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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007

^[3]Passed away on June 14, 2008



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MicroRNA signatures in liver diseases

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INTRODUCTION

Genomic studies have demonstrated that many portions of the human genome do not encode conventional protein-coding genes but encode biologically active non-coding RNA species^[1]. With the development of small RNA interface techniques over the past decade, it becomes clear that many small RNA molecules could regulate gene and protein expression. One class of such small non-coding RNAs is microRNAs (miRNAs), a group of regulatory RNAs of 19-22 nucleotides involved in control of gene expression at the post-transcriptional level^[2]. Recent studies suggest that miRNAs are involved in regulating cell fate (cell death and proliferation), initiation and progression of human cancer, developmental timing, and inflammatory responses^[3-6]. Modulation of miRNA expression *in vitro* as well as *in vivo* has revealed an important role of miRNAs in liver functions. In this review, we appraise the recent findings on miRNAs in liver physiology and disease. We will also discuss the development and use of miRNA antagonists (antagomirs) to target miRNAs *in vivo*, which may translate into novel therapeutic strategies for liver disease in the future.

Abstract

MicroRNAs (miRNAs) are an emerging class of highly conserved non-coding small RNAs that regulate gene expression at the post-transcriptional level. It is now clear that miRNAs can potentially regulate every aspect of cellular activity, including differentiation and development, metabolism, proliferation, apoptotic cell death, viral infection and tumorigenesis. Recent studies provide clear evidence that miRNAs are abundant in the liver and modulate a diverse spectrum of liver functions. Deregulation of miRNA expression may be a key pathogenetic factor in many liver diseases including viral hepatitis, hepatocellular cancer and polycystic liver diseases. A clearer understanding of the mechanisms involved in miRNA deregulation will offer new diagnostic and therapeutic strategies to treat liver diseases. Moreover, better understanding of miRNA regulation and identification of tissue-specific miRNA targets employing transgenic/knockout models and/or modulating oligonucleotides will improve our knowledge of liver physiology and diseases.

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Key words: MicroRNAs; Non-coding RNAs; Liver; Tumorigenesis; Gene regulation

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miRNAs ARE REGULATORY NON-CODING SMALL RNAs IMPORTANT TO POST-TRANSCRIPTIONAL GENE REGULATION

miRNAs are endogenous, single-stranded RNA molecules consisting of approximately 22 non-coding nucleotides that regulate target genes^[2,3]. miRNA molecules have been identified in over 80 species including those encoded by viral genomes. There are approximately 500-1000 different mammalian miRNA genes; a complete list and details about the nomenclature of the miRNAs can be viewed at Sanger mirBase 10.1 (<http://microrna.sanger.ac.uk/sequences/>)^[7]. Up to 600 miRNAs have been identified in humans^[2,3].

Most miRNAs are generated by RNA polymerase

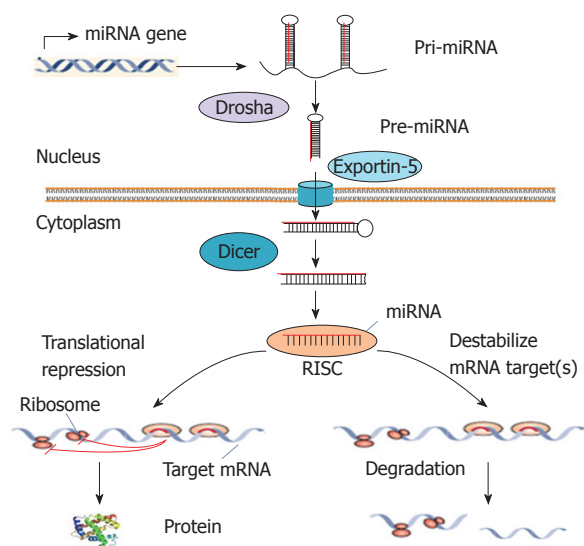


Figure 1 miRNA biogenesis and function in animal cells. Animal genomes have specific genes that encode miRNAs. Primary miRNA transcripts (pri-miRNAs) are processed into precursor miRNA (pre-miRNAs) stem-loops of approximately 60 nucleotides in length by the nuclear RNase III enzyme Drosha. These pre-miRNAs are transported to the cytoplasm via exportin-5 and are further processed by the ribonuclease Dicer. Mature miRNAs are then incorporated in the RNA-induced silencing complex (RISC) and interfere with the regulation of mRNA translation by targeting mRNAs resulting in mRNA degradation or translational repression.

as long primary transcripts (pri-miRNAs) that form a stem-loop structure^[8-11]. In the nucleus, pri-miRNAs are processed into 70-100 nucleotide-long hairpin pre-miRNAs by the RNase III Drosha. These pre-miRNAs are then exported into the cytoplasm by exportin-5^[11]. They are then further processed by another RNase III, Dicer. The resultant approximately 22-nucleotide RNA duplexes contain the mature miRNA and the passenger miRNA strand^[12,13]. The mature miRNA can be incorporated into the so-called RNA-induced silencing complex (RISC). This miRNA-mRNA interaction either blocks translation initiation, induces the endonucleolytic cleavage of the target mRNA, or both (Figure 1)^[12-14]. In mammal cells, the sequence of the miRNAs loaded in the complex targets the RISC to specific binding sites in the 3'-untranslated region (UTR) of mRNA transcripts. Each mRNA can be regulated by several miRNAs, and one miRNA can recognize several targets^[12,13,15]. Intriguingly, miRNAs may also lead to an upregulation of gene expression^[16]. However, the exact mechanism is currently unknown but may be the result of direct effects, such as chromatin remodeling, or indirect effects, e.g. suppression of transcriptional repressors. It is believed that expression of 30% of human genes may be regulated by miRNAs^[17].

miRNAs ARE ABUNDANT AND FINELY REGULATED IN THE LIVER

One of the first clues of the existence of miRNAs in mammals came from studies on genetic alterations in liver tumors. An unusual transcript, named hcr, was described

and characterized as liver-specific, essentially non-coding, specifically nuclear, and processed by endonucleases in one of woodchuck liver tumors investigated in 1989^[18]. Later on, the hcr transcript was found to encompass the so-called "pri-miRNA" for miR-122^[19]. miRNA-122 was later described as a liver specific miRNA and has been reported in mouse, woodchuck and human livers, in human primary hepatocytes, and in cultured liver derived cells^[19,20]. Besides miR-122, many other miRNAs, such as miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, miR-143, and the *let-7* family, are also abundantly expressed in adult liver tissue. While miR-122 appears as the most highly expressed miRNA in adult liver, miR-92a and miR-483 seem to be more specifically expressed in the fetal liver (Table 1)^[21]. Thus, the liver displays a differential miRNA expression profile during its development.

The liver contains many cell types including parenchymal cells (i.e. hepatocytes) and "non-parenchymal cells" which include endothelial cells, stellate cells, lymphoid cells, and biliary epithelial cells (cholangiocytes). Each cell type may have completely distinct miRNA expression profiles. However, miRNA expression profile in those cell types, in particular from humans, is not fully tested yet. Current understanding is limited to available human cell lines. For example, a distinct expression profile of miRNAs has been identified in H69 cells, a cell line of SV40 transformed human cholangiocytes^[22,23].

The molecular mechanisms underlying transcriptional regulation of miRNA genes in the liver remain largely unknown. Hepatocyte nuclear factor 1 α (HNF-1 α) is a hepatocyte-enriched transcription factor. Manipulation of HNF-1 α function through RNA interference causes reciprocal changes in miR-107 expression and thus, may be involved in the regulation of miR-107 transcription in the liver^[24]. The myocyte enhancer factor-2 transcription factor can increase the expression of miR-1-2 and miR-133a-1^[25]. The transcription factor Myc can up-regulate the expression of miR17-92 cluster and down-regulate several other miRNAs in tumorigenesis^[26]. However, whether these transcription factors are also involved in the transcriptional regulation of those miRNAs in the liver is unclear. In addition, decrease of *let-7* family expression in human cholangiocytes in response to microbial stimulation appears to be nuclear factor (NF)- κ B-dependent^[23]. On the other hand, the functional expression of transcription factors can also be regulated by miRNAs, as it has recently been shown for signal transducer and activator of transcription 3 (STAT-3)^[22].

miRNAs MAY BE KEY PLAYERS IN THE REGULATION OF LIVER FUNCTIONS

For a comprehensive understanding of miRNA function and potential therapeutic use in liver physiology and disease, identification and validation of miRNA targets are of fundamental importance. Several bioinformatic methods have been developed to predict miRNA targets^[7]. Arora and Simpson adapted the 'ranked ratio

Table 1 Repertoire of miRNAs in human liver

	Atlas	Adult	Fetal		Atlas	Adult	Fetal
miR-122	+++	+++	++	miR-154	+	/	/
miR-126	+++	/	/	miR-193a	+	/	/
miR-16	++	++	/	miR-193b	+	/	/
let-7a	++	+++	/	miR-195	+	/	/
miR-22	++	+++	+	miR-199a	+	+++	+
miR-125b	++	+++	+	miR-21	+	+++	+
miR-143	++	+++	/	miR-223	+	/	/
let-7b	++	++	/	miR-25	+	++	/
miR-99a	++	++	/	miR-27a	+	++	/
let-7c	++	++	/	miR-29b	+	/	/
miR-181a	++	/	/	miR-29c	+	/	/
miR-194	++	+++	/	miR-3-c	+	+++	+
miR-451	++	/	/	miR-32-	+	/	/
miR-3-d	++	++	/	miR-377	+	/	/
miR-15b	++	/	/	miR-378	+	/	/
miR-193a-5p	++	/	/	miR-424	+	/	/
miR-24	++	+++	++	miR-425	+	/	/
miR 29a	++	/	/	miR-486-5p	+	/	+
miR-23b	++	+	+	miR-5-5	+	/	/
miR-26b	++	/	/	miR-874	+	/	/
miR-27b	++	++	/	miR-885-5p	+	/	/
miR-3-a	++	++	/	miR-1-7	Traces	/	/
miR-92a	++	++	+++	let-7e	Traces	/	/
miR-13-a	++	/	/	miR-98	Traces	/	/
miR-15a	++	++	/	miR-3-b	--	++	/
miR-186	++	/	/	miR-34	--	++	/
miR-191	++	++	/	miR-1-6a	--	++	/
miR-26a	++	/	++	miR-125a	--	++	/
miR-28	++	++	/	miR-148	--	++	/
miR-1- -	++	/	/	miR-149	--	++	/
let-7d	++	/	/	miR-189	--	++	/
miR-17	++	/	+	miR-199b	--	++	/
miR-185	++	/	/	miR-21-	--	++	/
miR-192	++	++	/	miR-321	--	+++	/
miR-3-e	++	/	/	miR-145	--	++	+
miR-381	++	/	/	miR-93	--	+	+
miR-99b	++	/	+	miR-483	--	/	+++
miR-1-3	++	+	/	miR-484	--	/	+
miR-23a	++	/	/	miR-485	--	/	+
let-7f	+	/	/	miR-487	--	/	+
let-7g	+	/	/	miR-2-	--	/	++
miR-139-5p	+	+	/	miR-18	--	/	+
miR-14-	+	/	/	miR-19b	--	/	+
miR-142	+	++	/	miR-1-6b	--	/	+
miR-144	+	/	/	miR-345	--	/	+
miR-151	+	/	+	miR-41-	--	/	+

The minus sign is used to indicate very low to undetectable levels and one plus to three pluses indicate a gradual expression from low levels to very high levels. The slash stands for the miRNAs that were not assayed. This figure is modified from Girard *et al*^[21] with the permission of the authors and the publisher.

(RR)' described by Yu *et al*^[27] and predicted the genes that are potentially targeted miRNAs in the liver^[28]. This RR value is an indicator of the distribution of miRNA target genes within a single mRNA population. A high RR indicates low expression in a greater proportion of target genes and is, therefore, indicative of miRNA expression in that tissue. The RR values for all miRNAs were calculated for the miRNAs in the liver^[28] and are shown in Figure 2. Clearly, many mRNAs could be the targets for miRNAs in the liver.

Validation of those potential targets for each miRNA requires experimental studies both *in vitro* and *in vivo*.

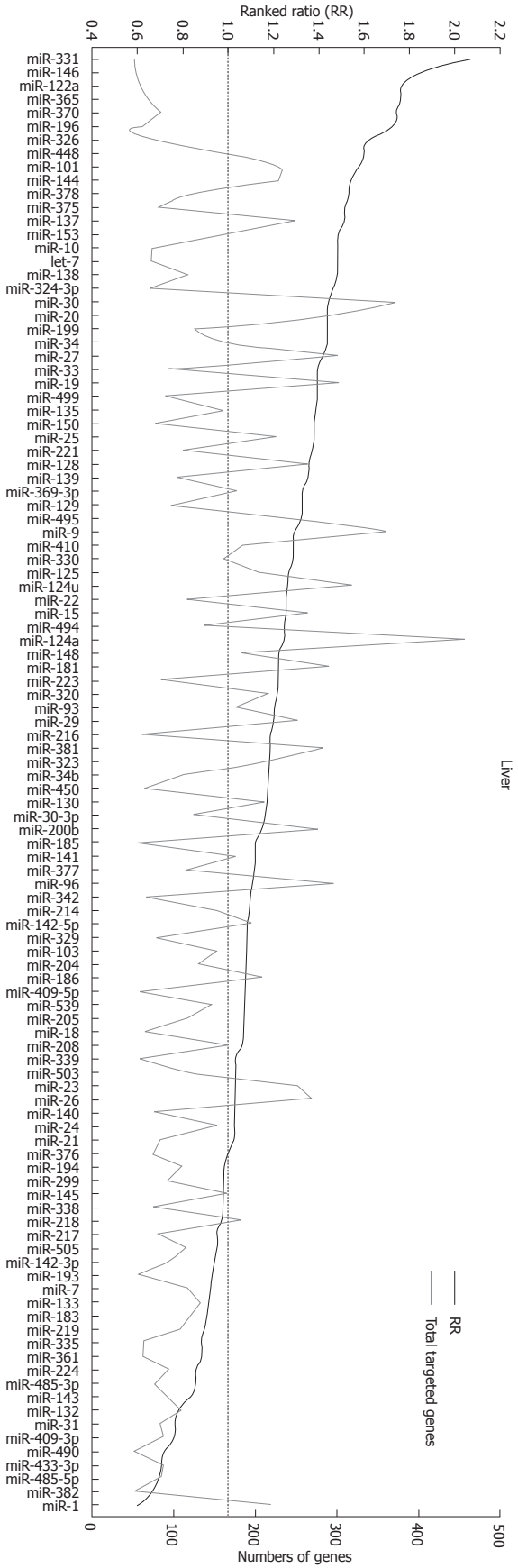


Figure 2 Prediction of targets by miRNAs in the liver. The miRNAs are ordered by RR values (upper-axis). The higher values reflect lower expression of predicted target genes and are, therefore, indicative of miRNA activity. The numbers of genes predicted to be targeted by each miRNA (lower-axis) are indicated by the grey line. This figure is reprinted from Arora and Simpson^[28] with the permission of the authors and the publisher.

Several groups have tested the overall importance of miR-122 in the regulation of metabolism in the liver^[29,30]. Through an antisense strategy to knock down miR-122, Esau *et al*^[30] observed that several genes which regulate lipid metabolism, specifically the key enzyme phosphomevalonate kinase, were down-regulated. Remarkably, silencing miR-122 in high-fat fed mice resulted in a significant reduction of hepatic steatosis, which was associated with reduced cholesterol synthesis rates and stimulation of hepatic fatty-acid oxidation^[30]. Functional inhibition of miR-122 by an “antagomir” resulted in increased expression of several hundred genes including those that are normally repressed in hepatocytes^[29]. In addition, Chang *et al*^[20] identified a binding site for miR-122 in the 3'-UTR of the cationic amino acid transporter (CAT-1) mRNA using the Lewis-based miRNA targets approach^[31]. Consistently, an inversed pattern of expression of CAT-1 and miR-122 was noted at all stages of liver development. Interestingly, miR-122-induced inhibition through the CAT-1 3'-UTR was efficiently relieved upon amino acid starvation, which validates CAT-1 as a target of miR-122^[32].

Several groups, including ourselves, have recently tested the role of miRNAs in the regulation of biliary epithelial immunity in the liver. Human cholangiocytes express *let-7* family members, miRNAs with complementarity to TLR4 mRNA. *let-7* regulates TLR4 expression *via* post-transcriptional suppression in cultured human cholangiocytes. Infection of cholangiocytes with *Cryptosporidium parvum* (*C. parvum*), a parasite that causes intestinal and biliary disease, results in decreased expression of primary *let-7i* and mature *let-7* in a MyD88/NF- κ B-dependent manner. The decreased *let-7* expression is associated with *C. parvum*-induced up-regulation of TLR4 in infected cells^[23]. miRNAs may also be involved in cholangiocyte responses to pro-inflammatory cytokines, such as interferon-gamma (IFN- γ), and actively participate in the regulation of biliary inflammatory response in the liver. Specifically, miR-513 regulates B7-H1 translation and mediates IFN- γ -induced B7-H1 expression in human cholangiocytes. B7-H1 (CD274, PD-L1) is a member of the B7 family of costimulatory molecules and plays a critical immunoregulatory role in cell-mediated immune responses. Resting human cholangiocytes express B7-H1 mRNA, but not B7-H1 protein. IFN- γ induces B7-H1 protein expression and alters miRNA expression profile in cholangiocytes. Of those IFN- γ -down-regulated miRNAs, miR-513 has complementarity to the 3'-UTR of B7-H1 and targeting of miR-513 to B7-H1 mRNA results in translational repression, but not B7-H1 mRNA degradation^[33].

miRNAS AND LIVER DISEASES

miRNAs and viral hepatitis

Genes encoding miRNAs have also been found in viruses and viral miRNAs have a regulatory effect on their protein-coding genes^[34]. This regulatory effect may be beneficiary to the virus toward maintaining its replication, latency and evading the host immune

system. Wu and colleagues analyzed the miRNA-encoding potential of the hepatitis B virus (HBV). Using computational approaches, the authors found that HBV putatively encodes only one candidate pre-miRNA. One viral mRNA was found to be targeted by the viral miRNA when they searched the target from viral mRNAs. Thus, HBV has evolved to use viral miRNAs as a means to regulate its own gene expression^[35].

miRNAs from the host cells may play a role in building up direct or indirect effect in regulating viral genes^[34,36,37]. Hepatitis C virus (HCV) is an enveloped RNA virus of the *Flavivirus* family, which is capable of causing both acute and chronic hepatitis in humans by infecting liver cells. miRNA-122 has been reported to facilitate the replication of HCV, targeting the viral 5' non-coding region^[38]. Indeed, HCV RNA can replicate in Huh 7 cells, which express miR-122, but not in HepG2 cells, which do not express miR-122. Silencing of miR-122 in hepatocytes resulted in a marked loss of replicating RNAs from HCV^[38]. A putative miR-122 binding site in the 50-end of HCV genome was identified, suggesting a direct role of miR-122 in HCV replication^[38]. An indirect effect of miR-122 inhibition on HCV regulation *via* the up-regulation of the cytoprotective enzyme heme-oxygenase 1 (HO-1) and the converse down-regulation of HO-1 repressor Bach1 was also characterized^[21]. In addition, HCV replication is associated with an increase in expression of cholesterol biosynthesis genes that are regulated by miR-122^[39].

On the other hand, Pedersen *et al*^[40] demonstrated IFN-mediated modulation of the expression of numerous cellular miRNAs in the treatment of hepatocytes infected with HCV. Expression of a total of 30 cellular miRNAs in hepatocytes was influenced by IFN- α/β or IFN- γ . Specifically, eight of the miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431 and miR-448), having nearly perfect complementarity in their seed sequences with HCV RNA genomes, were up-regulated. Importantly, these miRNAs are capable of inhibiting HCV replication and infection. This has opened the door to our understanding of novel host-defense mechanisms that exist in mammalian cells as well as the antiviral mechanisms employed by interferon.

miRNAs and human hepatocellular cancer (HCC)

Several studies have shown that specific miRNAs are aberrantly expressed in malignant HCC cells or tissues compared to non-malignant hepatocytes or tissue^[41-47]. Selected miRNAs such as miR-21, miR-224, miR-34a, miR-221/222, miR-106a, and miR-203 are up-regulated in HCC compared to benign hepatocellular tumors such as adenomas or focal nodular hyperplasia. Certain miRNAs have been noted to be decreased in HCC compared to non-tumoral tissue, such as miR-122a, miR-422b, miR-145, and miR-199a. Murakami *et al*^[48] showed a correlation between miR-222, miR-106a, miR-92, miR-17-5p, miR-20, and miR-18 and the degree of differentiation suggesting an involvement of specific miRNAs in the progression of the disease. Interestingly, the altered expression of some miRNAs was associated

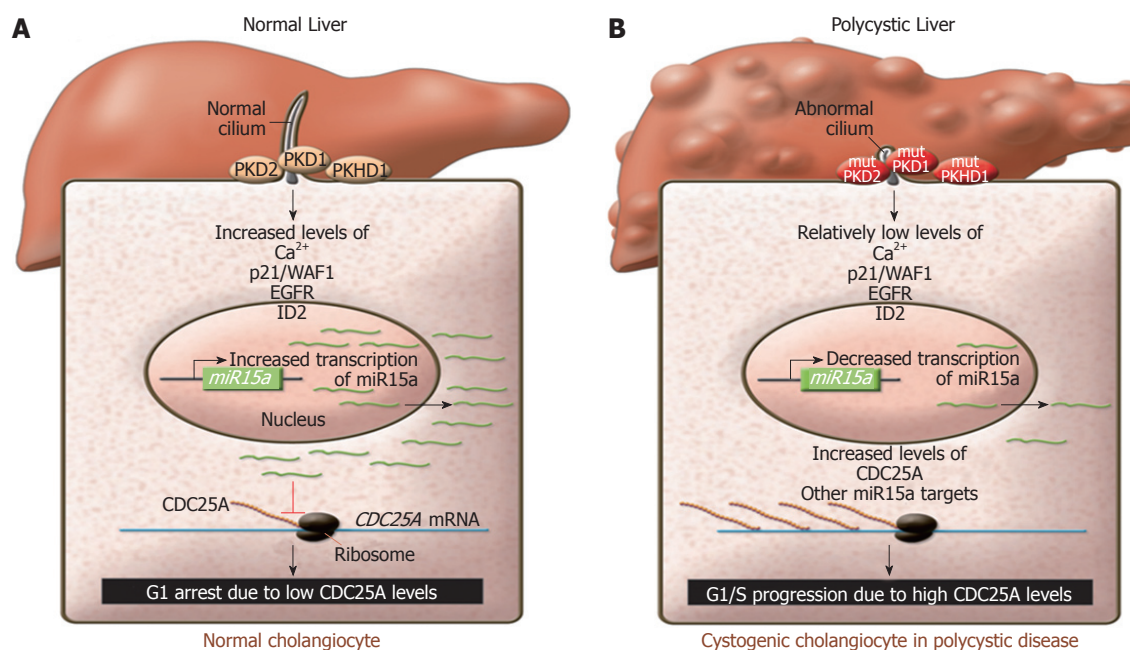


Figure 3 Model of miR15a function in hepatic cystogenesis. A: In normal cholangiocytes, ciliary signaling activates intracellular signaling pathways resulting in miR15a expression and CDC25A repression. Decreased levels of CDC25A then lead to G1 arrest, preventing cyst formation; B: In cholangiocytes of polycystic liver, miR15a level is reduced as a result of mutation of molecules important to the ciliary signaling. Reduction of miR15a results in elevated Cdc25A level, increased cell proliferation, and cyst growth. This figure is reprinted from Chu and Friedman^[49] with the permission of the authors and the publisher.

with distinctive risk factors, such as miR-96 with hepatitis B virus infection and miR-126* with alcohol use. Further investigations suggested the highly deregulated miR-223 and miR-222 could unequivocally distinguish HCC from adjacent non-tumoral liver, irrespective of viral association^[46]. In HCC patients with hepatitis C and liver cirrhosis, miR-122, miR-100, and miR-10a were overexpressed, whereas miR-198 and miR-145 were up to five-fold down-regulated in hepatic tumors compared to normal liver parenchyma^[47].

Alteration of expression profile in HCC for some miRNAs may be the consequence of malignant transformation. For other miRNAs, they may play a role in the transformation process. miRNA-122 was reported to be significantly and specifically down-regulated in HCC in humans as well as in rodents^[41,42]. Amongst the putative target genes of miR-122 that can be predicted using computational tools, at least three are of interest in tumorigenesis: the gene for N-Myc, which is frequently rearranged in woodchuck liver tumors by woodchuck hepatitis virus^[43], the gene referred to as “down-regulated in liver malignancy”^[44], and the gene for cyclin G1^[45]. In fact, miR-122 was shown to modulate cyclin G1 expression in HCC-derived cell lines, and an inverse correlation between miR-122a and cyclin G1 expression in primary liver carcinomas was further observed^[45]. These studies suggest an influence of the down-regulation of miR-122 and the converse expression of cyclin G1 in hepatocarcinogenesis^[21].

miRNAs and polycystic liver diseases

The polycystic liver and kidney diseases are a family of disorders with heterogeneous etiologies. Autosomal dominant polycystic kidney disease (ADPKD) is

associated with renal and liver cystogenesis that clinically manifests in adulthood, often leading to dialysis and renal transplantation. It is caused by mutations in either of two genes, *PKD1* and *PKD2*, which code for polycystin 1 and polycystin 2, respectively^[49]. Autosomal recessive polycystic kidney disease (ARPKD) can present in neonates with massive renal cysts, causing respiratory failure secondary to abdominal competition that subsequently leads to infant demise, although milder forms can present later in life. Proposed mechanisms of disease include ciliary dysfunction, excess cell proliferation, and altered cell-cell or cell-matrix interactions.

Lee and colleagues provide data to support a novel mechanism for cystogenesis involving miRNA. They demonstrate that levels of the miRNA miR15a are decreased in livers of patients with ARPKD and ADPKD, respectively, and congenital hepatic fibrosis as well as in the *PKC* rat model of ARPKD. This results in increased expression of the cell-cycle regulator Cdc25A, which is a direct target of miR15a, and increased cellular proliferation and cystogenesis *in vitro*. As a whole, the findings indicate that changes in miRNA expression contribute to the phenotypic changes found in cystic liver disease (Figure 3)^[49,50].

miRNAs FOR THE DIAGNOSIS OF LIVER DISEASES

miRNAs could be of diagnostic significance for many liver diseases but current literature has been focused on tumors in the liver. Hepatocellular tumors comprise diverse benign and malignant neoplasms. Although the phenotypes can be broadly distinguished histologically or immunologically, these tumors can vary widely

in their clinical behavior and prognosis. The use of miRNA-based classifications that correlate with etiology, pathogenetic changes, or malignant tendency will enhance molecular diagnosis and enable further definition of these phenotypes. In turn, this may yield clinically useful predictive markers of tumor behavior, as well as identify individual genetic and molecular contributors to tumorigenesis^[51,52]. Thus, miRNA profiling studies could be used for defining clinical phenotypes, as well as providing potentially useful molecular diagnostic markers.

Impairments in miRNA functioning seen in cancerogenesis can be used for determination of miRNA expression for diagnostics of tumor origin. Each type of cancer is characterized by a certain profile of miRNA expression^[52]. For example, the miRNA expression profiles in malignant hepatocytes differ from those of malignant cholangiocytes. Cluster analysis of miRNA expression profiles in tumors accurately determines not only type of the tissue (e.g. epithelium or hemopoietic system), but also discriminates tumors within the same type of tissue; this may reflect mechanism of transformation^[53]. Obviously, evaluation of miRNA profiles can be used for prognosis of the development of tumors^[54-56]. Such an approach for tumor diagnostics is very promising. However, it is not widely employed yet due to inadequately developed technology, lack of standards, requirements of very high purity of RNA samples, and not always reproducible results.

miRNAS AS THERAPEUTIC TARGETS FOR LIVER DISEASES

Development of miRNA/RNAi-based therapeutics requires several critical experimental steps, which include: (1) miRNA profiling of disease versus healthy tissue; (2) functional analysis of dysregulated miRNAs; and (3) *in vivo* studies with the use of different RNAi-based therapeutic methods to dysregulate miRNAs^[57]. The success of such strategies for gene therapy will provide clinicians with a larger repertoire that includes miRNA-therapeutic agents. For example, chemically engineered oligonucleotides, termed 'antagomirs', have recently been developed and proven to be efficient and specific silencers of endogenous miRNAs in mice^[29]. The silencing effect was considerably sustained over time probably because of a long half-life of endogenous miRNAs^[12]. In addition, induction of stable loss-of-function phenotypes for specific miRNAs by lentiviral-mediated antagomir expression has recently been described^[58].

Over the past several years, strategies based on targeting HBV, and to a lesser extent HCV, by both synthetic and expressed activators of the RNAi pathway have proved efficient to inhibit viral replication both *in vitro* and *in vivo*^[59,60]. The recent study by Pedersen *et al*^[40] provided great insights into validating sequence-predicted targets of cellular miRNAs within the HCV genome. miRNA-122 antagomir can down-regulate expression of several adult-liver genes^[29], providing

the potential to generate a new attractive expandable cell source for hepatocyte transplantation that would feature stem/progenitor cell phenotype. In addition, the effect of miR-122 antagomir in high-fat fed mice may be of therapeutic potential to reduce hepatic steatosis^[29].

The important breakthrough in the field of hepatocarcinogenesis came from the accurate correlation of alterations in miRNAs with tumor proliferation and differentiation. So far, there have been very limited insights into this characterization. Recent studies by Meng *et al*^[22,61] suggest a role for miRNAs in the influence of interleukin-6 in malignant cholangiocytes. MicroRNA-141, which showed strong overexpression in malignant cholangiocytes, was specifically localized in 12p, a region of known chromosomal aberration in biliary tract cancers^[42]. Inhibition of miR-21 sensitized the response of cholangiocarcinoma cell lines to chemotherapy^[42]. This observation gives rise to significant hope that miR-21 could serve as a biomarker for drug response in cholangiocarcinoma.

CONCLUSION

Study of miRNAs flourished during the decade after their discovery. It is now clear that miRNAs can potentially regulate every aspect of cellular activity, from differentiation and proliferation to apoptosis, and also modulate a large range of physiological processes from developmental timing to organogenesis^[1-6]. miRNAs also modulate a diverse spectrum of liver functions with developmental, (patho) physiological, and clinical implications. In the near future, the distinctive signature patterns of miRNA expression associated with liver cancer should allow classification of different stages in tumor progression^[21]. Further, creating artificial miRNAs with salutary effects by promoting the expression of beneficial gene products (e.g. tumor-suppressor proteins) or targeting viral genomes (e.g. molecules designed to specifically target HCV-genome sequences) may become part of our patient management and complement chemotherapy and antiviral treatments. Overall, unraveling the regulatory circuits of miRNAs in the liver is a great challenge, but may provide attractive targets for mechanism-based treatment of liver diseases.

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Chronic pancreatitis: Maldigestion, intestinal ecology and intestinal inflammation

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Abstract

Exocrine pancreatic insufficiency caused by chronic pancreatitis results from various factors which regulate digestion and absorption of nutrients. Pancreatic function has been extensively studied over the last 40 years, even if some aspects of secretion and gastrointestinal adaptation are not completely understood. The main clinical manifestations of exocrine pancreatic insufficiency are fat malabsorption, known as steatorrhea, which consists of fecal excretion of more than 6 g of fat per day, weight loss, abdominal discomfort and abdominal swelling sensation. Fat malabsorption also results in a deficit of fat-soluble vitamins (A, D, E and K) with consequent clinical manifestations. The relationships between pancreatic maldigestion, intestinal ecology and intestinal inflammation have not received particular attention, even if in clinical practice these mechanisms may be responsible for the low efficacy of pancreatic extracts in abolishing steatorrhea in some patients. The best treatments for pancreatic maldigestion should be re-evaluated, taking into account not only the correction of pancreatic insufficiency using pancreatic extracts and the best duodenal pH to permit optimal efficacy of these extracts, but we also need to consider other therapeutic approaches including the decontamination of intestinal lumen, supplementation of bile acids and, probably, the use of probiotics which may attenuate intestinal inflammation in chronic pancreatitis patients.

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Key words: Chronic pancreatitis; Exocrine pancreatic insufficiency; Leukocyte L1 antigen complex; Pancreatic elastase; Pancreatic extracts

INTRODUCTION

Exocrine pancreatic insufficiency caused by chronic pancreatitis results from various factors which regulate digestion and absorption of nutrients. Pancreatic function has been extensively studied over the last 40 years, even if some aspects of secretion and gastrointestinal adaptation are not completely understood. The pancreatic gland normally secretes more than 2 L of juice per day which is composed of water, bicarbonates and enzymes^[1]; protein secretion per gram of pancreatic tissue is elevated more than that of any other organ^[2], and more than 85% of the protein content is composed of enzymes which are able to digest lipids, proteins and carbohydrates^[3]. The pancreas normally produces more enzymes than are necessary for food digestion^[1], and normal digestion is guaranteed up to a loss of 95% of pancreatic secretory capacity^[4]. Recently, it has been demonstrated that gastric lipase can compensate pancreatic lipase even if it is not capable of complete lipolytic activity^[5]. Enzyme degradation in the intestinal lumen is the main factor controlling nutrient absorption. The activity of pancreatic enzymes progressively decreases during their progression in the intestinal lumen: 60% of active trypsin and chymotrypsin are present in the jejunum, whereas only 20% of these enzymes are present in the ileum; on the other hand, amylases and lipases are more stable^[6-8]. There are various explanations for the loss of enzymatic activity during progression in the intestinal lumen, including proteolytic degradation (chymotrypsin is the main lipase degradation factor)^[9], lipase acid inactivation (lipase is particularly sensitive to acid inactivation)^[10], and the brief half-life of some

enzymes, particularly lipase^[11]. This is the reason why, in patients with exocrine pancreatic insufficiency, fat maldigestion is more severe than that of carbohydrates and proteins. In addition to an optimal concentration of biliary acids and colipases in the intestinal lumen, good fat digestion requires an adequate blending of nutrients with the pancreatic juice and optimal intestinal motility. In pathological conditions, such as chronic pancreatitis, there is a deficit in bicarbonate production; a low duodenal pH determines biliary acid precipitation and the remaining lipase activity worsens. Finally, other causes of malabsorption may be an accelerated gastric emptying and a lower intestinal transit time^[12,13].

CLINICAL MANIFESTATIONS AND DIAGNOSIS OF EXOCRINE PANCREATIC INSUFFICIENCY

The main clinical manifestations of exocrine pancreatic insufficiency are fat malabsorption, known as steatorrhea, which consists of fecal excretion of more than 6 g of fat per day, weight loss, abdominal discomfort and abdominal swelling sensation. Fat malabsorption also results in a deficit of fat-soluble vitamins (A, D, E and K) with consequent clinical manifestations. The diagnosis of exocrine pancreatic insufficiency is based on these clinical symptoms and signs observed with direct and indirect tests. Some of these tests can be used to determine the degree of insufficiency which is usually classified as mild, moderate or severe. The most sensitive test is the secretin-cholecystokinin (CCK) or secretin-cerulein test; this test has a double-lumen tube capable of separately draining the gastric juice and the pancreatic juice. The test starts with pancreatic stimulation by secretin which produces the hydro-electrolyte pancreatic secretion and CCK or cerulein, which can stimulate enzymatic secretion. This test is highly sensitive and specific^[14] but is invasive, lengthy and expensive; moreover, it is only possible in patients with a normal gastrointestinal tract, and it is not useful in patients with an altered digestive anatomy. At present, fecal chymotrypsin and elastase 1 are more frequently used to diagnose exocrine pancreatic insufficiency^[15]. In particular, the determination of elastase 1 is more sensitive and specific than chymotrypsin determination. The advantage of these tests is that they can be used in patients who have undergone surgery involving the gastrointestinal tract, but they can not reveal a mild degree of exocrine pancreatic insufficiency^[15,16]. A cholesteryl-octanoate breath test is rarely used because of its high cost and possible interference with metabolic and pulmonary diseases^[17]. Fecal fat determination is useful in monitoring lipid malabsorption therapy. Pancreatic exocrine evaluation during magnetic resonance cholangiopancreatography with secretin administration is still under study and the results of the published studies seem to be promising^[18-20].

MALDIGESTION, INTESTINAL ECOLOGY AND INTESTINAL INFLAMMATION

The relationships between pancreatic maldigestion, intestinal ecology and intestinal inflammation have not received particular attention, even if in clinical practice these mechanisms may be responsible for the low efficacy of pancreatic extracts to abolish steatorrhea in some patients.

One mechanism which has been hypothesized between maldigestion and intestinal alterations relates to bacterial overgrowth in the small intestine; bacterial overgrowth is often seen in experimental models of exocrine pancreatic insufficiency^[21]. Furthermore, bacterial overgrowth has been observed in dogs with naturally occurring exocrine pancreatic insufficiency^[22]. The presence of bacterial overgrowth in human exocrine pancreatic insufficiency has been studied using non-invasive breath tests based on ¹⁴C-cholyglycine^[23], ¹⁴C-xylose^[24], glucose^[25,26] or by intubation followed by culture of intestinal aspirates^[27]. These studies indicated that bacterial overgrowth complicates 25%-50% of patients with exocrine pancreatic insufficiency, and it was suggested that bacterial overgrowth might either contribute to diarrhea or account for the persistence of diarrhea in patients with exocrine pancreatic insufficiency who receive adequate pancreatic enzyme supplementation. Furthermore, bacterial overgrowth might give rise to bile acid malabsorption and changes in intestinal permeability^[28].

However, Madsen *et al*^[29] found no bacterial overgrowth in any of their patients with exocrine pancreatic insufficiency, and these findings seem to conflict with previous observations seen in both humans and animals. In fact, in the study based on intestinal culture^[27], it was observed that bacterial overgrowth occurred in 50% of patients with severe pancreatic insufficiency who were not receiving enzyme replacement therapy, and studies in dogs indicated that bacterial overgrowth from ligation of the pancreatic duct can be reversed by bovine pancreatic extract replacement therapy; thus these results indicate that pancreatic enzymes might have an important influence on small-intestinal bacterial flora^[21]. Since all the patients studied by Madsen had oral enzyme supplementation, it is possible that enzyme substitution treatment normalized the luminal conditions of the small intestine, which otherwise would have facilitated bacterial overgrowth.

In a previous study, a wide range of bile salt malabsorption was observed in patients with exocrine pancreatic insufficiency secondary to alcoholic pancreatitis^[30]. Moreover, these data suggested that intraluminal factors, rather than a primary defect in the ileal mucosa, were responsible for bile salt malabsorption. The fecal loss of bile salts in their patients was markedly reduced by oral administration of pancreatic enzymes, indicating an important role for pancreatic enzymes in bile acid absorption. It has been postulated from studies performed *in vitro* that a lack

of pancreatic enzymes causes generalized maldigestion, which in turn may be responsible for persistent bile salt binding to maldigested protein, carbohydrate, or fiber in these patients^[31]. It is conceivable, therefore, that in patients with untreated pancreatic insufficiency, an exceeding low concentration of intraluminal pancreatic enzymes during the postprandial period gives rise to persistent binding of bile acids to undigested dietary components, resulting in bile acid malabsorption.

Low intraluminal pH in the upper small intestine might be another important factor in the pathogenesis of fecal loss of bile acids in pancreatic insufficiency. Thus, bile acid malabsorption in patients with chronic pancreatitis and exocrine dysfunction does not occur until bicarbonate output is below a certain level^[32], and cimetidine has been shown to reduce pH-induced precipitation of bile acids, thereby improving the micelle concentration of bile salts in the duodenum^[33]. Moreover, food residues seem to absorb more bile salts at pH values less than 6.0^[34].

The study by Madsen^[29] also evaluated intestinal permeability in exocrine pancreatic insufficiency due to chronic pancreatitis in adult patients; these authors found that patients receiving enzyme replacement therapy had reduced urinary excretion of mannitol. The exact character of the underlying epithelial impairment was at that time not obvious, as the pathways for permeation of both smaller molecules such as mannitol and larger molecules are not yet known. It is possible that larger pores located in the crypts of intestinal epithelium permit absorption of larger molecules, while smaller molecules pass through both these pores and smaller pores located in the villi^[35,36]. According to this hypothesis, it is suggested that a defect localized in the villi of the intestinal epithelium may exist. It is possible, however, that more pronounced disturbances in intestinal permeability would have been revealed, if urine excretion of the test substances had been properly corrected for variations in the small-intestinal transit rate^[37]. Since bacterial overgrowth which often gives rise to defects in intestinal permeability was not found in any of our patients, it is suggested that pancreatic disease *per se* and not enzyme supplementation therapy caused the permeability defect. In a recent study, we evaluated fecal calprotectin in a total of 90 subjects; 22.2% with chronic pancreatitis, 16.7% with pancreatic cancer, 6.7% with chronic non-pathological pancreatic hyperenzymemia, 17.8% with non-pancreatic diseases and 25.6% with no detectable diseases^[38]. Calprotectin is a cytoplasmic antimicrobial component prominent in granulocytes, monocytes, and macrophages. It accounts for approximately 60% of the total protein in the cytosol. The release of calprotectin is most likely a consequence of cell disruption and death^[39] and is stable in stools for more than 7 d at varying temperatures, as well as being resistant to proteolysis even after transportation and storage^[40]. Calprotectin can inhibit bacterial proliferation both as a component of the innate immune response and through its iron-binding capacity^[41]. Fecal calprotectin determination has been demonstrated to

be useful in diagnosing various inflammatory diseases of the gastrointestinal tract^[42-46]. We found that patients with chronic pancreatitis had abnormally high fecal calprotectin concentrations in 55% of cases and most of these patients (40%) had pancreatic insufficiency^[38]. It is possible that, in these patients, pancreatic insufficiency may determine an alteration in intestinal ecology, and in intestinal inflammation. In the population studied, multivariate analysis showed that patients with abnormally low fecal elastase had more than a five-fold risk of increased fecal calprotectin suggesting that, in patients with pancreatic disease, the determination of fecal calprotectin may be useful in evaluating the possible presence of intestinal inflammation which may worsen the intestinal absorption of nutrients. Thus, our data further support the hypothesis that pancreatic insufficiency may cause intestinal inflammation probably due to a modification in intestinal ecology.

CONCLUSION

Pancreatic extracts are the basic treatment for pancreatic insufficiency. However, we need to explore the possibility that other drugs used to treat pancreatic insufficiency such as proton pump inhibitors or H₂-blockers should be administered to our patients in order to modify the duodenal pH and to permit optimal efficacy of pancreatic extracts. Furthermore, we need to explore the possibility that other therapeutic approaches including the decontamination of intestinal lumen, supplementation of bile acids and the use of probiotics may attenuate intestinal inflammation permitting optimal efficacy of pancreatic extracts as well as the control of clinical signs and symptoms of pancreatic insufficiency.

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Jose JG Marin, Professor, Series Editor

Bile-acid-induced cell injury and protection

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Abstract

Several studies have characterized the cellular and molecular mechanisms of hepatocyte injury caused by the retention of hydrophobic bile acids (BAs) in cholestatic diseases. BAs may disrupt cell membranes through their detergent action on lipid components and can promote the generation of reactive oxygen species that, in turn, oxidatively modify lipids, proteins, and nucleic acids, and eventually cause hepatocyte necrosis and apoptosis. Several pathways are involved in triggering hepatocyte apoptosis. Toxic BAs can activate hepatocyte death receptors directly and induce oxidative damage, thereby causing mitochondrial dysfunction, and induce endoplasmic reticulum stress. When these compounds are taken up and accumulate inside biliary cells, they can also cause apoptosis. Regarding extrahepatic tissues, the accumulation of BAs in the systemic circulation may contribute to endothelial injury in the kidney and lungs. In gastrointestinal cells, BAs may behave as cancer promoters through an indirect mechanism involving

oxidative stress and DNA damage, as well as acting as selection agents for apoptosis-resistant cells. The accumulation of BAs may have also deleterious effects on placental and fetal cells. However, other BAs, such as ursodeoxycholic acid, have been shown to modulate BA-induced injury in hepatocytes. The major beneficial effects of treatment with ursodeoxycholic acid are protection against cytotoxicity due to more toxic BAs; the stimulation of hepatobiliary secretion; antioxidant activity, due in part to an enhancement in glutathione levels; and the inhibition of liver cell apoptosis. Other natural BAs or their derivatives, such as choly-N-methylglycine or cholylysarcosine, have also aroused pharmacological interest owing to their protective properties.

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Key words: Apoptosis; Cholestasis; Liver; Necrosis; Oxidative stress; Ursodeoxycholic acid

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INTRODUCTION

Bile acids (BAs) are the major organic solutes in bile, and are involved in several important functions in the liver and the intestine. However, the retention of hydrophobic BAs in pathophysiological conditions, such as cholestatic diseases, is believed to play an important role in liver injury by inducing apoptosis or necrosis of hepatocytes^[1]. Primary BAs are directly synthesized from cholesterol by hepatocytes, by the addition of hydroxyl groups and the oxidation of its side chain to form a more water soluble end product. The hydroxylation is always on one side of the molecule resulting in an amphipathic molecule. In humans, the most abundant BA species are the primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA) (Figure 1), and the secondary BAs deoxycholic acid (DCA) and

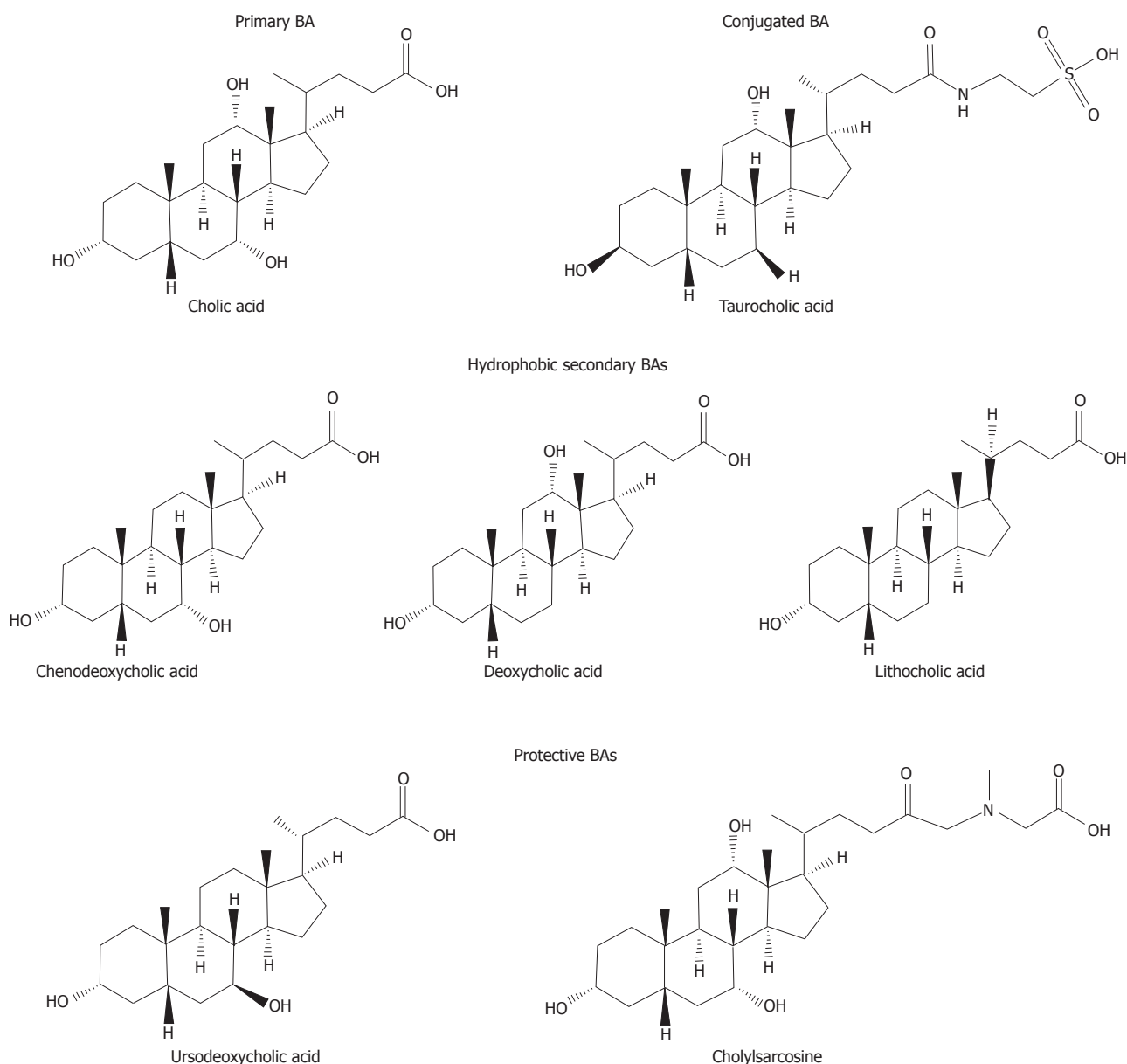


Figure 1 Molecular structures of different potentially toxic or protective natural bile acids and synthetic bile acid analogues.

lithocholic acid (LCA) (Figure 1), generated in the intestine by bacterial biotransformation of CA and CDCA, respectively. Small amounts of other secondary BAs such as ursodeoxycholic acid (UDCA; Figure 1), currently used in the treatment of cholestatic liver diseases, are also present in the human BA pool^[2,3].

BA hydrophobicity is an important determinant of the toxicity and protection of BAs, two biological properties of these compounds which will be reviewed in this manuscript. BA hydrophobicity depends on the number, position and orientation of the hydroxyl groups, as well as amidation at the C-24 position. Regarding the magnitude of hydrophobicity of BAs the order would be UDCA < CA < CDCA < DCA < LCA^[4]. Before being secreted into bile, most BA molecules are conjugated with glycine or taurine (Figure 1) within hepatocytes to more hydrophilic amidated forms. Under normal conditions, the amount of BAs undergoing further biotransformations to dianionic

glucuronidated or sulfated derivatives is negligible, although these may become important in cholestasis^[5].

BILE-ACID-INDUCED HEPATOCYTE INJURY

The retention and accumulation of hydrophobic BAs, such as CDCA and DCA, inside hepatocytes during cholestasis have long been implicated as a major cause of liver damage in this disease^[1]. Experimentally, hydrophobic BAs are known to induce injury to isolated hepatocytes^[6], cultured hepatocytes^[7], and whole liver^[8], but the mechanisms involved in this toxicity are not fully understood. In animal models^[9] and human cholestatic disorders^[10], hepatocyte swelling and the intracellular accumulation of bile pigments have been reported to occur. Moreover, swelling, pleomorphism and abnormal cristae have also been reported in

mitochondria from cholestatic hepatocytes^[11]. This injury to parenchymal cells, which occurs early on in the course of cholestasis, can be responsible for many of the subsequent inflammatory and fibrinogenic responses of non-parenchymal cells. Thus, altered hepatocytes may release molecules, including growth factors, cytokines, chemokines and lipid peroxide products, able to amplify the inflammatory response, stimulate fibrogenesis by hepatic stellate cells, or directly injure other nearby cells^[12].

Several mechanisms may account for the cytotoxicity associated with the most hydrophobic BAs in cholestatic liver diseases^[1]. BAs could disrupt cell membranes through their detergent action on lipid components^[13] and promote the generation of reactive oxygen species (ROS) that, in turn, oxidatively modify lipids, proteins, and nucleic acids, and eventually cause hepatocyte apoptosis^[14]. Additionally, they can activate Kupffer cells to generate ROS, which may further contribute to the liver cell insult^[15].

BILE-ACID-INDUCED OXIDATIVE STRESS

Several studies have suggested that oxidative stress may play an important role in the pathogenesis of hepatic injury during cholestasis in rats^[16] and humans^[17]. Thus, the antioxidant α -tocopherol is able to reduce both hydrophobic BA-induced ROS generation and injury to hepatocytes *in vitro*^[18] and *in vivo*^[8]. Hepatic mitochondria have been proposed as a major source of the oxidative stress induced by these BAs. In this respect, it has been demonstrated that hepatic mitochondria undergo lipid peroxidation during experimental cholestasis and BA toxicity in rats^[8,16]. Hydrophobic BAs impair respiration and electron transport in hepatic mitochondria. These compounds decrease the activities of several enzyme complexes involved in the electron transport chain, such as complexes I, III and IV, whereas complex II is not affected in isolated rat liver mitochondria^[19]. Moreover, toxic BAs decrease the mitochondrial membrane potential developed upon succinate energization^[20]. These compounds decrease state three and enhance state four respiration in mitochondria^[20]. The decrease in state three respiration is probably related to an inhibitory action of these compounds on the phosphorylation system, although by means of a mechanism that has yet to be defined. The reported stimulation of state four by hydrophobic BAs is associated with an enhanced permeability of mitochondria to protons^[20]. The observed uncoupling may be the consequence of several interactions of these compounds with the structure of mitochondria. These BAs induced membrane permeability to protons, either by acting as protonophores or by disruption of the structural organization of membrane components. Hydrophobic BAs can stimulate the generation of ROS in rat hepatic mitochondria and hepatocytes^[18,21] as well as in human hepatic mitochondria^[22]. Intracellular ROS generation by mitochondria appears to be an early event in hydrophobic BA-induced hepatocyte

toxicity^[21]. As occurs in cholestatic rats, this can lead to a depletion of antioxidant defences, including total hepatic and mitochondrial glutathione contents, and the concentrations of substances involved in electron transport, such as ubiquinone-9 and ubiquinone-10^[23].

Hydrophobic BAs can induce the mitochondrial permeability transition (MPT)^[22,24,25], a critical intracellular event that triggers both the apoptotic and necrotic forms of cell death in hepatocytes^[26]. MPT involves a rapid increase in the permeability of the mitochondrial membrane to low-molecular-weight solutes, caused by the opening of a channel composed of the adenine nucleotide translocator of the inner membrane, the voltage-dependent anion channel of the outer membrane, and mitochondrial cyclophilin D^[26]. Induction of MPT is associated with mitochondrial swelling, collapse of the mitochondrial membrane potential, reduced oxidative phosphorylation, rupture of the outer mitochondrial membrane, cytochrome c release, and generation of ROS^[26]. Hydrophobic BA-induced MPT has been associated with mitochondrial ROS generation in rat hepatocytes^[27] and in isolated mitochondria^[14]. An elevation of the cytosolic free calcium concentration induced by hydrophobic BAs^[28] may also be an important permissive factor that allows oxidant stress to open the permeability pore.

BILE-ACID-INDUCED CELL DEATH PATHWAYS

BA accumulation within the hepatocyte can result in cell injury and death through two mechanisms; lower BA concentrations induce hepatocellular apoptosis^[25,29-31], whereas higher concentrations induce necrosis^[7,21]. Both types of cell death seem to play a role in cholestatic liver injury, although the contribution of each is controversial. The role of apoptosis in cholestatic injury has been demonstrated^[32]. However, in certain models of BA toxicity in the mouse, it has been reported that hepatocyte necrosis is the predominant form of cell death^[33].

Interestingly, at the level of apoptosis induction, cytotoxicity of BAs does not always correlate with their hydrophobicity, which is not the case in necrosis. Apoptosis induction is indeed dependent on the BA, its concentration, or its conjugation state^[29].

Necrosis

In rat hepatocytes, cellular necrosis is characterized by cell swelling and disruption of the intracellular and plasma membranes. BA-induced lethal hepatocellular injury has been attributed to direct membrane damage due to the detergent-like properties of hydrophobic BAs^[13], as well as to depletion of ATP, ion dysregulation, mitochondrial and cellular swelling, plasma membrane failure, and cell lysis, releasing intracellular contents^[6]. In the liver of humans with cholestatic disorders, the histological features of hepatocyte necrosis, such as massive swelling of hepatocytes containing accumulated bile and elevated serum hepatocellular aminotransferase

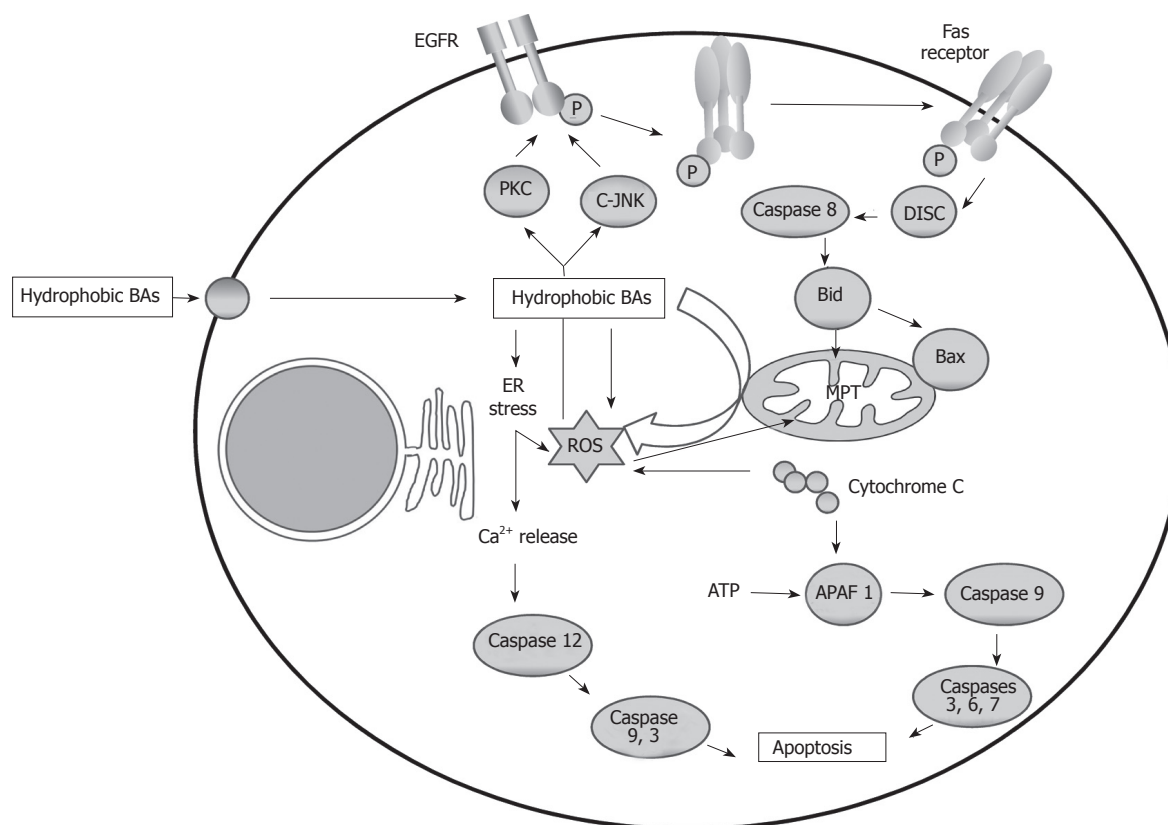


Figure 2 Intracellular mechanisms of bile acid-induced hepatocyte apoptosis. In this schema, further transduction after activation of death receptors and formation of the DISC, direct mitochondrial toxicity and ER stress are implicated.

enzymes, have been observed^[10]. Antioxidants have been shown to prevent hepatocellular necrosis and reduce oxidant stress in isolated hepatocytes exposed to hydrophobic BAs^[18,21] and to inhibit the dissipation of mitochondrial membrane potential^[14]. Thus, it has been proposed that MPT induction by ROS generated in hepatocyte mitochondria^[27] is a critical event promoting BA-induced hepatocyte necrosis. In contrast to BA-induced apoptosis, only high concentrations of BAs are able to induce hepatocyte necrosis^[7,21]. The generation of ROS can result from a direct detergent effect of BAs on membrane enzymes, such as phospholipase A₂, which upon activation release arachidonic acid. Mitochondrial damage due to BAs can arise from several causes, including the endogenous generation of arachidonic acid^[34].

