-test were conducted to assess the significance of between and within group differences using SAS statistical software. Statistical significance was indicated by $P < 0.05$.

RESULTS AND DISCUSSION

Effects of octreotide on MSAP and FPP

The initial MSAP in PHPH and IHPH rats was lower than that in the unmodeled/untreated normal control rats (111.1 ± 2.6 mmHg and 126.9 ± 6.6 mmHg *vs* 137.3 ± 9.8 mmHg). In addition, the initial MSAP of the PHPH rats was significantly lower than that of the IHPH rats ($P <$ 0.05). Octreotide treatment had no effects on MSAP. FPP in IHPH and PHPH rats was higher than that in the unmodeled/untreated normal control rats (14.01 ± 0.56 mmHg and 13.79 ± 0.31 mmHg *vs* 8.37 ± 0.10 mmHg). Octreotide treatment decreased FPP in both the IHPH rats (11.29 ± 0.64 mmHg) and PHPH rats (11.70 ± 0.36 mmHg).

Effects of octreotide on splanchnic hemodynamics

Table 1 presents the effects of octreotide on splanchnic hemodynamics. Portal venous inflow (PVI) of the IHPH and PHPH rats was significantly increased as compared with that of the unmodeled/untreated normal rats, indicating hyperhemodynamics in portal hypertension. Octreotide markedly reduced PVI in both the IHPH and PHPH rats ($P <$ 0.05). Splanchnic vascular resistance (SVR) in the IHPH and PHPH rats was much lower than that in unmodeled/untreated normal rats ($P <$ 0.05), and octreotide increased SVR in both; however, the changes in SVR in the IHPH rats were not significantly different from the SVR in the unmodeled/untreated normal rats while the SVR in the PHPH rats was still lower than that of unmodeled/untreated normal ones. Portal venous resistance (PVR) was significantly increased in the IHPH rats ($P <$ 0.05) but showed no change in the PHPH rats, fitting with the observation of the magnitude of portosystemic shunt (PSS) being much higher in the PHPH rats than in the IHPH rats. However, FPP of the PHPH rats was still higher than that of the unmodeled/untreated normal rats, possibly related to both the increased PVI and collateral resistance observed in the PHPH rats. The octreotide treatment led to significantly increased PVR in the PHPH rats ($P <$ 0.05). PSS was 34.29% ± 11.72% and 94.4% ± 1.3% in the IHPH and PHPH rats, respectively. The magnitude of PSS was much greater in the PHPH rats than in the IHPH rats. However, the octreotide treatment produced no effects on PSS in either the IHPH or the PHPH rats.

Effects of octreotide on splanchnic blood flow

Table 2 presents the effects of octreotide treatment on splanchnic blood flow. Blood flow of the stomach, small and large intestines, mesentery and pancreas was markedly increased in both the IHPH and PHPH rats as compared with the unmodeled/untreated normal rats. Furthermore, the splanchnic blood flow was increased to a greater extent in the PHPH rats than in the IHPH rats. Due to splenomegaly in the IHPH and PHPH rats, the blood flow/1 g spleen weight was not significantly different in the IHPH rats, the PHPH rats and the unmodeled/untreated normal rats, and the hepatic artery flow (HAF)/1 g liver weight in the IHPH rats was lower than that in the unmodeled/untreated normal rats ($P <$ 0.05). In the PHPH rats, HAF was greater than that in the unmodeled/untreated normal rats because of the hepatotrophy in the former. The ratio of liver weight to body weight in the PHPH rats was only decreased by 5:2% when compared with that of the unmodeled/untreated normal rats (2.40% ± 0.21% *vs* 2.53% ± 0.11%). However, the HAF/1 g liver weight was increased by 70.7% in PHPH rats when compared with that in the unmodeled/untreated normal rats (0.99

Table 2 Effects of octreotide on splanchnic blood flow (mL•g•min)

	Normal Cont	IHPH Cont	IHPH Thera	PHPH Cont	PHPH Thera
Stomach	0.59 ± 0.1	0.76 ± 0.2[a]	0.59 ± 0.1[c]	0.71 ± 0.1[a]	0.53 ± 0.1[c]
Intestine and mesentery	1.36 ± 0.1	1.65 ± 0.1[a]	1.11 ± 0.1[ac]	1.83 ± 0.2[a]	1.21 ± 0.1[ac]
Pancreas	0.84 ± 0.1	0.97 ± 0.1[a]	0.62 ± 0.1[c]	1.27 ± 0.2[a]	1.01 ± 0.2[c]
Spleen	0.98 ± 0.4	0.78 ± 0.2	0.46 ± 0.2[ac]	0.96 ± 0.2	0.72 ± 0.2[c]
Liver	0.58 ± 0.1	0.38 ± 0.1[a]	0.28 ± 0.1[ac]	0.99 ± 0.1[ae]	0.62 ± 0.1[c]

[a]$P < 0.05$ *vs* unmodeled/untreated normal controls; [c]$P < 0.05$ *vs* control group; [e]$P < 0.05$ *vs* the IHPH control group.

Table 3 Effects of octreotide on plasma glucagon levels (ng/L)

	Peripheral Vein	Portal Vein
Normal Cont	19.28 ± 2.5	44.25 ± 6.1
IHPH Cont	49.37 ± 10.6[a]	180.25 ± 31.2[a]
IHPH Thera	40.12 ± 14.6[a]	140.96 ± 41.9[a]
PHPH Cont	69.82 ± 17.2[a]	143.60 ± 25.6[a]
PHPH Thera	50.08 ± 7.6[a]	106.33 ± 37.9[a]

[a]$P < 0.05$ *vs* unmodeled/untreated normal controls.

± 0.14 mL *vs* 0.58 ± 0.12 mL). These results indicate that, due to the decrease of portal blood flow, HAF compensatorily increased in the PHPH rats. The octreotide treatment significantly decreased PVI in both the IHPH and PHPH rats, and decreased the HAF/1 g liver weight by 26.3% in the IHPH rats (0.28 ± 0.1 mL *vs* 0.38 ± 0.7 mL) and by 37.4% in the PHPH rats (0.99 ± 0.14 mL *vs* 0.62 ± 0.14 mL).

Effects of octreotide on the concentration of plasma glucagon

Table 3 presents the effects of octreotide on the concentrations of plasma glucagon. The IHPH and PHPH rats showed significantly higher concentrations of plasma glucagon in both the peripheral and portal veins as compared with that in the unmodeled/untreated normal rats ($P < 0.05$), indicating hyperglucagonemia as part of the portal hypertension condition. However, the octreotide-induced decrease in plasma glucagon levels in the peripheral and portal veins did not reach statistical significance for either the IHPH rats or the PHPH rats.

In summary, the results of the present study demonstrate that octreotide significantly decreases PVI and HAF and increases SVR and PVR in rats with IHPH and PHPH. However, the octreotide-induced reduction in plasma glucagon concentrations in both the peripheral and portal veins did not reach statistical significance. These data suggest that the reduction of splanchnic blood flow produced by octreotide predominantly results from direct vasoconstriction, whereas the decrease of plasma glucagon levels is less important. However, the octreotide treatment was unable to produce a decrease in either PVI or FPP to normal levels in the portal hypertensive rat models, indicating that other factors in addition to glucagon may be responsible for the increased splanchnic blood flow in portal hypertension.

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ORIGINAL RESEARCH

Overproduction of nitric oxide inhibits vascular reactivity in portal hypertensive rats

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Abstract

AIM: To evaluate the relationship between nitric oxide (NO) and hyperdynamic circulatory status in portal hypertension.

METHODS: Twenty male Sprague Dawley rats (weighing 200 ± 20 g) were randomized into two groups: portal hypertension group (n = 12) and sham-operated control group (n = 8). The portal hypertensive model was established by means of graded constriction of the portal vein. The concentrations of nitrite (NO^-_2) in the portal vein and peripheral blood were measured by fluorometric assay to reflect NO levels. The reactivity of isolated abdominal aortic rings from rats with partial portal vein constriction and controls was determined by assessing response to administration of potassium chloride (KCl) (10–80 mmol/L) and phenylephrine (10^{-9}-10^{-4} mol/L) with or without preincubation with NO synthase inhibitor Nω-nitro-L-arginine (L-NNA).

RESULTS: Serum concentrations of NO^-_2 in the portal vein blood (0.766 ± 0.097 μmol/L) and peripheral blood (0.687 ± 0.092 μmol/L) were elevated in portal hypertensive rats, as compared with the concentrations in controls (0.613 ± 0.084 μmol/L and 0.591 ± 0.045 μmol/L respectively, both $P < 0.01$). In addition, the rates of NO^-_2 in portal vein blood were markedly higher than those in peripheral blood ($P < 0.05$) in the portal hypertensive rats. Abdominal aortic rings from rats with portal vein constriction exhibited significantly impaired contractility to phenylephrine and KCl, as compared with the control rats. The EC^{50} values of KCl were markedly higher in the portal hypertensive rings (26.5 ± 0.9 mmol/L) than in the control rings (22.3 ± 1.7 mmol/L, $P < 0.01$), as were the EC^{50} values of phenylephrine (37.2 ± 0.4 nmol/L *vs* control rings: 28.1 ± 0.2 nmol/L, $P < 0.01$). After preincubation of rings with L-NNA, the difference in EC^{50} values between portal hypertensive and control rings was no longer statistically significant for either KCl (20.18 ± 0.8 mmol/L *vs* 19.4 ± 1.2 mmol/L, $P > 0.05$) or phenylephrine (22.4 ± 1.8 nmol/L *vs* 21.8 ± 1.4 nmol/L, $P > 0.05$). However, the maximal concentrations of KCl and phenylephrine for inducing contractions were still significantly lower in the portal hypertensive rings (1.08 ± 0.1 g and 1.43 ± 0.14 g) than in the control rings (1.21 ± 0.11 g and 1.72 ± 0.11 g respectively, both $P < 0.05$). Thus, addition of the NO synthase inhibitor L-NNA could partially restore contractile responses to KCl and phenylephrine in portal hypertensive rings.

CONCLUSION: NO overproduction inhibits the vascular reactivity to vasoconstrictors, and it might be one of the main causes of vasodilatation and hyperdynamic circulatory status in portal hypertension.

Key words: Portal hypertension; Nitric oxide; Vascular reactivity; Hyperdynamic circulatory status; Vasodilatation

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INTRODUCTION

Mechanisms of portal hypertension are complicated. In addition, circulatory abnormalities may affect the portal venous systemic circulation regardless of the presence or absence of defective liver structure and function. Hyperdynamic circulation can perpetuate and aggravate portal hypertension, possibly resulting in a series of grave cardiovascular and non-cardiovascular complications[1]. In recent years, nitric oxide (NO), a newly identified neurotransmitter, cell-killing factor and cellular messenger molecule, has attracted the attention of biologists and medical workers. Evidence has shown that NO plays an important role in cardiovascular function and is involved in the physiology of vascular tone, blood flow velocity and vascular resistance. Recent studies have also indicated that NO is involved in the pathophysiology aof portal hypertension[2].

In order to evaluate the relationship between NO and hyperdynamic circulation in portal hypertension, we used rats with partial portal vein constriction and rats that were sham-operated (controls) to investigate the reactivity of isolated vascular rings to potassium chloride (KCl) and to phenylephrine with or without pre-exposure to the NO synthetic inhibitor Nω-nitro-L-arginine (L-NNA). We determined that the serum concentrations of NO^-_2 detected by

Table 1 Baseline values in rats with portal hypertension and sham-operated control rats

Parameter	Portal hypertension (n = 11)	Control (n = 8)
Body weight (g)	208 ± 17	201 ± 21
PVP (kPa)	1.644 ± 0.142[b]	1.034 ± 0.113
Serum Na^+ (mmol/L)	135 ± 1[b]	141 ± 2
Serum K^+ (mmol/L)	4.1 ± 0.2	4.3 ± 0.2
ALT (U/L)	147.6 ± 38.4	141.7 ± 53.3
Albumin (g/L)	16.43 ± 4.12	17.21 ± 3.84
A/G	0.64 ± 0.04	0.66 ± 0.08

[b]$P < 0.01$ *vs* control.

fluorometric analysis reflect NO levels. The results of this study provide new insights that may help towards developing new treatments of portal hypertension.

MATERIALS AND METHODS

Animal model

Twenty male Sprague-Dawley rats (weighing 200 ± 20 g) were randomized into two groups: the portal hypertension model group (n = 12) and the sham-operated control group (n = 8). The portal hypertensive model was established by means of graded constriction of the portal vein. Specifically, after the rats were anesthetized with ketamine hydrochloride (0.15 mg/100 g, intra-abdominal), the portal vein was isolated and a stenosis was created by means of a single ligature (3-0 silk) placed around both the portal vein trunk and a needle (blunt, external diameter 0.8 mm). Removal of the needle facilitated production of a consistent extrahepatic portal hypertension. The sham operation consisted of dissection and visual inspection of the portal vein.

Sample preparation

Two weeks after the surgeries, the animals were re-anesthetized and portal venous pressure was measured. Blood samples from the portal vein and abdominal vein (2.0-3.0 mL) were obtained by direct puncture and centrifuged at 3000 rpm for 10 min to separate the serum. The serum samples were then stored at -40 ℃ for subsequent detection of NO^-_2. Whole blood samples (1.5 mL) were also obtained for other assays to detect serum electrolytes and markers of liver function. The abdominal aorta was dissected, placed in oxygenated Krebs solution, stripped of all fatty tissues and adventitia, and then cut into 3 mm rings. the length and external diameter of which were measured.

Observation of vascular reactivity

The prepared rings from both groups of animals were mounted horizontally between two stainless-steel stirrups in individual organ bath chambers filled with 10 mL of modified Krebs solution (mmol/L concentrations: NaCl 118.3, KCl 14.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.1, and EDTA 0.026, pH 7.4) and constantly bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at 37 ℃. After 60 min of equilibration, the rings were allowed to adjust gradually with 30 mmol/L KCl until the optimal resting tension was obtained. All the experiments were the performed on the rings in the optimal resting tension, which was approximately 1.5 g. The contraction responsiveness to cumulative additions of KCl (10, 20, 30, 40, 50, 60, 70 and 80 mmol/L) and phenylephrine (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^-4 mol/L) were measured with force displacement transducers (JZ-BK) and recorded with a multichannel paper recorder (XWTD464). The rings were then washed and equilibrated for 30 min, and then preincubated for 30 min with 10^{-5} mol/L of the specific NO biosynthesis inhibitor L-NNA. Contraction responses were evoked and measured with the range of cumulative concentrations of KCl and phenylephrine listed above in the presence of L-NNA. At the end of the experiments, the rings were weighed.

Quantification of NO^-_2

In principle, NO^-_2 can react with 2, 3-diaminonathalen (DAN) in acid solution and produce a fluorescent substance, the intensity of which

Table 2 Serum levels of NO^-_2 in rats with portal hypertension and sham-operated control rats (x ± s, $\times 10^{-6}$ mol/L)

Group	n	Portal vein	Peripheral vein
PH	11	0.766 ± 0.097[ac]	0.687 ± 0.092[a]
Control	8	0.613 ± 0.084	0.591 ± 0.045

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* peripheral vein blood.

Table 3 Physical characteristics of aortic rings from rats with portal hypertension and sham-operated control rats (x ± s)

Parameter	Portal hypertension	Control
ED (mm)	1.51 ± 0.04	1.49 ± 0.03
Length (mm)	3.09 ± 0.04	3.05 ± 0.07
Weight (mg)	1.59 ± 0.08	1.54 ± 0.08

ED: External diameter.

can be measured by a spectrofluorometer and used to indicate $NONO^-_2$ content. To quantitate NO^-_2, we first made a standard curve by putting 100 μL aliquots of 0, 3.2×10^{-7}, 6.4×10^{-7}, 1.28×10^{-6}, 1.92×10^{-6}, 2.56×10^{-6}, 3.2×10^{-6} and 3.84×10^{-6}-mol/L $NaNO_2$ into 5 mL test tubes, and then adding 10 μL of DAN-HCl solution, mixing well and allowing to react for 10 min at 20 ℃. The reaction was stopped by adding 5 μL of 2.8 mol/L NaOH. Double-distilled water was then added to bring the final volume up to 3 mL, and the fluorescence intensity was measured at Ex365nm, Em420nm (RF-5000 Spectrofluorometer, Japan). Finally, the standard curve of fluorescence intensity versus the concentration of $NaNO_2$ was generated. To measurement the concentration of serum NO^-_2, 200 μL aliquots of serum were placed in test tubes and mixed with 200 μL chloroform. After standing for 5 min, each tube was centrifuged for 15 min at 3500 r/min. The resultant supernatant was then mixed with 20% of its volume of 20% sodium sulfosalicylate to remove proteins. After centrifuging for 15 min at 3500 r/min, 100 μL of the resultant supernatant was mixed with 50 μL of 0.01 mol/L EDTA and then 40 μL of DAN-HCl solution was added. After reacting at 20℃ for 10 min, 5 μL of 2.8 mol/L NaOH was added to stop the reaction. Double-distilled water was added up to a final volume of 3 mL, and the fluorescence intensity was measured. The concentration of NO^-_2 was calculated from the standard curve.

Statistical analysis

Dose-response contraction curves were made with the experimental data. The EC^{50} values and the Rmax were calculated from each dose-response contraction curve by means of a non-linear regression method with a curve-fitting program. Rmax and EC^{50} values were averaged and the results were assessed statistically using either the paired or unpaired Student's t-test as appropriate. The data of NO^-_2 were analyzed with Student's t-test, and the results are presented as mean ± SEM. Statistical significance was set at a P level of 0.05 or less.

RESULTS

All the animals survived the modeling and sham operations and subsequent experimental course, except for one. No rats in the control or portal hypertensive model group showed any appreciable alteration in hepatic histology. Body weight, age, serum electrolytes, liver function and portal venous pressure are shown in Table 1. No differences were found between the partial portal vein constricted and control rats for the parameters of body weight, age, serum potassium, alanine aminotransferase (ALT) and albumin. However, compared with the controls, the portal hypertensive rats showed marked hyponatremia and elevated portal venous pressure (both $P < 0.01$).

Serum NO^-_2 levels are shown in Table 2. Serum concentrations of NO^-_2 in portal vein blood (0.766 ± 0.097 μmol/L) and peripheral blood (0.687 ± 0.092 μmol/L) were markedly elevated in the portal hypertensive rats, as compared with the levels in controls (0.613 ± 0.084 μmol/L and 0.591 ± 0.045 μmol/L respectively, P <0.05). The level of NO^-_2 in portal vein blood of portal hypertensive rats

Table 4 Rmax and EC^{50} values for KCl and phenylephrine in aortic rings from rats with portal hypertension and sham-operated control rats with and without L-NNA treatment ($\bar{x} \pm s_{\bar{x}}$)

Agent	EC^{50}		Maximal contractions (g)	
	Untreated	L-NNA	Untreated	L-NNA
KCl (mmol/L)				
PH	26.5 ± 0.9^{ba}	20.2 ± 0.8	0.72 ± 0.05^{ba}	1.08 ± 0.1^{b}
Control	22.3 ± 1.7^{a}	19.4 ± 1.2	0.98 ± 0.05^{a}	1.21 ± 0.11
PE ($\times 10^{-8}$ mol/L)				
PH	3.72 ± 0.4^{ba}	2.24 ± 0.18	1.04 ± 0.08^{ba}	1.43 ± 0.14^{a}
Control	2.81 ± 0.2^{b}	2.18 ± 0.14	1.34 ± 0.39^{a}	1.72 ± 0.11

$^{b}P < 0.01$ *vs* control; $^{a}P < 0.05$ *vs* L-NNA.

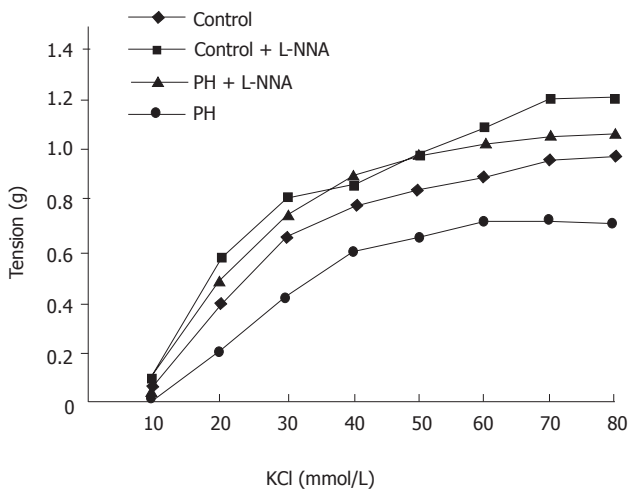

Figure 1 Dose response contraction curves for KCl in the presence of 10^{-5} mol/L L-NNA in control and portal hypertensive abdominal aortic rings.

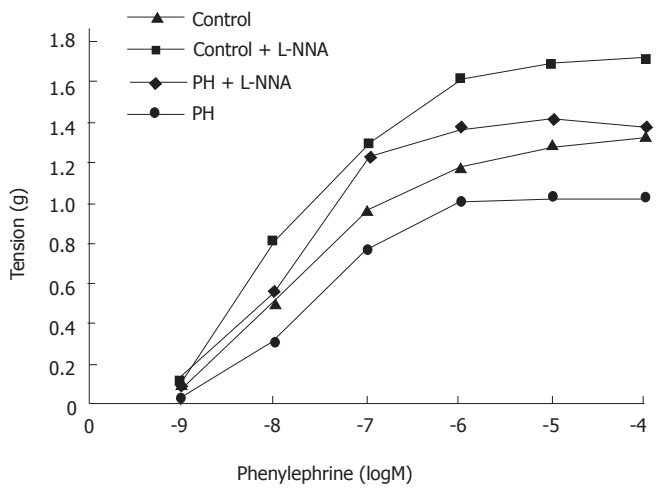

Figure 2 Dose-response contraction curves for phenylephrine in the presence of 10^{-5} mol/L L-NNA in control and portal hypertensive abdominal aortic rings.

was significantly higher than that in peripheral blood ($P < 0.05$). The physical parameters of the aortic rings are shown in Table 3. The aortic rings of the portal hypertensive rats and the control rats showed no differences in external diameter, length or weight ($P > 0.05$).

Contractile responses of abdominal aortic rings to KCl

Contractile responses to KCl were attenuated in rings from the portal hypertensive rats, as compared with those from the control rats, as evidenced by the dose response curves being shifted to the right (Figure 1). The EC^{50} values were markedly higher in the aortic rings from the portal hypertensive rats (26.5 ± 0.9 mmol/L) than in those of the control rats (22.3 ± 1.7 mmol/L, $P < 0.01$). Maximal contraction was significantly diminished in the aortic rings of the portal hypertensive rats (0.72 ± 0.05 g), as compared with those of the control rats (0.98 ± 0.05 g, $P < 0.01$). After preincubation of the rings with L-NNA (10^{-5} mol/L), the KCl-induced contractile curves were shifted to the left (compared with those made in the absence of L-NNA) for both the portal hypertensive and control rings. The difference in EC^{50} values of the aortic rings was no longer statistically significant between the portal hypertensive rats (20.18 ± 0.8 mmol/L) and the control rats (19.4 ± 1.2 mmol/L, $P > 0.05$). However, the maximal KCl-induced contractions were still lower in the aortic rings from the portal hypertensive rats (1.08 ± 0.18 g) than in those from the control rats (1.21 ± 0.11 g, $P < 0.05$) (Table 4).

Contractile responses of abdominal aortic rings to phenylephrine

Phenylephrine induced contractile curves were shifted to the right for the aortic rings from the portal hypertensive rats (Figure 2). The EC_{50} values were markedly higher for the aortic rings from the portal hypertensive rats (37.2 ± 4 nmol/L) than those from the control rats (28.1 ± 2 nmol/L, $P < 0.01$). Maximal contractions were markedly lower in the aortic rings from the portal hypertensive rats (1.04 ± 0.08 g) than in those from the controls (1.34 ± 0.39 g, $P < 0.01$). After the rings were preincubated with L-NNA (10^{-5}mol/L), the phenylephrine-induced contractile curves were shifted to the left for both portal hypertensive and control rings (Figure 2). The difference in EC_{50} values of the aortic rings was no longer statistically significant between the portal hypertensive rats (22.4 ± 1.8 nmol/L) and the control rats (21.8 ± 1.4 nmol/L, $P > 0.05$). The maximal contractions were still lower in the portal hypertensive rings (1.43 ± 0.14 g) than in the control rings (1.72 ± 0.11 g, $P < 0.05$) (Table 4).

DISCUSSION

NO is synthesized from L-arginine by the enzyme NO synthase (NOS). There are two different types of NOS; the constitutive from (cNOS) is dependent on calcium and calmodulin for its activity, while the inducible from (iNOS) has no calcium or calmodulin requirement. cNOS is continuously present in the internal surface of the endothelial cell membrane, but iNOS represents a newly synthesized enzyme that is expressed in response to specific stimuli of endotoxin and cytokines in serial cell types, including vascular endothelium, vascular smooth muscle cells, hepatocytes and macrophages[3].

To date, no ideal technique is available to detect the NO content in tissues. The metabolic products of NO^{-}_{3}/NO^{-}_{2} are used to assess the NO level indirectly. The Griees reaction has been widely applied to measure NO^{-}_{2}, and it is relatively simple but its sensitivity is low[4]. In the present study, a new fluorometric analysis assay was developed and used to detect serum content of NO^{-}_{2}. Because NO^{-}_{2} plus 2, 3-diaminonaphthalene make a kind of fluorescent substance of 1-(H)-naphthotriazole, and the detection of the fluorescence intensity can reflect NO^{-}_{2} levels, the sensitivity of detection was markedly improved. The results of this study showed that serum levels of NO^{-}_{2}, especially those in portal vein blood, were elevated in rats with partial portal vein constriction, as compared with those in sham-operated controls. These findings indicated that there was an increased production of NO in portal hypertensive rats.

NO is an important regulator of systemic and local hemodynamics in physiological conditions, and is involved in the physiology of vascular tone, blood flow, velocity and vascular resistance. The mechanisms underlying these processes involve NO diffusion into the vascular smooth muscle cell, where it activates soluble guanylate cyclase and generates a subsequent increase in cGMP to cause relaxation of the vascular smooth muscle by binding of free intracellular calcium. Patients and experimental animals with portal hypertension reportedly present with hyperdynamic circulatory dysfunction characterized by splanchnic and systemic

arterial vasodilation[5]. The pathogenesis of arterial vasodilation correlated with NO overproduction in portal hypertension. These findings collectively suggest that NO is an important mechanism of the hyperkinetic circulation associated with prehepatic portal hypertension.

L-NNA is an L-arginine analog, and competitively inhibits the activity of both iNOS and cNOS, subsequently attenuating NO release[6]. Our study found reduced contractile responses to both KCl and phenylephrine in abdominal aortic rings from partial portal vein constricted rats as compared with those from the sham-operated controls. Addition of the NOS inhibitor L-NNA (10^{-5} mol/L) significantly increased the contractility to KCl and phenylephrine in both the control and portal hypertensive rings, but the contractile responses increased proportionally more in the portal hypertensive aortic rings than in the control rings in the presence of L-NNA. However, the difference in EC_{50} values between the portal hypertensive and the control groups lost significance. These results suggest that vascular endothelium produces and releases some NO to maintain vasodilation under normal circumstances, while overproduction of NO in portal hypertensive vascular endothelium inhibits the vascular contractile response to KCl and phenylephrine. However, although the maximal KCl^- and phenylephrine-induced contractions were increased in rings from both portal hypertensive rats and control rats in the presence of L-NNA, the maximal contractions were still significantly lower in the portal hypertensive rings than those of the control rings. Thus, other factors, such as alteration in vascular smooth muscle, may play an important role in the pathogenesis of vasodilation and hyperdynamic circulation in portal hypertension.

The mechanism of increased vascular production of NO in rats with partial portal vein constriction cannot be shown from this study. The likely explanation is up-regulation of cNOS in the vascular endothelium, because iNOS is not expressed in isolated vessels which are not able to be stimulated by endotoxin and cytokines. In fact, increased cNOS activity has been reported by measuring NOS activity directly in homogenized blood vessels in animal models of portal hypertension with and without cirrhosis[7,8]. Whether the up-regulation of cNOS and subsequent NO overproduction is a primary or secondary event in the arteriolar vasodilation in portal hypertension could not be ascertained. Shear stress has been shown to be a powerful stimuli of cNOS[3]; therefore, overproduction of NO could be consequent to shear stress and other factors related to the hyperkinetic circulation. NO overproduction by endothelial cells would amplify and perpetuate arteriolar vasodilation. The effective inhibition of cNOS may reverse hyperdynamic circulation.

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ORIGINAL RESEARCH

Plasma D (-)-lactate as a new marker for diagnosis of acute intestinal injury following ischemia-reperfusion

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Author contributions: All authors contributed equally to the work.

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Abstract

AIM: To observe the kinetics of D (-)-lactate alteration in both portal and systemic circulation systems, and its relationship with intestinal injury in rats subjected to acute intestinal ischemia-reperfusion.

METHODS: Anesthetized rats underwent a 75-min superior mesenteric artery occlusion followed by a 6-h reperfusion. Plasma D (-)-lactate levels were measured by an enzymatic spectrophotometric assay.

RESULTS: Intestinal ischemia for 75 min resulted in a significant elevation of D (-)-lactate levels in the portal vein, as compared with the baseline values ($P < 0.05$). Plasma D (-)-lactate levels had a tendency to further increase after reperfusion, up to 6 h. Similar alterations in D (-)-lactate were also found in systemic circulation, and there were no significant differences between the portal and systemic circulations at any time point. Moreover, the macropathological evaluation scores were significantly correlated to the portal D (-)-lactate levels in animals at various time points ($r = 0.415$, $P < 0.01$). In addition, there was a remarkable rise of endotoxin concentration within the portal vein at the end of the 75-min ischemic period ($P < 0.05$), reaching a peak at 2 h post-reperfusion.

CONCLUSION: Acute intestinal ischemia is associated with failure of the mucosal barrier resulting in increased plasma D (-)-lactate levels in both portal and systemic blood. The subsequent reperfusion might further increase D (-)-lactate levels, which are correlated to the macropathological alterations. Plasma D (-)-lactate may be a useful marker of intestinal injury following both ischemia and reperfusion insults.

Key words: D (-)lactate; Endotoxin; Intestinal injury; Reperfusion injury

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INTRODUCTION

Major trauma and shock may initiate a cascade of events leading to sepsis with subsequent multiple organ failure. Gut mucosal barrier dysfunction is assumed to play an important role in causing the septic process[1]. From our and other studies, it has been suggested that enteric organisms and/or their toxins might translocate across the intestinal mucosa, entering the systemic circulation *via* the lymphatic or portal systems and resulting in the development of remote organ damage in animals and patients with shock following hemorrhage, trauma, or burns[1-3].

The intestine is one of the most sensitive tissues to ischemia-reperfusion injury. Reperfusion of the intestine is often associated with increased mucosal permeability and ulceration. Mucosal injury has been assessed either morphologically or by measuring the permeability of the mucosal barrier to small or large solutes; however, there exists no reliable serum marker for the early diagnosis of acute intestinal insult in clinical practice.

