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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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Contents

Contents

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Contents

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EDITORIAL

Islet transplantation and antioxidant management: A comprehensive review

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Abstract

Islet transplantation as a promising treatment for type 1 diabetes has received widespread attention. Oxidative stress plays an essential role in cell injury during islet isolation and transplantation procedures. Antioxidants have been used in various studies to improve islet transplantation procedures. The present study reviews the role of oxidative stress and the benefits of antioxidants in islet transplantation procedures. The bibliographical databases Pubmed and Scopus were searched up to November 2008. All relevant human and animal *in-vivo* and *in-vitro* studies, which investigated antioxidants on islets, were included. Almost all the tested antioxidants used in the *in-vitro* studies enhanced islet viability and insulin secretion. Better control of blood glucose after transplantation was the major outcome of antioxidant therapy in all *in-vivo* studies. The data also indicated that antioxidants improved islet transplantation procedures. Although there is still insufficient evidence to draw definitive conclusions about the efficacy of individual supplements, the benefits of antioxidants in islet isolation procedures cannot be ignored.

Key words: Antioxidant; Diabetes; Free radical; Islet; Transplant

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INTRODUCTION

Diabetes mellitus which is characterized by hyperglycemia has become an important disorder with major costs and complications worldwide. In a genetically susceptible person with an environmental trigger such as viruses and toxins, autoimmune destruction of β cells can occur causing type 1 diabetes usually in childhood and in young adults[1]. Type 2 diabetes usually results from dysfunction of β cells and peripheral insulin resistance. It is accepted that oxidative stress is increased in both type 1 and type 2 diabetes, and it has been shown in many studies that biochemical markers of oxidative stress are higher in tissue samples and in the pancreas of diabetic patients[2]. However, secondary complications comprising micro- and macro-vascular disorders which result in frequent amputation, end-stage renal failure, and blindness have motivated various investigators to identify new therapeutic approaches to cure diabetes. With this aim, whole pancreas transplantation was first carried out in 1966 by Kelly and Lillehei at the University of Minnesota and was then performed worldwide. Marked morbidity following pancreas transplantation prompted researchers to find other possible ways of curing this disease. As reviewed by Fontaine *et al*[3], since 1974 when the first human islet transplantation was conducted by Sutherland and his colleagues, and then up to 1999, among approximately 1000 patients who received islet allotransplantations, most of the results were disappointing and only 10% remained insulin-independent for longer than one year.

In 1999, use of the Edmonton protocol with its steroid-free immunosuppressive regimen was an impressive leap in achieving insulin-independence after islet transplantation[4]. Although the Edmonton protocol

succeeded in achieving insulin-independence, two or more donors are still needed to achieve normoglycemia following islet transplantation.

GREAT EFFORTS TO OPTIMIZE ISLET TRANSPLANTATION

Recent years, many studies have been carried out to optimize the Edmonton protocol to obtain the final goal of one-donor islet transplantation. Donor characteristics and pancreas procurement are the first steps. The quality of the donor pancreas depends largely on donor factors, such as age, body mass index, serum glucose levels, and hemodynamic stability[5]. In the procurement phase, improved surgical techniques such as *in-vivo* atraumatic dissection and *in-situ* separation have made sufficient advancements in minimizing warm ischemia before isolation. Moreover, using perfluorocarbon (PFC) and oxygen in the University of Wisconsin (UW) solution during cold ischemic preservation, which is identified as the Two layer method (TLM), has enhanced the final quality of islets[6].

One of the most important areas of research in islet transplantation is the isolation procedure, which remains a major topic of islet transplant investigations. At present, the maximum rate of purified islets at leading centers is about 50% to 70%[7]. The largest reduction in islet yield occurs during the islet isolation phase. Below we will look in detail at the islet isolation phase, which has been the focus of a large number of studies.

Other efforts have focused on new approaches relating to the best site of transplantation, better revascularization of islet-grafts, visualization after engraftment, and further anti-rejection strategies which are not covered in this review. For the latest progress in these aspects of islet transplantation, readers are referred to two recently published review articles[8,9].

ISLET ISOLATION AND ROLE OF OXIDATIVE STRESS

Successful islet transplantation would enable patients to live without tedious multiple insulin injections and reduce the risk of hypoglycemia. As previously mentioned, the main restriction in this procedure is the loss of healthy islets at the end of the operation due to the inevitable prolonged time required for islet isolation. In the clinical setting, the isolated islets are transplanted immediately or within a few days after harvesting from donors. Isolated islets are avascular and are therefore ischemic from the time of isolation through to the period required for revascularization. Prolonged ischemia has profound deleterious effects on the islets, resulting in a significant loss of islet cells. Two major factors which are expected to cause potential cell damage include hypoxia and enzymatic/mechanical trauma related to the experimental procedures.

Reactive oxygen species (ROS) in physiological concentrations provide normal conditions to protect cells, for instance they are important within white cells to allow effective defense against infection. Nevertheless, whenever ROS are accumulated in excess and for long periods they can destroy cells. Free radicals cause damage to cellular proteins, membrane lipids and nuclear nucleic acids. The only protective mechanism present in the body to protect cells against excessive free radicals is the antioxidant enzyme system. Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) are the main antioxidant enzymes. Hypoxia which occurs during the islet transplantation procedure initiates a cascade of biochemical reactions which results in the production of ROS causing necrosis and apoptosis *via* intracellular pathways.

Another important point which can lead to worsening of this condition is that β cells contain low levels of antioxidative enzymes such as CAT, SOD and GPx and thus they can only weakly defend against oxidative stress[10-12]. These findings have demonstrated the major destructive role of oxidative stress in islet transplantation and have encouraged investigators to use antioxidants during the isolation phase and the entire transplantation process to overcome the final lack of healthy islets.

Various strategies such as modifications in enzymatic digestion[13], purification with iodixanol instead of ficoll[14], incubation of purified islets in culture medium, genetic manipulation for overexpression[15,16] or silencing[17] of specific genes, employment of different anti-inflammatory or other supplements such as small intestinal submucosa[18] and serecin[19] have been considered in an attempt to increase islet quality and yield. In a previous publication we hypothesized that using phototherapy could improve islet function before transplantation[20].

This review focuses on antioxidant management in islet transplantation procedures and evaluated both *in-vitro* and *in-vivo* studies.

ANTIOXIDANT RECRUITMENT IN ISLET TRANSPLANTATION STUDIES

To perform a comprehensive survey and obtain all related studies we searched Pubmed and Scopus databases up to November 2008. Search terms were "antioxidant", "islet", "transplant" and "oxidative stress". Individual antioxidants such as vitamin E or C were also searched with the term "islet".

There were many studies in which various methods other than utilizing antioxidants were used to increase function and viability of islets which were not included in this review. Table 1 lists the various antioxidant agents which have been added to islets during the isolation procedure or to the culture medium. Table 2 shows the *in-vivo* studies.

Metabolite and vitamin antioxidants

Vitamin E or tocopherol was the supplement most used to enhance the viability of islets. This lipid-soluble

Table 1 ***In-vitro*** **effects of antioxidants on pancreatic islets**

Authors	Substance/Dose	Sample	Study design	Duration	Assessments
Campbell *et al*[21], 2008	Sodium selenite (30 nmol/L)	Rat	Islets were incubated with/without Na_2SeO_3	72 h	Insulin content ↑ Insulin secretion ↑ Glucose-stimulated insulin secretion[1]
Kanitkar *et al*[22], 2008	Curcumin (10 μmol/L)	Rat	Cryopreservation of islets with/without curcumin Assessments were performed after 24 h culture post-thawing	Cryopreserved	Intact islets ↑ ROS ↓ Insulin Secretion ↑ Expression of Hsp70 and HO-1 ↑
Thomas *et al*[23], 2007	Peptide SS-31 (1 nmol/L)	Mice	SS-31 was added to all reagents which were used for islet isolation	Isolation procedure	Islet apoptosis ↓ Islet yield ↑
		Human	Islets were incubated in culture medium with without SS-31	72 h	Islet apoptosis ↓
Hara *et al*[24], 2007	Epigallocatechin-3-gallate (EGCG) (36, 72, 360 μmol/L)	Rat	Islets cultured under normal or H/R condition with/ without EGCG	48 h	Islet apoptosis ↓ LDH ↓ Insulin secretion ↑
Xiong *et al*[25], 2006	Puerarin (10, 50, 100 μmol/L)	Rat	Islets cultured under normal or H_2O_2 stress conditions with/without Puerarin	24 h	Islet apoptosis ↓, Islet viability ↑ CAT & SOD activity ↑, ROS ↓ Insulin secretion ↑
Marzorati *et al*[26], 2006	Glutathione (1, 5, 10 mmol/L) Vitamin E (2×10^{-5} mmol/L) Ascorbic acid (0.3 mmol/L)	Human	Antioxidant agents were added to culture medium individually	48 h	Glutathione : CCL2/MCP-1 release ↓ Insulin secretion[1] Vitamin E: CCL2/MCP-1 release[1] Ascorbic acid: CCL2/MCP-1 release[1]
Amoli *et al*[27], 2006	Curcumin (10, 20 μmol/L)	Rat	Curcumin was added to culture medium	18 h + 24 h	MCP-1 release ↓
Rao *et al*[28], 2005	MCI – 186	Rat	Islets were treated with H_2O_2 in the presence or absence of MCI-186	18 h	Cell death ↓
Avila *et al*[29], 2005	L-glutamine (5 mmol/L)	Human	Pancreas were manually perfused through the main pancreatic duct with either the standard HBSS or with HBSS + L-glutamine	0 → 24 h	Islet yield ↑ Lipid peroxidation (MDA) ↓ Glutathione (GSH) ↑ Viability[1] Insulin secretion[1]
Brandhorst *et al*[30], 2005	Free L-glutamine (2.5 & 5 mmol/L/L) Stable L-glutamine [NALG] (2.5 & 5 mmol/L)	Pig	Islets pretreated with free L-glutamine or NALG for 24 h and then stressed with H_2O_2, ETA or cytokine mix	24 h	Viability: L-glutamine ↑ NALG[1]
Giovagnoli *et al*[31], 2005	Encapsulated enzymes (100 mg/35 mL)	NPCCs	NPCCs were co-cultured with/without entrapped enzymes	9 d	Viability ↑, Insulin secretion ↑ Insulin/DNA ratio ↑ mRNA expression of insulin and Glut-2 ↑
Bottino *et al*[32], 2004	MnTDE (34 μmol/L)	Human	MnTDE was added as a supplement to culture or isolation medium	60 h	Islet yield ↑, Insulin secretion ↑ NF-κB DNA-binding ↓ IL6 & MCP-1 ↓, IL8[1] PARP activation ↑
Arata *et al*[33], 2004	Ascorbic Acid-2 Glucoside (AA2G) (100 μg/mL)	Human	Cryopreservation of islets with UW solution or AA2G + UW solution	Cryopreserved	Viability ↑ Insulin secretion ↑ Insulin gene expression ↑
Luca *et al*[34], 2003	Free vitamin D3 (2 μmol/L) Encapsulated vitamin D3 (20 μmol/L)	Rat	Islets were treated with/ without vitamin D3	9 d	Insulin secretion ↑
Hardikar *et al*[35], 2001	Taurine (0.3 & 3 mmol/L)	Rat	Cryopreservation of islets with/without taurine	Cryopreserved	Viability ↑ Lipid peroxidation ↓
Luca *et al*[36], 2000	Vitamin D3 Vitamin E	NPCCs	NPCC cells were treated with/without VitD3 & VitE during their maturation and differentiation process	16 d	Large & intact islets ↑ Insulin secretion ↑
Tajiri *et al*[37], 1999	Vitamin E (50 μg/mL)	Rat	Islets were co-cultured with/ without vitamin E	1 d	Insulin secretion ↑
Shewade *et al*[38], 1999	Riboflavin	Rat	Inclusion of riboflavin in the cryopreserved medium	Cryopreserved	Viability ↑ Lipid peroxidation ↓ GSH ↑, Insulin secretion ↑
Jindal *et al*[39], 1996	Zinc (20 μmol/L)	Rat	Preservation of islets in UW and Hanks solution with/without Zinc	1, 3, 6 d	Viability[1]

↑: Significant increase compared with non-treated group; ↓: Significant decrease compared with non-treated group; [1]No significance difference between groups. Hsp70: Heat shock protein 70; HO-1: Heme oxygenase-1; H/R: Hypoxia/Reoxygenation; LDH: Lactate dehydrogenase; CCL2/MCP-1: Monocyte chemoattractant protein 1; HBSS: Hanks balanced salt solution; NALG: N-acetyl-L-alanyl-L-glutamine; MDA: Malondialdehyde; MnTDE: Manganese (Ⅲ) 5,10,15,20-tetrakis (1,3-diethyl-2imidazoyl) porphyrin; NF-κB: Nuclear factor-κB; PARP: Poly (ADP-ribose) Polymerase indicative of ongoing cell damage and death; UW: University of Wisconsin; NPCCs: Neonatal pancreatic porcine cell clusters.