Apoptosis

The impairment of apoptosis in hepatocytes and bile duct epithelial cells has been implicated in the pathogenesis of many liver diseases^[32]. It is well known that hydrophobic BAs can induce apoptosis by activating the death receptor or extrinsic pathway^[35,36], and through the mitochondrial or intrinsic pathway^[25,27,30] (Figure 2). More recently, it has been demonstrated that hydrophobic BAs can also induce apoptosis in hepatocytes by causing endoplasmic reticulum (ER) stress (Figure 2). In contrast to BA-induced cell necrosis, apoptosis is characterized by the maintenance of cellular ATP content^[29].

Extrinsic pathway of apoptosis: BA-associated hepatocyte apoptosis has been shown to occur through the death receptors Fas^[35] and TRAIL-R2. In contrast, BAs do not appear to enhance tumor necrosis factor (TNF)- α /TNF-R1 cytotoxicity^[36]. Toxic BAs cause cell death, partly due to the activation of a protease cascade. The proximal signaling protease caspase 8 appears to be activated by toxic BAs in a Fas-receptor-dependent manner. After caspase 8 activation, cathepsin B activity also increases. Inhibition of either protease attenuates apoptosis *in vitro*, suggesting that they both play a critical role in BA-induced apoptosis^[35]. Moreover, toxic BAs can induce Fas aggregation on the plasma membrane *via* a Fas-ligand-independent mechanism^[37]. These compounds appear to promote Fas activation by altering the cellular trafficking of this death receptor. The shuttling of Fas and the resulting apoptosis can be inhibited by Golgi-disrupting agents and microtubule poisons. By inference, a Golgi-associated and microtubule-dependent pathway appears to be involved in the trafficking of Fas to the cell surface during BA cytotoxicity^[38].

Oxidative stress has been implicated in the stimulation of Fas translocation induced by BAs. It has been demonstrated that BA-induced oxidative stress may trigger the activation of c-Jun-N-terminal kinases (JNKs) and protein kinase C (PKC). These are responsible for activating the epidermal growth factor receptor (EGFR), which associates with Fas in a JNK-dependent manner. The resulting phosphorylation of Fas induces its mobilization to the plasma membrane^[39].

The increased density of Fas on the cell surface also sensitizes hepatocytes to cell death induced by Fas agonists^[38]. Thus, toxic BAs also promote Fas-ligand-dependent hepatocyte apoptosis.

After death receptor activation and death-inducing signaling complex (DISC) formation, caspase 8 is activated and the pro-apoptotic protein Bid is cleaved and translocated to the mitochondria, which results in opening of the MPT pore, and the release of cytochrome c and other proapoptotic intermembrane space small molecules. Cytosolic cytochrome c leads to the binding of apoptosis activating factor-1 (APAF 1) with procaspase 9, resulting in the activation of caspase 9. A caspase cascade is then initiated and, finally, activation of the effector caspases, which leads to irreversible hepatocyte death^[40].

The death receptor TRAIL-R2 has been also suggested to be involved in the apoptosis induced by BA. Enhanced expression and oligomerization of this death receptor has been described in glycochenodeoxycholic acid (GCDCA)-induced apoptosis in Fas-deficient cell lines^[36].

Intrinsic pathway of apoptosis: BAs are able also to induce apoptosis through the mitochondrial or intrinsic pathway, in which intracellular stress causes mitochondrial dysfunction and the subsequent release of proapoptotic factors^[25,27,30]. As mentioned above, BAs can directly induce the generation of ROS by mitochondria^[18,21] and mitochondrial membrane potential depolarization^[27,30,31] in rat hepatocytes. MPT induction in apoptosis induced by several BAs has also been demonstrated^[24,25]. Antioxidants prevented GCDCA-induced apoptosis in rat hepatocytes through a mechanism involving the MPT^[25]. It has also been demonstrated that DCA-triggered apoptosis, involved in decreased mitochondrial membrane potential and alterations in Bax subcellular distribution, exhibits increased mitochondrial-associated Bax protein levels^[31]. These results suggest that BA-induced MPT is the initial event, and the initial cytochrome c release stimulates Bax translocation to the mitochondria, accomplishing further cytochrome c release^[31]. *In vivo* studies show that 3 d after bile-duct-ligation, Bax expression is increased but then decreases over time. Bax translocation to the mitochondria and cytochrome c release are also found in these conditions^[41].

Mitochondrial dysfunction can also occur in death-receptor-mediated apoptosis, especially the so called “type II cells”, such as hepatocytes. Mitochondrial cytochrome c release is (Fas-associated death domain) FADD/caspase 8-dependent during the death receptor-mediated apoptosis of type II cells^[42].

ER stress-induced apoptosis: More recently, it has been demonstrated that hydrophobic BAs can also induce apoptosis in hepatocytes through another intracellular pathway of cell death involving ER stress. Thus, GCDCA induces ER stress which, in turn, induces apoptotic signalling in a time-dependent manner in isolated rat hepatocytes. This BA caused Ca^{2+} release

from ER, which in turn induced extracellular Ca^{2+} influx followed by the activation of calpain and caspase-12. In this study it is suggested that ER stress induced by GCDCA may trigger the activation of both ER mediated apoptosis and mitochondria-mediated apoptosis in isolated rat hepatocytes by cross-talk between ER and mitochondria using calcium ions as signal substances^[43,44]. Recent studies have suggested that ER stress might be involved in hepatocyte cell death caused by cholestasis or BAs^[45,46]. It has been demonstrated that cholestasis induces ER stress mediated by CHOP, a key component in ER stress mediated apoptosis, and triggers hepatocyte cell death. Moreover, CHOP deficiency attenuates this cell death and subsequent liver fibrosis. The results demonstrate an essential role of CHOP in development of liver fibrosis due to cholestatic liver damage^[46].

BILE-ACID-INDUCED DAMAGE IN THE PLASMA MEMBRANE

In hepatocytes, high concentrations of BAs can cause damage to the basolateral membrane, cell organelle membranes, and because they are more concentrated in bile, these compounds might be particularly harmful to the outside layer of the canalicular membrane. Hydrophobic BA-induced structural and functional injury of hepatocyte membranes plays an important role in the pathogenesis of cholestatic liver diseases in humans^[47]. Conjugated-BAs are more hydrophilic and not usually cytotoxic until their concentrations approach their critical micellar concentration^[48]. The effect of BAs on cell membranes is induced by binding to the membrane components^[49]. The authors carried out experiments using large unilamellar vesicles and showed that at very low BA concentrations, BA/lipid aggregates are formed in the outer membrane monolayer, with a size and BA-binding strength that depended on the species of BA and lipids involved. As BA concentrations increased, binding to membranes also rose until a certain threshold was reached and the BA-membrane interaction then resulted in the formation of transient membrane holes^[49], which caused the disruption of plasma membrane integrity and subsequently lysis of hepatocytes^[1]. The efficiency of BAs to solubilise membrane lipids, such as phospholipids, cholesterol or fatty acids, is generally enhanced with increasing BA hydrophobicity^[1,13]. CDCA and DCA have critical micellar concentrations lower than that of CA; therefore at any given concentration they are more cytotoxic^[48]. In the hepatocyte, when the concentration of hydrophobic BAs exceeds the binding capacity of the cytosolic proteins, these compounds possibly damage organelle membranes, especially in the case of mitochondria, which leads to mitochondrial damage and ultimately to apoptosis or necrosis^[48].

The amount of BAs present in bile as monomers depends on their degree of association with phospholipids and cholesterol to form mixed micelles. Such mixed micelles are formed at concentration

values well below cytotoxic levels, which accounts for the lack of BA-induced injury in the biliary system and small intestine under physiological circumstances. In *Mdr2*-knockout mice and patients lacking the canalicular phosphatidylcholine transporter MDR3 (*Mdr2* in rodents), phospholipid secretion is markedly impaired, leading to an increase in the concentration of monomeric BAs, which causes damage to biliary epithelial cells^[48].

BILE-ACID-INDUCED INJURY IN OTHER CELL TYPES

Biliary cells

In addition to the cytoprotective role of biliary phospholipids against the detergent activity of BAs, other mechanisms prevent cell damage, necrosis and apoptosis in the biliary epithelium even through this is exposed to high concentrations of these compounds. In immortalized mouse cholangiocytes it has been demonstrated that hydrophobic BAs cause apoptosis when they are taken up and accumulate inside the cell. This is, in part, inhibited by the activity of Mrp3, an export pump able to reduce intracellular BA concentrations^[50]. However, when the accumulation of hydrophobic BAs occurs in chronic or subchronic cholestasis, this triggers cholangiocyte proliferation^[51]. This stimulation capacity has been observed both in normal cholangiocytes and in cholangiocarcinoma cells^[52,53]. Hydrophobic BA-induced cholangiocellular proliferation occurs by transactivation of EGFR^[37,54], which stimulates a phosphatidylinositol 3-kinase-dependent pathway^[55].

Gastrointestinal cells

In humans, an increased incidence of cancer of the laryngopharyngeal tract, esophagus, stomach, pancreas, small intestine and colon is associated with high levels of hydrophobic BAs^[34]. DCA has been proposed to be a cancer promoter in these organs^[56]. These BAs cause DNA damage in several human colon cancer cell lines^[57,58], probably through an indirect mechanism involving the induction of oxidative stress and the production of ROS. Repeated DNA damage is likely to increase the mutation rate in several genes, including those coding for tumour suppressor factors and oncogenes. Moreover, hydrophobic BAs may behave as selection agents for apoptosis-resistant cells. The subpopulation of cells with a reduced apoptosis capability may be selected to survive and proliferate versus the more fragile normal cells. Indeed, long-term exposure of a human colonic epithelial cell line to sublethal concentrations of DCA has been reported to result in the selection of a population of cells partially resistant to DCA-induced apoptosis^[59].

Kidney and lung cells

Regarding extrahepatic tissues, BA accumulation in the systemic circulation may contribute to endothelial

injury in the kidney and lungs^[60]. Hydrophobic BAs cause oxidative damage to tubular cell membranes by stimulating the generation of ROS from mitochondria, as well as promoting their release from neutrophils and macrophages. Oxidative stress can promote the formation of a variety of vasoactive mediators including endothelin-1, cysteinyl leukotrienes, F2-isoprostanes and endogenous products of lipid peroxidation. These mediators can affect renal function directly by causing renal vasoconstriction or by decreasing the coefficient of glomerular capillary ultrafiltration, and thus reducing the glomerular filtration rate. Collectively, these factors contribute to the onset of renal failure in patients with biliary obstruction^[61]. Disturbances in the biochemical functions of the kidney glomeruli and tubules have been reported in patients with intrahepatic cholestasis during pregnancy^[62].

BAs may reach the lung accidentally by aspiration of gastrointestinal contents or by direct uptake from blood. Bile aspiration produces a severe chemical pneumonitis in a porcine lung model^[63] and intratracheally injected BAs have been shown to produce severe pulmonary edema in rabbits^[64]. When intratracheal instillation of taurocholic acid (TCA, Figure 1) in rabbits was studied, microscopic evidence of widespread atelectasis, the pooling of eosinophilic substances in the intra-alveolar spaces, and the formation of hyaline membranes were found. The authors speculated that surfactant activity might be inhibited by BAs^[65].

Placental and fetal cells

The accumulation of BAs may also have deleterious effects on the fetal-placental unit. The excretion of BAs produced by the fetus is performed by the placenta and the maternal liver, therefore impairment in biliary excretion may lead to the accumulation of BAs, in particular in the fetal liver-placenta-maternal liver trio^[66]. An interesting issue that has recently been addressed is how this accumulation of BAs can affect fetal and placental tissues. In cholestatic pregnant rats, hydrophobic BA accumulation induces impairment of the placental antioxidant system and oxidative damage. These alterations are accompanied by enhanced activation of the mitochondrial pathway of apoptosis^[67]. Moreover, the accumulation of hydrophobic BAs in the fetal compartment also causes marked oxidative damage and apoptosis in the fetal liver^[68]. This is evidenced by enhanced lipid peroxidation and protein carbonylation, a pro-apoptotic imbalance in the Bax- α /Bcl-2 ratio, caspase-3 activation, and DNA fragmentation. Different sensitivities to BAs have been reported in fetal and maternal hepatocytes in short-term primary culture^[69]. Although the basal production of ROS by fetal hepatocytes has been found to be higher than by maternal hepatocytes, ROS production is higher in maternal hepatocytes in response to exposure to relatively high concentrations of GCDCA^[69].

BA accumulation can cause other complications in extrahepatic fetal tissues. TCA (Figure 1) impairs neonatal rat cardiomyocyte function by altering calcium

dynamics and impairing the function of gap junctions in these cells, resulting in dysrhythmia. This has been proposed as a mechanism for intrauterine fetal death in obstetric cholestasis^[70]. Recently, it has been reported that BAs can induce lung injury in newborn infants because these compounds are detectable in the bronchoalveolar lavage fluid of newborns from mothers with intrahepatic cholestasis during pregnancy affected by respiratory distress syndrome. Elevated serum BA levels in these infants can reach the lung after uptake from the circulation. These findings support the concept of a role of BA in the etiopathogenesis of some types of pneumonia^[71].

BILE-ACID-INDUCED CELL PROTECTION

UDCA: protective effects and mechanisms of action

UDCA (Figure 1) and its taurine-conjugated derivative, tauroursodeoxycholic acid (TUDCA) are hydrophilic BAs that have become highly popular owing to their low toxicity and efficiency in the treatment of several cholestatic liver diseases, such as cholelithiasis, primary biliary cirrhosis, primary sclerosing cholangitis, cystic fibrosis and intrahepatic cholestasis of pregnancy^[2,3]. These BAs may be also useful to protect organs other than the liver, such as the brain^[72] or placenta^[67,73-75].

UDCA is a major primary BA in some species of bears^[76]. In fact, for centuries dried bear bile has been used in traditional Chinese medicine as a remedy for liver disorders^[76]. In humans, UDCA is considered as a minor secondary BA because it is formed by 7 β -epimerization of CDCA in the gut by intestinal bacteria. The abundance of UDCA in the total BA pool is less than 3%^[77]. Despite its clinical efficacy, the precise mechanism by which UDCA improves liver function during cholestasis is still under study. It is considered that the choleretic effect of UDCA, together with its ability to cause a marked shift in the composition of the BA pool towards hydrophilicity, accounts for the beneficial properties of UDCA in the treatment of liver disorders^[2,78]. Moreover, it has recently become evident that UDCA and TUDCA are capable of exerting direct protective effects at the cellular and molecular level, including the stabilization of hepatocyte membranes, the enhancement of defences against oxidative stress and the inhibition of apoptosis induced by several agents^[2]. Other proposed mechanisms of action for UDCA, such as the immunomodulation and stimulation of bile secretion by hepatocytes and bile duct epithelial cells, may also contribute to the cytoprotective effects of this hydrophilic BA^[78]. Nevertheless, the predominant mechanism of action of UDCA may vary, depending on the pathophysiology of the underlying liver disease.

Protection against oxidative stress: UDCA has shown to play an important role in the prevention of the oxidative injury induced by several agents, either through a direct antioxidant effect or an increase in antioxidant defences. The enhancement of glutathione levels, which has been demonstrated using isolated rat

hepatocytes^[79] and rats with bile-duct-ligation-induced secondary biliary cirrhosis^[80], can be included among the beneficial effects of UDCA treatment, and may be due to a higher expression of the enzymes involved in glutathione synthesis. In this sense, an enhancement of γ -glutamylcysteine synthetase at the transcriptional level has been found in isolated rat hepatocytes treated with UDCA, which allows these cells to be more resistant to cadmium- or hydrogen-peroxide-induced oxidative injury^[79]. UDCA treatment of chronic bile-duct-ligated rats also leads to an up-regulation of γ -glutamylcysteine synthetase and prevents the marked increase in the production of mitochondrial peroxide and of hydroxynonenal-protein adducts observed during chronic cholestasis^[80]. The activity of methionine S-adenosyltransferase, another enzyme involved in glutathione (GSH) biosynthesis, has also been found to be increased by UDCA in rat livers^[81].

Pre-treatment of hepatocytes with UDCA also increases the amount of thiol-containing proteins such as metallothioneins, which efficiently scavenge hydroxyl radicals (OH \cdot), the most highly reactive ROS^[79]. In human hepatoblastoma HepG2 cells, UDCA has been shown to be able to activate the metallothionein IIA promoter^[82].

During rat development, there is an excessive mitochondrial response to pro-oxidant stimuli, together with less well developed antioxidant protection mechanisms. This may account for the particularly high sensitivity of the foetal liver to lipid peroxidation^[83]. In rats, obstructive cholestasis during pregnancy (OCP) increases the degree of lipid peroxidation and protein carbonylation in placenta^[67] and in foetal liver^[68]. Treatment of rats with UDCA during pregnancy prevents oxidative injury in the placenta^[67] and foetal liver^[68]. The drop in the activities of the enzymes involved in the mechanisms of resistance against oxidative stress, such as catalase, glutathione peroxidase and glutathione-S-transferase^[67], increases GSH content and the GSH/glutathione disulfide ratio. The impairment in liver structure and function in 4-wk-old pups born from rats with OCP is also partially prevented if the mothers are treated with UDCA^[75].

Hydrophobic BAs stimulate Kupffer cells, increasing their capacity to generate ROS, which in turn attack nucleic acids, thiol proteins or membrane lipids, causing lipid peroxidation. UDCA can block hydrophobic-BA-induced cellular phenomena, therefore, it could also antagonise macrophage activation by hydrophobic BAs to blunt their capacity to generate ROS^[15].

UDCA has direct antioxidant properties, which are evident at therapeutically relevant drug concentrations and are especially relevant against Fe³⁺- and OH \cdot -induced oxidative damage^[84]. The OH \cdot -scavenging efficiency of UDCA appears remarkable, considering that its rate constant for reactions with this radical species is about 10-fold higher than that of the typical pharmacological scavenger (mannitol) and of the physiological scavengers glucose and histidine^[84]. Thus, together with the high therapeutic concentrations of UDCA reached in

human bile, the drug could readily act as an effective OH[•] scavenger, especially in the biliary milieu^[84]. It is therefore possible that UDCA could act not only as a site-specific OH[•] scavenger, but also as an antioxidant against iron (IV)-induced oxidative damage.

Inhibition of apoptosis: UDCA and TUDCA have been shown to prevent *in vitro* apoptosis induced by several agents in both hepatic and non-hepatic cells^[27]. In rats with OCP, UDCA also prevents apoptosis in the placenta^[67] and fetal liver^[68]. In patients with primary biliary cirrhosis treated with UDCA, this drug shows a potential effect in reducing nuclear DNA fragmentation in biliary epithelial cells^[85]. It has been suggested that UDCA could function as a therapeutic agent in the treatment of neurodegenerative disorders associated with increased levels of apoptosis^[72]. Indeed, UDCA is neuroprotective in pharmacological and transgenic animal models of Huntington's disease^[86]; it inhibits unconjugated bilirubin-induced apoptosis in both glial and neuronal rat cells in culture^[87], improves graft survival in Parkinsonian rats^[86], and protects against neurological injury after acute ischemic and hemorrhagic stroke^[86]. The ability of UDCA to prevent apoptosis induced by a wide variety of compounds, such as hydrophobic BAs, ethanol, transforming growth factor beta 1 (TGFβ1), Fas ligand, and okadaic acid, suggests a mechanism that is common to each of the different apoptotic pathways^[27]. A possible explanation for this ubiquitous antiapoptotic effect of UDCA seems to involve a blockage of mitochondrial dysfunction, mitochondria having a role as integrators of apoptosis signalling pathways^[24,30]. UDCA is involved in both short- and long-term mechanisms in the prevention of the MPT responsible for hepatocyte cell death^[24,30]. UDCA is able to reduce BA-induced disruption of the mitochondrial membrane potential, ROS production, and Bax protein abundance in mitochondria^[30]. UDCA treatment also prevents the marked decrease in cardiolipin levels, which modulate apoptotic processes by inhibiting the MPT of damaged and primarily apoptotic hepatocytes during biliary cirrhosis induced by chronic cholestasis in rats^[88]. Additionally, UDCA prevents the release of cytochrome c from mitochondria to the cytoplasm after mitochondrial injury and the subsequent cytosolic caspase activation and cleavage of the nuclear enzyme poly(ADP-ribose) polymerase^[31].

Nevertheless, the mechanisms by which UDCA prevents cell death involve molecular targets other than mitochondria, such as the ER. In an ER stress model in the liver-derived cell line Huh7, it has been shown that stress in this organelle activates caspase-12 and triggers apoptosis without the involvement of mitochondria^[89]. TUDCA is able to abolish the typical morphological changes of ER stress preceding apoptosis, including activation of caspase-3 and -7, DNA fragmentation, and cleavage of poly(ADP-ribose) polymerase. TUDCA also blocks one of the calcium-mediated apoptotic pathways by a reduction in calcium efflux and the inhibition of

caspase-12 activation^[89].

Apoptosis can be inhibited, not only by blocking pro-apoptotic pathways, but also by promoting survival signals, including the cAMP, Akt, nuclear factor kappaB (NF-κB), mitogen-activated protein kinases (MAPK), and phosphatidylinositol 3-kinase (PI3K) signalling pathways^[90,91]. The antiapoptotic effect of TUDCA against hydrophobic BA-induced apoptosis in rat hepatocytes is independent of caspase-8 inhibition, but results from the activation of the p38 MAPK, extracellular signal regulated-kinase (ERK) MAPK, and PI3K survival pathways^[91]. UDCA stimulates the activation of the intracellular MAPK pathway through the activation of the epidermal growth factor receptor (EGFR)^[90]. TGFβ1-induced hepatocyte apoptosis is associated with the activation of E2F transcription factors and p53 stabilization through its inhibitor Mdm-2. UDCA interferes in the E2F-1 transcriptional activation of apoptosis, thus modulating p53 stabilization, NF-κB activation, and the expression of Bcl-2 family members^[92], through a mechanism that appears to involve nuclear glucocorticoid and mineralocorticoid receptors^[93].

Stimulation of bile flow and detoxification of cholephilic compounds: The impairment of bile formation in cholestasis results in the accumulation of potentially toxic BAs and other biliary constituents in the liver, which can result in necrosis, apoptosis, fibrosis, and ultimately liver cirrhosis^[94]. UDCA stimulates the biliary excretion of BAs and other cholephilic organic anions, even in situations of impaired bile secretion, such as primary biliary cirrhosis and primary sclerosing cholangitis, two disorders in which the effects of UDCA have mostly been studied^[3]. In patients with these diseases, UDCA treatment decreases the serum levels of bilirubin and the accumulation of hydrophobic BAs^[95], probably due to stimulation of the expression of the export pumps involved in the detoxification process of cholephilic organic compounds by hepatocytes^[96], which are down-regulated in cholestatic liver diseases^[94,96].

Transient latent cholestasis in young rats born from mothers with OCP has been reported^[97]. UDCA treatment of rats with OCP has long-term beneficial effects on their offspring by partially preventing the congenital impairment in hepatobiliary function of the pups that affects their biliary lipid secretion^[73]. UDCA also has a beneficial effect on BA transport mechanisms in placentas from patients with intrahepatic cholestasis of pregnancy^[73] and rats with OCP^[74]. UDCA partially prevents OCP-induced impairment in the placenta-maternal liver tandem excretory pathway, by preserving trophoblast structure and function^[74].

In rodents fed for several weeks with diets supplemented with CA, CDCA and LCA, a down-regulation of the expression of xenobiotic-metabolizing enzymes, such as hepatic glutathione S-transferase in mice^[98] and intestinal UDP-glucuronyltransferase in rats^[99], has been found. The prevention by UDCA of the decrease in the activity of phase-II-metabolizing

enzymes by hydrophobic BAs helps to maintain detoxification processes of cholephilic compounds^[98,99].

The secretory capacity of hepatocytes is mainly determined by the number and activity of carrier proteins in the apical membrane. In mouse liver, UDCA stimulates the gene expression of both the canalicular bile salt export pump (Bsep) and the canalicular multidrug resistance associated protein 2 (Mrp2)^[100]. UDCA also stimulates the alternative basolateral ATP-binding cassette proteins Mrp3^[101] and Mrp4^[102], which represent a compensatory overflow system under cholestatic conditions when the function of canalicular export pumps is impaired. UDCA also stimulates murine renal (Mrp2 and Mrp4) and intestinal (Mrp2 and Mrp3) efflux transport proteins, resulting in an increased overall elimination capacity for potentially toxic biliary compounds^[101,102].

Besides the long-term effects of UDCA on the regulation of gene transcription, post-transcriptional events such as increased targeting of transport proteins to the canalicular membrane *via* stimulation of vesicular exocytosis, can be accounted for by the increased expression of hepatobiliary transporters under treatment with UDCA or its conjugated derivatives^[78]. Calcium⁻^[103], protein kinase C (PKC)⁻^[104] and MAPK-dependent mechanisms^[105] mediate the enhancement of the secretory capacity of cholestatic hepatocytes by TUDCA. Indeed, TUDCA favors the insertion of rat Mrp2 into apical membranes *via* PKC-dependent mechanisms^[104]. Furthermore, p38 MAPK signalling pathways are involved in the enhanced insertion of both rat Bsep^[105] and human BSEP^[106] into the canalicular membrane of hepatocytes by TUDCA. Since the Golgi apparatus may serve as a BSEP pool and since p38 MAPK regulates BSEP trafficking from the Golgi apparatus to the plasma membrane, the activation of p38 MAPK by TUDCA can recruit Golgi-associated BSEP and insert it into the canalicular membrane^[106].

Besides the up-regulation of synthesis, apical targeting and insertion, direct activation of key canalicular transporters through modification of their phosphorylation status may contribute to the anticholestatic action of UDCA. This has been shown for mouse Bsep, whose transport capacity is increased by TUDCA *via* PKC α -mediated phosphorylation^[107].

Protection of cholangiocytes against toxic effects of hydrophobic BAs: The enrichment of bile with UDCA contributes to the prevention of toxic effects of more hydrophobic BAs, because this compound renders bile more hydrophilic, modifying the structure and composition of mixed phospholipid-rich micelles^[108]. In addition, UDCA and its conjugates exert a direct effect on membranes by stabilizing their structure. This effect appears to be due to the incorporation of UDCA into the non-polar domain of the lipid bilayer and of its conjugates, into the interface^[109].

An experimental model in which the *in vivo* effects of UDCA on the protection of cholangiocytes have been studied is the *Mdr2*-knockout mouse. These

animals lack the ability to secrete phospholipids into bile and develop a chronic cholangitis resembling human chronic cholestatic liver disease^[110]. The feeding of mice with UDCA or TUDCA decreases the degree of cholangiocellular injury, portal inflammation, and ductular proliferation^[110]. 24-norursodeoxycholic acid seems to be superior to UDCA in the treatment of cholangitis in *Mdr2*-knockout mice^[111]. Similar beneficial effects of UDCA have been found in bile-duct-ligated rats^[112]. Likewise, in patients with primary cholestatic liver diseases under treatment with UDCA, the inflammatory reaction around bile ducts has been reported to be less severe^[113].

In addition to stabilizing cell membranes, other molecular mechanisms responsible for the protective effects of UDCA on cholangiocytes have been described recently. BAs are taken up by cholangiocytes mainly *via* the apical sodium-dependent BA transporter (ASBT). The accumulation of BAs in chronic cholestasis triggers cholangiocyte proliferation and secretion through a PI3K-dependent pathway^[55]. UDCA and TUDCA reduce ASBT expression in cholangiocytes isolated from bile-duct-ligated rats, and they inhibit cellular growth and secretion^[114] through Ca²⁺- and PKC α -dependent mechanisms^[115]. TUDCA also inhibits the growth of the cholangiocarcinoma cell line Mz-ChA-1 through a signal-transduction pathway involving MAPK p42/44 and PKC α , which suggests that this compound may be a candidate for the treatment of cholangiocarcinoma^[116].

Immunomodulation: The immunomodulatory mechanism of action of UDCA accounts for the beneficial properties of treatment with this BA against several autoimmune liver diseases, such as primary biliary cirrhosis and chronic viral hepatitis. Among the *in vitro* experimental evidence of the immunomodulatory activity of UDCA reported is its ability to decrease the secretion of interleukins 2 and 4, TNF- α and interferon- γ from activated T lymphocytes, and immunoglobulin production from B lymphocytes^[117]. The suppression of cytokine and immunoglobulin production and T-cell-mediated cytotoxicity in mice by the treatment not only with UDCA^[118] but also with CDCA^[119] has been observed. In patients with primary biliary cirrhosis, the administration of UDCA down-regulates the expression of abnormal major histocompatibility complex (MHC) class I molecules in periportal hepatocytes, whereas the expression of abnormal MHC class II molecules in bile-duct epithelial cells does not change^[120]. However, UDCA suppresses the interferon- γ -mediated induction of MHC class II gene expression *via* the glucocorticoid-receptor-mediated pathway^[121].

Protective effects of other bile acids

Other natural BA molecules or their derivatives have also aroused pharmacological interest owing to their protective properties. Besides the efficiency of CDCA in cholesterol cholecystolithiasis^[122] and cerebrotendinous xanthomatosis^[48,123], BA replacement therapy with a

mixture of this BA together with CA is used in the treatment of inborn errors of BA biosynthesis involving the A ring^[48]. This treatment suppresses the synthesis of cytotoxic BA precursors and restores the input of primary BAs into the enterohepatic circulation^[48]. Cholyl-N-methylglycine or cholylsarcosine (Figure 1) is a synthetic BA analog that has been shown to prevent the severe fat malabsorption seen in patients with short-bowel syndrome due to a BA deficiency, which leads to impaired micellar solubilization in the proximal intestine^[124].

CONCLUSION

Rapid advances in the understanding of the cellular and molecular pathophysiology of BAs have led to a better knowledge of hepatocyte injury caused by the retention of hydrophobic BAs in cholestatic diseases. These BAs can damage cell membranes and promote the generation of ROS, and eventually cause necrosis and apoptosis. Hydrophobic BAs can also trigger hepatocyte apoptosis by the activation of death receptors, through the mitochondrial pathways, and by the induction of ER stress. The accumulation of hydrophobic BAs in the systemic circulation may have also deleterious effects on extrahepatic tissues such as kidney, lung, placenta, and foetal cells.

UDCA is a hydrophilic BA useful for the treatment of cholestatic liver diseases by its ability to modulate hydrophobic-BA-induced injury in hepatocytes. Underlying mechanisms of their beneficial effects are only now being clarified, and include protection against cytotoxicity due to more toxic BAs, stimulation of bile secretion, immunomodulation, protection against oxidative stress, and the inhibition of apoptosis. Other natural BAs or their derivatives, such as CA, CDCA and cholylsarcosine, have also aroused pharmacological interest owing to their protective properties in several diseases.

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REVIEW

Body weight, lifestyle, dietary habits and gastroesophageal reflux disease

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Abstract

While lifestyle modifications are currently used as first-line treatment for subjects with gastroesophageal reflux disease (GERD), the pathogenetic role of lifestyle factors and consequently, the efficacy of lifestyle measures is controversial. Our aim was to systematically review the pathogenetic link between overweight/obesity, dietary habits, physical activity and GERD, and the beneficial effect of specific recommended changes, by means of the available literature from the 1999 to the present. Obesity, in particular, abdominal obesity, plays a key role in determining GERD symptoms and complications through mechanical and metabolic effects. Controlled weight loss (by diet or surgery) is effective in improving GERD symptoms. No definitive data exist regarding the role of diet and, in particular, of specific foods or drinks, in influencing GERD clinical manifestations. Moderate physical activity seems to be beneficial for GERD, while vigorous activity may be dangerous in predisposed individuals. In conclusion, being obese/overweight and GERD-specific symptoms and endoscopic features are related, and weight loss significantly improves GERD clinical-endoscopic manifestations. The role of dietary behavior, mainly in terms of specific dietary components, remains controversial. Mild routine physical activity in association with diet modifications, i.e. a diet rich in fiber and low in fat, is advisable in preventing reflux symptoms.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is defined as an abnormal reflux of the gastric contents into the esophagus at least once a week, leading to symptoms, such as heartburn and/or acid regurgitation, and/or esophageal mucosal damage, which may also provoke long-term complications, such as Barrett's esophagus^[1,2]. GERD represents a common disorder, particularly in the Western world (about 10%-20% in Western countries and under 5% in Asia) and its prevalence appears to be increasing^[3,4]. The incidence rate, reported by two longitudinal studies^[5,6], was 4.5 and 5.4/1000 people per year, respectively.

GERD is a multifactorial disease in which anatomical and functional factors both play a pathogenetic role. The main pathogenetic mechanism of GERD is considered to be transient lower esophageal sphincter relaxation (TLESR)^[7] which may account for the majority of reflux episodes, in patients with esophagitis and in those with non-erosive reflux disease (NERD). An increased number of TLESR episodes, combined or not with an impaired LES basal tone or with gastric or esophageal motor dysfunction, may lead to GERD, but the underlying causes of these functional disorders are still partially unknown^[2].

However, the role of genetic factors was suggested by twin studies^[8,9] wherein heritability accounted for 31%-43% of the likelihood of reflux disease, which suggests that genetic and environmental factors both

play an important role. Among the environmental factors, lifestyle factors, in particular being overweight/obese, incorrect dietary habits, the lack of regular physical activity and smoking have frequently been suggested to be possible GERD risk factors. However, the exact pathogenetic role of these factors is still under debate and the beneficial effect of specific recommended changes in lifestyle habits is also controversial^[10].

A comprehensive search of the literature (Medline/PubMed databases 1999-June 2008, using the following keywords singly and in different combinations: GERD, food intake, food questionnaire, energy intake, motor activity, exercise, obesity, abdominal obesity) was carried out and the evidence available was critically reviewed.

RELATIONSHIP BETWEEN OVERWEIGHT/OBESITY AND GERD

The observation of a consensual increase in the frequency of obesity and GERD^[7,11,12] in Western countries has suggested a possible pathogenetic link between these two diseases, and it has generated great interest in elucidating the mechanisms demonstrating this association. However, although the relationship between GERD and obesity has been the subject of several studies, conflicting results have been obtained.

In a meta-analysis of epidemiological studies regarding the association between obesity and GERD-related disorders, it was found^[13] that being overweight (BMI, 25-30 kg/m²) and being obese (BMI, > 30 kg/m²) were associated with GERD symptoms (OR, 1.43; 95% CI, 1.158-1.774 and OR, 1.94; 95% CI, 1.468-2.566, respectively), erosive esophagitis (OR, 1.76; 95% CI, 1.156-2.677 in overweight subjects) and esophageal adenocarcinoma (OR, 1.52; 95% CI, 1.147-2.009 and OR, 2.78; 95% CI, 1.850-4.164, respectively). A cross-sectional study on 206 consecutive patients not on acid-suppressing medications who underwent 24-h pH measurement showed^[3] a significant ($P < 0.005$) association between a BMI > 30, a high waist circumference and acid reflux episodes. An additional meta-analysis of clinical studies on the relationship between obesity and reflux symptoms, esophagitis or GERD-related hospitalization documented a positive association between BMI (OR, 1.57; 95% CI, 1.36-1.80 in overweight and OR, 2.15; 95% CI, 1.89-2.45 in obese subjects) and GERD in studies carried out in the USA, but the results were heterogeneous in those carried out in Europe^[14].

Different explanations have been proposed in order to interpret these geographical differences. In fact, the relationship between BMI and the percentage of body fat differs between ethnic groups^[15].

Since there are probably multiple pathogenetic mechanisms of GERD (TLESR, esophageal and gastric motor function, and gastric secretion), it is possible that not all of them are related to, or influenced by, the presence of obesity^[16]. Furthermore, different definitions of GERD (endoscopic, symptom reports by self-administered or validated questionnaires) have

been utilized in the different studies, as well as different measures of obesity (BMI, central adiposity).

RELATIONSHIP BETWEEN BEING OVERWEIGHT/OBESITY AND GERD: SUGGESTED PATHOGENETIC MECHANISMS

The exact pathophysiological mechanisms that demonstrate the association/relationship between being overweight/obese and GERD have not been fully identified, but some hypotheses have been suggested.

It has long been hypothesized^[17-20] that visceral adiposity, expressed by an increased abdominal waist circumference, could be associated with increased intra-abdominal pressure which would, in turn, promote GERD by increasing intragastric pressure (IGP).

Using high-resolution manometry, it was found^[17] that IGP as well as the gastroesophageal pressure gradient (GEPG), during expiration and inspiration, was significantly higher ($P < 0.0001$) in obese and overweight patients, as compared to those with a normal BMI. This showed an IGP increase of 0.3 mmHg per unit of increase in BMI and the association was stronger in men than in women. Similar results were obtained in a recent retrospective analysis^[18] in patients with typical GERD symptoms who underwent pH-monitoring and esophageal manometry. By means of multiple regression analysis, an increase of BMI was independently associated with IGP and with an increase of GEPG during inspiration; furthermore, BMI, IGP and GEPG were strong independent predictors of hiatal hernia, and IGP and GEPG were not independently associated with abnormal acid exposure or esophagitis.

A further pathogenetic mechanism has been suggested by Pandolfino *et al*^[17] who observed that obesity was associated with a separation between LES and the extrinsic crural diaphragm, a disruption which could predispose obese subjects to hiatal hernia. It is known that hiatal hernia is commonly associated with symptomatic GERD, and patients with abnormal esophageal acid exposure have a significantly higher prevalence of hiatal hernia^[21,22]. Furthermore, in patients with hiatal hernia, esophagitis or abdominal low distal esophageal pH are more common than in those without hiatal hernia^[23]. In a retrospective case-control study on 1389 patients, obesity was found to represent an independent risk factor for hiatal hernia^[24]. Therefore, it is possible that obesity, through alteration of normal IGP and separation between LES and the extrinsic crural diaphragm, predisposes to hiatal hernia and consequently, to GERD.

Another plausible mechanism for the association between obesity and GERD is represented by slower esophageal acid clearance, as shown by Quiroga *et al*^[25] in a case-control study using esophageal manometry in normal weight and obese patients with GERD and in healthy subjects. All subjects with GERD showed altered

Table 1 Association between being overweight/obese and GERD-related symptoms

Author	Year	Country	Study design	Population size	Obesity index	Method of data collection	Association
Nilsson <i>et al</i> ^[42]	2003	Norway	Population-based	65 363	BMI	Questionnaire	Yes
Nandurkar <i>et al</i> ^[41]	2004	USA	Population-based	211	BMI	Validated questionnaire	Yes
Hampel <i>et al</i> ^[13]	2005	USA	Meta-analysis		BMI	Validated symptoms score	Yes
Corley <i>et al</i> ^[14]	2006	USA	Meta-analysis		BMI	Questionnaire	Yes
Jacobson <i>et al</i> ^[38]	2006	USA	Cross-sectional, women	10 545	BMI	Questionnaire	Yes
Nocon <i>et al</i> ^[39]	2006	Germany	Population-based	7124	BMI	Interview	Yes
Corley <i>et al</i> ^[37]	2007	USA	Cross-sectional	80 110	BMI and abdominal diameter	Questionnaire	Yes
Nocon <i>et al</i> ^[40]	2007	Germany	Population-based	6215	BMI	Validated questionnaire	Yes
Zheng <i>et al</i> ^[43]	2007	Sweden	Swedish twin registry	27 717	BMI	Questionnaire, telephone interview	Yes
Andersen <i>et al</i> ^[44]	1991	Denmark	Population-based	1321	BMI	Validated questionnaire	No
Lagergren <i>et al</i> ^[46]	2000	Sweden	Cross-sectional	820	BMI	Interview	No
Talley <i>et al</i> ^[45]	2004	Australia	Cross-sectional	777	BMI	Questionnaire	No
Zagari <i>et al</i> ^[47]	2008	Italy	Population-based	1033	BMI	Questionnaire	No

esophageal motility and obese patients also had impaired esophageal acid clearance.

In obese patients, other esophageal motor abnormalities, such as hypotensive LES pressure, nutcracker esophagus and non-specific motility disorders^[26-29] have been observed.

However, the most important reflux mechanism in obese subjects seems to be TLESR^[7]. The main stimulus for generating TLESR episodes is gastric distension, which leads to intense stimulation of both stretch and tension mechanoreceptors in the proximal stomach^[30,31]. In fact, in a recent study^[32], three groups of subjects without GERD (28 obese, 28 overweight and 28 normal) underwent BMI measurements, upper endoscopy, manometry and pH recordings for both the fasting and the postprandial period and were given a symptom questionnaire. During the 2-h postprandial period, both overweight and obese individuals showed a significantly ($P < 0.001$) higher rate of TLESR episodes, and a higher proportion of TLESR episodes accompanied by acid reflux and total acid exposure than normal weight subjects. A direct correlation between increasing BMI, an increased number of TLESR episodes, and an increased number of TLESR episodes associated with acid reflux was identified. Therefore, it seems that obese subjects have a higher postprandial IGP, which provokes more postprandial TLESR episodes.

Another mechanism by which obesity can cause GERD is related to the visceral component of abdominal obesity. In fact, visceral fat is metabolically active^[33] and it has been associated with low serum levels of protective cytokines, such as adiponectin, and high levels of inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6. An increase in these inflammatory cytokines in patients with erosive esophagitis and Barrett's esophagus has also been observed^[34].

In conclusion, although conflicting and non-definitive results exist, it is likely that GERD and obesity are in some way linked; in particular, abdominal obesity seems to play a key role in determining GERD symptoms and complications through mechanical and metabolic effects. Consistent with this evidence, it is

possible to hypothesize that GERD may be a curable condition through the control of body weight and, in particular, by reducing abdominal obesity.

OBESITY AND GERD-RELATED SYMPTOMS

Different studies have analyzed the possible relationship between obesity and GERD by evaluating the clinical manifestation of the disease since reflux symptoms (mainly, heartburn and acid regurgitation) represent the main target for diagnostic evaluation and treatment in GERD patients. However, the factors responsible for the generation of symptoms, in normal or overweight subjects, have not been clearly identified.

Sensitization of esophageal chemoreceptors, either directly by intermittent exposure to refluxed acid or indirectly through esophagitis-associated inflammatory mediators, is thought to be one of the most important mechanisms responsible for symptom generation in GERD^[35,36].

Several authors have evaluated the relationship between obesity and the symptoms of GERD, both to confirm the association between obesity and GERD and to find potential risk factors for symptom generation (Table 1). In all the studies available, only typical GERD symptoms (i.e. heartburn and regurgitation) have been taken into account, with the use of interviews or structured questionnaires.

In a large cross-sectional study^[37] on abdominal obesity, GERD symptoms and ethnicity performed on 80 110 members of a health organization, it was found that increased abdominal diameter, adjusted for BMI, was an independent risk factor for reflux symptoms (OR, 1.85; 95% CI, 1.55-2.21) in the white population but not among blacks and Asians, and this aspect was not influenced by gender.

A recent large cohort study in 10 545 women reported a significant ($P < 0.001$) dose-dependent relationship between increasing BMI and frequent reflux symptoms; this relationship was present even in the normal range of BMI^[38].

Table 2 Association between overweight/obesity and reflux esophagitis

Author	Year	Country	Study design	Population size	Obesity index	Method of data collection	Association
Ruhl <i>et al</i> ^[49]	1999	USA	Cohort	12349	BMI	Upper endoscopy	Yes
Hampel <i>et al</i> ^[13]	2005	USA	Meta-analysis		BMI	Upper endoscopy	Yes
Nocon <i>et al</i> ^[40]	2007	Germany	Population-based	6215	BMI	Upper endoscopy	Yes
Kim <i>et al</i> ^[48]	2007	South Korea	Population-based	27319	BMI	Upper endoscopy	Yes
Kang <i>et al</i> ^[51]	2007	South Korea	Population-based	2457	BMI, waist circumference	Upper endoscopy	Yes
Lee <i>et al</i> ^[50]	2008	South Korea	Population-based	3363	BMI, waist-to-hip ratio	Upper endoscopy	Yes
Chung <i>et al</i> ^[52]	2008	South Korea	Cross-sectional	7078	BMI, waist circumference, visceral adipose tissue	Upper endoscopy	Yes
Furukawa <i>et al</i> ^[58]	1999	Japan	Cross-sectional	6010	BMI	Upper endoscopy	No
Baldi <i>et al</i> ^[59]	2008	Italy	Cohort	1542	BMI	Upper endoscopy	No
Zagari <i>et al</i> ^[47]	2008	Italy	Population-based	1033	BMI	Upper endoscopy	No

Two large population studies^[39,40] and a case-control study^[41] have found a positive association between BMI and reflux symptoms. The same results were previously obtained in a large population-based study carried out in Norway^[42]; the authors found a stronger association among women, especially premenopausally, and that the use of hormone therapy strengthened the association, which suggested that estrogens may play an important role in the etiology of reflux disease. Another recent study^[43] performed in Sweden on a large number of twins, confirmed that BMI seems to be a risk factor for GERD symptoms in monozygotic twins.

However, the association between obesity and GERD symptoms was not confirmed in subjects with benign esophageal disease^[44]. Furthermore, a cohort study from New Zealand found no association between BMI and reflux symptoms^[45] but, as its authors noted, the cohort consisted of young adults, and GERD symptoms do not usually appear until middle age. The same results were produced by a nationwide case-control study in Sweden^[46] and by a recent population-based epidemiological study in Italy^[47].

No data are available yet about the possible correlation between obesity and atypical GERD symptoms.

In conclusion, although further studies are probably needed, most of the available evidence suggests a relationship between obesity, mainly abdominal obesity, and GERD-related typical symptoms.

OBESITY AND REFLUX ESOPHAGITIS

Endoscopic investigation, alone or combined with information regarding specific symptoms obtained by means of structured questionnaires, has been carried out in order to evaluate the possible correlation between GERD-related disorders, such as esophagitis, and obesity (Table 2).

The above mentioned meta-analysis by Hampel *et al*^[13] has documented the association between a BMI ≥ 25 kg/m² and erosive esophagitis. This association was confirmed by Nocon *et al*^[40] using a symptomatic questionnaire and upper endoscopy in 6215 patients and by Kim *et al*^[48] who observed, in 27319 subjects, an association between an increasing BMI and abnormal endoscopic findings, such as erosive gastritis, gastric

ulcer, duodenal ulcer (OR, 1.31; 95% CI, 1.22-1.40; $P < 0.0001$ for overweight subjects; OR, 1.40; 95% CI, 1.14-1.72; $P < 0.001$ for obese patients) and reflux esophagitis (OR, 1.61; 95% CI, 1.42-1.83; $P < 0.001$ for overweight subjects, OR, 2.23; 95% CI, 1.59-3.11; $P < 0.001$ for obese patients). In a long-term follow-up study (median 18.5 years) on 12349 subjects^[49], increased hospitalization rates for esophagitis and hiatal hernia were documented in patients with a BMI > 25 kg/m². Other population-based studies^[50,51] have also confirmed the correlation between obesity, mainly abdominal obesity, and erosive esophagitis.

Furthermore, an association between the metabolic syndrome and reflux esophagitis has recently been documented^[52] in a cross-sectional case-control study on 7078 subjects. In particular, it was observed that, among the single components of the metabolic syndrome, abdominal obesity (in particular visceral fat) and elevated serum triglycerides represented independent risk factors for reflux esophagitis (OR, 1.60; 95% CI, 1.03-2.48). Furthermore, it has been demonstrated that only visceral adipose tissue, evaluated by cross-sectional computed tomography, was an independent risk factor for reflux esophagitis. This tissue, in fact, is recognized to be metabolically active and it has been associated with elevated serum levels of pro-inflammatory adipokines (IL-6, TNF- α and adiponectin), compounds which may play a role in GERD development^[53,54]. It seems that visceral adipose tissue plays a key role in increasing lipolysis and free fatty acid, leading to insulin resistance, which is considered the major pathophysiological factor for the development of the metabolic syndrome^[33,55-57]. It has been suggested that these substances might alter the LES pressure or affect the esophageal clearance of refluxate^[52].

However, the association between BMI and esophagitis has not been confirmed in a recent Japanese prospective study^[58], as well as in an epidemiological survey^[47] and a multicenter Italian observational study^[59]. The different percentages of body fat in different ethnic groups may represent a possible explanation for these different results^[15].

In conclusion, as occurs for specific GERD symptoms, most of the evidence available also suggests a positive correlation for the association between being

overweight/obese and GERD-related morphological lesions.

WEIGHT CHANGES AND GERD-RELATED SYMPTOMS

The observed relationship between weight gain and an increase in GERD-related symptoms^[60,61], as well as that between weight reduction and a decrease in GERD-related symptoms^[62-64], represents additional evidence of the close relationship between obesity and GERD. However, results from studies of weight loss for the control of GERD-related symptoms in obese subjects are not conclusive^[65].

In an uncontrolled study on selected patients with a BMI > 25 kg/m², Fraser-Moodie *et al*^[64] found that weight loss, induced by general dietary advice, had a beneficial effect on GERD symptoms, as evaluated by a structured questionnaire. Different results were obtained^[66,67] in obese patients with reflux disease who were on a very-low-calorie diet, in which no reduction in reflux symptoms or changes in reflux episodes measured by 24 h pH-monitoring were documented. However, these studies have presented important limitations since they included patients with hiatal hernia, which represents an irreversible condition that contributes to the occurrence of GERD despite the weight loss, and they were carried out on very small numbers of patients, 20 and 15, respectively.

The effect of weight loss induced by endoscopic or surgical procedures has been evaluated. A significant reduction in esophageal acid exposure was documented during weight loss induced by an intragastric balloon^[62,63] in a randomized, double-blind, sham-controlled study. An improvement in GERD symptoms was also found after weight loss induced by bariatric surgery^[68]. In particular, the Roux-en-Y gastric bypass seems to represent the surgical procedure that is most effective in improving GERD symptoms in obese subjects^[68-72]. Roux-en-Y gastric bypass may be successful at reducing GERD symptoms by diverting bile away from the esophagus^[68,73], eliminating acid production in the gastric pouch^[73], and reducing the volume of acid refluxate^[70]. This hypothesis is supported by the observation of rapid symptom improvement after the surgical procedure; long-term symptom improvement is likely to be the result of weight loss^[68].

Studies evaluating other types of bariatric surgery, such as laparoscopic gastric banding or vertical banded gastroplasty, have produced conflicting results^[26,74-78]. In particular, laparoscopic adjustable gastric banding has no, or minimal, effect on GERD-related symptoms but, over time, symptoms increase toward baseline or beyond. This effect is likely caused by slippage of the band distally, which results in more stomach above the band. Therefore, the impact of laparoscopic adjustable gastric banding on GERD is independent of weight loss; instead, it is the direct anatomical alteration of the band that impacts on GERD symptoms^[65].

However, these studies are still limited by small sample sizes, lack of randomization, failure to use control groups, use of retrospective data and inconsistent timing of postoperative re-evaluation. Furthermore, it has yet to be clarified whether the surgical technique itself, and/or weight loss, represents the mechanism of action for symptom improvement.

In obese subjects undergoing weight loss, the effect of changes in meal composition on reflux symptoms has also been investigated^[79,80], although in small studies. In a study on eight obese volunteers, a lower carbohydrate diet reduced reflux symptoms and reflux episodes, which was evaluated by means of 24-h pH monitoring^[79]. A higher frequency of reflux symptoms with a high-fat diet as compared to a low-fat diet has also been demonstrated^[80], using four different calorie/fat composition diets, in 15 patients with GERD. Furthermore, esophageal acid exposure was higher with the high-calorie diet as compared to the low-calorie diet.

Together, these data indicate an effective role for weight loss in improving GERD symptoms as well as the effect of differing meal compositions on reflux disease.

DIET AND GERD

It is a common belief that some foods may induce or worsen GERD symptoms; in fact, in daily clinical practice, this belief leads to advising patients to avoid the suspect foods^[81]. Furthermore, since GERD symptoms are most commonly reported postprandially, the role of diet components in inducing symptoms has been suggested. However, different and conflicting results exist in the literature for identifying the most "refluxogenic" foods (Table 3).

Old experimental and clinical studies have shown a decrease in LES pressure and an increase in esophageal acid exposure in response to the ingestion of food rich in fats, chocolate and carminatives^[82-85]. Nebel *et al*^[86] have demonstrated that fried foods, spicy foods and alcohol are the most common precipitating factors of heartburn; however, this study had no control group and did not quantify the intake of dietary items.

In order to elucidate the association of different nutrients with the risk for GERD, El-Serag *et al*^[87] carried out a cross-sectional study on 915 employers, using a dietary questionnaire to estimate the average food consumption over the previous year, and a GERD questionnaire together with an upper endoscopy for assessing GERD severity. A positive association between high fat intake, and GERD symptoms and erosive esophagitis was observed, while a high-fiber diet seemed to reduce reflux symptoms. However, the effects of fat on GERD symptoms and erosive esophagitis were dependent on BMI since this is statistically significant only in overweight individuals. Furthermore, a higher daily intake of fats and proteins was observed in those participants with erosive esophagitis.

More recently, Shapiro *et al*^[88] observed in 58 subjects with typical heartburn symptoms that increased consumption of cholesterol, saturated fatty acids and an

Table 3 Dietary intake and GERD-related clinical manifestations

Author	Year	Country	Study design	Population size	Method of data collection	Effects
Fatty foods, chocolate, carminatives						
Nebel <i>et al</i> ^[84]	1972	USA	Case series	10	Infused open-tipped system 24-h pH-metry	Lowered LESP
Becker <i>et al</i> ^[82]	1989	USA	Case-control	20		Increased esophageal acid exposure
Nebel <i>et al</i> ^[86]	1976	USA	Population-based	1004	Symptom questionnaire	Worsened reflux symptoms
Iwakiri <i>et al</i> ^[89]	1996	Japan	Population-based	20	pH-monitoring	
Holloway <i>et al</i> ^[90]	1997	Australia	Case-control	23	Esophageal manometry and pH-monitoring	
Meyer <i>et al</i> ^[91]	2001	USA	Population-based	11	Esophageal perfusion with HCl and duodenal perfusion with fat and saline, symptoms interview and scales	
El-Serag <i>et al</i> ^[87]	2005	USA	Cross- sectional	371	Dietary and symptom questionnaire and upper endoscopy	No association with GERD symptoms, reflux episodes or lower esophageal sphincter pressure
Shapiro <i>et al</i> ^[88]	2007	USA	Case series	50	GERD symptoms checklist, upper endoscopy, 24-h pH-metry, dietary intake records	
Ruhl ^[49]	1999	USA	Epidemiological	12349	Upper endoscopy, symptoms interview, total dietary servings of high fat foods	
Phel ^[92]	1999	Germany	Case series	12	Esophageal manometry, pH-metry	
Colombo ^[93]	2002	Italy	Case series	13	pH-metry in three solid/liquid meals	
Nandurkar ^[41]	2004	USA	Population-based, nested case-control	211	GERD, energy expenditure, dietary intake questionnaires	
Zheng ^[43]	2007	Sweden	Swedish Twin Registry	27717	Questionnaire, telephone interview	
High caloric load						
Colombo ^[93]	2002	Italy	Case series	13	pH-metry in three solid/liquid meals	
Fox ^[80]	2007	UK	Case series	15	pH-monitoring in four dietary conditions	
Fruits, vegetable, high fiber intake						
El-Serag <i>et al</i> ^[87]	2005	USA	Cross- sectional	371	Dietary and symptom questionnaire and upper endoscopy	Improved symptoms
Zheng <i>et al</i> ^[43]	2007	Sweden	Monozygotic co-twin study based on Swedish Twin Registry	27717	Questionnaire, telephone interview	No association
Alcohol						
O'Leary <i>et al</i> ^[97]	2003	Ireland	Randomized, double-blind, placebo-controlled trial	56	Symptom questionnaires and drug-diet-alcohol records	Worsened reflux symptoms
Rosaida <i>et al</i> ^[98]	2004	Malayia	Cross-sectional	1000	Upper endoscopy and symptom and risk factor questionnaire	No association
Wang <i>et al</i> ^[96]	2004	China	Epidemiological	2789	Symptom and risk factor questionnaire	
Mohammed <i>et al</i> ^[95]	2005	UK	Population-based	1533	Symptom and lifestyle questionnaire	
Talley <i>et al</i> ^[102]	1994	USA	Cross-sectional	1644	Symptom and lifestyle questionnaire	
Stanghellini <i>et al</i> ^[101]	1999	Multinational	Cross-sectional	5581	Symptom and lifestyle questionnaire	
Nilsson <i>et al</i> ^[99]	2004	Norway	Case-control	> 40000	Symptom and lifestyle questionnaire	
Zheng <i>et al</i> ^[43]	2007	Sweden	Swedish Twin Registry	27717	Questionnaire, telephone interview	
Shapiro <i>et al</i> ^[88]	2007	USA	Case series	50	GERD symptoms checklist, upper endoscopy, 24-h pH-metry, dietary intake records	Reduced perception of acid reflux
Coffee						
Price <i>et al</i> ^[103]	1978	USA	Population-based	66	Intraesophageal infusion of coffee, orange juice, spicy tomato drink and HCl	Worsened reflux symptoms
Stanghellini <i>et al</i> ^[101]	1999	Multinational	Cross-sectional	5581	Symptom and lifestyle questionnaire	No association
Boekema <i>et al</i> ^[104]	1999	Netherlands	Randomized controlled crossover study	15	24-h pH-monitoring and coffee and water drinking	
Wang <i>et al</i> ^[96]	2004	China	Epidemiological	2789	Symptoms and risk factor questionnaire	Protective factor
Zheng <i>et al</i> ^[43]	2007	Sweden	Swedish Twin Registry	27717	Questionnaire, telephone interview	

higher percentage of calories from fats was significantly associated with an increased likelihood of having reflux events. The role of fats in symptom generation has been confirmed by some^[89-91], but not by others^[41,49,92,93]. Ruhl *et al*^[49] failed to document an association between dietary fat intake and erosive esophagitis and reflux symptoms,

although higher reflux disease hospitalization rates were associated with an increased BMI. These results are consistent with those produced by Nandurkar *et al*^[41], who analyzed potential risk factors for reflux among 211 community subjects and concluded that only BMI, and not diet, may influence symptomatic GERD.