D (-)-lactate is produced by indigenous bacteria found in the gastrointestinal tract, and mammals do not possess the enzyme systems to rapidly metabolize it[4]. Therefore, an increase in D (-)-lactate might reflect an efflux of bacteria and/or their products into circulation as a result of mucosal injury. Recently, a report was published showing that serum D (-)-lactate was increased in animal models of acute intestinal ischemia and simple obstruction[5]; yet, it remains unclear whether this process is associated with a significant dysfunction in mucosal barrier following reperfusion.

The present study was designed to determine the kinetics of plasma D (-)-lactate changes, and to examine whether D (-)-lactate levels are correlated to intestinal damage in rats caused by acute intestinal ischemia-reperfusion injury.

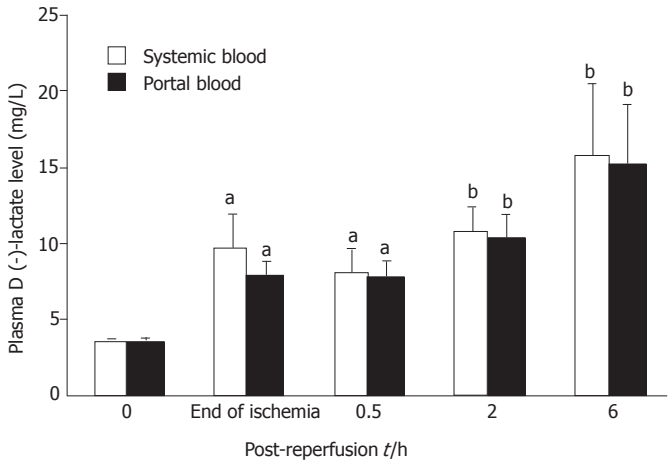

Figure 1 Kinetics of plasma D (-)-lactate alteration in portal and systemic circulation systems. Six to nine animals were assessed for each time point. Data are expressed as x ± s_x. [a]*P* < 0.05 and [b]*P* < 0.01 *vs* baseline values.

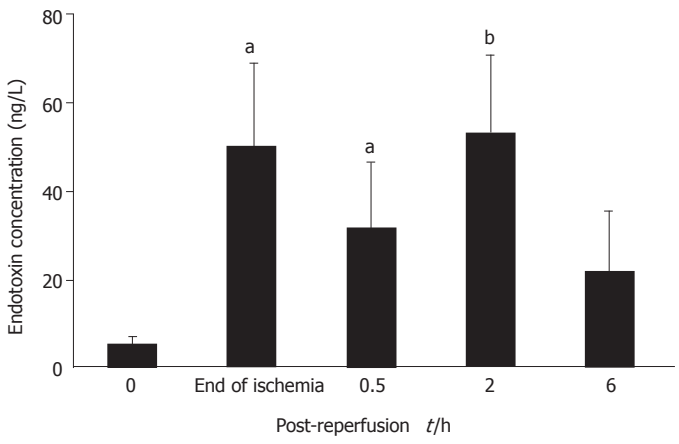

Figure 3 Changes in plasma endotoxin concentration in portal vein following acute intestinal ischemia-reperfusion injury. Six to nine animals were assessed for each time point. Data are expressed as x ± s_x. [a]*P* < 0.05 and [b]*P* < 0.01 *vs* baseline values.

MATERIALS AND METHODS

Animal model

Adult male Sprague-Dawley rats purchased from Charles River Wiga GmbH (Sulzfeld, Germany) were used in this study. Following anesthesia (ketamine/xylazine: 112/15 mg/kg), the right femoral artery was cannulated under aseptic conditions using a polyethylene catheter connected to a blood pressure monitor. After a midline laparotomy was performed, the superior mesenteric artery (SMA) was isolated from the surrounding connective tissues near its aortic origin. The SMA was then occluded with an atraumatic clip applied to the root of the mesentery. After 75 min of ischemia, the arterial clip was removed, allowing reperfusion. Reperfusion was confirmed by the return of pulsations to the mesenteric vascular arcade[6]. The laparotomy incision was closed, and anesthetized animals were monitored during an additional 6 h of reperfusion.

Study design

Six to nine animals were sacrificed at each of following time points: before occlusion, at end of ischemia, and at 0.5 h, 2 h and 6 h after release of the clamp. Portal blood samples were obtained by portal vein puncture before the animals were sacrificed at each time point, and the collection was limited to once per animal to avoid potential contamination. Plasma was separated by centrifugation and stored at -30 ℃ until analysis.

Plasma D (-)-lactate measurement

Plasma D (-)-lactate levels were measured by an enzymatic spectrophotometric assay with slight modification using a centrifugal analyzer (Cobas-Fara)[7]. For analysis, the supernatant plasma without deproteinization was added to tubes and mixed with carbonate buffer containing 4.6 mmol/L of NAD^+. The reaction was initiated by adding 50 μL of D-lactic dehydrogenase (D-LDH; Sigma Chemical Co., St. Louis, MO, USA) to each tube and the absorbance change at 340 nm was measured against the reagent blank tubes with 50 μL water instead of D-LDH. Serial concentrations of D-lactate (D-lactic acid; Sigma Chemical Co.), from 3.125 to 50 mg/L, were analyzed in order to prepare the standard curve.

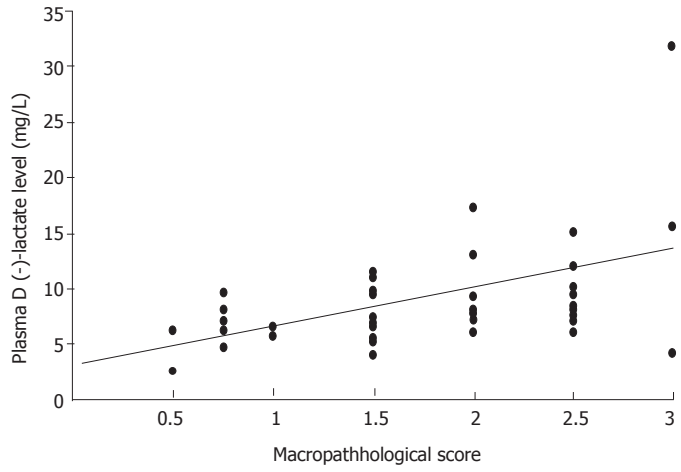

Figure 2 Correlation between the macropathological evaluation scores of intestine and the portal D (-)-lactate levels in animals subjected to intestinal ischemia-reperfusion injury.

Plasma endotoxin determination

The endotoxin content of plasma samples was assayed by the Limulus amebocyte lysate test with a kinetic modification of the test kit procedure[2]. The method has a detection limit of 0.75 ng/L in 1: 10 plasma dilution.

Morphological evaluation

In a blind manner, a postmortem examination was performed together with a macropathological evaluation based on a pre-established four-grade scoring system[8]. For histological examination, tissue samples from small intestine were taken and fixed in 10% buffered formalin for analysis as light microscopic sections stained with hematoxylin-eosin.

RESULTS

As shown in Figure 1, intestinal ischemia for 75 min resulted in a significant elevation in D (-)-lactate levels in portal vein as compared with the baseline values ($P < 0.05$). Plasma D (-)-lactate levels had a tendency to further increase after reperfusion, up to 6 h. Similar alterations in D (-)-lactate were also found in systemic circulation (Figure 1), there were no significant differences between the portal and systemic circulations at any time point.

The animals were found to have no abnormal changes in bowel functions before the induction of intestinal ischemia. SMA occlusion for 75 min caused marked intestinal damage, which was much worse upon reperfusion. The macropathological evaluation scores were 2.0 ± 0.2, 2.2 ± 0.2 and 2.7 ± 0.1 at 0.5, 2, and 6 h after reperfusion. The macropathological evaluation scores of intestine were significantly correlated to portal D (-)-lactate levels in animals at various time points ($r = 0.415$, $P < 0.01$; Figure 2).

The endotoxin concentration within portal vein rose remarkably in the control animals, being 9.5-fold over the baseline values at the end of 75-min ischemia ($P < 0.05$). It reached a maximum at 2 h after clamp release, and decreased gradually up to the end of the observation period (Figure 3).

DISCUSSION

In the present experiment, we observed that ischemia-reperfusion of the small bowel resulted in a significant elevation in D (-)-lactate levels in both portal and systemic blood. This was potentially associated with failure of the intestinal mucosal barrier leading to the subsequent escape of indigenous endotoxin/bacteria to the circulation systems. Our data also suggested that an early and sustained rise in systemic D (-)-lactate might occur during mesenteric ischemia, even without further reperfusion injury[5].

The normal gut epithelium provides an anatomical and

physiological barrier to the toxic intestinal contents. Conditions which lead to mesenteric hypoperfusion, such as those seen in hemorrhage and other shock states, have been shown to cause breakdown of the intestinal mucosal barrier[1-3,9]. In this study, the status of the mucosal barrier was assessed quantitatively using a plasma marker, namely D (-)-lactate. D (-)-lactate is strictly a product of bacterial fermentation, and it is known to be produced by many species of bacteria found in the gut flora[4,5], while mammalian tissue does not produce D-lactic acid, which may not or only slowly be metabolized. D-lactic acid accumulating in the blood can generally be considered as a result of active bacterial metabolism due to systemic infections or some gastrointestinal disorders. In fact, D-lactate acidosis has been described in humans with short bowel syndrome, as well as bacterial infections[10]. More recently, in a clinical study, Murray *et al*[11]demonstrated that patients found to have mesenteric ischemia at laparotomy had significantly elevated D (-)-lactate levels in peripheral blood as compared with the patients with other acute or normal abdominal conditions. Thus, circulating D (-)-lactate could aid in diagnosing acute mesenteric ischemia[5]. From the current experiment, it is clearly revealed that marked elevation in plasma D (-)-lactate occurred during the occlusive episode, which had a tendency to further increase after release of the clamp and was correlated with a progressive disintegration in the histology of the small bowel in response to reperfusion injury. Therefore, plasma D (-)-lactate appears to be a useful marker reflecting gut barrier damage in the setting of acute ischemia-reperfusion insult.

The mechanisms by which intestinal ischemia-reperfusion leads to a sustained elevation in plasma D (-)-lactate are not entirely understood from this experiment; although, it is possible that acute intestinal insult might cause substantial mucosal injury and increase permeability, thereby inducing an efflux of bacteria and their metabolic products into the portal circulation. In this study, the pathological examination showed marked intestinal lesions during ischemia, which became worse upon reperfusion. Another possible explanation would be that an increased gut permeability might attribute to release of inflammatory mediators, particularly tumor necrosis factor, which is considered to be induced by gut-derived endotoxin/bacteria, as well as hypoxia. These mediators may directly damage the mucosal barrier function[6]. In addition, it has been reported that ischemia-reperfusion injury to the intestine is associated with overgrowth of the residing microbial flora, and normally low plasma D (-)-lactate levels could be elevated markedly as a result of increased release of D-lactate by bacterial proliferation concomitant to increased mucosal permeability. Our previous observation that the overgrowth of Gram-negative bacteria in the intestinal tract was associated with an increase in intraluminal bacterial products, such as endotoxin following hemorrhage and resuscitation[3], gives further support to this opinion. Finally, since D-lactate is not metabolized in the liver; persistent elevation of D-lactate level in both portal and systemic circulation systems following intestinal ischemia-reperfusion injury resulted in similar D (-)-lactate concentration in the peripheral and portal vein throughout the observation period. Therefore, our data might be of potential importance, serving as the experimental basis for the favorable clinical use of D (-)-lactate in the diagnosis of acute intestinal disorders related to ischemia-reperfusion injury.

In summary, these data suggest that acute intestinal ischemia is associated with failure of the mucosal barrier resulting in increased plasma D (-)-lactate levels in both portal and systemic blood, and it is also remarkably enhanced by the subsequent reperfusion. Plasma D (-)-lactate may be a useful marker of intestinal injury following both ischemia and reperfusion insult.

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ORIGINAL RESEARCH

Establishment and application of an experimental model of human fetal hepatocytes for investigation of the protective effects of silybin and polyporus umbellalus polysaccharides

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Author contributions: All authors contributed equally to the work.

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Abstract

AIM: To establish a new experimental model system of human fetal hepatocytes to study the mechanisms underlying the protective effect of silybin and polyporus umbellalus polysaccharides (PSP) on the cellular ultrastructure.

METHODS: Human fetal hepatocytes were obtained from the liver of a human fetus that resulted from a medically necessary induced labor; the mother provided informed consent for sampling, experimental use and publication of findings. The hepatocytes were cultured and then pretreated with silybin or PSP or without either (control), after which the treated cells were exposed to CCl_4 for 4 h. Changes in cellular ultrastructure were observed by scanning electron microscopy and transmission electron microscopy, and changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST) and superoxide dismutase (SOD) were assayed.

RESULTS: Levels of ALT and AST were significantly decreased, and level of SOD was elevated in the two pretreatment groups following CCl_4 exposure, as compared to the control group. The cellular integrity and ultrastructure were well preserved in the two pretreatment groups but were seriously damaged in the control group.

CONCLUSION: The CCl_4-induced hepatotoxic cell model system of human fetal hepatocytes is an effective tool for studying the hepatoprotective effect of drugs and may be applicable for studies to screen medicines for treatment of hepatitis.

Key words: Fetal hepatocytes; Experimental model; Silybin; Polyporus umbellalus polysaccharides

Wang MR, Le MZ, Xu JZ, He CL. Establishment and application of an experimental model of human fetal hepatocytes for investigation of the protective effects of silybin and polyporus umbellalus polysaccharides. *World J Gastroenterol* 1997; 3(4): 228-230 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/228.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.228

INTRODUCTION

The protective effects of drugs against hepatic injury, such as that induced by viruses or chemical substances, were traditionally studied by using animal model systems. The animals — most frequently dogs or rats — were pretreated with the drugs under investigation either by oral administration or injection and then administered hepatotoxic substances — usually CCl_4 or D-galactosamine[1]. The protective effects of the drug were then determined by biochemical assay of serum and the pathological changes observed in the liver. However, there are some drawbacks to the animal-based approach, including a long experimental period and complicated procedure and inconsistent results due to the inherent individual differences of the animals and animal-human interspecies differences. Effects of a drug on an animal's liver may not precisely or comprehensively reflect effects on the human liver. Moreover, the animal models are limited in their ability to indicate whether any protective effects of a drug result from a direct or an indirect action, specifically because the protective effects of a drug on liver can be produced by stimulating the humoral factors. In order to overcome each of these challenges of the animal model system approach, we established a model system based on primary culture of human fetal hepatocytes and the applied it to the study of the protective effects of silybin and polyporus umbellalus polysaccharides (PSP) on liver using scanning and transmission electron microscopy (SEM and TEM, respectively).

MATERIALS AND METHODS

Isolation and culture of hepatocytes

Hepatocytes were obtained from the liver of a human fetus that had resulted from a medically necessary induced labor; the mother provided written informed consent for sampling, experimentation and publication of the related findings. The hepatocytes were isolated by means of the modified collagenase method[2]. The viability of the final parenchymal cell suspension was determined by

Table 1 Levels of alpha-fetoprotein and transaminase in culture medium at different culture times (x ± s)

	n	Activity (U)		
		1 h	16 h	24 h
aFP/ng·L^{-1}	3			18.3 ± 2.0
ALT/IU·L^{-1}	3	8.2 ± 2.3	10.0 ± 1.6	12.0 ± 2.1
AST/IU·L^{-1}	3	10.3 ± 1.6	12.4 ± 1.8	13.5 ± 1.5

Table 2 Alanine aminotransferase and aspartate aminotransferase activity in culture medium of hepatocytes (x ± s)

Group	*n*	ALT activity (U)			AST activity (U)		
		1 h	4 h	16 h	1 h	4 h	16 h
Control	3	40 ± 3.1	63 ± 2.5	71 ± 3.0	108 ± 5.6	165 ± 4.5	178 ± 2.4
PSP (0.5 g·L^{-1})	3	48 ± 2.2	35 ± 2.5	27 ± 3.4	95 ± 2.8	126 ± 2.3	101 ± 3.1
Silybin (0.5 g·L^{-1})	3	40 ± 2.5	46 ± 3.0	34 ± 3.3	93 ± 3.2	143 ± 4.0	123 ± 3.8

Table 3 Superoxide dismutase level in the culture medium of hepatocytes (x ± s)

Group	*n*	SOD (nmol/L)		
		1 h	4 h	16 h
Control	3	2.3 ± 0.8	3.0 ± 0.6	3.3 ± 1.2
PSP (0.5 g·L^{-1})	3	3.1 ± 1.8	4.8 ± 1.6	7.9 ± 2.1
Silybin (0.5 g·L^{-1})	3	2.4 ± 1.6	3.7 ± 1.1	5.9 ± 0.7

trypan blue exclusion assay, and found to be > 90% with a < 5% contamination of non-parenchymal cells. The cells were seeded at a density of 1 × 10^{12}·L^{-1} into 24-well plates (Nuclon) and cultured at 37 ℃ in a humidified atmosphere of 5% CO_2 in 2 mL RPMI-1640 (pH 7.0, Gibco). Each well was then supplemented with 4 mmol·L^{-1}-L-glutamine, 10% fetal calf serum, penicillin (at 100 IU/mL) and streptomycin (at 100 μg/mL).

Determination of bioactivity of the cultured hepatocytes

The medium was exchanged at 4 h after plating, and levels of alpha-fetoprotein (aFP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the medium were measured at 1, 4 and 16 h of culture.

Exposure of hepatocytes to CCl_4

Ten hours after the first medium change, a co-culture of silybin or PSP (or normal saline, for a control group) was started with hepatocytes at a final concentration of 0.5 g·L^{-1}. The hepatotoxic injury was then induced at 1, 4 and 16 h by addition of 1 mol·L^{-1} CCl_4 directly into the medium, with hepatocytes at a final concentration of 20 μg·L^{-1}. After 4 h, the levels of ALT, AST and superoxide dismutase (SOD) in culture medium were measured and the cells were collected for SEM and TEM.

Preparation of hepatocytes for electron microscopy

The primarily cultured hepatocytes were collected for SEM and TEM observation after 4 h exposure to CCl_4 for the groups pretreated with silybin or PSP and the normal saline control group. The methods for the hepatocyte preparation were carried out as previously described[3]. The changes of hepatocytes were observed under scanning (Philips SEM-50) and transmission electron (Japanese H-300) microscopes.

RESULTS

Bioactivities of isolated hepatocytes

The isolated human fetal hepatocytes possessed good bioactivities, as evidenced by the detection of aFP in medium after 24 h of culture. The levels of ALT and AST were lower in the culture medium (Table 1) and the hepatocytes were less damaged than in the control group, and showed normal integrity of cellular membrane under SEM (Figures 1 and 2).

Activities of transaminases and SOD in culture medium

As shown in Tables 2 and 3, the activity of ALT and AST in the medium was significantly decreased and the level of SOD was elevated in the silybin and PSP pretreatment groups as compared with the control. The affects peaked at 16 h for the cultured hepatocytes pretreated with silybin and PSP.

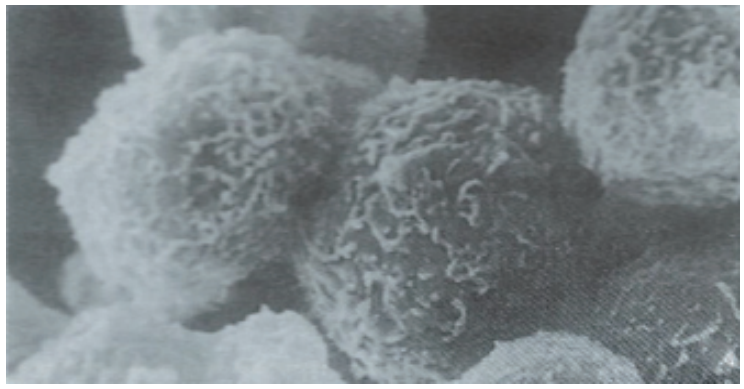

Figure 1 Normal human fetal hepatocytes. The cellular membrane is intact and microvilli can be clearly seen. × 7500

Figure 2 Normal human fetal hepatocytes. × 4500

Ultrastructural changes of human fetal hepatocytes

Ultrastructure of the human fetal hepatocytes that had been pretreated with silybin or PSP remained well preserved following exposure to CCl_4, as compared with that of the control group. The control group showed a remarkable amount of dead cells and irregularly shaped hepatocytes (Figure 3), with the cellular membranes showing mesh-like appearance. In the silybin (Figure 4) and PSP pretreatment groups (Figure 5), the surface structure of the cellular membrane was slightly damaged and the morphological integrity was indistinguishable from that of the normal fetal hepatocytes (Figure 1). In the control group, most hepatocytes showed necrosis and serious damage to the cellular membranes (Figure 6). In contrast, the hepatocytes pretreated with silybin or PSP appeared generally similar to the normal fetal hepatocytes (Figure 2), with only a few showing swelling of the mitochondria, increased chromatin, reduced glycogen granules and slight dilatation of endoplasmic reticulum (Figures 7 and 8).

DISCUSSION

In this study, the hepatoprotective effects of silybin and PSP were demonstrated using the newly established experimental model system based on human fetal hepatocytes with toxic injury induced by CCl_4 exposure. In particular, pretreatment with silybin or PSP protected the ultrastructure of the hepatocytes CCl_4-induced damage. Moreover, the pretreated cells showed lower transaminases and higher SOD in the culture medium. Thus, these two drugs were able to effectively protect the cultured primary human fetal hepatocytes from chemical injury of CCl_4. The lower transaminase activity in the culture medium may have been due to the preserved integrity of the cellular membrane system and/or of the mitochondria. The preserved integrity itself may have been brought about by the elevated SOD, which normally acts to prevent cellular membrane damage caused by free radicals.

To date, there has been no report of an experimental model system using primary cultured human fetal hepatocytes to study

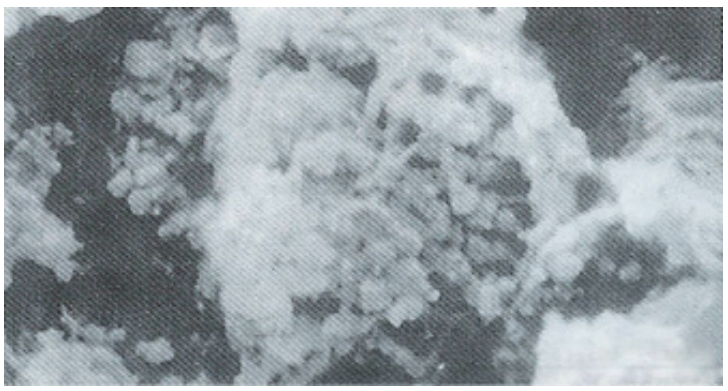

Figure 3 Hepatocytes in the control group following exposure to CCl_4. The cellular membrane appears mesh-like. × 10500

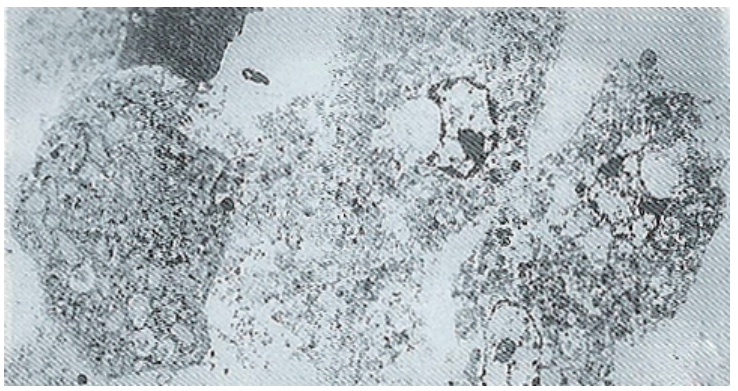

Figure 6 Hepatocytes in the non-pretreated control group after exposure to CCl_4. × 4500

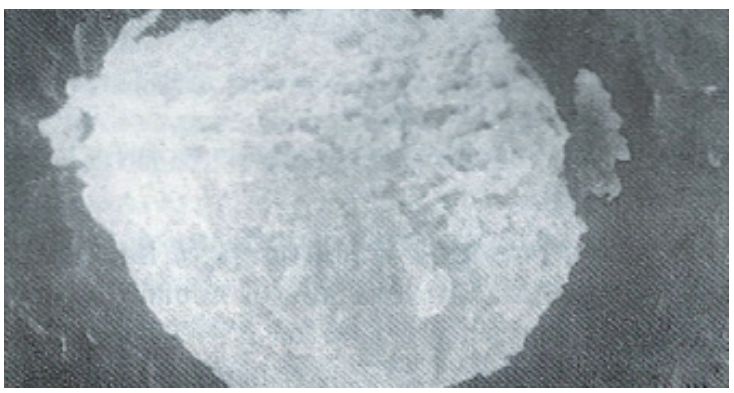

Figure 4 Hepatocytes pretreated with silybin and exposed to CCl_4. The changes of the cellular membrane are similar to Figure 5. × 8000.

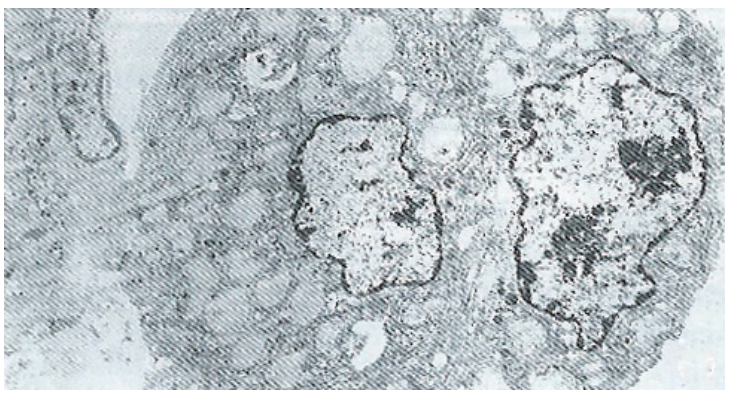

Figure 7 Hepatocytes in the PSP pretreatment group. The ultrastructure is well preserved. × 4500

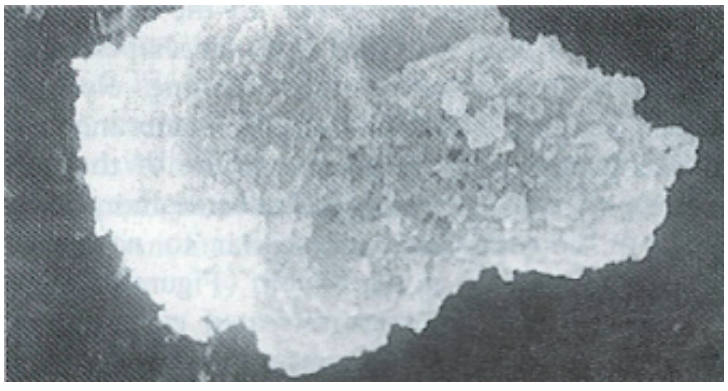

Figure 5 Hepatocytes pretreated with PSP and exposed to CCl_4. The cellular membrane is intact and microvilli swelling is present. × 7500

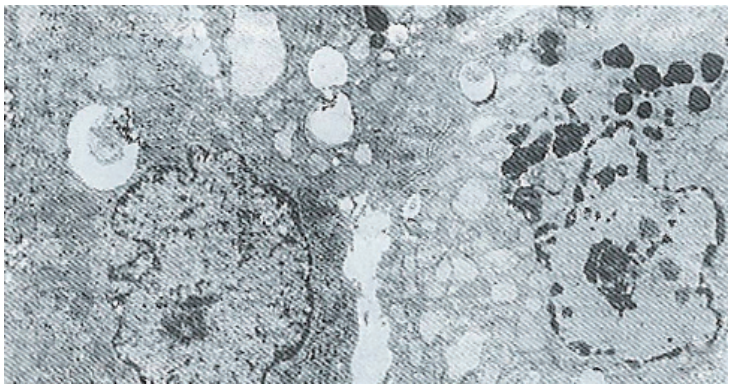

Figure 8 Hepatocytes in the silybin pretreatment group. The changes are similar to those in Figure 7. × 4500.

the hepatoprotective effects of drugs using assessment by SEM and TEM. The design of this model system was thought to be reasonable. The parenchymal hepatocytes were isolated from the liver of a human fetus by using collagenase digestion, and this process provided a high purity and good bioactivity, with sensitivity to CCl_4. Cell systems offer many advantages as compared to whole animal model systems. The experimental time in the cell system is shorter, the procedures are simpler, and the cost is lower. Additionally, there exists no cellular individual difference between the hepatocytes used in experimental group and control group because they are of the same origin; thus, the results are more reliable. Moreover, the results of silybin and PSP treatment of the human fetal hepatocytes in this study were similar to those observed with silybin and PSP treatment of whole animals[4,5]. Therefore, this model system will be useful for studying the effects of drugs on human liver cells.

The model system established in this study could also be applied to the following studies: comparative analysis of several drugs on human hepatocytes at the same time, due to large amounts of hepatocytes being obtained from a single liver from a human fetus; study of the mechanisms and the localization of the action of medicines against hepatitis by observation of the ultrastructural changes of hepatocytes combined with biochemical and enzymological changes in culture medium; and screening of new drugs against viral hepatitis or alcoholic hepatitis or cirrhosis[2].

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ORIGINAL RESEARCH

Comparative study of different interventional therapies for primary liver cancer

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Abstract

AIM: To compare the therapeutic effect of three types of interventional management for primary liver cancer.

METHODS: A total of 468 patients with primary liver cancer were randomly allocated to the following three groups: 138 cases treated with chemotherapy alone using mitomycin C, adriamycin and 5-FU (group A); 158 cases treated with chemoembolization using lipiodol (group B); and 172 cases with chemoembolization using lipiodol and gelfoam (group C). All patients were angiographically and sonographically followed-up.

RESULTS: In group C, 67.5% patients had AFP value decreased by > 50%, which was much higher than the 43.3% in group B and 32.2% in group A. Tumor size reduction by ⩾ 50% occurred in 20.3% of patients in group A, 41.2% of patients in group B and 44.8% of patients in group C. The intergroup differences between group A and group B or C were significant ($P < 0.01$). The 1-year and 3-year survival rates were 20.5% ± 3.6% and 1.9% ± 2.4% for group A, 51.3% ± 4.4% and 10.1% ± 4.9% for group B, and 63.0% ± 2.4% and 13.9% ± 5.0% for group C, respectively. The differences between all three groups were significant ($P < 0.05$). The mean survival time for patients in groups A, B and C were 9.6 mo, 16.1 mo and 17.9 mo, respectively.

CONCLUSION: Chemoembolization with lipiodol and gelfoam was the most effective therapy for primary liver cancer in this study. The position of the embolization should be far and middle sections of the hepatic artery, and the proximal section should be reserved as the route of the next intra-arterial chemoembolization.

Key words: Liver neoplasms/therapy; Chemoembolization; Therapeutic; Mitomycine; Adriamycin; Lipiodol; 5-fluorouracil gelfoam

Liu Q, Jia YC, Tian JM, Wang ZT, Ye H, Yang JJ, Sun F. Comparative study of different interventional therapies for primary liver cancer. *World J Gastroenterol* 1997; 3(4): 231-233 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/231.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.231

INTRODUCTION

In recent years, interventional therapies have been widely applied to the treatment of inoperable patients with middle and late stage liver cancer. These therapies include, but are not limited to, simple intra-arterial drug infusion, lipiodol embolization, gelfoam embolization, and microsphere chemoembolization. The literature has not provided definitive evidence as to which modalities are most efficacious. In order to summarize the experience in this field, we analyzed and compared our data as follows.