Table 2 ***In-vivo*** **effects of antioxidants on pancreatic islets**

Author	Substance/Dose	Sample	Procedure	Output
Thomas *et al*[23], 2007	Peptide SS-31 (3 mg/kg)	Mice → Mice	Injection of SS-31 to donor mouse + adding SS-31 during islet isolation	Better glucose control after transplant
Avila *et al*[29], 2005	L-glutamine (5 mmol/L)	Human → Rat	Transplantation of human islets which were treated before with/without intraductal L-glutamine to nude rats	Normoglycemia percentage ↑ (83% *vs* 26%) Time to reach normoglycemia ↓ (1.83 d *vs* 7.6 d)
Brown *et al*[40], 2005	Pyruvate Vitamin E Vitamin C	Rat → Rat	Oral administration of pyruvate, vitamin E and vitamin C during perioperative period	Pyruvate enhanced engraftment & functionality of suboptimal islet mass Vitamin E and vitamin C failed to enhance
Olcott *et al*[41], 2004	Salen-manganese [EUK-8] (100 mg/kg)	Mice → Mice	Daily treatment with/without EUK-8 (IP injection) to recipient NOD mice	Better glucose control until 20 d after transplant
Winter *et al*[42], 2002	Vitamin A (500 μg) Ascorbic Acid (40 mg) Vitamin E (10.6 mg) Selenium (2 μg)	Rat → Rat	Oral antioxidants, 24 h prior to transplantation	Slight better glucose control
Bottino *et al*[43], 2002	SOD mimetic compound (34 μmol/L)	Human → Mice	Culture of isolated islet in the presence of SOD mimetic for at least 2 h before transplantation	Better glucose control
Hardikar *et al*[35], 2001	Taurine (0.3 & 3 mmol/L)	Rat → Mice	Transplantation of islets which were previously cryopreserved with/without taurine	No significant difference was observed
Tajiri *et al*[37], 1999	Vitamin E (40 mg/kg)	Rat → Rat	Every other day IP injection of vitamin E to transplanted rats	Treatment for 2 not for 6 wk enhanced basal insulin release and arginine induced insulin release
Vajkoczy *et al*[44], 1997	Vitamin E (150, 8000 mg/kg)	Rat → Rat	Oral vitamin E after xenograft	Reduction of xenograft leukocyte-endothelium interaction at day 6 Adequate development of functional capillary density
Gembal *et al*[45], 1993	Allopurinol Vitamin E Chlorpromazine	Rat → Rat	Transplantation of islets which were pretreated with/without combination of allopurinol, alpha-tocopherol and chlorpromazine.	Better glucose control after 3 d

↑: Significant increase compared with non-treated group; ↓: Significant decrease compared with non-treated group.

vitamin protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction[46]. Luca *et al*[36] and Tajiri *et al*[37] have shown that vitamin E can increase secretion of insulin from islets when added *in-vitro* to the culture medium. Likewise, when vitamin E was administered before, after, or during the transplantation period[37,42,44], the islet transplantation outcome was better and glucose control was slightly superior compared with control groups. Another study demonstrated that digestion buffer which contained vitamin E, resulted in healthy isolated islets, however, this study was not designed to evaluate the effect of vitamin E and therefore a control group was absent. An investigation into the effect of a mixture of allopurinol, vitamin E and chlorpromazine on islet transplantation concluded that a combination of free radical scavengers, antioxidants and membrane stabilizing drugs may be used to increase the effectiveness of islet transplantation[45].

In contrast to the above-mentioned positive effects of vitamin E, Brown and colleagues[40] showed that vitamin E and C could not enhance the function of rat engrafted islets; although the authors did not specify the dose which they applied. In a different study[26], the use of vitamin E and ascorbic acid did not decrease the release of monocyte chemoattractant protein 1 (MCP-1) from human islets. MCP-1 is a chemokine secreted from pancreatic islets to recruit the immune system and plays an important role in the clinical outcome of islet transplantation because of its proinflammatory property[47,48]. Nevertheless, this review did not assess the secretion of insulin or the viability of islets following the use of vitamin E, thus we can not conclude that islets may or may not benefit from vitamin E.

Ascorbic acid or vitamin C is a monosaccharide antioxidant. This water-soluble vitamin is a reducing agent and can neutralize oxygen species. This metabolite antioxidant in the form of ascorbic acid-2 glucoside (AA2G) supplies a stable form of vitamin C in the culture medium and cryopreservation solution and thus improves viability, secretion and expression of insulin in cryopreserved human islets[33]. In the study by Winter *et al*[42], the combined administration of vitamin C and E, but not vitamin A and selenium, led to a significant improvement in functional islet graft survival, associated with augmented islet engraftment. In contrast, Brown *et al*[40] and Marzorati *et al*[26] showed that the combination of vitamin E and C was unable to improve graft survival or decrease *in-vitro* MCP-1 release, respectively.

Glutathione (GSH), as a cysteine-containing peptide, is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other

metabolites and enzyme systems as well as reacting directly with oxidants in the cells. When GSH was added to culture medium, in contrast to vitamin E and C, it reduced MCP-1[26]. L-glutamine as a precursor of GSH was shown to increase islet yield when added intra-ductally during human pancreatic islet isolation. This study indicates that intra-cellular GSH levels can be increased by means of intra-ductal glutamine administration prior to the isolation procedure. Consequently, authors found lower cellular lipid peroxidation in islets isolated from glutamine-pre-treated pancreata, indicating less oxidative damage. Although they could not detect any differences in cell viability and islet function *in vitro*, the islets isolated from glutamine-pre-treated pancreata performed significantly better than controls after transplantation in diabetic nude mice[29]. Supporting this study, Brandhorst *et al*[30] demonstrated that pig islet culture will significantly improve if L-glutamine is administered in an unbound (free) form compared with the stable compound N-acetyl-L-alanyl-L-glutamine (NALG).

Taurine (2-amino ethanesulfonic acid) an end-product of sulfur amino acid metabolism, is one of the most abundant free amino acids in the body. The membrane stabilizing, free radical scavenging, and osmoregulatory roles of taurine have been well documented[49,50]. In one study, taurine enhanced viability and reduced lipid peroxidation in cryopreserved islets. However, no significant difference was observed in the islet insulin content between groups following cryopreservation, and taurine could not enhance glucose control following transplantation of treated islets[35].

1, 25 dihydroxyvitamin D3, the active form of vitamin D3 is a membrane antioxidant and can prevent lipid peroxidation at the cell membrane[51]. Luca and colleagues treated neonatal porcine cell clusters (NPCCs) with vitamin D3 at a certain time in their maturation and differentiation process. Insulin recovery showed that vitamin D3, unlike untreated controls, resulted in preservation of islet function for significantly long periods of time. Furthermore, this group exhibited sustained release vitamin D3 which entrapped vitamin D3 in microspheres. They showed that this form of vitamin D3 caused more insulin secretion compared with free vitamin D3 and both forms caused more insulin secretion than the untreated control group[34,36].

Another study evaluated the benefit of 1, 25 dihydroxyvitamin D3 (1, 25 D3) on cytokine-induced human pancreatic islets. Addition of 1, 25 D3 significantly reduced nitrite release, IL-6 production and MHC class I expression which were not significantly different from controls. The authors suggested that vitamin D3 could affect this *via* reduction of cytotoxic challenges. Hence, it might play a role in the prevention of islet allograft rejection. However, more *in-vivo* studies are required to affirm this suggestion.

Riboflavin (vitamin B2) although it is not a pure antioxidant, has been used in one study[38]. The authors showed that inclusion of this vitamin in the cryopreservation medium could protect islets comparable to healthy fresh isolated islets.

Trace elements

Selenium as an essential trace element plays an important role in the expression of some selenoproteins and selenoenzymes such as GPx. The antioxidative property of this element has been confirmed in many different cell types[52]. The insulin-mimetic effect of selenium has been found both *in-vitro* and *in-vivo*[53]. To ameliorate islet function, Campbell *et al*[21] showed that sodium selenite could increase islet content and secretion but not glucose-stimulated insulin secretion in culture media. In the study by Winter, when a combination of vitamin E, C, A and selenium were administered orally to transplanted mice, no significant improvement in functional islet graft survival was seen when compared with mice administered only vitamin C and E.

Furthermore, selenium in the insulin-transferrin-selenium (ITS) compound is a commercially available media supplement for the culture of mammalian cells under serum-free or near serum-free conditions. In islet culture, when Fraga and colleagues used ITS supplement instead of FBS, ITS was capable of maintaining viable islets for up to two months[54].

Zinc, another trace element antioxidant has a role in the storage, synthesis, and secretion of insulin in islet cells. In one study[39] when investigators added zinc to cold preservation UW and Hanks solution, no difference in islet viability was seen between the groups. The authors concluded that despite the integral role of zinc in islet metabolism, they were unable to find a beneficial role for zinc in cold storage solutions for the purposes of islet preservation.

Manganese as a trace element is a component of antioxidant enzymes and is used in new SOD mimetic compounds which will be discussed later.

Herbs

Curcumin is a polyphenolic compound commonly found in the dietary spice turmeric[55,56]. Curcumin is an inhibitor of nuclear factor-κB (NF-κB) and has various biological activities such as anti inflammatory, antioxidant, antiseptic and anticancer effects[57,58]. In two different studies, curcumin was shown to decrease MCP-1 release from islets[27], decrease generation of ROS, increase secretion of insulin, and present more intact islets[22].

Puerarin, the main isoflavone glycoside found in the Chinese herb, Radix of Pueraria lobata (Willd) Ohwi, has been used for various medicinal purposes in traditional Chinese medicine. It has been shown that puerarin has antioxidant activities such as radical scavenging and increasing SOD activity as well as antihyperglycemic effects[59]. Xiong *et al*[25] found that if islets pretreated with puerarin for 48 h were exposed to H_2O_2, this did not result in loss of islet viability. They showed that this protective effect resulted from inhibition of free radical generation. Puerarin was also found to increase CAT and SOD activities.

Epigallocatechin-3-gallate (EGCG) is the main ingredient found in green tea. Anticarcinogenic,

antioxidant, and antiangiogenic activities have been attributed to EGCG as a constituent of green tea[60]. In the study by Hara and colleagues, EGCG was shown to protect islets from hypoxia/reoxygenation injury. Insulin secretion was increased and apoptosis was inhibited by EGCG[24].

Enzymes and new antioxidants

Two forms of enzymes, encapsulated slow release SOD and CAT and SOD-mimetic compounds were shown to increase viability, islet yield, and to decrease the release of MCP-1 and IL-6 from islets.

Encapsulated SOD and CAT in Poly D, L-lactide-co-glycolide (PLGA) microspheres were analyzed with NPCC cells. These powerful antioxidizing agents were shown to significantly improve morphology, viability and function, as assessed by microscopy, molecular, biochemical and functional studies on the incubated NPCCs[31].

SOD mimetics: There are four main classes of SOD mimetics including desferrioxamine, macrocyclics, salen compounds, and mesoporphyrins[61]. Two types of these compounds have been employed to possibly enhance islet survival by counteracting oxidative stress.

MnTDE is a manganese-porphyrin pentachloride, a synthetic porphyrin protein, which has been prepared as a SOD mimetic[62]. Synthetic probes with MnTDE (AEOL10113 or AEOL10150) were used in the culture medium of human islets to ameliorate islet yield and insulin secretion. When treated islets were transplanted into diabetic mice, glucose control was better than in the non-treated group[32,43].

MnTMPyP Mn (Ⅲ) tetrakis (1-methyl-4-pyridyl) porphyrin, is a composition of porphyrin SOD mimetics. MnTMPyP preserved islet viability upon exposure to a nitric oxide donor in culture medium[61].

EUK-8 a salen compound SOD mimetic is one of a new class of synthetic salen-manganese compounds with SOD, peroxidase, and CAT activities. EUK-8 treatment prolonged the survival of islet allografts in newly diabetic non-obese diabetic (NOD) mice[41].

Peptide SS-31 (D-Arg-2-, 6-dimethyltyrosine-Lys-Phe-NH2) is a novel peptide shown to target the inner mitochondrial membrane and prevent oxidative damage to cells. It has been shown to decrease islet apoptosis and increase islet yield[23].

MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5one; Edaravone) is a new free radical scavenger produced for use in some clinical conditions. It is a strong scavenger of hydroxyl radicals and was shown to have benefits in myocarditis and cerebral infarction. MCI-186 prevented islet cell death dose-dependently when cells were treated with H_2O_2[28].

DISCUSSION

Most studies on antioxidants and diabetes in the literature have evaluated the possible effects of antioxidants in preventing β cell glucose toxicity and cytokine-mediated cell damage in the field of type 1 diabetes pathophysiology. The role of antioxidants in type 2 diabetes is still undetermined due to scientific conflict. Vitamin E and C, selenium and the majority of antioxidant trace elements, as well as herbs and drugs have been used or are being used to try to manage diabetes and its complications[2,63]. Moreover, there are, as seen in this review, few studies available which used antioxidants to improve islet transplantation outcome. However, a review of the existing studies indicates that many antioxidants are able to enhance cellular defense mechanisms against oxidative stress in islet cells. With deeper inspection of the presented studies, the effects of these drug compounds can be divided into two separate sub-categories.