Also Pehl *et al*^[92] did not find differences in reflux parameters comparing a high-fat meal with a low-fat meal; similar results were obtained by Colombo *et al*^[93], although they found that a caloric load increased esophageal acid exposure.

Furthermore, none of the dietary items evaluated (i.e. vegetables, fruits, fish, meat, rice, milk, grilled and fried food), including alcohol, was associated with the risk of GERD symptoms in the above-mentioned monozygotic co-twin study based on the Swedish Twin Registry^[43], which investigated lifestyle factors that potentially cause GERD.

Considering the recommended advice of controlling alcohol drinking and reducing, or avoiding, coffee to prevent GERD symptoms^[81,94], several studies investigated the role of alcohol and coffee in GERD. While some authors have suggested that alcohol is an independent risk factor for GERD-related symptoms^[95-98], others have not found such a relationship^[99-102]. In their study about the lifestyle habits and GERD symptoms of the participants in two consecutive public health surveys in Norway (> 40000 people), Nilsson *et al*^[99] did not find that alcohol, coffee or tea were risk factors for reflux-related symptoms. Shapiro *et al*^[88] not only confirmed these results, but also documented that alcohol was associated with a reduced perception of intra-esophageal acid reflux events.

Despite the observation that the intraesophageal infusion of coffee in patients with acid sensitivity may induce heartburn^[103], two large epidemiological studies have found no association between coffee drinking and GERD^[96,101]. Boekema *et al*^[104] have found that coffee does not alter postprandial acid reflux time or the number of acid reflux episodes, and others^[41] have noted that coffee consumption is lower in subjects with reflux symptoms. However, the latter result might reflect avoidance of coffee by those who suffer from reflux because the beverage aggravates symptoms.

Furthermore, in their recent study of twins, Zheng *et al*^[43] have found that coffee intake might be a protective factor for GERD symptoms in men, but not in women. The authors have suggested that the differences observed might be caused by sex differences regarding caffeine metabolism. In fact, it has been demonstrated that the conversion of caffeine to paraxanthine, which accounts for 84% of the primary degradation of caffeine in humans^[105], is markedly inhibited by exogenous estrogen in women taking oral contraceptives^[106] or in postmenopausal women on hormone replacement therapy^[107]. Given the conflicting data reported, the relationship between coffee and GERD remains unclear; as a consequence, there is insufficient evidence to support the routine recommendation of avoiding such beverages for patients with GERD.

In conclusion, no definitive data exist regarding the role of diet and, in particular, of specific foods or drinks, in GERD clinical manifestations. Despite the insufficient evidence to support an association between dietary behavior and GERD, some dietary interventions continue to be recommended as first-line therapy^[81]. Larger prospective controlled trials are required to

conclusively recommend dietary modifications in the treatment of GERD.

SMOKING AND GERD

Population-based and epidemiological studies have suggested that tobacco smoking may represent a risk factor for GERD^[9,39,108-113]. Studies using specific questionnaires have reported that smoking is significantly associated with GERD-related symptoms (OR, 1.35; 95% CI, 1.01-1.82)^[114-116]. Furthermore, a recent monozygotic co-twin study^[43] has provided compelling evidence that tobacco smoking increases the risk for the occurrence of frequent GERD symptoms, and a case-control study^[99] on 3153 patients with severe GERD-related symptoms has shown that the duration of smoking was associated with increasing reflux symptoms (OR, 1.7; 95% CI, 1.5-1.9 in subjects who had smoked for > 20 years). Different mechanisms have been suggested to justify the association between smoking and GERD. Cigarette smoking can reduce the LES pressure^[117-119] and decrease salivary bicarbonate secretion, thus reducing the physiological neutralizing effect of saliva on intraesophageal acid and prolonging acid clearance^[120,121]. Furthermore, abrupt increases in intra-abdominal pressure, as occur during coughing or deep inspiration, have been associated with reflux symptoms in smokers^[122]. However, studies that have examined acid perfusion using the Bernstein test or esophageal pH^[123-125] have reported that smokers compared to non-smokers do not show an increased esophageal acid exposure time, despite having more "reflux episodes"^[126]. Furthermore, two old case-control studies evaluating, on very small samples, the effect of smoke cessation on GERD outcomes^[126,127] were unable to document an improvement in GERD symptoms after the cessation of tobacco use.

PHYSICAL ACTIVITY AND GERD

Since previous investigations have demonstrated that strenuous exercise may induce GERD^[128-130] and that GERD symptoms are common among athletes^[131], it has been suggested that physical activity represents another risk factor for GERD. However, available evidence indicates that a positive association between exercise and GERD is present in vigorous, but not in moderate, exercise^[41,132,133]. In fact, Clark *et al*^[134] have reported that running, cycling and weight lifting increase GERD in asymptomatic volunteers. Furthermore, these authors have found that specific types of exercise are more likely to induce reflux symptoms, with running and resistance exercises being more refluxogenic than cycling.

Similar results have been obtained by Peters^[135], in addition, this author has found increased reflux using high-carbohydrate sport drinks with respect to water, which demonstrates a possible role of sport drinks in facilitating reflux symptoms. Therefore, it seems that a hierarchy of exercises in inducing reflux symptoms exists^[134]. However, there is no general agreement with respect to the mechanism by which vigorous exercise

Table 4 Physical activity and GERD-related clinical manifestations

Author	Year	Country	Study design	Population size	Method of data collection	Association/Effect
Vigorous/agonistic exercise						
Clark <i>et al</i> ^[134]	1989	USA	Crossover	12 asymptomatic volunteers	pH-monitoring, 1 h exercise period (bicycling, running and weight routine)	Yes
Schoeman <i>et al</i> ^[132]	1995	Australia	Randomized controlled crossover	10 healthy subjects	Perfused sleeve sensor for 24 h during moderate physical activity, rest and sleep, standardized meals, and standardized exercise	Yes
Peters <i>et al</i> ^[135]	2000	Netherlands	Randomized controlled crossover	7 males triathletes	pH-monitoring, 50 min of running, cycling and supplementation of conventional sport drinks and tap water	Yes
Collings <i>et al</i> ^[128]	2003	USA	Case series	30 athletes	pH-monitoring, evaluation of clinical symptoms during standardized exercise	Yes
Pandolfino <i>et al</i> ^[129]	2004	USA	Case-control	20	pH-monitoring, 60 min of exercise (running and resistance exercise), upper endoscopy and manometry	Yes
Nandurkar <i>et al</i> ^[41]	2004	USA	Population-based, nested case-control	211	GERD, energy expenditure, dietary intake questionnaires	Yes
Ravi <i>et al</i> ^[130]	2005	Ireland	Randomized controlled crossover	135	Esophageal manometry and pH-monitoring before, during and immediately after moderate exercise	Yes
Physical activity at work/postprandial exercise						
Zheng <i>et al</i> ^[43]	2007	Sweden	Swedish Twin Registry	27717	Questionnaire, telephone interview	Yes
Emerenziani <i>et al</i> ^[140]	2005	Belgium-Germany	Clinical trial	37 GERD pz	pH-impedance-monitoring, upper endoscopy, scintigraphic gastric emptying	Yes

induces reflux. Exercise may alter esophageal motility and worsen symptoms of the upper gastrointestinal tract^[136]. Clark *et al*^[134] have speculated that “body agitation” may be important in inducing reflux. Soffer *et al*^[137] have focused on a decreased duration, amplitude and frequency of esophageal contractions with increasing exercise intensity. This suggestion has not been confirmed by Choi *et al*^[138] who reported increased frequency, but not duration or amplitude, of peristaltic contractions. Furthermore, Pandolfino *et al*^[129] have suggested that the anatomical compromise of the esophagogastric junction, as a consequence of frequent abdominal straining associated with strenuous exercise, may predispose to exercise-induced reflux. Other studies have suggested that GERD may be increased in athletes because of a decreased gastrointestinal blood flow, alterations of hormone secretion, changes in the motor function of the esophagus and the ventricle, and constrained body position during exercise^[139].

All these studies suggest that a specific physical activity, i.e. an agonistic activity, plays a possible pathogenetic role in inducing GERD symptoms. However, these results should not be extended to normal physical activity, which has been demonstrated to have a protective effect against GERD^[99] (Table 4). In particular, in a large population-based study^[99], a protective effect of physical activity was observed, documenting a correlation between the number of exercise sessions lasting at least 30 min and a decreased risk of GERD symptoms (OR, 0.5; 95% CI, 0.4-0.7). Therefore, a mechanism of an exercise-strengthened antireflux barrier, possibly constituted by striated muscle was suggested. The same results were produced by Nocon *et al*^[39], who have also found that subjects with typical GERD symptoms are physically less active than those without symptoms.

Furthermore, in their monozygotic co-twin study, Zheng *et al*^[43] have provided evidence that physical activity at work increases the risk of GERD symptoms, whereas physical activity at leisure time decreases this risk. The authors have suggested that physical activity at work might be linked with postprandial exercise, which has been found to be a risk factor for the development of GERD symptoms^[140]. Indeed, physical exercise at leisure time is predominantly performed at times without a feeling of stomach fullness and is, therefore, most unlikely to be a reflux-provoking postprandial exercise^[140].

In conclusion, the relationship between exercise and GERD is also controversial. It may be a consequence of differences in the populations studied (age, race), evaluation of exercise (short-term, long-term), assessment of physical activity (different questionnaires) and diagnosis of the disease (symptom scale or pH-metry). However, mild routine physical activity in association with diet modifications, i.e. a diet rich in fiber and poor in fat, seems to be advisable to prevent reflux symptoms.

CONCLUSION

There is sufficient evidence to support the relationship between being obese/overweight and GERD, expressed as specific symptoms and endoscopic features. Furthermore, available evidence suggests that controlled weight loss (by diet or surgery) is able to induce a significant improvement in GERD symptoms and/or in GERD clinical-endoscopic manifestations. Definitive data still do not exist regarding the association between dietary behavior, mainly in terms of specific dietary components and GERD manifestations. Moderate

physical activity seems beneficial, while vigorous activity may be dangerous in predisposed individuals. However, owing to the evidence that incorrect dietary habits and the absence of regular physical activity represent important risk factors for the development of the so-called “non-communicable diseases”^[141], lifestyle changes are recommended in patients with or at high risk for GERD. According to the recent proposal by a panel of international experts of a new algorithm for GERD management^[142], life-style factors (i.e. meal size and timing, not lying down after a meal or lying down where the head is in a non-elevated position, not smoking, not consuming alcohol, not eating heavily spiced or fatty food and having a physically active life) are important instruments for the overall management of GERD. Additional clinical studies are required.

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REVIEW

Reactive oxygen species: A double-edged sword in oncogenesis

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INTRODUCTION

Reactive oxygen species (ROS) are molecules or ions formed by the incomplete one-electron reduction of oxygen. These reactive oxygen intermediates include singlet oxygen, superoxides, peroxides, hydroxyl radical, and hypochlorous acid. They contribute to the microbicidal activity of phagocytes, regulation of signal transduction and gene expression, and induce oxidative damage to nucleic acids, proteins, and lipids. Peroxidation by ROS alters the amounts of unsaturated fatty acids and proteins in the cell membrane and thus affects membrane fluidity. In addition, with aging, humans tend to show an increased affectability of lipid peroxides caused by ROS^[1]. Recent research has indicated that ROS also play a critical role in the energy dysfunction of mitochondria caused by ethanol-induced gastric mucosa injury^[2]. In addition, oxidative damage caused by ROS and other free radicals is involved in a number of pathological conditions including cancer. Data presented herein is consistent with this opinion. Yagoda *et al*^[3] found erastin interacted with voltage-dependent anion channel proteins to induce mitochondrial dysfunction, release of oxidative species and, ultimately, non-apoptotic, oxidative cell death. This process has a degree of selectivity for cells with activated Ras-Raf-MEK signaling. ROS production also involves the induction of autophagy, which contributes to caspase-independent macrophage cell death^[4]. ROS, produced in the redox cycle, contribute to p53 mutations, which are dominated by G-to-T transversions. These mutations are suppressed by ROS attenuators^[5,6]. The mutations of p53, a well characterized tumor suppressor, are believed to relate to carcinogenesis. Since oxidative stress comprehensively

Abstract

Reactive oxygen species (ROS) are molecules or ions formed by the incomplete one-electron reduction of oxygen. Of interest, it seems that ROS manifest dual roles, cancer promoting or cancer suppressing, in tumorigenesis. ROS participate simultaneously in two signaling pathways that have inverse functions in tumorigenesis, Ras-Raf-MEK1/2-ERK1/2 signaling and the p38 mitogen-activated protein kinases (MAPK) pathway. It is well known that Ras-Raf-MEK1/2-ERK1/2 signaling is related to oncogenesis, while the p38 MAPK pathway contributes to cancer suppression, which involves oncogene-induced senescence, inflammation-induced cellular senescence, replicative senescence, contact inhibition and DNA-damage responses. Thus, ROS may not be an absolute carcinogenic factor or cancer suppressor. The purpose of the present review is to discuss the dual roles of ROS in the pathogenesis of cancer, and the signaling pathway mediating their role in tumorigenesis.

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Key words: p38 mitogen-activated protein kinases; Reactive oxygen species; Signal transduction; Tumorigenesis

damages cells and tissues, it is reasonable that factors which induce ROS would contribute to the occurrence and development of tumors, while antioxidant agents that scavenge ROS may inhibit this process. To the best of our knowledge, the former inference is consistent with previous reports^[7,8], however, there is little supportive evidence for the latter^[9,10]. Vitamins C and E, two reducing agents and antioxidants, show no additional benefit in the chemoprevention of gastric cancer^[11,12]. Inadequate dose, heterogeneous research, poor compliance and multiple effects of antioxidants may lead to this paradox. Is there anything more to be elucidated on this subject?

REGULATION OF ROS PRODUCTION BY RAS

Oxidative stress and Ras activation lead to the production of ROS^[13]. Introduction of ROS by Ras may occur at the transcription level. GATA-6 is a component of the specific protein-DNA complexes at the nicotinamide adenine dinucleotide phosphate oxidase (Nox) 1 promoter, and is able to trans-activate the Nox1 promoter. GATA-6 is phosphorylated at serine residues by MEK-activated extracellular signal-regulated kinase (ERK), which enhances GATA-6 DNA binding. The site-directed mutation of the consensus ERK phosphorylation site (PYS(120)P to PYA(120)P) of GATA-6 abolishes its trans-activation activity, suppressing the growth of CaCo-2 cells. By MEK-ERK-dependent phosphorylation of GATA-6, oncogenic Ras signaling enhances the transcription of Nox1^[14]. A regulatory subunit, Rac, of the NADPH oxidase complex also involves the regulation of ROS^[15]. Other factors that regulate the production of ROS will not be discussed here.

ROS INVOLVE TUMORIGENESIS THAT RELATES TO THE RAS-RAF-MEK-ERK PATHWAY

Growth factors, cellular stress, and γ radiation stimulate oncogenic Ras-Raf-MEK signaling, which plays a crucial role in tumorigenesis. As an important mediator of physiological and pathological signal-transduction pathways, ROS is also involved in Ras-Raf-MEK signaling (Figure 1). The functions of ROS in tumorigenesis relating to this pathway include the following. (1) In cells with activated Ras-Raf-MEK signaling, released ROS cause non-apoptotic, oxidative cell death, as previously mentioned^[3], and the Ras-ERK pathway is critical in mediating protection against apoptotic cell death induced by increased oxidative stress^[16]. (2) The activity of the ROS-generating enzyme Nox1 is required for vascular endothelial growth factor (VEGF), a potent stimulator of tumor angiogenesis. However, if extracellular signal-regulated kinase (ERK)-dependent phosphorylation of the transcription factor

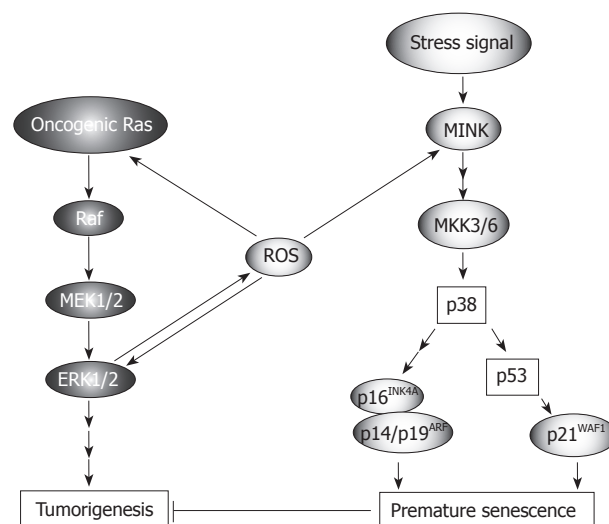


Figure 1 Roles of ROS in the Ras-Raf-MEK-ERK signaling and p38 pathway (Modified from Ref. 31, with permission).

Sp1 and Sp1 binding to a VEGF promoter is inhibited, this activity does not occur. Nox1 mediates oncogenic Ras-induced upregulation of VEGF and angiogenesis by activating Sp1 through Ras-ERK-dependent phosphorylation of Sp1^[18]. (3) Ras (p19) interaction with p73 β , a structural and functional homolog of p53, amplifies p73 β -induced apoptotic signaling responses including Bax mitochondrial translocation, cytochrome c release, increased production of ROS and loss of mitochondrial transmembrane potential. After taxol treatment, endogenous expression of Ras and p73 β significantly increase, and taxol-enhanced endogenous p73 β transcriptional activities are further amplified by p19, which markedly increases cellular apoptosis in the p53-null SAOS2 cancer cell line^[17]. (4) In human U937 monocytes, hydrogen peroxide (H₂O₂) evokes Ca²⁺ influx through TRPM2 to activate Ca²⁺-dependent tyrosine kinase Pyk2 and amplify ERK signaling *via* Ras GTPase. TRPM2 Ca²⁺ influx controls the ROS-induced signaling cascade responsible for chemokine production, which aggravates inflammation^[18].

In contrast, the released ROS have complicated effects on Ras-Raf-MEK signaling, which may occur on several levels (Figure 1). ROS directly enhance the activation of Ras^[19], and augment ERK1/2^[20]. Melatonin, a natural antioxidant, inhibits the activation of Ras in H4IIE hepatoma cells^[21]. Generation of ROS is required for Ras transformation phenotypes including anchorage-independent growth, morphological transformation, and tumorigenesis^[22]. In diabetes-related angiogenesis of the retina, activation of H-Ras and its downstream signaling pathway may be under the control of superoxide, and H-Ras activation in diabetes can be prevented by inhibiting superoxide accumulation^[23]. H₂O₂ activates H-Ras and its downstream signaling pathway, including Raf-1 and phosphorylation of p38 MAP kinase. Inhibition of superoxide significantly attenuates glucose-induced activation of H-Ras, Raf-1 and p38 MAP kinase^[23]. PI3K is a mediator in the

E-Ras-PI3K-Akt signaling pathway, which leads to tumor-like properties in embryonic stem cells. AKR1C2 and AKR1C3 mediated prostaglandin D(2) metabolism augments the PI3K/Akt proliferative signaling pathway in human prostate cancer cells^[24]. Activated Ras is usually associated with cancer, but it also produces paradoxical premature senescence in primary cells by inducing ROS followed by the accumulation of tumor suppressors p53 and p16^{INK4A}^[25].

ROS INVOLVE TUMOR SUPPRESSION VIA THE P38 PATHWAY

Oncogenic Ras sequentially activates MEK, p38 and two p38 downstream kinases, MAPK-activated protein kinase2 (MK2) and p38 regulated/activated protein kinase (PRAK), which in turn suppress Ras-induced cell proliferation by blocking activation of Jun N-terminal kinase (JNK). Increased intracellular levels of ROS, induced by the Ras-Raf-MEK-ERK signaling cascade, may mediate the activation of the p38 pathway and act as an intermediate signal between the MEK-ERK and MKK3/6-p38 pathways (Figure 1). On the one hand, the activation of p38 mitogen-activated protein kinase (MAPK) is a prerequisite for ROS-mediated functions such as apoptotic cell death in cancer cells^[26], and adrenal steroidogenesis^[27]. On the other hand, inhibiting or scavenging ROS may attenuate the activation of p38-dependent pathways^[28,29]. Since Ras induces the production of ROS and the latter activates p38, a conclusion can be derived theoretically that the inhibition of Ras may weaken the tension of p38. This inference is supported by research which involved H4IIE hepatoma cells^[21]. However, in some cases, it is not certain that increased intracellular ROS should enhance the activation of p38^[30]. The p38 MAPK pathway negatively regulates cell proliferation and tumorigenesis. The involvement of the p38 pathway in the regulation of cellular processes that directly contribute to tumor suppression includes oncogene-induced senescence (OIS), replicative senescence, contact inhibition and DNA-damage responses, which have been discussed in detail^[31]. Recently, we found that p38 also plays an important role in inflammation-induced cellular senescence^[32], which is believed to be a process related to tumor suppression. Several reports have shown that ROS mediate OIS *via* p38-dependent pathways^[33-35]. The accumulation of intracellular ROS induced by oncogenic Ras is ERK-dependent during the activation of p38 and the induction of senescence. After sensing the oxidative stress induced by activated Ras, p38 directs cells to undergo apoptosis^[36]. Human cancer cell lines with high ROS levels display enhanced tumorigenicity and impaired p38 α activation by ROS. p38 α has also been reported to antagonize oncogenic transformation induced by activated N-Ras in murine fibroblasts^[37] and by activated K-Ras in colon cancer cell lines^[38]. Activated components of the p38 pathway phosphorylate multiple residues on p53, including Ser33 and Ser46 (by p38),

Ser37 (by PRAK), and possibly others, leading to increased transcriptional activity of p53 and induction of a transcriptional target of p53 and p21^{WAF1}^[31]. Through an unknown mechanism, activated p38 also induces the expression of p16^{INK4A} and p14/p19^{ARF}, which, together with the p53-p21^{WAF1} cascade, cause premature senescence that serves as a tumor-suppressing defense mechanism both in cell culture and *in vivo*^[31]. Ras also involves senescence, in which Seladin-1 acts as a key mediator of oxidative stress^[39]. Seladin-1 has previously been implicated in Alzheimer's disease and cholesterol metabolism. Following oncogenic and oxidative stress, Seladin-1 binds to the p53 amino terminus and displaces E3 ubiquitin ligase Mdm2 from p53, thus resulting in p53 accumulation. Ablation of Seladin-1 causes the bypass of Ras-induced senescence in rodent and human fibroblasts, and allows Ras to transform these cells. Wild-type Seladin-1, but not mutants that disrupt its association with either p53 or Mdm2, suppresses the transformed phenotype. The same mutants are also inactive in directing the p53-dependent oxidative stress response^[39]. p38 related replicative senescence, contact inhibition and DNA-damage responses will not be discussed here, refer to Ref. 31.

ROS INVOLVE APOPTOSIS THAT RELATES TO THE P38 PATHWAY

Numerous researchers have shown that ROS relate to apoptosis that is processed through the mitochondrial pathway, which depends on the activation of p38 (Figure 2)^[40-44]. Apoptosis signal-regulating kinase 1 (ASK1) is an evolutionarily conserved mitogen-activated protein 3-kinase that activates both JNK and p38 MAPK, which may also be triggered by ROS^[45-49]. However, activation of MAPKs (JNK, p38, ERK) is differentially regulated by cleavage size (40 kDa and 36 kDa) of mammalian sterile 20-like kinase 1, which is controlled by caspase-7 and -3^[50]. ASK1-induced and ROS-dependent activation of MAPKs is crucial for apoptosis^[43,51], and for TLR4-mediated mammalian innate immunity^[52]. In the case of oxidative stress, a positive feedback may form in the ASK1-p38-TNF- α pathway, which enhances ROS-mediated apoptosis (Figure 2). Ask1 activates both JNK and p38 MAPK, then the activated p38 translocates into the nucleus and stimulates the expression of MK2. After moving out of the nucleus, MK2 increases TNF- α production. On the other hand, enhanced TNF- α and ROS activate ASK1 activity^[46], which leads to the activation of JNK. JNK abrogates Bcl-2, which is believed to be a protector away from mitochondria-related apoptosis, although Bcl-2 may manifest opposing phenotypes in text of interacting with other proteins^[53]. In addition, this positive feedback is required for ROS-mediated apoptosis (Figure 2). Functional analyses have revealed that the initial ROS-independent activations of JNK, Bax, and caspase-3 are not sufficient for cell death, and thus, should be re-activated by ROS in order to kill the cells^[54]. ROS do

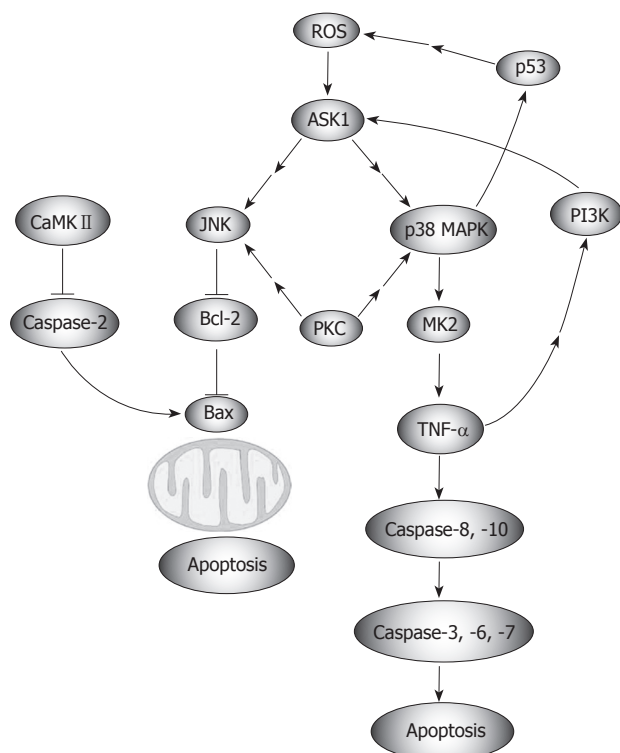


Figure 2 ROS-mediated apoptosis.

not simply mediate the lethal action of γ radiation, but actually amplify it by forming a feedback loop between a downstream effector (caspase) and the upstream initiation signals leading to the activation of JNK. This role of ROS appears to allow Bcl-2 to block the signaling events, which are initially induced upstream^[54]. p38 α MAPK contributes to the further activation of p53, which also leads to a positive feedback loop, p38 α MAPK/p53. The p53/ROS/p38 α MAPK cascade is essential for cisplatin-induced cell death in HCT116 cells, and the subsequent p38 α /p53 positive feedback loop strongly enhances the initial p53 activation^[55]. Of interest, p38 may stimulate indirectly the production of ROS *via* p53. Glioma pathogenesis-related protein 1 (GLIPR1), a novel p53 target gene, is down-regulated by methylation in prostate cancer and has p53-dependent and -independent pro-apoptotic activities in tumor cells. Overexpression of GLIPR1 in cancer cells leads to suppression of colony growth and induction of apoptosis. Mechanistic analysis indicates that GLIPR1 up-regulation increases the production of ROS, leading to apoptosis through activation of the JNK signaling cascade^[56]. However, in p38-related apoptosis that is independent of ROS generation, JNK seems to execute a reverse function. Inhibition of JNK by SP600125 significantly enhanced apoptosis^[57].

Via regulation of MAPKs, the protein kinase C (PKC) δ -mediated pathway also involves ROS related apoptosis. As a tentative stimulator of p38, JNK1/2 and MEK/ERK signaling^[58], PKC δ regulates cell apoptosis and survival in diverse cellular systems. Knock down of PKC δ suppresses p38 MAPK phosphorylation. *Via* p38 MAPK, activated PKC δ

regulates the phosphorylation of heat shock protein (HSP) 27. Attenuated phosphorylation of HSP27 correlates with tumor progression in patients with hepatic cell cancer^[59]. PKC δ translocates to different subcellular sites in response to apoptotic stimuli. The localization of PKC δ differentially affects the activation of downstream signaling pathways. PKC δ -cytosol increases the phosphorylation of p38, whereas PKC δ -nucleus increases c-JNK phosphorylation. Moreover, p38 phosphorylation plays a role in the apoptotic effect of PKC δ -cyto, whereas c-JNK activation mediates the apoptotic effect of PKC δ -Nuc^[60]. Recent evidence has shown that calcium/calmodulin (Ca^{2+} /CaM)-dependent protein kinase II (CaMK II) activity is also enhanced by pro-oxidant conditions. CaMK II is activated by angiotensin II-induced oxidation, leading to apoptosis in cardiomyocytes both *in vitro* and *in vivo* (Figure 2)^[61].

Besides apoptosis, ROS also relate to proliferation. In mice lacking Nrf2 transcription factor, oxidative stress-mediated activation of p38, Akt kinase and downstream targets is impaired, resulting in enhanced death and delayed proliferation of hepatocytes^[62]. p38 MAPK, p53, and p21 also act as molecular mediators on the way from increased ROS levels to the observed growth arrest^[63].

CONCLUSION

Besides their well-known roles, recent studies have demonstrated additional functions of ROS in tumorigenesis. However, the evidence comes from studies performed in cell culture, in addition to data from human tumors. In addition, these cell lines are generally kept in room air, whereas hyperoxic oxygen levels may favor enhanced ROS formation, which is well known. The relevance of ROS in all these events *in vivo*, especially in humans, is not clear. ROS seem to have dual roles in tumorigenesis, cancer promoting and cancer suppressing. ROS participate in both Ras-Raf-MEK1/2-ERK1/2 signaling and the p38 MAPK pathway. However, these two pathways may have inverse functions in tumorigenesis. The former is related to cancer promotion, whereas the latter is associated with a variety of cellular responses such as OIS, replicative senescence, contact inhibition and DNA-damage responses. Thus, regarding ROS as an absolute "carcinogenic factor" or "cancer suppressor" seems to be inappropriate. It seems that more extensive investigations are needed to determine the integrity of ROS in human cancer development. Two aspects of research remain to be carried out in the future. Firstly, we should determine whether ROS directly mediate the Ras-Raf-MEK1/2-ERK1/2 and p38 MAPK signaling pathways, or whether other mediators are needed. Secondly, the definite shunting mechanism, which controls the steering from triggering Ras-Raf-MEK1/2-ERK1/2 signaling to triggering p38 MAPK signaling and *vice versa*, should be determined. The relationship between ROS and p38-pathway-mediated OIS is of particular interest because several reports indicate that part of the OIS pathway is intact at least in certain cancer cells, and that senescence

responses improve the outcome of chemotherapy. Drugs which artificially trigger senescence in tumor cells will thus improve cancer treatment^[31]. Studies on the shunting mechanism would facilitate research on the roles of ROS in tumorigenesis, and could shed light on drug discovery.

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ORIGINAL ARTICLES

Laser capture microdissection and genetic analysis of carbon-labeled Kupffer cells

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Abstract

AIM: To develop a method of labeling and micro-dissecting mouse Kupffer cells within an extraordinarily short period of time using laser capture microdissection (LCM).

METHODS: Tissues are complex structures comprised of a heterogeneous population of interconnected cells. LCM offers a method of isolating a single cell type from specific regions of a tissue section. LCM is an essential approach used in conjunction with molecular analysis to study the functional interaction of cells in their native tissue environment. The process of labeling and acquiring cells by LCM prior to mRNA isolation can be elaborate, thereby subjecting the RNA to considerable degradation. Kupffer cell labeling is achieved by

injecting India ink intravenously, thus circumventing the need for *in vitro* staining. The significance of this novel approach was validated using a cholestatic liver injury model.

RESULTS: mRNA extracted from the microdissected cell population displayed marked increases in colony-stimulating factor-1 receptor and Kupffer cell receptor message expression, which demonstrated Kupffer cell enrichment. Gene expression by Kupffer cells derived from bile-duct-ligated, *versus* sham-operated, mice was compared. Microarray analysis revealed a significant (2.5-fold, q value < 10) change in 493 genes. Based on this fold-change and a standardized PubMed search, 10 genes were identified that were relevant to the ability of Kupffer cells to suppress liver injury.

CONCLUSION: The methodology outlined herein provides an approach to isolating high quality RNA from Kupffer cells, without altering the tissue integrity.

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Key words: Kupffer cells; India ink; Laser capture microdissection; Bile duct ligation; DNA microarray

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INTRODUCTION

Kupffer cells, resident tissue macrophages that line the liver sinusoids, play a key role in modulating inflammation in a number of experimental models

of liver injury^[1]. Since Kupffer cells represent only a small portion of the entire liver cell population, greatly outnumbered by the parenchymal cells, Kupffer cell isolation faces major technical obstacles. Initial Kupffer cell preparations were heavily contaminated with other cell types or, if sufficiently purified, were only a small fraction of the total cell number and therefore not truly representative. Preparation improved drastically with the introduction of techniques that involved perfusion of the liver with collagenase and pronase^[2].

After enzymatic digestion of the liver, the non-parenchymal liver cells are purified by density gradient centrifugation or centrifugal elutriation^[3]. Neither of these methods is able to separate Kupffer cells from other non-parenchymal cell types since cell size and density exhibit considerable overlap^[4]. Furthermore, Kupffer cell purification can be achieved by adherence to plastic^[5], or positive selection using specific antibodies and magnetic beads^[6].

Laser capture microdissection (LCM) circumvents many of the limitations inherent in conventional isolation methods. LCM was created at the National Institutes of Health, Bethesda, MD, USA and further developed by Arcturus Engineering, Inc., Mountain View, CA, USA^[7]. The principals of LCM entail overlaying the tissue section with a transparent ethylene vinylacetate thermoplastic film. At the point of interest, the film is melted onto the tissue surface with a laser integrated with the microscope optics and then removed, capturing sample areas as small as 3–5 μm from intact tissue sections^[8].

For the analysis of gene expression, an important challenge for LCM remains the specific and rapid labeling of the target cell population, thus minimizing the degradation of RNA. In this regard, classic immunohistochemical staining methods for visualizing Kupffer cells are not generally applicable. In the present study, a well-established and efficient method using India ink to label Kupffer cells *in vivo* was described^[9]. Kupffer cells are mainly located in the periportal areas, where they have ready access to pathogens and particulate antigens entering the liver with portal-venous blood^[10,11]. In contrast to liver sinusoidal endothelial cells (LSECs), which mainly ingest soluble materials *via* pinocytosis, Kupffer cells take up particulate material *via* phagocytosis^[12]. It is relevant to note, therefore, that while colloidal gold ≤ 100 nm diameter particle size is internalized almost exclusively by LSECs, colloidal carbon is taken up primarily by Kupffer cells. This apparent contradiction is explained by the fact that blood platelets bind carbon and the platelet-carbon complexes are subsequently phagocytosed by Kupffer cells^[9]. Importantly, the phagocytic capacity of Kupffer cells is not altered by the ingestion of these complexes^[13].

The ability to isolate carbon-labeled Kupffer cells from intact tissue sections using LCM offers an attractive approach to studying gene expression under diverse conditions. The aim of this study was to validate this approach and to apply it to an animal model of cholestatic liver injury.

MATERIALS AND METHODS

Animals

Wild-type female, C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA) and used at 8–12 wk of age. The animals were treated in accordance with NIH publications entitled “Principles for Use of Animals” and “Guide for the Care and Use of Laboratory Animals.” The mice were housed in well-ventilated rooms maintained at 22°C and an alternating 12-h light and dark cycle; food and water were provided *ad libitum*.

Common bile duct ligation

Ligation of the common bile duct was performed as previously described^[6]. The abdomen of mice under deep anesthesia was disinfected with 70% ethanol. A midline upper abdominal incision was made and the abdominal wall was retracted. The common bile duct was identified, isolated and double-ligated with #4-0 braided silk sutures and divided in between. The fascia and skin of the midline abdominal incision were closed with #6-0 braided silk sutures. Control mice underwent sham operations in which the common bile duct was exposed, but not ligated.

Kupffer cell staining

Frozen, 6- μm thick liver sections were cut with a cryostat (Leica, Wetzlar, Germany). Slides were warmed to room temperature for 30 min, fixed by immersion in ice cold acetone (4°C) for 5 min, and air dried for 30 min. After rinsing the slides three times in PBS to remove the tissue freezing matrix, non-specific binding was blocked with 5% normal rabbit serum and 1% BSA for 60 min. This step was followed by 15 min of avidin blocking and 15 min of biotin blocking (Vector Laboratories, Burlingame, CA, USA). After each step, the slides were rinsed in PBS. The slides were stained first with a 1/10 dilution of rat IgG_{2a} anti-mouse F4/80 (a pan-specific macrophage marker; eBioscience, San Diego, CA, USA) monoclonal antibody for 60 min. Subsequently, the slides were washed three times for 10 min with PBS and then incubated for 45 min on a shaker with a 1/50 dilution of biotinylated goat anti-rat polyclonal antibody (Vector Laboratories). After rinsing the slides, pre-diluted streptavidin-Cy3 (Invitrogen, Carlsbad, CA, USA) was applied to the tissue sections and incubated for 30 min; the slides were then dried and mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). All the steps were performed at room temperature.

Processing of the liver tissue and LCM

Mice were inoculated *iv* *via* the tail vein with 200 μL India ink diluted 1:100 in saline 1 d prior to surgery. Immediately following euthanasia, the livers were perfused *in situ* with 20 mL Hank's buffered salt solution to eliminate blood cells, and dissected. Tissue wedges were flash frozen in Tissue-Tek® Optimum Cutting Temperature™ (OCT) compound and stored at -80°C.

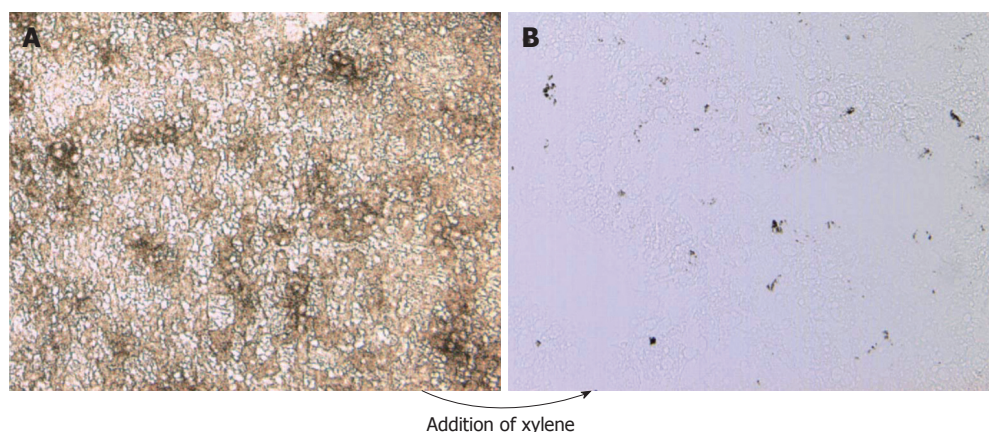


Figure 1 India-ink-positive cells revealed in tissue section overlaid with xylene. A non-stained liver section derived from a mouse inoculated iv with India ink was not mounted (A), or was overlaid with xylene (B).

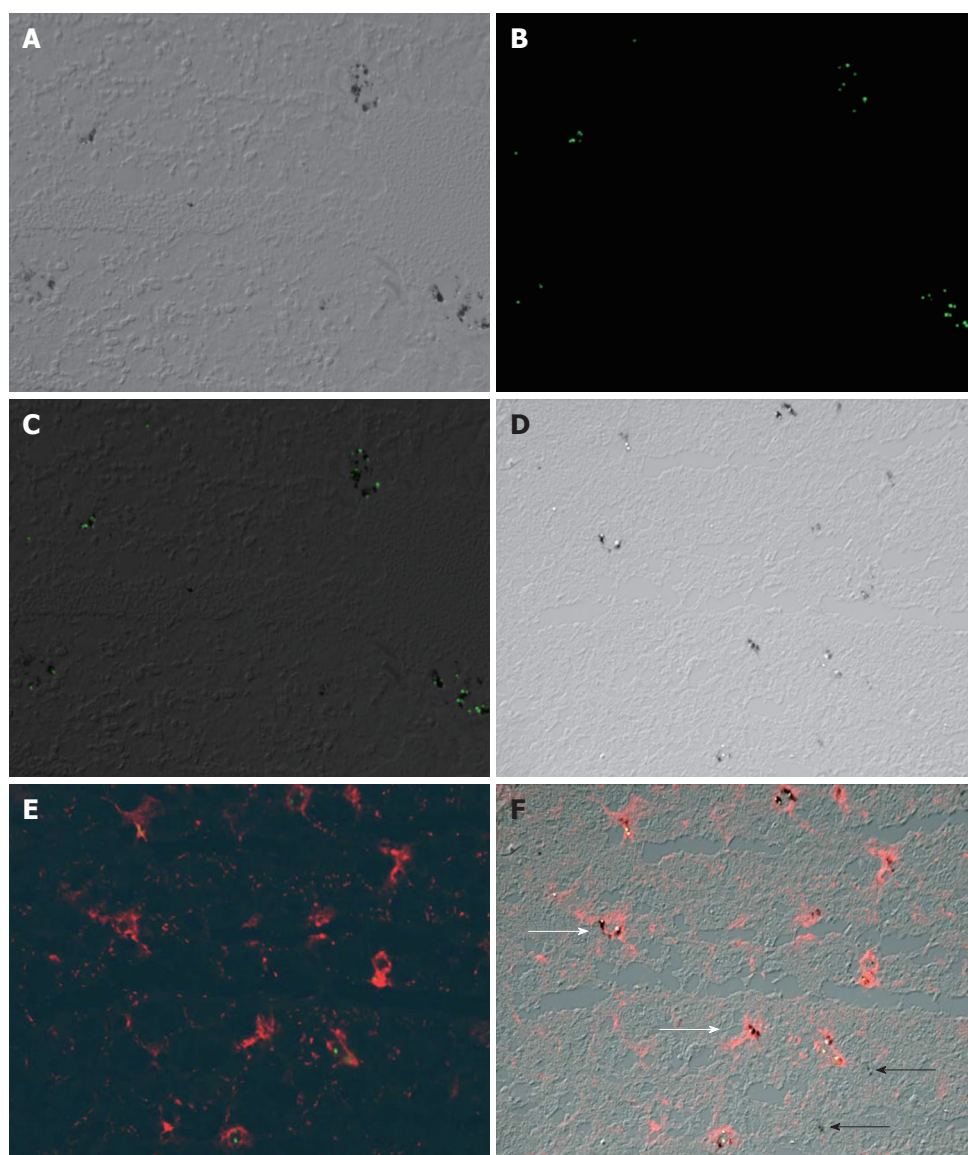


Figure 2 Carbon particles co-localize with ingested fluorescent latex beads and macrophages stained immunohistochemically. A-C: Kupffer cells lining the liver sinusoids were identified by the presence of carbon particles in mice inoculated iv with India ink. Fluorescent latex beads mixed with India ink co-localized with the carbon particles. D-F: A carbon-particle-containing liver section was stained sequentially with streptavidin-conjugated anti-pan macrophage marker F4/80 and biotin-Cy3, and visualized by fluorescence microscopy. An accumulation of carbon particles stained intensely with F4/80 (white arrows), whereas single carbon spots (black arrows) were not stained with F4/80.

Cryostat sections (6 μ m) were prepared under RNase-free conditions at -20°C , fixed immediately in acetone for 2 min at 4°C , dehydrated by sequential immersion for 30 s in 75%, 95% and 100% ethanol, immersed for 2 min in xylene, and then air dried. For each cutting session, new blades, single use staining jars, and fresh (RNase free) solutions were used.

LCM was performed using an AutoPix Automated

Laser Capture Microdissection System equipped with an infrared diode laser according to the protocols and methods provided by the manufacturer (Arcturus Engineering, Santa Clara, CA, USA). Air-dried slides were placed under the microscope of the LCM system and one drop of xylene was applied to visualize the carbon-containing Kupffer cells (Figure 1). A static image was taken and, after the xylene evaporated,

the liver sections were overlaid with thermoplastic membranes mounted on transparent, CapSure Macro LCM Caps (Arcturus). The carbon-labeled (Kupffer) cells were captured by laser activation (12–15 μm laser spot size, 60–80 mW power, 3.0 ms duration) and focal melting of the membrane. At least 300 cells were captured on each cap, and three different caps (equivalent to approximately 1000 cells) were prepared for each sample.

RNA extraction and purification

Cells captured on thermoplastic membranes were immersed in Lysis Buffer (RLT) (Qiagen Inc., Valencia, CA, USA). Subsequently, the RNA was extracted, purified using an RNeasy Micro Kit (Qiagen), and quantified using Lab-on-Chip technology (Agilent Technologies, Palo Alto, CA, USA). Intact 18S and 28S rRNA confirmed the integrity of the RNA extracted. RNA representative of cells constituting the whole liver was extracted from an entire (scraped) tissue section.

RNA amplification and cRNA labeling

Purified total RNA obtained from the microdissected cells was subjected to 1.5 rounds of linear amplification using T7 bacteriophage RNA-polymerase-driven *in vitro* transcription, with materials and protocol provided in the RiboAmpTM HS RNA Amplification Kit (KIT0205; Arcturus). The final amplification and labeling of dsDNA product was performed using the Enzo BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, Framingdale, NY, USA).

Microarray hybridization

Microarray analysis was performed using the GeneChip Mouse Genome[®] 430 2.0 array and the recommended instruments (GeneChip Scanner 3000), with reagents and protocols provided by the supplier (Affymetrix, Santa Clara, CA, USA). This GeneChip covers the transcribed mouse genome on a single array with 45 000 probe sets that analyze the expression level of over 39 000 transcripts.

Bioinformatics and data mining

Each group consisted of three samples; each sample was pooled from three sets of captured material derived from individual, sham-operated or bile-duct-ligated mice. The expression signals were normalized using the standardization and normalization of microarray data (SNOMAD) program^[14]. Concordantly absent expression signals were removed from analysis. A *t* test was used to perform paired comparisons of the gene expression levels between sham-operated and bile-duct-ligated animals. A 5% false discovery rate (FDR) correction controlled for multiple comparisons. The *q* value of a test measured the minimum FDR rate incurred when calling that test significant. *q* values were computed from the unadjusted *P* values, using the Q-VALUE program written by Storey and Tibshirani^[15]. Significant, differentially expressed genes were grouped into functional categories using the GenMAPP 2 (<http://www.GenMAPP.org>) and MAPPFinder

programs by integrating the annotations of the Gene Ontology Project (<ftp://ftp.geneontology.org/go/gene-associations>)^[16,17].

Quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR)

Purified RNA extracted from microdissected Kupffer cells was treated with DNase (10 U DNase I/ μg total RNA) and reverse transcribed with Sensiscript Reverse Transcriptase according to the protocols provided by the supplier (Qiagen). Quantitative gene expression analysis was performed using the MX4000 Multiplex Quantitation QPLR System (Stratagene, La Jolla, CA, USA) and SYBR green technology. QuantiTect SYBR Green PCR Master Mix (12.5 μL ; Qiagen) was mixed in 96-well optical plates with an equal volume of RNase-free water that contained 0.6 $\mu\text{mol/L}$ of the forward and reverse primers, and cDNA corresponding to 70 ng of total RNA input. The plates were heated for 2 min at 50°C, and then 15 min at 95°C to activate the HotStart *Taq* DNA polymerase. Subsequently, 45 cycles consisting of 30 s at 95°C followed by 1 min at 55°C and 30 s at 72°C were run. To verify the generation of single PCR products, melting curves were constructed by heating the samples to 95°C with a ramp time of 20 min at the end of the run. 18S rRNA was used as the housekeeping standard. The threshold cycle (i.e. the number of PCR cycles required in order for the fluorescent signal to reach a fixed intensity) was determined and the number of RNA copies was calculated from standard curves. The mean mRNA copies/ 10^5 18S rRNA copies \pm SD for samples derived from three mice treated comparably was reported. The PCR primers listed in Table 1 were designed using Primer3 software (Whitehead Institute, Cambridge, MA, USA) and purchased from Qiagen.

Statistical analysis

The results were analyzed using the SigmaStat statistics program (Jandel Scientific, San Rafael, CA, USA). Individual means were compared using a non-paired Student's *t* test or a Mann-Whitney rank sum test. Data derived from three or more groups were compared by one-way analysis of variance (ANOVA) followed by a Tukey test, to identify the groups that differed significantly ($P < 0.05$).

RESULTS

Carbon particles co-localize with fluorescent latex beads and anti-F4/80 staining

LCM and analysis of the genes expressed by a specific cell type in a heterogeneous population (i.e. Kupffer cells among other hepatic cells in the study reported here) depends upon the rapid identification of those cells. The intravenous inoculation of India ink and subsequent ingestion of carbon particles by Kupffer cells provides a well-documented means of rapidly labeling Kupffer cells *in vivo* for laser capture. At 18 h or more post-inoculation of India ink, the livers were perfused *in situ* with a balanced salt solution, dissected, and frozen at

Table 1 PCR primers

Gene	GenBank accession size	Orientation	Primer sequence	Amplicon size
KCR	D88577	Forward	AAATGACCTCAGCTCCCAGA	103
		Reverse	TTCACCAGCCCTTCCATAC	
CSF-1 receptor (CD115)	X06368	Forward	TGGCCTTCCTTGCTTCTAAA	114
		Reverse	ATGTCCCTAGCCAGTCCAA	
HMGCS1	NM_145942	Forward	GCGGCTAGAAGTTGGAACAG	196
		Reverse	AGCATATCGTCCATCCCAAG	
Lipocalin 2	NM_008491	Forward	CCAGTTCGCCATGGTATTTT	169
		Reverse	GGTGGGGACAGAGAAGATGA	
Hck	BC010478	Forward	GCCTCAAAAACAGAGCCAAG	150
		Reverse	GTACAGTGGACCAATGG	
18S rRNA	XR_000144	Forward	AATGGTGCTACCGGTCATTCC	192
		Reverse	ACCTCTCTACCCGCTCTC	

-80°C, and 6-μm liver sections were prepared from representative tissue wedges. The carbon particles were visualized microscopically by overlaying the sections with xylene (Figure 1). To ensure the particles were phagocytosed by Kupffer cells, mice were injected simultaneously with India ink and fluorescent latex beads (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), which are often used to track and label Kupffer cells *in vivo*. Using conventional light and UV light to visualize fluorescent emissions, two images were made with a confocal microscope, and both images were merged using Adobe Photoshop. As shown in Figure 2A-C, the carbon particles and fluorescent latex beads co-localized. The liver sections were also stained with a streptavidin-conjugated antibody specific for F4/80, a surface marker expressed by macrophages including Kupffer cells, and biotinylated Cy3 was used to visualize antibody binding. Confocal images of the carbon particles and bound antibody were merged and found to co-localize as well (Figure 2D-F). Notably, areas with an accumulation of carbon particles stained intensely with F4/80 (white arrows), whereas single carbon spots (black arrows) were not stained with F4/80. This observation was considered when selecting areas for LCM.

LCM of carbon-positive areas enriches for Kupffer cell receptor (KCR) and colony-stimulating factor-1 receptor (CSF-1R) mRNA

Labeling Kupffer cells with India ink *in vivo* prior to liver dissection negates *in vitro* staining prior to LCM. This drastically reduced the time between tissue sectioning and actually capturing the cells (approximately 15 min elapsed time) (Figure 3). To ascertain that material was captured, tissue sections and caps were examined by conventional microscopy. A liver section with carbon particles overlaid with xylene is shown in Figure 4A; the red-crossed-through circles show the location of carbon-positive areas on the static image obtained with the LCM system. After microdissection, the same section displayed gaps where the captured areas were removed (Figure 4B). Captured material on caps before RNA extraction with lysis buffer is shown in Figure 4C. Overlaying the cap with water revealed carbon particles associated with this material (Figure 4D).

The RNA was extracted from the captured material

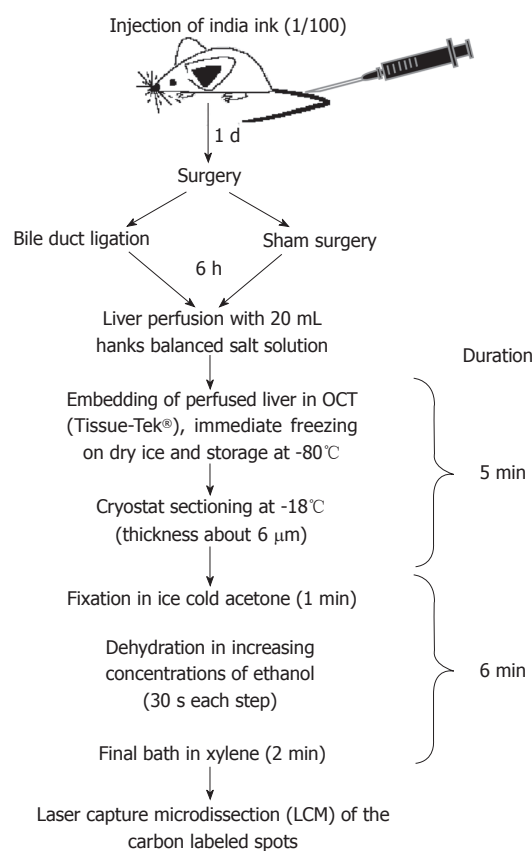


Figure 3 Experimental timeline. Schematic outline of the experimental approach used to isolate Kupffer cells labeled *in vivo* with carbon. Notably, the elapsed time between liver dissection, sectioning and LCM of carbon-labeled Kupffer cells was only about 11 min.

on caps, and the quality and quantity were assessed using a bioanalyzer and Lab-on-Chip technology (Agilent Technologies). Degradation appeared only slight relative to the RNA extracted from an entire tissue section scraped from a slide (Figure 5). Indeed, the quality of RNA obtained by LCM proved to be high and the quantity sufficient for subsequent analyses by real-time RT-PCR and microarray analysis. The amount of RNA isolated ranged from 100 to 1000 pg per 1000 Kupffer cells captured.

The carbon-labeled material obtained by LCM was significantly enriched in KCRs and CSF-1R RNA transcripts relative to the transcripts determined in the

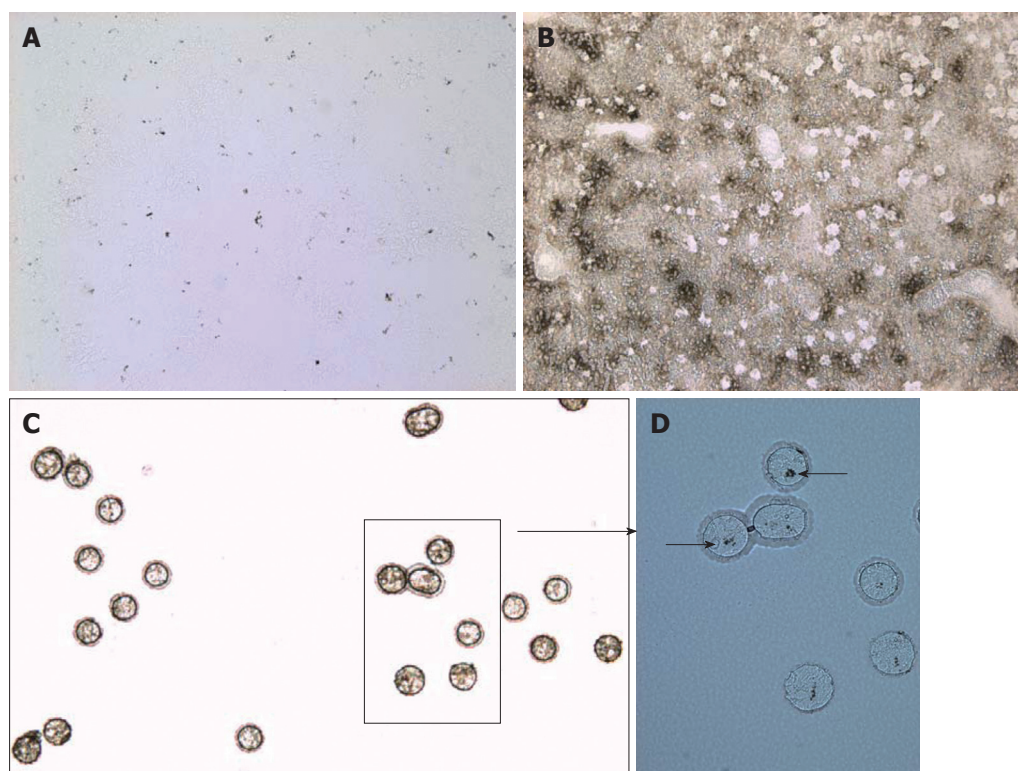


Figure 4 Images documenting LCM of carbon-labeled Kupffer cells. A: Static image of carbon-labeled liver section overlaid with xylene (10 × magnification); B: Same tissue section after the evaporation of xylene and capture of carbon-labeled cells (10 ×); C: Captured cells visualized on the CapSure LCM cap (20 ×); D: Same cap mounted with water and a coverslip showing carbon within microdissected cells (arrows, 40 ×).

tissue scrape (Figure 6). This further substantiates the association of carbon with and preferential dissection of Kupffer cells by LCM in accordance with our previous findings^[6].