MATERIALS AND METHODS

Over the past 4 years, we have treated nearly 1500 cases of liver cancer with 2764 transcatheter chemotherapies. In order to analyze the results of these collective cases more reliably, the cases from the most recent 1-year period were excluded and only cases with complete data were included. A total of 468 cases with complete data were analyzed comparatively; among these, 73 had been confirmed by surgery, 17 by puncture biopsy, and all others diagnosed by findings from AFP testing and/or B-ultrasound, CT and angiography examination. The 468 cases were divided randomly into the following three groups: 138 cases treated with simple intra-arterial drug infusion (TAI, group A), 158 cases treated with TAI combined with lipiodol embolization (TAI-Lp TAE, group B), and 172 cases treated with TAI-Lp TAE plus gelfoam embolization (TAI-Lp Gs-TAE, group C). In group C, 22 patients received microcapsule embolization (containing drugs). The three groups of patients were similar in clinical characteristics.

Celiac angiography was first performed according to Seldinger's method for all patients to determine the arterial distribution and tumor location, size and type. Then, the catheter was inserted into the feeding artery and infusion or embolization was performed. All patients were treated with ADM 30-50 mg, MMC 20 mg and 5-FU 1000 mg simultaneously. Occasionally, CP 200 mg was used instead of the MMC. Lipiodol 10-30 mL mixed with anti-cancer drugs was infused. Finally, 1-2 mm^3 particles of gelfoam sponge were added to the contrast medium and infused slowly.

Table 1 Changes in AFP level *n*(%)

Group	Positive	No. of comparison	Decreased		No change	Increased
			⩾ 50%	25%-50%		
A	89 (64.5)	59 (66.3)	19 (32.2)	9 (15.3)	15 (25.4)	16 (27.1)
B	102 (46.6)	79 (77.5)	34 (43.0)	12 (15.2)	10 (12.7)	23 (29.1)
C	104 (60.5)	83 (79.8)	56 (67.5)	10 (12.1)	4 (4.8)	13 (15.7)

Table 2 Changes in tumor size after treatment *n*(%)

Group	Reduced		No change	Increased > 25%
	⩾ 50%	25%-50%		
A	28 (20.3)	14 (10.2)	72 (52.2)	24 (17.4)
B	54 (41.2)	28 (17.7)	58 (36.7)	18 (11.4)
C	77 (44.8)	34 (20.0)	57 (33.1)	4 (2.3)

Table 3 Survival rate ± standard error *n*(%)

Year	Group A	Group B	Group C
1	20.5 ± 3.6	51.4 ± 4.4	63.0 ± 3.9
2	5.6 ± 2.9	18.1 ± 4.5	26.6 ± 4.2
3	2.0 ± 2.4	10.1 ± 4.9	13.9 ± 4.7

$P < 0.01$, group A *vs* groups B and C; $P < 0.05$, group B *vs* group C.

RESULTS

Angiographic results allowed for tumor types to be classified as single nodules, multiple nodules, massive (5-10 cm), gigantic (>10 cm), gigantic plus multiple nodules, and diffuse types. There was no obvious difference among the groups.

The criteria of improvement after the treatment of liver cancer were decreased AFP value, reduced tumor size, and prolonged survival. The therapeutic effects for the 3 groups are shown in Tables 1-3. The mean survival period of group A was 9.6 mo, of group B was 16.1 mo and of group C was 17.3 mo. The difference was statistically significant between group A and group B or C ($P < 0.01$), but not between group B and group C.

DISCUSSION

Interventional treatment of liver cancer in our hospital has advanced remarkably over the most recent decades. Early on, the fear of serious complications related to the embolization procedure and a lack of practical skill in application of the technique meant that most patients were offered only intra-arterial infusion chemotherapy. No patient with artery to portal vein fistula or cancer embolus in the portal vein was treated by embolization. With accumulated practical experience, however, the scope of embolization grew and our practitioners became comfortable with learning and adopting new methods. As a result, curative outcomes were increased obviously.

Changes of AFP value

Serum AFP value is one of the most important standards for evaluating the therapeutic effects. The positive rates among the three groups in the current study were 60%-65%. Among the cases receiving more than 2 times of treatment, the cases whose AFP value decreased by > 50% represented 67.5% of group C, which was much higher than the 43.0% of group B and 32.2% of group A. AFP levels reduced < 25% or increased < 25% were regarded as unchanged, cases of which were the lowest in group C (4.8%) and the highest in group A (25.4%). The total rate of cases with decreased was 47.5% in group A, 58.2% in group B, and 79.5% in group C, which was in general agreement with the results previously reported for our home country[1,2] and abroad[3]. High or low value of AFP was not compatible with the reduction of tumors. Four cases were found in our series in which the tumors decreased obviously but the AFP increased rapidly in a short period of time after embolization.

Changes of tumor size and necrosis rate

Generally, all tumors show a reduction or necrosis (to varying degrees) after embolization chemotherapy. The effective rates of therapy for groups A, B and C were 20.3%, 41.2% and 44.8% respectively, which were similar to the results reported by Imaeda *et al*[3] in which 6% of the TAI group and 36% of the TAE group had tumors reduced by > 50%. There were only three cases with tumors disappearing completely (complete remission, CR) in our series and all the tumors were single nodule and had diameters of < 5 cm (Figures 1 and 2). It is uncommon for massive or gigantic tumors (> 5 cm in diameter) to disappear completely after treatment. In our study, these tumors generally showed necrosis of the central area and reduced tumor blood vessels (Figures 3 and 4). The scope of necrosis varied greatly among the different therapies. Among the 22 cases examined pathologically in our hospital[4], the tumor necrosis rate was 30.0% ± 28.3% for the 7 cases with simple MMC TAI, and necrosis of > 50% accounted for 28.6% of these; the rates in the other groups were 81.4% ± 28.3% and 85.7% respectively for the 7 cases with MTXmc TAE and 25%-75% for the 8 cases with MMC-TAI-Lp-TAE. Takayasu *et al*[5] also compared the effects of different therapies and reported that simple Lp TAE in 6 cases had almost no treatment significance, but showed value for position diagnosis, ADM TAI-Lp-TAE in 15 cases had some effects but not obvious, ADM TAI-Lp Gs TAE in 10 cases gave a complete necrosis rate of 83% for the main tumors and 53% for the daughter nodules. The results showed that lipiodol itself was not a good embolization agent but was a carrier that delayed drug excretion, thereby enhancing therapeutic effects. Therefore, gelfoam or microcapsule chemoembolization was the most effective therapy.

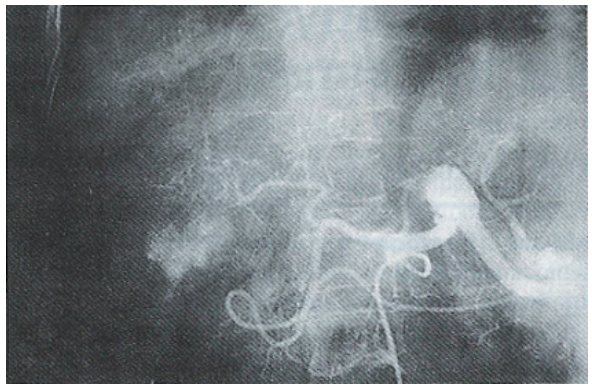

Figure 1 Primary liver cancer of the right lobe, with a hypervascular area diameter of 3 cm before treatment.

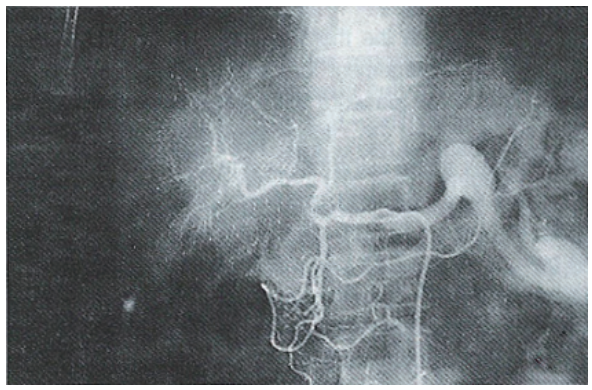

Figure 2 The same case as in Figure 1, showing the tumor stain having disappeared after the 6th treatment.

Survival rate and period

Therapeutic effects are closely related with treatment methods. Hirai *et al*[6] reported that the 1-year survival rates for 191 cases with TAI and 187 cases with TAI-Lp TAE were 22.0% and 66.2% respectively. In the report by Ohishi *et al*[7], the 1-year survival rates for 181 cases with TAI-Gs-TAE and 547 cases with TAI-Lp Gs-TAE were 44.0% and 60.4% respectively. In our series, the 1-year survival rates for groups A, B and C were 20.5%, 51.3% and 63.0%. Imaoka *et al*[8] reported that 146 patients with liver cancer tumors of < 3.6 ± 2.3 cm in diameter who received interventional treatment before operation had a 4-year survival rate of 53% in the TAI group,

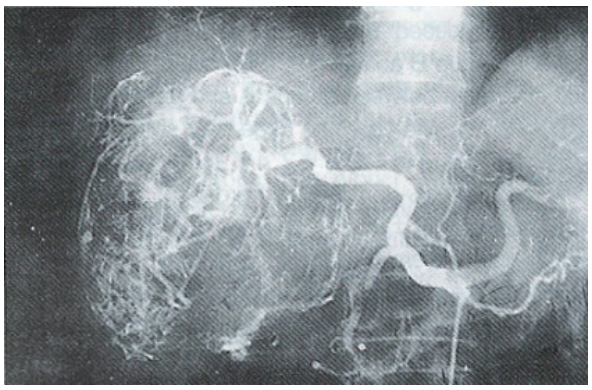

Figure 3 Celiac angiogram of gigantic nodular HCC with 12.5 cm diameter in the right lobe before treatment.

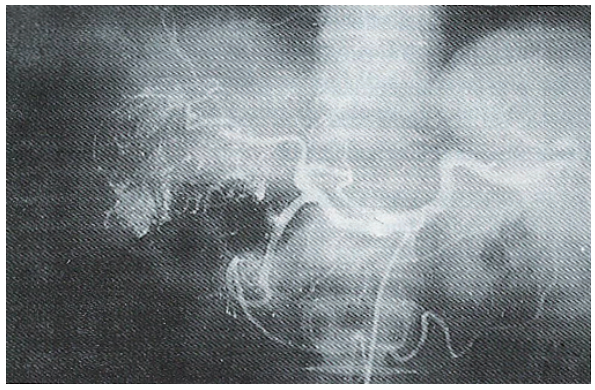

Figure 4 The same case as in Figure 3 after four treatments, showing necrosis in the center of the tumor and reduced presence of new vessels.

66% in the TAI-Lp Gs-TAE group and 83% in the sandwich therapy group. The survival period of the group with simple TAI was about 6 mo. Imaeda *et al*[3] reported that the mean survival period of the TAI group was 8 ± 0.6 mo and of the TAE group was 13.7 ± 0.7 mo, while the mean survival period of groups A, B and C in our series were 9.6, 16.1 and 17.9 mo respectively, with the difference being significant ($P < 0.01$).

The above data shows that the survival rate of patients treated with intra-arterial drug infusion is higher than that of patients treated with systemic chemotherapy (1-year survival rate of 5.4%), that the TAI-Lp TAE is better than simple TAI, that the effect of TAI-Lp Gs-TAE is better than the former methods, and that much better effects are achieved when sectional sandwich therapy is used, i.e. embolization of the peripheral liver artery with Lp, infusion of the anti-cancer drugs and embolization with Lp and gelfoam. Why are compound embolization chemotherapy and the sandwich method most effective? To answer this question, it is first necessary to explain it in terms of the microcirculation and physiological anatomy of liver[9]. The hepatic artery and portal vein are parallel and divided into two branches. The segment below the 8th class is segmentulum and the 12th class is the terminal hepatic arteriole (THA) of the classic lobule. The inner diameter of the THA is 20-50 μm, and that of the terminal portal vein (TPV) is 35 μm. The pressure of THA is 30-35 mmHg, which is 8 times that of TPV. Because the pressure difference between THA and TPV is high, drugs and Lp fill the THA and sinusoid, and flow reversely into the TPV when they are used at a volume exceeding a certain level, so both the THA and TPV are chemoembolized. Gelatin sponge particles are also injected into the small arteries to achieve the synergic effect of microtumor necrosing completely.

Traditionally, embolization was divided into the central and peripheral type. Arteries of the two far sections belonged to the peripheral type, and peripheral arteries constituted over 10 sections. We consider that the range of the peripheral type is too extensive, unfavorably limiting embolization. We suggest then a classification for far, middle and proximal sections, with class 7 and above being the far section (peripheral type), classes 3-6 being the middle section, and classes 1 and 2 being the proximal section or central type. In treatment of liver cancer, the better effect can be achieved if Lp or drug microcapsules are used first for embolization of the far section and then if gelfoam particles are used for embolization of the middle section. Large arteries of the proximal section are regarded as the route of intra-arterial chemoembolization and not appropriate for embolization.

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ORIGINAL RESEARCH

Anti-human AFP variant monoclonal antibody in radioimmunodetection of primary hepatocellular carcinoma

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Abstract

AIM: To investigate the affinity of AFP-R-LCA monoclonal antibody (AFP-R-LCA McAb) for AFP-positive primary hepatocellular carcinoma (HCC) cells.

METHODS: AFP-R-LCA McAb was labeled by ^{131}I. Eleven cases of HCC with AFP positivity, 6 with AFP negativity, and 4 with hepatitis B-related cirrhosis were investigated by radioimmunodetection.

RESULTS: The ^{131}I-AFP-R-LCA McAb immunoreacted with 9 of the HCC AFP-positive cases (9/11), but with none of the 6 AFP negative HCC cases or of the 4 cirrhosis patients. ^{131}I-AFP-R-LCA McAb at a small dose (7.4×10^7 Bq/300 μg) was associated with no side effects as determined by the liver function test, prothrombin time (Pt) test and thyroid gland function test ($P > 0.05$). Two cases of AFP-positive HCC were not imaged because of large tumor size (diameter > 10 cm) and higher AFP concentration in serum (20000 μg/L).

CONCLUSION: AFP-R-LCA McAb has a strong and special affinity to AFP-positive HCC cells and may be useful as a carrier for radioimmunodetection and radioimmunotherapy.

Key words: Liver neoplasms; AFP; McAb; Radioimmunodetection

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INTRODUCTION

Radioimmunodetection (RAID) and radioimmunotherapy using antibodies against tumor-associated antigens (TAA) are hot topics of tumor targeting research. Since the 1980s, several articles describing such anti-tumor monoclonal antibodies (McAb) have been published. Successful RAID and radioimmunotherapy with the anti-human AFP variant McAb (AFP-R-LCA McAb) against human hepatocellular carcinoma (HCC) were carried out in our laboratory using nude mice xenografts[1]. Based on our findings from those animal experiments, we next used the AFP-R-LCA McAb labeled by ^{131}I to immunodetect 17 human cases of HCC and 4 cases of hepatitis B-related liver cirrhosis.

MATERIALS AND METHODS

Cases and groups

HCC was diagnosed according to clinical findings from CT or MRI, B ultrasonography or AFP concentration in serum, and pathological examinations (after operation). Liver cirrhosis was diagnosed according to clinical findings from B ultrasonography, CT or MRI, liver function test, *etc*. The pre-RAID clinical characteristics are shown in Table 1. All patients included in this study had been admitted to our hospital between March 1, 1995 and September 1, 1996.

Preparation of ^{131}I-AFP-R-LCA McAb

The purification procedure for AFP-R-LCA McAb from ascites fluid was adopted from an earlier study[1]. Briefly, the monoclonal immunoglobulin IgG-containing ascites fluid was precipitated by saturated ammonium sulfate and the purified IgG was separated with a DEAE-Sephacel column. The monoclonal IgG was radioiodinated to a high specific activity with ^{131}I using the chloramine-T method. The product of ^{131}I-AFP-R-LCA McAb was isolated by a Sephadex-G column and then passed through a 0.22 μm filter to remove any bacterial contaminants. After cultivation and Limulus testing to ascertain the absence of bacteria and pyrogens, the product was prepared for clinical use. The labeling rates were 51% to 60%, and the specific activities were 0.11 to 0.33 GBq/mg.

Liver function, thyroid gland function, and prothrombin time (Pt)

Periphery vein blood was collected at 1-3 d before and 2 wk after the RAID.

Methods of RAID

Seven days before and 1 d before the RAID, patients were administered a compound iodine solution to block the thyroid gland. Thirty min before the RAID, patients were injected with 25 mg of phenergan intramuscularly. The radioiodine-labeled monoclonal antibodies, ^{131}I-AFP-R-LCA McAb, were injected intravenously through peripheral veins ($3.7\text{-}7.4 \times 10^7$ Bq/300 μg). The upper abdomen was scanned using emission CT (ECT ILC 3700; Siemens, Germany) and photograms were taken (Omega 500 γ camera) at 24 h, 48 h, 72 h, 120 h and 144 h later.

Table 1 Pre-radioimmunodetection clinical characteristics of hepatocellular carcinoma and control groups

Parameter	HCC group	Control group	
		AFP-negative HCC	Liver cirrhosis
Case	11	6	4
Sex, male/female	11/0	6/0	4/0
Median age in years	52	54	54
Age range in years	35-65	35-65	35-65
Tumor mass of ⩽ 10 cm	9	6	
Tumor mass of > 10 cm	2		
AFP of 2000-10000 μg/L	9		0
AFP of > 10000 μg/L	2		0

Table 2 Differences in liver function and Prothrombin time of patients with hepatocellular carcinoma or liver cirrhosis, before and after intravenous injection of 131I-McAb (x ± s)

Group	*n*	Liver function						Pt, *t*/*s*	
		TB, cB/μmol·L^{-1}		ALT, λB/nmol·S^{-1}·L^{-1}		A/G			
		Before	After	Before	After	Before	After	Before	After
AFP-positive HCC	11	8.1 ± 2.3	8.2 ± 2.1	756 ± 108	748 ± 112	1.4 ± 0.3	1.4 ± 0.2	11.0 ± 2.3	11.0 ± 2.2
AFP-negative HCC	6	6.2 ± 2.0	6.5 ± 2.1	781 ± 116	768 ± 105	1.4 ± 0.2	1.4 ± 0.4	13.0 ± 2.5	13.0 ± 2.6
Liver cirrhosis	4	7.1 ± 2.5	7.5 ± 2.4	914 ± 152	926 ± 146	1.3 ± 0.4	1.3 ± 0.5	12.0 ± 3.1	12.0 ± 3.2

ALT, alanine aminotransferase; HCC, Hepatocellular carcinoma; Pt, Prothrombin time; TB, Total bilirubin.

Statistical analysis

All the clinical parameters are expressed as x ± s, and the Student's *t*-test was used to determine statistical significance of differences.

RESULTS

The ^{131}I-AFP-R-LCA McAb (^{131}I-McAb) was detected in human HCC of 9 of 11 patients with positive AFP status. The imaging of tumors began at 72 h after intravenous infusion of ^{131}I-McAb. After 120 h, the tumor image became clear, but disappearing gradually at 144 h. Besides the detection in the tumor tissues, high concentrations of ^{131}I-McAb were detected in tissues of the heart and spleen. Two patients with AFP-positive HCC were not imaged, as 1 had a large tumor (diameter > 10 cm) and the other had an excessively high concentration of AFP in serum (> 100000 μg/L). There was no positive detection in any of the 6 HCC cases with AFP-negative status or in any of the 4 cases of liver cirrhosis.

Liver function and Pt

There were no obvious differences in liver function or Pt when the levels of before and after intravenous injection of ^{131}I-McAb were compared (Table 2).

Thyroid gland function

There were no obvious differences in thyroid gland function when the levels from before and after intravenous injection of ^{131}I-McAb were compared (Table 3).

The relationship between AFP level and radioimmunoimaging findings of the patients with HCC is presented in Table 4.

DISCUSSION

Radiolabeled antibodies against TAA are gaining acceptance as tools for the detection of neoplasms using the technologies of external scintigraphy and RAID[2,3]. Many reports of RAID for human liver neoplasms, carried out using radioiodine-labeled monoclonal antibodies[4,5], have appeared in the literature. Here, we describe our most recent study that successfully used ^{131}I-AFP-R-LCA McAb for RAID of human liver neoplasms.

We selected AFP-R-LCA McAb labeled by ^{131}I with intravenous delivery. After 72 h, tumor images were obtained, but the findings were more distinct after 120 h. Radioactivity of the tumor tissue and other organs in the area scanned by ECT showed that the tumor was more radioactive than the other tissues (with exception of the heart and spleen). Thus, AFP-R-LCA McAb showed a strong and special affinity for AFP-positive HCC cells. AFP-R-LCA McAb was detected in local area of tumors, with increased intensity over time (within 120 h).

Table 3 Differences in serum FT3 and FT4, before and after intravenous injection of 131I-McAb (x ± s, cB/pmol•L^{-1})

Group	*n*	FT3		FT4	
		Before	After	Before	After
AFP-positive HCC	11	5.2 ± 1.3	5.1 ± 1.2	15.2 ± 3.2	15.1 ± 3.4
AFP-negative HCC	6	5.6 ± 1.5	5.5 ± 1.4	16.2 ± 3.1	16.4 ± 3.0
Liver cirrhosis	4	5.4 ± 1.2	5.3 ± 1.3	15.4 ± 4.5	15.4 ± 4.1

HCC, Hepatocellular carcinoma.

Table 4 Relationship between AFP level and radioimmunoimaging findings of hepatocellular carcinoma patients

AFP, ρB/μg·L^{-1}	*n*	Positive imaging, *n*	Positive percentage, %
2000-10000	6	6	100
10000-100000	3	2	66.7
> 100000	2	1	50

The heart and spleen have a large blood supply, and the blood and spleen belong to the reticuloendothelial system and have a strong non-specific affinity to ^{131}I-AFP-R-LCA McAb. Therefore, the heart and the spleen showed densely under ECT imaging.

Two of the AFP-positive HCC cases in the current study were not imaged because of large tumor size (diameter > 10 cm) with poor blood supply or related necrosis and higher serum AFP concentration (200000 μg/L); these features can thwart ^{131}I-AFP-R-LCA McAb competitively to produce a large amount of immune complexes. The immune complex, itself, can hinder ^{131}I-AFP-R-LCA McAb from getting into the tumor area. Thus, a clear image depends not only on the TAA concentration in serum but also on the tumor's blood supply.

Goldenberg *et al*[6] reported that the serum concentration of AFP may have no influence on RAID, possibly because AFP on the surface of HCC cells is different from AFP circulating in the blood. This conclusion, however, is not consistent with ours.

Successful localization of a radioisotope-labeled monoclonal antibody to a tumor *in vivo* depends not only on the affinity of the antibody for the target cells but also on the speed with which the immunoconjugate passes through the physiological barriers (*i.e.* resistance of the blood vessel wall and phagocytosis by histiocytes) and the speed of its entering into the tumor tissues, as well as the antigen concentration in serum[7,8]. It is a generally accepted practice that tumor images be obtained at 72 h after intravenous infusion of the antibody ligand, but that more distinct images are found within 120 h. Our experimental result agreed with this.

In conclusion, AFP-R-LCA-McAb has a strong and special affinity to AFP-positive HCC cells. The detection of AFP-R-LCA-McAb in tumor tissues of HCC suggests its potential as a carrier for RAID and radioimmunotherapy.

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ORIGINAL RESEARCH

Comparative study on proliferation activity in small hepatocellular carcinoma related to hepatitis virus B and C

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Abstract

AIM: To compare the proliferation activity of small hepatocellular carcinoma (HCC) related to hepatitis B virus (HBV) and hepatitis C virus (HCV).

METHODS: Sixty liver biopsy specimens were obtained from patients with small HCC (≤ 3 cm in diameter) and examined immunohistochemically using anti-proliferating cell nuclear antigen monoclonal antibody. Of the 60 specimens, 30 were HBV-related and 30 were HCV-related. The 60 patients providing the samples for study were matched by sex and morphologic features of the HCC specimens.

RESULTS: The labeling index of proliferating cell nuclear antigen was 7.9% in the HBV-related HCC specimens and 12.5% in the HCV-related HCC specimens. There was no statistically significant difference between the two groups ($P > 0.05$).

CONCLUSION: In the early phase, or small stage, of HCC, HBV-related HCC shows similar proliferating activity to that of HCV-related HCC; this finding suggests that in the early phase, HBV-related HCC has similar malignancy to HCV-related HCC.

Key words: Liver neoplasms; Carcinoma; Hepatocellular; Hepatitis B virus; Proliferating cell nuclear antigen

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most common malignant tumors and its development is known to be closely associated with hepatitis B virus (HBV) infection and hepatitis C virus (HCV) infection[1,2]. However, no reports in the literature have yet described a comparative analysis of the tumor behaviors of these two kinds of HCCs.

Proliferating cell nuclear antigen (PCNA) is a nuclear protein related to cell proliferative activity[3], and its immunohistochemical detection is a useful adjunct to morphologic features, providing insight into the understanding of tumor behavior. In this study, we compared the proliferation activity of HBV-related small HCC with that of HCV-related small HCC by means of immunohistochemical staining using a monoclonal antibody against PCNA to provide information about their clinical features.

MATERIALS AND METHODS

Formalin-fixed and paraffin-embedded liver biopsy specimens were obtained from the archives of the Second Department of Internal Medicine at Kurume University School of Medicine, Japan. These specimens included 30 HBV-related HCCs (male:female ratio of 25:5; mean age of 57.6 years) and 30 HCV-related HCCs (matched by sex ratio and morphologic features; mean age of 60.1 years). The size of the tumors was < 3 cm in diameter and the number of tumors per patient ranged from 1 to 4. Histopathological diagnoses were made according to the General Rules for Cancer of the Liver (Liver Cancer Study Group of Japan)[4]. Of the 60 cases involved in this study, 32 were well-differentiated, 24 were moderately differentiated, and 4 were poorly differentiated.

Immunohistochemistry

Three μm thick sections were prepared from paraffin blocks and deparaffinized by soaking in a graded ethanol series, after which the processed sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Normal sheep serum was then applied for 20 min to reduce non-specific antibody binding. Monoclonal antibody to PCNA (clone 5A10) was applied as a 1:250 dilution and allowed to incubate for 1 h in a moist chamber. Biotinylated sheep anti-mouse IgG was then applied as the linker molecule and allowed to incubate for 30 min, followed by application of a streptavidin-horseradish peroxidase complex (Vector) and incubation for 1 h. All steps were carried out at room temperature and followed by washing in phosphate buffered saline (PBS). Diaminobenzidine-hydrogen peroxide was used as a chromogen and a hematoxylin counterstain was made. Sections were then processed in an alcohol gradient series, cleared in xylene, and mounted in DPX. A negative control was generated by replacing the primary antibody with PBS.

Assessment of PCNA

PCNA labeling indices were calculated after counting PCNA-positive nuclei per 500 nuclei in 2-5 fields of each HCC case at high power (× 200).

Table 1 Clinical parameters associated with prognosis

Parameter	HBV-related HCC	HCV-related HCC	*P* value
AFP in μg/L, mean	80.22	99.04	0.743
Survival duration in days, mean	1353.4	1341	0.947

Other clinical parameters

Alpha-fetoprotein (AFP) concentration and survival duration were analyzed retrospectively. Statistical analysis was made by Student's *t*-test.

RESULTS

Proliferating cell nuclear antigen LI

PCNA staining was confined to the nuclei. All identifiable staining was considered positive, regardless of the staining intensity. The PCNA LI was 7.9% in HBV-related HCCs and 12.5% in HCV-related HCCs. There was no statistically significant differences between these two groups ($P = 0.33 > 0.05$).

Other clinical parameters (Table 1)

DISCUSSION

HBV-related HCC and HCV-related HCC are clinically distinct. In the early phase, or small stage, of the HCC tumor development, these two kinds of HCC show similar growth rates, survival rates and prognosis, whereas in the advanced stage, the HBV-related HCC has poorer prognosis than that of HCV-related HCC (unpublished data). In this study, we retrospectively analyzed the survival duration of patients with small HCC who were treated with percutaneous ethanol injection therapy (PEIT) and confirmed that the survival duration was similar for the two forms.

Proliferation activity of tumors that is defined by PCNA immunohistochemical analysis has been reported to be related to metastatic potential, recurrence and overall prognosis[5,6]. Therefore, PCNA LI expression in the tumor reflects, at least partially, the degree of malignancy. The current study showed no significant difference in the expression of PCNA LI in patients with HBV-related small HCC and HCV-related small HCC; this finding suggests that in the early phase HBV-related HCC has similar malignancy to HCV-related HCC. It may therefore help to understand the clinical features of these two kinds of HCCs. Additionally, it has been previously reported that serum AFP values are not only of diagnostic value but also of prognostic significance in patients with HCC[7]. In the current study, we found that the serum AFP values were not significantly different between HBV-related small HCC and HCV-related small HCC. This finding is also in accordance with the clinical features of HBV-related HCC and HCV-related HCC in the early stage.

In conclusion, our results indicate that, in the early stage, the proliferation activity of HBV-related HCC is similar to that of HCV-related HCC. This information provides a better understanding of the clinical features of these two kinds of HCC.

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BRIEF REPORT

Effect of garlic on micronuclei frequency in peripheral blood lymphocytes of rats with N-methyl-N'nitro-N-nitrosoguanidine-induced gastric carcinoma and precancerous lesions

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Abstract

AIM: To investigate the effects of garlic on micronuclei frequency (MNF) in peripheral blood lymphocytes (PBLs) of Wistar rats with N-methyl-N'nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinoma (GC) and precancerous lesions (PLs).

METHODS: Wistar rats were exposed to MNNG at 1.25 mg/5 mL per day for 10 mo to induce GC and PLs (MNNG group, n = 30); rats not exposed to MNNG served as unmodeled controls (control group, n = 16). The MNNG rats were randomly divided into a prevention treatment group (n = 30; receiving 10 mL of a 10% garlic solution per day) and an untreated model control group (n = 20; receiving tap water). MNF in PBLs were detected at experiment months 10 and 16 mo by the microculture technique.

RESULTS: The MNNG group had similar MNF levels at months 10 and 16. Compared to the control group, the MNNG, prevention and untreated model control groups had remarkably higher MNF levels (P < 0.01). The level of PLs was significantly lower in the prevention treatment group than in the untreated model control group (P < 0.01). The prevention group showed significantly lower MNF than the MNNG group (P < 0.01), and the MNF level was reduced in month 16 compared to month 10 (P < 0.01). However, the difference in MNF levels in groups given prevention or treatment was not significant.

CONCLUSION: MNNG exposure exerted continuous mutagenicity and carcinogenicity properties on PBLs, and garlic exerted a remarkable anti-mutagenic and anti-carcinogenic effect. MNF in PBL may be a novel marker of early-stage GC.

Key words: Garlic/Pharmacology; Stomach neoplasms/zhongyi-yaoliaofa; Adenocarcinoma/Zhongyiyaoliaofa; Precancerous conditions/zhongyiyaoliaofa; Lymphocytes/Drug effects; Micronuclei/Drug effects

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ORIGINAL RESEARCH

A multicenter randomized study on Me-CCNU, 5-FU and ADM versus ACNU, 5-FU and ADM for treatment of advanced gastric cancer

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Abstract

AIM: To compare the efficacy of a combined chemotherapy regimen of 5-fluouracil (5-FU) and adriamycin (ADM) with nimustine hydrochloride (ACNU; brand name Nidran), a new nitrosourea agent, or with methyl-CCNU for advanced gastric cancer.