The first sub-category concerns the direct antioxidant effects of drug compounds on islet viability (direct inactivation of free oxygen species). For instance, vitamin E and C were shown to increase viability of islets but they failed to decrease MCP-1 release from the cells. This shows that these vitamins do not act *via* inflammatory pathways. Enzyme antioxidants also promote islet yield and activity by direct inactivation of free radicals. The role of nitric oxide (NO) in early islet transplantation rejection should not be overlooked. NO was shown in experimental studies to harm islet cells in culture medium early after transplantation. N (G)-monomethyl-L-arginine (NMA) (a reversible inhibitor of NO synthase) prevented the dysfunction or destruction of cultured islets and markedly decreased the time needed to restore euglycemia after intraportal transplantation of islets in diabetic rats[64]. Direct antioxidants such as vitamin E and salen-manganese compounds can prevent NO and nitrite radicals as well as ROS[65,66] thus may play a protective role.

The second sub-category of effects concerns the action of these drug compounds on the modulation of beta cell apoptosis initiation and signaling. One potential ROS-dependent target molecule is the nuclear transcriptional factor NF-κB. It is now known that NF-κB is a key transcription factor involved in regulating proinflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes. Some antioxidants block the effects of NF-κB including curcumin[22,27], glutathione and MnTDE porphyrin[32] and have been shown to ameliorate islet yields by reducing MCP-1 release as a consequence of NF-κB blockade.

However, the two above-mentioned sub-categories may change following further investigations. Newer studies, especially those after 2004, have focused on the possible pathways by which antioxidants may improve the function of islets.

As previously stated, antioxidant enzymes are found in low levels in islets compared with other tissues. In addition, minimal amounts of GPx protein and mRNA expression as well as GPx activity in islets have been detected[67], showing that GPx is low compared with SOD and CAT in islets. It is important to develop methods to increase islet survival and the number of islets during isolation and transplantation, thus

the suggestion of using antioxidants to improve islet transplantation is reasonable.

However, based on current data from *in-vitro* studies, we can conclude that the addition of an antioxidant during islet isolation procedures would result in better islet function. Furthermore, the overexpression of intracellular antioxidant enzymes and proteins[68], as well as transgenic islets[15] has been found to improve the function of islets, which is in agreement with our conclusions.

Interestingly, all the *in-vivo* studies with the exception of one on taurine[35] showed better control of blood glucose using antioxidant supplementation mainly in the early stages of islet transplantation. However, similar to *in-vitro* studies, there was no significant advantage of any one antioxidant on glucose control following transplantation. Of course, the lack of human studies limits our conclusions regarding *in-vivo* research and necessitates further investigations to establish the benefits of antioxidants in human islet transplantation. Fortunately, there is sufficient evidence on the existence of oxidative stress in diabetes and the significant role of antioxidants in the reduction of diabetic complications[69-76].

CONCLUSION

The collective data reviewed here show that different antioxidants improve islet transplantation procedures both *in-vitro* and *in-vivo*. We recommend antioxidant supplementation in islet isolation protocols, however, there is still insufficient evidence to draw definitive conclusions about the efficacy of individual supplements and to support profitable antioxidant management *in-vivo* particularly in humans.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Alcohol and liver

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FROM THE EDITOR

Liver is a primary site of ethanol metabolism, which makes this organ susceptible to alcohol-induced damage. Alcoholic liver disease (ALD) has many manifestations and complicated pathogenesis. In this Topic Highlight, we included the key reviews that characterize new findings about the mechanisms of ALD development and might be of strong interest for clinicians and researchers involved in liver alcohol studies.

Being the primary site of alcohol metabolism, liver is severely influenced by alcohol drinking. The combination of toxic effects of alcohol and numerous predisposing factors usually form the basis for ALD development. This disease has many manifestations, which are triggered by multiple pathogenic factors, causing progression in liver damage from steatosis to liver cirrhosis and hepatocarcinoma. The progression between various stages of ALD is driven by so-called "second hits", which trigger ALD development. In 2007, in *World Journal of Gastroenterology*, we published Topic Highlight: Alcohol liver injury: Pathological feature and models. There, we reviewed the role of alcohol in changes of iron metabolism, proteasome function, immune response, signaling mechanisms, transmethylation reactions, as well as apoptosis and mitochondrial damage. Current Topic Highlight is a logical continuation of the previous one, which further expands our understanding of the mechanisms of ALD progression and complexity of ALD pathogenesis, thereby providing important information for hepatologists about the modern directions in alcohol research.

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2 Dolganiuc A, Szabo G. *In vitro* and *in vivo* models of acute alcohol exposure. *World J Gastroenterol* 2009; 15: 1168-1177
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TOPIC HIGHLIGHT

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Nuclear effects of ethanol-induced proteasome inhibition in liver cells

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Abstract

Alcohol ingestion causes alteration in several cellular mechanisms, and leads to inflammation, apoptosis, immunological response defects, and fibrosis. These phenomena are associated with significant changes in the epigenetic mechanisms, and subsequently, to liver cell memory. The ubiquitin-proteasome pathway is one of the vital pathways in the cell that becomes dysfunctional as a result of chronic ethanol consumption. Inhibition of the proteasome activity in the nucleus causes changes in the turnover of transcriptional factors, histone modifying enzymes, and therefore, affects epigenetic mechanisms. Alcohol consumption has been associated with an increase in histone acetylation and a decrease in histone methylation, which leads to gene expression changes. DNA and histone modifications that result from ethanol-induced proteasome inhibition are key players in regulating gene expression, especially genes involved in the cell cycle, immunological responses, and metabolism of ethanol. The present review highlights the consequences of ethanol-induced proteasome inhibition in the nucleus of liver cells that are chronically exposed to ethanol.

Key words: Alcohol liver injury; Proteasome inhibition; Epigenetic mechanisms

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INTRODUCTION

Proteasomes of mammalian cells exhibit a complex heterogeneity. Different proteasome variants exist and perform subtle activities. The 20S proteasome catalytic core binds to different activators (19S, 11S and PA200), and the resulting complexes perform specific functions, such as ATP-ubiquitin proteolysis, immunological response, cell cycle regulation, nuclear factor (NF) κB activation, response to hypoxia, and transcription[1]. Upon immune response, or interferon (IFN)γ treatment, the three catalytic subunits, β1, β2 and β5, are replaced by three different subunits, LMP2, LMP7 and MECL-1, respectively, which form the immunoproteasome[2]. Several chemical compounds, proteins and regulatory complexes, function as activators or inhibitors of the catalytic core 20S proteasome[3]. Some of them are termed gate-openers because they provoke the opening of the gate at the alpha-type subunits of the 20S proteasome[4], and facilitate the access of designated proteins to be degraded to the catalytic chamber formed by the beta-type subunits. The best known of these activators are the regulatory complex 19S, which is involved in the ubiquitin-proteasome pathway (UPP), the PA28α/β and PA28γ complexes, which, among other functions, are important for the generation of epitope peptides presented to the major histocompatibility complex[5], and PA200, which is implicated in DNA repair[6]. Recently, the hybrid form of the 26S proteasome, which contains both 19S and PA28 complexes associated at the two opposite sides of the 20S core particle, has also been characterized[7], and other activators and proteins that modulate the proteasome activity are yet to be discovered.

As complex as is the UPP, it does not work alone. These regulatory complexes and proteasome interacting proteins (PIPs) complement the UPP to perform specific functions. Proteasome failure occurs in the liver cells because chronic ethanol exposure interferes with

the binding of the 20S catalytic core to its regulatory complex and to its PIPs. The 26S proteasome is responsible for the ATP-ubiquitin degradation of short-lived cellular proteins and the elimination of damaged and misfolded proteins, in addition to providing basic housekeeping functions[8-10]. Therefore, damaged and ubiquitinated proteins accumulate, causing protein aggregation, such as Mallory-Denk body (MDB) formation[11-13], when, because of chronic ethanol ingestion, the 20S is unable to bind to the 19S to form the 26S proteasome, which leads to cell death and tissue damage[14].

PROTEASOMES AND TRANSCRIPTION

The UPP plays an important role in a variety of cellular functions, primarily *via* its proteolytic activity. However, recent studies have implicated this pathway in the direct regulation of specific transcriptional factors[15,16]. It is now believed that the proteolytic activity of the proteasome is critical in promoting the exchange of transcriptional factors on chromatin, and possibly facilitating multiple rounds of transcription initiation, hence controlling gene expression[17]. Proteasomes are also now widely accepted as being essential for promoting the exchange of transcriptional factors on chromatin[18,19]. A growing body of evidence demonstrates that the components of the UPP are directly, and mechanistically, involved in transcription and in regulating the gene expression of the DNA repair system[20,21], cell cycle[22,23], and chromatin modifying enzymes[17]. Although the role of the UPP in regulating many transcription factors, such as p53, NFκB and hypoxia-inducible factor 1 α, is well established, the role of the UPP in regulating epigenetic mechanisms has only recently been given attention[24].

Ethanol-induced oxidative stress in nuclei is believed to damage DNA and proteins, thereby disrupting genomic integrity[25]. Oxidative stress has also been shown to cause adduct formation with lysine located in the N-terminal histone tails[26]. These lysine residues are the site of a number of post-translational modifications that regulate chromatin function. Therefore, chronic accumulation of oxidative stress adduct modifications may cause epigenetic changes, which may have important implications in terms of histone function and genomic integrity. Oxidatively damaged histones are degraded by a nuclear 20S proteasome in an ATP- and ubiquitin-independent manner[27], which indicates that damaged histones accumulate when the activity of the proteasome is inhibited and causes alteration of the epigenetic mechanisms.

However, the effect of ethanol-induced proteasome inhibition in the nucleus, and thus in regulating gene expression, is still not well known. Inhibition of proteasome function has been widely reported in models of alcoholic liver disease (ALD)[28,29], but how proteasome dysfunction may enhance hepatotoxicity is not well defined. It has been reported that chronic ethanol exposure leads to post-translational modifications of the alpha type subunits of the 20S proteasome that constitute the opening of the gate to the catalytic chamber of the 20S proteasome[29], thus blocking the opening of the gate and causing proteasome activity to decrease. Recently, proteasome activity has been measured in the isolated nuclei from liver cells of rats fed ethanol chronically, and the proteasome was found to be significantly inhibited (personal observation, manuscript submitted to *World Journal of Gastroenterology*, 2008). The inhibition of proteasome activity in the nucleus is therefore etiologically involved in the accumulation of damaged proteins in the nucleus, and in the deregulation of transcription.

Thus, it seems reasonable to postulate that the nucleus should have a more important proteasomal activity than other cellular compartments. It is possible that proteasome inhibition, caused by chronic ethanol feeding in the nucleus, may be greater than the inhibition caused in the cytosolic compartment[29].

EPIGENETIC MECHANISMS AND PROTEASOME INHIBITION

Chronic ethanol exposure causes an alteration in liver cell memory by changing the epigenetic mechanisms, thus causing significant liver pathology that persists after ethanol withdrawal[30]. Dr. French's group has shown that cells forming MDBs have a memory of the exposure to the drug even four months after drug withdrawal[31]. This memory is primarily associated with histone modifications that are recovered when s-adenosylmethionine (SAMe) is added to the drug-primed mouse diet[32].