Comparison of microarray data generated from Kupffer cells dissected from sham-operated and from bile-duct-ligated mice

Kupffer cells play a vital role in suppressing tissue damage that occurs in a mouse model of cholestatic liver injury. To identify the genes involved, groups of mice inoculated iv with India ink 18 h previously underwent bile-duct ligation or sham operation. The livers were dissected at 6 h post-surgery and sectioned; the carbon-labeled cells were obtained by LCM; the RNA was extracted, purified and amplified; and DNA microarray analysis was performed using the GeneChip Mouse Genome[®] 430 2.0 array. A total of 493 genes were found to be more than 2.5-fold up- or down-regulated (q value < 10) when the microarray data from bile-duct-ligated and sham-operated animals were compared. Interestingly, most of the genes that exhibited significant change were involved in cell growth and maintenance (Table 2: Gene ontology). Sixteen of the genes identified were highly up-regulated or down-regulated (> 5-fold change with a q value < 0.5), and the focus of further analyses. Our primary interest in evaluating these genes was to ascertain their role in maintaining the integrity of the liver during periods of cholestasis. Therefore, the 16 highly up- or down-regulated genes were subjected to a standardized PubMed search (<http://www.ncbi.nlm.nih.gov/sites/entrez>) with terms related to our model (Table 3). Abstracts dating back 15 years were reviewed and the publications relevant to our model were selected

Table 2 Significantly up- or down-regulated genes ontology

	Number	Percentage (%)
Cytoplasm	105	16.7
Cell growth and/or maintenance	87	13.8
Integral to membrane	85	13.5
Nucleus	57	9.0
Nucleoside and nucleic acid metabolism	54	8.6
Protein metabolism	54	8.6
Biosynthesis	34	5.4
Purine nucleotide binding	33	5.2
DNA binding	30	4.8
Signal transduction	26	4.1
Response to external stimulus	24	3.8
Transferase activity, transferring phosphorus containing groups	24	3.8
Lipid metabolism	23	3.7
Plasma membrane	19	3.0
Catabolism	18	2.9
Immune response	18	2.9
Phosphorus metabolism	18	2.9
Peptidase activity	16	2.5

(articles of interest). Based upon this search algorithm, the number of genes of interest was further reduced to 10 (Table 4). Three of these genes, i.e. 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HM GCS1), lipocalin 2, and hemopoietic cell kinase (Hck), were of particular interest in light of the role of Kupffer cells in abrogating cholestatic liver injury.

DISCUSSION

Non-specific phagocytosis of particles taken up in the liver is mediated primarily by Kupffer cells. Kupffer cells constitute 20%-30% of the non-parenchymal cells of

Table 3 Standardized Pubmed search with terms relevant to Kupffer cells abrogating liver injury during cholestasis: Each gene up- or down-regulated > 5-fold

Gene	Fold change	Search terms									Articles of interest
		Gene	Kupffer cells	Cholestasis	Apoptosis	Cell death	Survival	Inflammation	Liver	Liver repair	
Acetyl-coenzyme A synthetase 2	9.61	179	-	-	-	1	2	-	19	-	2
Hydroxysteroid (17-β) dehydrogenase 2	8.26	668	-	-	4	2	10	3	70	-	1
RIKEN cDNA 1110025G12	7.61	-	-	-	-	-	-	-	-	-	-
HM GCS1	5.81	181	-	-	6	6	2	-	67	-	3
RBP 4	5.76	1044	2	2	10	10	25	42	195	2	3
RIKEN cDNA 1200011D03 gene	5.75	-	-	-	-	-	-	-	-	-	-
Inter-alpha trypsin inhibitor, heavy chain 2	5.46	113	2	-	1	-	3	19	63	-	5
Lipocalin 2	5.35	375	-	1	24	25	15	48	17	1	15
Hck	-5.67	4	-	-	-	-	-	-	-	-	3
Hypothetical protein 5930431H10	-6.15	-	-	-	-	-	-	-	-	-	-
Leucine-rich repeat-containing 5	-6.26	22	-	-	1	1	-	-	1	-	2
SAM domain and HD domain, 1 (SAMHD1)	-6.81	5	-	-	-	-	-	-	-	-	-
Myristoylated alanine rich protein kinase C substrate	-7.14	458	-	-	5	7	10	5	11	-	3
Heterogeneous nuclear ribonucleoprotein A/B (SCAN-KRAB-) zinc finger gene 1	-7.94	50	-	-	2	2	2	-	5	-	3
Inactive X specific transcripts	-9.05	6	-	-	1	-	1	-	-	-	1
	-12.21	3	-	-	-	-	-	-	-	-	-

Table 4 Overview of function, source and involvement of the 10 genes identified by Pubmed-search: Degree of up- or down-regulation

Gene	Fold change	Biological function	Primary cell source	Involvement	Reference
Acetyl-Coenzyme A synthetase 2	9.61	Mitochondrial matrix enzyme involved in CoA ligation	Mitochondrial matrix	Activation during fasting	[50,51]
HM GCS1	5.81	Cholesterol biosynthesis		Inhibition induces apoptosis, up-regulation involved in liver regeneration	[25,26]
RBP4	5.76	An α2-globulin that transports vitamin A from the liver	Parenchymal liver cells, little in Kupffer cells	Known marker for hepatocellular necrosis	[22,23]
Inter-alpha trypsin inhibitor, heavy chain 2	5.46	Plasma protease inhibitor	Kupffer cells	Endothelial growth factor	[27-29]
Lipocalin 2	5.35	Iron-siderophore-binding protein	Macrophages, neutrophils	Suppression of inflammation, tissue involution, apoptosis, differentiation of myeloid cells	[31-40]
Hck	-5.67	Protein-tyrosine kinase	Granulocytes, monocytes	Mitogenesis, differentiation, survival, migration	[41-44]
Leucine-rich repeat-containing 5	-6.26	Interaction with cellular G-proteins		Enzyme inhibition, cell adhesion, cellular trafficking, proliferation and activation of lymphocytes and monocytes	[45,52]
Myristoylated alanine rich protein kinase C substrate	-7.14	Substrate of protein kinase C		Intracellular signaling, brain development, cellular migration and adhesion, phagocyte activation, phagocytosis	[46,47,53-55]
Heterogeneous nuclear ribonucleoprotein A/B (SCAN-KRAB-) zinc finger gene 1	-7.94	Chromatin-associated RNA-binding protein	Ubiquitous	RNA handling, proliferation arrest	[56,57]
	-9.05	Protein interaction domain	Ubiquitous	Cell survival, differentiation	[48,49]

the liver, where they reside within the sinusoidal vascular space, predominantly in the periportal area. Here, they

are perfectly situated to clear endotoxins from the blood, and to phagocytose microorganisms and debris. For

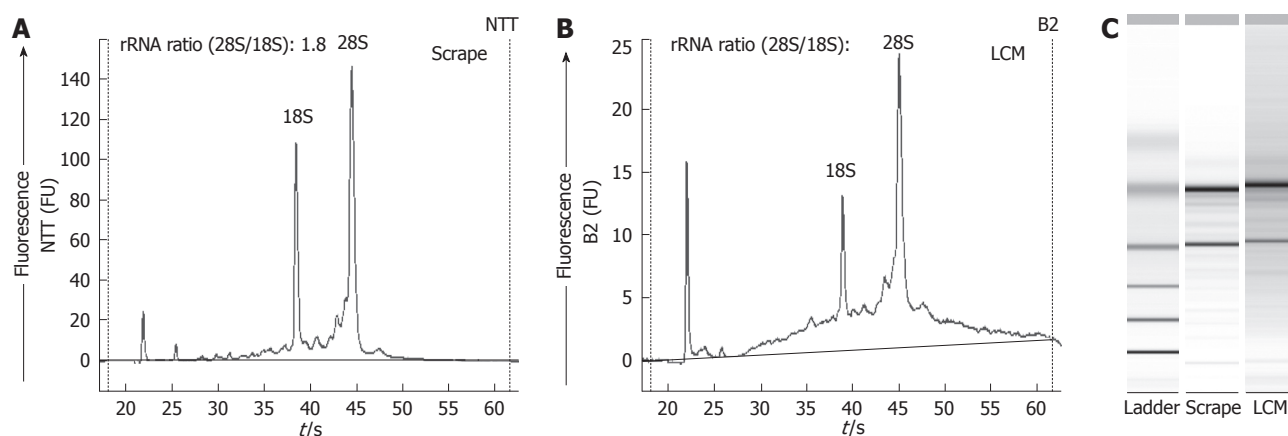


Figure 5 High quality RNA is extracted from material obtained by LCM. RNA size and quality were analyzed with the Agilent 2100 Bioanalyzer. A, B: Profiles of total RNA extracted from scrapes of the entire liver section (A) and from laser-captured cells (B); C: Electrophoresis gel of the same RNA.

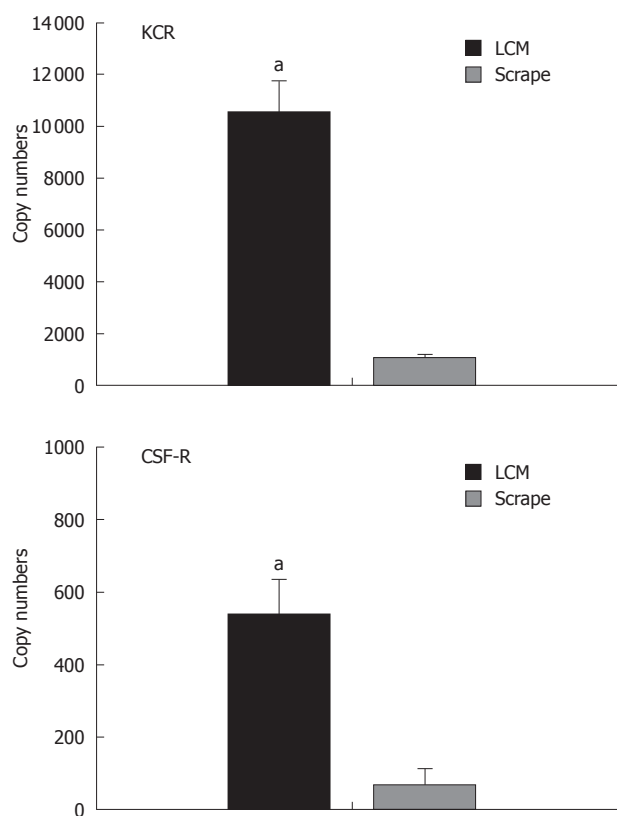


Figure 6 Kupffer-cell-specific mRNA transcripts are enriched in the laser-captured material. Carbon-labelled cells or total liver section scrapes were obtained from three mice; Kupffer cell receptor and CSF-1 receptor mRNA transcripts were quantified by real-time RT-PCR. ^aSignificantly more than extracted from total tissue scrapes ($P < 0.005$; non-paired Student t test).

example, latex particles inoculated *iv* distinguish a latex-labeled Kupffer cell population that does not change over a 3-mo period, substantiating the relatively long life span of these resident tissue macrophages^[18]. While endothelial cells lining the blood vessels internalize soluble materials *via* pinocytosis, Kupffer cells generally ingest particulate matter, e.g. colloidal carbon, *via* phagocytosis. The restricted ability to internalize carbon particles was used herein to label and subsequently dissect Kupffer cells by LCM. The specificity of

this approach is demonstrated by co-localization of the particles with fluorescent latex beads inoculated simultaneously *iv*, and with anti-F4/80-stained hepatic macrophages (Figure 2). The specificity is further documented by the elevated levels of macrophage-associated RNA transcripts (i.e. CSF-1R and KCR messages) extracted from the carbon-labeled material obtained by LCM, relative to those levels extracted from whole liver sections.

Unlike the majority of cells that constitute the mononuclear phagocyte system, Kupffer cells synthesize interleukin (IL)-10 (an anti-inflammatory cytokine) when exposed to lipopolysaccharide (LPS), rather than proinflammatory cytokines such as IL-12 and IL-18^[19]. This finding suggests that Kupffer cells, which are continuously exposed to LPS derived from the intestines *via* the hepatic portal vein, generally impose an immunosuppressive effect on the hepatic environment. In this regard, it is relevant to note that the ingestion of endotoxin and debris occurs coincidentally with the ability of Kupffer cells to induce tolerance^[20]. Moreover, in addition to tolerance, our studies involving a mouse model of cholestasis indicate that Kupffer cells play a general role in suppressing inflammation and liver injury^[6].

Differentiating Kupffer cells from non-resident macrophages, which infiltrate the liver during periods of chronic inflammation, and which may or may not display similar effector functions, represents a major challenge in characterizing the activity of Kupffer cells. The relatively short time period between bile-duct ligation and liver dissection enabled the specific identification and isolation of carbon-labeled Kupffer cells in the study reported here. Whether this approach permits the differentiation of Kupffer cells from inflammatory macrophages recruited to cholestatic livers 2-3 d after biliary obstruction is a matter of ongoing investigations in our laboratory.

LCM allows the isolation of tissue sections as small as 8-10 μm ^[8] and avoids procedures such as collagenase digestion that, conceivably, can alter the gene expression profile of Kupffer cells. Since no staining is necessary,

sections derived from the livers of sham-operated and bile-duct-ligated mice could be processed and subsequently dissected within an extremely short period of time. Moreover, while the effects of histological staining on the integrity of RNA are well documented^[21], the quality of RNA obtained using our approach proved to be high and the quantity sufficient enough for subsequent analyses (Figure 5).

Microarray analysis comparing Kupffer cells derived from bile-duct-ligated versus sham-operated mice revealed a total of 493 genes that were differentially expressed (2.5-fold, q value < 10). In an effort to restrict further analysis to genes specifically relevant to Kupffer cells and their response to cholestasis, those genes that were up- or down-regulated more than five-fold were subjected to a standardized PubMed search (Table 3), and 10 genes of interest were selected (Table 4). Notably, the expression of four of these genes [retinol binding protein 4 (RBP4), inter-alpha trypsin inhibitor heavy chain 2, lipocalin 2, and Hck] was previously described in Kupffer cells or cells of the mononuclear phagocyte lineage. Of these, only RBP4 appears more common in hepatocytes than in Kupffer cells^[22]. RBP4 is often up-regulated in response to hepatocellular necrosis^[23] and thus, its expression in Kupffer cells may derive readily from the ingestion of apoptotic hepatocytes^[24]. Two of the other up-regulated genes are involved in tissue regeneration: HMGCS1 is critical for hepatocyte regeneration after partial hepatectomy^[25,26], and inter-alpha trypsin inhibitor, a plasma protease inhibitor, promotes endothelial cell growth^[27-29].

In addition to promoting tissue regeneration and cell growth, Kupffer cells may induce apoptosis by infiltrating inflammatory cells (e.g. neutrophils) and thus, suppress their contribution to cholestatic liver injury^[30]. In this regard, lipocalin 2, the product of one of the up-regulated genes expressed by Kupffer cells present in cholestatic livers induces apoptosis by leukocytes, but not other cell types^[31-34]. Moreover, lipocalin has been implicated in myeloid cell differentiation and innate host defenses to bacterial infections^[35-40].

In contrast to the genes discussed immediately above, five genes expressed by Kupffer cells were significantly down-regulated in response to cholestasis. Hck, a member of the highly conserved sarcoma family of protein-tyrosine kinases that is preferentially expressed by cells of the myeloid lineage, influences cell migration, adhesion, differentiation and survival^[41-44]. Secondly, leucine-rich repeat containing protein 5 promotes the proliferation and/or activation of lymphocytes and monocytes^[45]. Thirdly, myristoylated alanine-rich C kinase substrate (a substrate of protein kinase C contained in large amounts in macrophages) has been implicated in phagocyte activation, endocytosis, exocytosis, phagocytosis, and mobility^[46,47]. Finally, the SCAN domain is a highly conserved motif found near the N terminus of C(2)H(2) zinc finger transcription factors. Some family members play a role in the transcriptional regulation of genes involved in cell differentiation and survival^[48,49].

In summary, LCM offers an effective approach to analyzing gene expression by Kupffer cells without altering liver integrity. Pre-labeling the cells with carbon *in vivo* renders further staining procedures unnecessary and allows the isolation of sufficient amounts of intact RNA. Thus, the contributions of Kupffer cells to various models of liver injury can be delineated. In this regard, using DNA microarray analysis, we identified 16 genes expressed by Kupffer cells that were highly up- or down-regulated following bile-duct ligation in a mouse model of cholestatic liver injury. A standard PubMed search conducted using terms relevant to the model determined 10 of these genes to be of specific interest and germane to the role of Kupffer cells in suppressing tissue damage. These genes are the subject of ongoing investigation in our laboratory.

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COMMENTS

Background

Kupffer cells, resident tissue macrophages that line the liver sinusoids, play a key role in modulating intrahepatic inflammation. Since Kupffer cells represent only a small portion of the entire liver cell population, greatly outnumbered by the parenchymal cells, Kupffer cell isolation faces major technical obstacles. Laser capture microdissection (LCM) offers a method of isolating a single cell type from specific regions of a tissue section. LCM is an essential approach used in conjunction with molecular analysis to study the functional interaction of cells in their native tissue environment.

Research frontiers

LCM circumvents many of the limitations inherent in conventional isolation methods. LCM was created at the National Institutes of Health (Bethesda, MD, USA) and further developed by Arcturus Engineering (Mountain View, CA USA). The principles of LCM entail overlaying the tissue section with a transparent ethylene vinylacetate thermoplastic film. At the point of interest, the film is melted onto the tissue surface with a laser integrated with the microscope optics and then removed, capturing sample areas as small as 3-5 μ m from intact tissue sections.

Innovations and breakthroughs

Described herein is a method of labeling and microdissecting mouse Kupffer cells within an extraordinarily short period of time. Kupffer cell labeling is achieved by injecting India ink intravenously, thus circumventing the need for *in vitro* staining. This method provides an approach to isolating high quality RNA from Kupffer cells without altering the tissue integrity.

Applications

The ability to isolate carbon-labeled Kupffer cells from intact tissue sections using LCM offers an attractive approach to studying gene expression under diverse conditions. Thus, the specific contributions of Kupffer cells to various models of liver injury can be delineated.

Terminology

LCM permits under direct microscopic visualization rapid isolation of selected cell populations from a tissue section. A transparent thermoplastic film is applied to the surface of the tissue section, and a laser pulse then specifically activates the film above the cells of interest. The film is melted onto the tissue surface and then removed, capturing the pre-selected cells. Kupffer cells are resident tissue macrophages that line the liver sinusoids. Here, they are perfectly situated to clear endotoxins from the blood, and to phagocytose microorganisms and debris. Kupffer cells play a key role in modulating inflammation in the liver.

Peer review

In the current study, a new method of isolating Kupffer cells is presented based upon labeling the cells with India ink inoculated iv and microdissection of the carbon-labeled cells by laser capture. The study is well-controlled with regard to the specificity of staining of Kupffer cells with India ink. The method was further validated in a model of cholestasis.

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Potential therapeutic significance of increased expression of aryl hydrocarbon receptor in human gastric cancer

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RESULTS: AhR expression was significantly increased in GC tissues and GC cell lines. IHC results indicated that the levels of AhR expression gradually increased, with the lowest levels in CSG, followed by CAG, IM, AH and GC. AhR expression and nuclear translocation were significantly higher in GC than in precancerous tissues. TCDD inhibited proliferation of AGS cells *via* induction of growth arrest at the G1-S phase.

CONCLUSION: AhR plays an important role in gastric carcinogenesis. AhR may be a potential therapeutic target for GC treatment.

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Key words: Apoptosis; Aryl hydrocarbon receptor; Cell cycle; Cell proliferation; Gastric cancer

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Abstract

AIM: To determine the functional significance of aryl hydrocarbon receptor (AhR) in gastric carcinogenesis, and to explore the possible role of AhR in gastric cancer (GC) treatment.

METHODS: RT-PCR, real-time PCR, and Western blotting were performed to detect AhR expression in 39 GC tissues and five GC cell lines. AhR protein was detected by immunohistochemistry (IHC) in 190 samples: 30 chronic superficial gastritis (CSG), 30 chronic atrophic gastritis (CAG), 30 intestinal metaplasia (IM), 30 atypical hyperplasia (AH), and 70 GC. The AhR agonist tetrachlorodibenzo-para-dioxin (TCDD) was used to treat AGS cells. MTT assay and flow cytometric analysis were performed to measure the viability, cell cycle and apoptosis of AGS cells.

INTRODUCTION

Gastric cancer (GC) is the fourth most common malignancy and the second most frequent cause of cancer-related death in the world. It is often diagnosed at advanced stages when treatment options are limited, leading to a poor prognosis^[1]. The development of human GC is a multi-step process where normal mucosa progresses to chronic gastritis, precancerous lesions (including gastric atrophy, intestinal metaplasia, dysplasia), and invasive cancer^[2,3]. The carcinogenesis of GC involves numerous genetic and epigenetic alterations, as well as many environmental risk factors^[4]. Environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and halogenated hydrocarbons (HAHs) are well-known carcinogens that play important roles in GC development^[5,6]. The toxic effects of PAHs and HAHs are mediated by a conserved signaling

pathway that binds and activates the aryl hydrocarbon receptor (AhR)^[7].

AhR is a ligand-activated transcription factor of the basic helix-loop-helix/Per-Arnt-Sim family. PAHs and HAHs are exogenous AhR ligands, among which 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) is the most potent^[8]. The ligand-AhR complex is translocated to the nucleus and heterodimerizes with the AhR nuclear translocator. The complex binds to the cognate enhancer sequence and subsequently activates downstream gene expression. AhR regulates genes that code for xenobiotic metabolizing enzymes, such as cytochrome P450 1A1 (CYP1A1), cytochrome P450 1B1 (CYP1B1), and growth-regulatory proteins^[9]. Inappropriately modified expression, and/or abnormally sustained expression of critical genes by the xenobiotic-activated AhR leads to various toxicities which were observed in exposed organisms: teratogenicity, immunotoxicity, tumor promotion, as well as various metabolic dysfunctions^[10,11].

Many studies in recent years have demonstrated a close relationship between AhR and mammary gland tumorigenesis^[12,13]. AhR gene polymorphisms have been linked to an increased risk of lung and breast cancers^[14,15]. Increased expression of AhR has been reported in lung, breast, and pancreatic cancers in humans^[9,12,16]. Studies also suggest that constitutively active AhR may promote hepatocarcinogenesis in mice^[17]. On the other hand, more and more studies have indicated that AhR-mediated responses are anti-proliferative in some cell types and that AhR might function as a potential target for cancer treatment^[16,18]. However, the role of AhR in gastric tumorigenesis is still unclear. Andersson *et al*^[19-21] reported that constitutively active AhR could induce stomach tumors and mediate down-regulation of osteopontin gene expression in a mouse model. In our previous study, we found increased expression of AhR in two human GC cell lines (RF1 and RF48) using microarray analysis^[22]. A recent study suggested that concurrent expression of AhR and CYP1A1 is correlated with GC development^[23].

The aim of our current study was to further determine the functional significance of AhR in gastric carcinogenesis, and to explore the potential role of AhR as a therapeutic target for GC treatment.

MATERIALS AND METHODS

Tissue specimens

Tissues of chronic superficial gastritis (CSG), chronic atrophic gastritis (CAG), intestinal metaplasia (IM) and atypical hyperplasia (AH) were obtained from 120 patients undergoing upper gastrointestinal endoscopy. Tissues of gastric tumors and their corresponding adjacent non-tumor tissues were collected from 70 GC patients who underwent GC surgery. Written, informed consent was obtained from all patients before sample collection. None of the GC patients had received preoperative chemotherapy or radiotherapy. Tissue samples were fixed in 10% neutralized formalin and embedded in paraffin for histological processing or

Table 1 Clinical and histological characteristics of the study population

Histology type	Patient number	Gender		Age (yr) mean \pm SD
		Male	Female	
CSG	30	20	10	50.47 \pm 11.63
CAG	30	12	18	53.27 \pm 16.36
IM	30	16	14	52.38 \pm 10.26
AH	30	15	15	55.67 \pm 16.88
GC	70	37	33	56.59 \pm 13.24
i-GC	32	17	15	57.27 \pm 14.56
d-GC	38	20	18	55.91 \pm 11.62

CSG: Chronic superficial gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; AH: Atypical hyperplasia; GC: Gastric cancer; i-GC: Intestinal-type gastric cancer; d-GC: Diffused-type gastric cancer.

snap-frozen in liquid nitrogen and stored at -80°C for RT-PCR and Western blot analysis. All tissue specimens were histologically verified by a pathologist. Chronic gastritis specimens were classified according to the updated Sydney System^[24]. GCs were classified according to the WHO classification^[25] and Lauren's classification^[26]. The clinical and histological characteristics of the study population are shown in Table 1. The study was approved by the Ethics Committee of the university hospital.

GC cell lines

Five GC cell lines- MKN28, MKN45, AGS, NCI N-87 (N87), and KATO III-were obtained from the Riken Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (ATCC, Rockville, MD, USA). All five cell lines were maintained in RPMI-1640 medium (Hyclone, USA) supplemented with 2 mmol/L glutamine, 100 mL/L fetal bovine serum (Hyclone, USA), 1×10^5 U/L of penicillin, and 0.1 g/L of gentamycin. The cellular environment was maintained at 50 mL/L CO_2 and 37°C . Cells were harvested from the exponential growth phase and total RNA and protein were prepared as described below.

RNA isolation, RT-PCR and real-time PCR

Gastric tissue specimens and cell pellets were homogenized with an ultrasound homogenizer. Total RNA in cells and tissues was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA was synthesized with 1 μg total RNA using reverse transcriptase, ReverTra AceTM (Toyobo Co., Osaka, Japan) under the following conditions: 30°C for 10 min, 42°C for 20 min, 99°C for 5 min, and 4°C for 5 min. PCR of cDNA was carried out in a reaction mixture (30 μL) containing 2 μL of template cDNA, 2.5 mmol/L MgCl_2 , 200 $\mu\text{mol/L}$ dNTPs, 0.3 $\mu\text{mol/L}$ primer 1 and 2, and 1 U of Taq DNA polymerase (New England Biolabs, China). Amplification was performed using the following conditions: 94°C for 5 min, followed by 25-32 cycles (denaturation for 45 s at 94°C , annealing for 30 s, and extension for 30 s at 72°C), and then 72°C for 7 min. Details of primers, annealing temperature, amplification cycles, and

Table 2 Primer sequences and PCR amplification conditions

Gene	Primers (5'→3')	Annealing temperature (°C)	Cycles	Product size (bp)
AhR	S: ACTCCACTTCAGCC-ACCATC A: ATGGGACTCGGCAC-AATAAA	55	25	204
CYP1A1	S: CCATGTCGGCCAC-GGAGTT A: ACAGTGCCAGGTG-CGGGT	59	32	174
β-actin	S: CTCGCTGTCCAC-CTTCCA A: GCTGTCACCTTCA-CCGTT	52	30	256

S: Sense primer; A: Antisense primer.

PCR product size for each gene are listed in Table 2. The PCR products were electrophoresed on 15 g/L agarose gel, stained with ethidium bromide, and visualized with an UV transilluminator. The positive rate of mRNA expression was calculated. mRNA expression levels of AhR were further detected by quantitative real-time PCR with beta-actin as the internal reference, using the Stratagene MX3000P system (Stratagene, USA). cDNA was mixed with SYBR Green QPCR master mix (Stratagene) and primers. The thermal cycling comprised of an initial step at 95°C for 10 min, then 40 intermediate cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 30 s), and one final cycle (95°C for 1 min, 55°C for 30 s, and 95°C for 30 s). Real-time PCR was performed using AhR primers (5'-TACCCTGGACTTGCCTCTGC-3' and 5'-TGAAGCCAGTCAGCACCTC-3'), and beta-actin primers (5'-TCATGAAGTGTGACGTGGACATC-3' and 5'-CAGGAGGAGCAATGATCTTGATCT-3'). Relative quantitation was calculated using the comparative threshold cycle (C_T) method. C_T indicates the fractional cycle number at which the amount of amplified target genes reaches a fixed threshold within the linear phase of gene amplification, and is inversely related to the abundance of mRNA transcripts in the initial sample. Mean C_T of duplicate measurements was used to calculate ΔC_T as the difference in C_T for target and internal reference (β -actin) genes. ΔC_T for each sample was compared to the corresponding ΔC_T of the experiment control and expressed as $\Delta\Delta C_T$. Relative quantitation was expressed as fold changes of the gene of interest compared to the experimental control according to the formula $2^{-\Delta\Delta C_T}$: fold change = $2^{-\Delta\Delta C_T}$.

Western blot analysis

Gastric tissue specimens and cell pellets were homogenized in a lysis buffer containing 20 mmol/L HEPES, 1 mmol/L EGTA, 50 mmol/L β -glycerophosphate, 2 mmol/L sodium orthovanadate, 100 mL/L glycerol, 10 mL/L Triton X-100, 1 mmol/L DTT, and 1 × Protease Inhibitor Cocktail (Roche, Mannheim, Germany). The lysate was centrifuged at 13000 g and 4°C for 10 min.

The supernatant was the total cell lysate. Protein concentration was measured using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA). Thirty micrograms of protein was loaded per lane, separated by 100 g/L SDS-PAGE, and transferred onto equilibrated polyvinylidene difluoride membrane by electroblotting. Membranes were blocked with TBS-T buffer containing 50 g/L non-fat dry milk. AhR, CYP1A1, and beta-actin were detected for 2 h using antibodies against AhR (SC-5579, Santa Cruz Biotechnology, USA, working dilution 1:150), CYP 1A1 (AB1258, Chemicon International, USA, working dilution 1:500), and beta-actin (4970, Cell Signaling Technology, USA, working dilution 1:1000). After secondary antibody incubation (working dilution 1:2000), enhanced chemiluminescence (Pierce Biotechnology, Inc., USA) was determined by exposure to x-ray film. Band intensities in Western blotting were quantified using Quantity One imaging analysis software. Band intensities of AhR and CYP 1A1 were normalized with corresponding band intensities of beta-actin. Data was reported as mean \pm SD.

Immunohistochemistry

Paraffin sections (4 μ m thickness) were dewaxed in xylene and rehydrated in graded alcohols. Antigen retrieval was performed by heating the sections for 10 min at 100°C in 0.01 mol/L citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with 30 mL/L H_2O_2 for 15 min and non-specific staining was reduced using a blocking serum for 10 min. The sections were then incubated with rabbit anti-human AhR antibodies (SC-5579, Santa Cruz Biotechnology, working dilution 1:100) overnight at 4°C. The next day, a two-step detection method (EnVision™ Detection Kit, Gene Tech Company Limited, China) was used according to the manufacturer's instructions. Briefly, after incubation with primary antibodies the tissues were incubated with the ChemMate™ EnVision™/HRP for 30 min at room temperature. The reaction was visualized using the CheMate™ DAB plus Chromogen. Hematoxylin was used as a counterstain. Negative controls were carried out using a similar process, however, the first antibodies were omitted.

A scoring system with two categories was used to evaluate the immunohistochemical results^[27]. Category A documented the number of immunoreactive cells: 0 (< 5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (> 75%). Category B documented the intensity of the immunostaining: 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). A final score was calculated by adding the individual scores for each category. The staining results were measured semi-quantitatively based on the final combined score: 0 (score less than 2), 1+ (score range from 2 to 3), 2+ (score range from 4 to 5), and 3+ (score range from 6 to 7). Immunostaining was assessed by an experienced histopathologist who was blinded to the clinical data of the patients.

Treatment of cells

TCDD and resveratrol were purchased from Sigma

Chemical Company (Bellefonte, PA, USA). Cells were plated on 60 mm diameter plates (for RNA preparation) and 100 mm diameter plates (for cytosolic preparation) at 80%-90% confluence in RPMI-1640. After incubating for 24 h, one group of cells was treated with TCDD at different concentrations (0, 0.01, 0.1, 1, 10, 100 nmol/L) for 24 h. A second group was also treated for an additional 24 h with TCDD (1 nmol/L) plus resveratrol (0, 1, 5, 10, 20 μ mol/L). Another group was treated with TCDD (1 nmol/L) for different time intervals (0, 1, 6, 24, 48, 72 h), respectively. All drugs were dissolved in dimethyl sulfoxide (DMSO). Control cells received 1 mL/L DMSO only.

MTT Assay

A total of 1×10^4 trypsin-dispersed cells in 0.1 mL culture medium were seeded into each well of a 96-well plate and cultured for 24 h. Next, the cells were incubated with medium alone or with medium plus TCDD at different concentrations (0, 0.01, 0.1, 1, 10, 100 nmol/L) for another 12, 24 or 48 h. Then, 20 μ L of MTT (5 g/L, Sigma) was added to each well and the incubation was continued for 4 h at 37°C. Finally, the culture medium was removed and 200 μ L of DMSO was added to each well. The absorbance was determined with an ELISA reader at 490 nm. The cell viability percentage was calculated as: Viability percentage (%) = (Absorption value of TCDD treatment group) / (Absorption value of control group) \times 100%

Flow cytometric analysis

For flow cytometric analysis, AGS cells were plated on 60-mm diameter culture plates and treated with TCDD at different concentration (0.01, 0.1, 1, 10, 100 nmol/L) for 48 h. The control contained 1 mL/L DMSO only. Prior to harvesting, the cells were washed twice with 0.01 mol/L PBS, trypsinized, and pelleted. The cells were then fixed with ice-cold 700 mL/L ethanol at 4°C overnight. Finally, the cells were washed twice with PBS and dyed with propidium iodide (PI). The DNA content was analyzed with a flow cytometer (Beckman-Coulter, USA). The cell cycle and apoptosis of AGS cells were analyzed using MULTICYCLE and winMDI2.9 software (Phoenix, AZ, USA). The final data was reported as the mean \pm SD for each of the three independent experiments.

Statistical analysis

All quantitative data were expressed as mean \pm SD and analyzed using Student *t*-tests. Immunohistochemical results were analysed using the Kruskal-Wallis test and the Mann-Whitney test. The differences in positive rates were evaluated by Fisher's exact test. All statistical analyses were carried out using the SPSS statistical software package (version 11.0, SPSS Inc.). $P < 0.05$ was considered statistically significant.

RESULTS

Expression of AhR in gastric cancer and pre-malignant tissues

RT-PCR and Western blotting were performed to

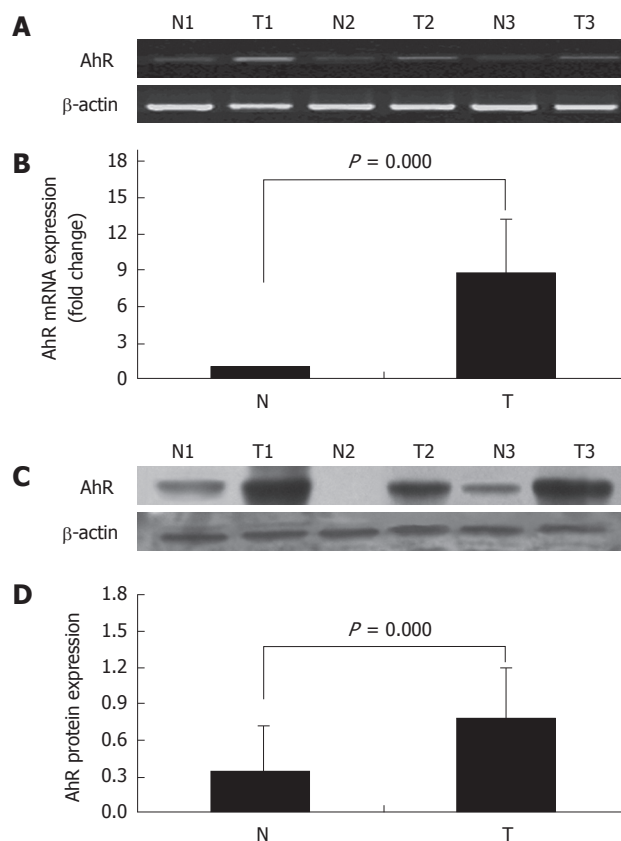


Figure 1 AhR mRNA and protein expression in GC tissues (T) and their corresponding adjacent non-cancerous tissues (N). A: AhR mRNA was detected by RT-PCR; B: AhR mRNA was detected by real-time PCR; C and D: AhR protein expression was detected by Western blotting and band intensities of AhR were normalized with corresponding band intensities of β -actin. A and C represent three cases; B and D summarize the overall mRNA and protein expression levels of AhR in all 39 cases.

analyze AhR mRNA and protein expression in 39 GC tissues and their corresponding adjacent non-cancerous tissues. Five GC cell lines (MKN28, MKN45, AGS, N87, and KATO III) were also analyzed. Compared with non-cancerous tissues both AhR mRNA and protein expression were significantly increased in cancer tissues. The AhR mRNA positive rate was significantly higher in GC tissues compared with their corresponding adjacent non-cancerous tissues (92.31%, 36/39 *vs* 66.67%, 26/39, $\chi^2 = 7.863$; $P = 0.005$). Quantitative real-time PCR and Western blotting results indicated that both AhR mRNA (Figure 1A and B) and protein levels (Figure 1C and D) in cancer tissues were significantly higher than levels in corresponding adjacent non-cancerous tissues ($P < 0.01$). AhR expression was high in MKN28, MKN45, AGS and KATO III cells, but very weak in N87 cells (Figure 2A-D). The five GC cell lines were derived from different sources: MKN45 and AGS were derived from poorly differentiated primary carcinoma of the stomach; MKN28 was derived from a moderately differentiated primary gastric carcinoma; N-87 was derived from a liver metastasis of a well-differentiated carcinoma; KATO-III was derived from metastasis of gastric carcinoma. Tumor stage did not appear to correlate with the level of AhR expression.

Expression of AhR was further detected by

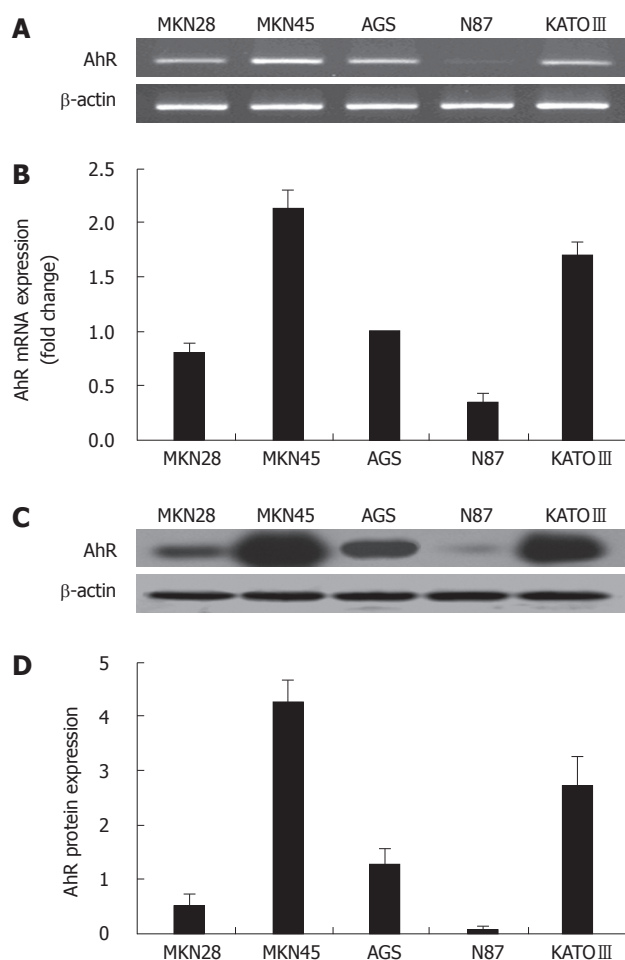


Figure 2 AhR mRNA and protein expression in five GC cell lines. A: AhR mRNA was detected by RT-PCR; B: AhR mRNA was detected by real-time PCR, AhR mRNA of AGS cells was used as the experimental control to calculate the fold changes; C and D: AhR protein expression was detected by Western blotting and band intensities of AhR were normalized with corresponding band intensities of beta-actin.

immunohistochemistry in 190 GC and pre-malignant gastric tissues: 30 CSG, 30 CAG, 30 IM, 30 AH, and 70 GC. Among the 70 GC patients, 38 suffered from Lauren diffuse type and 32 had intestinal type GC^[25]. There were no significant differences in gender or age in the different groups in this study population ($P = 0.095$) (Table 1). Strong nuclear expression and weak cytoplasmic distribution of AhR were observed in epithelial cells of both GC and pre-malignant tissues. Interestingly, AhR expression was also found in some stroma cells of both GC and pre-malignant tissues (Figure 3). The levels of AhR expression gradually increased, with the lowest levels in CSG, followed by CAG, IM, AH and GC (Table 3). Considering the fact that AhR needs to move into the nucleus to trigger expression of its target gene, evaluation of nuclear translocation of AhR may be of more importance than assessing the overall AhR expression in both the nucleus and cytoplasm. Therefore, we further calculated nuclear expression of AhR in GC and pre-malignant tissues. As with overall AhR expression, nuclear translocation of AhR also showed an increasing trend, with the lowest expression in CSG, followed by CAG,

Table 3 Expression of AhR in gastric cancer and pre-malignant tissues

Histology type	Patient number	AhR expression				AhR positive rate (%)
		-	+	++	+++	
CSG	30	16	1	13	0	46.67
CAG	30	8	7	15	0	73.33
IM	30	7	8	15	0	76.67
AH	30	5	5	18	2	83.33
GC	70	2	7	35	26	97.14 ¹
i-GC	32	1	3	16	12	96.88
d-GC	38	1	4	19	14	97.37

¹Compared with CSG, CAG, IM and AH, $P < 0.05$.

Table 4 Nuclear translocation of AhR in gastric cancer and pre-malignant tissues

Histology type	Patient number	Nuclear expression of AhR				AhR nuclear positive rate (%)
		-	+	++	+++	
CSG	30	20	1	9	0	33.33
CAG	30	14	5	11	0	53.33
IM	30	13	6	11	0	56.67
AH	30	5	5	18	2	83.33 ²
GC	70	4	6	34	26	94.29 ¹
i-GC	32	2	3	15	12	93.75
d-GC	38	2	3	19	14	94.74

¹Compared with CSG, CAG, IM and AH, $P < 0.05$. ²Compared with CSG, CAG and IM, $P < 0.05$.

IM, AH and GC (Table 4). Both AhR expression and nuclear translocation were significantly higher in GC than in precancerous tissues. There were no significant differences in AhR expression and nuclear translocation between diffuse type (d-GC) and intestinal type gastric cancers (i-GC) (Tables 3 and 4).

Effects of AhR signal pathway activation in AGS GC cell line

To investigate the potential role of the AhR signal pathway in gastric carcinogenesis, we first treated the GC cell line AGS with a specific AhR agonist, TCDD. CYP1A1, a classic target gene of AhR, was utilized as the indicator of AhR signal pathway activation. Although a baseline level of CYP1A1 expression was observed in AGS cells, RT-PCR and Western blot analysis showed that both CYP1A1 mRNA and protein expression in AGS cells were increased in a dose- and time-dependent manner following TCDD treatment (Figure 4A-D). After TCDD treatment, while CYP1A1 protein expression increased, AhR protein in the total cell lysates gradually decreased (Figure 4C and D). To further confirm the activation of the AhR signal pathway in gastric carcinogenesis, we treated AGS cells with a specific AhR antagonist, resveratrol^[28,29]. Controls included AGS cells treated with DMSO only. Experimental samples included AGS cells treated with resveratrol (10 $\mu\text{mol/L}$) only or TCDD (1 nmol/L) plus different concentrations of resveratrol (0, 1, 5, 10, 20 $\mu\text{mol/L}$), respectively for 24 h (Figure 5). In concordance with previous results, treatment of AGS cells with 1 nmol/L TCDD caused a

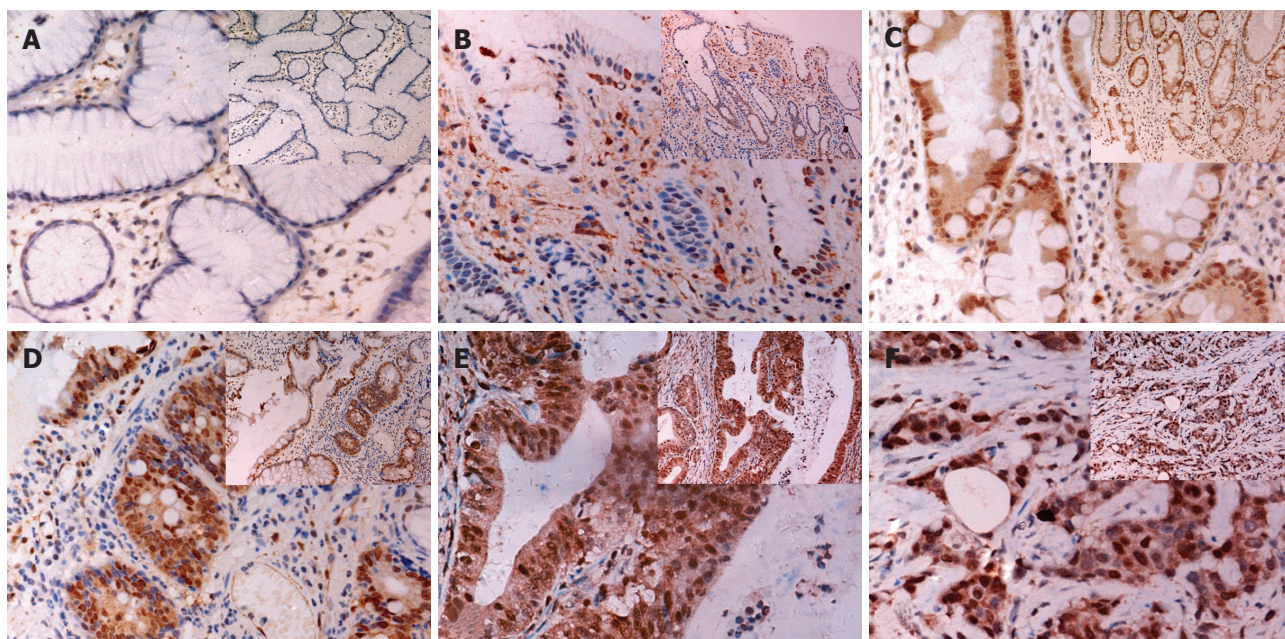


Figure 3 Immunohistochemical staining of AhR in gastric tissues. A: CSG; B: CAG; C: IM; D: AH; E: i-GC; F: d-GC (Original magnification $\times 400$ and $\times 200$). Strong nuclear expression and weak cytoplasmic distribution of AhR were observed in epithelial cells and some stroma cells of both GC and pre-malignant tissues.

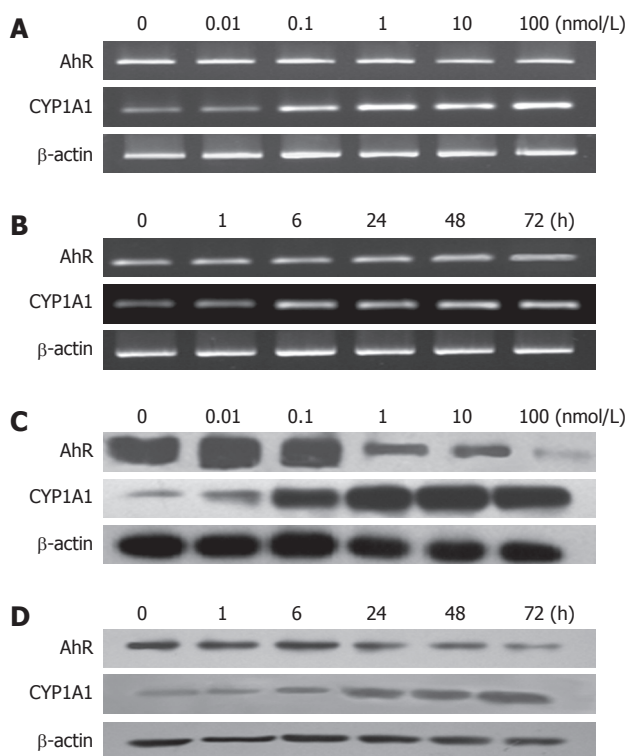


Figure 4 AhR and CYP1A1 expression in AGS cells after TCDD treatment. A and B: RT-PCR; C and D: Western blotting. Treatment of AGS cells with specific AhR agonist TCDD resulted in a dose- (A and C) and time-dependent (B and D) induction of CYP1A1 expression. The results shown are representative of three independent experiments.

remarkable increase in CYP1A1 expression. However, this TCDD-induced CYP1A1 expression was partially reversed by resveratrol in a dose-dependent manner (Figure 5A and B).

Effects of AhR activation by TCDD on the

proliferation, cell cycle and apoptosis of AGS cells were further analyzed by MTT assay and flow cytometry. MTT assay demonstrated that the viability of AGS cells was significantly decreased in a dose- and time-dependent manner after TCDD treatment (Figure 6). Flow cytometric analysis demonstrated that TCDD caused a dose-dependent alteration in the cell cycle distribution of AGS cells 48 h after treatment. TCDD increased the proportion of cells in the G1 phase and correspondingly decreased the proportion in the S phase of the cell cycle. The proportion of cells in the G2 phase showed no significant change after TCDD treatment (Table 5, Figure 7). However, apoptosis of AGS cells was unable to be detected in this assay (Table 6, Figure 8). Thus, these results suggest that TCDD inhibits proliferation of AGS cells via induction of growth arrest at the G1-S phase.

DISCUSSION

AhR is an evolutionarily conserved ligand-activated transcription factor bound and activated by ubiquitous environmental pollutants. Historically, AhR has been studied for its transcriptional regulation of genes encoding xenobiotic metabolizing enzymes such as cytochrome P450 enzymes, which metabolize many of these chemicals into mutagenic and toxic intermediates. Therefore, it has been suggested that AhR may play a role in oncogenic processes, especially those initiated by environmental carcinogens^[11-13]. Environmental carcinogens such as PAHs and HAHs are well-known exogenous AhR ligands that play important roles in GC^[5,6]. In addition to synthetic and environmental chemicals, numerous naturally occurring dietary and endogenous AhR ligands have also been identified

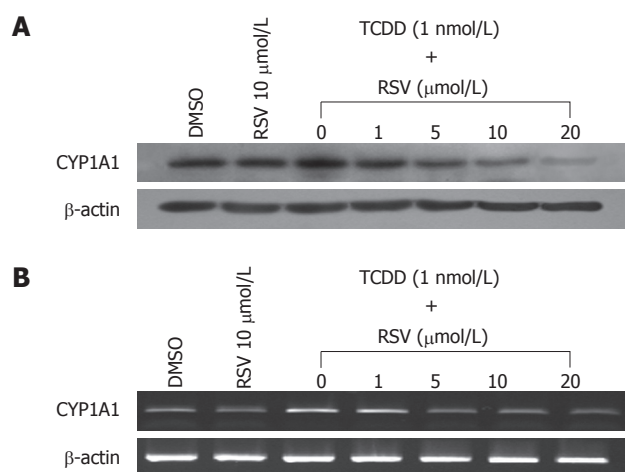


Figure 5 Inhibition of TCDD-induced CYP1A1 mRNA and protein expression by resveratrol. A: CYP1A1 protein was detected by Western blotting; B: CYP1A1 mRNA was detected by RT-PCR. The results shown are representative of three independent experiments. Treatment of AGS cells with 1 nmol/L TCDD caused a remarkable increase in CYP1A1 expression. This TCDD-induced CYP1A1 expression was partially reversed by resveratrol in a dose-dependent manner.

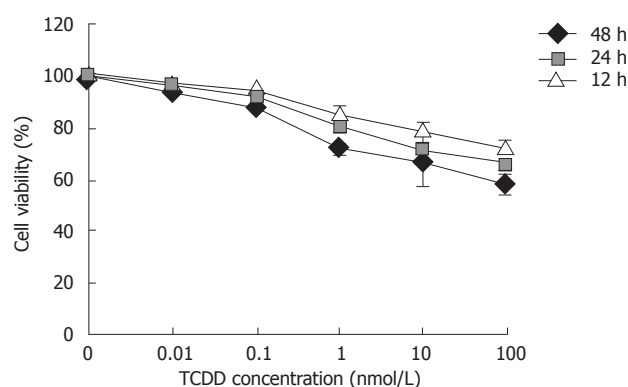


Figure 6 Viability of AGS cells after TCDD treatment was assessed by MTT assay. Viability of AGS cells was significantly decreased in a dose-dependent manner after TCDD treatment.

recently^[8,10]. Since gastric epithelium may be constantly exposed to both exogenous and endogenous AhR ligands, it would be of significance to shed light on the essential role of AhR in gastric tumorigenesis. Andersson *et al.*^[19-21] first suggested that constitutively activated AhR could induce stomach tumors in a transgenic mouse model. In our previous study, we found increased expression of AhR in two human GC cell lines (RF1 and RF48) using microarray analysis^[22]. A recent study by Ma *et al.*^[23] reported that concurrent expression of AhR and CYP1A1 is correlated with GC development. However, the role of AhR in human gastric tumorigenesis is still unclear.

In the current study, we first detected AhR mRNA and protein expression in 39 GC tissues and five GC cell lines using RT-PCR and Western blot analysis. Compared with their corresponding adjacent non-cancerous tissues, both AhR mRNA and protein expression were significantly increased in cancer tissues. Moreover, significantly different AhR levels in GC

Table 5 The effect of TCDD on AGS cell cycle

TCDD concentration (nmol/L)	Percentage of cell cycle (%)		
	G ₀ /G ₁	S	G ₂ /M
Control	54.47 ± 0.45	39.10 ± 1.39	6.43 ± 1.48
0.01	60.47 ± 3.11 ^a	33.20 ± 2.51	6.33 ± 1.12
0.1	66.07 ± 0.80 ^b	28.67 ± 3.08 ^b	5.33 ± 2.34
1	67.53 ± 2.57 ^b	25.73 ± 4.56 ^b	6.73 ± 2.06
10	67.20 ± 4.33 ^b	25.03 ± 5.31 ^b	7.77 ± 1.99
100	68.57 ± 5.57 ^b	25.10 ± 7.41 ^b	6.33 ± 1.96

Values given are the mean ± SD of three independent experiments. ^a*P* < 0.05, ^b*P* < 0.01, compared with respective control value.

Table 6 The effect of TCDD on AGS cell apoptosis

TCDD concentration (nmol/L)	Sub-G ₁	<i>P</i>
Control	8.33 ± 1.59	
0.01	9.10 ± 2.46	0.583
0.1	8.20 ± 1.65	0.924
1	7.97 ± 0.31	0.792
10	6.30 ± 1.71	0.161
100	9.57 ± 1.52	0.382

Values of Sub-G₁ given are the mean ± SD of three independent experiments.

cell lines from different derivations suggest that AhR expression may not be correlated with tumor stage. Since the development of human GC is a multi-step process, we further detected the expression and distribution of AhR using immunohistochemistry in a series of GC and pre-malignant gastric tissues. Ma *et al.*^[23] performed similar examinations in their study. However, their study included only 39 GC tissues, 17 pre-malignant tissues, and six non-cancerous mucosa samples which were detected using immunohistochemistry. In addition, atypical hyperplasia, the most important pre-malignant histology type, was not included in their study. The small sample size in that study may not accurately reflect the real expression pattern of AhR in GC and pre-malignant tissues. In our study, we included a larger sample size and included 30 atypical hyperplasia tissues. Similar to the findings of Ma *et al.*^[23], our data also demonstrated a close correlation of AhR with tumor formation via enhanced expression levels and frequent nuclear translocation from pre-malignant lesions to GC. Significantly increased nuclear translocation of AhR was found even early in AH (Table 4). There were no significant differences in AhR expression and nuclear translocation between i-GC and d-GC. Our findings suggest that activation of AhR signaling may be an early event in gastric carcinogenesis. Interestingly, besides strong expression of AhR in epithelial cells, AhR expression was also found in some stroma cells of both GC and pre-malignant tissues. Trombino *et al.*^[30] reported similar findings in their study of mammary tumorigenesis. Using a rat model of PAH-induced mammary tumorigenesis, they demonstrated that AhR expression levels were significantly elevated in PAH-induced mammary tumors as well as in stroma elements surrounding these tumors. Since stroma

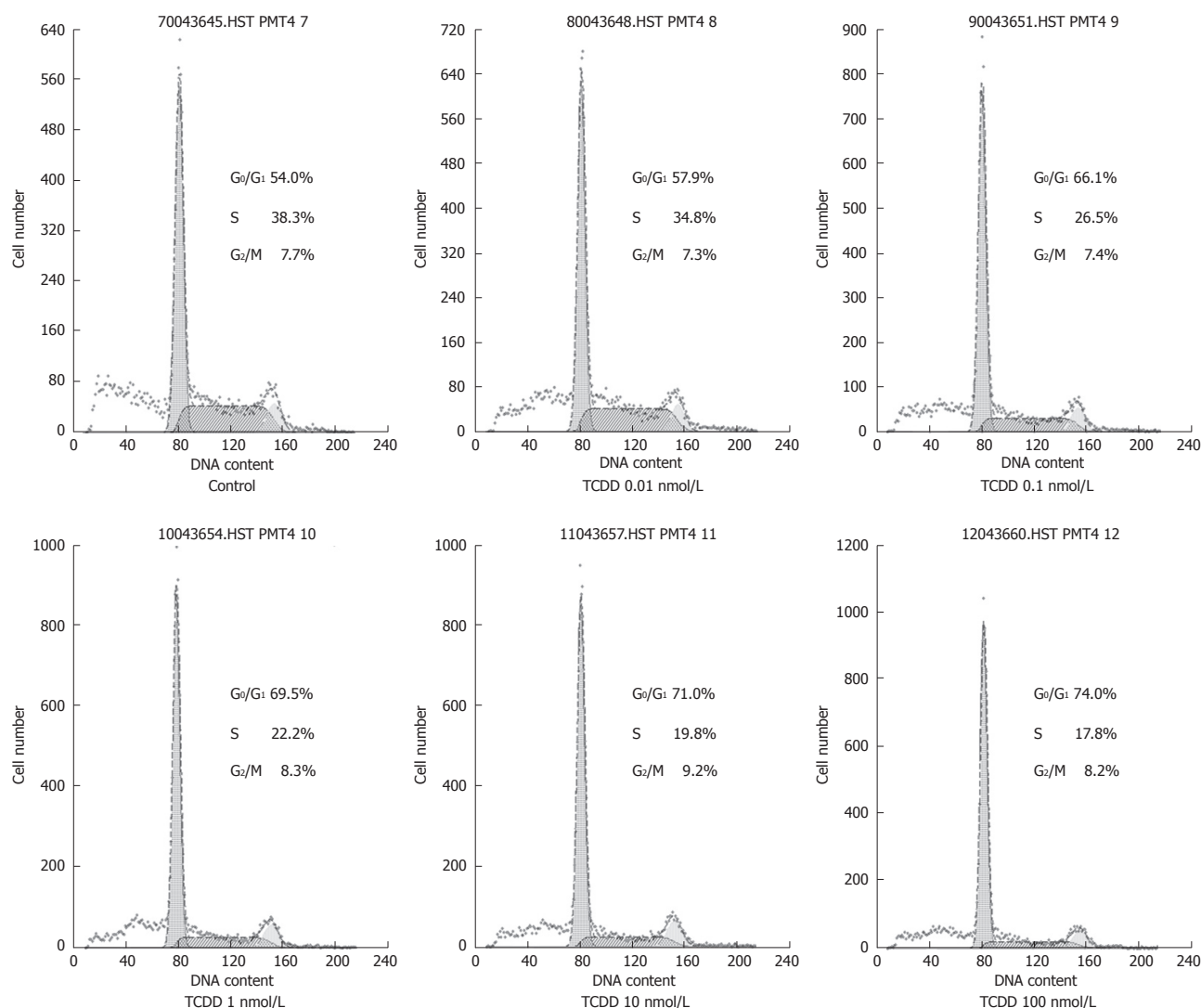


Figure 7 The effect of TCDD on AGS cell cycle distribution. AGS cells were treated with different concentrations of TCDD and subjected to flow cytometric analysis. The percentage of each phase is indicated in each panel. The results shown are representative of three independent experiments.

elements play important roles in maintaining the microenvironment and regulating growth of epithelial cells, expression of AhR in stroma cells may have a bearing on malignant transformation of gastric epithelial cells.