METHODS: One-hundred-and-three cases of advanced gastric cancer were randomly allocated into Group A (Me-CCNU, 5-FU and ADM combination) and Group B (ACNU, 5-FU and ADM combination). The quality of life (QOL) questionnaire, composed of 11 ordinal categorical items, was used to collect data from these patients.

RESULTS: Group A had no case of complete remission (CR) or partial remission (PR), while Group B had no CR but 8 PR (8/46 cases), for a response rate of 0% in Group A and 17.4% in Group B. The median survival time in Group A was 108 d and in Group B was 112 d. Both groups tolerated the treatment well and there were no serious adverse effects. QOL evaluations showed better psychological and physical feelings of tiredness for Group B than for Group A, and scores based on facial scaling showed a more pleasant inclination for the former.

CONCLUSION: ACNU combination is superior to the Me-CCNU combination for advanced gastric cancer patients.

Key words: Stomach neoplasms; Adriamycin; 5-fluouracil; Mitomycin C; Nimustine hydrochloride

Xiao SD, Li DH, Zhang DZ, Shen MJ, Zhu XT, He GF, Zhao TP, Xi LP, Deng XC, Wang M, Wang XL, Chen Q, Zhang YP, Yao CL, Bao JG, Tong GW, Zhu LF, Jiang H, Minoru K. A multicenter randomized study on Me-CCNU, 5-FU and ADM versus ACNU, 5-FU and ADM for treatment of advanced gastric cancer. *World J Gastroenterol* 1997; 3(4): 238-241 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/238.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.238

INTRODUCTION

In the 1960s, combined chemotherapy regimens with 5-fluouracil (5-FU), adriamycin (ADM) and mitomycin C, known as FAM, and with 5-FU, ADM and methyl (Me)-CCNU, known as FAMe, were regarded as effective for the treatment of advanced gastric cancer. However, later massive serial studies of FAM showed low response rates, namely 33% for FAM[1] and 18% for FAMe[2].

ACNU or nimustine hydrochloride (brand name Nidran, manufactured by Sankyo Co. Ltd., Japan) is a new nitrosourea antitumor agent applicable to various malignant tumors. It becomes even more effective when used in combination with other chemical agents. The Shanghai nimustine hydrochloride (ACNU) Cooperative Study Group carried out a randomized controlled study on Me-CCNU, 5-FU and ADM combination versus ACNU, 5-FU and ADM combination for advanced gastric cancer, and evaluated the efficacies by observing response rates, toxicities, adverse effects, and quality of life (QOL).

Table 1 General data of evaluable patients in Groups A and B

Parameter	Group A	Group B
No. of cases	47	46
Sex, M	36	32
Sex, F	11	14
Age (yr), -40	4	7
41-50	9	4
51-60	8	14
61-70	16	17
71-80	10	4
Performance status grade 1	1	0
Performance status grade 2	5	11
Performance status grade 3	5	5
Performance status grade 4	35	26
Unclear	1	4
Histology		
Papillary adenocarcinoma	3	1
Tubular adenocarcinoma 1	4	8
Tubular adenocarcinoma 2	17	12
Low differentiated adenocarcinoma	13	8
Mucocellular carcinoma	2	1
Signet ring cell carcinoma	0	2
Others	8	14

MATERIALS AND METHODS

General data

A study on ACNU combination therapy versus Me-CCNU combination therapy was carried out by the Shanghai ACNU Cooperative Study Group from October 1992 through April 1994, using 103 patients with advanced gastric cancer. Among them, 102 cases were eligible and 93 of them had complete records; therefore, the participation rate was 90.3%. The patient data for age, sex, performance status (PS), and histological classification are presented in Table 1.

Patient population

Patients in the study had primary gastric cancers that were histologically confirmed by biopsy and no patient had received chemotherapy and/or radiotherapy prior to study enrollment. All patients had unresectable or recurrent primary foci with metastatic lesions. The age of the patients ranged from 35-75 years and had PS of 1-4. All of these patients had no active duplicate cancers, no severe disturbance in bone marrow, hepatic, renal or cardiac functions, and were able to be treated with oral medication.

Methods

Allocation Patients meeting the requirements of the study from Zeach were enrolled and their data obtained and recorded using standardized forms and telephone interview conducted by the cooperative study group office. After all the reported details were checked for comprehensiveness, the patient participants were randomly allocated into either treatment Group A or Group B.

Treatment methods Group A (control group) consisted of patients who received Me-CCNU 100 mg/d p.o. on day 1, ADM 30 mg/m^2 *i.v.* on day 1, and 5-FU 300 mg/m^2 *i.v.* from days 1-5. Group B (treatment group) consisted of patients who received ACNU 50-75 mg/d *i.v.* on day 1, ADM 30 mg/m^2 *i.v.* on day 1, and 5-FU 300 mg/m^2 *i.v.* from days 1-5. Thus, 5 d were taken as one course and successive courses were repeated at an interval of 4-5 wk. The total ADM doses did not exceed 500 mg/m2.

QOL questionnaire[3] Measurement of QOL was made by a self-assessment report by the patients. The patients themselves, their relatives, or care nurses filled in the QOL questionnaire once a week at regular intervals. Doctors were forbidden from filling in the questionnaire in order to avoid confounding by subjective assumption. The QOL questionnaire was composed of 11 ordinal categorical items, consisting of 3 basic items covering mental feelings (appetite, psychology and sleepiness), physical feelings (tiredness, pain and worry) and social relationships (family, friends or co workers).

QOL Questionnaire

Question 1. How good is your appetite?
a. Very good
b. Pretty good
c. OK
d. Not too good
e. Not good at all
Question 2. How well do you sleep?
Question 3. How often do you feel tired (psychological)?
Question 4. How often do you feel tired (physical)?
Question 5. How much and how often do you feel pain?
Question 6. How much does your family understand and support you?
Question 7. What is your relationship with your family and co-workers?
Question 8. How often do you worry about your disease?
Question 9. Would you like to continue the current therapy?
Question 10. How much help do you need to accomplish your daily activity?
Question 11. Please rate your overall condition today by marking 0 around the number.

Table 2 Courses of treatment in Groups A and B patients

Groups	No. of courses								Total
	1	2	3	4	5	6	7	8	
Group A	16	20	5	2	0	1	0	0	47
Group B	8	23	6	2	0	0	1	1	46
Total	24	43	11	4	0	1	1	1	93

Observations Blood cell counting was performed every week. Hepatic and renal functions were tested every 2 wk. Imaging examination, including barium X-ray surveys or endoscopic and ultrasonic B and/or CT scanning, were performed every 4-8 wk. Patients who had considerable adverse effects and could not proceed to a second course were given a barium X-ray check-up 4 wk after the first course.

Details of all examinations, as well as of any adverse effects, were carefully recorded by means of standardized forms. Each patient was followed-up until death.

Evaluation of efficacy A clinical evaluation committee was organized to estimate the following 4 items periodically in accordance with the Criteria of Evaluating the Efficacy of Chemotherapy for Solid Tumors proposed by the Japanese Research Society for Gastric Cancer: Qualification and completion of materials; extent of development of lesions and response to the treatment; survival time; and quality of life.

Statistics

The data of treatment efficacy was compared using the Chi-square test and Fisher's exact test. Principal component analysis and correlation analysis were used to assess the quality of life data.

RESULTS

Completion of courses

Each case underwent at least one course of combination therapy. In Group A 44 cases underwent a total of 85 courses, and in Group B 41 cases received a total of 95 courses (Table 2).

Efficacy

Complete response (CR) and partial response (PR) were regarded as effective. No CR occurred in either groups. As shown in Table 3, there was no PR in Group A (0/47 cases) and 8 in Group B (8/46; for a response rate of 17.4%, $P < 0.01$). The anti-tumor efficacy of the treatments in the two groups is shown in Table 4. The X-ray barium examinations of 2 PR cases before and after ACNU combination therapy are shown in Figures 1-4.

Survival time

In group A the median survival time from the beginning of the treatment was 108 d. Three out of 47 cases in Group A, survived > 1 year, with the longest survival time being 740 d (Case No. 36). In

Table 3 Data on partial remission cases

Case No.	Group	Sex	Age	Performance status	Pathohistology	No. of courses	Effective site
22	B	M	54	4	Tubular adenocarcinoma	4	Liver
35	B	M	46	4	Tubular adenocarcinoma	3	Stomach
45	B	M	65	4	Low differentiated adenocarcinoma	7	Liver
67	B	F	60	4	Low differentiated adenocarcinoma	4	Spleen
71	B	F	66	3	Tubular adenocarcinoma	3	Stomach
82	B	F	72	2	Low differentiated adenocarcinoma	2	Liver spleen
83	B	M	74	4	Tubular adenocarcinoma	8	Stomach
100	B	M	65	4	Tubular adenocarcinoma	3	Liver

Table 4 Anti-tumor effect in Group A and B patients

Group	A	B
Partial response (%)	0 (0%)	8 (17.4%)
No change (moderate response)	16 (1)	17 (2)
Progressive lesion	31	21
95% reliability	0, 6.2%	6.4%, 28.4%
Fisher's test	$P > 0.01$	$P > 0.01$
Median survival time	108 d	112 d
Average course	1.9	2.3

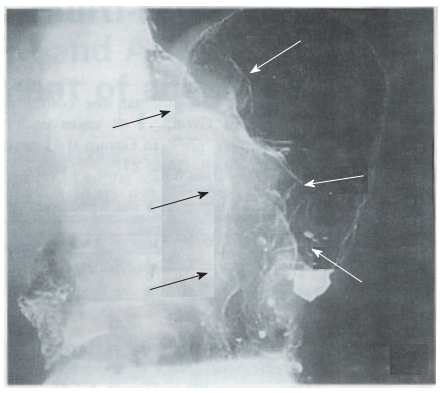

Figure 1 Huge filling defect found between the angulus and fundus.

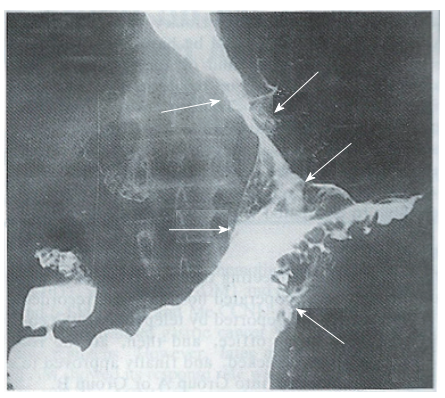

Figure 2 The lesion became remarkably smaller 3 mo after receipt of the combination chemotherapy with nimustine hydrochloride.

Group B the median survival time was 112 d. Of the 46 total cases, 6 survived > 1 year, with the longest survival time being 690 d (Case No. 53).

Toxicity

Both groups tolerated the respective treatment well and very few adverse effects were seen and those occurred only in the early days of chemotherapy. No remarkable leukopenia, thrombopenia or decreased hemoglobin occurred, and there were no serious impairments of hepatic, renal or cardiac functions (Table 5).

QOL

An overall investigation into appetite, sleepiness, tiredness (psychological and physical), pain, social relationships, mood, daily activities and facial expressions showed that the reports of psychological tiredness (Q3), physical tiredness (Q4) and facial expression (Q11) were better for Group B than for Group A (Figure 5).

Table 5 Adverse effects in Group A and B patients

	Group A		Group B	
	Cases	%	Cases	%
Loss of appetite	3	6.5	4	8.7
Nausea	3	6.5	3	6.5
Vomiting	9	19.6	8	17.4
Fatigue	1	2.2	1	2.2
Muscular disability	4	8.7	3	8.7
Alopecia	4	8.7	3	6.5
Fever	1	2.2	2	4.3
EKG abnormality	1	2.2	1	2.2
Leukopenia	2	4.3	5	10.9
Decrease of hemoglobin	1	2.2	0	0.0
Elevation of GPT	1	2.2	2	4.3
Total No. of side effects	30		33	
Total cases with side effects	23	48.9	22	47.8
Total cases without side effects	24	50.1	24	52.2

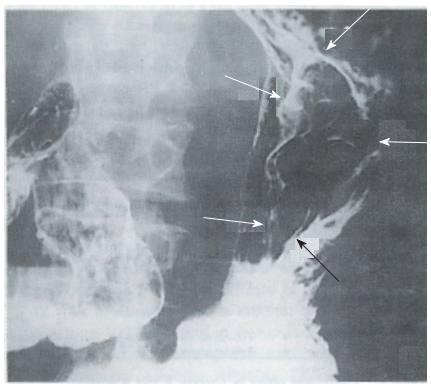

Figure 3 Filling defect in the upper part of the gastric corpus.

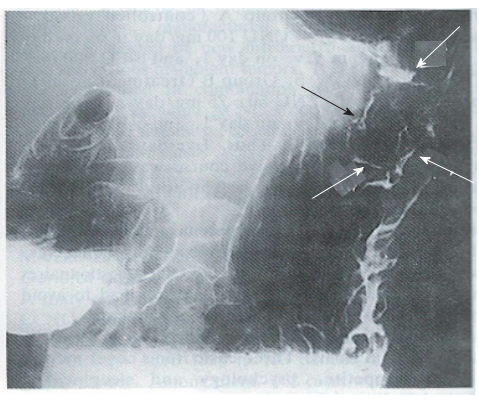

Figure 4 Nine months after combination chemotherapy, the lesion was much smaller.

DISCUSSION

ACNU is a newly developed nitrosourea anti-cancer agent. Different from other kinds of nitrosourea agents, it is water-soluble, capable of being administered through the vein and artery. Moreover, it is able to penetrate the blood brain barrier to reach into brain cancerous tissues and exert a full anti-tumor action. The mechanism of ACNU acts strongly across a broad spectrum against various tumors

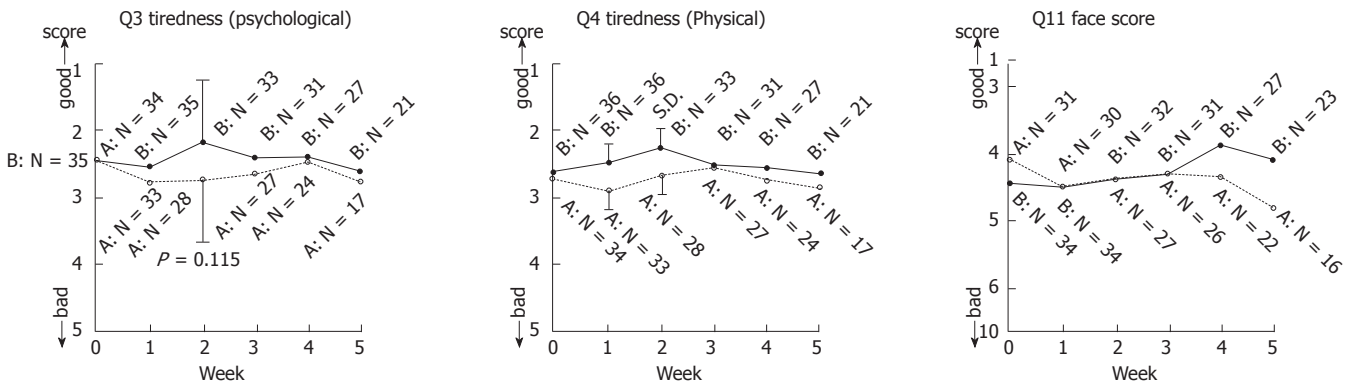

Figure 5 Comparison of psychological tiredness (Q3), physical tiredness (Q4), and face scores (Q11) between Groups A and B.

by way of alkylation of cellular DNA, which causes depolymerization of DNA and results in inhibition of DNA synthesis. Laboratory experiments showed that it could rapidly distribute systemically to organs including brain. Administration of ACNU to mice solid FM3A tumors inhibited tumor growth and reduced tumor size[5].

Our clinical study on advanced gastric cancer showed that the Group B treatment (ACNU combination) was significantly superior to the Group A treatment (Me-CCNU combination) in terms of efficacy, QOL, survival time, etc. As for survival time, 6 cases in Group B survived > 1 year, but only 3 cases did so in Group A. The median survival time was 112 d for patients in Group B, but 108 d for those in Group A. However, the survival time in neither group was long, which might be related to the fact that 70% of the cases had performance status of Grade 4 upon enrollment.

As to dosage of ACNU, 14 cases were given 50 mg/d, and 2 cases reached PR, accounting for a response rate of 14.3%. When the dosage was increased to 75 mg/d for 32 cases, 6 reached PR, accounting for a response rate of 18.3%. Neither dosage caused significant adverse effects. Therefore, we suggest that 75 mg/d should be used at the very beginning or at least on condition that no significant adverse effects appear, dosage could be adjusted to 75 mg/d. Moreover, an even higher dosage could be considered as an approach to yield more ideal results.

Recently QOL has become accepted worldwide as an important assessment tool for use in clinical practice. It is also regarded as an important factor for evaluating anti-cancer agents. The QOL questionnaire that we used had been proven clinically at the Tokyo Women's Medical College for its practicability, suitability and reliability. The current study proved that the overall QOL features of Group B were better than those of Group A, which is consistent with the results of efficacy of ACNU.

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ORIGINAL RESEARCH

Comparison of preoperative TN staging of gastric carcinoma by endoscopic ultrasonography with CT examination

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Abstract

AIM: To assess the accuracy and limitations of endoscopic ultrasonography (EUS) in the preoperative staging of gastric carcinoma in comparison with computed tomography (CT).

METHODS: According to the new (1987) TN staging, 62 patients with gastric carcinomas were examined preoperatively by EUS and the results compared with those of postoperative pathological TN staging. CT of abdomen was performed before surgery for 32 of the patients.

RESULTS: The overall accuracy of T staging was 83.9% for EUS and 28.1% for CT. For the detection of regional lymph node metastases, EUS accuracy was 79.0%, sensitivity 80.0% and specificity 87.5%, versus 50.0% accuracy for CT. The coincidence of perigastric infiltration was 90.0% for EUS and 41.2% for CT. The most frequent causes of misdiagnosis by EUS were microscopic tumor invasion and peritumorous inflammatory or fibrous changes.

CONCLUSION: EUS is a reliable method for the clinical evaluation of locoregional extension of gastric cancer and more accurate than CT in the preoperative staging of gastric carcinoma.

Key words: Stomach neoplasms/radiography; Stomach neoplasms/ultrasonography; Neoplasm staging; Tomography, X-ray computed; Endoscopy

Guo W, Zhang YL, Li GX, Zhou DY, Zhang WD. Comparison of preoperative TN staging of gastric carcinoma by endoscopic ultrasonography with CT examination. *World J Gastroenterol* 1997; 3(4): 242-245 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/242.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.242

INTRODUCTION

Gastric carcinoma is one of the most common malignant human tumors and carries a very poor prognosis with a 5-year survival rate of 6%-25%, depending on the tumor stage[1]. Surgical resection of the tumor and the involved lymph nodes remains the only historically proven curative treatment. An accurate preoperative staging of the most significant prognostic factors of gastric cancer, such as depth of invasion (T staging) and involvement of lymph nodes (N staging), would allow better planning of appropriate treatment. The decision as to which patients should either undergo primary operation or prior to chemotherapy, or should be treated by only palliative methods is greatly influenced by the results of preoperative tumor staging. The overall goal is to prevent under or overtreatment, to minimize the inherent morbidity and mortality rates.

Conventional endoscopy permits an examination of only the surface of the stomach, and important data, such as infiltration of the wall, local spread and lymph node involvement, are not provided by this technique. Computed tomography (CT) is currently used in the preoperative staging of gastric cancer, but the results are not completely satisfactory. The major difficulty encountered with CT is its inherent incapability for correct staging of the depth of tumor penetration of the gastric wall and its capacity for correct staging of regional lymph node involvement is also limited.

The purpose of our study was to assess the role of endoscopic ultrasonography, a relatively new modality, in the preoperative TN staging of gastric cancer.

MATERIALS AND METHODS

From February 1996 to February 1997, 62 patients (35 men and 27 women; from 27 to 76 years of age, with mean age of 61-year-old) with gastric cancer diagnosed by upper gastrointestinal endoscopy and biopsy underwent preoperative evaluation by endoscopic ultrasonography (EUS). CT of abdomen was performed before surgery for 32 of the patients. Tumors were located predominantly in the fundus and cardia region (n = 20), the body (n = 16) and antrum (n = 23), and finally throughout the whole stomach (n = 3). Fourteen of the patients had linitis plastica. All of the patients

Table 1 Relationship between endoscopic ultrasonography and anatomic layers in the normal gastric wall

EUS	Histology
1st hyperechoic layer	Water interface and superficial mucosa
2nd hypoechoic layer	Deeper mucosa
3rd hyperechoic layer	Submucosa
4th hypoechoic layer	Muscularis propria
5th hyperechoic layer	Subserosa, serosa and the interface echo

EUS: Endoscopic ultrasonography.

Table 2 TNM classification

T_1	Invasion of mucosa or submucosa
T_2	Invasion of muscularis propria or subserosa
T_3	Invasion of serosa
T_4	Invasion of adjacent structures
N_0	No lymph node metastasis
N_1	Metastasis in perigastric lymph node (s) within 3 cm of the edge of the tumor
N_2	Metastasis in perigastric lymph node (s) > 3 cm from the edge of the primary, or in lymph node along the left gastric, common hepatic, splenic or celiac arteries
M_0	No distant metastasis
M_1	Distant metastasis

Table 3 Accuracy of endoscopic ultrasonography in the preoperative T staging in 62 patients with gastric carcinoma

Histopathological T stage	*n*	EUS T stage (*n*)				EUS accuracy (%)	EUS overstaging (%)	EUS understaging (%)
		T_1	T_2	T_3	T_4			
T_1	5	5	0	0	0	100.0	0.0	0.0
T_2	11	0	9	2	0	81.8	18.2	0.0
T_3	24	0	2	18	4	75.0	16.7	8.3
T_4	22	0	0	2	20	90.9	0.0	10.0
Total	62					83.9	9.7	6.5

EUS: Endoscopic ultrasonography; T staging: Depth of invasion.

Table 4 Accuracy in the preoperative determination of the N stage in 62 patients with gastric carcinoma

Histopathological N stage	*n*	EUS N stage (*n*)			EUS accuracy (%)
		N_0	N_1	N_2	
N_0	32	28	4	0	87.5
N_1	18	5	13	0	72.2
N^2	12	1	3	8	66.7
Total	62				79.0

EUS: Endoscopic ultrasonography; N staging: Involvement of lymph nodes.

were operated on within 3 wk after the findings upon EUS and CT examination. No complication was encountered during this study.

CT was carried out with a Somatom Plus (Siemens Corp, Germany). Scanning of abdomen was done with 10 mm sections using intravenous and oral contrast.

EUS studies were made using an echoendoscope GF-UM20/EUM20 (Olympus Corp., Japan), which had a rotating 360° transducer with swithchable frequencies of 7.5 MHz and 12 MHz. The patients were examined in a left lateral position after oropharyngeal anesthesia and given premedication with 5 mg diazepam and 20 mg 654.2 intramuscularly. The ultrasonic endoscope was introduced and advanced into the stomach. The stomach was filled with 300-500 mL de-gassed water and the balloon filled with water. The transducer was pulled back from the pylorus to the cardia, moving the tip of the endoscope along all parts of the stomach to detect the tumor, its contiguous structures and perigastric lymph nodes. The findings were recorded with a Polaroid camera.

The normal gastric wall is made up of five ultrasonographic layers of different echogenicities (Table 1). Tumors were identified by thickening and disruption of the typical five-layered configuration of the wall appearing as a hypoechoic mass. The assessment of tumor infiltration depth was based on the generally accepted five-layered structure of the gastric wall. Lymph nodes with a homogeneous or inhomogeneous hypoechoic echo pattern and sharply delineated boundaries were considered malignant, whereas hyperechoic lymph nodes with indistinct contours were classified as benign[2].

The findings of EUS and CT in terms of the diagnosis of depth of invasion by the primary tumor, local invasion, and lymph node metastasis were compared with postoperative pathological determination according to the new (1987) TNM staging system (Table 2)[3].

RESULTS

A total of 62 patients underwent endoscopic ultrasonography for gastric cancer staging. All tumors were diagnosed as adenocarcinomas by histologic examination. The depth of infiltration of the tumor was displayed by EUS in all cases (Table 3). A T_1 carcinoma was correctly diagnosed with EUS as a hypoechoic pattern limited to the mucosa in 1 out of 2 patients (M type), or to the submucosa (SM-type) in all 3 patients. Overstaging occurred in 1 patient as a result of inflammatory changes surrounding the lesion, which was suggested as M-type or SM-type tumor sonographically, but all the 5 patients were eventually correctly diagnosed as having stage T_1. Nine of 11 T_2 tumors were correctly staged, whereas 2 cases were overstaged as stage T_3. A T_3 carcinoma was correctly diagnosed with EUS in 18 out of 24 patients. Understaging occurred in 2 patients, and overstaging in 4. For stage T_4, EUS diagnosed 20 out of 22 lesions correctly and understaged the other 2.

The staging accuracy of EUS was 100% for T_1 tumors, 81.8% for T_2, 75% for T_3, and 90.9% for T_4. The overall accuracy rate was 83.9% (52/62). Understaging occurred in 6.5% (4 cases), and overstaging in 9.7% (6 cases). The total mis-staged rate was 16.1%. The coincidence of perigastric infiltration was 90.9% (20/22).

Thirty-two patients were examined by CT, including 5 cases of T_2, 10 cases of T_3 and 17 cases of T_4 diagnosed by post-operative pathologic examination. CT correctly predicted 2 out of the 10 T_3 and 7 of the 17 T_4 lesions according to findings of thickening of gastric wall or accompanying infiltration of the pancreas or the adjacent liver lobe; however, the examinations failed to assess the depth of wall penetration in the remaining T_2-T_4 lesions. The overall accuracy rate of CT for T staging was 28.1% (9/32). The coincidence of perigastric infiltration was 41.2% (7/17).

Table 4 summarizes the results of EUS and histopathology for assessing perigastric lymph node involvement. EUS was able to display enlarged lymph nodes of > 3 mm in diameter. The display rate of enlarged lymph nodes was 88.9% (40/45). N_0 stage was correctly diagnosed with EUS in 28 (87.5%) of 32 patients; EUS misdiagnosed benign lymph nodes as metastatic lesions in 4 (12.5%) patients. N_1 was correctly diagnosed with EUS in 13 of 18 (72.2%) patients; metastasis was misinterpreted as benign lesions in the other 5 patients (27.8%). N_2 was correctly predicted in 8 of 12 (66.7%) patients and misinterpreted as N_1 in 3 (25.0%) patients and as N_0 in 1 (8.3%) patient. The sensitivity and specificity of EUS in determining the *n* stage was 80.0% and 87.5%, respectively. The accuracy of EUS in staging lymph nodes was 87.5% for N_0, 72.2% for N_1, and 66.7% for N_2. The overall accuracy rate was 79.0% (49/62).

CT correctly predicted 9 out of 19 patients with cancer positive lymph nodes, for an accuracy rate of 47.4%, and correctly diagnosed the absence of nodal spread in 7 patients that were deemed N_0 histologically. The overall accuracy rate was 50.0%.

DISCUSSION

This prospective study showed that EUS was not only highly accurate in determining the depth of invasion of gastric carcinoma, but also in detecting regional lymph node metastases, and that EUS was more accurate than CT for T staging (83.9% *vs* 28.1%) and *n* staging (79.0% *vs* 50.0%). This is in accordance with the results of previous studies[4].

The ability of EUS to image the gastrointestinal tract wall in detail provides a great advantage for staging the depth of tumor invasion (*i.e.* T). The high resolution of the individual layers of the

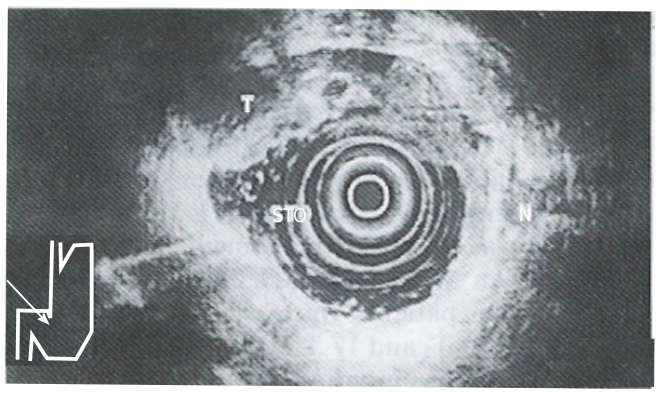

Figure 1 Endoscopic ultrasonography-T_2 cancer. Endoscopic ultrasonography shows a hypoechoic tumor (T) adjacent to an ulcerative lesion (U) invading the muscularis propria (Mp).

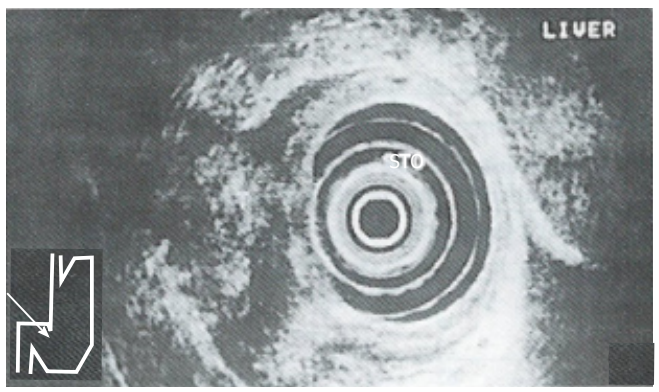

Figure 3 Endoscopic ultrasonography-T_4 cancer. Endoscopic ultrasonography shows a transmural hypoechoic tumor with penetration into adjacent structures.

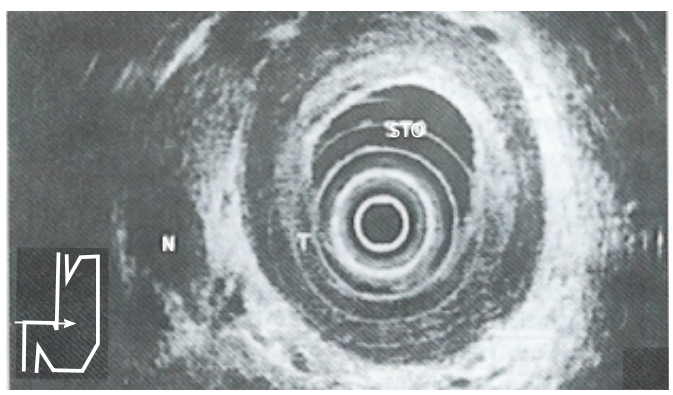

Figure 2 Endoscopic ultrasonography-T_3 cancer. Endoscopic ultrasonography findings in linitis plastica. Hypoechoic diffuse wall thickening is shown, with preservation of layers as distinctive structures. Note invasion of all layers but absence of invasion of adjacent organs.