Epigenetic mechanisms are involved in development, phenotype determination and maintenance, aging, and cancer. These epigenetic mechanisms are based on histone modifications controlled by histone acetyltransferases (HATs) and histone methyltransferases, and by DNA methylation, controlled by a regulated balance between DNA methyltransferase and demethylase. These epigenetic events can change rapidly in response to changes in cell signaling, which are initiated by environmental influences, such as inflammation, metabolism, or toxic injury. These changes cause the genome to respond globally or in a restricted manner, depending on the nature of the signals generated. During these responses, the histone modifications themselves are rapidly changed as well[33]. The dynamics of this process are pervasive and extremely complex, which makes it difficult to connect the epigenetic responses to the final manifestations. The identification of key epigenetic changes, which are linked to alcohol liver injury, is of high interest and is at its very beginning. Histone modifying enzymes are the key target to regulate the histone modifications. Histone deacetylase (HDAC) inhibitors and demethylating agents are currently used in clinical trials to treat cancer. HDAC inhibitors, like trichostatin A, repress

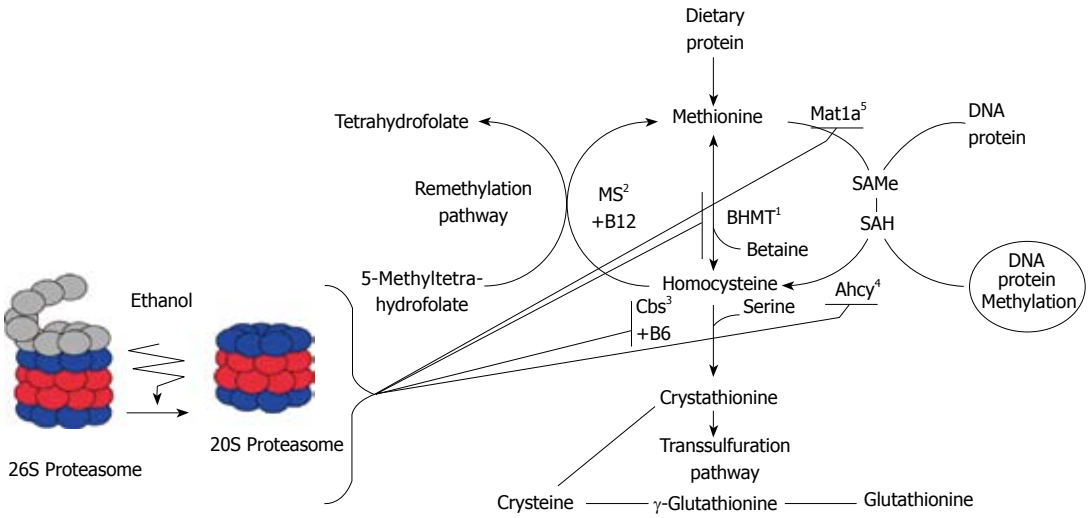

Figure 1 Illustration of methionine metabolism enzymes system and the effects of proteasome inhibition in the remethylation pathway. 1-BHMT: Betaine homocysteine Methyltransferase; 2-MS: Methionine synthase; 3-Cbs: Cystathionine beta-Synthase; 4-Ahcy: S'-adenosylhomocysteine transferase; 5-Mat1a: Methionine adenosyltransferase.

the expression of genes that induce growth arrest, cellular differentiation and apoptosis[34]. Likewise, DNA methyltransferase inhibitors, like 5-azacytidine (Aza), are being used in clinical trials to treat cancer. Aza, a potent inhibitor of Dnmt 1 and Dnmt3a (not Dnmt3b), induces demethylation and reactivation of silenced tumor suppressor genes[35]. Moreover, proteasome and HDAC inhibitors may be a prominent combination in future cancer therapy[36], and specifically, in gastrointestinal cancer[37].

HISTONE MODIFICATIONS AND HISTONE MODIFYING ENZYMES

The nucleosome surface interacts with several histone modifying enzymes that are responsible for histone tail lysine or arginine modifications[38], operating as part of a predictive and initiated epigenetic code that defines patterns of gene expression[39]. Histone modifications control the accessibility of DNA elements for transcription factors, which influences gene expression. These modifications control the recruitment of proteins involved in DNA methylation and affect chromatin structure and function[40].

Histone modification alterations can change long-term effects on gene expression patterns by influencing chromatin structure throughout the cell cycle, and from one cell generation to the next[41]. For instance, trimethylation of H3K9me3 and depletion of acetylation leads to gene silencing, because H3K9me3 acts as a binding site for heterochromatin-forming protein HP1. In the nuclear extract from liver cells of rats fed ethanol chronically, H3K9me3 is decreased and H3K9 acetylation is increased[42,43]. These histone modifications are linked to DNA modifications and affect gene expression because DNA methylation is dependent on prior histone methylation[44-47].

More epigenetic mechanisms still need to be elucidated with respect to alcoholism, particularly histone modifications, such as methylation, phosphorylation and ubiquitinylation of lysine and arginine residues. The task will be to identify the modifications and the specific modifying enzymes that regulate gene expression and account for the cellular memory observed when ethanol is fed chronically[30]. For example, it has been reported that a global increase in histone H3 lysine 9 acetylation, histone H3 lysine 4 dimethylation and H3 lysine 27 trimethylation is found in the liver of rats fed ethanol chronically[48], which correlates with an increase and a decrease, respectively, in transcriptionally active promoters. However, the genes linked to these changes are still to be identified. Microarray analysis has shown that hundreds of genes are up-regulated and hundreds are down-regulated when rats are fed ethanol for 1 mo, which indicates that ethanol causes several epigenetic changes that affect gene expression[25]. More specifically, gene expression of ALDH1a4[48] and ADH[49] are up-regulated, which suggests a change in the epigenetic events that regulate the expression of these genes in the liver of rats fed ethanol chronically. However, the linkage of ethanol-induced proteasome inhibition to the changes in the expression of these genes still needs to be determined. Data mining of microarray analysis of proteasome inhibition has shown that, similar to ethanol-treated rat liver, several genes are changed, which indicates an epigenetic phenomenon c proteasome inhibition[17,50].

PROTEASOME INHIBITION AND REMETHYLATION PATHWAY

The changes in gene expression in the remethylation pathway, particularly for the enzymes that regenerate SAMe, cause deregulation of cellular methylation, thus resulting in abnormal cellular methylation.

Hyperhomocysteinemia (HHcy) is a leading cause of liver injury in ALD. A lack of the activity of the enzymes responsible for homocysteine metabolism,

particularly cystathionine b-synthase (Cbs) or 5,10-methy lenetetrahydrofolate reductase, results in severe forms of HHcy. Additionally, nutritional deficiencies in B vitamin cofactors required for homocysteine metabolism, including folic acid, vitamin B6 (pyridoxal phosphate), and/or B12 (methylcobalamin), can induce HHcy (Figure 1).

Proteasome inhibition is believed to be involved etiologically in the changes in gene expression of these enzymes responsible for the hepatic transmethylation reactions. Indeed, analyses performed on the livers of rats given PS-341 (bortezomib, Velcade®), a dipeptide boronic acid used to inhibit proteasome activity, and used today as an anticancer agent in human myeloma[51], have shown down-regulation of gene expression for methionine metabolizing enzymes. Proteasome inhibition causes a decrease in methionine adenosyltransferase (MAT1a), in S-adenosylhomocysteine hydrolase gene expression, and a marked decrease in gene expression of betaine-homocysteine methyltransferase[50,52] and Cbs[53], which affects cellular methylation. Rats fed ethanol chronically also exhibit a relatively low level of MAT1a, which is associated with low levels of SAMe and faster growth[54]. These observations indicate that SAMe is an attractive agent for both chemoprevention and treatment of alcohol associated liver cancer[54].

Proteasome inhibition, induced by ethanol feeding, is associated with histone modifications, and is involved in the regulation of histone modifying enzymes, such as the HAT p300[42]. It is believed that proteasome inhibition affects gene expression, particularly genes of the transmethylation pathway, *via* an epigenetic mechanism that involves modified histone and transcriptional factors.

CONCLUSION

Regulation of gene expression of the transmethylation pathway is one of the promising approaches to counteract proteasome inhibition effects. Investigating the changes in the remethylation pathway, particularly the enzymes that regenerate SAMe, the major methyl group donor, is essential to identify the key enzyme that is changed by chronic ethanol exposure and proteasome inhibition.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

In vitro and *in vivo* models of acute alcohol exposure

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Abstract

Alcohol abuse is a global problem due to the financial burden on society and the healthcare system. While the harmful health effects of chronic alcohol abuse are well established, more recent data suggest that acute alcohol consumption also affects human wellbeing. Thus, there is a need for research models in order to fully understand the effect of acute alcohol abuse on different body systems and organs. The present manuscript summarizes the interdisciplinary advantages and disadvantages of currently available human and non-human models of acute alcohol abuse, and identifies their suitability for biomedical research.

Key words: Models; Acute alcohol abuse; Human; Non-human; Progress

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INTRODUCTION

Alcohol abuse is widely spread around the globe[1-9]. Alcohol is the third leading cause of preventable death in the United States and the third leading cause of healthy years lost to death and disability in developed nations[9]. Humans use and abuse alcohol acutely or chronically, when alcohol consumption is frequent and dependence has developed[10]. Although significant progress was made in the area of alcohol research during the last decades, the pathogenesis of alcohol use and abuse is not fully understood. Further, most research was focused on alcoholism, which is an advanced stage of alcohol abuse, involving chronic alcohol consumption, alcohol dependence and severe health and social consequences[1-13]. Thus, research models are emergent in order to detail what drives human desire to consume alcohol, how the body responds to alcohol, and most important, what are the beneficial and harmful effects of acute alcohol consumption on the human body.

ACUTE ALCOHOL ABUSE (AAA): HOW BIG THE PROBLEM REALLY IS?

In the USA, a "drink" is defined as an equivalent of 14 g alcohol, which equals roughly 1 shot [1.25 oz of 40% (80-proof) liquor], 1 (12 oz) beer (4.2 mL/L, Ethanol), or 1 (4 oz) glass of wine (12 mL/L, Ethanol)[14]. In other countries, the alcohol content of a serving is measured in "units". One unit (about 25 mL of a 40% 80-proof liquor) contains 7.9 g of pure ethanol[8,15]. However, in many countries the "standard drink" is used to quantify alcohol intake. More importantly, the standard drink varies significantly from country to country, from 10 mL (7.9 g) of alcohol in the UK to as high as 25 mL (19.75 g) in Japan[16]. Current use includes at least one drink in the past 30 d; binge drinking is defined as five or more drinks on the same occasion within 2 h at least once in the past 30 d; and heavy use is defined as five or more drinks on the same occasion on at least 5 different days in the past 30 d[11-13]. The 0.08% blood alcohol level (BAL) is the legal limit for most states in the US and it is achieved with consumption of five or more drinks for an adult male and four or more drinks for an adult female[11-13].

Traditionally medical research focuses on the mechanisms of chronic alcohol abuse; this is due to the significant financial burden that society encountered primarily from chronic alcohol abusers[1-13]. However, more recently acute alcohol abuse has emerged as a

Table 1 The characteristics of *in vitro* and *in vivo* models of AAA

AAA model	Advantages	Disadvantages	Area of research
In vitro	Low cost	Limited alcohol metabolism	Behavioral and biomedical
	Technically easy to perform	Limited complexity at cellular and tissue levels	
	Large number of experimental groups		
	Pure cell populations	Limited areas of research, not suitable for behavioral and social studies.	
	Single cell type or multi-cell type co-culture		
	Strictly controlled settings yielding reproducible results		
In vivo	Availability of physiological routs of alcohol administration	Ethical concerns	All areas of research including biomedical, behavioral and social.
	Complex interactions of all bodily organs and systems, including complex metabolism	High cost	
		Limited information about the effect on one separate cell population.	
	Controlled settings, caloric and composition controls		
	Indications to individual and population variability		

social problem[17]. The National Survey on Drug Use and Health (NSDUH) estimated that in the USA about 4.4 million persons had used alcohol for the first time in 2004, which lead to about 12 000 "new recruits" per day; this was significantly greater than in 2002 (3.9 million) and 2003 (4.1 million). Most (86.9%) of the 4.4 million recent alcohol initiates were younger than 21 years of age at the time of encounter. More than one fifth (22.%) of people age 12 or older participated in binge drinking at least once in the 30 d prior to the survey in 2004[12]. Acute alcohol intake in the form of binge drinking in 2004 was highest for the 18- to 25-year-old age group compared with other age groups, with the peak rate occurring at age 21[1,5-7,11-13]. The statistics also show that illness and death among young adults primarily result from lifestyle choices and behaviors, including excessive alcohol use[18,19].

AAA: BIOMEDICAL IMPACT

The known biological effects of AAA include those of the central nervous system (CNS) and non-CNS origin. Alcohol use is characterized by symptoms of CNS intoxication, impaired brain activity, poor motor coordination, and behavioral changes[20,21]. AAA leads to impaired CNS activity due to alcohol's effect on synthesis[22], release[23] and signaling[23,24] of neurotransmitters, including serotonin[25,26], glutamate[27], GABA[28], endocannabinoids[29,30] and their receptors. AAA causes damage and functional impairment of the gastrointestinal (GI) tract, including luminal GI[31-38], liver[39-55], and pancreas[56-62]; it also affects the protein, carbohydrate, and fat metabolism[58,63-66]. AAA leads to insufficient immune system responses to infections; such deficiency was observed both in organ-specific[67-69] and systemic infections[70-72]. Acute alcohol intoxication impairs the ability of the host to counteract hemorrhagic shock[73], augments corticosteroid release[74] and delays wound healing[75-78], thus contributing to higher morbidity and mortality[79] and prolonged recovery from trauma[80]. The pathogenesis of AAA effects on human health is not fully understood.

MODELS OF AAA

Research of acute alcohol consumption/abuse is entirely based on models, due to their advantage of controlled settings. Currently there are *in vitro* and *in vivo* models of AAA; their characteristics are defined in Table 1. In contrast to chronic alcohol abuse, the research of AAA has not benefited from population studies due to recall bias[81-84].

One important feature of AAA models is the definition of biologically meaningful levels of alcohol, either *in vitro* or *in vivo*, and their relationship to blood alcohol levels (BAL) in humans. This is an important requirement of the research models of AAA, because BAL can be detected as soon as minimal amounts of alcohol are ingested[85], however measurable affects of alcohol on physiology and/or behavior is established at 0.08% or above this level, with individual variations depending on the species, metabolic particularities, age, gender and genetic background[86-97]. It is also important to identify that AAA models differ by their route of alcohol delivery to achieve alcohol intoxication, some of them being physiological, such as oral administration, while others being non-physiological, when ethanol is administered by parenteral routes. Nevertheless, current research shows that the BAL levels, rather than the route of alcohol administration play a major role in the establishment of the biological effects of alcohol[97].