To further investigate the potential role of the AhR signal pathway in gastric carcinogenesis, we treated GC cell line AGS with the most potent AhR agonist, TCDD, and chose CYP1A1, a classic target gene of AhR, as the indicator of AhR signal pathway activation. Although both CYP1A1 and CYP1B1 are classic target genes of AhR, cell-specific expression of these two genes have been reported previously^[30-32]. Over-expression of CYP1A1, but not CYP1B1 in GC has been reported by Ma *et al*^[23] and Zhang *et al*^[33]. Baseline levels of CYP1A1 expression were also observed in AGS cells in the present study. However, expression of CYP1A1 was significantly increased in a dose- and time-dependent manner after TCDD treatment, indicating the activation of AhR. Interestingly, while CYP1A1 protein expression increased, AhR protein in the total cell lysates gradually decreased (Figure 4C and D). Similar phenomena have

been reported by several other groups^[34-36]. Recent studies have demonstrated that the down-regulation of AhR following ligand binding is ubiquitin mediated and occurs *via* the 26S proteasome pathway following nuclear export of AhR. The degradation of AhR is the endpoint and would be one of the key factors controlling gene regulations by the AhR signal pathway^[37]. To confirm the activation of the AhR signal pathway by TCDD, we treated AGS cells with a specific AhR antagonist, resveratrol. Previous studies suggested that resveratrol can regulate the transcription of AhR targeted genes by preventing AhR from binding to the enhancer sequences of the gene promoter^[28,29]. Our results showed that TCDD-induced CYP1A1 expression was partially reversed by resveratrol in a dose-dependent manner. The incomplete reversal of CYP1A1 expression by resveratrol may be due to the fact that AhR is not the only regulator of CYP1A1 transcription^[38,39]. Taken together, these results suggest that the AhR signal pathway could be activated in GC cells and that abnormal activation of the AhR signal pathway may be involved in gastric carcinogenesis.

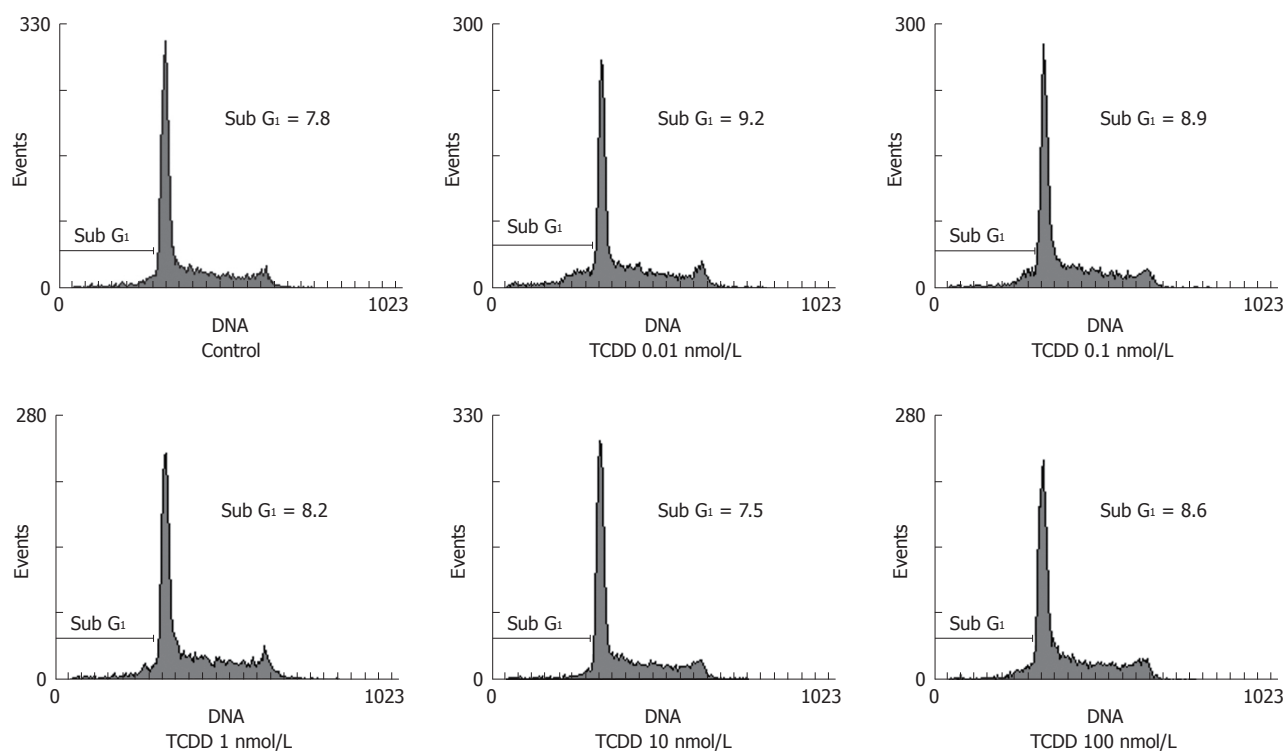


Figure 8 The effect of TCDD on apoptosis of AGS cells. AGS cells were treated with different concentrations of TCDD and subjected to flow cytometric analysis. Cellular apoptosis was evaluated by fragmented DNA (sub-G₁) analysis using winMDI2.9. The results shown are representative of three independent experiments.

Since AhR is significantly up-regulated in GC and may be involved in the early stage of gastric carcinogenesis, regulation of the AhR pathway may have a potential role in the treatment of GC. Interestingly, our MTT assay demonstrated that the viability of AGS cells was significantly decreased in a dose- and time-dependent manner after TCDD treatment. Further flow cytometric analysis indicated that TCDD inhibited growth of AGS cells *via* induction of growth arrest at the G₁-S phase. As far as we know, this is the first report suggesting an inhibitory role of AhR agonists on human GC cell growth. Similar results have been reported in the treatment of pancreatic cancer and mammary tumors by AhR agonists^[16,18,40]. However, previous studies by Andersson *et al.*^[19,20] showed that constitutively active AhR may result in significant proliferation in the parietal/chief cell region of glandular gastric mucosa in transgenic mice. These contradictory outcomes indicate that AhR appears to contribute to processes in both cell cycle arrest as well as cell proliferation. Recent studies on the cellular signal pathway may partly explain this complex phenomenon. As an evolutionarily conserved transcription factor, outside its well-characterized role in the induction of xenobiotic metabolizing enzymes, AhR also functions as a modulator of cellular signaling pathways. By interacting with different signal pathway effectors, AhR activation may result in completely different effects on cell growth^[12,13]. Our present findings on the inhibitory effect of TCDD on GC cell growth suggest that AhR may be a potential therapeutic target for gastric cancer.

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COMMENTS

Background

The carcinogenesis of gastric cancer (GC) involves numerous genetic and epigenetic alterations, as well as many environmental risk factors. Environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and halogenated hydrocarbons (HAHs) are well-known carcinogens that play important roles in GC development. The toxic effects of PAHs and HAHs are mediated by a conserved signaling pathway that binds and activates the aryl hydrocarbon receptor (AhR).

Research frontiers

AhR is a ligand-activated transcription factor and can mediate the carcinogenic and other toxic effects of a variety of environmental pollutants. Many studies in recent years have demonstrated a close relationship between AhR and tumorigenesis. However, the role of AhR in gastric tumorigenesis is still unclear. In this study, the authors demonstrate a close correlation of AhR with GC formation and the potential role of AhR as a therapeutic target for GC treatment.

Innovations and breakthroughs

AhR functions as a modulator of cellular signaling pathways. By interacting with different signal pathway effectors, AhR activation may result in completely different effects on cell growth. This is the first report suggesting an inhibitory role of AhR agonists on human GC cell growth. Furthermore, the present findings suggest that AhR may be a potential therapeutic target for GC.

Applications

By understanding how cell growth of human GC is influenced by AhR activation, this study may represent a future strategy for therapeutic intervention in the treatment of patients with GC.

Terminology

AhR is a ligand-activated transcription factor of the basic helix-loop-helix/Per-

Arnt-Sim family. As an evolutionarily conserved transcription factor, outside its well-characterized role in the induction of xenobiotic metabolizing enzymes, AhR also functions as a modulator of cellular signaling pathways.

Peer review

The authors investigated the functional significance of AhR in gastric carcinogenesis. This paper is interesting and written well.

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ORIGINAL ARTICLES

Effect of Bu-Zhong-Yi-Qi-Tang on deficiency of N-glycan/nitric oxide and islet damage induced by streptozotocin in diabetic rats

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Abstract

AIM: To investigate the effect of Bu-Zhong-Yi-Qi-Tang (Decoction for Reinforcing Middle Jiao and Replenishing Qi) on deficiency of N-glycan/nitric oxide (NO) and islet damage induced by injecting two medium doses of streptozotocin (STZ).

METHODS: Diabetes was induced by intraperitoneal injection of STZ at 55 mg/kg on day 1 and day 8. Islet damage was evaluated using a scoring system. Nitrite, nitrate, α -mannosidase and amylase activities were measured by colorimetry. N-glycan patterns of amylase were determined with lectin [ConA, pisum sativum agglutinin (PSA), peanut agglutinin (PNA), and lens culinaris agglutinin (LCA)] affinity precipitation method.

RESULTS: Severe islet necrosis and mild islet atrophy were observed in diabetic rats. The number and size of islets, the activities of α -mannosidase, amylase and nitrite were decreased, while the binding of PNA and LCA to amylase was increased. All of which were improved after treatment with Bu-Zhong-Yi-Qi-Tang. Islet damage was significantly correlated with nitrite, nitrate, α -mannosidase, amylase and the binding of LCA, PNA, and PSA to amylase.

CONCLUSION: STZ-induced islet damage is related to N-glycan deficiency in proteins by blocking α -mannosidase activity and no deficiency, accumulation of unfolded proteins, and endoplasmic reticulum stress and activation of cellular signals, all of which are improved after treatment with Bu-Zhong-Yi-Qi-Tang.

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Key words: N-Glycan; Nitric oxide; Diabetic rats; Islet damage; Alpha mannosidase; Bu-Zhong-Yi-Qi-Tang

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Liu XQ, Wu L, Guo XJ. Effect of Bu-Zhong-Yi-Qi-Tang on deficiency of N-glycan/nitric oxide and islet damage induced by streptozotocin in diabetic rats. *World J Gastroenterol* 2009; 15(14): 1730-1737 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1730.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1730>

INTRODUCTION

N-glycan plays an important role in the quality control (QC) of glycoprotein folding both in endoplasmic reticulum (ER) lumen and in ER-associated degradation (ERAD) of proteins by cytosolic proteasomes^[1]. Alpha-mannosidase, a key enzyme converting precursor high-mannose-type N-glycans to matured complex-type structures, contributes to the establishment of an equitable glycoprotein quality control standard, by which the efficiency of Asn-linked glycoprotein conformational maturation results in dislocation of misfolded glycoproteins into the cytosol, where proteins are degraded in proteasomes and maintain the homeostasis of ER^[2].

The unfolded protein response (UPR) is a conserved cellular response designed to alleviate damage and promote survival of cells experiencing stress by either shutting down translation to reduce the protein flow into ER, or by up-regulating molecules that protect cells or clear misfolded proteins from the ER, thus alleviating ER stress. Nitric oxide (NO) is a known activator of the

UPR^[3-5], and NO-induced UPR activation may function to assist in the recovery of β -cells from NO-mediated damage, which is associated with enhanced expression of heat shock proteins and chaperones that assist in protein folding.

Islet β cells have a well-developed ER and secrete a large amount of insulin and glycoproteins. Islet β cells may be at risk of developing ER stress and increase misfolded glycoproteins. Inability to clear misfolded proteins causes early accumulation of unfolded proteins, and ER stress and activation of cellular signals lead to cell death, thus resulting in loss of β cell secretion potential and diabetes.

Xiong *et al.*^[6] showed that the pathogenesis of diabetes is closely correlated to Pixu (insufficiency of the spleen), a major pathogenic factor for diabetes syndrome. Pixu is therefore considered in the treatment of diabetes. Diabetes mellitus is caused by Pixu, when diabetes progresses, deficiency of Yin and dryness-heat exist simultaneously, and when diabetes prolongs, deficiency of Yin becomes the key pathogenesis, finally resulting in impaired Yin and Yang^[7].

The levels of nitrite and nitrate are significantly decreased in stimulated crude whole saliva of diabetic patients with Pixu^[8]. Bu-Zhong-Yi-Qi-Tang, a traditional Chinese medicine, tonifies qi, strengthens the stomach and spleen, and increases sunken Yang qi. It is prescribed mainly for Pixu by improving the deficiency of spleen and stomach qi. It was reported that the lower levels of nitrite, nitrate, and peroxynitrite in gastric mucosa of rats with Pixu can return to normal after treatment with Bu-Zhong-Yi-Qi-Tang^[9].

This study was to examine the effect of Bu-Zhong-Yi-Qi-Tang on deficiency of N-glycan/NO and islets damage in rats with diabetes mellitus induced by streptozotocin (STZ).

MATERIALS AND METHODS

Ethics

This study was approved by Guangdong Science and Technology Committee and Guangdong Management Committee for Medical Experimental Animals.

General procedure

Sprague-Dawley rats were obtained from the Laboratory Animal Research Center of Guangzhou University of Traditional Chinese Medicine. The animals were housed in a plastic cage in an air-conditioned room at $20 \pm 2^\circ\text{C}$ with a humidity of $58\% \pm 5\%$ in a 12-h light and dark cycle, with free access to standard rat food and tap water. The rats were divided into normal control, and diabetic model groups after 1 wk.

Diabetic model

A diabetic model of rats was established by intraperitoneal injection of STZ at 55 mg/kg on day 1 and day 8. The rats were bred for 4 wk. Blood was sampled from the tail vein and blood glucose was determined with the one touch ultra blood glucose monitoring system (LifeScan,

Inc. Milpitas, CA, USA). Animals with their non-fasting blood glucose less than 16.65 mmol/L were excluded from the study. The rats were divided into diabetics groups and Bu-Zhong-Yi-Qi-Tang treatment group.

Preparation and administration of Bu-Zhong-Yi-Qi-Tang

Bu-Zhong-Yi-Qi-Tang was made of *Radix Astragali*, *Codonopsis pilosula*, *Glycyrrhiza uralensis* f. *isch*, *Rhizoma Atractylodis Macrocephalae*, *Radix Angelicae Sinensis*, *Rhizoma Cimicifugae Foetidae*, *Radix Bupleuri Chinensis*, and *Pericarpium Citri Reticulatae*, which were put into a 20-fold volume of distilled water, decocted from 80°C to 100°C , filtered and concentrated at 40°C - 80°C , and stored in a refrigerator until use. Bu-Zhong-Yi-Qi-Tang was administered by gavage, 8 g/kg per day for 4 wk.

Samples

At the end of treatment, the animals were killed by exsanguination from the carotid artery. Blood samples were taken immediately and centrifuged at 3000 r/min for 5 min. Serum was separated for the measurement of nitrite, nitrate, α -mannosidase and amylase activities, and determination of the N-glycan patterns of amylase. The pancreas was removed from each rat and dissected longitudinally. One half was dried and homogenized. Supernatant was removed for the measurement of nitrite, nitrate, α -mannosidase and amylase activities, and determination of the N-glycan patterns of amylase. The other half was fixed in Bouin's solution for 24 h. The pancreatic tissue, embedded in paraffin wax, was cut into 3-4- μm -thick sections which were stained with hematoxylin and eosin (HE) for histopathological examination.

Histopathological examination of pancreatic islet damage

Bouin's-solution-fixed pancreatic tissue was cut into eight sections (approximately 1 mm \times 1 mm \times 1 mm). Four sections were randomly selected, stained with HE, and observed under a microscope with a digital camera (C170 D425, Olympus Imaging Corp). Digital micrographs were analyzed for estimating the number, size, necrosis and atrophy of islets and insulinitis by two observers unaware of the status and/or treatment modalities.

The number of islets was calculated as previously described^[10] with some minor modifications and graded as 0, no visible islets; 1, < 5 islets; 2, 6-10 islets; 3, 11-20 islets; 4, > 20 islets.

The size of islets was measured as previously described^[11] with some minor modifications and graded as 1, 1-40 cells per section; 2, 41-100 cells per section; 3, 101-200 cells per section; 4, 201-400 cells per section; 5, > 400 cells per section.

Insulinitis was evaluated by examining at least 10 islets from each treatment group and graded as 0, no mononuclear cell infiltration; 1, very mild mononuclear cell infiltration in and around the islets; 2, mild cell infiltration in and around the islets; 3, moderate cell infiltration in and around the islets; 4, marked cell infiltration observed as the structure of islets was

destroyed. Pancreas was graded for insulinitis according to the most severely involved islets in each pancreas.

Necrosis of islets was graded as 0, no islet necrosis; 1, very mild islet necrosis; 2, mild islet necrosis; 3, moderate islet necrosis; 4, marked islets necrosis.

Atrophy of islets was graded as 0, no islet atrophy; 1, very mild islet atrophy; 2, mild islet atrophy; 3, moderate islet atrophy; 4, marked islet atrophy.

Determination of nitrite and nitrate in serum and pancreas tissue

Pancreatic tissue was homogenized, supernatant and serum were initially deproteinized with Somogyi reagent^[12] (using 2.0 mL 55 mmol/L NaOH followed by 2.0 mL 75 mmol/L ZnSO₄). After centrifugation, aliquots of supernatant were mixed with an equal volume of Griess reagent^[13]. The absorbance was measured at 546 nm. Nitrite level was measured using 120 μ mol/L sodium nitrite solution diluted to 10 μ mol/L intermediate dissolution. Results were expressed as μ mol/L per gram wet tissue, and serum nitrite was expressed as μ mol/L.

Pancreatic tissue was homogenized, supernatant and serum were initially deproteinized with Somogyi reagent^[12] (using 2.0 mL 55 mmol/L NaOH followed by 2.0 mL 75 mmol/L ZnSO₄). After centrifugation, excess nitrite was eliminated with 10 mg/mL ammonium sulfamate and acidified with 1 mol/L hydrochloric acid which prevented interference with hydroxide or carbonate. Aliquots of supernatant nitrate were determined by dual-wavelength ultraviolet spectrophotometry^[14,15]. Nitrate absorbance was calculated by subtracting the absorbance at 275 nm for background correction from the absorbance at 220 nm. Nitrate level was measured using 120 μ mol/L standard sodium nitrate solution diluted to 10 μ mol/L intermediate dissolution. Results were expressed as μ mol/L per gram wet tissue, and serum nitrate was expressed as μ mol/L.

Determination of α -mannosidase activity in serum and pancreas tissue

The animals were killed by exsanguination from the carotid artery. Blood samples were taken immediately. The serum was stored at -18°C. Pancreas was removed, fragmented and stored at -20°C until required. Extracts from pancreatic tissue were prepared by homogenization in 0.05 mol/L sodium phosphate (pH 6.5) containing 5 mmol/L MgCl₂ and 0.1% Triton X-100 (a DY89-1 tissue homogenizer) at 4°C. Tissue homogenate was centrifuged at 16000 r/min for 30 min. Clear supernatant and serum were used to determine the enzyme activity. The level of α -mannosidase was measured by spectrophotometry as previously described^[16] with some minor modifications. Fifty microliters of supernatant (or serum) was added to 50 μ L 4 mmol/L p-nitrophenyl- α -D-mannopyranoside substrate (Fluka, Bucks, Switzerland) in 0.1 mol/L sodium acetate-acetic acid buffer (pH 5.8). Reaction mixtures were incubated at 37°C for 60 min. The reaction was terminated by adding

3 mL glycine-sodium hydroxide (0.1 mol/L, pH 10.14). The amount of liberated p-nitrophenol was determined by spectrophotometry at 405 nm. One international unit of enzyme was defined as the amount required for catalyzing the release of 1 μ mol p-nitrophenol from p-nitrophenyl- α -D-mannopyranoside substrate per hour at 37°C under the conditions described above. Tissue enzyme activities were expressed as U/g wet tissue, and serum enzyme activities were expressed as U/mL serum.

Determination of amylase activity and binding of lectin to amylase in serum and pancreas tissue

Extracts from pancreatic tissue were prepared by homogenization in 9 g/L NaCl (a DY89-1 tissue homogenizer) at 4°C. Tissue homogenate was centrifuged at 16000 r/min for 30 min. Clear supernatant and serum were used to determine the amylase activity and binding of lectin to amylase. Amylase N-glycan patterns were analyzed with lectin [ConA, pisum sativum agglutinin (PSA), peanut agglutinin (PNA), lens culinaris agglutinin (LCA)] affinity precipitation method. The precipitation procedure was performed as previously described^[17,18]. The clear supernatant and serum were mixed with 400 μ L of an aqueous solution of ConA, PSA, PNA, LCA (5 g/L in distilled water). The mixture was incubated for 30 min at 37°C and centrifuged at 2000 r/min for 15 min. Without disturbing the precipitate, we removed the supernatant and measured its amylase activity. Binding of lectin to amylase was calculated by subtracting the corrected value from the total amylase activity. ConA, PSA, PNA, LCA were purchased from Sigma (St. Louis, MO, USA).

Statistical analysis

All data were presented as mean \pm SD. Statistical significance was calculated using unpaired Student's *t* test. The correlation coefficient between islet histomorphometry and nitrite, nitrate, binding of lectin to amylase, activity of α -mannosidase and amylase was calculated. *P* < 0.05 was considered statistically significant. All analyses were performed using Excel 2003.

RESULTS

Body weight and blood glucose level

The body weight of normal rats increased over the 12 wk experimental period. However, diabetic rats lost 22.1% of their body weight within 12 wk, the non-fasting blood glucose level increased from 16.62 mmol/L to 31.63 mmol/L 4 wk after injection of STZ, and remained significantly elevated for 8 wk thereafter.

Histopathological changes in pancreatic islets

Relatively well documented pancreatic islets and tightly arranged islet cells were observed in the normal control group. Severe islet necrosis and mild islet atrophy were detected in the diabetic group, and improved after treatment with Bu-Zhong-Yi-Qi-Tang compared to the diabetic group (Figure 1). Histomorphometrical analysis

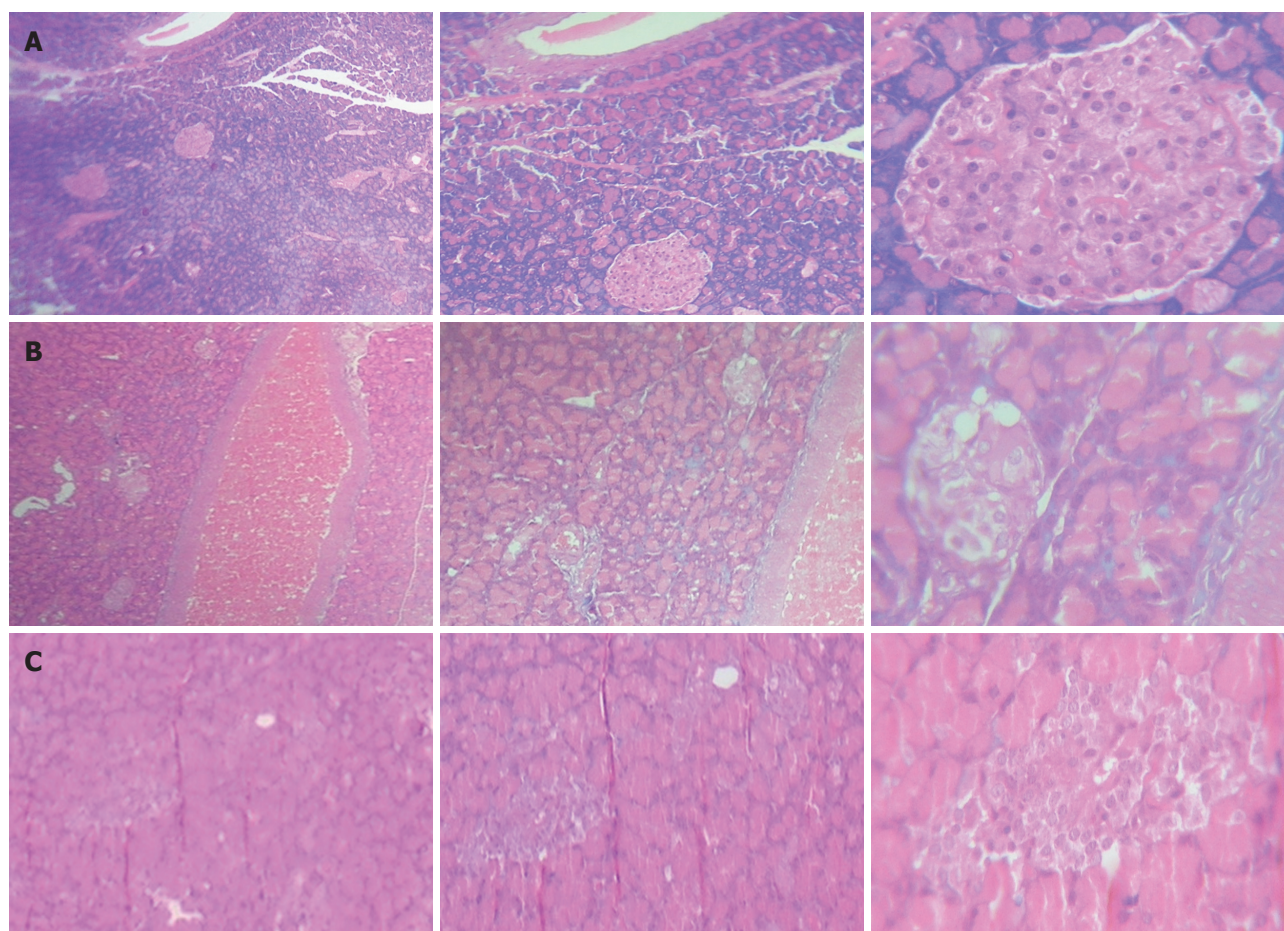


Figure 1 Histological profiles for pancreatic islets in normal rats (A), diabetic rats (B), Bu-Zhong-Yi-Qi-Tang-treated rats (C). Relatively well documented pancreatic islets and tightly arranged islet cells were observed in normal rats. Severe necrosis and mild atrophy of islets were found in diabetic rats, which were improved after treatment with Bu-Zhong-Yi-Qi-Tang. Stained sections showing islet morphology in sections stained with HE. The left region: Low-power magnification view; Middle region: Middle-power magnification view; Right region: High-power magnification view.

Table 1 Changes in histomorphometry of pancreatic islets of diabetic rats after treatment with Bu-Zhong-Yi-Qi-Tang (mean \pm SD)

Treatment	Islet number islets/pancrease section	Islet size cells/islet section	Necrosis	Atrophy	Cell infiltration
Normal rats	3.50 \pm 0.84	3.83 \pm 0.45	0	0.17 \pm 0.41	0
Diabetic rats	1.08 \pm 0.29 ^b	1.00 \pm 0.0 ^b	3.25 \pm 0.45 ^b	2.08 \pm 0.29 ^a	0
Bu-Zhong-Yi-Qi-Tang-treated rats	1.17 \pm 0.41 ^b	2.5 \pm 0.55 ^{b,d}	0.33 \pm 0.52 ^d	1.17 \pm 0.41 ^{a,d}	0

^a P < 0.05, ^b P < 0.01 *vs* normal control rats; ^d P < 0.01 *vs* diabetic rats.

Table 2 Levels of nitrite and nitrate in serum and pancreas tissue of diabetic rats after treatment with Bu-Zhong-Yi-Qi-Tang (mean \pm SD)

Treatment	Nitrite μ mol/L serum	Nitrate μ mol/L serum	Nitrite μ mol/L per gram wet tissue	Nitrate μ mol/L per gram wet tissue
Normal rats	21.33 \pm 0.25	98.78 \pm 49.20	146.70 \pm 24.77	13.29 \pm 10.73
Diabetic rats	14.00 \pm 0.19 ^b	85.76 \pm 30.08	95.60 \pm 28.89 ^b	11.59 \pm 9.54
Bu-Zhong-Yi-Qi-Tang-treated rats	25.98 \pm 0.74 ^{b,d}	114.78 \pm 30.09 ^d	132.82 \pm 54.03 ^d	21.30 \pm 18.73

^a P < 0.05, ^b P < 0.01 *vs* normal rats; ^d P < 0.01 *vs* diabetic rats.

showed that the number and size of islets were significantly decreased, and the necrosis and atrophy of islets were significantly exacerbated in the diabetic group compared to the normal control group. However, the size of islets was significantly increased, and the necrosis and atrophy of islets were significantly improved in the Bu-Zhong-Yi-Qi-Tang treatment group compared to the diabetic group (Table 1).

Levels of nitrite and nitrate in serum and pancreas tissue

The levels of nitrite and nitrate in serum and pancreas tissue of the diabetic group were significantly lower than those in the normal control group, and significantly increased in the Bu-Zhong-Yi-Qi-Tang treatment group compared to the diabetic group (Table 2).

Table 3 Activity of α -mannosidase and amylase in serum and pancreas tissue of diabetic group after treatment with Bu-Zhong-Yi-Qi-Tang (mean \pm SD)

Treatment	Serum enzyme activities (U/mL serum)		Tissue enzyme activities (U/g wet tissue)	
	α -mannosidase	Amylase	α -mannosidase	Amylase
Normal rats	3.60 \pm 2.43	28.71 \pm 11.07	81.78 \pm 30.34	19.53 \pm 3.84
Diabetic rats	3.17 \pm 7.07	17.35 \pm 13.76 ^a	20.26 \pm 20.42 ^b	13.15 \pm 0.98 ^a
Bu-Zhong-Yi-Qi-Tang-treated rats	1.53 \pm 1.72	25.14 \pm 8.76 ^c	44.79 \pm 26.93 ^{b,d}	17.94 \pm 2.20 ^d

^a $P < 0.05$, ^b $P < 0.01$ vs normal rats; ^c $P < 0.05$, ^d $P < 0.01$ vs diabetic rats.

Table 4 Binding of lectin to amylase in serum of diabetic group after treatment with Bu-Zhong-Yi-Qi-Tang (mean \pm SD)

Treatment	ConA-binding% (O-binding%)	LCA-binding% (O-binding%)	PNA-binding% (O-binding%)	PSA-binding% (O-binding%)
Normal rats	22.18 \pm 42.27 (71.43)	2.61 \pm 6.78 (85.71)	3.89 \pm 9.92 (85.71)	6.52 \pm 14.22 (78.57)
Diabetic rats	31.18 \pm 43.91 (58.33)	27.84 \pm 44.15 ^a (58.33)	32.86 \pm 41.01 ^a (50.0)	25.0 \pm 45.23 (75.0)
Bu-Zhong-Yi-Qi-Tang-treated rats	1.20 \pm 3.62 ^{a,c} (87.50)	16.41 \pm 34.06 (75.0)	1.78 \pm 5.0 ^c (87.50)	9.09 \pm 16.44 (62.50)

^a $P < 0.05$ vs normal rats; ^c $P < 0.05$ vs diabetic rats.

Table 5 Binding of of lectin to amylase in pancreas tissue of diabetic group after treatment with Bu-Zhong-Yi-Qi-Tang (mean \pm SD)

Treatment	ConA-binding% (O-binding%)	LCA-binding% (O-binding%)	PSA-binding% (O-binding%)
Normal rats	23.53 \pm 27.83 (40.0)	7.06 \pm 15.78 (80.0)	20.0 \pm 44.72 (80.0)
Diabetic rats	20.0 \pm 35.46 (62.5)	47.30 \pm 40.53 ^a (12.5)	14.87 \pm 27.68 (75.0)
Bu-Zhong-Yi-Qi-Tang-treated rats	16.82 \pm 31.18 (50.0)	35.91 \pm 37.08 ^a (30.0)	22.0 \pm 41.46 (50.0)

^a $P < 0.05$ vs normal rats.

Activity of α -mannosidase and amylase in serum and pancreas tissue

The activity of α -mannosidase and amylase in pancreas tissue in the diabetic group was significantly lower than that in the normal control group. However, the serum α -mannosidase activity was not significantly different between the two groups. The activity of α -mannosidase and amylase in pancreas tissue was significantly increased in the Bu-Zhong-Yi-Qi-Tang treatment group compared to the diabetic group (Table 3).

Binding of lectin to amylase in serum and pancreas tissue

Binding of LCA, and PNA to serum amylase and binding of LCA to pancreas amylase in the diabetic group were significantly higher than those in the normal control group, while the binding of ConA and PSA to amylase was not significantly different between the two groups. After treatment with Bu-Zhong-Yi-Qi-Tang, the binding of ConA, PNA and LCA to amylase was significantly lower than that in the diabetic group (Tables 4 and 5).

Correlation between the number, size, necrosis and atrophy of islets and the activity of α -mannosidase, amylase, nitrite, nitrate, and binding of lectin to amylase

The number, size, necrosis and atrophy of islets were significantly correlated with the activity of nitrite, nitrate, α -mannosidase, amylase and binding of LCA, PNA, and PSA to amylase (Table 6).

DISCUSSION

In the present study, severe necrosis and mild atrophy of islets were observed in diabetic rats. The number and size of islets. The activity of α -mannosidase and amylase were decreased, and the binding of PNA and LCA to amylase were increased. All of which were improved after treatment with Bu-Zhong-Yi-Qi-Tang.

Islet damage was found to be related to the levels of nitrite, nitrate, α -mannosidase and amylase, and the binding of LCA, PNA and PSA to amylase.

Trimannosyl oligosaccharide is a unique moiety recognized by concanavalin A lectin (ConA) for high affinity and extended site binding. ConA primarily binds to the outer trimannosyl region of high mannose and bisected hybrid-type glycopeptides rather than to the central trimannosyl region of complex glycopeptides^[19]. Fucosyl residues in the outer chain moieties have a shielding effect on the neighboring α -mannosyl residues and can eliminate this type of interaction^[20]. PNA is a lectin with a high binding affinity for galactose-galactosamine disaccharide^[21]. NeuAc caps the galactose-terminated chains^[22]. Like ConA, LCA is able to bind (or not bind) to the same glycopeptides. LCA additionally requires the fucosyl- N-glycan-asparaginyl core for high-affinity binding. The presence of a core fucose residue greatly enhances recognition of N-linked sugar chains by LCA. Exposure of terminal N-acetylglucosamine (GlcNAc) residues to glycopeptides can enhance the binding of glycopeptide to LCA^[23]. Like ConA, PSA is able to bind (or not bind) to the same glycopeptides. PSA

Table 6 Correlation between the number, size, necrosis and atrophy of islets and levels of α -mannosidase, amylase, nitrite and nitrate and binding of lectin to amylase

Biochemistry index	<i>n</i>	Islet number	Islet size	Necrosis	Atrophy
Serum nitrite	50	0.404 ^b	0.736 ^b	-0.319 ^b	-0.866 ^b
Serum nitrate	50	0.181	0.194	-0.126	-0.245 ^a
Pancreas tissue nitrite	50	0.30 ^a	0.456 ^b	-0.222	-0.480 ^b
Pancreas tissue nitrate	50	0.101	0.157	-0.041	-0.317 ^b
LCA-binding of amylase in serum	61	-0.254 ^a	-0.290 ^a	0.077	0.228 ^a
ConA-binding of amylase in serum	61	-0.048	-0.10	-0.011	-0.016
PNA-binding of amylase in serum	61	-0.114	-0.261 ^a	0.283 ^a	0.041
PSA-binding of amylase in serum	61	-0.143	-0.26 ^a	0.165	0.153
LCA-binding of amylase in pancreas tissue	43	-0.442 ^b	-0.253 ^a	0.174	0.183
ConA-binding of amylase in pancreas tissue	43	-0.117	-0.095	-0.192	0.232
PSA-binding of amylase in pancreas tissue	43	-0.095	-0.037	-0.288 ^a	0.106
The activity of α -mannosidase in serum	61	0.067	0.050	0.026	-0.108
The activity of α -mannosidase in pancreas tissue	43	0.638 ^b	0.747 ^b	-0.508 ^b	-0.714 ^b
The activity of amylase in serum	61	0.158	0.365 ^b	-0.215 ^a	-0.310 ^b
The activity of amylase in pancreas tissue	43	0.428 ^b	0.639 ^b	-0.314 ^a	-0.631 ^b

^a*P* < 0.05, ^b*P* < 0.01, test of significance of correlation coefficient.

additionally requires the fucosyl-N-glycan-asparaginyl core for high affinity binding. The presence of a core fucose residue greatly enhances recognition of N-linked sugar chains by PSA. Exposure of terminal mannose residues to glycopeptides can enhance the binding of glycopeptide to PSA^[23].

These findings indicate that N-glycan processing is deficient in diabetic rats. Amylase core-fucosylate, high-mannose-type, hybrid-type sugar chains are increased while terminal sialic acid and fucose on the sugar chain are decreased, thus resulting in exposure of terminal galactose and GlcNAc residues to PNA and LCA.

N-glycan plays an important role in the quality control of glycoprotein folding in lumen and ERAD of proteins by cytosolic proteasomes^[1]. Alpha-mannosidase, a key enzyme converting precursor high-mannose-type N-glycan to matured complex-type structure, contributes to the establishment of an equitable glycoprotein quality control standard, by which the efficiency of Asn-linked glycoprotein conformational maturation results in dislocation of misfolded glycoproteins into the cytosol where proteins are degraded in the proteasome and maintain the homeostasis of ER^[2].

Lin *et al*^[24] cultured neurons derived from embryonic chicken brains with tunicamycin (TM), an inhibitor of N-linked glycosylation, and found that the light neurons resembled necrotic cells, but the dense neurons exhibited distinct morphological features of necrosis and apoptosis, indicating that TM has an irreversible toxicity to the neurons and a different mechanism underlying neuron death.

Finnie *et al*^[25] showed that cultured rat hepatocytes exposed to TM have degenerative changes characterized by marked cytoplasmic lipid accumulation and dilatation of cisternae of rough ER or necrosis.

Shi *et al*^[26,27] reported that inhibition of 6A8 alpha-mannosidase causes oncosis-like death of BJAB cells with no apoptotic bodies, annexin-V staining DNA fragmentation assay cannot show any evidence of apoptosis in these cells. However, binding of ConA

to the cells transduced with the antisense 6A8 DNA is increased, but ConA-binding to the cells transduced with the sense 6A8 DNA is decreased.

The UPR is a conserved cellular response designed to alleviate damage and promote survival of cells experiencing stress by either shutting down translation to reduce the protein flow into the ER or by up-regulating molecules that protect cells or clear misfolded proteins from the ER and alleviate stress. NO, a known activator of UPR^[3-5], may function to assist in the recovery of β -cells from NO-mediated damage, which is associated with enhanced expression of heat shock proteins and chaperones that assist in protein folding.

Islet β cells have a well-developed ER, reflecting their role in secreting a large amount of insulin and glycoproteins. Islet β cells may be at risk for ER stress. Inability to clear misfolded proteins causes accumulation of unfolded proteins, ER stress and activation of cellular signals, leading to cell death, loss of β cell secretion potential, and diabetes.

These findings indicate that STZ induces islet damage, which might be related to the N-glycan processing deficiency of proteins. Blocking of α -mannosidase activity and NO deficiency can easily cause accumulation of unfolded proteins, ER stress and activation of cellular signals leading to cell death.

Diabetes (Xiaoke) was firstly recorded in the Yellow Emperor's Canon of Medicine (722-221 B.C.), a classic medical book, in which Xiaozhong (polyorexia) and Xiaodan (diabetes) are used to describe diabetes. It points out that the etiologic factors excess fat and sugar, and imbalance of emotion. The General Treatise on the Etiology and Symptomology (618-907 AD) say that diabetes patients often suffer from carbuncle and deep-root carbuncle. The Medical Secrets of an Official (670-755 AD) describes that urine of patients with diabetes is sweet, indicating that glucose can be found in the urine of diabetes patients early.

It has been shown that the pathogenesis of diabetes is closely related to Pixu^[6], a major pathogenic factor

for diabetes syndromes. Improving Pixu is therefore appropriate in the treatment of diabetes. In this study, the nitrite and nitrate levels were significantly decreased in diabetic rats, which is consistent with the reported findings in patients with Pixu^[8].

Bu-Zhong-Yi-Qi-Tang, a traditional Chinese medicine, tonifies qi, strengthens the stomach and spleen, and increases sunken Yang qi, and is prescribed mainly for the deficiency of spleen and stomach qi in the treatment of Pixu. It was reported that the levels of nitrite, nitrate, and peroxynitrite in gastric mucosa are lower in Pixu rats, and return to normal after treatment with Bu-Zhong-Yi-Qi-Tang^[9].

In conclusion, Bu-Zhong-Yi-Qi-Tang can improve these abnormal conditions of diabetic rats by increasing the levels of nitric oxide and mannosidase activation, promoting N-glycosylation of proteins, assisting in the quality control of glycoprotein folding in ER lumen, and preventing accumulation of unfolded proteins, ER stress, activation of cellular signals.

COMMENTS

Background

N-glycans, α -mannosidase and nitric oxide (NO) play an important role in the quality control of glycoprotein folding in endoplasmic reticulum (ER) lumen, promote ER homeostasis and prevent ER-stress-related cell damage. There is evidence that ER stress plays a role in the pathogenesis of diabetes, contributing to loss of pancreatic beta-cells and insulin resistance. However, little attention has been paid to islet damage associated with the deficiency of N-glycan/nitric oxide in diabetes mellitus patients. It has been shown that the pathogenesis of diabetes is closely related to Pixu, a major pathogenic factor for diabetes syndromes. Improving Pixu is therefore appropriate in the treatment of diabetes. Bu-Zhong-Yi-Qi-Tang, a traditional Chinese decoction, is prescribed mainly for Pixu. However, little attention has been paid to its activity in islet damage and related mode of action in patients with diabetes.

Research frontiers

N-glycans, α -mannosidase and NO play an important role in the quality control of glycoprotein folding in ER lumen, promote ER homeostasis and prevent ER-stress-related cell damage. There is evidence that ER stress plays a role in the pathogenesis of diabetes, contributing to loss of pancreatic beta-cells and insulin resistance. The results of this study indicate that streptozotocin induces islet damage, which might be related to the N-glycan processing deficiency of proteins. Blocking of α -mannosidase activity and nitric NO easily causes accumulation of unfolded proteins, ER stress and activation of cellular signals leading to cell death. Bu-Zhong-Yi-Qi-Tang could improve these abnormal conditions of diabetic rats by increasing the levels of NO and mannosidase activity, promoting N-glycosylation of proteins, assisting in quality control of glycoprotein folding in ER lumen, and preventing accumulation of unfolded proteins, ER stress, activation of cellular signals leading to cell death.

Terminology

Bu-Zhong-Yi-Qi-Tang: a traditional Chinese medicine, consisting of *Radix Astragali*, *Codonopsis pilosula*, *Glycyrrhiza uralensis* f. *isch*, *Rhizoma Atractylodis Macrocephalae*, *Radix Angelicae Sinensis*, *Rhizoma Cimicifugae Foetidae*, *Radix Bupleuri Chinensis*, *Pericarpium Citri Reticulatae*, is prescribed mainly for deficiency of spleen and stomach qi in treatment of Pixu. Streptozotocin (STZ): 2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose, is actively transported into pancreatic β cells via the Glut-2 glucose transporter. Alpha-mannosidase: a key enzyme converting precursor high-mannose-type N-glycans to matured complex-type structure, contributes to the establishment of an equitable glycoprotein quality control standard, by which the efficiency of Asn-linked glycoprotein conformational maturation results in dislocation of misfolded glycoproteins into the cytosol, where proteins are degraded in proteasome and maintain the homeostasis of ER.

Peer review

This is a good descriptive study. Experiments were well designed and the data

contained novelty. The authors investigate the deficiency of N-glycan/nitric oxide in diabetic rats, and evaluated the effects of Bu-Zhong-Yi-Qi-Tang on diabetics, showing that STZ can induce islet damage, which might be related to the N-glycan processing deficiency of proteins. Blocking of α -mannosidase activity and nitric oxide deficiency could easily cause accumulation of unfolded proteins, ER stress and activation of cellular signals leading to cell death. Bu-Zhong-Yi-Qi-Tang was found to be effective against these abnormal conditions.

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ORIGINAL ARTICLES

Expression and location of α -fetoprotein during rat colon development

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Author contributions: Liu XY and Dong D performed the animal treatment, RT-PCR, immunofluorescent staining and wrote the paper; Sun P did part of RT-PCR and immunofluorescent staining; Du J conducted Western blot and statistical analysis; Ge YB designed the experiment and wrote the paper; Gu L and Ge YB contributed equally to this work.

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demonstrated that AFP is localized in the mesenchyme of rat colon from the embryo to the weaning stage by immunofluorescence and presents 72-kDa isoform in the developing rat colons by Western blotting. The dynamic expression of AFP in the various developmental stages of the colon indicates that AFP might be involved in many aspects of colon development.

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Key words: Alpha-fetoprotein; Development; Mesenchyme; Colon; Rat

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Abstract

AIM: To investigate the expression of α -fetoprotein (AFP), a cancer-associated fetal glycoprotein, and its involvement during rat colon development.

METHODS: Colons from Sprague-Dawley rat fetuses, young and adult (8 wk old) animals were used in this study. Expression levels of AFP in colons of different development stage were detected by reverse-transcriptase PCR (RT-PCR) and Western blotting. To identify the cell location of AFP in the developing rat colons, double-immunofluorescent staining was performed using antibodies to specific cell markers and AFP, respectively.

RESULTS: The highest levels of AFP mRNA were detected in colons of rats at embryonic day 18.5 (e18.5). Compared to e18.5 d, the AFP expression was significantly decreased during rat development [85% for e20.5, $P < 0.05$, 58% for postnatal day 0.5 (P0.5), $P < 0.05$, 37% for P7, $P < 0.05$, 24% for P14, $P < 0.05$, and 11% for P21, $P < 0.05$] and undetected in adult rats. Only the 72-kDa isoform of AFP was detected by Western blotting, the expression pattern was similar to AFP mRNA and conformed to the results of mRNA expression. The AFP positive staining was identical to different distribution patterns in fetuses, young and adult animals and positive staining for both AFP and vimentin was overlapped in mesenchymal cells at each stage tested.

CONCLUSION: This study has for the first time

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INTRODUCTION

The mammalian gut epithelium is a highly organized and dynamic system that requires continuous, controlled proliferation and differentiation throughout life. Identification of the growth factors controlling these processes is crucial since the molecular mechanisms regulating organogenesis are often the same as those necessary for repair following injury. Furthermore, mis-regulations of embryonic signaling pathways are often associated with neoplastic diseases.

We assumed that accurate transcriptional profiles over gut development interval could provide fundamental information about underlying mechanisms, and characterized candidate regulators of cell interactions and mucosal differentiation. This resource also can be applied to address long-standing questions about reactivation of fetal genes in cancer.

A number of transcription factors, growth factors, and their receptors have been found to be expressed in the gastrointestinal epithelium or mesenchyme. However, little is known about their specific functions in gastrointestinal development. For those factors where a mutation has been generated by gene targeting,

gastrointestinal development either proceeds normally^[1-3] or the embryos die too early to allow assessment of the gene functions in gut development^[4,5].

Mammalian α -fetoprotein (AFP) is a single-chain glycoprotein with molecular mass ranging from 66 to 72 kDa and 3%-5% carbohydrate (glycan) content. This protein, which expresses at high levels in the fetal liver and yolk sac, constitutes 0.1% of the total mRNA in the fetal gut^[6]. At birth, AFP mRNA declines precipitously in both liver and gut to levels that are barely detectable^[7]. The gut development during late gestation and early neonatal period is accompanied by changes in the synthesis of AFP^[8], and abundance declines significantly during gut development. In this case, AFP is considered as an important growth factor with a specific function in gastrointestinal development.

The ontogeny of AFP gene expression has been examined in the fetal and adult mouse gastrointestinal tract^[9] to understand the basis of the ontogeny of AFP transcription in the gut and its regulatory elements. However, little is known about the expression pattern of AFP genes or its involvement during rat colon development.

MATERIALS AND METHODS

Specimens

Colons from Sprague-Dawley rat fetuses embryonic day 18.5 (e18.5 and e20.5 gestation), young (0 d and 1, 2 and 3 wk old) and adult (8 wk old) animals were used in this study. Five rats were used at each age stage. The embryonic age was determined according to Kaufman^[10]. Mating was performed by housing a male and a female rat together in the same cage overnight. The presence of a vaginal plug was determined the next morning (0.5 d gestation). Rats were housed in plastic cages in an air-conditioned and light controlled room at $24 \pm 2^\circ\text{C}$ and $60\% \pm 5\%$ humidity. The study protocol was approved by the Nanjing Medical University Animal Care and Use Committee.

Reverse-transcriptase PCR (RT-PCR)

Total RNA was extracted from tissues at each time point with TRIZOL reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada), according to the manufacturer's instructions. The quality of the RNA was verified by agarose gel electrophoresis using ethidium bromide staining. For each PCR, 2 μg DNA-free total RNA with oligo (deoxythymidine) primers and reverse transcriptase were used. PCR was performed in 50- μL reactions containing 2.5 ng cDNA, 1 μL each primer pair, and 25 μL Premix Taq (TaKaRa, CA, USA). PCR was carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Quebec, Canada) to determine the annealing temperature for each pair of primers^[11]. The AFP primer pairs used were: 5'-GCTGAACCCAGAGTACTGCAC-3' (forward), and 5'-GACACGTC GTAGATGAACGTG-3' (reverse). Amplification reactions were carried out for 30 cycles at 94°C for 30 s, 58.4°C for 30 s and at 72°C for 1 min.

The amplified products were 443 bp and analyzed on 1% agarose gels and visualized by ethidium bromide staining. Controls omitting RT cDNA or DNA polymerase showed no reaction bands. The data were normalized by 18S RNA.

Western blot analysis

The tissues were homogenized in a lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride and Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany). The lysate was then centrifuged at $12000 \times g$ for 25 min at 4°C . The total protein concentration of each sample was analyzed by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). An equal amount of protein samples, 60 μg , from each specimen was boiled in $3 \times$ loading buffer (10 mmol/L Tris-HCl, pH 6.8 including 3% SDS, 5% β -mercaptoethanol, 20% glycerol and 0.6% bromophenol blue) for 3 min and separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After transfer, membranes were blocked with 5% fat-free milk in Tris-buffered saline plus 0.05% Tween 20 (TBS-T) overnight at 4°C . The membranes were then incubated with the primary antibody (sc-8108, an affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of AFP, diluted 1:500; Santa Cruz, Biotechnology CA, USA) for 2 h at room temperature. After washing in TBS-T three times, the membranes were incubated with the peroxidase-linked rabbit antigoat IgG conjugates (Santa Cruz Biotechnology) for 1 h at room temperature. At the end, they were washed again in TBS-T, incubated in enhanced chemiluminescence reagents (Pierce) for 2 min, and exposed to X-Omat BT film (Eastman Kodak, Rochester, NY, USA). Signal intensity was quantified using a Bio-Rad image analysis system and the results were normalized to band intensities at e18.5. Loading controls of presumably and constantly expressed proteins such as β -actin were used; however, their variability and increase in development precluded their use^[12]. For negative controls, the primary antibody was omitted.

Double fluorescence immunohistochemistry

Tissues were fixed in 4% paraformaldehyde overnight at 4°C followed by a standard protocol of dehydration and paraffin embedding, and 5- μm sections were cut. The paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol and distilled water. The non-specific binding sites were blocked in 1% bovine serum albumin (BSA) for 30 min. For AFP and vimentin double immunofluorescence, the goat anti-AFP primary polyclonal antibody was applied and revealed using fluorescein isothiocyanate (FITC)-labeled rabbit antigoat IgG (1:400, sc-2777; Santa Cruz Biotechnology). Mouse anti-vimentin primary monoclonal antibody (1:1000, CBL202; Chemicon International, Inc. Temecula, CA,

USA) was then applied and revealed by rhodamine-labeled anti-mouse IgG (1:400, AP192C; Chemicon International, Inc.). Sections were placed in gel aqueous mounting medium (G0918; Sigma, St. Louis, MO, USA) with a cover glass and were examined under an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan). Controls were treated by omitting the primary or secondary antibody. No staining was observed under the negative control conditions. Images were taken at a magnification $\times 200$.

Statistical analysis

All experiments were done in triplicate. Analysis of the experimental data was carried out using PDQuest 7.0 software (Bio-Rad Laboratories) and one-way analysis of variance and paired *t* test were used. Data are presented as mean \pm SD. *P* < 0.05 was considered statistically significant.

RESULTS

Temporal expression of AFP in the developing rat colons

We carried out RT-PCR and Western blotting to detect the expression of AFP using samples extracted from the colons of fetal e18.5 and e20.5, postnatal day 0 (P0), P7, P14 and P21 and adult rats. As shown in Figure 1, the highest levels of AFP mRNA were detected in colons of rats at e18.5. The AFP mRNA levels in colons declined steadily during rat development and were undetected in adult rats (*P* < 0.05) (Figure 1). The level of AFP mRNA in e20.5 colon was significantly decreased compared with that in e18.5 colon. AFP mRNA in P0 colon was lower than those in e20.5 colon (*P* < 0.05), and AFP mRNA in P7 colon was also lower than in P0 (*P* < 0.05). There was no difference in the levels of AFP mRNA between P14 and P21. The AFP protein had four isoforms: 72, 60, 48 and 37 kDa. In our study, only the 72-kDa isoform of AFP was detected in rat colon (Figure 2A). From the results of the densitometric quantification (Figure 2B), it was seen that the total AFP was the highest at e18.5, after which expression decreased steadily, being the lowest in the adult colons. This result was similar to those of AFP mRNA expression.

Regional and temporal localization of AFP in the developing rat colons

In e18.5 fetus, the colonic mucosa was lined by a stratified epithelium and AFP positive staining was detected in the epithelium and mesenchymal tissue (Figure 3A). In e20.5 fetus, the epithelium has transformed into a simple columnar one. The AFP positive cells located at the bottom of crypt-like structure and the number of positive cells decreased markedly at this time (Figure 4A). After birth, positive cells were scattered on the epithelium during the first 7 d (Figure 4B). By P14 and P21, when the adult crypt structure replaced the villi, positive cells became largely restricted to the base of the crypts, no positive staining of AFP in the adult colonic epithelium was observed (Figure 3C and D).

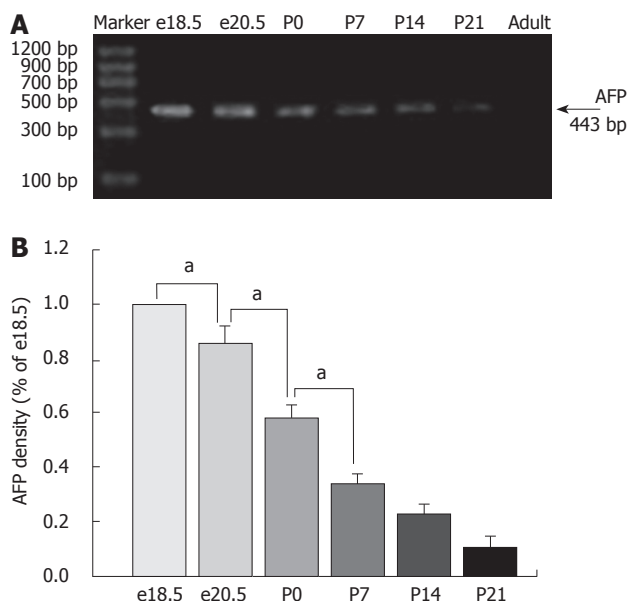


Figure 1 Expression of AFP mRNA using RT-PCR analysis in the developing rat colons as indicated in lanes e18.5, e20.5, P0, P7, P14, P21 and adult rats. The position of molecular weight markers is indicated on the left. A: The highest levels of AFP mRNA were detected in colons of rats at e18.5 and declined steadily during rat development and were undetected in adult rats; B: Results are indicated in percentages above the e18.5 value and are representative of three independent experiments. ^a*P* < 0.05 vs e18.5, e20.5 and P0, respectively.