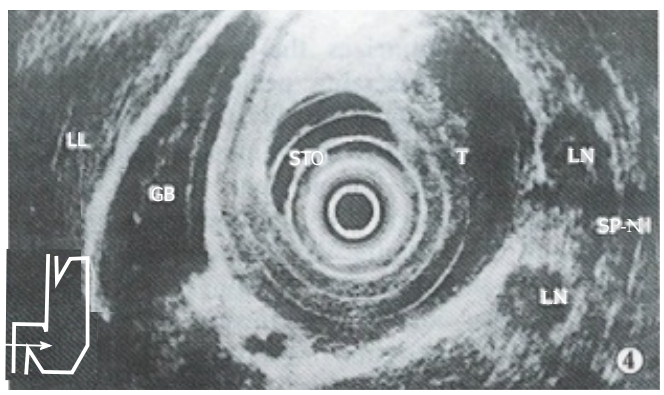

Figure 4 Peritumorous lymph node metastasis.

gastrointestinal wall seen with EUS is a direct result of the relatively high frequency transducers used (7.5 MHz and 12 MHz). The degree of tumor penetration into each layer can be documented as a focal disruption. Early gastric cancer (*i.e.* T_1) is visualized as a lesion with a hypoechoic echo pattern limited to the mucosa (*i.e.* M type) and/or submucosa (*i.e.* SM type). Advanced cancer is visualized as a structure with a hypoechoic pattern penetrating into (*i.e.* T_2) (Figure 1) and/or through the muscularis propria (*i.e.* T_3) (Figure 2) and/or into the adjacent structures (*i.e.* T_4) (Figure 3). Endosonographically, early carcinoma can be distinguished from advanced carcinoma. With respect to CT, the size and location of a gastric mass can be documented as areas of gastric wall thickening, with the normal thickness in a distended stomach defined as less than 8 mm. Furthermore, a lack of the fat layer between the gastric mass and an adjacent organ indicates direct invasion. On the other hand, CT cannot differentiate between single layers of the gastrointestinal wall. This limits the use of CT in separating early (*i.e.* T_1) from advanced tumor stages (*i.e.* T_2/T_3).

In our study, 16.1% carcinomas were mis-staged by EUS. Whereas microscopic tumor invasion was the most common cause of understaging, overstaging was mainly due to peritumorous inflammation or scar tissue which could not be distinguished from the tumor. In our experience, an association of the carcinoma with scar tissue should be suspected when a fusion of the 2nd and 4th layer is seen on EUS. Sometimes, overstaging in gastric T_2 carcinoma is due to the fact that in patients without ascites, differentiation between serosa (T_3) and subserosal (T_2) infiltration is not possible. Furthermore, not all the parts of the stomach are covered by serosa; it is absent in some areas, such as the lesser curvature and the anterior wall of the antrum. On the other hand, in T_4 cancers, EUS did not detect organ invasion (especially in the colon), leading to understaging. In addition, anatomic limitations inhibit perfect endosonographic examinations of two regions of the stomach: The prepyloric antral area and the proximal portion of the lesser curvature distal to the cardia.

In staging nodal diseases, EUS is also superior to CT, with an accuracy rate of 79.0% versus 50.0%. Lymph node metastases were determined by CT as enlarged lymph nodes of > 8 mm in diameter. We found the size of imaged lymph nodes to be an unreliable criterion of malignancy. Malignant nodes as small as 3 mm can be imaged with EUS, and our error in overstaging nodes by EUS occurred in nodes > 2 cm in diameter. The echo pattern and borders of lymph nodes seen on EUS were found to be reliable features for evaluating metastatic lymph node involvement. We have found that malignant nodes (Figure 4) tend to be rounded, sharply defined and hypoechoic compared to benign nodes that tend to be elongated, hazier in outline and more echogenic. Inflammatory changes and micrometastases were the most important causes of false positive and false negative results. These shortcomings can probably be resolved by using the echoendoscope equipped for EUS-guided cytological aspiration[5].

EUS is limited in evaluation of distant metastasis (*i.e.* M staging). Due to its limited depth of penetration, high frequency EUS can be used to scan the left liver lobe but only a portion of the right lobe, and it is unable to evaluate pulmonary metastases and distant peritoneal metastases, such as retroperitoneal and mesenteric nodes below the level of the superior mesenteric artery. CT is superior to EUS in this regard. In our experience in staging gastric cancer, the greatest accuracy for overall staging is achieved with combination of CT and EUS, namely using CT for M, and EUS for T and N.

In summary, our study has shown that EUS is superior to CT in staging T and *n* categories of gastric carcinoma. EUS is a reliable method for the clinical evaluation of locoregional extension of gastric cancer, and will be helpful in guiding management decisions, selecting appropriate surgical procedures and facilitating research aimed towards improving prognosis for this highly lethal disease. Therefore, EUS should become an important, if not essential, diagnostic procedure for clinical TNM staging.

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BRIEF REPORT

Radioimmunoassay-detected basal level of epidermal growth factor in gastric juice of 86 healthy Chinese volunteers

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INTRODUCTION

Epidermal growth factor (EGF) is a low molecular weight polypeptide consisting of 53 amino acid residues[1]. EGF is primarily produced by the submaxillary glands and Brunner's glands, but its distribution pattern spans the gastrointestinal tract with a particularly high concentration in gastric juice. To date, however, the precise quantitative value of EGF in gastric juice of healthy subjects has not been reported for a study cohort of Chinese. In the study presented herein, we used radioimmunoassay (RIA) to measure the basal level of EGF in 86 healthy Chinese volunteers of various age.

MATERIALS AND METHODS

Subjects

Eighty-six healthy volunteers, including 24 children (male/female, 18/6; age range: 8-9 years) 28 young adults (male/female, 16/12; age range: 18-20 years) and 34 adults over the age of 40 (male/female, 20/14).

Methods

Special capsules designed for collecting minute quantities of gastric juice were swallowed by each of the 86 subjects under basal condition (*i.e.* before 8 am in the morning, representing the fasting state). After 20 min in the stomach, the capsules were manually extracted; the volume of gastric juice in each ranged from 0.3 mL to 0.5 mL. Each specimen was preserved individually by freezing at -40 ℃ until use. In preparation for measurement and analysis, the specimen was centrifuged at 2000 r for 30 min at 4 ℃. A total of 0.1 mL of the resulting supernatant was used to measure EGF by RIA, according to the method described by Lu *et al*[2].

RESULTS

The average EGF concentration in the gastric juice of the 86 healthy volunteers under basal condition was 0.62 ± 0.15 μg/L. The values of EGF by age group were as follows: Children, 0.61 ± 0.14 μg/L; young adults, 0.65 ± 0.14 μg/L; older adults, 0.59 ± 0.13 μg/L. The EGF level among males in the study (n = 52) was 0.61 ± 0.14 μg/L and among females was 0.62 ± 0.15 μg/L. No statistically significant differences were noted between the different age and sex groups.

DISCUSSION

The primary sources of EGF are the submaxillary glands and Brunner's glands of the duodenum. It is believed that EGF can inhibit secretion of gastric acid and stimulate DNA synthesis, and that it plays a role in protection of gastrointestinal mucosa[3]. Although, EGF is known to be of higher concentration in the gastric juice, the exact concentration of EGF in normal gastric juice had not yet been defined, partially due to the inconvenience of collecting specimens. Conventional gastric tubing and gastroscopy causes discomfort, but the newly developed capsule (taken orally) can more easily obtain minute specimens of gastric juice from both children and adults. These capsules are a promising innovation for research science since EGF level might be an important variable in evaluating health status. The study described herein indicates that the capsules will be useful for future investigations into various benign and malignant diseases of the gastrointestinal tract.

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ORIGINAL RESEARCH

Treatment of cancerous ascites and radical gastrectomy with intraperitoneal hyperthermic double-distilled water and cis-diaminodichloro-platinum perfusion

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Author contributions: All authors contributed equally to the work.

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Abstract

AIM: To study the therapeutic effect of intraperitoneal hyperthermic double-distilled water and cis-diaminodichloro-platinum (DDP) perfusion for cancerous ascites and radical gastrectomy.

METHODS: LACA mice were injected peritoneally with H_{22} cancer cells (2×10^7 tumor cells). Five days later, the mice received treatments with either intraperitoneal perfusion of 37 ℃ isotonic fluid (group Ⅰ), or 43 ℃ simple hyperthermic double-distilled water (group Ⅱ), isotonic fluid (group Ⅲ), DDP (group Ⅳ) or a combination of the hyperthermic double-distilled water with DDP (group Ⅴ). A clinical experiment with intraperitoneal hyperthermic double-distilled water perfusion with DDP was carried out from September 1991 through September 1993 with 32 advanced gastric cancer patients who had undergone radical gastrectomy.

RESULTS: In comparison with the untreated control group of cancer cell-bearing LACA mice, the mice in all treatment groups showed near complete obliteration of cancer cells in the peritoneal cavity, markedly reduced ascites, prolonged survival times, and reduced growth of peritoneal cancerous nodes. In the clinical experiment, all 32 patients with advanced carcinoma had achieved satisfactory results at the 1-year follow-up, but had unsatisfactory results at the 2-year follow-up.

CONCLUSION: The intraperitoneal hyperthermic double-distilled water perfusion with DDP inhibited the occurrence of ascites in LACA mice bearing cancer cells, and prolonged the lifetime of patients with gastric cancer who had undergone radical gastrectomy.

Key words: Gastrectomy; Stomach neoplasms; Ascites; Cis-diaminodichloro-platinum; Perfusion

Chen ZX, Chen JP, Chen Z, Peng DS, Zhen JX, Tan JS. Treatment of cancerous ascites and radical gastrectomy with intraperitoneal hyperthermic double-distilled water and cis-diaminodichloro-platinum perfusion. *World J Gastroenterol* 1997; 3(4): 246-248 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/246.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.246

INTRODUCTION

The five-year survival rate of gastric carcinoma after radical gastrectomy is about 30%-40%. Among the various causes of death, peritoneal metastasis recurrence accounts for about 50%. Recent reports from abroad[1-4] have demonstrated the therapeutic efficacy of intraperitoneal hyperthermic isotonic anticarcinogen fluid perfusion, but the long-term outcome has not yet been reported. In this study, we investigated the treatment of intraperitoneal hyperthermic double-distilled water and cis-diaminodichloro-platinum (DDP; the cisplatin chemotherapy drug) perfusion to prevent peritoneal metastasis and treat cancerous ascites in a mouse model and in patients with gastric cancer who had undergone radical gastrectomy.

MATERIALS AND METHODS

Materials

LACA mice and ascites tumor cells (liver cancer cell ascites, H_{22}) were provided by the Cancer Institute of West-China University of Medical Sciences. Double-distilled water (DDW) and physiological saline were provided by our pharmaceutical department. DDP (2 mL/tube, containing 10 mg DDP) were procured from the manufacturer, Gejiu Biochemical Pharmaceutical Factory (Yunnan, China).

Methods

Seventy mice were given an intraperitoneal injection of 2×10^7 tumor cells; the male: female ratio was 35:35 and the range of

Table 1 Management strategies for ascites cancer cells in LACA mice

Management	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ
Injection of tumors cells	+	+	+	+	+
37 ℃	+	-	-	-	-
43 ℃	-	+	+	+	+
0.9% normal saline	+	-	+	+	-
DDW	-	+	-	-	-
DDP	-	-	-	+	+

+: Management; -: Without management. DDP: double-distilled water and cis-diaminodichloro-platinum; DDW: Double-distilled water.

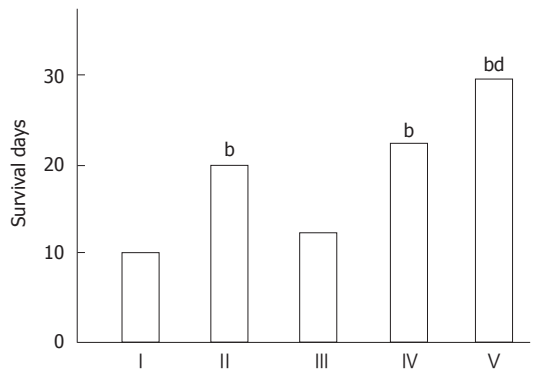

Figure 1 Survival days for all groups. $^{b}P < 0.01$ *vs* group Ⅰ, $^{d}P < 0.01$ *vs* groups Ⅱ, Ⅳ and Ⅴ.

body weight was 22-24 g. The injected mice were randomly divided into the following 5 groups for treatment at day 5 post-injection: Physiological saline at 1.5 mL[5] (group Ⅰ, control group); hyperthermic double-distilled water (group Ⅱ); isotonic fluid (group Ⅲ); DDP (2.5 mg/kg)[6] (group Ⅳ); and hyperthermic double-distilled water combined with DDP (group Ⅴ). All treatments were delivered as intraperitoneal perfusion, and the various features of the perfusions are presented in Table 1.

Observation and analysis of indexes

Average survival days of mice and quantity of ascites The exact time of death was recorded, after which a small incision was made in the abdominal wall in order to remove the ascitic fluid by suction. The collected fluid was used in subsequent analysis as described below.

Peritoneal cancer nodes examination An autopsy was performed to observe the distribution, number and size of the cancer nodules. All data were recorded for subsequent statistical analysis.

Number of living tumor cells Samples of the collected ascitic fluid from each mouse were diluted with physiological saline and stained with TaiPan blue to distinguish the living tumor cells. The total number were counted and recorded.

Tumor cells under optical and electron microscopic observation A smear was made using the precipitate obtained by centrifugation. After ultra-thin sectioning and staining with hematoxylin-eosin and counterstaining with uranyl, acetate and lead citrate, the sections were observed under both optical and electronic microscopy. *F* and *Q* tests were used for statistical comparison.

RESULTS

Ascites tumor cells and survival rate of tumor cells

In the control group, ascites were obvious within 1 wk, while the managed groups (groups Ⅱ-Ⅴ) presented a slower appearance of ascites and a reduced amount of ascitic fluid. Four mice in group Ⅴ developed no ascites. Managed groups Ⅱ, Ⅳ and Ⅴ, with the exception of group Ⅲ, showed a significantly low number of tumor cells ($P < 0.01$ *vs* control group I). When group Ⅴ was compared with groups Ⅱ and Ⅳ, the tumor cells were found to be drastically lower in the former and the survival rate to be markedly improved (Table 2).

Table 2 Ascites, tumor cells and survival rate of tumor cells for all groups

Group	*n*	Ascites (mL)	Tumor cells (× 10^7/mL)	Survival rate of tumor cells (%)
Ⅰ	14	22.13 ± 3.16	21.43 ± 3.42	89.42 ± 3.21
Ⅱ	14	10.61 ± 2.85[b]	8.20 ± 3.41[b]	69.21 ± 7.22[b]
Ⅲ	14	17.29 ± 2.93	18.26 ± 3.12	87.86 ± 1.68
Ⅳ	14	10.86 ± 1.86[b]	6.54 ± 3.75[b]	75.14 ± 6.89[b]
Ⅴ	14	5.36 ± 4.28[bd]	4.57 ± 2.90[bd]	45.14 ± 22.64[bd]

$^{b}P < 0.01$ *vs* group Ⅰ, $^{d}P < 0.01$ *vs* groups Ⅱ and Ⅲ.

Table 3 Detected peritoneal cancerous nodes for all groups

	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ
Proportion of peritoneal cancerous nodes	7/14	3/14	5/14	4/14	1/14[a]
Incidence of peritoneal cancerous nodes	50.00	21.43	35.71	28.57	7.64[a]

$^{a}P < 0.05$ *vs* group Ⅰ.

Survival days of mice

With the exception of group Ⅲ, the management strategies were associated with significantly improved survival ($P < 0.05$ *vs* control group Ⅰ). When group Ⅴ was compared with groups Ⅱ, Ⅲ and Ⅳ, the former showed significantly greater prolonged survival (Figure 1).

The data for effects of the different management strategies on growth of peritoneal cancerous nodes are presented in Table 3.

Light and electron microscopy observation of ascites tumor cells

Under the light microscope, the tumor cells of group Ⅰ were found to be larger in number, gathered in clots, and presenting with large and deeply-stained nuclei. The tumor cells of group Ⅲ were found to be reduced in number. The tumor cells of groups Ⅱ and Ⅳ showed a portion with water-like degeneration and necrosis. The tumor cells of group Ⅴ showed a sharp sharply reduction in number, with the present cells showing necrotic pathological changes, cytoplasm dissolution and nucleus disappearance. Under the electron microscope, the tumor cells of group Ⅴ showed no complete structures, while those of groups Ⅱ and Ⅳ showed degeneration in various degrees, with distension of mitochondria and nucleoplasm.

CLINICAL APPLICATION

The clinical experiment was conducted from September 1991 to September 1993 and involved 60 cases of advanced gastric cancer. The patients, once enrolled, were divided randomly into the following two groups. The first group was a control (28 cases) and was treated with operation only; the second group was experimental (32 cases) and was treated with an intraperitoneal perfusion of 43 ℃ double-distilled water combined with DDP.

For each patient, the abdominal cavity was opened for perfusion and washing with 200 mL of 37 ℃ normal saline, which was then sucked out in order to search for the detouched tumor cells. Patients allocated to the second group received the following additional treatment: Before closure of the abdomen, an intraperitoneal perfusion of hyperthermic (44-46 ℃) sterile double-distilled water (2500-3000 mL) was delivered by circulating perfusion for 30 min, after which 700-1000 mL of perfusate was allowed to remain in the abdomen, and 300 mg of DDP (200 mg/m^2) was added[7]; the abdomen was then closed with a tube in place to facilitate post-operative drainage. After postoperative clamping for 4 h, the tube was opened for drainage. At the same time of the perioperative perfusion, a rapid (20 min) *i.v.* drip of 12 g of sodium thiosulfate was administered, followed by a gradual (8 h) *i.v.* drip of 24 g of sodium thiosulfate in 1000 mL of 5% G.S.[8].

In the second group (prevention group), 13 (48.1%) of the 27 patients were positive for detouched tumor cells, and all 13 became negative after treatment. In the first group (control group), 9 patients were positive and 6 remained positive after the operation.

The prevention group had a 1-year survival rate of 96.9%

(31/32), which was significantly higher than that of the control group (46.1%, 13/28; $P < 0.01$), but a 2-year survival rate that was similar to the control group (75.0%, 24/32 *vs* 42.9%, 12/28; $P > 0.05$).

The incidence of complications of intraperitoneal perfusion was 65.6% (21/32) and included nausea (43.8%, 14 patients) and vomiting (25.0%, 8 patients). These cases were mild, and easily relieved by paspertin or ondansetron zofran. The levels of serum creatinine and urea nitrogen were higher after perfusion than before the operation, but still within normal limits. Six patients showed a slight decrease in white blood cell count after perfusion, but in all cases the count was above 3.0×10^9. All patients showed normal findings from liver function tests; there were no instances of peritoneal nerve injury, delayed healing of the incision, anastomotic leakage, or intestinal adhesion.

DISCUSSION

When the serosa of the gastric tumor is invaded, the tumor cell may detach spontaneously or a result of manipulation during operation, thereby entering the free peritoneal cavity or the pelvic fossae[9]. Kaibara[3] reported that the 5-year survival rate in patients free from peritoneal metastasis was 51.4% but only 18.7% for their counterparts with peritoneal metastasis. Thus, it is important to prevent and treat peritoneal metastasis.

Thermochemotherapy is able to eliminate recurrence at the excision site and helps to avoid peritoneal seeding. Moreover, Kaibara[3] reported that it might raise the 5-year survival rate. In our experiment, on day 5 after intraperitoneal injection of H_{22} cancer cells into the peritoneal cavity of LACA mice, the tumor cells presented with continuous division and proliferation activity, and seeding was present. When the treatment of intraperitoneal perfusion of hyperthermic (43 ℃) hypotonic (double-distilled water) fluid combined with DDP was compared with perfusion of normal temperature hypotonic fluid, isotonic fluid and DDP, the former showed the best effect, probably due to the synergic action of the agents when used as a combination therapy.

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CASE REPORT

Pancreatitis associated with herpes zoster: A case report

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INTRODUCTION

The etiology of acute pancreatitis has been well studied and is known to be diverse, but pancreatitis caused by herpes zoster (HZV) has rarely been reported. We encountered a case of pancreatitis associated with HZV in our hospital and report it below.

CASE REPORT

A 28-year-old woman was admitted to our hospital with complaints of anorexia, nausea and epigastric pain that had lasted for 6 d. The patient denied recent alcohol ingestion, previous food intolerance, jaundice or abdominal pain, and she had no history of peptic ulcer disease, hyperlipidemia, abdominal trauma or recent use of medication. Physical examination revealed umbilicated vesicles on an erythematous base at the right hypogastric region and extending dorsally along the right ribs. There was marked tenderness in the epigastric region, and auscultation revealed diminished bowel sounds. No abnormal mass or enlargement of the liver or spleen was detected by palpation. Both the Grey-Turner's sign and Cullen's signs were absent. Pelvic examination revealed no tenderness of the cervix or cul-de-sac. Initial laboratory tests included a white blood cell count of 7.7×10^9/L, hemoglobin level of 123 g/L, serum amylase of 603 U/L (normal, < 180 U/L), urine amylase of 3864 U/L (normal, < 1200 U/L), serum lipase of 2780 U/L (normal, < 100 U/L) and positive serum HZV (by PCR).

Abdominal ultrasound showed a normal liver, gallbladder, and pancreas. Bile ducts were not dilated. There was a small amount of fluid detected in the peritoneal cavity. The serum potassium level was 2.3 mmol/L, but the serum levels of calcium, cholesterol and triglycerides were normal. The diagnostic impression was acute pancreatitis and HZV. Treatment included intravenous fluids, electrolyte replacement, interferon and nasogastric suction. A week later, the patient's abdominal pain disappeared gradually and she resumed eating. Repeated abdominal B-ultrasonography showed no fluid in the peritoneal cavity. The vesicles presence resolved.

DISCUSSION

Acute pancreatitis is a process of autodigestion of the pancreas caused by premature activation of zymogens, leading them to become active proteolytic enzymes. The etiology of acute pancreatitis is diverse and reported causes include the mumps virus, rubella virus, Coxsackie B virus, Epstein Barr virus and hepatitis A virus; however, the association between HZV and pancreatitis has rarely been reported.

The pathogenic mechanism underlying HZV-related pancreatitis remains unknown. One plausible mechanism for virus-associated acute pancreatitis is inflammation and destruction of pancreatic acinar cells directly induced by the virus. In the case described herein, HZV was detected in the serum by PCR and other coincidental etiologies, such as alcohol abuse, gallstone disease, drug-induced pancreatitis, trauma, hyperlipidemia, and ulcer diseases, were excluded by clinical examination and history taking.

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ORIGINAL RESEARCH

Pharmacokinetics of four 5-FU preparations administered rectally to rats and rabbits

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Abstract

AIM: To compare the pharmacokinetic characteristics of four preparations of fluorouracil (5-FU) administered rectally using a rat model.

METHODS: Concentrations of 5-FU were measured in plasma, the rectal wall and mesentery lymph tissues of rats and rabbits by high performance liquid chromatography. Differences between the main pharmacokinetic parameters were compared by statistical analysis.

RESULTS: The 5-FU concentrations in the rectal wall and mesenteric lymph tissues were significantly higher than the concentration in blood following rectal administration for all four of the preparations ($P < 0.01$). The drug level in the rectal wall was higher in the animals received delivery of an emulsion, compared to those who received delivery as a suppository ($P < 0.05$). Moreover, the animals who received a lipophil-based suppository had lower plasma level of drug than those who received a hydrophil-based suppository, and the animals who received the simple (o/w) emulsion had lower plasma level than those who received the complex (w/o/w) emulsion. The differences found in the rat model were confirmed in rabbits ($P < 0.01$).

CONCLUSION: The lipophil-based suppository and the simple emulsion of 5-FU might be more suitable for rectal administration for treatment of rectal cancers.

Key words: Fluorouracil/phamarcokinetics; Rectal administration; Rectal neoplasms/drug therapy

Zhang X, Wang CS, Wu GZ, Ling BD, Liang RH, Fang XS, Xin TY. Pharmacokinetics of four 5-FU preparations administered rectally to rats and rabbits. *World J Gastroenterol* 1997; 3(4): 249-250 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/249.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.249

INTRODUCTION

Fluorouracil (5-FU) is one of the most effective medicines for chemotherapy of large bowel cancer; unfortunately, intravenous administration does not allow for a sufficiently high concentration to reach the cancer tissues and is associated with frequent serious toxic reactions and side effects[1]. However, a higher 5-FU concentration in cancer tissues accompanied by a relative lower level in plasma could be obtained if the drug is administered rectally[2]; as such, this strategy would also reduce the risk of adverse reactions, such as arrest of bone marrow functions.

We generated four different preparations of 5-FU for rectal administration, and compared their pharmacokinetic characteristics in the rat and rabbit whole animal systems.

MATERIALS AND METHODS

Animals

Female and male rats (weight range: 150-200 g) and rabbits (weight range: 2-3 kg) were provided by the animal facility of North Sichuan Medical College.

Compounds

5-FU raw drug (lot number: 910732) was procured from the manufacturer, Shanghai 12th Pharmaceutical Factory. All reagents used in the study were of AR grade.

Preparations

Crystalline grain (o.d. 10 μm) was generated by the solvent-transformation method. The hydrophil-based (polyethylene glycols) suppositories (HBS) and lipophil-based (semi-synthetic fatty glyceride) suppositories (LBS), each containing 250 mg 5-FU (for rabbits) or 25 mg (for rats), were generated by the heating-melt method. Two kinds of emulsions containing 5-FU (5%) (wt/vol)) were generated using an aqueous solution, namely the simple (o/w) emulsion (SE)

Table 1 Comparison of tissue concentrations in rats among the four 5-FU preparations ($\bar{x} \pm s$, $n = 5$)

Group	Concentration		
	Plasma (mg/L)	Rectal wall (mg/g)	Mesenteric lymph nodes (mg/g)
HBS	27.10 ± 15.06[c]	71.97 ± 21.64[b]	75.92 ± 21.81[b]
LBS	16.60 ± 15.03	46.82 ± 20.84[b]	41.93 ± 17.22[b]
SE	14.39 ± 9.40	154.11 ± 46.54[a,b]	41.29 ± 15.30[b]
CE	19.47 ± 8.71[c]	156.60 ± 42.31[a,b]	46.45 ± 13.41[b]

[b]$P < 0.01$ *vs* plasma level; [a]$P < 0.05$ SE group *vs* HBS group, CE group *vs* LBS group; [c]$P > 0.05$ HBS group *vs* LBS group, CE group *vs* SE group. HBS: hydrophil-based (polyethylene glycols) suppositories; LBS: Lipophil-based (semi-synthetic fatty glyceride) suppositories SE: Simple (o/w) emulsion; CE: Complex (w/o/w) emulsion.

Table 2 Main pharmacokinetic parameters in rabbits after rectal administration of four 5-FU preparations ($\bar{x} \pm s$, $n = 3$)

Group	T1/2 (h)	Vd (L)	AUC [(mg·g)/L]	Cmax (mg/L)	Tmax (h)
HBS	1.19 ± 0.52	2.25 ± 1.01	95.41 ± 45.21[b]	39.56 ± 19.02	0.84 ± 0.41
LBS	1.92 ± 0.80	16.90 ± 7.44	20.44 ± 9.82	8.56 ± 3.70	0.55 ± 0.29
SE	4.54 ± 1.75	22.98 ± 10.51	35.60 ± 15.03	9.95 ± 3.97	0.40 ± 0.17
CE	2.10 ± 0.98	3.00 ± 1.42	126.19 ± 73.35[b]	30.48 ± 15.24	0.99 ± 0.49

[b]$P < 0.01$ HBS group *vs* LBS group, CE group *vs* SE group. HBS: hydrophil-based (polyethylene glycols) suppositories; LBS: Lipophil-based (semi-synthetic fatty glyceride) suppositories SE: Simple (o/w) emulsion; CE: Complex (w/o/w) emulsion.

and the complex (w/o/w) emulsion (CE).

Instruments and requirements of assay

High performance liquid chromatography (HPLC) was carried out with the chromatographic column-Zorbax ODS (Gilson Corporation, France) using the following parameters: 4.6 mm × 150 mm; graininess 5 μm; mobile phase, phosphate buffer solution (0.025 mol/L, pH 3.0); and flow rate, 1 mL/min.

Experiments

Twenty rats were randomly divided into four groups, with total weight balanced in each. The rats in the four groups were rectally administered 5-FU as HBS, LBS, SE or CE at a dose of 25 mg. In order to prevent leakage of the drug solution from the anus, the orifice was clipped closed after the drug delivery. At 1 h post-delivery, blood samples (2 mL each) were drawn from the tail vein and the rats were sacrificed for immediate tissue harvesting (rectal and mesenteric lymph nodes). The rectal tissues were first cleaned by distilled water and blotted on filter paper, then 30 mg were weighed out for the subsequent analyses. The blood samples (containing heparin) were centrifuged and 1 mL plasma was collected for analysis. The 5-FU concentrations in the tissues and plasma were determined by HPLC assay as previously described but with slight modification[3].

Twelve rabbits were randomly divided into four groups and rectally administered 5-FU as HBS, LBS, SE or CE at a dose of 250 mg. At 0.5, 1, 1.5, 2, 4 and 6 h post-delivery, blood samples (1.5 mL) were drawn from the posterior auricular arteries and processed for analysis as described above.

RESULTS

Data for the main pharmacokinetic parameters are presented in Tables 1 and 2.

DISCUSSION

5-FU is one of the most common anticancer drugs used in clinical practice today, and its strong killing effects on cancer cells are well recognized. Specifically, 5-FU damages proliferating cells, thereby reducing the tumor mass in size and preventing tumor cells from spreading and undergoing metastasis. However, if the drug is administered intravenously, its curative effects are inadequate because it achieves a lower concentration in the rectal wall and mesenteric lymph nodes and instead has a relatively higher concentration in blood[4]. High blood concentrations lead to serious side effects that preclude the patients' ability to tolerate the treatment.

In this study, the 5-FU concentrations in the rectal wall and mesenteric lymph nodes were significantly higher than that in blood at 1 h after administration to rats, for all four of the different preparation types ($P < 0.01$). Comparison of the four types of preparations showed that the emulsions provided higher levels of 5-FU in the rectal wall than did the suppositories ($P < 0.05$). The drug concentrations in the blood was higher in the rats given HBS than in those given LBS, and higher for CE than for SE ($P > 0.05$). The differences were confirmed in the rabbit system as well ($P < 0.01$).

Thus, LBS and SE provided higher 5-FU concentrations in the rectal wall and mesenteric lymph nodes, and a lower concentration in blood. Rectal administration can reduce toxic and side effects and increase anticancer effects; therefore, the two preparations, LBS and SE, are more suitable for clinical application.

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BRIEF REPORT

Influence of diet intake on liver function test

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Abstract

AIM: To study whether dietary intake influences liver function test.

METHODS: Blood samples were obtained from patients liver diseases (n = 100) and controls (without liver diseases; n = 100) first at 07: 00 in the morning (fasting state) and then 2 h after a meal (fed state). The Hitachi-7150 automatic biochemistry analyzer was used to assess the following liver function indexes: Serum bilirubin, thymol turbidity test, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, gamma glutamyl transferase, SP, A and G. Statistical significance of differences between inter-group values was determined using SAS software.

RESULTS: None of the indexes showed statistically significant differences between the fasting state and the fed state (P = 0.476-0.978).

CONCLUSION: Liver function test can be performed after a meal.

Key words: Dietary proteins; Dietary fats; Liver function tests

Cheng SQ, Zhang JF, Zhang ZF, Qian MY, Guo XL, Shang WZ, Li DJ. Influence of diet intake on liver function test. *World J Gastroenterol* 1997; 3(4): 250 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/250.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.250

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ORIGINAL RESEARCH

Determination of β-glucuronidase in human colorectal carcinoma cell lines

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Abstract

AIM: To study the relationship between β-glucuronidase and the invasiveness of human colorectal carcinoma cell lines.