Thus, optimal AAA models should fulfill several criteria: (1) Define the length of alcohol exposure. *In vitro* the length of acute alcohol treatment is variable in diverse published experimental settings and range from seconds to hours; it is currently accepted that treatment with alcohol for up to 24 h is considered as an acute setting[98-106]. *In vivo* the consumption of alcohol in one setting implies that the entire dose of alcohol is consumed at once, while a 'binge' is defined by NIAAA as an excessive pattern of alcohol drinking that produces BAL greater than 0.08% within a 2-h period and may, or may not, be associated with dependence[11,12,17,18]. Thus any model using consumption of biologically active amounts of alcohol within 2 h is considered an acceptable model of AAA[81,107-121]. (2) Establish an exposure to an accurate concentration of ethanol. For *in vitro* studies the 10-100 mmol/L ethanol range is considered physiological, with 25 mmol/L ethanol being close to 0.08% BAL achieved *in vivo* after 4-5 drink equivalents[7,11,12,98-106]. For the *in vivo* studies an 0.08% BAL or above this level yields

signs of intoxication and it is employed in the majority of biomedical studies[107-121]. (3) Recruit individuals who are currently not and never have been alcohol abusers for *in vivo* studies and employ alcohol-naïve primary cells or cell lines for *in vitro* studies. Alcohol use habits of the study participants are usually determined by questionnaires[122]. Among most frequently used questionnaires are those that incorporate the AUDIT and CAGE tests[123-125]; the study parameters are usually permissive for males who had alcohol use of fewer than nine drinks/week, females < 6 drinks/week.

IN VITRO AAA MODEL

The *in vitro* alcohol treatment model is based on supplementation of culture media with pure alcohol, usually 200-proof ethanol. Currently supplementation of cell culture with a wide variety of alcohol concentrations, ranging from 1 to 500 mmol/L, is reported in the bio-medical literature. One of the major concerns with the *in vitro* alcohol treatment using concentrations above 100 mmol/L is the direct cytotoxic effect of alcohol on cells[40,100]. At lower concentrations (< 100 mmol/L), alcohol changes the redox status of the cells and alters intercellular junctions[33,126], increases the membrane fluidity of cells[127-129] and affects the composition of lipid rafts[106,130,131], all of which may contribute to alcohol-mediated increase in transcellular and paracellular permeability[132,133] and thus affect cell function[106,130-134]. Alcohol also affects the expression of adhesion molecules[135], which may be a concern when using adherent cell types due to possible cell detachment. Additional concerns arise from the possibility of modified *ex vivo* function of some primary cells, including hepatocytes, stellate cells and their precursors, due to limited *ex vivo* environment compared to *in vivo* conditions[136-138].

From a technical point, the acute alcohol exposure of cells *in vitro* may be hampered by alcohol evaporation. To avoid the fluctuation of alcohol concentration due to evaporation, investigators used settings where ethanol was added into the culture media and the cell culture plates were maintained for the entire duration of stimulation in a microclimate chamber at 37℃ with gas mixture and an alcohol atmosphere[139]. For example, if the desired alcohol concentration in the cell culture is 25 mmol/L, a Petri dish with 2 × the alcohol amount (50 mmol/L) was placed on the bottom of the chamber to ensure the saturation of the gas in the chamber); such conditions maintain the initial alcohol concentration ± 15% over a 24 h period[139]. However, depending on the scientific question of the study, the declining alcohol levels *in vitro* may be desired to mimic the alcohol elimination *in vivo*; in these situations the *in vitro* experiments are disadvantaged by the absence/limitation of alcohol metabolism[76,134].

The *in vitro* AAA model offers the possibility of primary *in vitro* exposure of alcohol-naive cells to alcohol alone or its combinations with diverse pharmacological or naturally-derived substances[24,28,31,35,36,42,55,68,71,72,96,103], but also the investigation of the effects of *in vivo* exposure to alcohol followed by *ex vivo* exposure to other stimulants[110,113,115] or *vice versa*. One other main characteristic of the *in vitro* AAA model is its simplicity, often considered as an advantage or disadvantage depending on the research goal. Most of the *in vitro* research involves culture of a single cell type[134,139-142] or co-culture of several cell types[143]; while such an approach brings forward the differential effect of alcohol on pure cell populations, and/or their intercellular interaction; it lacks the systemic alcohol metabolism and intercellular interactions. More recently significant efforts were invested in establishment of more complex *in vitro* systems, such as culture of cells in three dimensional systems[100], organ slices[144] or organ explants[145]; while such systems are informative in the setting of chronic alcohol exposure to date there is no report of their use as an AAA model.

IN VIVO AAA MODELS

The *in vivo* models of AAA are more informative compared to the *in vitro* model due to complex physiological impact of alcohol on all bodily organs and systems, but also due to the availability of systemic alcohol metabolism. Currently there are human and non-human models of AAA, and the later include use of invertebrates[146-147] and vertebrates[21,25,37,44,46,47,53,65,72,86,93,94,98,104,110,111]. The invertebrate models (Drosophila melanogaster[146,147], Caenorhabditis elegans[105]) and those using lower vertebrates (Zebra fish Danio rerio)[98] are invaluable for research of the effect of alcohol on behavior, development and maintenance of memory, and on basic signaling mechanisms. These models offer the advantage of a well-defined genetic background, high-turnover rate of experiments due to short life cycle and relatively low-cost; in light of these advantages they constitute an excellent resource for research of signaling pathways and are highly desirable for their drug-screening capacity. On the downside, significant differences in the structure and function of organs and systems compared to humans limit the informative value of invertebrate and lower vertebrate models of AAA.

The vertebrate models are preferred to those using invertebrates due to closer resemblance of their bodily structure, function, and metabolism to that of humans. However, because of intrinsic differences between humans and other vertebrates, no single non-human model is perfect since none of the models can represent all features of the complex human trait, such as motivation for social occasional or binge alcohol consumption, development of alcohol dependence and establishment of the impact on health. Further, the controlled setting of research models may not be completely satisfactory for psychology and social research, since they may not fully reproduce the social component, the motivation and the spontaneity of alcohol abuse. However, research models are invaluable for the understanding of the effects of alcohol and its

mechanisms of action on hardwired bodily systems, including the brain and all other organs and systems.

HUMAN MODELS OF AAA

Human alcohol intake in the experimental setting is the best available model of AAA, because it offers the advantage of the physiological route of alcohol consumption, the possibility to investigate human pathobiology and the availability of relatively large amounts of physiological bodily fluids for research. The disadvantages of human models of AAA include ethical concerns related to potential harmful health effects due to excessive or repeated intoxication, and the theoretical possibility of development of dependence or tolerance even after a one-time drinking session. Published models of human AAA are based on consumption of alcoholic beverages containing either distilled ethanol or wine; these models are physiological, as they involve alcohol drinking, and achieve a biologically meaningful BAL[87,92,107,110-113,115]. The majority of the reported *in vivo* models of human AAA strictly control for the amount of alcohol based on the constant volume of alcohol per kg body weight, includes placebo-treated age and gender matched controls. However, most of these studies design the consumption of the alcohol beverage during a 2 h period of time[92,107,110-113], which based on recent NIAAA and NSDUH classification qualifies as binge drinking[11-13,17]. Thus the major disadvantage of the human models of AAA is that they (1) do not clearly distinguish between one-time and the binge alcohol consumption pattern, and, (2) for ethical reasons, do not allow longer binge sessions which are often observed in real-life and account for the majority of the heavy alcohol intake in young adults[5,7,11-13,17-19].

To fulfill the requirement for an AAA model, the human studies usually include nonalcoholic individuals, who did not drink any alcohol at least 24 h prior to the study. Depending on the study design, some AAA human models require that the study participants did not take any medication, while others accept individuals taking moderate doses of anti-hypertensive medication and oral contraceptives[107,110]. The study participants are usually required to abstain from food for at least 6 h before alcohol consumption and are allowed free access to water and a light meal before or shortly after the study[107]. The human model of AAA is currently used for research in physiology[86,92,111,122], hematology[107,128] and immunology[110,113,115].

CONSUMPTION OF DISTILLED ETHANOL MODEL

In this model the study individuals drink distilled alcohol (usually 80-proof vodka) in amounts of about 0.5-0.6 g/kg body weight, which is an equivalent of about 2 mL vodka/kg body weight in a standardized total volume of liquid (300-450 mL of water or orange juice)[92,107,110-113].

CONSUMPTION OF NON-DISTILLED ETHANOL MODEL

In this model the study individuals drink wine to an equivalent of a pre-determined amount of ethanol/kg BW (for example, Fehr *et al*[107] reported use of 4.36 mL of red wine/kg of body weight as an equivalent of 0.5 g ethanol/kg BW to lead to a peak BAL of about 15 mmol/L in the first 2 h), while the control individuals are exposed to the same volume of fluid by mouth (usually water) per individual in a randomized way. The major disadvantage of this model is the use of controlled volumes of liquids that are not matched by calorie intake or by composition, which is technically challenging to achieve due to restricted availability of equivalent alcohol-free compounds. To bypass the bias concern some studies employ a cross-over approach, where each subject serves as its own control and repeats the study at least 2 wk after the first experiment with either alcohol or placebo consumption according to the cross-over design[107].

NON-HUMAN AAA MODELS

Among non-human vertebrates commonly involved in alcohol research are primates[90,91,148], pigs[104,120], dogs[114,121], mice[70,72,74,86,89,96,109,118,119,141], rats[88,94,108,149,150] and rabbits[132]. The rodent AAA models (mice and rats) are used most frequently due to their relatively well-defined genetic background and the availability of diverse genetic traits, including those coding for high or low alcohol consumption[88,89,96,109]. Most non-human AAA models currently in use[93,95] examine relative oral self-administration from a bottle containing alcohol versus one[86,94,108] or multiple bottles[119] containing water (preference drinking) or administration of alcohol against the will, either by physiological (by mouth using gavage)[54,71,72] or by non-physiological (parenteral)[67,68] routes. Voluntary consumption of alcohol may be an optimal animal model of AAA, due to physiological route and pattern of alcohol consumption. However, in the self-administration models it is not clear when or if the animals drink to pharmacologically significant levels because the drinking is episodic and often occurs over a 24-h period. Nevertheless, these models are invaluable for research of neurobiology of acute intoxication with alcohol and for establishment of mechanisms of addiction. The AAA models using administration of alcohol against-the-will bypass all the above-mentioned inconveniences of AAA models using voluntary consumption. Alcohol administered either by physiological (by mouth using gavage) or by non-physiological (parenteral) routes yields comparable physiological effects on the central nervous system and on organs/systems that are not affected directly by the route of alcohol administration, such as muscle and brain[97]. However, administration of alcohol per os is more physiological compared to administration via parenteral routes, yields meaningful levels of BAL and shows signs of acute alcohol intoxication[54,71,72,132,149].

Table 2 The effect of acute alcohol abuse on GI system

GI segment	Effect of acute alcohol exposure
Oral cavity	Unknown
Esophagus	Low concentrations of alcohol (up to 5%) cause alterations in ion transports and affect the barrier function
	Concentrations of alcohol of 10% and above cause injury of mucosa
	Co-carcinogenic potency
	Motor dysfunction: decrease in lower esophageal sphincter pressure and amplitude
Stomach	Motor dysfunction: Inhibition of gastric emptying
	Mucosal damage, impaired barrier function, increased epithelial permeability
	Pro-inflammatory reaction: decreased gastric blood flow, vascular damage, polymorphonuclear neutrophils (PMN) dependent- and independent-mucosal damage
	Aggravation of *H pylori* infection
Intestine	Disruption of barrier function
	Epithelial apoptosis
	Enhanced bioavailability of some alcohol-soluble drugs and impaired absorption of key nutrients
	Increased paracellular intestinal permeability to toxins
Liver	Hepatocytes:
	Amplification of Fas-mediated hepatocyte death
	Generation of oxidative stress
	Hepatic mitochondrial dysfunction
	Increased free iron levels
	Imbalanced fatty acid metabolism
	Inhibition of IFN-α-induced antiviral response towards hepatotropic viruses including hepatitis C virus favors hepatitis C virus repli con expression
	Induced histone H3 acetylation leading to increased gene expression in the liver
	Limited hepatic protein synthesis
	Arrest of liver regeneration early after partial hepatectomy and suppression of hepatic stimulator substance (HSS) activity by induc tion of liver cell cycle arrest
	Kupffer cells:
	Suppressed LPS-mediated priming for enhanced CC-chemokine release in vitro; up-regulated expression of CC-chemokine mRNA; primed the KC for enhanced RANTES release
	Desensitized HIV-1 gp120-induced CC-chemokine production
	Downregulates HIV-1 glycoprotein 120-induced KC and RANTES production
	Regulates production of reactive oxygen species
	Modulate the tolerance to LPS
	Stellate cells:
	Imbalanced redox potential owed to increased generation of reactive oxygen species upon GSH depletion
Pancreas	Stimulates islet blood flow, amplifies insulin secretion, induces hypoglycemia
	Lower baseline amylase output of acinar pancreatic cells, with the difference being significantly exacerbated by cerulein stimulation
	Interference with release of oxidized proteins in acinar cells
	Predisposes the pancreas to postprandial cholinergic stimulation that triggers cellular events leading to pancreatic inflammation
	Impaired apical exocytosis and redirected exocytosis to less efficient basolateral plasma membrane sites
	Augments elevated-[Ca^{2+}]-induced trypsin activation in pancreatic acinar zymogen granules, leading to premature activation of trypsin and tissue damage.