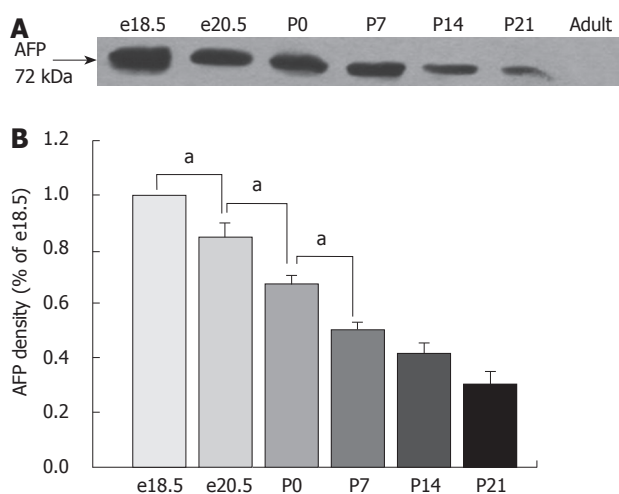


Figure 2 Expression of AFP protein using Western blot analysis in the developing rat colons as indicated in lanes e18.5, e20.5, P0, P7, P14, P21 and adult rats. A: Western blot analysis using AFP (C-19), an affinity purified goat polyclonal antibody against a peptide mapping at the C-terminus of AFP, revealed a 72-kDa isoform. The highest levels of AFP protein were detected in colons of rats at e18.5 and declined steadily during rat development and were undetected in adult rats. B: Results are indicated in percentage above the e18.5 value and are representative of three independent experiments. ^a*P* < 0.05 vs e18.5, e20.5 and P0, respectively.

Cells localization of AFP in the developing rat colons

The pattern of AFP-expressing cells and mesenchymal cells were very similar, which suggested a relationship between them. To identify the cells, an antibody against vimentin, which has been used as a marker for the mesenchymal cells, was used. Double-immunofluorescent staining for the vimentin and AFP showed a complete

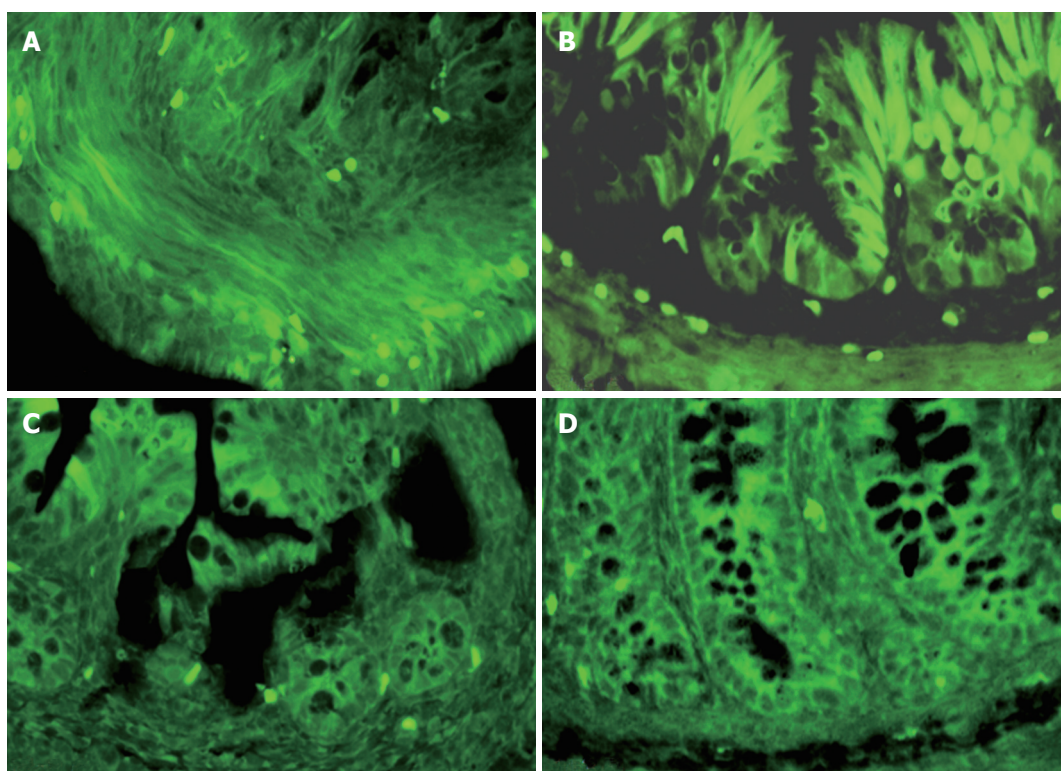


Figure 3 Immunofluorescence localization of AFP in the developing rat colons. A: In e18.5, AFP positive staining can be detected in the epithelium and mesenchymal tissues; B: At P0, positive cells were located at the base of the crypts and scattered on the epithelium; C, D: Only a few positive cells restricted to the base of the crypts between 14 and 21 d, and no positive cells can be detected in adult rat colons. ($\times 200$).

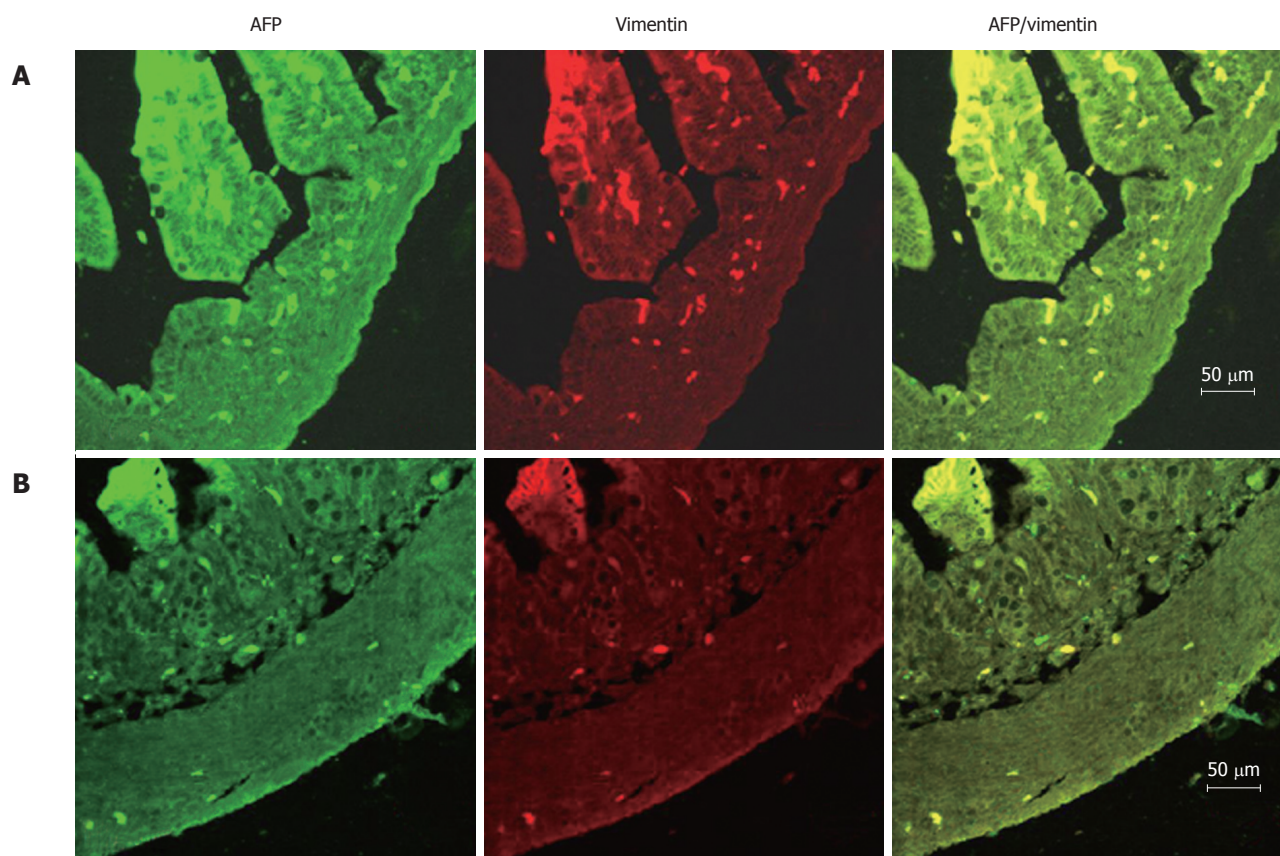


Figure 4 Immunofluorescence localization of AFP and vimentin in the developing rat colons. Labeling by the AFP antibody was detected with an FITC (green)-labeled secondary antibody. Labeling of vimentin was detected with a rhodamine- (red)-labeled secondary antibody on the same section. The overlap of AFP (green) and vimentin (red) labeling appeared orange in color. Double-labeling revealed complete localization of AFP and vimentin in the same colon cells at both e20.5 and P7. A: e20.5; B: P7.

overlap between the AFP positive cells and the antibody staining for vimentin at each stage tested (Figure 4A and B).

DISCUSSION

AFP is known to be associated with the successful completion of term pregnancies in mammals and even minute amounts of AFP may still be necessary during human pregnancy^[13]. The capability of both up and down modulation of growth and differentiation as a dose-dependent function of AFP has been demonstrated in a multitude of cell types including placental, ovarian, uterine, lymphoid, epidermal, endothelial, testicular, breast, and liver^[14-18]. The rat colon undergoes rapid growth and differentiation during the last few days of gestation and the first 2 wk after birth^[19-22]. This maturation process is accompanied by changes in the composition of AFP, which indicates that AFP might be involved in many aspects of colon development. Liu *et al*^[23] studied the changes of the protein levels of AFP in rat pancreas during development also by Western blot analysis and immunohistochemistry. Their results have demonstrated that the expression of AFP protein in the rat pancreas was increased in e18.5 rats and down-regulated after birth. They found a the similar possibility to ours, that the pancreatic cells, which went through dramatic growth, differentiation and proliferation, result from the dynamic expression of AFP in the various developmental stages^[23].

The genetic variants of rat AFP mRNA consist of sizes ranging from 2.2 to 1.35 kb, representing translated proteins ranging from 72 kDa down to 37 kDa, respectively^[24]. The smaller AFP isoforms are found to be truncated from the amino-terminal end. Molecular variants of AFP expressed in liver have long been reported in biomedical studies. All isoforms of the 72-kDa, 60-kDa and 48-kDa AFP protein may be involved in different aspects of liver cell behavior^[25,26]. Using a polyclonal antibody against a peptide mapping at the C terminus of AFP^[27-29], we found that only the 72-kDa isoform in the developing rat colons was detected by Western blotting. These results indicate that the 72-kDa isoform of AFP, with the highest expression in embryonic and regenerated liver, may also play an important role in colon cell proliferation and organ maturation.

Tyner *et al*^[9] have reported that AFP is expressed in a subset of enteroendocrine cells expressing chromogranin A, which suggested that they could be of enteroendocrine origin. AFP is a soluble glycoprotein that is able to bind many ligands including fatty acids, metals, steroids (estrogens), thyroxine, and tryptophan^[6,30]. The identity of the cells that express AFP in the colon cells was tested by double antibody staining with antibodies to chromogranin A (an enteroendocrine cell marker), cytokeratin (a epithelial cell marker), proliferating cell nuclear antigen (a proliferative cell marker) and vimentin (a mesenchymal cell marker and AFP) at each stage in rat development, respectively (data

not shown). Only an overlap of positive staining of AFP and vimentin was found in the same cell, indicating that AFP is indeed expressed and produced in mesenchymal cells. Liu *et al*^[23] reported that, in rat developing pancreas, AFP was also co-expressed with the vimentin, which was similar to our results. These results demonstrated that mesenchymal cell-derived AFP can act as a potent paracrine regulator of colon cell proliferation and organ maturation. The epithelial-mesenchymal interactions play an essential role in the control of gastrointestinal epithelial growth and differentiation not only in fetal stages, but also in adults^[31,32], but the mechanism has not been fully understood. Characterization of AFP expression in mesenchymal cells may help us discern a function in the gastrointestinal tract.

Cancer cells display immature features and dysregulated gene expression, with attenuation of tumor suppressors and aberrant expression of genes that are inactive in normal adult tissues. Since many down-regulated genes during development are re-expressed in tumors, understanding their respective cellular roles can provide information about both development and cancer biology. Despite some recent advances^[33], the extent of embryonic gene expression by tumor cells and the significance of this phenomenon are still unknown. AFP is one such gene and is reactivated in human tumors of the same fetal origin. Further studies are necessary to identify elements in the AFP gene that contribute to its expression. Numerous data support the hypothesis that AFP repression is a part of a global scheme of liver differentiation prematurely activated by glucocorticoids^[34]. The involvement of glucocorticoids in AFP expression in the gut remains an open question. Studies are now in progress in our laboratory using animal and tissue culture models.

In summary, our present study has, for the first time, demonstrated that AFP is localized to the mesenchyme in rat colon from the embryo to the weaning stage (up to 21 d after birth) by immunofluorescence, and presents a 72-kDa isoform in the developing rat colon by Western blotting. The dynamic expression of AFP in the various developmental stages of the colon indicates that AFP might be involved in many aspects of colon development. The exact function of AFP in colon development remains to be determined.

COMMENTS

Background

Mammalian α -fetoprotein (AFP) is a single-chain glycoprotein, developmentally down-regulated and re-expressed in tumors. The authors assumed that its accurate profiles over gut developmental interval could provide fundamental information about underlying mechanisms. This resource also can be applied to address longstanding questions about reactivation of fetal genes in cancer.

Innovations and breakthroughs

This study demonstrated that AFP presented a 72-kDa isoform and localized to the mesenchyme in the developing rat colon. The expression of AFP in the various developmental stages is dynamical. AFP might be involved in many aspects of colon development.

Applications

This animal model is a useful tool for the studies of gastrointestinal mucosal

proliferation and differentiation mechanism *in vivo*. The authors will identify elements in the AFP gene that contribute to its expression.

Terminology

AFP is a single-chain glycoprotein with molecular mass ranging from 66 to 72 kDa and a 3%-5% carbohydrate (glycan) content.

Peer review

The study investigated the expression of AFP and its involvement during rat colon development. It was well designed and conducted adequately.

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ORIGINAL ARTICLES

Recombinant vascular basement-membrane-derived multifunctional peptide inhibits angiogenesis and growth of hepatocellular carcinoma

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Abstract

AIM: To investigate the anti-angiogenic and anti-tumor activities of recombinant vascular basement membrane-derived multifunctional peptide (rVBMDMP) in hepatocellular carcinoma (HCC).

METHODS: HepG2, Bel-7402, Hep-3B, HUVE-12 and L-02 cell lines were cultured *in vitro* and the inhibitory effect of rVBMDMP on proliferation of cells was detected by MTT assay. The *in vivo* antitumor efficacy of rVBMDMP on HCC was assessed by HepG2 xenografts in nude mice. Distribution of rVBMDMP, mechanism by which the growth of HepG2 xenografts is inhibited, and microvessel area were observed by proliferating cell nuclear antigen (PCNA) and CD31 immunohistochemistry.

RESULTS: MTT assay showed that rVBMDMP markedly inhibited the proliferation of human HCC (HepG2, Bel-7402, Hep-3B) cells and human umbilical

vein endothelial (HUVE-12) cells in a dose-dependent manner, with little effect on the growth of L-02 cells. When the IC₅₀ was 4.68, 7.65, 8.96, 11.65 and 64.82 μ mol/L, respectively, the potency of rVBMDMP to HepG2 cells was similar to 5-fluorouracil (5-FU) with an IC₅₀ of 4.59 μ mol/L. The selective index of cytotoxicity to HepG2 cells of rVBMDMP was 13.8 (64.82/4.68), which was higher than that of 5-FU [SI was 1.9 (8.94/4.59)]. The VEGF-targeted recombinant humanized monoclonal antibody bevacizumab (100 mg/L) did not affect the proliferation of HepG2, Bel-7402, Hep-3B and L-02 cells, but the growth inhibitory rate of bevacizumab (100 mg/L) to HUVE-12 cells was 87.6% \pm 8.2%. Alternis diebus intraperitoneal injection of rVBMDMP suppressed the growth of HepG2 xenografts in a dose-dependent manner. rVBMDMP (1, 3, 10 mg/kg) decreased the tumor weight by 12.6%, 55.9% and 79.7%, respectively, compared with the vehicle control. Immunohistochemical staining of rVBMDMP showed that the positive area rates (2.2% \pm 0.73%, 4.5% \pm 1.3% and 11.5% \pm 3.8%) in rVBMDMP treated group (1, 3, 10 mg/kg) were significantly higher than that (0.13% \pm 0.04%) in the control group (P < 0.01). The positive area rates (19.0% \pm 5.7%, 12.2% \pm 3.5% and 5.2% \pm 1.6%) of PCNA in rVBMDMP treated group (1, 3, 10 mg/kg) were significantly lower than that (29.5% \pm 9.4%) in the control group (P < 0.05). rVBMDMP at doses of 1, 3 and 10 mg/kg significantly reduced the tumor microvessel area levels (0.26% \pm 0.07%, 0.12% \pm 0.03% and 0.05% \pm 0.01% *vs* 0.45% \pm 0.15%) in HepG2 xenografts (P < 0.01), as assessed by CD31 staining.

CONCLUSION: rVBMDMP has effective and unique anti-tumor properties, and is a promising candidate for the development of anti-tumor drugs.

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Key words: Hepatocellular carcinoma; Recombinant vascular basement membrane-derived multifunctional peptide; Proliferating cell nuclear antigen; CD31; Therapeutic action

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INTRODUCTION

Hepatocellular carcinoma (HCC), the fifth most common cancer in the world, is responsible for over 600 000 deaths annually^[1]. The majority of patients with HCC die within 1 year after the diagnosis. Unfortunately, HCC is often diagnosed at its late stage when potentially curative therapies are least effective. For such patients, medical treatment modalities, including chemotherapy, chemoembolization, ablation, and proton beam therapy, remain disappointing. Most patients show recurrent HCC that rapidly progresses to its advanced stage with vascular invasion and multiple intrahepatic metastases and their 5-year survival rate is only 7%^[2]. Patients with surgically resectable localized HCC have a better prognosis, but their 5-year survival rate is only 15%-39%^[3], showing that new therapies for this aggressive disease are urgently needed.

Angiogenesis plays a critical role in the development of HCC. Antiangiogenesis therapy, which inhibits blood vessel formation, may be a promising treatment modality for HCC, because HCC depends on a rich blood supply^[4].

Tumstatin, a 28-kDa (244 amino acids) peptide fragment derived from the NC1 domain of $\alpha 3$ chain of type IV collagen, is an endogenous angiogenesis inhibitor, and has two binding sites for $\alpha v\beta 3$ integrin. One is in the N-terminal region of the molecule consisting of amino acids 74-98, which is associated with the anti-angiogenic property. The other is in the C-terminal region consisting of amino acids 185-203, which is associated with the antitumor activity^[5-7]. The peptide fragment of tumstatin consisting of amino acids 74-98 binds to both endothelial and melanoma cells, but only inhibits the proliferation of endothelial cells. However, the anti-tumor activity of amino acids 185-203 is not realized until this peptide region is exposed by truncation, a requirement not essential for the anti-angiogenic activity of amino acids 74-98^[5].

By targeting proliferating tumor cells and endothelial cells in a previous study^[8], we have constructed a fusion gene of the human IgG3 upper hinge region with two tumstatin-derived specific sequences, which exhibit anti-proliferation and anti-angiogenic activities. The human IgG3 upper hinge region is composed of 11 amino acids, and has a good flexibility, thus not affecting the spatial conformations of the connected peptides. The fusion sequence is named vascular basement membrane-derived multifunctional peptide (VBMDMP)^[9]. Recombinant

VBMDMP (rVBMDMP) can significantly inhibit tumor growth and metastasis in a mouse lung carcinoma model^[10]. Moreover, rVBMDMP selectively inhibits the proliferation of endothelial and human colon cancer cells, as well as induces apoptosis of endothelial cells *in vitro* and suppresses the growth of human colon cancer xenografts in Balb/c-nude mice^[11]. However, whether rVBMDMP inhibits tumor growth and angiogenesis of human HCC xenografts in a nude mouse model is unknown.

In the present study, we showed that rVBMDMP selectively inhibited the proliferation of HCC cells, using *in vitro* models of tumor growth, and also potently inhibited tumor neoangiogenesis of HepG2 xenografts in a nude mouse model, suggesting that rVBMDMP can be used as a potential agent in the treatment of human HCC.

MATERIALS AND METHODS

Cell culture and reagents

HepG2, Bel-7402, Hep-3B, HUVE-12 and L-02 cell lines, were purchased from the China Center for Type Culture Collection (CCTCC), were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μ g/mL streptomycin (Life Technologies) in an incubator containing 50 mL/L CO₂ at 37°C. rVBMDMP was over-expressed in *Escherichia coli* with pGEX-4T-1-VBMDMP and purified as previously described^[10] with a purity of over 95%. Synthetic peptide CNYYSNSYSFWLASLNPER (amino acid 185-203 of tumstatin, T4 peptide) and its rabbit polyclonal antibody were provided by Xi'an Huacheng Biotechnology Co., Ltd (China). Bevacizumab was purchased from Roche (Avastin®, Basel, Switzerland). Mouse monoclonal antibodies against proliferating cell nuclear antigen (PCNA) and CD31, as well as peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

MTT assay

Cells were seeded in a 96-well plate at a density of 1000 cells/well as described previously^[12]. Different concentrations of drugs were added to each well and cultured for 48 h, followed by incubation with 0.5 g/L MTT for 4 h. The supernatant was removed after centrifugation. Finally, 100 μ L DMSO was added and the absorbance at 570 nm wavelength (A_{570}) was measured with an enzyme-labeling instrument (ELX-800 type). Relative cell proliferation inhibition rate (IR) = (1-average A_{570} of the experimental group/average A_{570} of the control group) \times 100%. The IR was analyzed using the CalcuSyn program to determine the IC₅₀.

Tumor xenograft experiments

Balb/c-nude female mice (Vital River Laboratory Animal Technology Co., Ltd), used in *in vivo* study, were housed in a sterile room at Institute of Cancer Research, University of South China, with free access to food and water.

Tumors were generated by harvesting HepG2 cells

from mid-log phase cultures using 0.25% trypsin (Life Technologies). Cells were resuspended in PBS to a final cell count of $2.5 \times 10^7/\text{mL}$. A cell suspension (0.2 mL) was subcutaneously injected into the back of each mouse. The mice received a total of 10 injections of 1, 3, and 10 mg/kg body weight rVBMDMP (i.p) every other day when their average tumor volume reached 200 mm³.

Tumor dimensions and body weight were recorded every 5 d from the beginning of treatment. Tumor length and width were measured using a Vernier caliper, and tumor volume was calculated as described previously^[13]. Upon termination of treatment, the mice were weighed and sacrificed, and their tumors were excised. The mean tumor weight per group was calculated. The ratio of the mean of the treated tumor weight to the mean of vehicle control tumor weight $\times 100$ was subtracted from 100% to give the tumor growth inhibition rate for each group.

Immunohistochemical staining and quantification

Immunohistochemical staining of paraffin tumor tissue sections was done with rabbit polyclonal anti-T4 peptide antibody (Xi'an Huacheng Biotechnology) at a dilution of 1:50 using the DAB system from DAKO (Carpinteria, CA, USA) according to the manufacturer's instructions.

The tumor tissue sections were viewed at $\times 100$ magnification and images were captured with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA), and analyzed under four fields, excluding peripheral connective tissue and necrotic regions. The total tissue area in each section was 2.576 mm². Areas of rVBMDMP, PCNA or CD31-positive objects were quantified using ImagePro Plus version 3.0 (Media Cybernetics, Silver Spring, MD, USA). Percentage of microvessel area (MVA) in each field was calculated as (area of CD31-positive objects/measured tissue area) $\times 100$. Percentage of rVBMDMP or PCNA-positive staining in each field was calculated as (area of rVBMDMP or PCNA-positive objects/measured tissue area) $\times 100$. Mean values of MVA- or VBMDMP or PCNA-positive area in each group were calculated from six tumor tissue samples.

Statistical analysis

Experimental data in each group were expressed as mean \pm SD. Analysis of variance was performed with SPSS software for Windows 15.0 using one way ANOVA and pairwise comparison with Student's *t* test. *P* < 0.05 was considered statistically significant.

RESULTS

Effects of rVBMDMP on proliferation of HCC, endothelial cells (ECs) and L-02 cell lines

MTT assay showed that rVBMDMP markedly inhibited the proliferation of human HCC (HepG2, Bel-7402, Hep-3B) cells and human umbilical vein endothelial (HUVE-12) cells in a dose-dependent manner, with

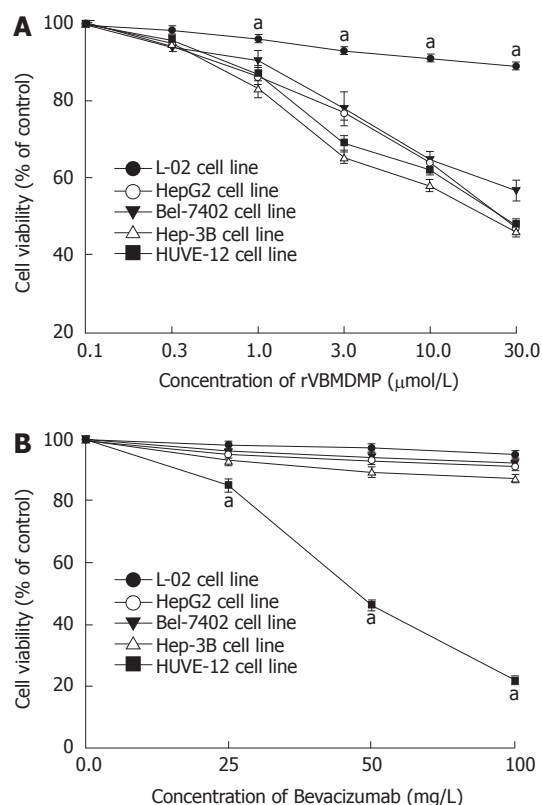


Figure 1 Proliferation of HCC (A) and ECs (B) selectively inhibited by rVBMDMP (mean \pm SD, *n* = 9). ^a*P* < 0.05 vs other cells.

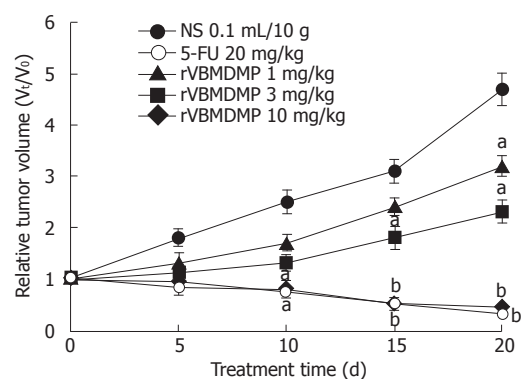


Figure 2 Robust efficacy of rVBMDMP against HepG2 xenografts in nude mice (mean \pm SD, *n* = 6). ^a*P* < 0.05, ^b*P* < 0.01 vs NS.

little effect on the growth of L-02 cells (Figure 1A). When the IC₅₀ was 4.68, 7.65, 8.96, 11.65 and 64.82 μmol/L, respectively, the potency of rVBMDMP to HepG2 cells was similar to that of 5-FU with an IC₅₀ of 4.59 μmol/L. The selective index of cytotoxicity to HepG2 cells of rVBMDMP was 13.8 (64.82/4.68), which was higher than that of 5-FU with a SI of 1.9 (8.94/4.59). Bevacizumab (100 mg/L) did not affect the proliferation of HepG2, Bel-7402, Hep-3B and L-02 cells, but its growth inhibitory rate for HUVE-12 cells was 87.6% \pm 8.2% (Figure 1B).

In vivo efficacy of rVBMDMP against HepG2 xenografts

rVBMDMP inhibited the growth of implanted HepG2 tumor xenografts in nude mice in a dose-dependent

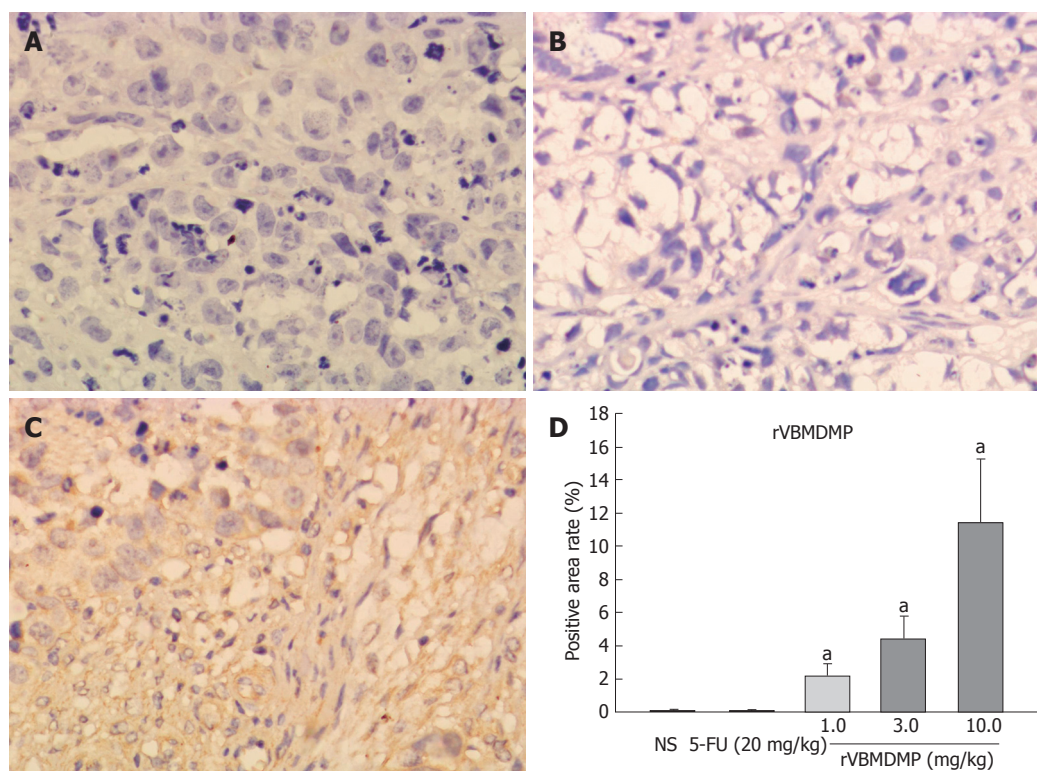


Figure 3 Accumulation of rVBMDMP in HepG2 xenografts in nude mice after treatment. A: NS; B: 20 mg/kg of 5-FU; C: 10 mg/kg of rVBMDMP; D: Quantification of rVBMDMP-positive areas in HepG2 xenografts. The data are expressed as mean \pm SD ($n = 6$). ^a $P < 0.05$ vs NS.

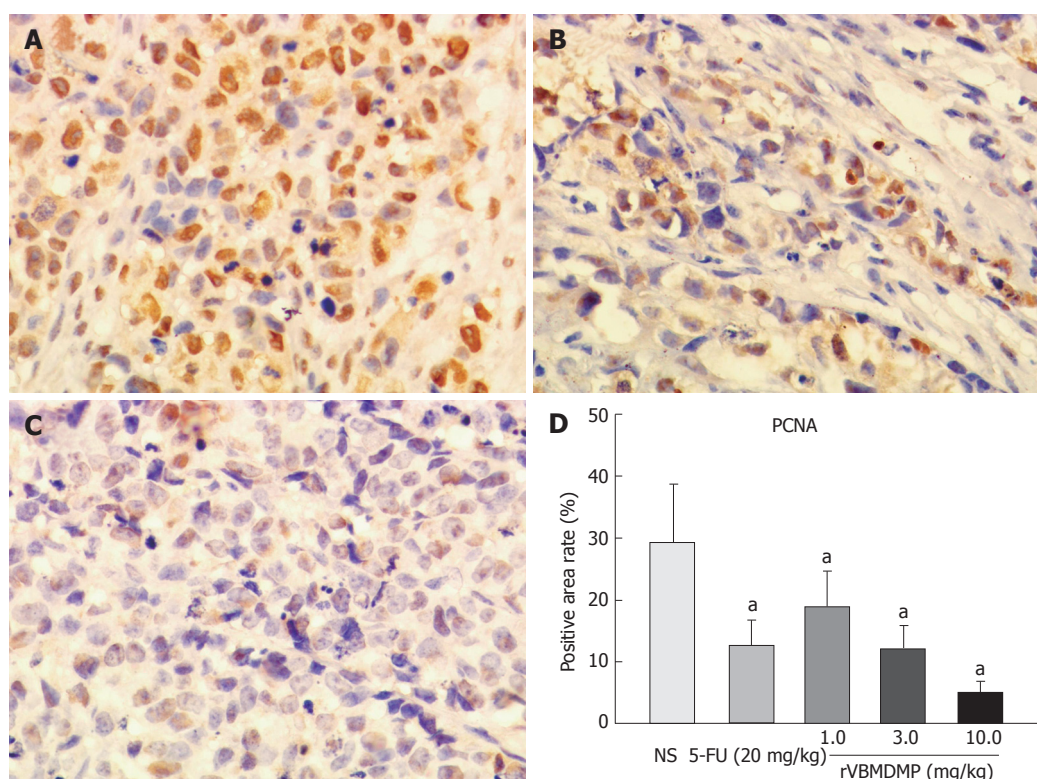


Figure 4 Expression of PCNA in HepG2 xenografts in nude mice after treatment. A: NS; B: 20 mg/kg of 5-FU; C: 10 mg/kg of rVBMDMP; D: Quantification of PCNA-positive areas in HepG2 tumors. The data are expressed as mean \pm SD ($n = 6$). ^a $P < 0.05$ vs NS.

manner (Figure 2). Different doses of rVBMDMP (1, 3, 10 mg/kg) decreased the tumor weight by 12.6%, 55.9% and 79.7%, respectively, compared with the vehicle control.

Distribution of rVBMDMP in HepG2 xenografts

Immunohistochemical staining of rVBMDMP showed that the positive area rates ($2.2\% \pm 0.73\%$, $4.5\% \pm$

1.3% and $11.5\% \pm 3.8\%$) were significantly higher in rVBMDMP treated group (1, 3, 10 mg/kg) than that ($0.13\% \pm 0.04\%$) in the control group ($P < 0.01$, Figure 3), indicating that rVBMDMP can accumulate in HepG2 xenografts nude mice.

PCNA expression in HepG2 xenografts

After intraperitoneal injection of rVBMDMP every other

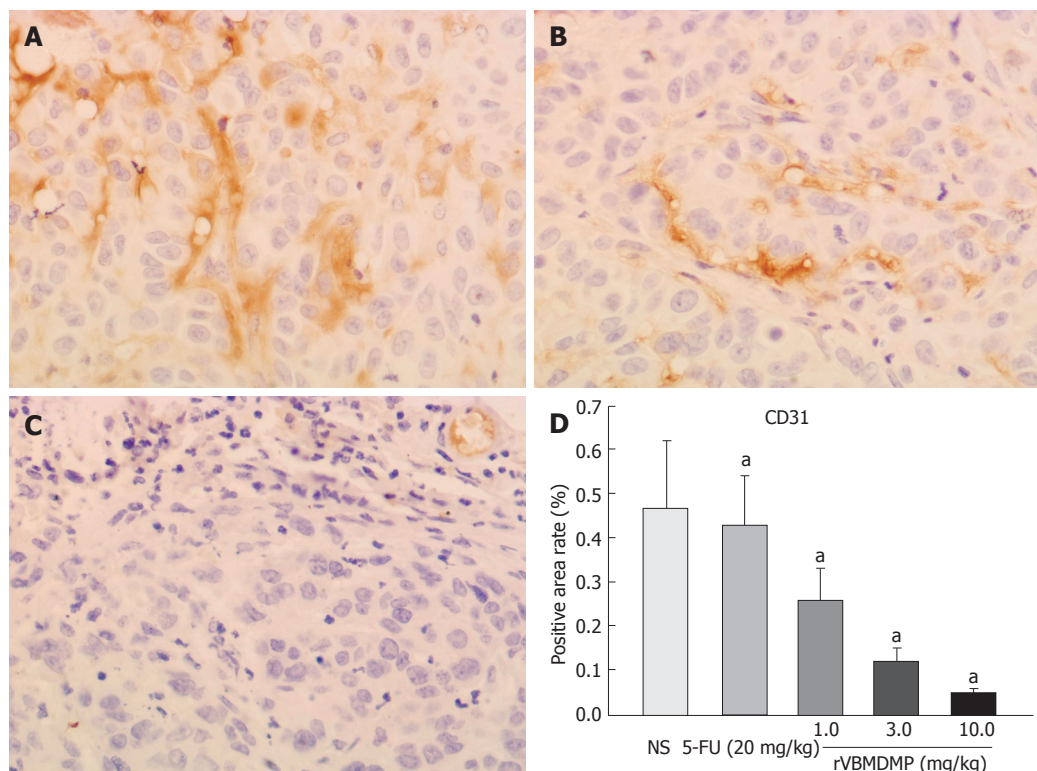


Figure 5 Microvessel density of HepG2 xenografts in nude mice after treatment. A: NS; B: 20 mg/kg of 5-FU; C: 10 mg/kg of rVBMDMP; D: Quantification of microvessel density (CD31 staining) positive areas in HepG2 xenografts in nude mice. The data are expressed as mean \pm SD ($n = 6$). ^a $P < 0.05$ vs NS.

day, the positive area rates ($19.0\% \pm 5.7\%$, $12.2\% \pm 3.5\%$ and $5.2\% \pm 1.6\%$) of PCNA in rVBMDMP treated group (1, 3, 10 mg/kg) were significantly lower than that in the control group ($29.5\% \pm 9.4\%$) ($P < 0.05$, Figure 4), suggesting that rVBMDMP inhibits the proliferation of tumor cells in HepG2 xenografts in nude mice.

Effect of rVBMDMP on angiogenesis of HepG2 xenografts

The tumor MVA rates ($0.26\% \pm 0.07\%$, $0.12\% \pm 0.03\%$ and $0.05\% \pm 0.01\%$) were significantly lower in the HepG2 xenografts of the rVBMDMP-treated group assessed by CD31 staining (1, 3 and 10 mg/kg) than that ($0.45\% \pm 0.15\%$) in the control group ($P < 0.01$, Figure 5), demonstrating that rVBMDMP inhibits angiogenesis of HepG2 xenografts in nude mice.

DISCUSSION

It was recently reported that angiogenesis inhibitors may not work well in monotherapy^[14,15]. In contrast, studies conducted in preclinical tumor models showed that angiogenesis inhibitors in combination with cytotoxic chemotherapeutic agents or radiation therapy produce additive or synergistic anti-tumor activities^[12,16,17]. The positive effects of combined chemotherapy with angiogenesis inhibitors have been reported^[18-22], suggesting that the combination therapy of a cytotoxic agent and an angiogenesis inhibitor may be a fruitful topic in future clinical research^[23,24].

In this report, rVBMDMP inhibited the proliferation of human HCC cells selectively *in vitro*. Our previously research also showed that rVBMDMP could inhibit the proliferation of colon cancer cells, but have no effect on the proliferation of normal cells^[11], suggesting that

rVBMDMP can maintain the selective anti-tumor activity of tumstatin amino acids 185-203 fragment, which is consistent with the previously reported findings^[23]. The specific inhibitory effect of rVBMDMP on the proliferation of tumor cells strongly suggests that rVBMDMP functions *via* a tumor-specific cell surface protein or its receptor.

Tumor neoangiogenesis has recently been recognized as an important factor in defining subsets of cancer patients with a poor outcome^[25-27]. A number of angiogenesis inhibitors, discovered in recent years, can inhibit tumor growth by targeting proliferating and migrating ECs. Targeting ECs supports growth of tumor rather than tumor cells directly, which is particularly promising because these ECs are genetically stable and do not develop drug resistance. In this study, rVBMDMP suppressed reduplication in human endothelial HUVE-12 cells, like bevacizumab. By immunostaining of CD31 in tumor tissues, we found that rVBMDMP significantly decreased the microvessel density of human HCC xenografts in a mouse model. It was reported that rVBMDMP can significantly inhibit the proliferation of endothelial cells, blood vessel formation, and tumor growth in *in vitro* and *in vivo* models of angiogenesis, as well as induce EC-specific apoptosis^[11]. These anti-angiogenic properties of rVBMDMP, coupled with its anti-tumor activities, strongly indicate that rVBMDMP acts as a novel inhibitor of angiogenesis and tumor growth.

Since the proliferation velocity of ECs is higher in tumor tissue than in normal tissue, angiogenesis inhibitors may be accumulated in tumor^[28]. Our results show that rVBMDMP was significantly accumulated in human HCC xenografts in a mouse model, indicating

that rVBMDMP is selectively distributed in tumor tissue.

Maeshima *et al.*^[5] demonstrated that tumstatin amino acids 185-203 fragment does not show anti-tumor activity until the peptide region is exposed to truncation, which is not required for the anti-angiogenic activity of tumstatin amino acids 74-98 fragment. A shorter fragment comprising seven N-terminal residues 185-191 (CNYYSNS) shares the same inhibitory profile. The three-dimensional structures of CNYYSNS and tumstatin amino acids 185-203 fragment show a β -turn at the YYSNS (188-191) sequence level, which is crucial for its biological activity^[29]. In our study, analysis of the structures of rVBMDMP using the AntheProt software indicated that both ends of the IgG3 upper hinge region sequence were a rarefaction structure, suggesting that rVBMDMP acts as a potent and specific agent against tumor progression^[11].

It has been shown that $\alpha_v\beta_3$ integrin is a putative receptor of tumstatin^[30,31]. Tumstatin fails to suppress neovascularization of Matrigel plugs in β integrin-deficient mice, and tumors in β integrin-deficient mice grow much faster than tumors in wild-type mice^[30,31], strongly suggesting that tumstatin acts via $\alpha_v\beta_3$ integrin as a negative regulator of angiogenesis. We speculate that the anti-tumor activity of rVBMDMP might also be mediated by $\alpha_v\beta_3$ integrin^[32].

In conclusion, rVBMDMP is a novel inhibitor of angiogenesis and tumor growth. Targeting both endothelial and tumor cells can enhance the efficacy of anti-tumor therapy. The mechanism of its action requires further investigation.

COMMENTS

Background

Tumstatin, a 28-kDa (244 amino acids) peptide fragment, derived from the NC1 domain of $\alpha 3$ chain of type IV collagen, is an endogenous angiogenesis inhibitor. Tumstatin has two binding sites for $\alpha_v\beta_3$ integrin. One is in the N-terminal region of the molecule consisting of amino acids 74-98, which is associated with the anti-angiogenic property. The other is in the C-terminal region consisting of amino acids 185-203, which is associated with the antitumor activity. However, the anti-tumor activity of amino acids 185-203 is not realized until this peptide region is exposed to truncation, a requirement not essential for the anti-angiogenic activity of amino acids 74-98.

Research frontiers

Angiogenesis plays a critical role in the development of hepatocellular carcinoma (HCC). Antiangiogenesis therapy, which inhibits blood vessel formation, may be promising treatment modality for HCC, because HCC depends on a rich blood supply. The strategy of targeting both proliferating tumor and endothelial cells can improve the effectiveness of therapy for HCC.

Innovations and breakthroughs

It was recently reported that rVBMDMP significantly inhibits tumor growth and metastasis in a mouse lung carcinoma model and selectively inhibits the proliferation of endothelial and human colon cancer cells. In this study, recombinant vascular basement membrane-derived multifunctional peptide (rVBMDMP) selectively inhibited the proliferation of HCC cells in *in vitro* and *in vivo* models of tumor growth. Furthermore, our *in vivo* studies suggested that rVBMDMP was significantly accumulated in human HCC xenografts and potentially inhibited tumor neoangiogenesis in HepG2 xenografts in nude mice.

Applications

rVBMDMP is a novel inhibitor of angiogenesis and tumor growth. Targeting both endothelial and tumor cells can enhance the efficacy of anti-tumor therapy and can be used as a treatment modality for HCC.

Terminology

VBMDMP is a fusion gene in the human IgG3 upper hinge region with two tumstatin-derived specific sequences (amino acids 74-98 and amino acids 185-203), which exhibits anti-proliferation and anti-angiogenic activities. Recombinant VBMDMP (rVBMDMP) is produced as a recombinant molecule in *E. coli*.

Peer review

The authors demonstrated that rVBMDMP selectively inhibited the proliferation of HCC cells, and was significantly accumulated in human HCC xenografts, and potentially inhibited tumor neoangiogenesis in HepG2 xenografts in a nude mouse model by examining the effects of rVBMDMP on tumor growth and angiogenesis of HCC *in vitro* and *in vivo*. The results are interesting and may represent the strategy of targeting both proliferating tumor and endothelial cells, and provide a new treatment modality for HCC.

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Exogenous phosphatidylethanolamine induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway

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affect the cell cycle, but induced apoptosis. PE significantly decreased $\Delta\Psi_m$ at 0.25, 0.5 and 1 mmol/L, respectively, suggesting that PE induces cell apoptosis by decreasing the mitochondrial transmembrane potential. The Bcl-2 expression level induced by different concentrations of PE was lower than that in control groups. However, the Bax expression level induced by PE was higher than that in the control group. Meanwhile, PE increased the caspase-3 expression in a dose- and time-dependent manner.

CONCLUSION: Exogenous PE induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway.

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Key words: Apoptosis; Bcl-2; Bax; Caspase-3; Phosphatidylethanolamine; Human hepatoma HepG2 cell

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Abstract

AIM: To investigate the signaling pathways implicated in phosphatidylethanolamine (PE)-induced apoptosis of human hepatoma HepG2 cells.

METHODS: Inhibitory effects of PE on human hepatoma HepG2 cells were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell cycle, apoptosis and mitochondrial transmembrane potential ($\Delta\Psi_m$) were analyzed by flow cytometry. Immunocytochemical assay and Western blotting were used to examine Bcl-2, Bax and caspase-3 protein levels in HepG2 cells treated with PE.

RESULTS: PE inhibited the growth of HepG2 cells in a dose- and time- dependent manner. It did not

INTRODUCTION

Phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM), are the dominant lipid constituents of the membranes of animal cells, in which they are distributed in an asymmetrical fashion and act as the matrix for the support and organization of different membrane proteins^[1]. In addition to this structural role, many individual phospholipid constituents are known to be involved in specific signaling functions necessary for cells to respond to external stimuli^[2-4].

Some studies have provided evidence that membrane

phospholipid asymmetry is disturbed in one of the early stages of apoptosis^[5-7]. Specifically, a translocation of PS and PE from the internal to external surface of the plasma membrane appears to be a fundamental mechanism through which apoptotic cells are recognized and eliminated by phagocytic macrophages^[8-10]. PS and PE externalization could arise either through inactivation of aminophospholipid translocase (APTL), whose normal function maintains the asymmetric distribution of PS and PE in cells^[11,12] or by accelerating reversal of movement of the phospholipid. Inhibition of APTL could induce apoptosis of central nervous system (CNS)-derived HN2-5 and HOG cells, activating caspase-3, indicating that abnormal distribution of cell membrane phospholipid can induce apoptosis^[13]. PS externalization is a characteristic feature of the apoptotic cells. It has been shown that the externalized PS serves as a marker for detecting the apoptotic cells, and PE exposed to the cell surface forms lipid rafts with PS during apoptosis^[14].

It was recently reported that overexpression of Raf kinase inhibitor protein (RKIP), a member of the phosphatidylethanolamine-binding protein (PEBP) family, can inhibit the Raf-ERK1/2 pathway^[15]. However, the effects of PE on cell proliferation or apoptosis remain unclear. Our results in this study suggest that exogenous PE induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway.

MATERIALS AND METHODS

Materials and agents

RPMI-1640 medium and fetal calf serum were purchased from GIBCO (Canada). PE, MTT and propidium iodide (PI) were purchased from Sigma. Annexin V-FITC apoptosis detection kit was from BD Biosciences (USA). Monoclonal antibodies were obtained from Cell Signaling Technology (USA).

Cell culture

Human hepatoma cell line HepG2 was obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). SMMC7721, HEK293 and HeLa cells were provided by Molecular Biology Center of the First Affiliated Hospital, Xi'an Jiaotong University. HepG2 cells (5.0×10^4 cells/mL) were cultured in RPMI-1640 supplemented with 100 mL/L fetal bovine serum, containing 2.0 mmol/L glutamine and 20 μ g penicillin-streptomycin/mL in 50 mL/L CO₂ at 37°C, and allowed to adhere for 24 h. The experiments were divided into four groups: control group, 0.25 mmol/L PE, 0.5 mmol/L PE, and 1 mmol/L PE treatment groups.

MTT assay for cell viability

HepG2 cells (2×10^4 cells/well) were seeded onto 96-well plates and incubated with test substances for an indicated time at 37°C in an atmosphere containing 50 mL/L CO₂. Then, 20 μ L MTT solution (5 g/L) was added into each well and incubated for another 4 h. Supernatants

were removed and formazan crystals were dissolved in 200 μ L dimethylsulfoxide. Finally, optical density was determined at 490 nm by POLARstar + OPTIMA (BMG Labtechnologies, Germany).

Cell cycle analysis by flow cytometry

DNA content per duplicate was analyzed by flow cytometry (BD Biosciences). Adherent cells were harvested by brief trypsinization, and washed with PBS, fixed in 700 mL/L ethanol, stained with 20 μ g/mL PI containing 20 μ g/mL RNase (DNase free) for 30 min, and analyzed by flow cytometry. The number of cells at the G0/G1, S, and G2/M phases was calculated.

Detection of HepG2 cell apoptosis by annexin-V/PI staining

HepG2 cells were treated with PE at 37°C in an atmosphere containing 50 mL/L CO₂ for 48 h, then harvested and washed twice with PBS. The cells were labeled by incubation with 5 μ L FITC-annexin V and 10 μ L PI at 250 μ g/mL for 10 min in the dark at room temperature. The cells were washed with PBS again and examined by flow cytometry. Apoptosis was routinely quantified by counting the number of cells stained with FITC-labeled annexin V.

$\Delta\Psi$ m examination

HepG2 cells (1×10^6 /mL) were washed twice with PBS, incubated with rhodamine 123 (10 μ g/mL) at 37°C for 30 min, then washed with PBS and analyzed by flow cytometry.

Immunocytochemical assay

HepG2 cells were grown on glass culture slides coated with poly-lysine in a 24-well plate and treated with PE. Slides with cells were fixed in 40 mg/L paraformaldehyde for 20 min, and then incubated with monoclonal antibody (anti-Bcl-2, anti-Bax, anti-caspase-3), which was labeled with FITC-conjugated goat anti-rabbit IgG. Positive-staining-area percentages were calculated under SP2 confocal microscope (Leica, Germany). Additionally, fluorescence intensity in 20 positive cells was evaluated.

Western blotting

HepG2 cells were plated at 5×10^4 cells/tissue culture dish in six-well plates with RPMI-1640. After exposure to the inhibitor at different concentrations, the cells were washed with PBS and subsequently lysed in 200 μ L of a lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1 mg/L sodium azide, 10 mL/L NP-40, 1 mmol/L phenylmethylsulfonyl fluoride. Insoluble material was removed by microcentrifugation at 13000 r/min for 15 min at 4°C. Cell lysates (80 μ g of protein/lane) were subjected to electrophoresis on 100 mg/L SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (NEN Life Science Products, Boston, MA, USA).

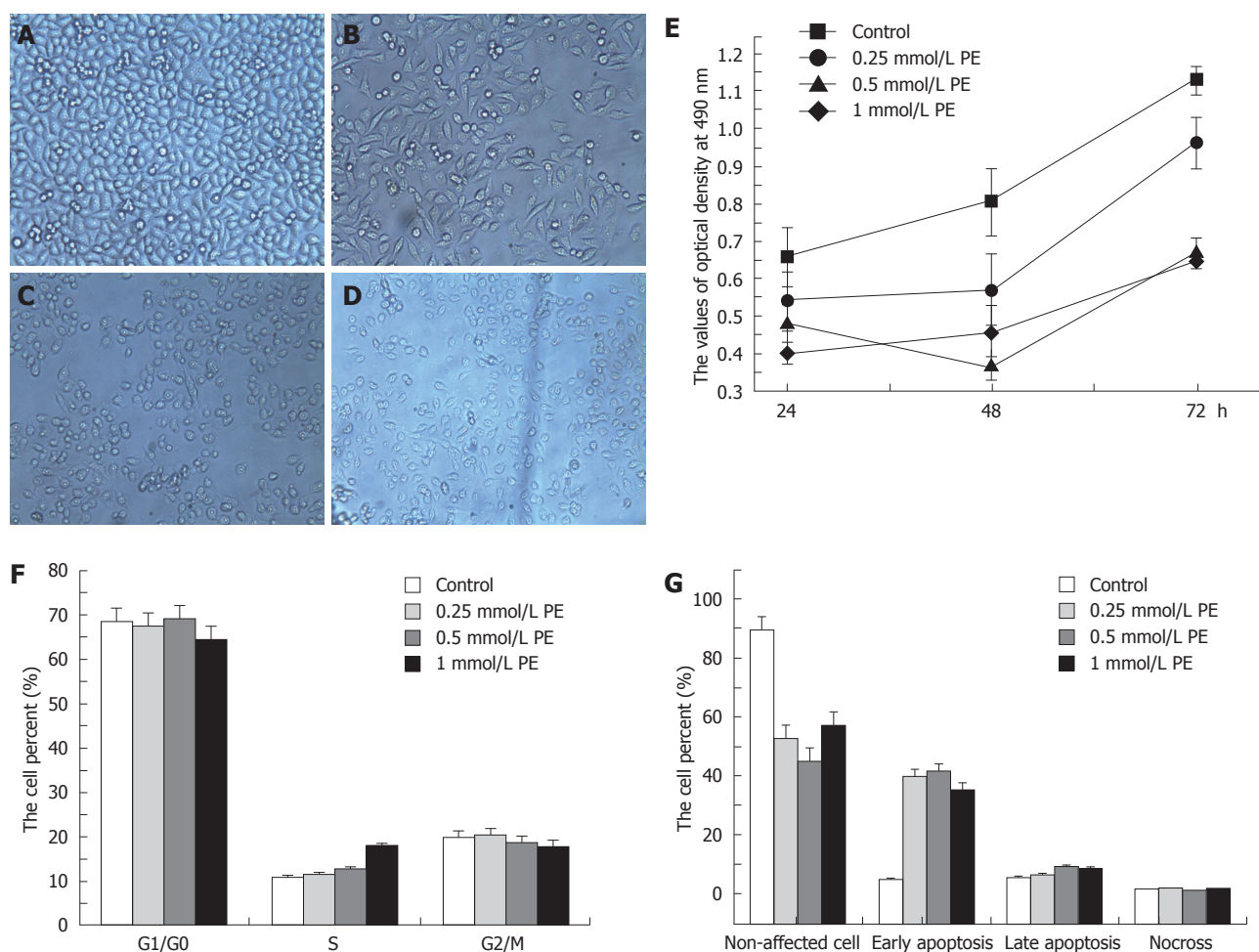


Figure 1 Effect of exogenous PE on the growth and apoptosis of human hepatoma HepG2 cells. A: Control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: 48 h after treatment, inhibition of cell growth shown by MTT assay; F: Cell cycle in human hepatoma HepG2 cells shown by PI staining 24 h after PE treatment, with data showing the cell percentages at G1/G0, S and G2 phases; G: Apoptosis of human hepatoma HepG2 cells shown by annexin- V/PI staining 24 h after PE treatment, with data showing the percentages of non-affected, early and late apoptotic cells and necrosis. The results are given as mean \pm SD from three experiments.

After blocked with Tris-buffered saline containing 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5 mL/L Tween 20, 10 mg/L bovine serum albumin, the membrane was incubated with different monoclonal antibodies (R&D Systems) and anti-glyceraldehyde-3-phosphate dehydrogenase, respectively. For chemiluminescence detection, WB membranes were incubated in the dark with ECL (Amersham) which is a luminol-based enhanced chemiluminescence substrate for horseradish peroxidase. The luminescent signal was recorded and quantified with the Syngene G box (Syngene, UK) which consists of a high-performance CCD videocamera with focus stabilized optics, in a mini darkroom enclosure. The instrument is linked to a computer that controls the instrument and handles the data. The luminescent signal is detected by the CCD camera and transmitted to the controller unit and the data are sent to the computer for analysis and documentation.

Statistical analysis

All data were expressed as mean \pm SD and analyzed by

SPSS 11.0 software. Analysis of data was performed using *t* test. *P* < 0.05 was considered statistically significant.

RESULTS

PE inhibited growth of HepG2 cells

The growth of SMMC7721, HEK293, HeLa and HepG2 cells was detected by MTT assay at different time points after treatment with PE at different concentrations. The results showed that PE inhibited the growth of these cells as well as normal cells (HEK293), suggesting that the inhibition is non-specific (Table 1). HepG2 cells were chosen in our study.