METHODS: Six colorectal carcinoma cell lines, including three well-differentiated (CX1, CCL187, and CCL229) and three poorly differentiated ones (CCL227, CCL228, and Clone A), were analyzed by Fischman's method to determine the concentration of β-glucuronidase in the medium.

RESULTS: Low levels of β-glucuronidase (activity range: 1.29 to 1.96 μg/106 cells·h) were associated with poor invasiveness. This finding was in contrast to the elevated levels of the enzyme (2.46-3.37 μg/106·h) detected in the medium derived from the more aggressively invasive cells (CCL 227, CCL 228, Clone A, and CCL 229).

CONCLUSION: Highly invasive colorectal carcinoma cells secreted higher levels of β-glucuronidase than the poorly invasive cells. Determination of secreted β-glucuronidase might represent a useful in vitro measurement tool to assess the invasiveness of colorectal carcinoma.

Key words: Colorectal neoplasms; β-glucuronidase invasiveness; Cell lines

Feng S, Song JD. Determination of β-glucuronidase in human colorectal carcinoma cell lines. *World J Gastroenterol* 1997; 3(4): 251-252 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/251.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.251

INTRODUCTION

The major cause of death in patients with malignant neoplasms is metastasis. The spread of tumor cells from the primary site to other organs involves a sequence of complex biologic events. Improving our knowledge about the mechanisms of metastasis, at a biologic level, may enable us to detect the pre-metastatic state and inhibit tumor invasion. The following multistep cascade contributes to the initiation of tumor metastasis: (1) tumor cells attach to the extracellular matrix by means of cell-surface receptors; (2) tumor cells degrade the matrix by secreting specific degradative enzymes; and (3) tumor cells migrate through the degraded matrix, facilitating metastasis[1]. Matrix degradative enzymes, such as urokinase, type Ⅳ collagenase, β-glucuronidase and Cathepsin B, play important roles in this process.

β-glucuronidase, a lysosomal acid enzyme that acts to degrade proteoglycan, the major component of basement membrane, participates in the processes of tumor invasion and metastasis. In this study, an attempt has been made to establish a relationship between β-glucuronidase secretion and the primitive nature of *in vitro* colorectal carcinoma. Accordingly, the β-glucuronidase secretory capacities of six colorectal carcinoma cell lines, representing varying degrees of differentiation and invasiveness, were compared. The results indicated that the poorly and highly invasive cells could be separated on the basis of β-glucuronidase secretion. This biochemical marker may prove useful in assessing the primitive characteristics of cultured colorectal carcinoma cells.

MATERIALS AND METHODS

Materials

Human colorectal carcinoma cell lines that had originated from tumor tissues and were well established in serial subcultures were used in this study. The cell lines CX1, CCL187, CCL227, CCL228, CCL229, and Clone A were generous gifts from the Dana Farber Cancer Institute of Harvard Medical School, USA.

Methods

The cell lines were maintained in Dulbecco's modified Eagle's medium (GIBCO, BRL) supplemented with 10% calf serum and 2 U/mL gentamycin. Cell cultures were incubated at 37 ℃ in a humidified atmosphere of 95% air and 5% carbon dioxide.

The culture medium was collected as follows. Colorectal carci-

Table 1 Conditions for measuring β-glucuronidase in culture medium

Medium (mL)	Water (mL)	Acetate buffer (mL)	pH	Substrate final	Molarity (mL)	Incubation time at 37 ℃ (h)	Alkalinizing reagent (mL)	Final pH	Coloremeter wavelength (nm)
0.2	0.4	0.2	4.5	0.2	0.006	18	2[1] + 3 mL H_2O	10.2	540

[1]Glycine-Duponal reagent: 15.01 g of glycine dissolved in 900 mL of H_2O and brought to pH 11.7 by addition of 50% NaOH solution. Duponal (sodium lauryl sulfate) was added to produce a final concentration of 0.2% and water was added to achieve a final volume of 1 L.

Table 2 Activity of secreted β-glucuronidase in culture medium of six colorectal carcinoma cell lines

Cell line	Differentiation degree[3]	Invasiveness[3,4]	n	β-glucuronidase activity, $\bar{x} \pm s$
CX1	Good	Low	6	1.29 ± 0.17
CCL187	Good	Low	6	1.96 ± 0.28
CCL229	Good	High	6	3.37 ± 0.34[b]
CCL227	Poor	High	6	2.46 ± 0.18[b]
CCL228	Poor	High	6	2.73 ± 0.19[b]
Clone A	Poor	High	6	3.22 ± 0.38

[b]$P < 0.001$ *vs* CX1, CCL187.

noma cells were seeded in 100 mL flasks (2.5×10^5 cells/mL). After 3 d of culture, the medium was refreshed completely. After an additional day of culturing, the medium was harvested and the cells enumerated. The collected medium was condensed (mL/5×10^6 cells) and stored at 4 ℃ for future use. β-glucuronidase activity levels in the collected medium was determined by Fischman's method[2]. Phenolphthalein standard curve was set up in a range of 0 mg/L to 40 mg/L. The substrate was phenolphthalein mono-β-D glucosiduronic acid sodium salt. The conditions for measuring medium levels of β-glucuronidase are shown in Table 1. One enzyme activity unit equated to 1 μg of released phenolphalein/10^6 cells·h. The results were analyzed by Student's *t*-test.

RESULTS

The medium from each cell line was analyzed for activity of β-glucuronidase. The well differentiated and poorly invasive cell lines CX1 and CCL187 were found to be low secretors of the enzyme (activity range: 1.29-1.96 μg/10^6 cells·h). In contrast, the poorly differentiated and highly invasive cell lines CCL227, CCL228 and Clone A, as well as the well differentiated CCL229 with high invasiveness[3], were relatively more active in this respect, with β-glucuronidase activities ranging between 2.46 μg/10^6 cells·h and 3.37 μg/10^6 cells·h (Table 2).

DISCUSSION

Recent studies have highlighted the association of matrix degradative enzymes with malignant tumors, and have suggested that these enzymes may play a role in tumor invasion and metastasis. Although a lot of work has been done to investigate the effects of urokinase and type (WTBZ) Ⅳ (WTB1) collagenase on tumor invasion and metastasis[5,6], there are few reports about β-glucuronidase in this respect, especially in regards to colorectal carcinoma. β-glucuronidase, a lysosomal acid enzyme that can degrade proteoglycan, the major component of basement membrane, is known to participate in the process of tumor invasion and metastasis. Poole[7] reported that β-glucuronidase activity was high in experimental rat tumors and that the enzyme was present in the matrix ahead of the invading tumor. Dai *et al*[8] reported that the β-glucuronidase activity level in stomach cancer was higher than that in non-cancerous tissues. Nicolson *et al*[9] confirmed that highly metastatic melanoma cells secreted higher levels of β-glucuronidase and degraded subendothelial basement membrane at a higher rate than poorly metastatic melanoma cells. All these findings have supported the hypothesis that β-glucuronidase is closely related to tumor metastasis.

In order to illustrate the relationship between β-glucuronidase secretion and invasiveness of human colorectal carcinoma, we analyzed the culture medium from six cell lines to determine the activity of β-glucuronidase within. The results indicated that the highly invasive cell lines secreted higher levels of β-glucuronidase than the poorly invasive ones, supporting the notion that β-glucuronidase might contribute to colorectal carcinoma invasion and metastasis. Moreover, determination of secreted β-glucuronidase might represent a useful measurement tool for the invasiveness of *in vitro* colorectal carcinoma.

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BRIEF REPORT

AgNOR and rasp21 expression in gastric mucosal lesions caused by *Helicobacter pylori* infection

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Abstract

AIM: To investigate AgNOR and rasp21 expression levels in gastric mucosal lesions caused by *Helicobacter pylori* (Hp) infection in order to gain insight into the related biological processes (i.e. tumor-like behavior) and possible underlying mechanism supporting Hp pathogenesis.

METHODS: Hp infection was diagnosed in using the standard Campylobacter-like organism test along with Wathin-Starry staining. The expression of AgNOR was detected by the silver colloid staining technique. The expression of rasp21 was detected by monoclonal antibody and immunohistochemical staining using the ABC method. The study included a total of 278 patients with endoscopically- and pathologically-confirmed gastric mucosal lesions, representing chronic superficial gastritis (CSG), chronic atrophic gastritis (CAG), intestinal metaplasia, dysplasia, and gastric cancer. Among these, 146 of the patients were Hp-positive and 132 were Hp-negative.

RESULTS: The Hp-positive group of patients showed significantly greater AgNOR in the gastric mucosal lesions than the Hp-negative group, with the exception of the CSG sub-group ($P < 0.05$ or $P < 0.01$). The positive rate of rasp21 expression in gastric mucosal lesions in the Hp-positive group was also significantly higher than that in the Hp-negative group, with the exception of the CSG and CAG sub-groups ($P < 0.05$).

CONCLUSION: Hp-positive gastric mucosal lesions show biological behaviour of tumors. Hp may act as a promoter to activate the ras gene and to stimulate cell over-proliferation.

Key words: *Helicobacter pylori*/pathogenicity; Gastric mucosal/pathology; Stomach diseases/microbiology; Stomach neoplasms/microbiology; Nucleoproteins/metabolism; Proto-oncogene protein P21/ (RAS)

Gao HJ, Lu XZ, Zhang XY, Zhao ZQ. AgNOR and rasp21 expression in gastric mucosal lesions caused by *Helicobacter pylori* infection. *World J Gastroenterol* 1997; 3(4): 252 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/252.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.252

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ORIGINAL RESEARCH

Clinical significance of CA19-9 in diagnosis of digestive tract tumors

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Abstract

AIM: To evaluate the clinical value of CA19-9 in diagnosing and differentiating gastrointestinal tumors and in monitoring patients treated surgically.

METHODS: Patients with gastric cancer (n = 70), colorectal cancer (n = 90), pancreatic cancer (n = 7), esophageal cancer (n = 10) and benign disorders (n = 30), and normal adults (n = 111; used as healthy controls), were studied. Fasting blood samples were obtained from each study participant. The serum CA19-9 concentration was measured with radioimmunoassay.

RESULTS: The mean CA19-9 level was significantly higher in patients with gastric cancer (170.69 ± 91.45 kU/L) and patients with colorectal cancer (87.21 ± 39.55 kU/L) than in the healthy controls (11.254 ± 6.00 kU/L). Compared with the healthy controls, the CA19-9 level was also much higher in patients with pancreatic cancer (1266.58 ± 521.31 kU/L) (P < 0.01). However, the CA19-9 concentrations in patients with non-recurrent gastric cancer (12.63 ± 3.62 kU/L), colorectal cancer (14.14 ± 3.26 kU/L) and benign disorders (14.23 ± 2.60 kU/L) were statistically similar to those in the healthy controls (P > 0.05). The demarcation value of CA19-9 between negative and positive was < 31.0 kU/L. The sensitivity of CA19-9 for gastric, colorectal, pancreatic and esophageal cancers and for gastrointestinal benign disorders was 47.3%, 50.0%, 83.3%, 20.0% and 0%, respectively. The specificity of CA19-9 for digestive system malignant diseases was 100% for all.

Key words: Digestive system neoplasms; CA19-9; Tumor-related antigen; Stomach neoplasms; Colorectal neoplasms; Pancreatic neoplasms; Esophageal neoplasms

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INTRODUCTION

The CA_{19-9} antigen isolated by Koprowski and colleagues[1] in 1979 is a lacto-N-fucopentaose II-like substance and one of the tumor-associated antigens present in serum in the mucin fraction. Close attention has been paid to the role of CA_{19-9} in the diagnosis of digestive tract tumors[2,3]. After strictly inspecting the quality of a commercially available CA_{19-9} antigen kit, we assayed serum CA_{19-9} levels in 207 patients with gastrointestinal diseases and in 111 normal adults (for comparative analysis as healthy controls). These data was used to evaluate the clinical significance of CA_{19-9} in diagnosis of digestive tract tumors.

MATERIALS AND METHODS

Experimental equipment

The CA_{19-9} McAb solid phase IRMA kit (Tianjin SYTRON Biotech Inc.) and the FT-613 automatic counter of ^{125}I radioimmunoassay (Beijing) were used.

Normal CA_{19-9} value

CA19-9 concentration was determined in the sera of 111 normal adults from the Guangzhou area (55 males, 50 females; mean age of 47.7 years) and used as the normal referential values for the study.

Patients

Serum samples were obtained from 207 patients with malignant and benign diseases of the digestive system. All diagnoses were confirmed by clinical, laboratory and pathological examinations. Of the 207 cases, 70 were gastric cancer, 90 were colorectal cancer, 7 were pancreatic cancer and 10 were esophageal cancer; a total of 30 of the cases were benign disorders, including 6 cases of chronic superficial gastritis, 7 of antral gastritis, 5 of gastric ulcers, 10 of duodenal ulcers, and 2 of acute appendicitis. The patients consisted of 148 males and 59 females, with age range of 22 years to 85 years (average age: 52.9 years).

RESULTS

The mean serum CA_{19-9} concentration in the 111 healthy controls from the Guangzhou area was 11.254 ± 6.006 kU/L. The differ-

Table 1 Serum CA19-9 concentration in patients and healthy controls

	n	$\bar{x} \pm s$	Range	> 31.0 kU/L	PR, %
Gastric cancer					
Preoperation	46	170.69 ± 91.45[a]	10.3-3220.0	19	41.3
Postoperative stability	15	12.62 ± 3.26	0.2-22.8	0	0.0
Postoperative recurrence	9	393.17 ± 3.804	9.0-2843.8	7	77.8
Colorectal cancer					
Preoperation	50	152.69 ± 76.39[a]	0.2-3261.0	20	40.0
Postoperative stability	24	14.15 ± 2.25	0.0-31.0	0	0.0
Postoperative recurrence	16	87.21 ± 39.55[a]	0.5-657.8	11	68.7
Pancreatic cancer	7	1266.58 ± 521.31[b]	11.0-3220.0	6	83.3
Esophageal cancer	10	19.94 ± 6.31	0.0-53.0	2	20.0
Benign disorders	30	14.23 ± 2.60	0.0-27.2	0	0.0
Healthy controls	111	11.25 ± 0.57	0.0-27.5	0	0.0

[a]$P < 0.05$ *vs* healthy controls, [b]$P < 0.01$ *vs* healthy controls; PR, positive rate.

ence of CA_{19-9} value among the healthy controls was not statistically significant among groups divided by age or sex ($P > 0.05$). The demarcation value of CA_{19-9} between the negative and positive was < 31.0 kU/L in our laboratory. The serum CA_{19-9} concentrations in patients with malignant and benign diseases are listed in Table 1. Table 2 shows the evaluational indexes of CA_{19-9} for diagnosing some of the gastrointestinal tumors.

DISCUSSION

We have determined the normal referential value of CA_{19-9} (11.254 ± 6.006 kU/L) in 111 healthy controls from the Guangzhou area using the solid phase IRMA kit. This result was similar to the results obtained by testing with solid phase radioimmunoassay kits manufactured by the Abbott Company and ORIS Company (France). The demarcation value of CA_{19-9} between the negative and positive was < 31.0 kU/L. This value was slightly lower than that reported in the kit's accompanying documentation (< 34.0 kU/L).

The results of our study showed that serum CA_{19-9} levels in patients with gastric cancer, colorectal cancer and some postoperatively recurrent cancers were significantly higher than those detected in the healthy controls. The sensitivity of CA_{19-9} in diagnosing gastric and colorectal cancers was 47.7% and 50.0%, and the specificity and positive predictive value were both 100% for all. None of the 30 patients with benign disorders of the gastrointestinal tract had a higher serum CA_{19-9} level than the normal referential value. The sensitivity and specificity of $CA_{19-9}$for benign disorders were 0% and 100%, respectively, indicating that CA_{19-9} is a highly specific tumor marker for diagnosing gastric and colorectal cancers and suggesting its potential to play an important role in the differentiation of benign and malignant diseases of digestive tract.

It is worth noting here that of the 39 patients with gastric cancer or colorectal cancer in this study who had no recurrent tumor postoperatively, none had a higher CA_{19-9} level than the normal referential value. The mean value of CA_{19-9} of the 39 patients was similar to the normal referential value. However, the serum levels of CA_{19-9} in the 9 patients with recurrent gastric cancer and in the 16 patients with recurrent colorectal cancer postoperatively were significantly higher than the normal referential value. The positive rate of CA_{19-9} in recurrent gastric and colorectal cancers was 77.8% and 68.7%, respectively. The CA_{19-9} level reached 2500 kU/L in a few of the patients with recurrent tumors. These collective results suggest that CA_{19-9} is a good index for evaluating the effect of treatment and predicting the prognosis of gastric and colorectal cancers postoperatively.

Table 2 Evaluational indexes for CA19-9-based diagnosis of gastrointestinal benign and malignant diseases

Disease	*n*	Positive	Se, %	Sp, %	Ac, %	+PV, %	-PV, %
Gastric cancer							
Preoperation and recurrence	55	26	47.3	100.0	65.9	100.0	50.8
Colorectal cancer							
Preoperation and recurrence	66	33	50.0	100.0	65.6	100.0	47.6
Esophageal cancer	10	2	20.0	100.0	80.0	100.0	78.9
Pancreatic cancer	7	6	83.3	100.0	97.3	100.0	96.8
GI benign disorders	30	0	0.0	100.0	78.7		787

Se, Sensitivity; Sp, Specificity; Ac, Accuracy; +PV, Positive predictive value; -PV, Negative predictive value.

Elevated serum CA_{19-9} levels have been observed in many different malignant diseases. The test seems especially promising for detection of pancreatic cancer, as more than 80% of these patients have been reported to show increased serum CA_{19-9} concentration[4,5]; therefore, CA_{19-9} may be a good tumor marker for diagnosing pancreatic cancer and monitoring patients treated surgically[6]. Here, we assayed the CA_{19-9} levels in patients with pancreatic cancer, and the serum CA_{19-9} concentration was found to be > 85 kU/L in 6 of the patients, > 110 kU/L in 5, > 1900 kU/L in 3, and 3200 kU/L in 1. The sensitivity, specificity, accuracy, positive predictive value and negative predictive value were 83.3%, 100%, 97.3%, 100% and 96.8%, respectively.

The incidence of pancreatic cancer is increasing worldwide. In 1987, the Japanese Cancer Registries reported the 5-year survival rates for 177 resected cases of T_1 stage, 783 resected cases of T_2 stage, 463 resected cases of T_3 stage and 304 resected cases of T_4 stage cancer as being 39.8%, 21.7%, 14.3% and 13.4%, respectively[7]. These results indicate that it is important to diagnose early stage pancreatic cancer, so as to improve prognosis. However, using the current diagnostic methods, it is difficult to diagnose pancreatic cancer in the early stage and to distinguish it from benign conditions that resemble pancreatic cancer in many aspects. Although only 7 patients with pancreatic cancer received the CA_{19-9} test in the current study, the results still showed that the CA_{19-9} test seems to be a useful additional tool in the diagnosis of pancreatic cancer.

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ORIGINAL RESEARCH

Analysis of amino acid constituents of gallstones

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Abstract

AIM: To seek drugs that will efficaciously dissolve bilirubin, glycoprotein and black stones and that will represent improved lithotriptic agents to resolve cholesterol stones, and to study the amino acid constituents of gallstones.

METHODS: According to characteristics determined by infrared spectroscopy and to the contents of bilirubin determined by semi-quantitative chemical analysis, 30 of 148 cases of gallstones were selected and divided into 5 groups. Amino acids of the 30 cases were detected by high-speed chromatography.

RESULTS: The quantity of amino acids was highest in black stones (226.9 mg/g) and lowest in pure cholesterol stones (1.4 mg/g). In the 5 groups of gallstones, the quantity of amino acids followed the hierarchy of black stone > mixed bilirubin stone and glucoprotein stone > mixed cholesterol stone > pure cholesterol stone. The proportions were: 95.95:29.02 and 28.05:5.78:1. Aliphatic amino acids accounted for ~ 50% of the total amino acids in the gallstones, with glycine accounting for 15.3% of the total amount of the 17 kinds of amino acids.

CONCLUSION: For mixed stones, the higher level of bilirubin, the higher content of amino acids. Acidic amino acids were relatively higher in bilirubin stones than in cholesterol stones.

Key words: Gallstones; Amino acids/analysis; Bilirubin; Glycine

Chen Y, Wang LL, Xiao YX, Ni JH, Yu Y. Analysis of amino acid constituents of gallstones. *World J Gastroenterol* 1997; 3(4): 255-256 Available from: URL: http://www.wjgnet.com/1007-9327/full/v3/i4/255.htm DOI: http://dx.doi.org/10.3748/wjg.v3.i4.255

INTRODUCTION

Dissolution of cholesterol stones can be achieved by oral ursodeoxycholic acid or chenodehydrocholic acid, and by perfusion with methyl tertiary butyl ether as well. However, no effective means of dissolving bilirubin, glycoprotein and black stones has been reported. In order to identify better lithotriptic agents, the contents of amino acids in gallstones needs to be studied.

MATERIALS AND METHODS

Sample selection

Gallstones were obtained from 148 patients who were treated by surgery in our hospital during the years of 1988 to 1992. The specimens were subjected to qualitative assessment by infrared spectroscopy, and 48 cases were also subjected to semi-quantitative chemical analysis. According to the characteristics corresponding to the infrared spectrum and the constituents of bilirubin, 30 cases were selected and divided into the following 5 groups: Pure cholesterol stones (n = 10), mixed cholesterol stones (n = 7), mixed bilirubin gallstones (n = 10), glycoprotein stones (n = 2), and black stone (n = 1).

Sample treatment[1]

After pulverizing by agate mortar and drying, 20 mg of powder from each gallstone was added to 6 mL of HCl (6 mol), nitrogen sealed and baked at 110 ℃ for 24 h. The volume was brought to 25 mL with distilled water. After filtration, 4 mL was collected, dried in a rotary evaporator, and washed twice with distilled water. The remaining sample was dissolved in 2 mL distilled water, of which 50 μL or 100 μL was used to measure the 17 amino acids, and taurine and ammonia concentrations. A trace of tryptophane was detected in 2 cases.

Analytical methods

Amino acids were detected by high-speed chromatography (L-8500; Hitachi Corp, Japan). The column was 4.6 mm × 60 mm, and 5 buffer solutions were used for the stepwise wash-off, with resin of 2622 s.c. (Hitachi ion exchange resin was used). The standard amino acid samples were provided by Sodium Glutamate Corp. (Japan). The quantitative analysis was conducted with extensional calculation. The coefficient of variation (c.v.) was 1.5% in this experiment.

Table 1 Content of various amino acids in 5 groups (30 cases) of gallstones ($\bar{x} \pm s$, mg/g)

	Pure cholesterol		Mixed cholesterol		Mixed bilirubin		Glycoprotein		Black
	n	$\bar{x} \pm s$	*n*	$\bar{x} \pm s$	*n*	$\bar{x} \pm s$	*n*	$\bar{x} \pm s$	
Gly.	10	0.635 ± 0.498	7	2.333 ± 2.890	10	10.459 ± 4.451	2	19.185 ± 0.955	16.552
Ala.	8	0.109 ± 0.084	7	0.794 ± 0.622	10	4.048 ± 2.409	2	1.925 ± 2.298	13.551
Val.	9	0.133 ± 0.089	7	1.043 ± 0.674	10	4.202 ± 2.709	2	3.500 ± 0.948	15.383
Ile.	5	0.080 ± 0.044	7	0.496 ± 0.330	10	2.407 ± 1.374	2	1.590 ± 0.523	4.921
Leu.	9	0.150 ± 0.093	7	1.113 ± 0.671	10	5.914 ± 3.983	2	4.510 ± 0.410	16.834
Thr.	10	0.112 ± 0.077	7	0.743 ± 0.499	10	3.784 ± 2.874	2	3.080 ± 0.820	11.642
Ser.	10	0.117 ± 0.077	7	0.790 ± 0.565	10	2.836 ± 1.799	2	3.085 ± 0.870	11.643
Pro.	4	0.116 ± 0.054	5	0.621 ± 0.203	10	2.953 ± 2.167	2	2.560 ± 0.968	12.421
Sys.	2	0.060 ± 0.014	6	0.422 ± 0.211	10	2.018 ± 1.199	2	2.235 ± 0.813	9.943
Met.	4	0.038 ± 0.013	4	0.398 ± 0.479	10	1.080 ± 0.670	2	0.800 ± 0.070	3.309
Phe.	10	0.160 ± 0.089	7	0.797 ± 0.451	10	3.499 ± 2.397	2	3.060 ± 0.509	9.928
Thr.	8	0.108 ± 0.055	7	0.596 ± 0.351	10	2.483 ± 1.713	2	1.710 ± 0.453	6.907
Asp.	10	0.220 ± 0.162	7	1.374 ± 0.869	9	6.066 ± 4.039	2	5.250 ± 1.796	24.103
Glu.	10	0.293 ± 0.194	7	1.723 ± 1.088	10	8.595 ± 5.468	2	7.460 ± 1.950	34.358
Lys.	9	0.126 ± 0.076	7	0.680 ± 0.405	10	4.058 ± 3.195	2	2.405 ± 1.124	17.582
His.	9	0.071 ± 0.039	7	0.401 ± 0.258	10	2.041 ± 1.983	2	0.945 ± 0.247	4.316
Arg.	9	0.012 ± 0.078	7	0.716 ± 0.418	10	4.012 ± 2.492	2	3.010 ± 1.923	13.517
Tau.	5	0.052 ± 0.028	9	1.695 ± 1.697	9	1.695 ± 1.697	1	1.540	1.608
Ammon.	10	0.662 ± 0.232	10	5.332 ± 1.590	10	5.332 ± 1.590	2	6.130 ± 1.174	6.435

RESULTS

The various contents of amino acids for the 30 gallstone cases in the 5 groups are presented in Table 1. All 30 had glycine, glutamic acid, threonine, phenylalanine and ammonia; among these, glycine content was the highest, accounting for 15.34% of the total amount, followed by glutamic acid, accounting for 13.01%. Asparagine, serine, valine, leucine, lysine, histidine and arginine were detected in 29 of the gallstone cases. There was a strong correlation ($P < 0.01$) between the above-mentioned amino acids. In 29 gallstone cases, there were more acidic amino acids than alkaline amino acids (1.39-2.73:1), except for a single bilirubin mixed stone (1:1.77) which had the appearance of black mud and came from a patient with malignant changes in the gallbladder and liver metastasis detected in postoperative pathologic examinations. The content of amino acids in one sample of pure cholesterol stones was the lowest for the 30 cases of gallstones examined (1.37 mg/g). Six amino acids (glycine, glutamic acid, aspartic acid, serine, threonine, and phenylalanine), taurine and ammonia were detected in this case. In the 5 groups of gallstones, the constituents of amino acids of one case of black gallstone were the most complete and the total amount of amino acids also was the highest (226.93 mg/g), only the content of glycine was slightly lower than that of glycoprotein.

In 10 cases of pure cholesterol stones, the content of glycine was higher than that of glutamic acid and aspartic acid, and that of glutamic acid was higher than that of aspartic acid, with the differences being statistically significant ($P < 0.005$).

In 7 cases of mixed cholesterol stone cases, the content of glutamic acid was significantly higher than that of aspartic acid ($P < 0.01$), but the content of glycine was higher, but not significantly, than that of glutamic acid and aspartic acid ($P > 0.05$).

In 10 cases of mixed bilirubin stones, although the content of glycine was higher than that of glutamic acid and aspartic acid, and that of glutamic acid was higher than that of aspartic acid, there was no significant differences ($P > 0.05$).

DISCUSSION

The amino acid is the fundamental unit of protein constitution. Nowadays, it is known that there are 20 kinds of amino acids[2], which are controlled by genetic code in protein molecules. They are called living proteinic amino acids, and consist of glycine, alanine, valine, leucine, isoleucine, methionine, proline, phenylalanine, tryptophan, tyrosine, serine, threonine, cysteine, glutamic acid, aspartic acid, histidine, lysine, arginine, asparagine and glutamine. Of the 20 living proteinic amino acids, 17 kinds were detected in this study; asparagine and glutamine were not detected because during this experiment[3] they would be completely hydrolyzed to free aspartic acid and glutamic acid, leading to artifactually higher amounts. Moreover, the proteins in this study were heated and hydrolyzed with 6 mol HCl, and tryptophan was damaged; additionally, cystine, methionine, threonine and serine were also influenced by the method, so the results for each were expected to be low. It has been reported that cysteine only exists in bilirubin gallstones, but in our experiment cysteine was not detected and only a small amount of the bisulfide compound of cysteine cystine and taurine produced during the conversion of cysteine in liver was detected.

Our results indicate that protein amino acids generally exist in gallstones. The components of amino acids detected in the gallstones were: Aliphatic amino acid > Acidic amino acid > Alkaline amino acid > Aromatic amino acid > Sulfur amino acid. In the 5 groups of gallstones of this study, the contents of amino acids were: Black gallstone > Mixed bilirubin stones and glucoprotein stone > Mixed cholesterol stone > Pure cholesterol stone. Their proportions were: 95.95:29.02 and 28.05:5.78:1. Glycine was the most abundant among the 8 kinds of aliphatic amino acids or the 17 kinds of amino acids in gallstones. For mixed stones, the higher the content of bilirubin, the higher the content of amino acids. With the exception of the black gallstones, all gallstones showed higher amount of glycine than that of aspartic acid and glutamic acid. However, in the pure cholesterol stones, glycine content was higher than aspartic acid and glutamic acid content ($P < 0.005$); thus, it was clear that proportion of acidic amino acids in bilirubin stones was relatively higher than that in cholesterol gallstones. In 30 cases of gallstones, the components of acidic amino acids were higher than those of alkaline amino acids, except for the bilirubin mixed stones from a patient with malignant changes in the gallbladder and liver metastasis that was determined by postoperative pathologic examination. It is likely that the metabolic product of malignant tissue is not favorable for the stable existence of acidic amino acids. The mechanism underlying this observation, however, remains unknown.

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ORIGINAL RESEARCH

Comparative study of changing patterns of concanavalin A-binding proteins in early stage of cholesterol gallstone formation

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Abstract

AIM: To elucidate the importance and the changing patterns of biliary concanavalin A-binding proteins (CPs) in the early stage of cholesterol gallstone formation.

METHODS: CP concentration and nucleation activity were measured by lectin affinity chromatography in bile samples of patients with cholesterol gallstones, pigment gallstones, gallbladder cholesterosis and non-biliary diseases.

RESULTS: The concentrations of CPs were much higher in patients with cholesterol gallstones (0.39 ± 0.11 g/L, n = 36, P < 0.01) or gallbladder cholesterosis (0.40 ± 0.09 g/L, n = 9, P < 0.01) than in those with pigment gallstones (0.2 ± 0.12 g/L, n = 7) and/or non-biliary diseases (0.27 ± 0.09 g/L, n = 10). Pronucleating activities were much stronger in patients with cholesterol gallstones (nucleation time ratio: 0.57 ± 0.21, n = 5, P < 0.01 *vs* pigment gallstones and/or non biliary diseases) and gallbladder cholesterosis (nucleation time ratio: 0.44 ± 0.23, n = 5, P < 0.01 *vs* pigment gallstones or non-biliary diseases). The binding percentages of CPs to model biliary vesicles were also higher for patients with cholesterol gallstones (n = 6) than those with pigment gallstones (n = 6) (2.4% ± 0.9% *vs* 0.9% ± 0.5%, P < 0.01).