Among disadvantages of administration of alcohol per os are technical challenges, time consumption and high cost of the procedures. In contrast, alcohol administration by parenteral routes is relatively easy to perform technically and offers controlled settings (time, amount); on the downside, they may be less suitable for research of the effects of alcohol on organs/systems that are affected directly by the routes of alcohol administration. In this context, administration of alcohol by intraperitoneal route may be less suitable for research using peritoneal macrophages, or even liver and intestines, compared to other administration routs, such as intravenous or enteral. Further, some parenteral methods of alcohol administration are preferred over others, owing to differences in the level of technical difficulty of the procedure and the effect of alcohol on different cell types. For example, alcohol administration by intravenous route is known to affect the erythrocytes when present in high concentrations[128]. Thus alcohol administration by intravenous route is currently limited to creating an acute alcohol exposure during treatment of alcohol withdrawal symptoms[151], while administration by intraperitoneal injections is widely preferred in research settings.

Similar to human AAA models, the non-human *in vivo* models employ either distilled alcohol[53,67-72,74,89,90,96,109,116,119,121,132,135,141,152] or alcohol-containing beverages, such as wine[152,153] and beer[46]; the control groups are usually treated with alcohol-free caloric and composition equivalents. The vertebrate AAA models are widely used in research of biomedical effects of AAA, including brain[23-30,116], gastrointestinal [38-44,46-48,64-66,154], vascular[73,153], muscle[97] and immune[68-72,74] systems.

THE PARTICULARITIES OF AAA MODELS FOR RESEARCH IN GASTROENTEROLOGY

In contrast to the abundance of the literature about the

effects of chronic alcohol abuse on the gastrointestinal system, research of the effects of acute alcohol abuse on the gastrointestinal (GI) tract is limited to certain cell types, as outlined in Table 2.

Currently the state of scientific knowledge suggests a tight interplay between organs and systems. The GI system is dependent on blood circulation and systemic availability of metabolites, is closely governed by both the central and the autonomous nervous system[155,156] and contains a hallmark of resident and recruited immune cells[157,158]. Thus, it is conceivable that the direct effects of alcohol on either of these systems will indirectly affect the function of the gastrointestinal system; this area is currently largely unexplored.

From a technical point, the GI research may take advantage of both *in vitro* and *in vivo* AAA models; however some *in vivo* models, such as those using parenteral administration of alcohol by the intraperitoneal route, may be less suitable due to the non-physiological direct contact between high concentrations of alcohol and GI tissues.

Alcohol use/abuse is associated with acute life-threatening conditions, including acute alcoholic hepatitis[45] or acute pancreatitis[159]. The majority of these patients report acute alcohol abuse, which is often overlapping with withdrawal from or even discontinued chronic alcohol abuse, or it follows an episode of binge drinking[45,159]. As such, it is difficult to associate these diseases with the single-occasion AAA, yet they do not fit into the classic chronic alcohol abuse picture. This category of alcohol abuse, defined as "acute-on-chronic", is in need of modeling for GI research.

In prospective, we currently lack in-depth knowledge in regards to the effects of acute alcohol abuse on different segments of the luminal GI tract, on liver functions, and on pancreas, including its endocrine and exocrine functions. Further, we do not know if acute alcohol consumption affects the GI stem cells and/or is involved in development of GI-derived tumors.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Autophagy and ethanol-induced liver injury

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Abstract

The majority of ethanol metabolism occurs in the liver. Consequently, this organ sustains the greatest damage from ethanol abuse. Ethanol consumption disturbs the delicate balance of protein homeostasis in the liver, causing intracellular protein accumulation due to a disruption of hepatic protein catabolism. Evidence indicates that ethanol or its metabolism impairs trafficking events in the liver, including the process of macroautophagy, which is the engulfment and degradation of cytoplasmic constituents by the lysosomal system. Autophagy is an essential, ongoing cellular process that is highly regulated by nutrients, endocrine factors and signaling pathways. A great number of the genes and gene products that govern the autophagic response have been characterized and the major metabolic and signaling pathways that activate or suppress autophagy have been identified. This review describes the process of autophagy, its regulation and the possible mechanisms by which ethanol disrupts the process of autophagic degradation. The implications of autophagic suppression are discussed in relation to the pathogenesis of alcohol-induced liver injury.

Key words: Autophagy; Autophagosome; Ethanol metabolism; Hepatomegaly; Lysosomes; Signal transduction

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INTRODUCTION

Lysosomes are the primary hydrolytic organelles of higher eukaryotic cells and have been appropriately named the cell's "digestive system". A large number of acid hydrolases inhabits these organelles. Together, these enzymes catalyze the breakdown of all forms of biopolymers, ranging from intracellular and extracellular proteins to storage carbohydrates (e.g. glycogen) to nucleic acids. Macromolecular catabolism takes place inside the lysosomal lumen at hydrogen ion concentrations that are 500-fold higher (pH about 4.7) than that in the cytoplasm (pH about 7.4)[1]. Maintenance of such an acidic lysosomal interior occurs through the continuous action of ATP-dependent proton transporters that reside on its membrane. Lysosomes exhibit morphological and functional plasticity; they undergo continuous change as they mature from larger primary vesicles that segregate and carry hydrolase precursors, to mature hydrolytic organelles that execute intracellular digestion. As the catabolic organelles for protein degradation, lysosomes differ from proteasomes in their size, their complexity, their intracellular locations and in the types of substrates they degrade. While proteasomes have essentially supplanted lysosomes as the primary system that degrades intracellular proteins in higher eukaryotes, the crucial task of breaking down protein substrates that are more difficult to digest, as well as hydrolyzing all other forms of polymeric substrates, is left to lysosomes. Certainly, compelling evidence of their importance is the existence of a number of human abnormalities known as lysosomal storage diseases, in which afflicted individuals possess genetically defective lysosomal hydrolases. Clinically, this group of diseases exhibits a spectrum of impaired function, from neurodegeneracy to premature death[2]. Furthermore, deficiencies in the pathways that participate in lysosome

biogenesis or that have a crucial function in supplying lysosomes with their substrates, can be lethal to the organism[3]. Studies in the mammalian liver have provided a great deal of our current understanding of the lysosomal pathway, as this organ is highly responsive to changes in nutrient supply[4,5]. The liver is also the predominant organ that metabolizes ethanol and liver injury is the principal clinical complication of alcohol abuse[6]. This review will focus on the process of autophagy, its role in normal hepatic function and its alteration due to ethanol consumption. A portion of this review will consider how ethanol disrupts protein catabolism, which is believed to have a significant role in liver injury. The reader will note the citation of other excellent reviews that have been published on the process of autophagy and/or lysosomal proteolysis[3,7-9] The physiological and biomedical importance of autophagy is further underscored by the establishment of the international journal, *Autophagy*, the first issue of which was published in 2005.

FEEDING THE LYSOSOMAL APPARATUS; HETEROPHAGY AND AUTOPHAGY

In order for lysosomes to be "fed", substrates of high molecular weight must gain entry to the interior of the organelle through a membrane that is normally impermeable to large molecules. Such access is accomplished by a number of mechanisms, each involving the active participation of membranes. During heterophagy extracellular materials are imported into the cell by either receptor-mediated or fluid phase endocytosis or by bulk phagocytosis of larger particles, including whole cells such as bacteria and apoptotic cells. All these are accomplished by invagination of the plasma membrane, thereby transporting the membrane-enclosed material, via endosomes to their final destinations, which, in many (but not all) cases is degradation in lysosomes. Heterophagy has a critical function in the normal turnover of senescent plasma proteins and in adaptive immunity, the latter involving antigen presentation through the action of endosomal proteases by the MHC class Ⅱ pathway[10].

Autophagy is exclusively an intracellular lysosomal process, which literally means "self eating". It was originally described in the 1950s and later systematically analyzed by the classical work of de Duve and Wattiaux in rat liver. Autophagy of intracellular proteins occurs by three distinct mechanisms: Macroautophagy is the vacuolar engulfment by membranes of large portions of cytoplasm and collateral organelles, forming a double membrane vesicle called an autophagosome or autophagic vacuole. The outer membrane of the autophagosome later fuses with existing lysosomes to form an autolysosome in which the contents are degraded. Microautophagy describes membrane uptake by existing lysosomes of smaller portions of cellular constituents by lysosomal membrane invagination followed by rapid hydrolysis of those molecules. Chaperone-mediated autophagy (CMA) is the singular uptake and degradation by lysosomes of specific protein substrates bearing a recognizable peptide motif, KFERQ. The latter process is mediated by molecular chaperones, the most prominent of which is a cytoplasmic form of heat shock protein 70 (HSC 70), which recognizes and binds the peptide motif of the substrate protein[11,12]. The CMA substrate/chaperone complex then moves to the lysosome, where a specific receptor, the lysosome-associated membrane protein type-2A (LAMP-2A) recognizes and binds the complex; the substrate protein is then unfolded and translocated across the lysosome membrane and degraded in the lysosomal lumen. CMA is enhanced by oxidative stress, and, in addition to the proteasome, thereby serves a quality control function by degrading modified proteins. CMA also declines with age, largely due to the depletion of LAMP-2A. Recent studies have demonstrated that CMA can be restored in livers of aged mice that are transgenic for LAMP-2A. These animals, compared with their wild type littermates, showed improved cellular quality control and hepatic function[13]. While microautophagy and CMA are largely ongoing constitutive processes, macroautophagy is more highly regulated. In general, when the term autophagy is used here, it will be used synonymously with macroautophagy.

Autophagy is clearly important for survival of the organism, particularly in times of nutrient deprivation when degradation of macromolecular constituents becomes necessary to recycle essential carbon sources to maintain viability. Autophagy was initially characterized morphometrically and biochemically in rat liver, one of the most sensitive organs to changes in nutrient supply. An illustration of this is when rats are fasted for 48 h, they exhibit a 40% loss of liver protein and a significant loss of liver mass, both of which are rapidly restored to normal levels 12 h after food is replenished. These respective changes correlate with an induction of autophagy by fasting followed by its nutrient-induced suppression after refeeding[4,14,15]. A similar suppression of both autophagy as well as lysosome biogenesis was reported 24 h after partial hepatectomy in regenerating rat liver[16]. Thus, autophagy rapidly responds to these changes and is an important component of cell adaptation. In fact, the half-life of the autophagosome is less than 10 min[5], which indicates that the molecular machinery required to form autophagic vacuoles is constitutive for rapid degradation of cellular constituents. Furthermore, like the proteasome, autophagy has a significant role in removing misfolded proteins from the hepatocyte. Such is the case in alpha-1-antitrypsin (A-1AT) deficiency, in which the cellular accumulation of the mutated unsecretable form of A-1AT, known as ATZ (the isoform most prone to aggregation), is directed to autophagic vacuoles for degradation[17]. Similarly, other aggregation-prone proteins are reported to have the same fate[18,19]. It was recently demonstrated that inhibition of proteasome activity by treatment of mice with bortezomib caused Mallory-Denk-(MD)-like body formation. Activation of autophagy with

rapamycin treatment prevented the formation of these inclusions, indicating that proteasome inhibition causes the formation of M-D like bodies in livers of susceptible mice, while autophagosomes prevent M-D body accumulation by resorbing and degrading these insoluble inclusions[20]. Thus, autophagy, not only functions in cell survival but also provides an additional line of cellular defense, the latter by removing aggregated, potentially toxic proteins, presumably resistant to degradation by the proteasome.