Polygon or fusiform HepG2 cells were observed in the control group with intact and distinct peripheria (Figure 1A), but round HepG2 cells were found in the PE treatment groups with ambiguous peripheria (Figure 1C and D). PE inhibited the growth of HepG2 cells in a dose-and-time dependent manner (Figure 1E). PE did not affect the cell cycle (Figure 1F), but induced apoptosis (Figure 1G).

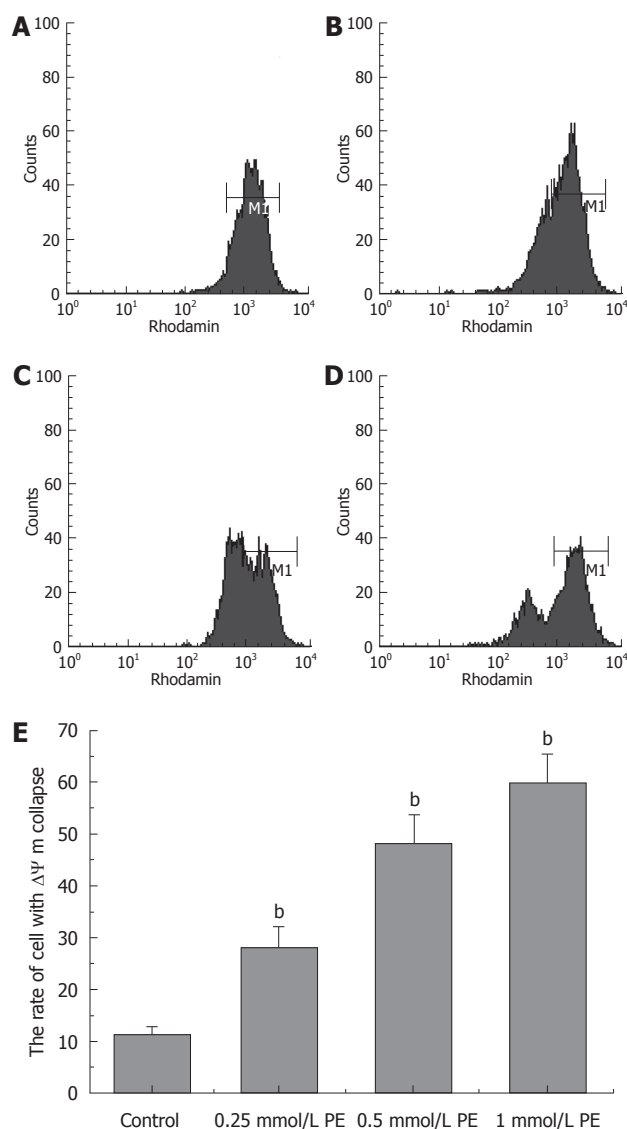


Figure 2 Effects of exogenous PE on the $\Delta\Psi_m$ of human hepatoma HepG2 cells. A: Control group; B, C, D: Groups treated with 0.25, 0.5 and 1 mmol/L PE at 24 h; E: Flow cytometry analysis of cells with $\Delta\Psi_m$ collapse in human hepatoma HepG2 cells shown by rhodamine staining at 24 h. The results are given as mean \pm SD from three repeat experiments. $^bP < 0.01$ vs control group.

Effect of PE on $\Delta\Psi_m$ of HepG2 cells

The uptake of rhodamine123, a lipophilic fluorescent dye absorbed by mitochondria, is positively correlated with $\Delta\Psi_m$. The descent of rhodamine can reflect the collapse of $\Delta\Psi_m$ ^[16]. In order to determine the effect of PE on $\Delta\Psi_m$, the uptake of rhodamine was detected by flow cytometry. PE significantly decreased the $\Delta\Psi_m$ at 0.5 mmol/L and 1 mmol/L (Figure 2C-E), suggesting that PE induces cell apoptosis by decreasing the $\Delta\Psi_m$.

PE induced apoptosis of Bcl-2/Bax in HepG2 cells

Bcl-2 and Bax are involved in the maintenance of mitochondria membrane stability^[17,18]. Immunocytochemical assay showed that Bcl-2 expression in HepG2 cells was significantly suppressed 24 h after treatment with

PE, showing a negative correlation with the PE dosage (Figure 3A-E), while Bax expression was significantly increased 24 h after treatment with PE, showing a positive correlation with the PE dosage (Figure 4A-E). The results of Western blotting and immunocytochemical assay were similar. The Bcl-2 expression level was lower in different PE treatment groups than in the control group at different time points (Figure 3F). However, the Bax expression level was higher in different PE treatment groups than in the control group (Figure 4F).

Involvement of caspase-3 in HepG2 cell apoptosis induced by PE

Caspase-3, a key regulatory protease from which many signaling pathways merge for the execution of apoptosis, participates in apoptosis induced by bcl-2/bax, p38 and JAK-STAT^[19,20]. We detected the caspase-3 expression in HepG2 cells after treatment with PE. Immunocytochemical assay showed that caspase-3 expression was significantly increased, showing a positive correlation with the PE dosage 24 h after treatment (Figure 5A-E). The caspase-3 expression level was higher in PE treatment groups than in the control group (Figure 5F). However, PE increased the caspase-3 expression in a dose- and time-dependent manner (Figure 5F).

DISCUSSION

PE is an important phospholipid component, which is involved in the formation of membrane asymmetry. PE locates at the intracellular leaflet of normal cell membranes, and is exposed to the cell surface during apoptosis. It has been shown that externalization of PE is a signal of early apoptosis^[21]. However, the effect of PE on cell apoptosis remains unclear.

In this study, PE inhibited the growth of HepG2 cells (Figure 1A-E) in a dose-dependent manner. Because the cell cycle and apoptosis are involved in the regulation of cell growth, they were detected with a flow cytometer 24 h after treatment with PE in our study. PE did not significantly affect the cell cycle (Figure 1F), but induced apoptosis of HepG2 cells (Figure 1G), suggesting that PE induces apoptosis by inhibiting the growth of HepG2 cells.

At the early stage of cell apoptosis, $\Delta\Psi_m$ was decreased before chromatin condensation and DNA fragmentation. It was recently reported that mitochondrial dysfunction is essential to the apoptotic pathway, and loss of $\Delta\Psi_m$ may be an early event in the apoptotic process^[22]. Reduced $\Delta\Psi_m$ induces cytochrome C release from the mitochondria, and causes apoptosis^[23-25]. In this study, 0.5 and 1 mmol/L of PE significantly decreased the $\Delta\Psi_m$ (Figure 2C-E), suggesting that PE induces apoptosis of HepG2 cells *via* the mitochondrial pathway. Loss of $\Delta\Psi_m$ was found to be closely associated with the expression of Bcl-2 and Bax. It is generally thought that the expression of Bax increases following death stimulation, and then translocates at the mitochondria to induce cytochrome

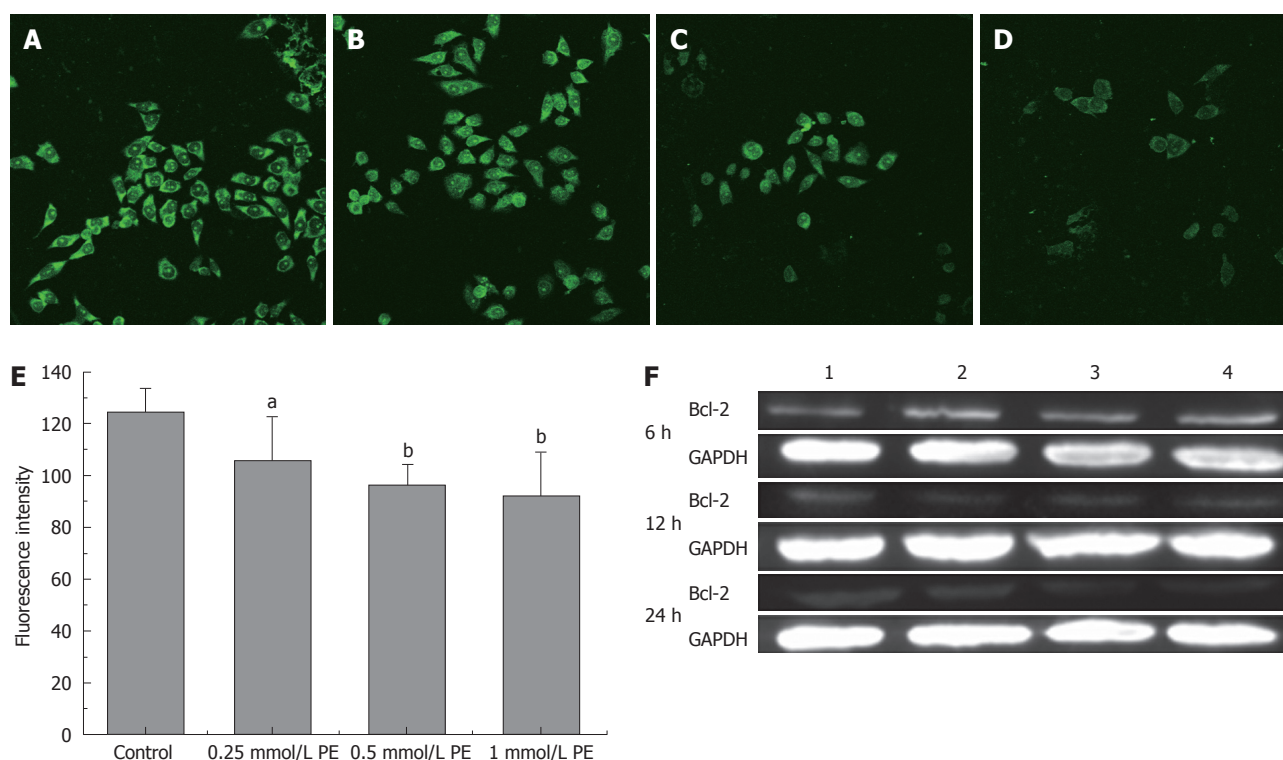


Figure 3 Inhibitory effect of exogenous PE on bcl-2 expression in human hepatoma HepG2. A: Cells in control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: Fluorescence intensity; F: 48 h after treatment and Western blotting. The results are presented as mean \pm SD. Lane 1: Control group; lanes 2-4: 0.25, 0.5 and 1 mmol/L PE treatment groups. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

Table 1 Inhibitory effect of PE on the growth of different types of cells (mean \pm SD)

Time of treatment	24 h				48 h			
	0	0.25	0.5	1	0	0.25	0.5	1
SMMC7721	0.75 \pm 0.06	0.59 \pm 0.03 ^b	0.42 \pm 0.02 ^b	0.30 \pm 0.02 ^b	0.96 \pm 0.03	0.42 \pm 0.02 ^b	0.36 \pm 0.02 ^b	0.30 \pm 0.02 ^b
HepG2	0.66 \pm 0.08	0.54 \pm 0.08 ^b	0.48 \pm 0.07 ^b	0.40 \pm 0.03 ^b	0.81 \pm 0.09	0.57 \pm 0.10 ^b	0.36 \pm 0.03 ^b	0.45 \pm 0.08 ^b
HEK293	0.63 \pm 0.02	0.39 \pm 0.10 ^b	0.40 \pm 0.01 ^b	0.35 \pm 0.02 ^b	0.80 \pm 0.03	0.40 \pm 0.02 ^b	0.31 \pm 0.02 ^b	0.24 \pm 0.03 ^b
HeLa	1.28 \pm 0.09	1.16 \pm 0.06 ^a	1.08 \pm 0.04 ^a	0.95 \pm 0.09 ^b	1.66 \pm 0.11	1.29 \pm 0.01 ^b	1.15 \pm 0.05 ^b	1.01 \pm 0.06 ^b

^a $P < 0.05$ and ^b $P < 0.01$ vs control group.

C release^[26,27]. It is also known that Bax undergoes post-translational modification during apoptosis of HepG2 cells in response to various stimuli with interferon alpha and chemotherapeutic drugs, and the cleaved form of Bax is a potent inducer of apoptosis^[28-30]. Anti-apoptotic Bcl-2 inhibits the pro-apoptotic function of Bax. In the present study, PE up-regulated the expression of Bax and down-regulated the expression of Bcl-2 in HepG2 cells in a dose-and time-dependent manner. Bax expression was observed 12 and 24 h after treatment with 0.5 and 1 mmol/L PE (Figure 4E). The expression of Bax was negatively correlated with decreased $\Delta\Psi_m$, suggesting that increased Bax expression may be involved in the decreased $\Delta\Psi_m$. Increased Bax/Bcl-2 proportion will lead to release of cytochrome C from the mitochondria, thus inducing cell apoptosis^[31]. Experiments *in vitro* have proved that PE plays a key role in cytochrome C transmembrane transport in liposomes composed of acid-phospholipid and neutrophospholipid^[32]. When the amount of the PE in PA/PE/PC system is increased, cytochrome C transmembrane

transport increases in liposome^[32].

Caspases are cystein proteases that play a key role in cascade activation during apoptosis induced by many stimuli^[33-36]. Activation of initiator of caspases (procaspases 8-10) leads to proteolytic activation of downstream effector caspases (caspase-3, -6, -7). The activation of caspase-3 is a common event in two major pathways, death receptor and mitochondrial pathways^[37-40]. In the present study, we proved that PE up-regulated caspase-3 expression in a dose-dependent manner, suggesting that exogenous PE induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway.

COMMENTS

Background

Phosphatidylethanolamine (PE) is one of the dominant lipid constituents in the membranes of animal cells in which it is distributed in an asymmetrical fashion. Many individual phospholipid constituents are known to be involved in specific signaling functions necessary for cells to respond to external stimuli, but the specific signaling function of PE is largely unknown.

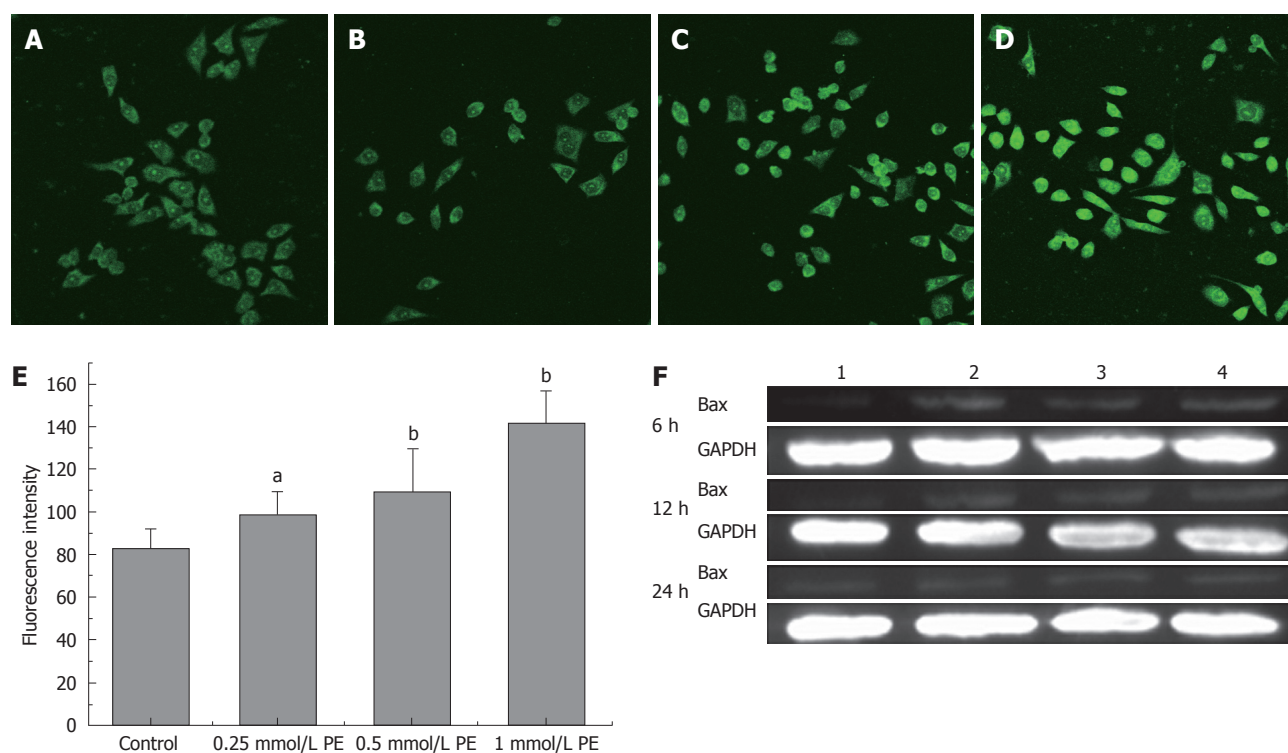


Figure 4 Inhibitory effects of exogenous PE on bax expression in human hepatoma HepG2 cells. A: Control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: Fluorescence intensity; F: 48 h after treatment and Western blotting. The results are presented as mean \pm SD. Lane 1: Control group; lanes 2-4: 0.25, 0.5 and 1 mmol/L PE treatment groups. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

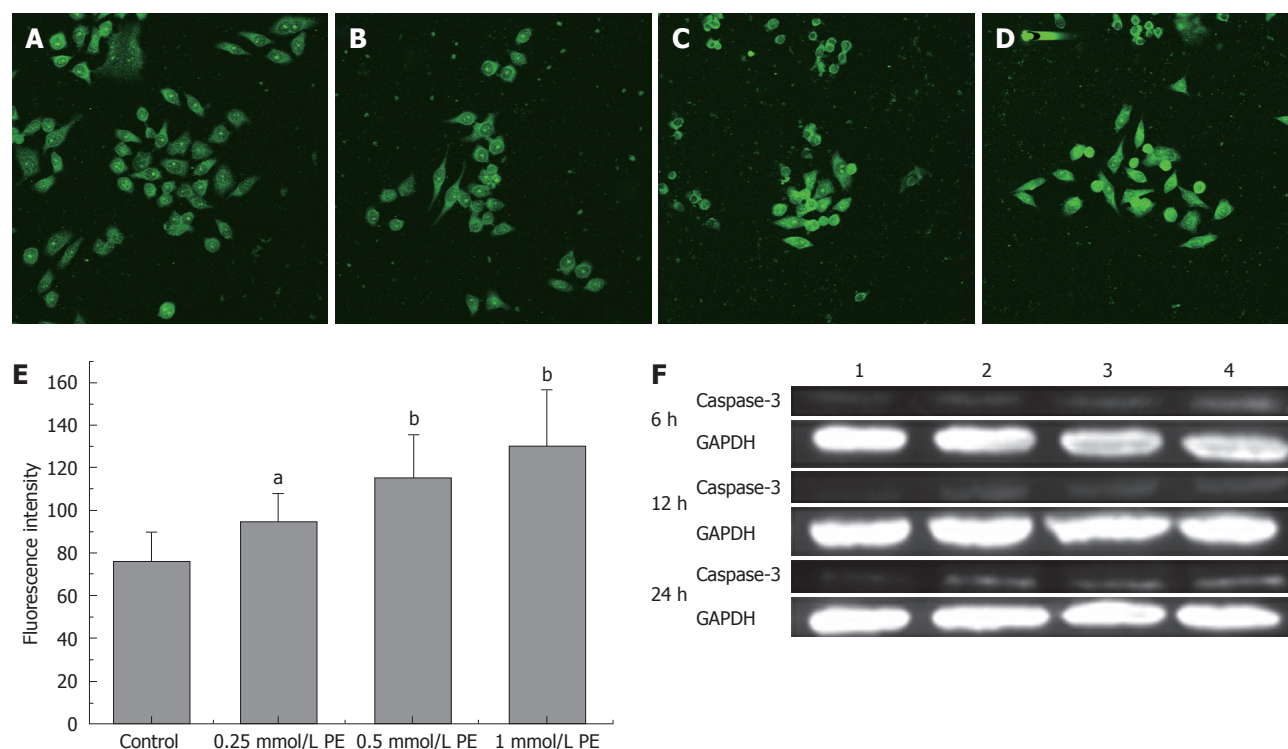


Figure 5 Inhibitory effect of exogenous PE on caspase-3 expression in human hepatoma HepG2 cells. A: control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: Fluorescence intensity; F: 48 h after treatment and Western blotting. The results are presented as mean \pm SD. Lane 1: Control group; lanes 2-4: 0.25, 0.5 and 1 mmol/L PE treatment groups. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

Research frontiers

Translocation of phosphatidylserine (PS) and PE from the internal to external surface of plasma membrane appears to be a fundamental mechanism underlying apoptotic cells. Aminophospholipid translocase involving the

asymmetric distribution of PE in cells was inhibited to induce apoptosis in our study, implying that PE externalization leads to apoptosis. The authors of this paper demonstrated that exogenous PE could induce apoptosis of human hepatoma HepG2 cells.

Innovations and breakthroughs

PS externalization is a typical feature of apoptotic cells. It has been shown that externalized PS serves as a marker for detecting apoptotic cells. PE exposed to the cell surface forms lipid rafts with PS during apoptosis. This is the first study to report that the bcl-2/bax pathway is involved in PE-induced apoptosis of HepG2 cells.

Applications

PE is a dominant component of liposome. This study revealed the mechanism of liposome cytotoxicity to cells.

Peer review

In the present work, the authors showed that PE, one of the important phospholipid components in cell membrane, inhibited the growth of HepG2 cells by inducing apoptosis, but did not change the cell cycle. Furthermore, PE down-regulated Bcl-2 expression, and up-regulated Bax expression in HepG2 cells, induced $\Delta\Psi_m$ collapse, and increased the caspase-3 expression level. Therefore, exogenous PE could induce apoptosis of human hepatoma HepG2 cells via the bcl-2/bax pathway. So it is an interesting work.

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Addition of senna improves quality of colonoscopy preparation with magnesium citrate

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However, abdominal cramps were observed more often under the senna protocol (29.2%) compared to the magnesium citrate only protocol (9.9%, $P < 0.0003$).

CONCLUSION: The addition of senna to the bowel preparation protocol with magnesium citrate significantly improves the cleansing outcome.

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Key words: Colonoscopy; Bowel preparation; Senna; Magnesium citrate; Polyp

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Abstract

AIM: To prospectively investigate the effectiveness and patient's tolerance of two low-cost bowel cleansing preparation protocols based on magnesium citrate only or the combination of magnesium citrate and senna.

METHODS: A total of 342 patients who were referred for colonoscopy underwent a colon cleansing protocol with magnesium citrate alone ($n = 160$) or magnesium citrate and senna granules ($n = 182$). The colonoscopist rated the overall efficacy of colon cleansing using an established score on a 4-point scale. Patients were questioned before undergoing colonoscopy for side effects and symptoms during bowel preparation.

RESULTS: The percentage of procedures rescheduled because of insufficient colon cleansing was 7% in the magnesium citrate group and 4% in the magnesium citrate/senna group ($P = 0.44$). Adequate visualization of the colonic mucosa was rated superior under the citramag/senna regimen ($P = 0.004$). Both regimens were well tolerated, and did not significantly differ in the occurrence of nausea, bloating or headache.

INTRODUCTION

Good bowel preparation is mandatory for optimal intraluminal visualization during colonoscopy. Inadequate bowel cleansing has a negative impact on completion rate^[1] and polyp detection rate^[1,2]. It also increases procedure time^[1,3] and difficulty^[1], and it may affect the procedure safety profile^[4]. All these factors negatively affect therapeutic efficiency and diagnostic accuracy and increase colonoscopy costs^[5].

Magnesium citrate is an osmotic saline agent that increases intraluminal volume resulting in a secondary increase of intestinal motility^[6]. According to a prospective study of colonoscopy practice in 68 hospitals in the UK, magnesium salts are used as bowel cleansing agents in 36.8% of colonoscopies^[7]. Magnesium citrate must be used cautiously in patients with impaired renal function.

Senna, an anthraquinone derivative, is a stimulant laxative stimulating intestinal motility^[8]. Senna has been reported to be a useful adjunct to polyethylene glycol

(PEG) colonoscopy preparation regimens^[9,10]. However, the role of senna as a potential adjunct of magnesium citrate for bowel preparation is yet to be explored.

In two prospective clinical audits, we compared the colon cleansing efficacy and patient tolerability of two colon preparation regimens for adults undergoing colonoscopy: (a) magnesium citrate; and (b) magnesium citrate combined with senna granules.

MATERIALS AND METHODS

Patients

A total of 345 consecutive adult out patients referred to the endoscopy unit of the John Radcliffe hospital for colonoscopy on routine clinical indications were prospectively invited to participate. Exclusion criteria were ileus or suspected bowel obstruction, significant gastroparesis or gastric outlet obstruction, toxic colitis or megacolon, pregnancy, or lactation. Patients included were referred mainly due to alarm symptoms such as altered bowel habit in patients > 45 years of age, blood per rectum, iron-deficiency anaemia, unintentional weight loss, and/or other symptoms suggestive of malignant disease. No patient refused to participate. Patients who could not be sedated for colonoscopy were excluded from further evaluation. All patients gave informed consent before participation.

From July to November 2006, we prospectively audited the performance of the large bowel cleansing with magnesium citrate (citramag-only group). The colon preparation protocol with magnesium citrate and senna (citramag/senna group) was audited from August 2007 to January 2008.

Bowel preparation regimens

All patients were asked to refrain from taking iron tablets 7 d prior to colonoscopy and any medications reducing gastrointestinal motility, e.g. loperamide, 4 d before colonoscopy, but continued all other medications. Two days before colonoscopy, all patients were instructed to eat a low-fiber diet such as white fish, chicken, white bread, eggs, cheese, or potato without skins. High-fiber kinds of food such as red meat, fruit, or vegetables, were to be avoided and patients were advised to consume plenty of fluids. On the day before colonoscopy patients, were instructed to have a low-fiber breakfast. Following that, patients were instructed not to eat any solid food until after colonoscopy and to consume plenty of clear fluids. On the day before colonoscopy at 5:00 pm, patients were instructed to dissolve one sachet of citramag in 200 mL of hot water, which was to be consumed half an hour later when cool. One sachet contains 11.6 g magnesium carbonate and 17.8 g anhydrous citric acid. At 7:00 pm, the patients were instructed to dissolve half of the second sachet of citramag in 100 mL of water and consume it once cooled. Patients were instructed to drink clear fluids (at least a cupful every 30 min) throughout the day and evening before colonoscopy. On the day of colonoscopy, at 6:00-7:00 am in the case of a morning appointment

or at 9:00-10:00 am in the case of an afternoon appointment, patients were instructed to consume the other half of a sachet of citramag as described above.

Patients in the citramag-only group underwent colonoscopy after bowel cleansing as described above (two sachets of citramag). Patients in the citramag/senna group were instructed to follow the above instructions but they were also asked to consume one sachet of senna in a cup of warm water and consume it at 2:00 pm on the day before colonoscopy (two sachets of citramag and one sachet of senna).

Colonoscopy

In order to eliminate interobserver variability, all colonoscopies were performed by the same experienced endoscopist who was, however, not blinded as to the cleansing regimen used. The colonoscopist rated the overall cleansing of the bowel on a 4-point Likert scale as in previous studies^[11-13]: 1 = "unacceptable" (large amounts of solid and semisolid fecal residue requiring additional cleansing resulting in rebooking); 2 = "poor" (enough feces or fluid to prevent a completely reliable examination); 3 = "satisfactory" (small amounts of feces or fluid not interfering with the exam); 4 = "good" (no more than small bits of adherent feces/fluid). For the primary efficacy variable, scores of 3 and 4 were considered "adequate" and scores of 1 or 2 were considered "inadequate".

Symptom score

Prior to colonoscopy, patients were asked to describe their tolerance to the cleansing protocol. Symptoms of nausea, vomiting, bloating, abdominal pain/discomfort, and headache were documented. Patients were asked to rate their symptom severity on a 5-point Likert scale as in previous bowel cleansing studies^[12-14]: 1 = "none"; 2 = "mild"; 3 = "moderate"; 4 = "severe"; and 5 = "extreme". Any adverse event reported during bowel cleansing and/or during the procedure and/or during stay in the recovery room after the procedure was also recorded.

Statistics analysis

Data are shown as mean and standard deviation or as median and ranges, as appropriate. The χ^2 test was used for comparisons between categorical variables and the Student's *t* test was used for comparisons between quantitative variables. All tests were two-tailed and conducted at a 5% significance level.

RESULTS

Overall, 345 patients underwent colonoscopy. Three patients in the citramag-only group could not be sedated for colonoscopy and were excluded from further evaluation. Data analysis was based on 160 patients who underwent colonoscopy after using the citramag bowel cleansing regimen and on 182 patients who performed bowel preparation for colonoscopy according to the combined citramag/senna regimen. Demographic and

Table 1 Demographic and clinical data

	Citramag-only (<i>n</i> = 160)	Citramag/senna (<i>n</i> = 182)
Male/female	76/84	97/85
Age	60 ± 13	58 ± 14
History of bowel resection	6	9
Sedation		
Fentanyl iv (µg)	75 (0-200)	75 (0-200)
Midazolam iv (mg)	4 (0-10)	4 (0-10)
Polyp detection rate	37 (23.1%)	63 (34.6%) ¹
Colorectal cancer	17	16
Completion rate	86% (adjusted 92%)	92% (adjusted 97%) ²

Data are presented as mean and standard deviation or *n* and percentage as appropriate. ¹*P* < 0.03, ²*P* = 0.07.

Table 2 Quality of colon cleansing as evaluated by the endoscopist *n* (%)

	Citramag-only (<i>n</i> = 160)	Citramag/senna (<i>n</i> = 182)
Good	20 (12.5)	41 (22.5)
Satisfactory	88 (55.0)	107 (58.8)
Poor	41 (25.6)	26 (14.3)
Unacceptable	11 (6.9)	8 (4.4)

clinical data of the patients are given in Table 1. The two groups did not differ regarding age, gender, previous history of colorectal surgery, caecal intubation rate, and sedation required (Table 1).

Patients following the citramag-only bowel cleansing regimen

In six of 160 patients (3.8%) using the citramag only cleansing protocol, bowel preparation was so poor with solid fecal residues that the procedure was abandoned in the rectosigmoid, and we did not attempt further insertion. In five other patients the cecum was reached but the view was unacceptable due to fecal residues, especially on the right side. Therefore, a repeat colonoscopy was indicated in these patients as well. Totally, 11/160 (6.9%) of the patients had to be rebooked for colonoscopy or virtual colonoscopy because of insufficient bowel cleansing after the citramag regimen.

Complete colonoscopy with visualization of the cecum was reached in 137/160 colonoscopies (86%). Adjustment for tumor strictures (4), cancellation of the procedure due to poor bowel preparation (6) or severity of colitis (1) raised the completion rate to 92% (137/149).

Patients following the citramag and senna bowel cleansing regimen

Five out of 182 patients (2.7%) were not examined further than the rectosigmoid because of insufficient bowel cleansing after taking the senna protocol, and three had to be rebooked because of insufficient views despite intubation of the cecum. Thus, eight of 182 patients (4.4%) had unacceptable bowel cleansing under the senna/citramag regimen.

The unadjusted completion rate for cecal intubation

was 92%. Adjustment for tumor strictures^[4] and exclusion of procedures abandoned in the rectosigmoid due to poor bowel preparation^[5] resulted in a completion rate of 97%.

Table 2 shows the quality of colon cleansing results for the two regimens used in the current study as assessed by the endoscopist. The combined citramag/senna regimen proved superior in bowel cleansing as it achieved “adequate” colon visualization (quality of colon cleansing rated as “good” or “satisfactory” in 148/182 (81.3%) compared to 108/160 (67.5%) colonoscopies using the citramag protocol (*P* = 0.004; Table 3). The colonic polyp detection rate was higher in the citramag/senna group compared to the citramag-only group (*P* < 0.03; Table 1).

Side effects and tolerability

Both protocols were well tolerated. None of the side effects observed were categorized as extreme, and five patients reported severe side effects during bowel preparation. The two bowel cleansing regimens did not significantly differ in the occurrence and intensity of nausea, vomiting, bloating or headache (Table 3). Abdominal cramps occurred more often in the citramag/senna group (*P* < 0.003; Table 3). Two patients reported “severe” abdominal pain/cramps both of whom had a stricturing tumor in the rectosigmoid.

DISCUSSION

According to our findings, the overall cleansing results were superior using the combination of senna compared to the citramag-only regimen. Although we did not perform a segmental evaluation of colon cleansing, the general impression was that particularly the right colon was better visualized in the citramag/senna group, while in the citramag-only group the cecum and ascending colon were often still covered in sticky solid fecal layers. Having reviewed the relevant bibliography and to the best of our knowledge, the current study is the first to evaluate the efficiency of senna as an adjunct to magnesium citrate for large bowel preparation prior to colonoscopy.

The rationale for using an osmotic agent such as magnesium citrate, together with a stimulant laxative such as senna for colonoscopy preparation, is that increased fluid bowel content produced by the osmotic agent may be more readily evacuated upon bowel stimulation by the stimulant agent^[15]. Previous studies have shown that the combination of PEG with stimulant bisacodyl allows for less volume of PEG to be used for colonic cleansing^[14,16]. Furthermore, the adjunctive use of senna with PEG has been shown to improve the quality of bowel preparation^[9] and to reduce the amount of PEG required for colonic cleansing^[10]. Similarly, we could demonstrate that the combination of senna with magnesium citrate was associated with improved quality in bowel preparation as assessed by a single experienced endoscopist. Although no cost-effectiveness analysis was undertaken, considering the relatively low price of senna^[13] and that bowel preparation has been reported

Table 3 Proportions of patients with side effects reported under bowel cleansing using citramag only or the combination of senna and citramag

Side effect	Treatment group	None (%)	Mild (%)	Moderate (%)	Severe (%)	Extreme (%)	P-value ¹
Nausea	Citramag	83.5	7.9	7.9	0.7	0	NS
	Citramag/senna	76.5	10.3	12.6	0.6	0	
Vomiting	Citramag	94.8	2.6	2.6	0	0	NS
	Citramag/senna	92.6	3.4	4	0	0	
Bloating	Citramag	88.1	5.3	6.6	0	0	NS
	Citramag/senna	81.7	7.4	10.3	0.6	0	
Abdominal pain	Citramag	90.1	6	3.9	0	0	P < 0.0003
	Citramag/senna	70.8	16	12	1.2	0	
Headache	Citramag	95.4	3.9	0.7	0	0	NS
	Citramag/senna	93.7	3.4	2.9	0	0	

¹P-value (significant at the 0.05 level) was obtained using a χ^2 -test to compare the frequency of side effects in treatment groups.

to have an impact on colonoscopy costs^[5], our results suggest that it may be a useful adjunct to magnesium citrate for outpatient colonoscopy preparation.

Bowel preparation was well tolerated in both the magnesium citrate-only and the magnesium citrate/senna group with no self-reported “extreme” symptoms during preparation in any one group. The prevalence of gastrointestinal symptoms did not differ between the two groups with the exception of an about three times increased frequency of abdominal pain noted in the magnesium citrate/senna group which, however, did not seem to affect compliance. No serious adverse events were recorded in either preparation group. Nevertheless it must be noted that no monitoring of laboratory values and electrolytes was performed, which is a limitation of the current study.

Although the majority of patients enrolled had alarm symptoms suggestive of colonic neoplasia (which may explain the relatively high polyp detection rate in both patient groups), our aim was not specifically to explore the potential role of the addition of senna to magnesium citrate in polyp detection. However, more colonic polyps were found in the group receiving bowel preparation with the addition of senna to magnesium citrate, which is in accordance with previous studies showing that the detection of polyps is dependent on bowel cleansing quality^[1,2].

There are certain limitations to the evaluation of the present audits. Although it was conducted in a prospective manner, no placebo was used and the endoscopist was not blinded to the patient group that was colonoscoped. Second, the scales used for the assessment of bowel cleansing and patient symptom severity were not previously validated. Third, no segmental assessment of bowel cleansing quality was performed, and finally, no monitoring of electrolyte levels was performed. As all consecutive patients underwent the same bowel preparation protocol during the audit periods, we can consider the allocation of the bowel cleansing regimen a block randomization.

In conclusion, the addition of senna to bowel preparation protocol with magnesium citrate significantly improves the cleansing outcome. It produces no major side effects but abdominal pains occur more often.

Senna might be a useful adjunct to magnesium citrate for colonoscopy preparation.

COMMENTS

Background

Adequate colon preparation is essential for the quality of colonoscopy. The completion and polyp detection rate as well as the complication risk and the duration of the colonoscopy are strongly affected by poor bowel cleansing.

Innovations and breakthroughs

As far as we know, this is the first study to evaluate the efficacy of senna as an adjunct to magnesium citrate for large bowel preparation prior to colonoscopy. Our findings demonstrate that the addition of senna to the bowel cleansing protocol with magnesium citrate significantly improves the cleansing outcome, but also increases the occurrence of side effects such as (mild) abdominal pains and discomfort.

Applications

Our study results suggest that senna can be used as a helpful adjunct to a cleansing protocol based on magnesium citrate and will increase the efficacy of the bowel preparation before colonoscopy.

Terminology

Magnesium citrate acts as an osmotic laxative which is often used for bowel preparation; senna is a stimulative agent increasing peristaltic movement.

Peer review

This interesting study evaluates how the addition of senna alters the cleansing outcome and the occurrence of side effects under a bowel preparation protocol based on magnesium citrate. The results suggest that senna might be a helpful adjunct to standard bowel preparation protocols.

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BRIEF ARTICLES

Prevalence of linked angina and gastroesophageal reflux disease in general practice

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CONCLUSION: The present study suggested that ischemic heart disease might be found although a patient was referred to the hospital with a complaint of GERD symptoms. Physicians have to be concerned about missing clinically important coronary artery disease while evaluating patients for GERD symptoms.

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Abstract

AIM: To evaluate the association between gastroesophageal reflux diseases (GERD) and coronary heart diseases.

METHODS: One thousand nine hundred and seventy consecutive patients who attended our hospital were enrolled. All of the patients who first attend our hospital were asked to respond to the F-scale questionnaire regardless of their chief complaints. All patients had a careful history taken, and resting echocardiography (ECG) was performed by physicians if the diagnostic necessity arose. Patients with ECG signs of coronary artery ischemia were defined as ST-segment depression based on the Minnesota code.

RESULTS: Among 712 patients (36%) with GERD, ECG was performed in 171 (24%), and ischemic changes were detected in eight (5%). Four (50%) of these patients with abnormal findings upon ECG had no chest symptoms such as chest pain, chest oppression, or palpitations. These patients (0.6%; 4/712) were thought to have non-GERD heartburn, which may be related to ischemic heart disease. Of 281 patients who underwent ECG and did not have GERD symptoms, 20 (7%) had abnormal findings upon ECG. In patients with GERD symptoms and ECG signs of coronary artery ischemia, the prevalence of linked angina was considered to be 0.4% (8/1970 patients).

INTRODUCTION

Chest pain often precedes echocardiographic coronary angiography, electrocardiography (ECG), and scintigraphy for the diagnosis of angina. Many patients presenting to a hospital emergency room for chest pain turn out not to have coronary artery disease (CAD)^[1]. According to data from a large university, 81%-86% of patients evaluated in an emergency room for acute chest pain did not have coronary ischemia^[2,3]. It is well known that non-cardiac chest pain is closely related to gastroesophageal reflux diseases (GERD)^[4,5]. Similarly, erosive esophagitis is not found in some patients with persistent GERD symptoms. Although GERD symptoms affect 10%-30% of the population in Western countries^[6], endoscopic esophagitis is less prevalent, and is reported to occur in up to 2% of individuals^[7-8]. Only one-third of GERD patients have endoscopic positive findings, while others have no obvious mucosal breaks, even though GERD symptoms are present^[9]. Chest pain of esophageal origin can be difficult to distinguish from that caused

by cardiac ischemia because the distal esophagus and the heart share a common afferent vagal supply, and GERD can cause episodes of non-cardiac chest pain that resemble ischemic cardiac pain^[10,11]. It is possible that GERD may be misclassified as angina pectoris and vice versa in clinical practice. The aim of this study was to evaluate the association between GERD and coronary heart disease and to clarify the presence of non-GERD heartburn.

MATERIALS AND METHODS

Ethics

The study was carried out in accordance with the Declaration of Helsinki, and approved by the ethical committee at Toho University.

Patients

Between October 2005 and May 2006, 1970 consecutive patients (934 men and 1036 women with a mean age of 43 years) who first attended the Outpatient Department of General Medicine and Emergency Care of Toho University Omori Hospital were enrolled. Informed consent was obtained from all the patients. None of the patients had a history of use of proton pump inhibitors, H₂-receptor antagonists, antibiotics, steroids, or nonsteroidal anti-inflammatory drugs for a period of at least 2 mo before the investigation. Patients who had a previous history of partial gastrectomy were also excluded from the study.

Questionnaire

All of the patients who attended our hospital were asked to respond to the F-scale questionnaire regardless of their chief complaints. The questionnaire is a self-report instrument, written in a simple and easy-to-understand language, which contained 12 questions. As reported previously by Kusano *et al.*^[12], the following definitions were used to identify symptoms in the F-scale: (1) "Do you get heartburn?"; (2) "Is your stomach bloated?"; (3) "Does your stomach ever feel heavy after meals?"; (4) "Do you sometimes subconsciously rub your chest with your hand?"; (5) "Do you ever feel sick after meals?"; (6) "Do you get heartburn after meals?"; (7) "Do you have an unusual sensation in your throat?"; (8) "Do you feel full while eating meals?"; (9) "Do some things get stuck when you swallow?"; (10) "Do you get bitter liquid coming up into your throat?"; (11) "Do you belch a lot?"; and (12) "Do you get heartburn if you bend over?". Symptoms frequency was measured on the following scale: 0, never; 1, occasionally; 2, sometimes; 3, often; and 4, always. A score of more than 7 points, was considered positive for GERD.

Electrocardiogram

All patients had a careful history taken, and resting ECG was performed by physicians if diagnostic necessity arose. Patients with ECG signs of coronary artery ischemia were defined as having ST-segment depression based on the Minnesota code^[13].

Table 1 Characteristics of study participants

Cardiogram	Abnormal	Normal	P
No. of patients	456	1514	
Age (yr)	48.2 ± 17.6 (16-91)	40.7 ± 15.7 (15-93)	< 0.001
Male/female	231/225	703/811	< 0.05
Hypertension	49/407	84/1430	< 0.001
Diabetes	36/420	45/1469	< 0.001
Hyperlipidemia	79/377	146/1473	< 0.001
Current smoker	156/300	488/1026	NS

Table 2 Relationship between GERD symptoms and ECG abnormalities

Cardiogram	Abnormal	Normal
GERD (+)	8	166
GERD (-)	20	261

Statistical analysis

All values are expressed as means ± SD. Comparisons of groups were made using Student's *t* test or chi-square tests as appropriate. *P* < 0.05 was considered statistically significant.

RESULTS

Overall, ECG was performed in 456 patients (23%, ECG group). The remaining 1514 patients were defined as controls. Patients in the ECG group were significantly older and the male to female ratio was significantly higher than in controls (Table 1). The prevalence of hypertension, diabetes mellitus, and hyperlipidemia was also significantly higher in the ECG group. There was no difference in the proportion of current smokers between the two groups.

Among 712 patients (36%) with GERD, ECG was performed in 171 patients (24%) and ischemic changes were detected in eight patients (5%). Four (50%) of these patients with abnormal findings upon ECG had no chest symptoms such as chest pain, chest oppression, or palpitations. These patients (0.6%; 4/712) were thought to have non-GERD heartburn, which may be related to ischemic heart disease. Of 281 patients who underwent ECG and did not have GERD symptoms, 20 (7%) had abnormal findings upon ECG (Table 2). No significant differences in the prevalence of ischemic changes were found between patients who underwent ECG and those who did not. As shown in Table 3, an exercise thallium test was performed in 12 GERD patients, of whom, one had ischemic coronary artery disease that was proven angiographically. In patients with GERD symptoms and ECG signs of coronary artery ischemia, the prevalence of linked angina, was considered to be 0.4% (8/1970 patients) (Figure 1). In contrast, patient with angina not related to GERD was found in 20 (1.0%).

DISCUSSION

Screening asymptomatic patients is an area of

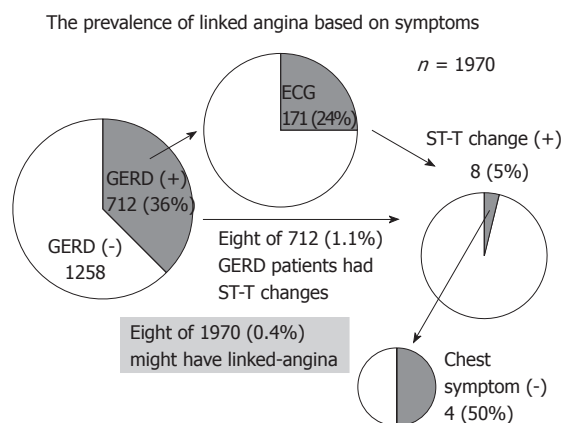
Table 3 Number of patients undergoing additional cardiological tests

	GERD (+) <i>n</i> = 171		GERD (-) <i>n</i> = 285	
	Abnormal findings (+)	Abnormal findings (-)	Abnormal findings (+)	Abnormal findings (-)
ECG	8	163	20	265
Exercised thallium test	2	10	5	13
Cardioangiography	1	0	4	3

considerable interest because silent CAD is an important cause of premature death; many of these patients have three-vessel CAD or left main coronary artery stenosis^[14]. Little is known, however, about the effects of intervention on asymptomatic disease, and screening strategies for asymptomatic CAD have been difficult to justify. Of patients with a low pre-test probability of CAD, only about 20% with a positive exercise test will have angiographically verified CAD^[15]. Little evidence exists currently to support doing resting ECGs^[16] or exercise tests^[17] in patients without clinical evidence of CAD or cardiovascular risk factors.

When coronary arteries appear normal upon angiography, particularly when there is no other cardiac disorder or objective evidence of ischemia, a diagnosis of non-cardiac chest pain is made. In that particular study^[18], cardiac mortality was 0.09% and coronary event rate was 0.65% per year, but 81% of patients had chest pain after 9 years follow-up. The clinician often is faced with the problem of a patient with comparatively mild coronary disease but persistent severe symptoms despite anti-anginal drugs. This subset of patients presents a difficult clinical problem because both the patient and physician usually perceive the pain to be of cardiac origin. It is probable that many of these patients have coexisting coronary disease and a symptomatic esophageal disorder. Symptoms of chest pain are a major source of concern for patients and physicians alike because they can sometimes be the harbinger of acute life-threatening events. However, many patients who describe chest pain that sounds identical to the type associated with significant cardiac disease can actually be free of such disease. After appropriate evaluations, about 40% of patients admitted for potential acute coronary syndromes turn out not to have CAD^[19,20]. A study at the Emergency Room Assessment of Sestamibi for Evaluating Chest Pain Trial (ERASE trial), and a second study at a large hospital center in Philadelphia, showed that 81%-86% of all patients presenting to the emergency department with chest pain did not have a final diagnosis of cardiac ischemia^[3,21]. Thus, differentiation between cardiac- and esophageal-origin chest pain is especially difficult since both organs have overlapping sensory pathways: Th1-Th4 for the heart and C8-TH10 for the esophagus. Moreover, they have autonomic nerve reflex, the so-called vagal visceral reflex function between the heart and organs of the gastrointestinal tract^[22].

Direct mechanical effects between the esophagus

**Figure 1** Prevalence of linked angina based on symptoms.

and the heart are thought to be compression of the left atrium by a huge hiatus hernia or extrinsic esophageal compression by cardiac enlargement. Other than these direct effects, a neural reflex, mediated by the vagus nerve, exists that allows changes in esophageal function to affect cardiac physiology^[22]. Atrial tachycardia can be triggered by swallowing and belching, although the precise neural mechanism remains unclear^[23]. Furthermore, bradycardia occurs in most people during balloon inflation within the esophagus and this may be blocked by atropine^[24]. It has been suggested that esophageal dysfunction can itself trigger myocardial-ischemia-linked angina^[25]. Smith *et al*^[25] have coined the term “linked angina”, which implies that gastrointestinal factors bring on attacks of genuine angina in patients with established CAD. They have explained this on the basis of cardiovascular changes, although gastroesophageal acid reflux may equally explain the phenomenon. Instillation of acid into the esophagus has been shown to significantly reduce the exertional angina threshold at which angina occurs or can provoke angina with ischemic ECG changes^[26]. A previous animal study has demonstrated the reduced coronary flow caused by distension of the stomach that does not occur after vagal section or after the administration of atropine. This suggests that reflex coronary vasoconstriction is initiated by vagal irritation in the gastrointestinal tract^[27]. Mellow *et al*^[28] have demonstrated that acid perfusion of the esophagus results in reduced coronary blood flow in patients with proven CAD. It should be emphasized that 67% of patients felt chest pain and two-thirds of them developed ischemic ST-segment changes upon ECG. Dobrzycki *et al*^[29] have also reported that GERD patients have a larger total ischemic burden and higher incidence of ST depression. Thus, spontaneous GER may have a role to play in the causation of or provocation of cardiac chest pain. Linked angina can be defined as that induced by GER. However, most patients with asymptomatic angina or symptoms, that do not reach the threshold required to define disease, do not refer to a hospital. Therefore, the present study was designed in the Department of General Medicine to evaluate the association between GERD and coronary heart disease in the general population because general

practice registers offer the best means of sampling the general population^[30]. We hypothesized that the prevalence of CAD in GERD patients who were referred for the first time to the gastroenterology outpatient clinic because of heartburn may be substantial.

The present study revealed a small number of patients with abnormal ECG findings but without chest symptoms such as chest pain, chest oppression, or palpitations. This suggests that the extra-esophageal condition causes GERD symptoms and that angina may be misclassified as GERD. Such patients are often treated with proton pump inhibitors and chest pain disappears, which suggests that CAD may be overlooked. The results may therefore have clinical relevance, as it has been reported in a previous population-based, nested case-control study that patients with GERD have an increased risk of angina pectoris in the year after GERD diagnosis^[31]. Actually, the incidence of reflux esophagitis is increasing as the population grows older, which makes it likely that such disease may be present in patients with CAD^[32]. Physicians have to be concerned about missing clinically important CAD while evaluating patients for GERD symptoms.

COMMENTS

Background

Screening asymptomatic patients is an area of considerable interest because silent coronary artery disease (CAD) is an important cause of premature death, although many patients presenting to a hospital emergency room for chest pain turn out not to have CAD. It is well known that non-cardiac chest pain is closely related to gastroesophageal reflux diseases (GERD) and GERD symptoms affect 10%-30% of the population. Little is known, however, about the prevalence of linked angina, which is defined as angina induced by GER.

Research frontiers

In patients with GERD symptoms and echocardiography signs of coronary artery ischemia, prevalence of linked angina, was considered to be 0.4% (8/1970 patients). The prevalence may be increased when more sensitive tests such as angiography are used for diagnosis of angina.

Innovations and breakthroughs

Recent reports have highlighted the association between non-cardiac chest pain and GERD because both organs have overlapping sensory pathways. However, there have been few reports that GERD symptoms may be caused by ischemic heart disease. The present study is believed to be the first to evaluate the presence of non-GERD heartburn. GERD symptoms induced by coronary artery ischemia, so-called linked angina, certainly exist.

Applications

The study results suggest that an extra-esophageal condition causes GERD symptoms and that angina may be misclassified as GERD. Since patients with GERD have an increased risk of angina pectoris in the year after GERD diagnosis, physicians have to be concerned about missing clinically important CAD while evaluating patients for GERD symptoms.

Terminology

Linked angina is defined as angina induced by GER. GERD symptoms typically include heartburn and regurgitation. GERD is diagnosed according to symptoms regardless of the presence of endoscopically proven esophagitis.

Peer review

The authors examined the prevalence of linked angina. The results suggest the possibility that GERD patients also have coronary heart disease.

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Lower gastrointestinal bleeding secondary to a rectal leiomyoma

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Abstract

The occurrence of leiomyoma of the rectum is uncommon. Most of these lesions are clinically silent and are found incidentally during laparotomy or endoscopic procedures for unrelated conditions. Symptomatic leiomyomas of the rectum are encountered less frequently, with only sporadic reports in the literature. We describe a case of a leiomyoma of the rectum presenting as recurrent lower gastrointestinal hemorrhage and secondary anemia.

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Key words: Rectal leiomyoma; Gastrointestinal bleeding; Endoscopy; Endoscopic ultrasonography; Immunohistochemistry

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De Palma GD, Rega M, Masone S, Siciliano S, Persico M, Salvatori F, Maione F, Esposito D, Bellino A, Persico G. Lower gastrointestinal bleeding secondary to a rectal leiomyoma. *World J Gastroenterol* 2009; 15(14): 1769-1770 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1769.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1769>

INTRODUCTION

Smooth muscle tumors may occur throughout the entire gastrointestinal tract, but are rarely seen in the colon and rectum. Most of these lesions are clinically silent and are found incidentally during laparotomy or endoscopic procedures for unrelated conditions. Symptomatic leiomyomas (LMs) of the rectum are encountered less frequently, with only sporadic reports in the literature. We describe a case of LM of the rectum, presenting as recurrent lower gastrointestinal hemorrhage and secondary anemia.

CASE REPORT

A 55-year-old woman presented to our unit complaining of recurrent rectal bleeding and secondary sideropenic anemia. Colonoscopy revealed the presence of a polypoid, submucous, ulcerated lesion in its vertex (2 cm from the anal margin) (Figure 1).

An endoanal ultrasound scan showed a mass located in the anterior wall of the rectum, approximately 7 cm in size, with no infiltration of perirectal fat (Figure 2). A biopsy was made and the pathological study showed a proliferation of fusiform, elongated spindle cells arranged in fascicles. The nuclei were elongated and cigar-shaped, and there was minimal nuclear pleomorphism. No mitotic figures were seen (Figure 3). Immunohistochemistry was positive for smooth muscle actin (SMA) and desmin and negative for CD117.

With a preoperative diagnosis of rectal LM, the mass was removed by local excision with preservation of the rectum. The patient is currently in the 12th mo of follow-up, and has no signs or symptoms of relapse.

DISCUSSION

Primary LMs present most commonly in the female genital tract and as skin lesions. This tumor is seldom encountered in the gastrointestinal tract. The most common localization is the stomach, followed by the small intestine. The colon, rectum and esophagus are less likely sites. LM of the anorectal region represent 3% of all gastrointestinal LM, and less than 0.1% of rectal tumors^[1-6].

Most reported LMs are sessile intraluminal or intramural tumors. They can also present as pedunculated extra luminal mass of the colon^[7]. LM often remain asymptomatic until they have reached a fairly large size. The clinical manifestations of these smooth muscle

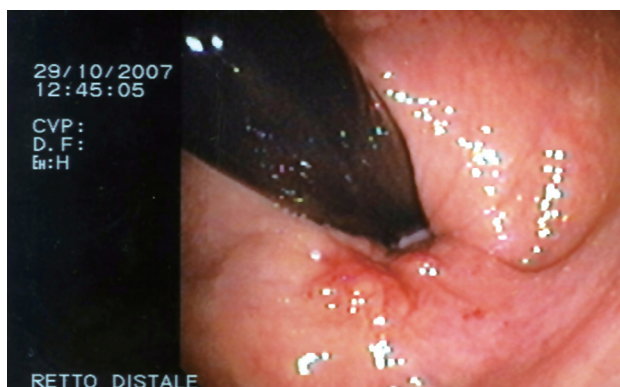


Figure 1 Endoscopic view of a polypoid, submucous, ulcerated lesion in its vertex.

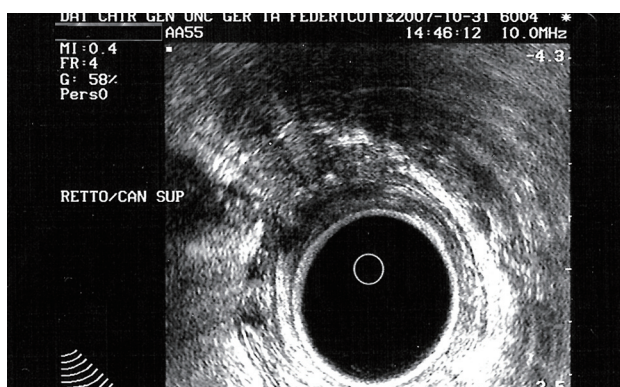


Figure 2 Endoanal ultrasound scan showing a mass located in the anterior wall of the rectum.

tumors depend on the location, size and direction of tumor growth. They include intestinal obstruction, hemorrhage, and perforation into the peritoneal cavity.

Intraluminal lesions can be detected earlier because of the earlier presentation of symptoms. Many of these tumors are discovered incidentally on routine endoscopic examination of the large bowel. Endoscopically, these tumors can present as pedunculated intramural or intraluminal polyps, and they may look like the more usual adenomas. Complementary investigation, such as with computed tomography, endoscopic ultrasonography, and magnetic resonance imaging, strongly corroborates the diagnosis. Endorectal ultrasound can help to define the extent of disease and may be a useful adjunct in deciding about the appropriate surgical procedure^[8].

The biological behavior of smooth muscle tumors varies from benign to locally aggressive and highly malignant. The biological behavior may not be reflected by the histology, as even benign-looking smooth muscle tumors may metastasize. Thus, a combination of site, tumor size, histological appearance and mitotic count give the best prediction of behavior^[9].

LM should be separated from gastro-intestinal stromal tumors (GISTs). LMs are positive for actin and desmin and negative for CD34 and CD117 (KIT), and

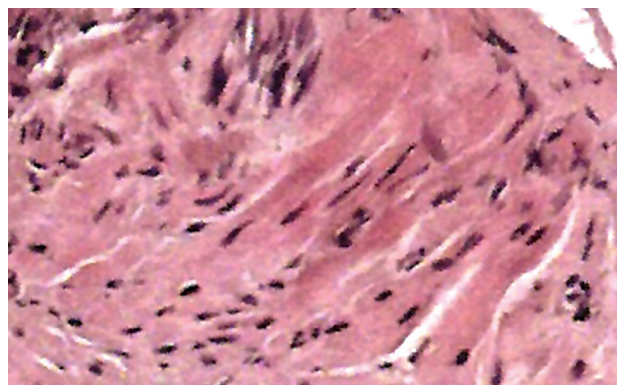


Figure 3 Microscopic findings showing a proliferation of fusiform, elongated spindle cells arranged in fascicles.