CONCLUSION: Hypersecretion of CPs, especially those in vesicular phase, may be an important change in the early stage of cholesterol gallstone formation.

Key words: Bile; Concanavalin A binding proteins; Cholesterol gallstone; Chromatography

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INTRODUCTION

Protein in bile has been considered for more than 3 decades as a contributing factor to the pathogenesis of gallstone formation[1]. But the essential progress in our understanding of this process was made just only 10 years ago, when Groen and colleagues[2] used lectin affinity chromatography to isolate and purify a potent pronucleating glycoprotein which has since been recognized as the bile-form aminopeptidase N. Since then, numerous nucleation promoting and inhibiting biliary proteins have been characterized[3-5]. But, as of yet, nearly nothing has been done in the research field of the most important or key nucleating proteins.

Concanavalin A-binding proteins (CPs), a group of biliary proteins containing almost all the well known pronucleating proteins, provided an opportunity to study the nucleation-effecting proteins systematically. Vesicular proteins are functionally location-specific pronuncleating proteins, their concanavalin A-binding regions may exert important actions in gallstone formation. In this article, the authors determined the concentrations and nucleation activities of total biliary proteins, biliary CPs and vesicles related to CPs, so as to elucidate the relationship between changes in quantity and quality of biliary CPs and gallstones formation and study systematically the importance of CPs in pathogenesis of gallstone formation.

MATERIALS AND METHODS

Patients

Sixty-six patients were enrolled in this study, including 43 with cholelithiasis (36 with cholesterol gallstones, 7 with pigment gallstones), 9 with gallbladder cholesterosis, 2 each with gallbladder adenomyomatosis and adenoma, and 10 with non-biliary diseases (5 with

Table 1 Concentrations of proteins in human gallbladder bile (g/L, $\bar{x} \pm s$)

Patient group	*n*	Total protein	CP concentration
Cholesterol gallstones	36	4.1 ± 1.5	0.39 ± 0.11
Pigment stones	7	3.2 ± 1.8	0.26 ± 0.12
Gallbladder cholesterosis	9	4.4 ± 1.6	0.40 ± 0.09
Gallbladder adenomyomatosis	2	3.8 ± 1.2	0.29 ± 0.19
Gallbladder adenoma	2	3.9 ± 1.1	0.26 ± 0.14
Non-biliary diseases	10	4.5 ± 1.6	0.27 ± 0.09

[a]$P < 0.05$ *vs* other groups: [b]$P < 0.01$ *vs* pigment gallstones and non-biliary diseases. CP: Concanavalin A-binding protein

Table 2 Nucleation time of human gallbladder bile ($\bar{x} \pm s$)

Patient group	*n*	Nucleation time (d)
Cholesterol gallstones	36	7.8 ± 4.2
Solitary	20	5.3 ± 2.7
Multiple	16	10.1 ± 5.6
Pigment gallstones	7	14.7 ± 6.0[1]
Gallbladder cholesterosis	9	6.1 ± 3.7
Stone-free diseases	14	16.3 ± 3.2[2]

Notes: Those ([1]3 cases, [2]8 cases) without nucleation after 21 d were recorded as 21 d.

colon cancer, 2 with gastric cancer, 1 each with peptic ulcer perforation and pancreatic pseudocyst). All the patients met the following criteria: No acute inflammation of the gallbladder, and no obstruction at the cystic duct or common bile duct; no abnormality in liver biochemistry, and negative bile culture; total lipid concentration in bile of > 50 g/L; serum protein concentration within normal range. Bile was sampled by aspiration according to the standard method[6] during operation and then stored at -20 ℃. Observation of cholesterol monohydrate crystal in bile was carried out immediately after sample obtainment.

Stone classification was based on gross inspection and cholesterol content. Cholesterol gallstone was identified by cholesterol dry weight percentage > 70%; pigment gallstone was identified if the cholesterol dry weight percentage was < 30%.

Isolation of biliary vesicles and quantitation of vesicular protein

Biliary vesicles were isolated from 10 mL bile of each patient according to the method described by Amigo *et al*[7], and further purified according to the method described by Stone *et al*[8]. Vesicular protein concentrations were determined using a pooled concentrated eluent that had undergone ultrafiltration.

Quantitative and nucleating activity analysis of CPs

CPs isolated from 1 mL bile were the bound fractions for concanavalin A affinity chromatography, and determined quantitatively using the Coomassie bright blue method. Nucleation activities were determined as follows: Lyophilized CPs prepared from 1 mL gallbladder bile as stated above were put into 1 mL artificial bile, and then incubated at 37 ℃. Nucleation time (NT) was determined according to Holan's method[9], and the NT ratio was calculated as the relative value to the blank control; final cholesterol crystal concentration (FCCC) was that after 3 wk at 37 ℃, and its relative percentage to the blank control was the FCCC percentage.

Preliminary study of the distribution of CPs in vesicles

To determine the distribution of CPs in the vesicular phase of native bile, concanavalin A was first linked to horseradish peroxidase (HRP) (con A-HRP, 1:1 molar ratio) according to Guo's report[10]. One drop of the con A-HRP solution was added into one drop of bile on a 200-mesh zinc grid that was covered with carbon film, and stored overnight in moist saturation at 37 ℃. The excess liquid was soaked out the next morning and dyed with 0.01% diaminobenzidine (DAB) solution (with dropwise addition of H_2O to bring the volume up to 20 mL) for 15 min. The prepared zinc grids were then gently washed 3 times with buffer solution, dried and examined by a JEM-1200EX electronic microscope at a magnification of 30000 and using an accelerating voltage of 80 KV.

The binding characteristics of CPs to vesicles and micelles in model bile were determined using gallbladder bile samples from 6 patients with cholesterol gallstones and 6 with pigment gallstones. The samples were processed to harvest CPs as described above; after 20 min of ultracentrifugation at 10000 rpm, the CPs were then added into model bile to a final concentration of 0.2 g/L. After incubation at 37 ℃ for 2 wk, the contents of vesicular and micellar protein in these solutions were analyzed.

Table 3 Cholesterol crystals and biliary proteins ($\bar{x} \pm s$)

Patient group	*n*	Total protein (g/L)	CPs (g/L)
Gallstones	36		
With crystal	29	4.9 ± 2.7	0.43 ± 0.14
Without crystal	7	3.4 ± 2.1	0.29 ± 0.16
Gallstone-free	30		
With crystal	14	4.1 ± 1.9	0.40 ± 0.17
Without crystal	16	3.2 ± 2.1	0.31 ± 0.17

Table 4 Influence of Concanavalin A-binding proteins on nucleating activities of model bile ($\bar{x} \pm s$)

Patient group	*n*	Nucleation time ratio	Final cholesterol crystal concentration percentage
Cholesterol gallstones	5	0.57 ± 0.21	143.4 ± 12.6
Pigment gallstones	5	0.71 ± 0.19	121.3 ± 27.4
Gallbladder cholesterosis	5	0.44 ± 0.23	152.7 ± 18.4
Non-biliary diseases	5	0.73 ± 0.11	130.4 ± 15.7

Preparation of model bile

According to the method described by Kibe *et al*[11], this model bile has a CSI of 1.2, total lipid concentration of 100 g/L and cholesterol/lecithin molar ratio of 4. The concentration of biliary protein was determined following the method described by Gallinger *et al*[12]. Dunnett's *t*-test was used to compare the mean values between test groups and control group, and the *U* test was used to compare the mean values between two groups. A probability of < 0.05 was considered significant.

RESULTS

Concentration of total biliary protein and nucleating activities in human gallbladder bile

Table 1 presents our observations of no significant differences in total biliary protein concentration for the patients with cholesterol gallstones and/or gallbladder cholesterosis as compared to other groups. But, a significantly higher concentration of biliary CPs was found in patients with cholesterol gallstones and/or gallbladder cholesterosis ($P < 0.05$). A very significant difference in biliary CPs was also found between patients with gallbladder cholesterosis and pigment gallstone as well as those with non-biliary diseases ($P < 0.01$). Gallbladder bile from patients with cholesterol gallstones and/or gallbladder cholesterosis nucleated more rapidly than those with pigment gallstone and/or other stone-free diseases ($P < 0.001$, Table 2).

Relationship between cholesterol crystals and biliary protein

As shown in Table 3, both the gallstone and stone-free patients with crystals have a higher concentration of total biliary protein than those without crystals, but significant differences were only found between gallstone patients with crystals and stone-free patients without crystals ($P < 0.05$). Biliary CP concentrations, however, were significantly higher in all patient groups with crystals than in the crystal-free patients ($P < 0.05$).

Nucleation activities of biliary CPs

The nucleation time induced by CPs from patients with cholesterol gallstones and gallbladder cholesterosis was significantly shorter than that from patients with non-biliary diseases and pigment gallstones ($P < 0.05$). An even more significant difference was found between patients with gallbladder cholesterosis and pigment gallstone as well as those with non-biliary diseases ($P < 0.01$). CPs from patients with cholesterol gallstones or gallbladder cholesterosis caused a significantly higher FCCC ratio than those with pigment

gallstones and non-biliary diseases ($P < 0.01$, Table 4).

Distribution of CPs in vesicle phase

Using a concanavalin A-affinity staining method, we observed many dark brown spherical vesicles of varied size in native bile. After incubation of artificial bile with CPs for 2 wk, the binding percentage of CPs to vesicles was 2.4% ± 0.9% in patients with cholesterol gallstones, which was significantly higher than that in patients with pigment gallstones (0.9% ± 0.5%, $P < 0.01$).

DISCUSSION

Both pro- and antinucleating proteins have been found in human bile, and dozens of pronucleating proteins and several antinucleating proteins have been discovered in recent years; however, which among them plays the most important or key role in cholesterol nucleation remains unknown. CPs are glycoproteins that contain almost all the nucleating proteins reported. Systematic studies of CPs may provide some approaches for identifying these key factors, but up to now few reports have dealt with this issue.

Theoretically, increase of nucleating proteins in bile would cause an elevation of total protein level[13], but inconsistent results have been reported because too many factors may influence the results. Our current data showed that patients with stone-free diseases had a higher level of biliary total protein than those with cholesterol gallstone (but with no statistical significance; Table 1). Nucleation time, however, was arbitrarily much faster in patients with cholesterol gallstones than those with pigment gallstones (Table 2), indicating that the differences between them arose from their differences in quantity and quality of nucleating proteins. Our results, as shown in Table 1, demonstrated a higher concentration of biliary CPs in patients with cholesterol gallstones and gallbladder cholesterosis than those with pigment gallstones and stone-free diseases, and a stronger nucleating activity for CPs from the former than the latter, indicating that the differences in CPs determined nucleation of cholesterol in bile.

The presence of cholesterol crystal in bile often indicates a high nucleating and crystal growth activity. In the current study, the concentration of biliary CPs in patients with cholesterol crystals was found to be higher than in those without the crystals; moreover, the concentration of biliary CPs in stone-free patients with crystals was higher than that in in gallstone patients without crystals (Table 3), which suggests that elevation of nucleating proteins was not secondary to gallstone formation.

Owing to their special location, vesicular proteins have been considered as a very important group of pronucleating proteins[14], and it was predicted that CPs, which include almost all the pronucleating proteins, also exist in the vesicular phase. We confirmed this prediction by an affinity staining method devised to display the presence of CPs in the vesicular phase. This investigation of CPs bound to artificial vesicles also demonstrated that only a small part of the CPs could bind to vesicles and the binding percentage was much higher for CPs from patients with cholesterol gallstones than that from patients with pigment gallstones. These results imply that vesicle-binding CPs may exert some special effects on cholesterol nucleation in human bile.

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ORIGINAL RESEARCH

A comparative study of biliary trace elements and clinical phenotypes in Wilson's disease

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Abstract

AIM: To further explore the etiological mechanism of Wilson's disease (WD) by comparing the changes of biliary trace elements and its clinical phenotype.

METHODS: WD patients with different types and conditions (n = 20), non-WD patients with chronic liver damage (n = 22), and healthy volunteers (n = 10; used as controls) were studied. Biliary samples were taken by duodenal drainage. Atom absorption spectrophotometer was used to assay the copper and zinc content of each sample.

RESULTS: In WD, the copper content and copper/zinc ratio of biliary juice were evidently lower than those of non-WD patients with chronic liver damage and of healthy controls (F = 14.76, 25.4; 14.92, 26.2 respectively; $P < 0.01$), while the biliary zinc level had no significant difference from the two non-WD control groups ($P > 0.05$). There were significant differences in biliary copper excretion among patients with different types and conditions (F = 3.75, $P < 0.05$; F = 6.20, $P < 0.01$).

CONCLUSION: Copper excretion by liver and the biliary system decreases obviously in WD, which plays a key role in the phenotypic copper retention, and the biliary copper retention is closely related with the severity of hepatic injury and illness.

Key words: Wilson's disease; Copper; Zinc; Duodenal drainage; Bile

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INTRODUCTION

Wilson's disease (WD) is an autosomal recessive disorder of copper transport, first described by Kinnear Wilson in 1992 as hepatolenticular degeneration. The disorder has a worldwide frequency of between 1/35000 and 1/100000 and a corresponding carrier frequency of 1/90. Its symptoms develop from the toxic build up of copper primarily in the liver, and subsequently in the brain, kidney, cornea and other tissues. The resulting liver cirrhosis and/or neurological damage are fatal if not treated with copper chelating agents[1,2].

The gene responsible for WD was independently identified by three research teams in 1993[3-5], and is located on chromosome 13q14.3. The gene codes for a putative intracellular copper transport protein (ATP7B, a member of the cation-transporting P-type ATPase family), spans > 80 kb of genomic DNA and consists of 22 exons[6]. Because of a defective ATP7B, the patients with WD have two fundamental disturbances of copper metabolism: A reduction in the rate of incorporation of copper into ceruloplasmin and a reduction in biliary excretion of copper. Although the role of copper in its pathogenesis has been known for several decades, few studies have been carried out to investigate the exact etiological mechanism of copper retention. Our study was designed to further explore the etiological mechanism of WD by observing the biliary trace element content of WD patients and non-WD patients in combination with its clinical phenotype.

MATERIALS AND METHODS

Subjects

Twenty in-patients with WD were chosen for this study, consisting of 13 men and 7 women with a mean age of 25-year-old. Their diagnoses all met the criteria proposed by Houwen *et al*[7]. Control subjects were chosen from among patients in the Department of Internal Medicine and healthy volunteers, all of who were carefully screened by clinical and copper metabolism examination for the

Table 1 Values of biliary trace elements of the Wilson's disease group and control groups ($\bar{x} \pm s$, μmol/L)

Group	n	Copper	Zinc	Copper/Zinc
Wilson's disease	20	4.42 ± 0.44	55.94 ± 3.14	0.13 ± 0.02
1st control	22	41.70 ± 1.97[a]	34.46 ± 1.85	1.54 ± 0.27[a]
2nd control	10	42.01 ± 2.63[a]	34.30 ± 2.84	1.56 ± 0.24[a]

[a]$P < 0.01$ *vs* Wilson's disease group.

Table 2 Comparison of biliary trace elements for different types of Wilson's disease ($\bar{x} \pm s$, μmol/L)

Type	n	Copper	Zinc	Copper/Zinc
Neurological	11	5.02 ± 0.61[b]	62.58 ± 22.84	0.13 ± 0.06
Psychiatric	5	4.78 ± 0.80[b]	57.41 ± 26.36	0.17 ± 0.14
Hepatic	4	2.31 ± 0.17	35.85 ± 11.51	0.10 ± 0.07

[b]$P < 0.05$ *vs* hepatic type.

Table 3 Variations of biliary trace elements in different severity of Wilson's disease ($\bar{x} \pm s$, μmol/L)

Grade	n	Copper	Zinc	Copper/Zinc
Ⅰ	7	5.67 ± 0.86[c]	82.31 ± 33.71	0.10 ± 0.05
Ⅱ	7	4.72 ± 0.55[c]	51.99 ± 19.57	0.17 ± 0.12
Ⅲ-Ⅳ	6	2.61 ± 0.22	29.76 ± 8.26	0.12 ± 0.07

[c]$P < 0.01$ *vs* Ⅲ-Ⅳ grades.

absence of clinical evidence of WD and then divided into two groups. The first control group consisted of 14 males and 8 females with mean age of 34-year-old, including 12 cases of chronic hepatitis, 6 of cirrhosis and 4 of primary biliary cirrhosis; the second control group of 10 healthy volunteers consisted of 5 men and 5 women with a mean age of 30-year-old.

Laboratory studies

All subjects maintained their regular diet throughout the study and underwent duodenal drainage to obtain a biliary sample after admission to the hospital. Eight of 20 patients with WD had no medicinal treatment before hospitalization, and the remaining 12 had histories of treatment with penicillamine, zinc sulfate, dimercaprol (BAL) and sodium dimercaptosuccinate (DMS); all of the patients with treatment histories were asked to stop taking the above-mentioned medicines for 4 wk prior to the sampling. None of the control subjects had taken any agents that may affect the metabolism of internal trace elements during the 4 wk before sampling. The biliary samples were all collected at 09:00 am. The Hitachi-208 atom absorption spectrophotometer was used to assay the copper and zinc content of each sample. All values are presented as $\bar{x} \pm s$. The *F*-test for variation analysis was employed to determine the statistical significance of differences between the means. *P* values of < 0.05 were considered significant.

Typing and grading

As WD patients have genetic heterogeneity, they were classified according to the following clinical symptoms: Neurological type, 11 cases with predominantly neurological symptoms; psychiatric type, 5 cases with mental symptoms; and hepatic type, 4 cases with liver symptoms[8]. The severity of disease was graded using the modified Goldstein method: Grade Ⅰ, 7 cases with mild extrapyramidal system symptoms, no liver symptoms or obstacles to daily life; Grade Ⅲ, 7 cases with obvious extrapyramidal system symptoms, no liver symptom or mild hepatosplenomegaly with normal liver function, and no hypersplenia; Grade Ⅲ, 4 cases with serious extrapyramidal system symptoms, obvious hepatosplenomegaly and/or liver function injury; and Grade Ⅳ, 2 cases with serious extrapyramidal system symptoms, bedridden, obvious hepatosplenomegaly or complicated with ascites and liver function injury. Typing and grading were accomplished independently by two experienced neurologists in our department, neither of who were aware of the results of biliary trace elements.

RESULTS

The biliary copper content and copper/zinc ratio of WD patients were notably lower than those of the first and second control groups and the differences were significant ($F = 14.76, 25.4; 14.92, 26.2$; $P < 0.01$). The comparison of biliary zinc content among the three groups showed no statistical significance ($F = 1.76, 1.98$, $P > 0.05$), indicating that the internal copper deposit of WD is directly related with the decrease of copper excretion by the liver and biliary system (Table 1).

The biliary trace elements of WD patients with different types are shown in Table 2. The biliary copper content in cases of neurological or psychiatric types was significantly higher than that in the hepatic type ($F = 3.75$, $P < 0.05$). In contrast, the biliary zinc content and copper/zinc ratio were similar among the three types ($F = 0.246$, 0.855, $P > 0.05$), showing that biliary copper excretion is consistent with the severity of hepatic injury of WD (Table 2).

The relationship between biliary trace elements and the severity of WD is shown in Table 3. The severity of the disease was classified into three groups (Grade Ⅰ, Grade Ⅱ and Grade Ⅲ-Ⅳ). The biliary copper content of Grade Ⅲ-Ⅳ WD patients was significantly lower than that of Grade Ⅰ-Ⅱ patients ($F = 6.20$, $P < 0.01$), but the biliary zinc content and copper/zinc ratio showed no significant differences ($F = 1.171, 1.081$, $P > 0.05$) among the 3 groups, illustrating that the severity of WD had a direct influence on biliary copper excretion but had no effect on biliary zinc excretion (Table 3).

DISCUSSION

Wilson's disease has various clinical manifestations caused by large amounts of deposition of internal copper resulting from abnormal copper metabolism. Patients with untreated WD are in positive copper balance. At present, there are many hypotheses to explain the etiological mechanism of copper retention, and the biliary copper excretion disturbance is considered as one of the main intrinsic mechanisms leading to the copper retention of WD[9]; however, the evidence for this remains deficient and there are few published studies of biliary copper excretion for WD. Many assumptions have failed to further explain its relations with the observed clinical conditions of the WD patients. An actual demonstration of its involvement in abnormal copper metabolism of WD remains to be further explored.

The biliary copper excretion speed for normal individuals averages 500-1300 μg/d, basically counteracting the copper absorption in gastrointestinal tract, while the excretion rate in urine and sweat is very small. Copper balance is maintained mainly through biliary copper excretion. In view of this, considerable emphases have been laid on the fact that biliary copper excretion abnormality may underlie the disorder. Indeed, many investigators have been trying to elucidate the exact point of breakdown in the copper metabolic chain of WD. Gibbs and Walshe[10] used 64-Cu to observe directly the excretion process of the biliary tracts of two WD patients with biliary tract fistula and non-WD patients, and found that the biliary copper content of the common bile duct and the copper excretion of the biliary tract for the former were much lower than those for the latter.

Courtoy *et al*[11] found that intravenously administered polymeric IgA in rats was bound to a receptor in the liver parenchymal cells and then transported *via* the endosomes to the bile canaliculus where it was excreted. Lyengar et al[12] further provided evidence that a high molecular weight copper-binding substance existing in the hepatic cells of normal subjects was absent in patients with WD by studying the cholecystokinin-stimulated biliary secretions. Afterwards, Hoof *et al*[13] pointed out that there was a similar mechanism for the transport of a copper protein to the human bile and that the genetic defect of this metabolic pathway in WD will lead to insufficient copper excretion.

Our results demonstrate that the biliary copper content and copper/zinc ratio of the patients with WD are significantly lower than those of non-WD patients with chronic liver damage and of healthy volunteers ($P < 0.01$, respectively). Additionally, we observed that the severity of liver lesions and patient conditions correlate well

with the decrease of biliary copper excretion, while the biliary zinc excretion has nothing to do with WD ($P > 0.05$). All these findings strongly suggest that liver and biliary pathways play important roles in the copper excretion dysfunction of WD and could, therefore, participate directly in the pathophysiology of copper retention. We think that due to a non-functional gene, the patients with WD lack a copper-transporting P-type ATPase (ATP7B) that would otherwise control endosome-mediated copper excretion to the bile canaliculus in the hepatic cells. Consequently, the copper will be routed to the lysosomes, where it will accumulate and fail to be discharged into the bile, leading to obvious decrease of biliary copper excretion and internal copper accumulation. The copper trapped in the hepatic cells further damages the endosomes, resulting in the vicious circle of disruption in copper transport and obvious hepatic damage[14].

This vicious circle may be the reason why healthy individuals and non-WD patients with chronic liver damage and other forms of biliary retention, such as chronic hepatitis or primary biliary cirrhosis, do not have the same outcome, and why the severity of hepatic damage in WD correlates well with the decrease of biliary copper excretion. Therefore, our findings, on one hand, have provided the experimental basis for our final understanding of WD pathogenesis, and, on the other hand, have indicated that if the biliary copper excretion can be promoted therapeutically, satisfactory results will be yielded both in maintaining the negative balance of copper metabolism and alleviating pathological damage to organs.

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BRIEF REPORT

Altered expression of tumor suppressors p16 and Rb in gastric carcinogenesis

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Abstract

AIM: To determine whether expression of the tumor suppressors p16 and Rb is altered in gastric carcinoma.

METHODS: Mucosal biopsies were endoscopically obtained from patients with superficial gastritis (n = 12), atrophic gastritis (n = 15), atypical hyperplasia (n = 20) and gastric cancer (n = 40). Upon obtainment, all samples were immediately fixed with 10% buffered formalin, embedded in paraffin, and sectioned serially. Protein expression of p16 and Rb was detected by immunohistochemistry (ABC method).

RESULTS: The gastric epithelium samples showed various degrees of nuclear immunostaining for p16 and Rb according to the different stage of lesion. Progressive pathology of the lesions was associated with a decreasing trend in positive immunostaining for p16 protein (83.3% > 73.3% > 30.0% > 27.5%) but an increasing trend for Rb protein (25.0% > 46.7% > 60.0% > 67.5%). A negative correlation was found between these two parameters and gastric cancer. Correlation analysis of the 40 cases of gastric cancer identified a negative correlation for 20 of the cases. When positive (n = 9) and negative tissues (n = 11) were compared, a statistically significant difference was found (50.0%, 22.5%, 27.5%) (P < 0.05).

CONCLUSION: Abnormal expression of p16 and Rb may play an important role in gastric carcinogenesis.

Key words: Genes, suppressor, tumor; Gene expression; Retinoblastoma protein/metabolism; Stomach neoplasms/metabolism; Carcinoma/metabolism

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Seropharmalogical effects of Fuzheng Huayu decoction on rat Ito cell morphology and function in culture

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Abstract

AIM: To investigate the mechanisms of anti-liver fibrosis actions of Fuzheng Huayu (FZHY) decoction, which acts to strengthen the body' s resistance and promote blood circulation.

METHODS: Ito cells were isolated from rats and cultured. Serum samples were collected from healthy (normal) rats after administration of FZHY decoction and added to the subcultured cells. The effects of FZHY decoction on the Ito cells were investigated by contrast microscopy (to observe cell morphology), [^{3}H]Pro incorporation assay (cell viability), [^{3}H]TdR incorporation and MTT colorimetric assay (cell proliferation), and [^{3}H]Pro incorporation and collagenase digestion (collagen synthesis rate).

RESULTS: The rat sera samples from rats treated with FZHY decoction had no influence on Ito cell morphology, but improved cell viability and markedly inhibited cell proliferation and collagen synthesis. The magnitude of these effects showed dependence on treatment dosage and drug concentration in serum.

CONCLUSION: The seropharmalogical method can be efficiently used to investigate the pharmacological mechanism of anti-fibrotic traditional Chinese herbs and formulae. Inhibition of Ito cell proliferation and collagen synthesis may be two of the major mechanisms underlying the anti-fibrosis actions of the FZHY decoction.

Key words: Fuzheng Huayu; Liver fibrosis; Ito cell; Collagen synthesis

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INTRODUCTION

Ito cells (also known as hepatic stellate cells, fat-storing cells or hepatic lipocytes) play a key role in liver fibrosis, the main hallmark of chronic liver diseases. In the chronic liver disease condition, Ito cells are activated, proliferative and synthesize large amounts of various components of the extracellular matrix, which may lead to liver fibrosis[1]. When cultured on uncoated plastic plates, Ito cells undergo activation and share the similar features of cell activation that are observed in the *in vivo* condition[2]. Our previous study[3] demonstrated that Fuzheng Huayu (FZHY) decoction, which acts to strengthen the body's resistance and promote blood circulation, exerts a protective effect on the liver in CCl_4-induced fibrotic rats, suggesting its potential for improving liver status and function in patients with cirrhosis. In order to investigate the actions of FZHY decoction on liver cells, the seropharmalogical method was applied to an *in vitro* (culture) system with rat Ito cells to determine the effects on cell morphology and function (*i.e.* cell viability, proliferation and collagen synthesis).

MATERIALS AND METHODS

Animals

Wistar male rats, weighing 350-500 g, were purchased from the Shanghai Science Academy.

Reagents

Minimum essential medium (MEM; developed by Eagle), 199 medium (M199) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco, USA. Pronase E, type IV collagenase, metrizimide and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Co., USA. L- [5-^{3}H] proline ([^{3}H]Pro) was purchased from Amersham Co., England.

Drug

The primary components of FZHY decoction are Cerdeceps, semen Persiciae and Radix Salviae Miltiorr hizae. The decoction fluid extract, containing 2.703 g of each of the above raw herbs per g of fluid, was supplied by the Shanghai Zhonghua Pharmaceutical Factory.

Table 1 Effect of drug sera on Ito cell viability ($\bar{x} \pm s$)

Group	*n*	[^{3}H]Pro (cpm/well)
Controls	4	3610.18 ± 99.47
G	4	6578.13 ± 1690.95[a]
G	4	9606.33 ± 950.85[a]
13.8 g	4	4560.26 ± 2026.83

[a]$P < 0.05$ *vs* controls.

Table 2 Effect of drug sera on Ito cell proliferation ($\bar{x} \pm s$)

Group	*n*	[^{3}H]TdR (cpm/well)	MTT (OD_{570})
Controls	4	2864.50 ± 239.15	0.4206 ± 0.016
G	4	1979.65 ± 76.76[a]	0.4625 ± 0.06
G	4	1882.05 ± 112.08[a]	0.3475 ± 0.040[a]
13.8g	4	1781.93 ± 49.69[a]	0.3268 ± 0.081[a]

[a]$P < 0.05$, *vs* controls. MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

Table 3 Effect of sera in different proportions to medium on Ito cell proliferation ($\bar{x} \pm s$)

Concentration	*n*	Controls	Drug sera	Inhibitive rate (%)
5%	4	1481.90 ± 168.03	703.03 ± 138.37[b]	52.56 ± 9.34
10%	4	2013.88 ± 628.87	637.33 ± 214.53[b]	68.35 ± 10.68
20%	4	2709.88 ± 788.19	480.20 ± 264.31[b]	82.27 ± 9.75

[b]$P < 0.01$ *vs* controls.

Table 4 Influence of drug sera on Ito cell collagen synthesis (%, $\bar{x} \pm s$)

Concentration	*n*	Intracellular		Extracellular	
		Controls	Drug sera	Controls	Drug sera
5%	6	0.42 ± 0.18	0.26 ± 0.10	4.02 ± 1.63	3.12 ± 30.33
10%	6	0.48 ± 0.16	0.25 ± 0.06[a]	4.49 ± 1.28	2.71 ± 1.04[a]
20%	6	0.58 ± 0.26	0.24 ± 0.08[a]	4.80 ± 1.47	2.59 ± 1.06[a]

[a]$P < 0.05$ *vs* controls.

Preparation of drug serum

The FZHY fluid extract was diluted to concentrations of 2.3%, 4.6% and 13.8% (w/v) with distilled water. Healthy (normal) rats were administered one of the dilutions orally at 10 mL/kg wt, twice, at an interval of 2 h. Control rats were given normal saline. One hour after the last administration, blood was collected from the inferior vena cava of the rats under sterile conditions. The samples were centrifuged at 1700 × *g* for 30 min at 4 ℃. The separated sera were then combined from rats that had received the same dosage of drug. The combined samples were mixed thoroughly, inactivated by incubating in a 56 ℃ water bath for 30 min, and stored in -70 ℃ freezer.

Cell isolation and culture

Ito cells were isolated from livers of Wistar rats and cultured according to the modified Friedman method as previously described[4]. Briefly, the cells were identified by immunofluorescent staining with desmin antibody and according to typical appearance under light microscopy. The recovery rate was 2.5 × 10^7 per liver, purity > 95%, and viability > 98%, as determined by the trypan blue exclusion assay. The primary Ito cells were expanded in culture, with passaging using 0.25% trypsin 0.02% EDTA; upon reaching confluence, the cells were subcultured with M199 containing 10% fetal calf serum in a humid CO_2 incubator with 5% CO_2 and 95% air.

Cell proliferation assay

The [^{3}H]TdR incorporation method[5] was used to assess cell proliferation. Confluent subcultured Ito cells in 24-well plates were incubated with M199 containing 5%, 10% or 20% (v/v) drug sera respectively. After 48 h, [^{3}H]TdR was pulsed in at 2.5 μCi/well and the cells were incubated for an additional 24 h, after which the cells were harvested for measurement of cpm by a Wallac 1410 Scintillator (Beckman, USA).