REGULATION OF AUTOPHAGY

Hormonal and nutrient regulators of autophagy include food deprivation or glucagon treatment and both are reliable autophagy inducers. Conversely, treatment with growth factors such as insulin or amino acids are well-known methods of autophagic suppression. The molecular mechanisms by which these agents actually induce or suppress autophagy have been investigated more recently and this has led to investigations of the protein components and signaling mechanisms that regulate macroautophagy to form the autophagosome. To date, over 30 autophagy-related gene products (Atgs) are now known. There are extensive molecular interactions that occur between these gene products in the early stages of autophagosome formation. The most well known mechanism is that of the class Ⅰ phosphoinositol-3-kinase (PI-3K) pathway, which initiates an upstream signaling cascade to regulate the activity of the mammalian target of rapamycin (mTOR), a kinase, which is a major regulatory anabolic protein, and a suppressor of autophagy. Activation of mTOR leads to inhibition of Atg1, a key signal in autophagy induction. If Atg1 is not inhibited by mTOR, it is free to recruit the autophagy proteins, Atg11, Atg13 and Atg17 to form a complex which signals the induction of autophagy. The cellular machinery that initiates the formation of the autophagosome occurs through the activation of Beclin-1 (Atg6), which interacts with the class Ⅲ PI 3K pathway and complexes with Atg 14. A second complex involves the interaction of Atg12, Atg16, Atg5, and Atg 7 complex, which is critical for the recruitment of Atg8, (also known as LC31) to the phagophore, the isolation membrane at which the macroautophagic response is initiated and the precursor of the autophagosome. Autophagic induction leads to cleavage of Atg8 (LC3-Ⅰ), which is covalently bound to phosphatidylethanolamine (PE) to form a distinct form of the protein, LC3-Ⅱ. The latter is associated with and is a commonly used marker for autophagosome membranes. Atg8 is believed to be involved in autophagosome closure as well as its relative size to surround portions of the cytoplasm for degradation[21]. Eventual fusion of the autophagosome and the lysosome requires the participation of the LAMP-2 protein. Figure 1 depicts the major regulatory proteins and signaling factors in the autophagic response.

ETHANOL CONSUMPTION AND THE HEPATIC AUTOPHAGIC/LYSOSOMAL SYSTEM

The majority of ethanol is metabolized in the liver and individuals who abuse alcohol by routinely drinking 50-60 g (about 4 to 5 drinks) of ethanol per day are at risk for developing alcoholic liver disease[6]. The pathogenesis of liver disease from alcohol abuse comes from the interaction of several factors, including the generation of oxidants and reactive metabolites from ethanol oxidation, which, in turn, causes other metabolic derangements. In addition, both acute and chronic ethanol administration cause enhanced formation of cytokines, especially TNF-alpha by hepatic Kupffer cells, which have a significant role in liver injury. This latter aspect is reviewed and described elsewhere[22-24]. Besides the development of fatty liver (steatosis), another early sign of excessive ethanol consumption is liver enlargement and protein accumulation, both of which are common findings in alcoholics and heavy drinkers. Baraona *et al*[25,26] originally described this in alcohol-fed rats. Later investigations sought to determine the origin of ethanol-elicited protein accumulation. They showed that chronic ethanol consumption slowed down the catabolism of long-lived proteins in rat liver[27] and also depressed hepatic protein synthesis[28]. Other studies confirmed that rats subjected to chronic ethanol feeding exhibited lower hepatic proteolysis than control rats. This was associated with reduced volume densities of autophagosomes and autolysosomes, as determined morphometrically[29]. Our later studies, using isolated lysosomes from chronically ethanol-fed rats also revealed a reduced capacity of these organelles for proteolysis of endogenous as well as exogenous (i.e. akin to CMA) substrates *in vitro*. Furthermore, there was an enhanced tendency of lysosomes from ethanol-fed rats to leak their contents, but the intact organelles still exhibited lower levels of cathepsins, B and L compared with those from pair-fed control rats. A later report showed that lysosomes from ethanol-fed rats exhibited altered sedimentation properties on density gradients. Pulse-chase analyses, using isolated hepatocytes demonstrated that the reduced lysosomal content of cathepsin L was the result of delayed trafficking of its nascent precursor and impaired processing to its mature catalytic form in cells from ethanol-fed rats[16,30,31]. Both flow cytometric and microscopy studies showed that vesicular movement and acidification of lysosomes in hepatocytes are both impeded after ethanol exposure[32,33].

MECHANISMS OF ETHANOL-ELICITED SUPPRESSION OF AUTOPHAGY

The exact mechanisms responsible for the aforementioned ethanol-induced changes in hepatic autophagy and lysosomal proteolysis are not clear but some cogent explanations have been advanced. One is that oxidants (e.g. peroxynitrite) and reactive species

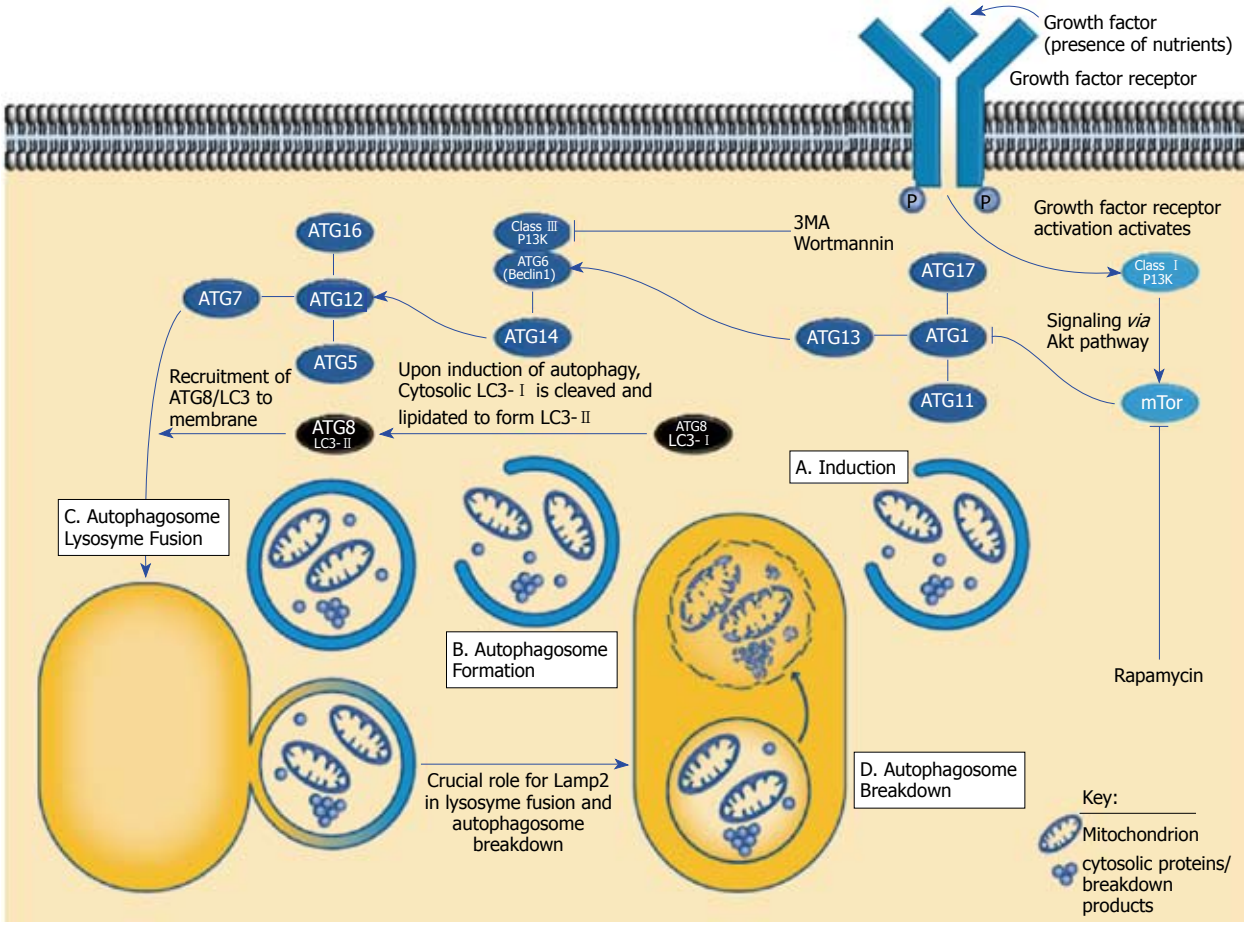

Figure 1 Interaction of gene products and pathways in the regulation of autophagy. Growth factors and nutrients activate the Class Ⅰ P13K proteins, which, in turn, signal, *via* the AKT pathway to activate mTOR. This leads to inhibition of ATG1 - the primary signal for autophagy. Nutrient deprivation or inhibition of mTOR by rapamycin allows ATG1 to recruit ATG11, ATG13 and ATG 17 to form a complex to initiate formation of the autophagosome. This is dependent on the formation of 2 complexes: ATG 6 (Beclin1) which interacts with the Class Ⅲ P13K protein complexes with ATG14. Another complex involves ATG12, ATG16, ATG5 and ATG7. The latter complex recruits ATG8 (LC3). Upon induction of autophagy, cytosolic LC3-I (ATG8) is cleaved and lipidated to form LC3-Ⅱ. LC3-Ⅱ is a marker for the autophagosome membrane. Fusion between the autophagosome and the lysosome and subsequent breakdown of the contents of the autophagic vacuole requires LAMP2 protein. Reproduced in modified form with permission (Abcam, Inc.) http://www.abcam.com/ps/pdf/cardiovascular/autophagic.pdf).

(e.g. acetaldehyde and malondialdehyde-acetaldehyde)[34] derived from ethanol metabolism may impair autophagy, similar to that which occurs with the proteasome[35-37]. Some of the current evidence for this is circumstantial, but nevertheless compelling as there is evidence of lysosomal damage, as judged by enhanced lysosomal fragility, which could result from either altered lipid metabolism, oxidative stress or both[15]. By analogy, other instances of lysosome fragility by iron-induced oxidative stress are documented[38,39]. However, ethanol administration has little to no effect on the activities of lysosomal hydrolases[31] and, because a significant number of cathepsins rely on a reduced sulfhydryl group for their catalytic activity in their active centers, the alteration of lysosomal glutathione or cysteine content by ethanol seems unlikely. It is worth noting that lysosomes and proteasomes seem to exhibit differential sensitivity to ethanol levels in the serum. Animal experiments have revealed that proteasome activity declines in animals that have higher (i.e. > 40 mmol/L) serum ethanol concentrations[40]. The lysosomal system, on the other hand, appears to be impaired by lower serum ethanol levels, as recently reported in livers of female ethanol-fed rats[41].

Ethanol-induced suppression of autophagy may result from alterations in hepatic amino acid pool sizes, especially those of leucine, phenylalanine, methionine, histidine, tryptophan, glutamine, proline, and tyrosine, which have been deemed regulatory amino acids and suppressors of macroautophagy[5]. L-leucine appears to be one of the strongest autophagic suppressors in this group. It is noteworthy that reports indicate that chronic ethanol administration in rats increases the intrahepatic levels of leucine by 1.4 to 1.8-fold over pair-fed controls[27,42]. Thus, the association of an ethanol-induced reduction in autophagy with higher levels of intrahepatic leucine may partially explain autophagic suppression in the ethanol-fed state. However, it remains to be conclusively demonstrated whether this association represents a causal relationship. It is also paradoxical that ethanol feeding results in higher levels of leucine during a slowdown in protein catabolism when one would expect the opposite situation. Still, leucine accumulation could reflect a reduced ability of the liver to synthesize proteins, which indeed occurs in ethanol-fed animals[42,43].

A third likely mechanism of autophagic suppression

by ethanol is its well-documented ability to disrupt protein trafficking in the liver. Autophagy requires the action of cytoskeletal elements, including the microtubules and microfilaments. Both are necessary for autophagosome formation and their fusion with other vesicular bodies, as demonstrated by blockage of these processes with specific inhibitors, including nocadazole and vinblastine (microtubules) and the cytochalasins (microfilaments)[44,45]. Disruption of vesicular movement within the hepatocyte by ethanol treatment occurs by mechanisms that are independent of the molecular motors, dyenin and kinesin, although there is evidence for alterations in the protein, dynamin[32]. Trafficking of exogenous proteins into the hepatocyte by endocytosis[46,47] and the intracellular delivery of proteases to lysosomes[31] are both inhibited by ethanol consumption. Furthermore, the anti-secretory properties of ethanol in the liver are well documented. Studies with liver slices and cultured cells indicate that ethanol metabolism is required for disruption of these protein trafficking events[48-51]. *In vitro* investigations also revealed that acetaldehyde, the initial product of ethanol oxidation, inhibits the polymerization of tubulin to form microtubules, indicating that the reactive metabolite may impair protein trafficking by forming adducts with tubulin subunits, thereby blocking their polymerization into microtubules[52]. Further, the finding that acetaldehyde can undergo secondary reactions with malondialdehyde (MDA) to form more bulky substituents on proteins, known as malondialdehyde acetaldehyde adducts (MAA) with proteins, makes this mechanism of autophagic suppression an attractive hypothesis.