GISTs have the opposite pattern^[10]. Surgical excision is the treatment of choice for most LMs. Snare polypectomy is an adequate treatment, but large LMs are believed to be best treated by surgical resection, because conventional colonoscopic resection of large and deep-seeded tumors poses a high risk of perforation^[11]. Ensuring the complete removal and follow-up are necessary precautions for tumors with any atypia or mitotic activity.

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Infliximab to treat severe ulcerative colitis

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Abstract

A 48-year-old female with severe ulcerative colitis refractory to conventional therapy was referred to our facility for management. The patient showed extensive ulcerative colitis since the age of 20 years and had failed therapy with 5-aminosalicylic acid agents and azathioprine. The disease remained active despite treatment with steroids and cyclosporine. The clinical and endoscopic parameters were consistent with severe disease. Infectious precipitants were ruled out. Given the severity of the disease and in order to avoid a colectomy, we started the patient on infliximab therapy. A dramatic clinical and endoscopic response was observed and she remained in remission at the end of a 1-year follow-up period. We discuss findings in the literature regarding the use of infliximab therapy in patients with ulcerative colitis who have failed steroids and cyclosporine.

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Key words: Cyclosporine; Infliximab; Treatment failure; Ulcerative colitis; Inflammatory bowel diseases

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INTRODUCTION

Infliximab is a monoclonal antibody against tumor necrosis factor alpha (TNF- α) used in the treatment of Crohn's disease and ulcerative colitis. It has demonstrated efficacy in patients with moderate to severe ulcerative colitis, as well as in those with severe disease who have failed intravenous (IV) steroids^[1]. According to historical data, patients with severe disease who fail IV steroids have a risk of colectomy of up to 60%. Currently, there are three therapeutic options: cyclosporine, infliximab and colectomy^[2]. Such patients are often treated empirically with cyclosporine or infliximab. If the patient fails to respond to either, colectomy is typically performed. Using infliximab to treat patients who have failed steroids and cyclosporine is controversial, since the risk of opportunistic infection is considered to be high. We report a case in which infliximab was safely introduced and successfully used in a patient with severe ulcerative colitis who would otherwise have undergone colectomy.

CASE REPORT

A 48-year-old female with ulcerative colitis presented to our facility for the management of refractory disease. She had extensive ulcerative colitis since the age of 20 years and had a number of flares requiring IV and oral (PO) steroids, having failed 5-aminosalicylic acid agents. In the prior 6 mo, the patient had been treated with azathioprine at 2 mg/kg per day and both IV and subsequent PO cyclosporine (8 mg/kg per day). She was referred for further management as she was passing in excess of six stools per day, as well as presenting bloody diarrhea and abdominal pain. She had severe anemia, with repeated need for blood transfusions. Sigmoidoscopy confirmed active ulcerative colitis (Figures 1 and 2). This clinical spectrum was despite current therapy with cyclosporine and azathioprine.

At the time of presentation, her blood pressure was 160/100 mmHg, with a pulse rate of 150 bpm, and an intense generalized abdominal pain. She had a white blood cell count of $161\,000/\text{mm}^3$, hematocrit of 13%, hemoglobin of 5.0 g/dL, erythrocyte sedimentation rate of 130 mm in the first hour, C-reactive protein of $> 5\text{ mg/dL}$, iron of 10 mg/dL, albumin of 2 g/dL, and alpha 1-acid glycoprotein of 230 mg/dL. A colonoscopy revealed severe ulcerative colitis extending from the rectum (Figure 1). Biopsies confirmed chronic active colitis.

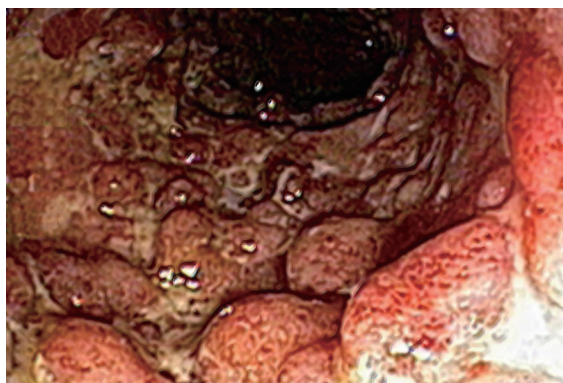


Figure 1 Rectal view during treatment with azathioprine.

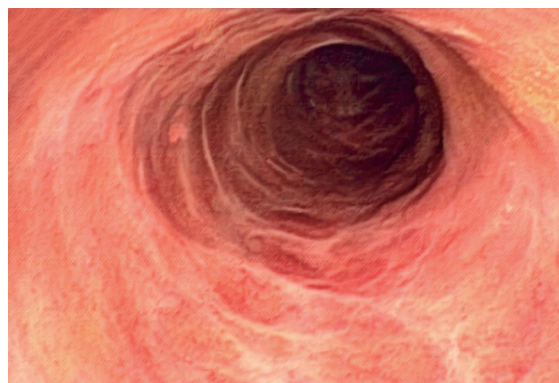


Figure 3 Colonoscopy: Rectal view after 18 mo of treatment with infliximab.



Figure 2 Colonoscopy: Sigmoid view during treatment with azathioprine.



Figure 4 Colonoscopy: Sigmoid view after 18 mo of treatment with infliximab.

Table 1 Laboratory test results by treatment regimen

	Azathioprine	Cyclosporine	Infliximab (after 18 mo of treatment)
Hemoglobin, g/dL	5.0	8.0	13.0
Hematocrit (%)	13	19	40
Erythrocyte sedimentation rate, mm in the first hour	130	230	7
Albumin, g/dL	2	2.5	3.5
Iron, mg/dL	10	15	55
α -1 glycoprotein, mg/dL	230	245	< 100
C-reactive protein, mg/dL	> 5	> 5	< 5

After 18 mo of infliximab treatment, the patient was in remission, with one stool per day and no abdominal pain. A repeat colonoscopy demonstrated marked endoscopic improvement (Figures 3 and 4). There was a dramatic improvement in the overall nutritional status of the patient and in the serum levels of all parameters, as shown in Tables 1 and 2. She remained in remission on maintenance infliximab and azathioprine at the last assessment.

DISCUSSION

Refractory ulcerative colitis is currently defined as an inadequate response to conventional treatment. In cases of ulcerative colitis, the symptoms used to determine whether an individual is refractory to treatment include

Table 2 Mayo scale score for ulcerative colitis by treatment regimen

	Azathioprine	Cyclosporine	Infliximab
Frequency of evacuations	3	3	0
Rectal bleeding	3	3	0
Endoscopic findings	3	3	0
Overall medical evaluation	3	3	0

fever, diarrhea three or more times per day, bleeding, and fecal urgency^[3]. Immunomodulators, such as azathioprine, have been used as adjuvant therapy in patients with ulcerative colitis who are classified as non-responders to oral steroids, the recommended initial dose being 2 mg/kg per day, which can be gradually increased if a satisfactory response is not achieved^[4].

Cyclosporine has been used in refractory patients, as well as in patients classified as non-responders, who typically present extensive or severe colitis. For such patients, treatment with cyclosporine appears to control the disease in approximately 90% of cases^[5-8]. In the present case, the patient did not exhibit a sustained response to cyclosporine, and there was recurrence within 1 mo, at which point infliximab was initiated.

Infliximab, a chimeric monoclonal antibody to TNF- α , was originally believed to bind biologically active TNF- α freely present in the lamina propria and expressed on inflammatory cells. However, it soon became clear that this binding phenomenon had to be

followed by complement binding and activation, leading to apoptosis of the activated inflammatory TNF- α -bearing cells, as well as inducing apoptosis of T cells^[9-11].

In two large-scale studies, designated Active Ulcerative Colitis Trial 1 and Active Ulcerative Colitis Trial 2, patients with ulcerative colitis were treated with infliximab, together with corticosteroids or the 6-mercaptopurin/azathioprine combination, and were monitored/evaluated using the Mayo scale^[12,13]. The authors found that the rate of remission was higher in patients treated with infliximab than in those receiving placebo.

Currently, the use of infliximab in patients with ulcerative colitis is recommended for those with corticosteroid dependence, refractory pouchitis and pouchitis in the maintenance phase, although there is still controversy regarding the appropriate duration of treatment^[14,15]. Treating patients with infliximab after failing steroids and cyclosporine is controversial, as one study reported a serious adverse event rate of 15%, due to opportunistic infections^[16-18]. In the case described here, we opted to maintain the infliximab, taking into account the quality of life that could be sustained without surgical intervention. The patient remained under treatment with infliximab every 8 wk for 2 years, at a dose of 5 mg/kg without adverse events and recurrence of the symptoms.

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CASE REPORT

A case of small bowel adenocarcinoma in a patient with Crohn's disease detected by PET/CT and double-balloon enteroscopy

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the small bowel appeared to be associated with the cancer development. Previous reports suggest the risk of SBA in patients with CD is higher than in the overall population. Since early diagnosis is extremely difficult in these cases, novel techniques, such as PET/CT and DBE, may be expected to help in making a preoperative diagnosis of the development of SBA in CD.

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Key words: Crohn's disease; Double-balloon enteroscopy; Positron emission tomography; Small bowel adenocarcinoma; Surveillance

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Kodaira C, Osawa S, Mochizuki C, Sato Y, Nishino M, Yamada T, Takayanagi Y, Takagaki K, Sugimoto K, Kanaoka S, Furuta T, Ikuma M. A case of small bowel adenocarcinoma in a patient with Crohn's disease detected by PET/CT and double-balloon enteroscopy. *World J Gastroenterol* 2009; 15(14): 1774-1778 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1774.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1774>

Abstract

Small bowel adenocarcinoma (SBA) in patients with Crohn's disease (CD) is quite rare, difficult to diagnose without surgery, and has a poor prognosis. Here, we report a 48-year-old man with SBA and a 21-year history of CD who was diagnosed by a combination of positron emission tomography/computed tomography (PET/CT) and double-balloon enteroscopy (DBE). Since the age of 27 years, the patient had been treated for ileal CD and was referred to our hospital with persistent melena. Multiple hepatic tumors were found by CT. PET/CT detected an accumulation spot in the small bowel. DBE revealed an ulcerative tumor in the ileum about 100 cm from the ileocecal valve. An endoscopic forceps biopsy specimen showed poorly differentiated adenocarcinoma. There were some longitudinal ulcer scars near the tumor, and the chronic inflammation in

INTRODUCTION

Previous studies have documented that patients with inflammatory bowel disease (IBD) are at increased risk of developing colorectal and intestinal cancer^[1-6]. However, because of its rarity, there is limited data on the precise cancer risk of small bowel adenocarcinoma (SBA) in patients with Crohn's disease (CD). To date, recommendations for screening and surveillance have been supported by very limited data^[7]. Even in extensive colitis, a recent report found that colonoscopy surveillance may not improve survival^[8], and other tools are needed to detect cancer development in the early stages. Here, we report a patient with a 21-year history of CD who developed a SBA that was detected by 18F-fluorodeoxyglucose (FDG) positron emission

tomography/computed tomography (PET/CT) and double-balloon enteroscopy (DBE) without conventional intestinal examinations.

CASE REPORT

A 48-year-old man who was diagnosed with ileal CD as a 27-year-old was referred to our hospital with persistent melena. His family history was negative for this disease. He had no evidence of gluten intolerance. He had been treated with 5-aminosalicylates (5-ASAs) combined with an elemental-diet (ED) therapy and had several hospitalizations for temporary melena in the past two decades. He had not agreed to have an intestinal examination at regular intervals. In January 2007, he was examined by abdominal CT due to persistent melena for 1 year, and multiple metastatic tumors were discovered in the liver (Figure 1A). Blood tests showed a slight anemia (Hb 11.8 g/dL), but he was negative for inflammatory factors. The serum carcinoembryonic antigen (CEA) was elevated (31.6 ng/mL; normal range, < 2.5 ng/mL), and wall thickening in part of the ileum was detected by CT (Figure 1B). PET/CT showed accumulations in the multiple hepatic tumors and the wall thickening of the ileum (Figure 2). DBE showed an ulcerative tumor in the ileum about 100 cm away from the ileocecal valve (Figure 3A). There were some longitudinal ulcer scars near the tumor (Figure 3B). An endoscopic forceps biopsy specimen showed microscopically poorly-differentiated adenocarcinoma (Figure 4A and B). Immunohistochemistry of the tumor cells was negative for p58 and positive for β -catenin (Figure 4C and D). Accordingly, he was diagnosed with SBA with hepatic metastases. Although he was treated by chemotherapy with S-1 and cisplatin (CDDP), there was no effective response. He died 4 mo after the diagnosis because of liver failure due to progression of the hepatic metastases.

DISCUSSION

Primary SBA is rare and the incidence is reported to be 1 to 5% of all gastrointestinal tract malignancies^[9,10], and the most important known risk factor for this malignant tumor is previous CD^[5,11]. SBA in CD was reported for the first time by Ginzburg in 1956^[12]. The risk for SBA is reportedly higher in patients with CD than in the overall population. Recent meta-analysis revealed the relative risk of SBA in patients with CD as 28.4 (95% CI, 14.5-55.7)^[13] to 33.2 (95% CI, 15.9-60.9)^[14]. In clinical settings, SBA in CD is difficult to diagnose, and most previous cases were diagnosed after surgery without suspicion of malignancy. Therefore, novel tools are needed to detect and survey the development of this cancer. In our case, we were able to diagnose SBA non-surgically by a novel approach using PET/CT and DBE.

Based on previous reports, Dossett *et al*^[3] summarized the 154 cases of SBA in CD reported in Europe and America. SBA in CD occurred more frequently in males than females (M:F ratio of 2.4:1). The age at diagnosis

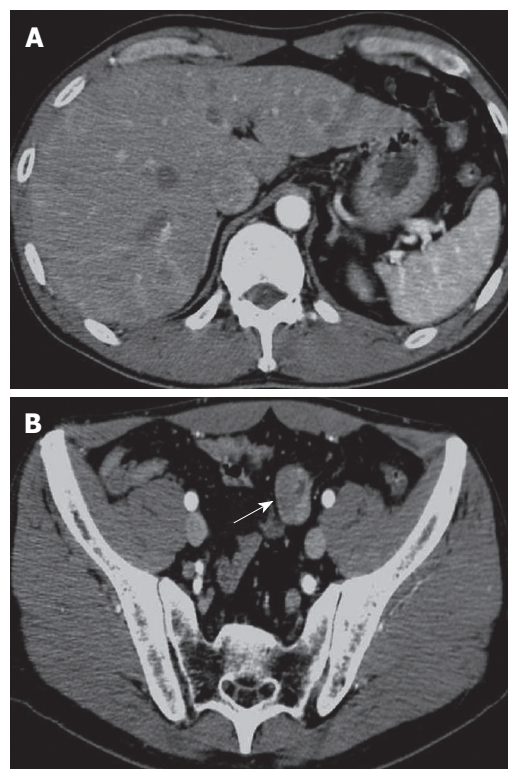


Figure 1 CT findings. A: Abdominal CT showed the multiple hepatic tumors with ring enhancement; B: Wall thickening of a part of the small bowel (arrow).

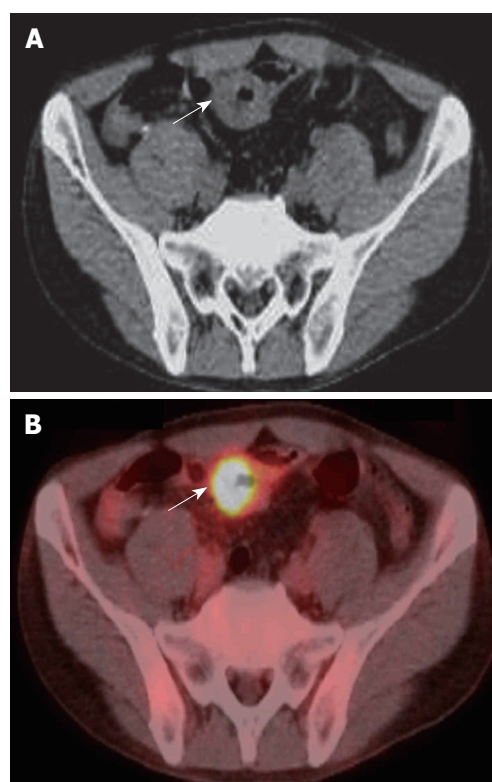


Figure 2 PET/CT findings. A: Wall thickening of a part of ileum (arrow). B: PET/CT showed ¹⁸F-FDG accumulation in the site of wall thickening of ileum (arrow).

ranged from 21 to 86 years (mean age, 51.3), and the average duration of CD was 24.5 years (0-45 years)^[3]. SBA in CD was observed at a younger age^[11] as compared

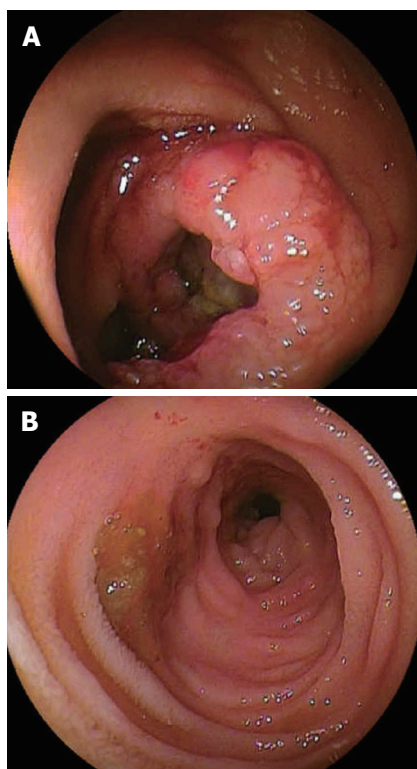


Figure 3 DBE findings. A: DBE showed ulcerative mass located in the ileum at about 100 cm away from ileocecal valve; B: Some longitudinal ulcer scars near the tumor.

with *de novo* cancers that were found in 60-69-year-olds^[15]. Tumors were found in the ileum at a rate of 75%. The presence of previously bypassed segments of the intestine was 20.3%. Obstruction was the most common manifestation (76%), whereas hemorrhage, fistula, and perforation were observed in 3.9%, 3.9%, and 5.4% of cases, respectively. The majority of diagnoses were made at the time of operation (35.4%) or postoperatively (61.5%). Only 3.1% of the cases could be diagnosed preoperatively. Survival rates after 1 and 2 years were 49.6% and 27%, respectively^[3].

In the present case, there were some longitudinal ulcer scars near the tumor, suggesting that chronic inflammation is associated with the development of SBA. Because it is well known that a combination of immunohistochemically strong p53 expression and absent or weak β -catenin expression in ulcerative colitis (UC) patients is evidence for colitis-associated dysplastic lesions^[16], we examined the immunohistochemical characteristics of the tumor^[17]. In this case, there was no detectable p53 expression, but strong β -catenin expression. Chemotherapy and radiation for SBA have produced disappointing results, and only a few studies have evaluated chemotherapy for unresectable SBA in CD^[18]. There have been no controlled studies recommending an effective treatment regimen. Although we treated this patient by systemic chemotherapy with S-1 and CDDP in agreement with the patient, the therapy was not effective and did not seem to prolong the survival time.

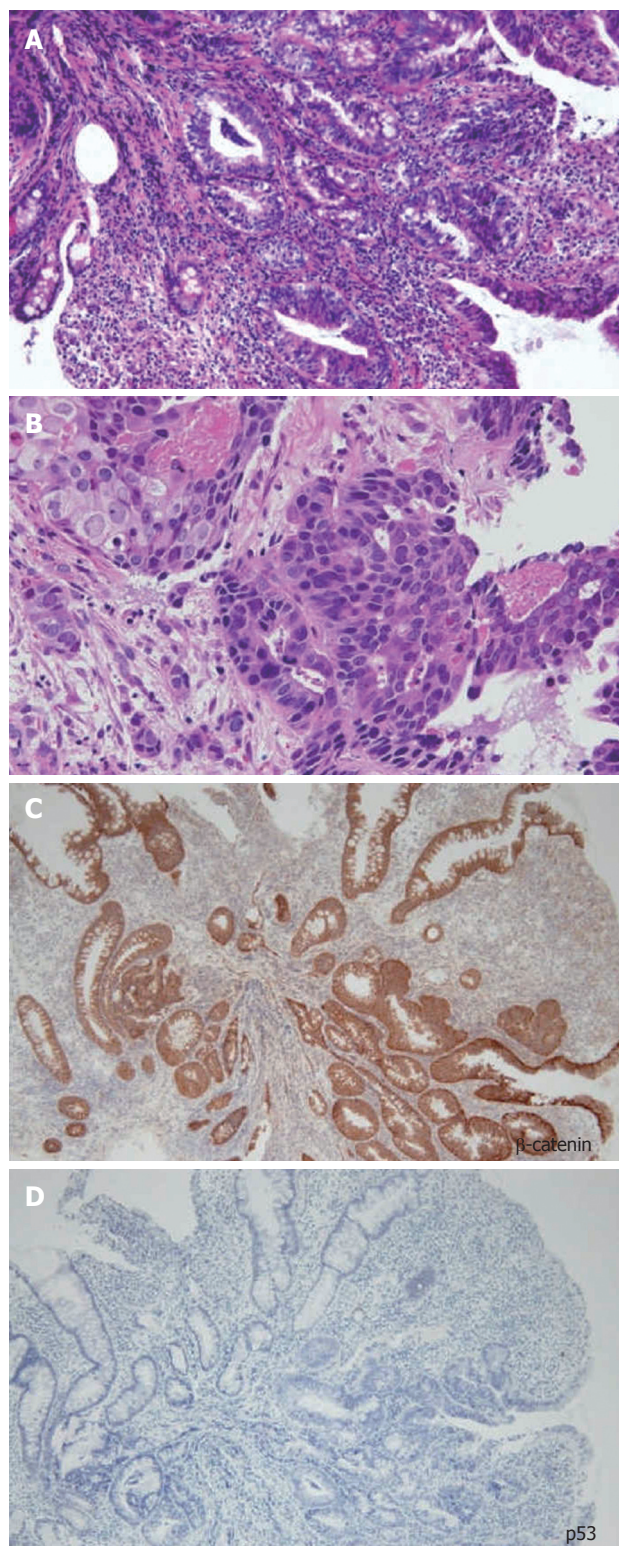


Figure 4 Histopathological findings of the biopsy specimen showed poorly differentiated adenocarcinoma. A: HE ($\times 100$), B: HE ($\times 400$), C: Immunohistochemical staining using β -catenin antibody ($\times 100$), D: Immunohistochemical staining using p53 antibody ($\times 100$).

There are several previous reports of long-standing risk factors for SBA, including onset of the disease before the age of 30 years, presence of a bypassed segment, chronic active course with stricture and fistulas, male gender, and smoking^[1-3,5,11,19-21]. Corticosteroid and

azathioprine therapy to treat CD are also considered potential risk factors. However, 5-ASAs prevent the development of intestinal adenocarcinoma in IBD^[1,22-25]. There is also a theoretical risk for an increased rate of malignancies due to antagonism of TNF- α , but to date, there is no clear proof of such an effect^[26,27].

Recommendations for screening and surveillance of SBA in CD have been supported by only very limited data^[8]. The overall risk of colorectal cancer for UC patients is estimated to be 3.7% (95% CI, 3.2%-4.2%); 2% at 10 years, 8% at 20 years, and 18% at 30 years^[28], whereas reported cancer in CD is 0.63%-3.1%^[29]. Although diagnostic investigations using conventional modalities such as small bowel series, double-contrast enteroclysis, and upper and lower gastrointestinal endoscopies have been performed on patients with a high cancer risk, a recent report found that colonoscopy surveillance may not improve survival even in studying extensive colitis, thus other tools are needed to detect cancer development^[8]. CT and MRI are now considered the common imaging modality of choice for abdominal malignancy. Endoscopy of the small intestine, especially capsule endoscopy and DBE, are promising new diagnostic tools^[30]. Capsule endoscopy is not invasive, but has a risk of retention for patients with CD who have a stenosis in the intestinal tract^[31,32]. DBE is a new endoscopic method that provides complete visualization, the ability to biopsy the small bowel, and provides diagnostic and therapeutic information^[33].

In this case, PET/CT was effective for the discovery of a small bowel tumor at the initial examination. Although the localization of lesions may not be completely accurate, PET/CT provides accurately fused morphological and functional imaging within a single examination. Today, PET imaging has a high sensitivity for detecting colorectal cancer (CRC) and is superior to conventional CT staging when CRC patients are assessed for local and distant metastases^[34,35]. PET/CT also offers a better non-invasive tool for identifying and localizing active intestinal inflammation in patients with CD^[36]. Although PET/CT is not able to replace conventional studies due to its high cost, it may be useful when conventional studies cannot be performed or are not completed in CD patients. In this case, the tumor was detected at an advanced stage with multiple liver metastases. Although it is still uncertain whether combining PET/CT and DBE will enable us to make an early diagnosis for SBA, this case certainly indicates the possibility of combining PET/CT and DBE without conventional modalities, such as a small bowel series, to detect a primary malignant tumor of the small bowel without surgery. One strategy to monitor high-risk patients might be to perform PET/CT at an initial examination, and then conduct a DBE examination for the patient who has abnormal regions suspected as being malignant.

The recent development of innovative imaging techniques involving PET/CT and DBE has opened a new area in the exploration of the small bowel in CD patients. Each of these techniques is characterized by

its own profile of favorable and unfavorable features^[30]. Future clinical studies are expected to demonstrate strategies to monitor SBA in CD patients.

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Successful treatment of multiple lung metastases of hepatocellular carcinoma by combined chemotherapy with docetaxel, cisplatin and tegafur/uracil

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Tsuchiya A, Imai M, Kamimura H, Togashi T, Watanabe K, Seki K, Ishikawa T, Ohta H, Yoshida T, Kamimura T. Successful treatment of multiple lung metastases of hepatocellular carcinoma by combined chemotherapy with docetaxel, cisplatin and tegafur/uracil. *World J Gastroenterol* 2009; 15(14): 1779-1781 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1779.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1779>

Abstract

We report the successful treatment of multiple lung metastases after hepatic resection for hepatocellular carcinoma (HCC) with combined docetaxel, cisplatin (CDDP), and enteric-coated tegafur/uracil (UFT-E). A 68-year-old man was diagnosed with multiple lung metastases of HCC 7 mo after partial hepatectomy for HCC. Oral UFT-E was given daily and docetaxel and CDDP were given intra-arterially (administered just before the bronchial arteries) every 2 wk *via* a subcutaneous injection port. One month after starting chemotherapy, levels of tumor marker, protein induced by vitamin K absence II (PIVKA-II), decreased rapidly, and after a further month, chest X-ray and computed tomography revealed the complete disappearance of multiple liver metastases. Two years after the combined chemotherapy, HCC recurred in the liver and was treated but no pulmonary recurrence occurred. In the absence of a standardized highly effective therapy, this combined chemotherapy with docetaxel, CDDP and UFT-E may be an attractive option for multiple lung metastases of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide^[1]. Prognosis has been improved by careful follow-up of the high-risk patient, progress in diagnostic techniques, and combined modality therapy such as surgery, chemotherapy including transarterial chemoembolization (TACE), percutaneous ethanol injection therapy, and radiofrequency ablation. While recurrence of HCC is generally intrahepatic, long-term therapy increases the risk of extrahepatic metastases, and more than 50% of these occur in the lung^[2,3]. While the most effective therapy for lung metastasis is surgical resection^[4-7], Kawamura *et al*^[5] reported that only 2.6% of patients with lung metastases can undergo this type of surgery. Most patients are not deemed suitable because of multiple lung metastases, uncontrolled intra-hepatic HCCs, and deterioration in liver function. Since the prognosis of extrahepatic tumors is poor, options are limited to palliation for many people. Most people will be forced to depend on the few available chemotherapy protocols suitable for this situation; there is no standardized effective chemotherapy regime. A recent pilot study found that S-1, a novel oral dihydropyridine dehydrogenase

(DPD) inhibitor, and interferon-alpha (IFN- α) were associated with objective response in three of 12 patients with lung metastasis of HCC^[8]. Accumulation of such reports may lead to a standard therapy being confirmed in the future. Ishikawa *et al*^[9] previously reported combined chemotherapy with docetaxel, combined docetaxel, cisplatin (CDDP) and enteric-coated tegafur/uracil (UFT-E) for lung metastases of HCC. This chemotherapy regime was effective for lung metastases of HCC but not for the primary hepatic lesion; however, the reasons for this difference remain unknown. We report a case in which multiple lung metastases of HCC were completely resolved by modified combined chemotherapy with docetaxel, CDDP and UFT-E.

CASE REPORT

A 68-year-old man diagnosed with multiple lung metastases of HCC was admitted to Saiseikai-Niigata Daini Hospital in February 2006. He had undergone right hepatic lobectomy to remove three hepatocellular carcinomas (6 cm, 5 cm and 4 cm in diameter) 7 mo before the admission. Pathological diagnosis was moderately differentiated HCC, and clinical stage at the time of surgery was stage IVa according to the TNM classification. Tests for hepatitis B and C viral markers were all negative, but examination of the resected liver indicated cirrhosis, possibly caused by alcohol. Liver function test disclosed the following values: alanine aminotransferase 137 IU/L, alkaline phosphatase 503 IU/L, serum albumin 4.4 g/dL, and PT-INR 1.19 (Child-Pugh class A). While there was no elevation of tumor marker alpha-fetoprotein (4.3 ng/mL), protein induced by vitamin K absence II (PIVKA-II) was markedly elevated at 6280 mAU/L. Chest X-ray and computed tomography (CT) showed at least 50 metastases spread diffusely throughout the lungs (Figure 1). Since no treatment guidelines were available, we modified the combined chemotherapy with docetaxel, CDDP and UFT-E reported by Ishikawa *et al*^[9] after obtaining informed consent. First, 400 mg/d of oral UFT-E was started. We then placed an intra-arterial catheter that delivered medication into the aorta just before the bronchial arteries, and docetaxel (80 mg/body initially, followed by 40 mg/body) and CDDP (50 mg/body initially, followed by 20 mg/body) were administered every 2 wk *via* a subcutaneous injection port (Figure 2). This was performed on an outpatient basis from the third injection. The serum level of PIVKA-II decreased 1 mo after starting chemotherapy and normalized after 4 mo. Two months after starting chemotherapy, chest X-ray and CT showed complete disappearance of the lung metastases (Figure 1). Adverse events during the therapy were grade 3 leukocytopenia (minimum white blood cell count was 2500/ μ L), grade 2 fatigue and anorexia, and grade 1 alopecia according to Common Terminology Criteria for Adverse Events v3.0 (CTCAE). Therapy was stopped after the fourth administration and only UFT-E was continued, in accordance with the patient's wishes. This was also stopped 5 mo after starting chemotherapy, when chest X-ray and CT remained

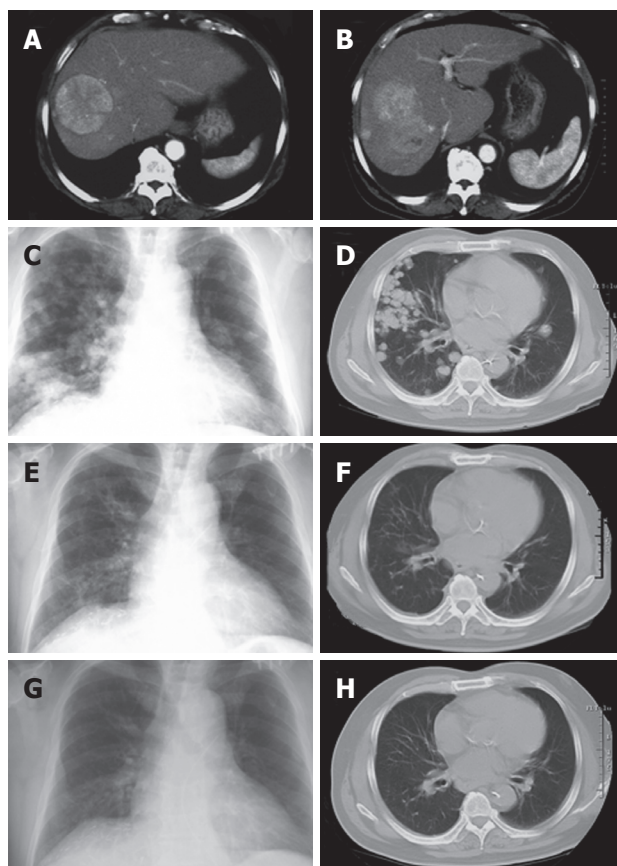


Figure 1 Chest X-ray and CT images before and after chemotherapy (A-H). Seven months before the chemotherapy, huge multiple HCCs were apparent in the right lobe of the liver (A, B). On admission before chemotherapy, multiple lung metastases were seen in the bilateral lung fields (C, D). Two months after starting chemotherapy, the multiple lung metastases had disappeared completely (E, F). Five months after starting chemotherapy, the tumors had not recurred (G, H).

negative. Approximately 2 years after hepatic surgery, HCC reappeared in the liver and was treated with TACE; however, lung metastasis did not recur. The patient remains well and is being followed-up as an outpatient.

DISCUSSION

The most effective therapy for lung metastasis of HCC is thought to be pulmonary resection^[4-7], and this has been reported to prolong life in some cases^[10]. However, the present patient was unsuitable for pulmonary resection because of multiple lung metastases. Reports of chemotherapy for lung metastasis of HCC are rare and most are case reports, indicating the absence of a highly effective standard therapy. Nonetheless, the literature contains reports of successful treatment of lung metastasis of HCC using tegafur/uracil^[11], 5-fluorouracil (5-FU) + IFN^[12], and 5-FU + CDDP + IFN^[13]. Moreover, a pilot study of S-1 and IFN- α in patients with pulmonary metastases of HCC showed that three of 12 patients responded objectively^[8]. There is also a report of the complete disappearance of multiple lung metastases with combined chemotherapy with docetaxel, CDDP and UFT-E^[9]. In the present case, multiple lung metastases also disappeared in response to modified

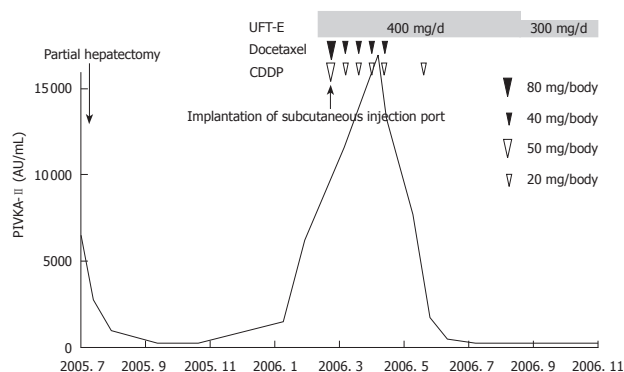


Figure 2 Clinical course of this patient. First, 400 mg/d of oral UFT-E was started. We then placed an intra-arterial catheter that delivered medication into the aorta just before the bronchial arteries, and docetaxel (80 mg/body initially, followed by 40 mg/body) and CDDP (50 mg/body initially, followed by 20 mg/body) were administered every 2 wk via a subcutaneous injection port. Levels of PIVKA-II, a tumor marker, decreased rapidly 1 mo after starting chemotherapy; levels continued to fall to normal and were maintained.

combined chemotherapy with docetaxel, CDDP, and UFT-E, suggesting that the effects of this combined chemotherapy are reproducible. We chose to modify the original intravenous combined chemotherapy including the docetaxel dose (original recommended dose was 60 mg/m² of docetaxel, 80 mg/m² of CDDP on day 1 and 400 mg/m² per day of tegafur/uracil in consideration of adverse effects^[9]. This original dose was determined by modifying the regime for advanced lung cancer^[14]) since two patients who received this could not be treated effectively because of adverse events attributed to docetaxel (personal communication). To reduce the adverse events and increase treatment efficacy, we used an intra-arterial catheter that delivered medication to just before the bronchial arteries. In this way, docetaxel and CDDP could be administered in high concentration to the lung.

The precise mechanisms underlying the effectiveness of this therapy for lung metastasis of HCC are not known. It has been reported previously that the growth inhibition of hepatoma cells induced by docetaxel was mediated through G2/M-phase arrest, caspase activation and DNA fragmentation^[15]. It is uncertain whether a single drug is sufficient, whether combinations of these agents are necessary, or whether docetaxel is the key drug. Furthermore, we do not know whether repeated doses or administration *via* the intra-arterial route improved the outcome for this patient. Further study is needed into the tumor characteristics of lung metastasis and immune responses in patients such as this one. While many issues must be resolved, this report is the second to document the effectiveness of combined chemotherapy with docetaxel, CDDP and UFT-E for lung metastasis of HCCs. This suggests that the present regime could be useful in this situation, as no highly effective standard therapy has been reported.

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CASE REPORT

Primary gastric teratoma on the cardiac orifice in an adult

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Abstract

Gastric teratoma (GT) is a seldom seen congenital abnormality. GT always occurs in children. The greater curvature and posterior wall of the stomach are the most common sites involving GT. We diagnosed a case of GT located on the inferior wall of the cardiac orifice in a 20-year-old man. To the best of our knowledge, this is the first case of GT located on the wall of the cardiac orifice in an adult in the English literature. We report this unusual case as an addition to this rare disease usually found in children. Computed tomography combined with endoscopic ultrasonography can be selected to diagnose GT.

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Key words: Adult; Cardiac orifice; Endoscopic ultrasonography; Stomach; Teratoma

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Liu L, Zhuang W, Chen Z, Zhou Y, Huang XR. Primary gastric teratoma on the cardiac orifice in an adult. *World J Gastroenterol* 2009; 15(14): 1782-1785 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1782.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1782>

INTRODUCTION

Gastric teratoma (GT) is a seldom seen congenital abnormality. It has been reported in the world literature that GT always occurs in children^[1,2], and an increasing mass in the epigastric region is the main sign of GT^[1,3,4]. Only six cases of GT in adults were reported in Medline^[5-8] between 1922 and 2007. Yoon *et al*^[9] reported the second case of GT in a child in Korea in 2000. GT is usually located on the posterior wall or greater curvature of the stomach^[3,7]. To the best of our knowledge, this is the first report of GT located on the wall of the cardiac orifice in an adult in the English literature. Calcifications in the cystic-solid mass may be features of GT on computed tomography (CT)^[3,4,10]. Endoscopic ultrasonography (EUS) may help to correctly diagnose GT before surgery.

CASE REPORT

The patient was a 20-year-old man. Due to slight pain and distention of the upper abdomen of 3 mo duration, he was admitted to our hospital. Physical examination was carried out and no mass was found in the epigastrium. The level of serum alpha-fetoprotein (AFP) was normal. CT showed a spherical mass on the inferior wall of the cardiac orifice of the stomach. The mass was about 4.8 cm × 5.2 cm, and there was no clear border between the mass and the wall of the stomach. The density of the mass was uneven. High-density and low-density substances were found in the mass (Figure 1). EUS demonstrated a giant mass on the inferior wall of the cardiac orifice. The mucosa of the mass was normal (Figure 2), and there was a lobulated polyp near the mass. The five-layer structure of the stomach was clearly manifested in the mass on ultrasonography (Figure 2). The mass had formed from the outer layer of the stomach. The mass was completely excised with a partial gastrectomy and the digestive tract was rebuilt. Macroscopically, the mass was about 5.0 cm × 5.3 cm × 2.3 cm, and was derived from the inferior wall of the cardiac orifice (Figure 3A). The mass contained small cystic tissue and a large solid area. Microscopically, the tumor was resected completely in one piece with a tumor cell-free margin. Cartilage, squamous cells and respiratory epithelium were observed in the mass (Figure 3B-F). No immature tissue was observed in the mass. The diagnosis of a primary mature GT derived from the cardiac orifice was established. The syndromes

Table 1 Five reported cases of GT in Medline between 1922 and 2007

Author (yr)	Age (yr)	Signs and symptoms	Location	Type	Size
Eustermann <i>et al</i> ^[6] 1922	31	?	Posterior wall	Mature	7 cm × 6 cm × 5 cm
Fadeeva <i>et al</i> ^[12] 1960	25	?	Anterior wall	Mature	4 cm × 3 cm
Gray <i>et al</i> ^[8] 1964	40	?	Lesser curvature	Mature	7 cm × 6 cm
Matsukuma <i>et al</i> ^[5] 1995	83	Tarry stool and anemia	Lesser curvature	Immature	12 cm × 10 cm × 6 cm
Joo <i>et al</i> ^[7] 1999	27	Fever, pain and vomiting	Greater curvature	Mature	9.5 cm × 7.5 cm × 5 cm

Note: Five patients were all male. Complete resection of the tumor was carried out in all patients and all patients recovered.

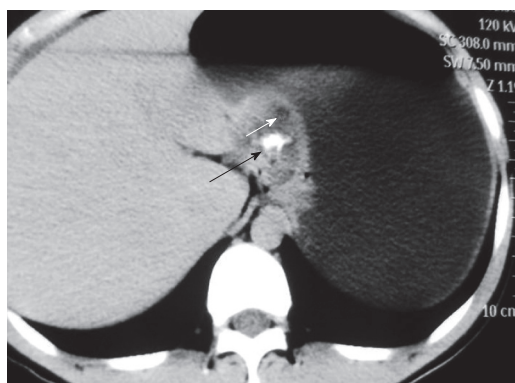


Figure 1 Axial CT shows a large soft tissue mass located on the inferior wall of the cardiac orifice of the stomach. The border of the mass is clear and the mass is from the stomach. The density of the mass is uneven. Low density (white arrow) indicates cystic tissue and the high density (black arrow) indicates calcifications.

in this patient disappeared within 1 wk after surgery. In the 10-mo follow-up period, the patient was well without abdominal discomfort.

DISCUSSION

Teratoma usually occurs in the gonads (mainly between the age of 6 mo and 15 years), the sacrococcygeal region (mainly in infants)^[1,2], and in the retroperitoneum, cranium or mediastinum^[5]. Teratoma derived from the stomach is very rare, and was first reported by Eustermann and Sentry in 1922^[6]. Up to 2002, only 107 cases of GT had been reported in the English literature^[1]. Surprisingly, GT always occurs in male children, especially around the age of 1 year^[1-3,5,11]. GT in an adult is unusual. To the best of our knowledge, there were just six cases of GT in adults reported in MEDLINE between 1922 and 2007, and five of these cases are listed in Table 1^[5-8,12]. GT is commonly located on the greater curvature and the posterior wall of the stomach^[3,7]. In the five cases listed in Table 1, two cases originated from the lesser curvature, and three cases from the posterior wall, anterior wall and the greater curvature, respectively. To the best of our knowledge, we report the seventh case of GT in an adult in the English literature and the first case originating from the cardiac orifice. Partly because of the rarity of GT in adults, the preoperative diagnosis of GT is difficult for surgeons^[7].

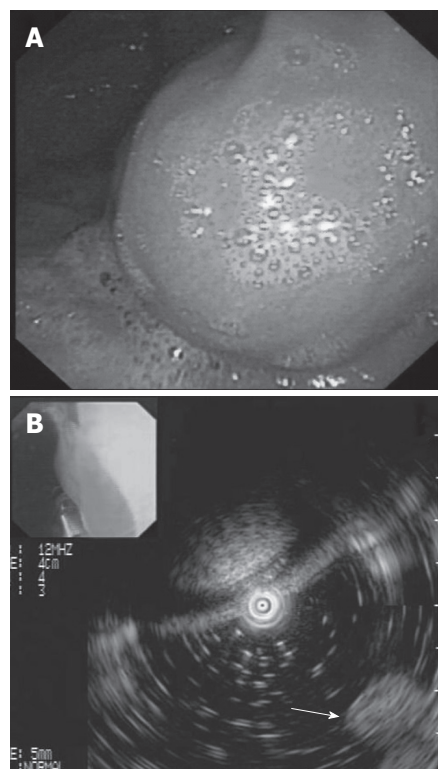


Figure 2 EUS was used to diagnose the gastric teratoma. A: A spherical mass was noted on the inferior wall of the cardiac orifice, and the mass mucosa was normal; B: The heterogeneous mass was formed from the outer layer and the five-layer structure of the stomach in the mass was clearly detected (white arrow).

Typical radiographic findings of GT^[3,4,10], such as calcifications, uneven density of the tumor and an intratumoral solid area with mixed cysts, were predominant in our case (Figure 1). EUS was first used to diagnose GT. A mass from the inferior wall of the cardiac orifice was observed. EUS demonstrated a heterogeneous mass from the outer layer of the stomach, and the five layers of the stomach were clearly detected (Figure 2). The echo pattern in the mass and the mass from the outer layer of the stomach were different from those of other common submucosal tumors, such as lipoma, myogenic tumor and lymphangioma. The use of EUS alone cannot help to diagnose GT qualitatively, but could provide useful information for preoperative diagnosis.

Cartilage, squamous cells and respiratory epithelium were found histologically (Figure 3F). According to pathological criteria, cartilage, squamous cells and respiratory epithelium in the mass are interesting

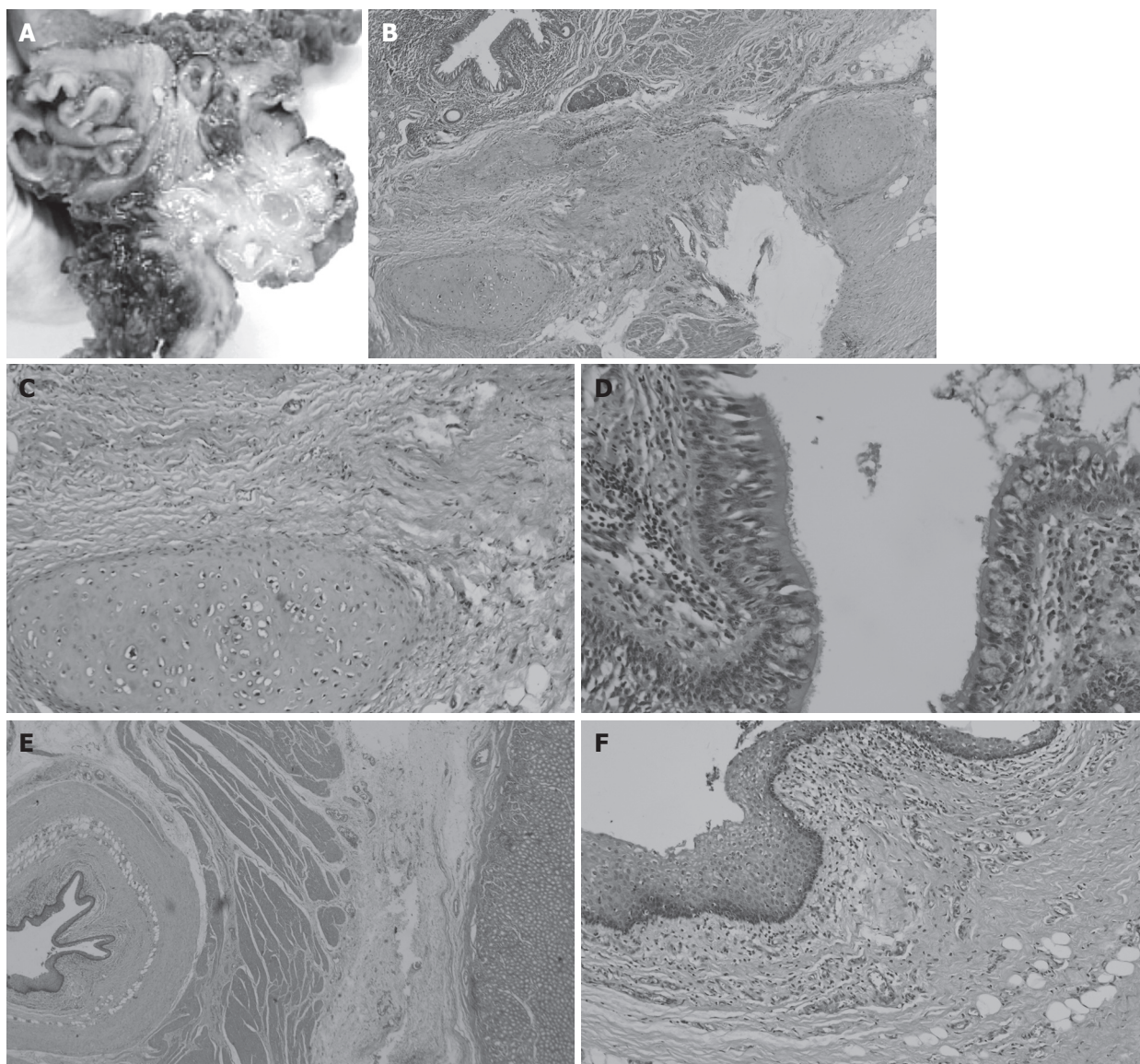


Figure 3 GT was pathologically examined under macroscopy and microscopy. A: The mass was about 5.0 cm × 5.3 cm × 2.3 cm, and it derived from the inferior wall of the cardiac orifice; B: Photomicrograph of an area composed respiratory epithelium and cartilage without immature teratoma components (HE, × 20); C, D: The cartilage (C) and the respiratory epithelium (D) were amplified (HE, × 100); E: The three layers, mucous layer, submucous membrane, and muscular layer, of the wall of the stomach were clear. The tissue of GT was derived from the muscular layer of the stomach (HE, × 20); F: The Squamous cell was amplified (HE, × 100).

histological features of teratoma. The diagnosis of GT from the cardiac orifice was therefore exclusively established. Complete resection is an effective method to treat both benign and malignant GT. Compared to traditional surgery, laparoscopic surgery has many advantages, and has been used to treat teratomas^[13], and will be used to treat GT in the future. AFP is a good indicator of the recurrence of malignant GT^[1,3,11]. Subsequent chemotherapy might be required when the level of AFP is high after surgery.

In conclusion, GT is rare and always occurs in infancy or childhood. The greater curvature and the posterior wall of the stomach are the most common sites involving GT. GT involving the cardiac orifice in an adult is seldom seen. To the best of our knowledge, this is the first report involving GT on the cardiac orifice in the English literature. EUS is helpful in diagnosing GT.

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LETTERS TO THE EDITOR

Hepatotoxicity associated with weight-loss supplements: A case for better post-marketing surveillance

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Abstract

There is a growing number of case reports of hepatotoxicity from the widely marketed weight-loss supplement Hydroxycut, which contains the botanical ingredient *Garcinia cambogia*. These case reports may substantially undercount the true magnitude of harm. Based on the past experience with harmful dietary supplements, US regulators should assume the more precautionary approach favored by Canada and Europe. Lacking effective adverse event surveillance for supplements, or the requirements to prove safety prior to coming to the market, case reports such as those summarized here assume added importance.

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Key words: Hydroxycut; Dietary supplements; *Garcinia cambogia*; Liver failure; Weight loss; Super citrimax; Hca-sx

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TO THE EDITOR

Dara *et al*^[1] report on a case series of two patients with hepatotoxicity associated with the weight-loss

supplement Hydroxycut, so named because it contains potentially hepatotoxic hydroxycitric acid derived from the tropical fruit *Garcinia cambogia*^[1]. Two earlier case reports in 2005 were also referenced^[2]. To this count should be added two additional case reports of hepatotoxicity associated with Hydroxycut^[3,4]. An estimated 15% of the US population uses dietary supplements for weight loss^[5], and Hydroxycut is the top selling product in this class and market, with roughly a million units sold per year^[6]. With such wide usage, these six cases may underestimate the true incidence of hepatotoxicity by several degrees of magnitude.

Each case report has similarities both in reported liver screening abnormalities and symptoms reported by patients, all of whom were otherwise healthy and experienced normalized hepatic function once they stopped taking the supplement. Table 1 synthesizes key laboratory findings and reported symptoms.

Poor regulation of dietary supplements in the US has been noted by consumer advocates, researchers and policymakers^[7-10]. US manufacturers of dietary supplements are not required to conduct trials establishing safety or efficacy prior to marketing; only provide a copy of their label for the Food and Drug Administration (FDA) to review^[7,9,11]. Ingredients do not need to be considered “generally regarded as safe” as pharmaceuticals or food additives do, and the FDA must prove that a supplement is harmful before taking regulatory action^[9,11]. This means consumers in effect become unwitting subjects in a large scale post-marketing trial of a product’s safety. Unfortunately, the FDA does a generally poor job of post-marketing monitoring of adverse events from supplements, only receiving reports of an estimated 1% of such events^[11]. A recent search of FDA’s adverse events surveillance database for “Hydroxycut”, “hydroxycitric acid”, “*Garcinia cambogia*”, or “SuperCitrimax” (the proprietary blend of *Garcinia cambogia* used in Hydroxycut) yielded no reports^[12]. Furthermore, the nation’s poison control centers, which receive far more supplement adverse event reports than the FDA, lack the necessary coordination to act in a surveillance role^[11]. Supplement manufacturers may not be forthcoming with information about emerging health risks from their products. The makers of the weight-loss supplement Metabolife 356, for example, withheld over 14000 reports they had received over 5 years documenting serious adverse events associated with

Table 1 Patients, symptoms and laboratory values reported with hydroxycut associated hepatotoxicity

Citation	Dara <i>et al</i> ^[1]		Jones <i>et al</i> ^[4]	Shim <i>et al</i> ^[3]	Stevens <i>et al</i> ^[2]	
Patient age (yr)	40	33	19	28	27	30
Patient gender	F	F	M	M	M	M
Reported symptoms/ duration	Fatigue, nausea, vomiting, cramping, fever, chills, anorexia/3 d	Fatigue, nausea, cramping, abdominal pain/2 wk	Nausea, vomiting, jaundice/6 d	Fatigue, dyspnea on exertion, jaundice/3 wk	Fatigue, jaundice/8 d	Fatigue, vomiting, jaundice, fever/10 d
Aspartate aminotransferase (U/L)	1020	934	1981	1049	1808	59
Alanine aminotransferase (U/L)	1150	570	1143	2272	3131	45
Serum alkaline phosphatase (U/L)	299	112	153	153	171	530
Serum bilirubin (mg/L)	6.7	209	117	181	78	78
Serum direct bilirubin (mg/L)	Not reported	142	68	90	Not reported	Not reported
Prothrombin time (s)	Not reported	Not reported	17.1	12.8	16	15

their ephedra-containing product, including myocardial infarction, stroke, seizure and death^[7]. (Though not legally mandated to report serious adverse events until 2007^[9,11], failure to act on such reports suggests an ethical lapse.) To be fair, the FDA has taken action to protect the public health from dangerous supplements, banning ephedra in 2004 due to its cardiac risk^[13]. Formerly a common ingredient in numerous weight-loss supplements, ephedra was banned only after 155 deaths were associated with its use^[14]. In contrast, regulators in Canada, the UK and Europe appear to take a more precautionary approach. The supplement kava-kava was banned in Canada in 2002 after 29 cases of hepatotoxicity were associated with its use^[15], and in the UK and Europe the following year after some 40 more cases were reported^[16]. In the US, the FDA has issued a consumer advisory about possible hepatotoxicity associated with kava-kava use, but has not banned the substance^[17].

Faced with the aforementioned difficulties ensuring the safety of widely used dietary supplements, reliable and transparent case reports such as those cited above assume added importance. They may also underscore the need for US regulators to adopt the more precautionary post-marketing practices of their European and Canadian counterparts. Authorities should also exercise their responsibilities to the public's health by being empowered to practise more stringent and evidenced-based regulation of these products, many of whom could be considered pharmaceuticals due to their clear pharmacological effects and potent risks^[9].

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Meetings

Events Calendar 2009

January 12-15, 2009
 Hyatt Regency San Francisco, San Francisco, CA
 Mouse Models of Cancer

January 21-24, 2009
 Westin San Diego Hotel, San Diego, CA
 Advances in Prostate Cancer Research

February 3-6, 2009
 Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
 Second AACR Conference
 The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
 Hyatt Regency Boston, Boston, MA
 Translation of the Cancer Genome

February 8-11, 2009
 Westin New Orleans Canal Place, New Orleans, LA
 Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
 Hong Kong Convention and Exhibition Centre, Hong Kong, China
 19th Conference of the APASL
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009
 Orlando, Florida
 AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
 Vienna, Austria
 EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
 Phoenix, Arizona
 AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
 Marriott Wardman Park Hotel
 Washington, DC
 13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
 Glasgow, Scotland
 British Society of Gastroenterology (BSG) Annual Meeting
 Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
 Silver Spring, Maryland
 2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
 Colorado Convention Center, Denver, CO
 AACR 100th Annual Meeting 2009

April 22-26, 2009
 Copenhagen, Denmark
 the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
<http://www.easl.ch/>

May 17-20, 2009
 Denver, Colorado, USA
 Digestive Disease Week 2009

May 29-June 2, 2009
 Orange County Convention Center
 Orlando, Florida
 45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
 Chicago, Illinois
 Endpoints Workshop: NASH

May 30-June 4, 2009
 McCormick Place, Chicago, IL
 DDW 2009
<http://www.ddw.org>

June 17-19, 2009
 North Bethesda, MD
 Accelerating Anticancer Agent Development

June 20-26, 2009
 Flims, Switzerland
 Methods in Clinical Cancer Research (Europe)

June 24-27 2009
 Barcelona, Spain
 ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
 Beijing International Convention Center (BICC), Beijing, China
 World Conference on Interventional Oncology
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009
 Snowmass, CO, United States
 Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
 Aspen, CO, United States
 Molecular Biology in Clinical Oncology

August 1-7, 2009
 Vail Marriott Mountain Resort, Vail, CO, United States
 Methods in Clinical Cancer Research

August 14-16, 2009
 Bell Harbor Conference Center, Seattle, Washington, United States
 Practical Solutions for Successful Management
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009
 Beijing International Convention Center (BICC), Beijing, China
 19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009
 Taipei, China
 Asian Pacific Digestive Week
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009
 Boston Park Plaza Hotel and Towers, Boston, MA, United States
 Frontiers in Basic Cancer Research

October 13-16, 2009
 Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
 Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
 Versailles, France
 Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
 Boston, MA, United States
 The Liver Meeting

November 15-19, 2009
 John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
 AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
 London, UK
 Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org



Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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Format

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- 1 Jung EM, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 ± 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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