Colorimetric MTT[6]

Confluently subcultured Ito cells in 96-well plates were incubated with 100 μL M199 containing drug serum. After 68 h, MTT solution (5 g/L in PBS) was added to each well at 10 μL per 100 μL medium and the cells were incubated for an additional 4 h. Next, acid-isopropanol (100 μL of 0.04 *n* HCI in isopropanol) was added, mixed thoroughly and allowed to incubate for a few minutes at room temperature to dissolve the dark blue crystals. The plates were read on an ELISA reader at wavelength of 570 nm.

Cell viability assay

Cell viability was indicated by intracellular protein synthesis[7]. Specifically, the cells were processed as described above for the [^{3}H]TdR incorporation method, but with use of the [^{3}H]Pro (2.5 μCi/well) instead of [^{3}H]TdR.

Cell collagen synthesis rate assay

Greets' method[8] was used to assess the collagen synthesis rate. Specifically, confluently subcultured Ito cells in 6-well plates were incubated with M199 containing the drug sera. After 48 h, the culture medium was replaced with DMEM containing 5 μCi/mL [^{3}H]Pro, 100 mg/L β-aminopropionitrile, and 50 mg/mL ascorbic acid. After 24 h, the new culture medium and cells were collected respectively. The cells were processed for whole cell extract. The samples were dialyzed thoroughly and reacted with collagenase. Measurement of the total radioactivity in the samples (cpm-t), and the radioactivity in the samples treated with collagenase (cpm-c) and without collagenase (cpm-b), was made by liquid scintillation spectrometry. The amount of collagen produced (*i.e.* the fraction of collagenous protein) was expressed as percentage of total radiolabeled protein and calculated using the formula:

$$\% \text{ collagen} = 100/[5.4 \times (cpm_t\text{-}cpm_c)/(cpm_c - cpm_b) + 1]$$

RESULTS

Effects of drug serum on Ito cell morphology

Both normal rat and drug serum could sustain cell survival and growth, and there was no significant difference in cell shape. Compared with the fetal calf serum control treatment, normal rat serum and drug serum also showed no different influence on cell shape.

Effects of drug serum on Ito cell viability

The drug serum from the rats treated with 2.3 g and 4.6 g doses improved cell [^{3}H]Pro incorporation, while the latter producing a greater improvement (Table 1).

Effects of drug serum on Ito cell proliferation

Effects of sera from rats treated with different drug doses All sera from the different dose groups were added to Ito cells in 10% (v/v) proportion of culture medium. The drug sera inhibited cell [^{3}H]TdR incorporation and transformation of MTT to formazan, and this inhibitive effect was dose-dependent (Table 2).

Effects of sera concentration on Ito cell proliferation The drug sera derived from the 4.6% (w/v) group were prepared in 5%, 10% and 20% (v/v) proportion to M199 and added to the Ito cells, respectively. The results showed that the inhibitive effect on cell proliferation was enhanced as the drug concentration in M199 increased (Table 3).

Effect of drug serum on Ito cell collagen synthesis

The drug sera derived from the 4.6% (w/v) group were added to cells in 5%, 10% and 20% (v/v) proportion to M199. The 10% and 20% proportions inhibited both intracellular and extracellular collagen synthesis, and showed a greater tendency of dose dependence (Table 4).

DISCUSSION

It is well known that Ito cells play a central role in liver fibrogenesis, and inhibition of Ito cell activation is one of the main targets for

anti-fibrosis therapy[9]. Although the optimal anti-fibrosis drug has not been found yet, some herbal decoctions have been reported to prevent fibrogenesis effectively. Traditional Chinese Medicine (TCM) has shown a brilliant future in its application, in particular. FZHY decoction has been shown to prevent extracellular matrix production and deposits in CCl_4- or DMN-induced fibrotic rats, with potential to improve fibrotic liver structure and function in patients with chronic hepatitis or cirrhosis[3].

Chinese herbs have very complicated components, and their pharmacokinetics remain unclear. Therefore, it is difficult to investigate their pharmacological actions *in vitro* by direct addition of herbs or their rough extracts to cultured cells, because the direct addition method could change the *in vitro* environment (such as medium osmotic pressure and pH value, *etc*.) or cause loss of effective metabolites of the herbs or exert an action of unabsorbed substances. In clinical practice, most herbs are administered orally and absorbed into the blood circulation where they exert their functions. Therefore, the serum pharmacological method of indirect addition of a drug serum to culture systems *in vitro* could overcome the above shortcomings; in this way, when the drug is applied the herbal actions can be studied and their real pharmacological effects determined[5].

We found that drug serum collected 1 h after two administrations of FZHY decoction in rats could be applied to perform convenient and efficient investigations[10]. In this study, the drug serum showed no obvious influence on Ito cell morphology, but could improve cell viability. Thus, the drug serum had no cytotoxicity, but produced an effect of "strengthening sufficiency". This also indicated that the seropharmalogical method could reflect the practical effect of Chinese herbs.

Ito cell activation was characterized in this study by increased total cell number (proliferation) and enhanced per cell fibrogenesis. [^{3}H]TdR incorporation represents the cellular DNA synthesis rate and the cleavage of MTT represents the activity of dehydrogenase enzymes in active mitochondria[6], both of which reflect cell proliferation. Cell collagen synthesis consists of two processes: Intracellular translation and hydroxization, followed by extracellular excretion and processing. The [^{3}H]Pro incorporation and collagenase digestion methods reflect both the intracellular and extracellular collagen production processes for individual cells. In this study, the drug serum decreased the Ito cells' [^{3}H]TdR incorporation and MTT cleavage, and inhibited both intracellular and extracellular collagen synthesis per cell. These effects were related to the drug dose and drug serum concentration. Thus, FZHY decoction could inhibit Ito cell proliferation and collagen synthesis, and prevent cell activation. This may be one of the major mechanisms of the anti-fibrotic actions of FZHY decoction.

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BRIEF REPORT

Influence of fever on biliary elements of guinea pigs

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Abstract

AIM: To study the influence of fever on biliary elements and gallstone formation in guinea pigs.

METHODS: Sixty guinea pigs were randomly divided and fed either a lithogenic diet (to induce gallstone formation) or a normal diet (for use as the non-gallstone controls), and each group was then subdivided into fever or non-fever subgroups. The fever condition was induced by subcutaneous injection of boiled non-fat milk (1 mL/kg, once a week for 4 wk). After 45 d, all the animals were euthanized for analysis; however, 36 h prior to euthanasia, the guinea pigs in the fever subgroups were injected subcutaneously with turpentine (1 mL/kg) to maintain the fever condition. Gallbladder lumens were examined and bile samples were analyzed.

RESULTS: Gallstone incidence was highest (40%, 6/15) in the group of animals that were fed the lithogenic diet and had fever. Compared to the non-fever subgroups, the fever subgroups had significantly higher total bile protein and bilirubin.

CONCLUSION: Fever influences biliary elements and may contribute to gallstone formation in guinea pigs.

Key words: Fever; Bile/metabolism; Bilirubin/metabolism; Cholelithiasis/etiology; Proteins/metabolism

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Clinical and experimental studies on stomach carcinoma treated with Yangwei Kangliu granules

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Abstract

AIM: To study the anti-cancer mechanism of Yangwei Kangliu (YWKL) granules from the view point of red blood cell (RBC) immunity and to investigate the relationship between RBC immunity and T lymphocyte immunity.

METHODS: Fifty patients with advanced gastric carcinoma were treated with a combination of YWKL granules and chemotherapy. Venous blood samples were obtained before treatment and after one course of treatment. The rosette rate of c-3b-receptor (RBC-C-3bRR), tumor and red cell (RRTR) and RBC immune complex (RBC-ICR) were measured under microscopy by counting the rosettes formed by sensitized or unsensitized yeast adherence. The T lymphocyte subset was observed by the method of APAAP. Control patients were treated with chemotherapy alone (n = 20). In addition, mouse tumor studies were performed to investigate the dynamic changes of RBC-C-3bRR, RRTR and RBC-ICR in response to treatment with YWKL granules (n = 30). Mice treated with chemotherapy alone (n = 30) or water alone (n = 30) were used as controls.

RESULTS: The clinical therapeutic effect of combination treatment with YWKL granules and chemotherapy (i.e. the treatment group) was markedly superior to that of chemotherapy alone (i.e. the control group) (P < 0.01). In the treatment group, the rosette rates of RBC-C-3bRR and of RRTR were significantly increased (P < 0.01) after treatment, the rate of RBC-ICR was markedly decreased (P < 0.01), and the ratio of CD4 to CD8 was obviously elevated (P < 0.01). Moreover, CD8 was much lower (P < 0.01) and the ratio of CD4 to CD8 was much higher (P < 0.01) than that in the control group. The RRTR rate was positively correlated with the ratio of CD4 to CD8. In mice, on day 9 of bearing cancer, the tumor weight in the group treated with YWKL granules alone was much lower than that of the tumors in the control mice groups; in addition, the YWKL treated mice showed higher RBC immune function than the mice of the two control groups. On day 13 of bearing cancer, however, the differences in both tumor weight and RBC immune function had disappeared.

CONCLUSION: The anti-cancer mechanism of YWKL granules may involve enhancement of RBC immunity and of T lymphocyte immune function, which is supported by the finding of RBC immune function being correlated with T lymphocyte immune function.

Key words: Stomach neoplasms; Yangwei Kangliu granules; RBC immunity

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INTRODUCTION

Several studies have provided experimental and clinical data demonstrating that the immune system plays a key role in controlling the occurrence and development of tumors. As a result, red blood cell (RBC) immunity has emerged as a new subdiscipline in the modern field of immunology[1]. Since then, studies have identified a close correlated between RBC immunity and prognosis of tumors, and have characterized the functions as involving clearance of the immune complex in circulation, controlling and regulating other immune cells and effector-like actions.

The current study was designed to investigate the anti-cancer effects of Yangwei Kangliu (YWKL) granules and its function of strengthening body resistance by supporting a healthy qi and dissipating blood stasis and toxic material; specifically, its effects on RBC immune activity were studied in humans and a mouse model system.

MATERIALS AND METHODS

Subjects

A total of 70 patients with pathology-confirmed gastric cancer who were admitted to our hospital or to the China-Japan Friendship Hospital for treatment were enrolled in our study and randomly divided

Table 1 Change of red blood cell immune function in the Yangwei Kangliu treatment group (%, $\bar{x} \pm s$)

	n	C-3b-RR	Immune complex	Tumor and red cell
Normal	30	17.3 ± 5.04	9.6 ± 1.72	40.6 ± 6.17
Pre-treatment	50	8.8 ± 2.37	13.2 ± 2.88	29.1 ± 6.32
Post-treatment	50	15.0 ± 2.71[b]	8.9 ± 1.87[b]	39.2 ± 1.96[b]

[b]$P < 0.01$ *vs* pre-treatment.

into the YWKL treatment group (for receipt of a combination therapy with YWKL granules plus chemotherapy) and the standard treatment control group (for receipt of chemotherapy alone). The YWKL treatment group consisted of 50 patients, including 41 males and 9 females ranging in age from 35 to 70-year-old, with a median age of 55-year-old. The treatment control group consisted of 20 patients, including 18 males and 2 females ranging in age from 30 to 60-year-old, with a median age of 51 years-old.

The Karnofsky performance status (KPS) ranged from 60 to 70 for 13 cases in the YWKL treatment group and for 6 cases in the control group, from 71 to 80 for 33 cases in the YWKL treatment group and for 12 cases in the control group, and > 80 for 12 cases in the YWKL treatment group and for 4 cases in the control group. According to the TCM differentiation, 33 cases were classified as stomach and spleen deficiency type, 7 as functional incoordination of liver and stomach type, and 2 as preponderance of pathogenic heat.

Therapeutic method

The YWKL granules used in therapy were composed of Rhizoma atractylodis, Radix astragali, Lignum sappan, Rhizoma paridis, Cordyceps, *etc*. (manufactured by the Great Wall Pharmaceutical Factory, Beijing). The YWKL drug was administered orally, three times a day, 1 packet each time (equivalent to 30 g of raw herbs). Chemotherapy was administered simultaneous to the CMF regimen, and delivered as an intravenous infusion of carboplation 400 mg on day 1, mitomycin 6 mg on days 7 and 14, and 5-fluorouracil 500 mg on days 7, 10, 14 and 17, for a total of 2-3 cycles (each cycle consisting of 4 wk, and one course consisting of 2-3 cycles).

The control group received the chemotherapy as described above, but without any YWKL.

Determination indices

The rate of c-3b-receptor (RBC-C-3bRR), tumor and red cell (RRTR) and RBC immune complex (RBC-ICR) were determined by the method described by Guo *et al*[2]. The T lymphocyte subset was observed by the method of APAAP.

Criteria for assessment of efficacy

Efficacy of the treatments was assessed according to the short-term effectiveness criteria set forth by the World Health Organization. For complete response (CR), the foci completely disappear, and no new foci are present. For partial response (PR), the foci size decreases by 50% or more, and no new foci are present. For stable disease (SD), the foci size decreases by < 25% or increases by < 25%. For progressive disease (PD), a single focus size or the total size of multifocal lesions increases by 25% or more, or new foci are present.

Clinical outcome

For the purposes of this study, short-term effectiveness was summarized as follows. In the YWKL treatment group, 8 cases achieved PR, 40 achieved SD, and 2 achieved PD. In the control group, 0 cases achieved PR, 16 cases achieved SD, and 4 cases achieved PD. The differences for each outcome between the two groups were statistically significant ($P < 0.05$).

The changes of RBC immune function are presented in Table 1. Thirty healthy persons served as normal controls. Before treatment, the RBC-C_{3b}RR and RRTR in patients with advanced gastric carcinoma were markedly lower than those in the normal controls ($P < 0.01$), while the RBC-ICR was much higher; after receipt of the combined treatment of YWKL and chemotherapy, the RBC-C_{3b}RR and RRTR were both significantly enhanced, and the RBC-ICR was decreased as compared with those observed at the pretreatment stage ($P < 0.01$).

Table 2 Change in T lymphocyte subset between the Yangwei Kangliu + chemotherapy (treatment) and chemotherapy only (control) group

Group	*n*	CD_3	CD_4	CD_8	CD_4/CD_8
Treatment group					
Pre-treatment	50	66.00 ± 6.81	38.00 ± 4.52	32.36 ± 2.91	11.23 ± 0.166
Post-treatment	50	66.27 ± 5.55	39.00 ± 4.15	26.45 ± 2.84[b]	1.48 ± 0.156[b]
Control group					
Pre-treatment	20	63.59 ± 8.32	38.18 ± 6.7	31.56 ± 2.82	1.26 ± 0.22
Post-treatment	20	62.18 ± 9.03	36.37 ± 3.39	32.47 ± 4.89[d]	1.21 ± 0.2[d]

[b]Compared with the treatment group in pre-treatment $P < 0.01$; [d]Compared with the treatment group in post-treatment, $P < 0.01$.

The changes in T lymphocyte immune function are presented in Table 2. After treatment, although the CD_3 and CD_4 levels were elevated, there were no difference from the pretreatment stage ($P > 0.05$). However, the level of CD_8 was significantly lower ($P < 0.01$) and the ratio of CD_4 to CD_8 was significantly greater ($P < 0.01$) than the pretreatment levels. The control group showed no differences of these indices from the pretreatment levels to the post-treatment levels. Comparison of the post-treatment indices of the treatment group and the control group showed no differences for the levels of CD_3 and CD_4 ($P > 0.05$). However, compared to the control group, the level of CD_8 in the treatment group was significantly lower ($P < 0.01$) and the ratio of CD_4 to CD_8 was also significantly higher ($P < 0.01$). The RRTR was positively correlated to the ratio of CD_4 to CD_8 ($Y = 1.14 + 0.005x$, $P < 0.01$).

EXPERIMENTAL STUDIES

Materials and methods

Tumor model and transplantation The fore-stomach carcinoma cell, which is a model of high pulmonary metastasis, were retrieved from storage in liquid nitrogen were thawed at 37-40 ℃ and inoculated subcutaneously into the right back of inbred 615-strain. When the resultant tumor had grown to 1.0-1.5 cm in diameter, the mice were sacrificed and the tumor tissues were resected and made into a single cell suspension. Then, this suspension was brought up to 0.2 mL with normal saline consisting of 1×10^6 tumor cells and was injected subcutaneously into the right back of a fresh mouse.

Grouping The transplanted mice were randomly divided into three groups of 30 for treatment with either TCM (administered 0.8 mL YWKL liquid, once daily, starting at 24 h after transplantation, with daily dose was equivalent to 1.4 g of raw drugs), chemotherapy (administered 25 mg/kg 5-fluorouracil, once every other day, intragastric), or control (administered 0.8 mL water, once daily, intragastric).

Determination index The inhibitory rates for tumor, RBC-C_{3b}RR and RBC-ICR were determined as described above.

Methods

A batch of mice were sacrificed respectively on post-inoculation day 3, 9 and 11; ten mice were sacrificed for each time point. The tumors were resected and weighed, and the inhibitory rate was calculated. Blood was collected by retro-orbital puncture and used to test the RBC-C_{3b}RR and RBC-ICR.

RESULT

Data for the dynamic changes of tumors in mice are shown in Table 3. After the latent stage (3-5 d of bearing tumors), the tumors grew rapidly; mice began to die on day 13 in all three groups. On day 3 post-inoculation, when the first batch of mice was sacrificed, no tumors were apparent. On day 9, the tumor weight was markedly lower in the TCM group than in the control or the chemotherapy group ($P < 0.01$ or $P < 0.05$). On day 13, there were no differences in tumor weights among the three groups ($P > 0.05$).

Table 3 Dynamic changes in tumor weight between experimental groups

Group	*n*	Day 9 post-inoculation	Day 13 post-inoculation
Control	10	1.27 ± 0.47	2.07 ± 0.42
TCM	10	0.74 ± 0.43[ab]	2.04 ± 0.21
Chemotherapy	10	1.196 ± 0.23	1.98 ± 0.35

[b]Compared with the control, $P < 0.01$; [a]Compared with chemotherapy, $P < 0.05$.

Data for the dynamic changes of RBC immune function in mice are shown in Table 4. With the development of tumor, the rate of RBC-C_{3b}RR decreased and that of RBC-ICR increased. On post-inoculation days 3 and 9, the RBC-C_{3b}RR of the TCM group was higher and the RBC-ICR was lower than that of the chemotherapy and control groups ($P < 0.01$); however, on day 13 there were no differences between the RBC-C_{3b}RR and RBC-ICR for the three groups ($P > 0.05$). At no time did the chemotherapy and control group show differences in RBC-C_{3b}RR and RBC-ICR.

DISCUSSION

Malignancy is closely correlated with RBC immunity. At present, RBC immune activity is often assessed by means of determination of RBC immune adhesion and the execution of RBC immune adhesion *via* the C_{3b} receptor[2,3].

Assuming that patients with advanced gastric carcinoma are always in a state of deficiency of healthy qi and stagnation of exogenous factor, YWKL granules would then function to support healthy qi, dissipating blood stasis and clearing away toxic materials. The prescription of YWKL used in this study included Rhizoma atractylodis and Radixs scutellariae, both of which can strengthen body resistance, Lignum sappan, which can activate blood circulation to dissipate blood stasis in the stomach and spleen, and Rhizoma paridis, which can clear away heat and toxic material. Our clinical and experimental study of this prescription showed that, in both patients with gastric cancer and mice bearing tumors, the RBC -C_{3b}RR and RRTR were low and the RBC-ICR was high before treatment. The experiment on mice further demonstrated that as the tumors grew, the red blood cell immunity gradually declined. On the ninth day of bearing tumor, the tumor weight in the YWKL treatment group was lower than that of the other groups; correspondingly, the RBC immunity was higher than that of the other two groups. On the thirteenth day of bearing tumor, there was no obvious difference of tumor weight, and no difference of RBC immunity existed among the three groups.

Clinical data had shown that the short-term effectiveness of

Table 4 Dynamic changes in red blood cell-c-3b-receptor and red blood cell-immune complex

Group	Red blood cell-c-3b-receptor			Red blood cell-immune complex		
	D3	D9	D13	D3	D9	D13
Control	5.27 ± 2.96	3.57 ± 1.04	2.88 ± 1.10	7.14 ± 1.93	9.80 ± 2.55	12.29 ± 4.83
TCM	13.40 ± 5.70[av]	7.20 ± 1.27[ac]	4.37 ± 1.12	3.96 ± 1.51[ac]	6.45 ± 1.03[ac]	11.52 ± 2.81
Chemotherapy	3.53 ± 1.17	2.94 ± 1.18	3.15 ± 1.16	7.89 ± 2.76	9.05 ± 2.85	14.30 ± 5.09

[a]Compared with the control, $P < 0.05$; [c]Compared with chemotherapy, $P < 0.05$; D: Day.

YWKL combined with chemotherapy was superior to that of chemotherapy alone and that the post-treatment RBC immunity was higher than that of the pre-treatment samples from the patients who were treated with the combination of YWKL granules and chemotherapy. According to the amount concept[4], the fewer the tumor cells the better the effect of the chemotherapy regimen, and of the immune therapy that is administered in conjunction or afterwards; moreover, when the number of tumor cells is below 10^6, the immune system may be able to eliminate it. Therefore, we believe that the anti-cancer mechanism of YWKL granules involves its improvement of immune activity, especially of the RBC immune activity, and this can make the treatment level effective in cases with 10^6 and $10^{7\text{-}8}$ tumor cells or even higher. This is also the theoretical basis that supports effectiveness of YWKL granules combined with chemotherapy is better than that of chemotherapy alone. Of course, whether YWKL granules can directly kill tumor cells as chemotherapy awaits further study. As to the cause underlying the observation that the inhibitory rate in the chemotherapy group was lower than that in the YWKL group, we speculate that 5-FU was not sensitive to Fc cells and it inhibited the immune function of the organism.

The anti-cancer effect of RBC immunity is due to its ability to not only clear the immune complex in circulation but also to control and regulate other immune cells, which is supported by the observed correlation between the RBC and T-lymphocyte subset.

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BRIEF REPORT

Effect of esophageal cancer- and stomach cancer-preventing vinegar on N-nitrosoproline formation in the human body

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INTRODUCTION

N-nitroso-compound (NNC) exposure can induce formation of many kinds of tumors in the human body, and as such represents a potential environmental cause of some human cancers. Many of the precursors of NNC are abundant in the modern environment, such as nitrate, mitrite and amino acid compounds, all of which can synthesize into corresponding NNCs, and the occurrence of stomach cancer has been associated with the ingestion of these precursors. Indeed, the gastric juice of patients with stomach cancer have been shown to have higher concentrations of both nitrates and compounds that can form introso compounds, compared to healthy individuals[1]. Ohshima *et al*[2] measured the concentration of N-nitrosoproline (NPRO) and of nitroso-L-proline (L-Pro) in urine following ingestion of nitrates with the aim of monitoring NNC synthesis in the human body and of studying the various kinds of factors influencing synthesis. Moreover, it has been suggested that blocking the synthesis of NNC in the human body may have important theoretical and practical significance in preventing cancers.

Vitamin C is well known for its efficacy in blocking NNC formation in the human body. Therefore, in this study, we tested whether esophageal cancer- and stomach cancer-preventing vinegar (OSCPV, China-produced patent vinegar [Patent No. ZL90102404], which is rich in vitamins) exerts it protective effect by blocking the synthesis of NPRO in the human body.

MATERIALS AND METHODS

Materials

Sodium nitrate AR was purchased from Xi'an Chemical Reagent Factory (Batch No. 83091). L-Pro chromatography depurant was purchased from Shanghai Biochemical Research Institute of the Chinese Academy of Sciences (Batch No. 8507169). NPRO and *N-nitrosopipecolic acid* (NPIC) (experimental quality) were purchased from IARC. Nitroso-methylurea, and OSCPV were also obtained.

Methods

All the subjects underwent gastroscopy. During the 2 d prior to the start of the experiment and during the experimental period, the subjects' food intake was restricted to rice and steamed buns. On the day when a urine sample was taken, the subjects were given a cooked wheat pastry for breakfast. The dietary rules strictly followed the requirements of the experiment. During the experiment, the subjects did not eat meat products or any other food that may contain NPRO, and they took no cigarettes, tea, beer, fruits, or medicines; only water was allowed for drinking.

The experiment course encompassed the following three stages. Stage A: On day 3 of the experiment, we tested the output of NPRO in the 24 h urine sample, which represented the background level. Stage B: At 7:00 a.m., the subjects drank 10 mL of water containing 300 mg $NaNO_3$ and another 10 mL of water containing 500 mg L-Pro as well as 15 mL of OSCPV, after which the counting time for urine collection was initiated. Twenty minutes later, the subjects ate breakfast. Stage C: At 48 h after stage B (this timing was used to ensure that the subjects had been able to completely discharge the NPRO gained in stage B), urine was collected. All urine samples were collected into individual polythene pails containing 10 mL of a solution of 3.6 *n* sulfosol in 20% ammonia sulfonate (AS solution). Each sample was tested respectively to measure output of NPRO corresponding to each stage of the experiment. To determine whether the urine collection was complete, we also tested the 24 h output of creatinine.

We used the methods of Ohshima *et al*[2] for extracting and testing NPRO in the urine. The main procedure is as follows: 25 mL urine is added to 400 mg NPIC (the internal standard) and 2 mL AS is added, after which the sample is extracted a total of 3 times using mixing with 30 mL, 20 mL and 20 mL ethyl acetate respectively. The final extract is dehydrated with anhydrous sulfate and placed into a rotary evaporator (inside temperature of 60 ℃). After evaporation,

Table 1 Effect of esophageal cancer- and stomach cancer-preventing vinegar in blocking synthesis of N-nitrosoproline in 76 patients

	Content of N-nitrosoproline (nmol/24 h urine)			
	Stage A	Stage B	Stage C	P
x	26.18	66.84	23.91	
s	5.78	17.29	4.37	< 0.001

Table 2 Effect of esophageal cancer- and stomach cancer-preventing vinegar in blocking synthesis of N-nitrosoproline in 76 patients according to disease subgrouping

Group	Average age, years	Sex, male/female	Content of N-nitrosoproline			Blockage rate, %
			Stage A	Stage B	Stage C	
Esophagus	40	9/3	21.30	60.17	28.21	82.22
Esophageal + cardia	46	9/6	30.15	81.42	23.98	122.03
Esophageal + non-typical hyperplasia	48	6/6	26.22	46.60	20.86	126.30
Superficial gastritis	46	9/6	28.63	73.10	25.11	107.91
Atrophic gastritis	54	9/3	32.86	89.63	28.37	107.90
Intestinal metaplasia + non-typical hyperplasia	54	6/5	17.90	50.13	16.90	103.10

Blockage rate, % = [(Stage B-Stage C)/(Stage B-Stage A)] × 100%

the solute is mixed with 2 mL ether and dissolved in diazomethane methyl methyl ester for 5 min. The methods of preparing diazomethane methane and dissolving with methyl ester involved putting about 1 g nitroso urea (synthesized in-house) into a 250 mL flask and then adding 60 mL ether, 20 mL methanol and 10 mL 60% KOH solution, and shaking the flask. In order to dissolve with methyl ester, the ether solution (which is filled with diazomethane methane) was placed into a 2-mouth flask. The diazomethane methane was combined with mitogen into the solution of ether containing dissolved NPRO and NPIC. The methyl ester dissolution was terminated when the solution of ether turned yellow. The procedure was conducted in a hood. Next, the solution was condensed to 10 mL with nitrogen and 0.5 mL of GC-TEA was added to test the content of methyl ester of NPRO. The content of methyl ester of standard NPRO, which was taken through the same treatment, was used as a quantitative reference. The chromatographic column used in the next step was a 2 mm × 3 mm stainless steel column with filling material of 60-80 order chromosorb WAW-DMCS and 10% Carbowax 20 M spread. The carrier was argon and the speed of the flow was 20 mL/min. The temperature of the column was 185 ℃, of the evaporator was 257 ℃, of the TEA pyrolysis was 500 ℃, and of the interface was 200 ℃. Under these conditions, the NPRO and NPIC were able to be well dissociated, with no other interfering peak produced. The lowest detection limit was < 0.5 ppb, and the recovery rate was 85%.

RESULTS

In the experiment, we took the output of NPRO—the level of endogenous nitrosification—as an index. After the 76 patients took 55 mg L-Pro, the output of NPRO in the 24 h urine sample rose from 26.18 nmol to 66.84 nmol, for an increase of 2.5 times. In contrast, after taking OSCPV and L-Pro, the NPRO in urine was reduced, to a concentration lower than the background level, which shows that OSCPV can block the synthesis of NPRO in the human body completely and suggests OSCPV as an ideal blockage agent for prevention of cancer (Tables 1, 2).

CONCLUSION

OSCPV is an effective agent that can block the synthesis of NNC. It is effective in blocking the synthesis of NPRO, which can induce cancer in the human body, making this agent capable of preventing human cancers in an effective manner.

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BRIEF REPORT

Gastric emptying and plasma levels of gastrointestinal hormones in patients with peptic ulcer

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Abstract

AIM: To study the plasma level of gastrointestinal hormones and the time of gastric emptying in patients with peptic ulcer.

METHODS: Thirty patients with gastric ulcer (GU), 29 patients with duodenal ulcer (DU), and 12 healthy controls were studied. Plasma levels of somatostatin (SS), vasoactive intestinal peptide (VIP) and substance P (SP) were measured by radioimmunoassay. Gastric emptying half-time ($GET_{1/2}$) was measured by the $TC\text{-}99^{m}$ resin/solid meal method.

RESULTS: $GET_{1/2}$ was significantly longer in the GU patients than that in the healthy controls (65.9 ± 14.8 min *vs* 53.3 ± 4.3 min, $P < 0.01$) and plasma VIP levels were significantly higher (37.5 ± 10.7 ng/L *vs* 18.4 ± 5.9 ng/L, $P < 0.05$).There was a significant positive correlation between $GET_{1/2}$ and plasma VIP levels ($r = 0.55$, $P < 0.01$). No significant differences were found in SS and SP levels when GU patients were compared with healthy controls ($P > 0.05$). $GET_{1/2}$ was markedly shorter in the DU patients than in the healthy controls (41.7 ± 10.2 min *vs* 53.3 ± 4.3 min, $P < 0.01$) and plasma SS levels were significantly lower (6.4 ± 2.5 ng/L *vs* 11.9 ± 3.4 ng/L, $P < 0.01$). There was a significant positive correlation between $GET_{1/2}$ and SS levels ($r = 0.56$, $P < 0.01$). Plasma SP levels in the DU patients were significantly higher than those in the healthy controls (54.4 ± 12.7 ng/L *vs* 41.6 ± 5.8 ng/L, $P < 0.01$). There was a significant negative correlation between $GET_{1/2}$ and SP levels ($r = -0.68$, $P < 0.01$). No significant differences were found in the plasma VIP levels when DU patient were compared to healthy controls ($P > 0.05$).

CONCLUSION: Elevation in VIP may contribute to occurrence of GU and its associated delay in $GET_{1/2}$. Increased SP and reduced SS may play important roles in $GET_{1/2}$ acceleration and in the pathogensis of DU.

Key words: Peptic ulcer/physiopathology; Gastric emptying; Gastrointestinal hormones/blood

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