ETHANOL-INDUCED ALTERATIONS IN CELLULAR SIGNALING: REGULATION OF AMP KINASE

While the foregoing mechanisms of autophagic suppression by ethanol are plausible, it is likely that ethanol and/or its metabolites impact signaling events that govern the autophagic response. There have been significant advances in our understanding of these processes at the molecular level, as recently reviewed[3]. Because autophagy is an energy-dependent process and requires ATP[53], it follows that ATP depletion naturally suppresses autophagy. Ethanol consumption has been reported to inhibit ATP production by mitochondria[54], in part by enhancing oxidative modification and inactivation of proteins within these organelles[55]. However, others have reported an increased efficiency of mitochondrial oxidative phosphorylation in livers of ethanol-fed animals[56], Thus, reduced ATP availability from mitochondria may not fully explain such downstream effects on autophagy. Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric protein that is, itself, activated by elevated ratios of AMP/ATP and is a significant regulator of a variety of metabolic and signal transduction pathways, including autophagy. Elevated AMP/ATP ratios are indicators of low intracellular energy charge. Therefore, when AMP activates the AMPK it generally down-regulates energy requiring pathways and stimulates catabolic pathways. Interestingly, however, inhibition of AMPK also suppresses autophagy[57]. These findings are consistent with the suppression of autophagy by ethanol consumption, which significantly reduces AMPK activity in liver[58]. Such a decline in AMPK activity would, in turn cause the release of suppression of mTOR activity by AMPK[59], thereby permitting mTOR to suppress proteolysis. Conversely, AMPK activation suppresses mTOR, thereby allowing autophagy to proceed. Recent work with cultured hepatoma cells determined that ethanol metabolism is required for suppression of AMPK phosphorylation. This occurs by an inactivation of two upstream kinases, LKB-1 and protein kinase C-zeta. Simultaneously, ethanol exposure activates protein phosphatase 2A (PP2A), which causes dephosphorylation (and inactivation) of AMPK[60]. The latter findings seem paradoxical, as AMPK is generally activated by reactive oxygen and nitrogen species[22,61]. Thus, while ethanol metabolism similarly generates oxidants and reactive species, including acetaldehyde, these molecules down-regulate upstream kinases and up-regulate the downstream phosphatase, PP2A, resulting in AMPK inactivation, which, in turn, can cause autophagic suppression. AMPK also has an important role in regulating lipid metabolism and AMPK suppression by ethanol allows activation of rate-limiting enzymes involved in lipid biosynthesis, which contribute to ethanol-induced fatty liver[62,63]. Figure 2 depicts the putative mechanisms of autophagic suppression.

PATHOLOGICAL CONSEQUENCES OF AUTOPHAGIC SUPPRESSION

Protein accumulation due to ethanol-elicited decline in protein catabolism probably contributes to the formation of Mallory Denk (M-D) bodies in liver cells. These inclusions are prominent histological hallmarks of liver disease in alcoholics[64]. M-D bodies contain the cytokeratins 8 and 18 as well as ubiquitin, and p62, an adaptor protein. As pointed out earlier, M-D bodies comprise an aggresome, consisting of filamentous ubiquitylated misfolded proteins that are believed to represent a failed attempt by the proteasome to degrade them. Recent evidence indicates that autophagy can degrade these insoluble complexes[20]. However, clearance of such cellular debris is hampered by an ethanol-elicited suppression of autophagy, a situation that may have a perilous outcome if drinking continues.

In addition to the accumulation of potentially toxic proteins, ethanol also causes mitochondrial damage. Changes ranging from morphologically detectable mitochondrial swelling, to DNA fragmentation, to depolarization of the inner membrane, (commonly known as the mitochondrial permeability transition or MPT) to disruption of mitochondrial gene products

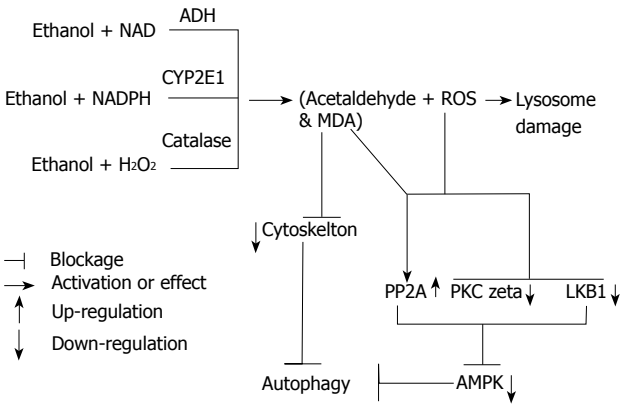

Figure 2 Putatative mechanisms of autophagy suppression by ethanol. Ethanol metabolism by the three major pathways, alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1) and catalase generates acetaldehyde, which can undergo secondary reactions with malondialdehyde (MDA) to form MAA. In addition, reactive oxygen species (ROS) are generated by CYP2E1 catalysis. Reactive oxidants (ROS) may also contribute to lysosomal damage. The reactive aldehydes are known to form adducts with tubulin and other cytoskeletal elements to block trafficking and movement of autophagic vacuoles and their formation. The combined formation of ROS and acetaldehyde putatively cause upregulation of protein phosphatase 2A (PP2A) and a downregulation of LKB1 and PKC zeta. The latter changes cause inactivation of AMPK, which in turn suppresses autophagy due to up regulation of mTOR (not shown) as described in the text.

as well as decreases in glutathione occur in livers of ethanol-fed animals[55,65-72]. It is crucial that such damaged organelles be removed from the cell and there is evidence that selective engulfment of mitochondria (called mitophagy) into autophagic vacuoles occurs in cells as a quality control device[73-75]. Similarly, there appears to be rather selective autophagy of ER components (ER-phagy) as well as peroxisomes (pexophagy) in cells under conditions where there is an accumulation of these organelles[76,77]. It remains to be demonstrated whether such selective autophagy represents actual recognition of damaged organelles or simply occurs because of mass action due to their accumulation, however, reports show that the permeability transition may act as a signal for autophagic destruction of the mitochondrion[74]. While there is no firm evidence of ethanol-elicited suppression of mitophagy, the detection of increased numbers of damaged mitochondria in livers of ethanol-fed animals provides circumstantial evidence of mitophagy inhibition. In this regard it is also worth noting that mitochondrial damage also occurs by non-oxidative products of ethanol metabolism, namely fatty acid ethyl esters (FAEE). These condensation products between ethanol and fatty acids are generated enzymatically and have been shown to cause mitochondrial damage[78]. Thus a mechanism for ethanol-induced injury in nonhepatic tissue such as pancreas, heart and brain could occur by the generation of FAEE.

CONCLUSION

This review has summarized the evidence for the ethanol-elicited suppression of autophagy as a mechanism by which liver cells can accumulate damaged proteins and organelles. Suppression of proteolysis in general can be lethal to cells, as normal turnover is disrupted and the removal of potentially toxic proteins is prevented. As an organ that is sensitive to nutrients as well as toxins, the liver is highly active in autophagy. The inability to respond to depletion of nutrient supply in the alcoholic state is a potentially perilous condition. Still, while there have been numerous investigations of hepatic autophagy, the actual mechanism(s) by which ethanol may influence this process remain(s) to be conclusively determined. From this summary of results from our laboratory and from the literature, we hypothesize that ethanol consumption probably influences pathways upstream of the autophagic process, including inhibition of AMPK, but this awaits experimental confirmation. Finally, while this review has specifically focused on autophagy in hepatic tissue, a recent report indicates that ethanol exposure also slows autophagy in neural cells[79], indicating that this effect of ethanol is not exclusively liver-specific. In conclusion, while autophagy has been recognized for decades as an indispensable means of macromolecular disposal, its roles in other cellular processes from differentiation to cell death are now being documented, thus rekindling new interest in this fascinating field of investigation.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Is the iron regulatory hormone hepcidin a risk factor for alcoholic liver disease?

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Abstract

Despite heavy consumption over a long period of time, only a small number of alcoholics develop alcoholic liver disease. This alludes to the possibility that other factors, besides alcohol, may be involved in the progression of the disease. Over the years, many such factors have indeed been identified, including iron. Despite being crucial for various important biological processes, iron can also be harmful due to its ability to catalyze Fenton chemistry. Alcohol and iron have been shown to interact synergistically to cause liver injury. Iron-mediated cell signaling has been reported to be involved in the pathogenesis of experimental alcoholic liver disease. Hepcidin is an iron-regulatory hormone synthesized by the liver, which plays a pivotal role in iron homeostasis. Both acute and chronic alcohol exposure suppress hepcidin expression in the liver. The sera of patients with alcoholic liver disease, particularly those exhibiting higher serum iron indices, have also been reported to display reduced prohepcidin levels. Alcohol-mediated oxidative stress is involved in the inhibition of hepcidin promoter activity and transcription in the liver. This in turn leads to an increase in intestinal iron transport and liver iron storage. Hepcidin is expressed primarily in hepatocytes. It is noteworthy that both hepatocytes and Kupffer cells are involved in the progression of alcoholic liver disease. However, the activation of Kupffer cells and TNF-α signaling has been reported not to be involved in the down-regulation of hepcidin expression by alcohol in the liver. Alcohol acts within the parenchymal cells of the liver to suppress the synthesis of hepcidin. Due to its crucial role in the regulation of body iron stores, hepcidin may act as a secondary risk factor in the progression of alcoholic liver disease. The clarification of the mechanisms by which alcohol disrupts iron homeostasis will allow for further understanding of the pathogenesis of alcoholic liver disease.

Key words: Alcohol; Hepatocyte; Kupffer cells; Oxidative stress; Second hit

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INTRODUCTION

Many eukaryotes require iron for critical biological processes[1,2]. However, excess iron is toxic, causing lipid peroxidation and oxidative stress[3]. Due to it being a "double edged sword", iron uptake and transport must therefore be tightly regulated[4-7]. Hepcidin, a circulatory peptide synthesized by the liver, performs this important task by regulating iron transport in different parts of the body including duodenum, spleen and bone marrow[8,9]. Alcohol has been shown to suppress hepcidin expression in the liver leading to an increase in intestinal iron transport[10-15]. Both iron and alcohol act synergistically to cause liver injury. Disruption of hepcidin synthesis by alcohol may therefore be one of the "second hit" mechanisms leading to the progression of alcoholic liver disease.

ALCOHOL-INDUCED DISTURBANCES IN IRON METABOLISM

Alcohol consumption has long been associated with

changes in iron homeostasis. The reported changes range from anemia to iron overload[16-22]. Blood loss is common in patients with alcoholic cirrhosis[23]. Anemia and iron deficiency can be due to gastrointestinal blood loss arising from the complications of alcohol abuse. Nutritional deficiencies, such as folate deficiency, can also be a common cause of anemia[24-27]. Megaloblastic and sideroblastic anemias, macrocytosis of alcoholism, elevated mean corpuscular volume, and iron deficiency have been reported to be common among hospitalized chronic alcoholics[23,27-30]. However, it has been demonstrated that anemia and megaloblastic and sideroblastic changes do not occur if an adequate diet is provided during chronic alcohol administration to human volunteers[25,31]. This has also been shown to be the case for relatively well-nourished alcoholics[25,26,32,33]. These findings therefore suggest that alcohol by itself does not induce iron deficiency or anemia.

In fact, wine has a high iron content and increases iron absorption[34]. Decreasing the alcohol content of red wine has been reported to significantly reduce the absorption of non-heme iron in human subjects[35]. Beer consumption has been suggested to have a more significant effect on serum ferritin levels than wine and spirits, both in males and females[18]. Alcohol consumption (up to 2 alcoholic drinks per day) has also been shown to exert a protective effect by reducing the risk for iron deficiency anemia in adult participants of the Third National Health and Nutrition Examination Survey[36]. On the other hand, heavy alcohol consumption (more than two alcoholic drinks per day) elevates the risk of iron overload[36]. In both male and female adolescents (aged 16-19 years), who participated in the First National Health and Nutrition Examination Survey, serum iron concentration was significantly related to drinking frequency[17]. Alcohol consumption was associated with elevated serum iron concentration in both male and female adolescents. Interestingly, male adolescents also exhibited increased transferrin saturation and hemoglobin concentration[17]. Drinking frequency was unrelated to dietary iron intake, poverty index or race[17]. A Danish population survey also reported a correlation between alcohol intake and elevated serum ferritin in healthy adult males and females (aged 30-60 years)[37]. African Americans who consume alcohol (4 drinks per day) exhibit a higher prevalence of ferroportin Q248H allele, which is implicated in iron accumulation, and alcohol has been suggested to contribute to higher body iron stores in this population[38]. Alcohol elevates iron absorption and patients with alcoholic cirrhosis often exhibit elevated liver iron content[39,40]. *In vivo* whole-body retention studies have demonstrated a two-fold increase in intestinal iron absorption in chronic alcoholics[41]. Alcoholic cirrhosis patients with higher liver iron content display increased mortality rates[42]. Experimental animal models of prolonged alcohol exposure also display increased liver iron deposition and hepatocellular injury[19,43]. In mild cases of alcoholic liver disease (ALD), iron has been reported to accumulate in hepatocytes[44]. However, in patients with advanced ALD, both parenchymal cells and Kupffer cells exhibit iron staining[20].

In some alcoholics, the elevated serum iron indices have been reported to significantly decrease within 2-6 wk of abstinence, suggesting a direct role for alcohol consumption[45]. However, the mechanisms by which alcohol disrupts iron metabolism are unclear. Recent studies indicate a role for hepcidin in this process.

HEPCIDIN

The discovery of the circulating peptide hormone hepcidin has revolutionized our understanding of iron metabolism. Hepcidin sequence is highly conserved between species, from fish to mammals. Hepcidin was first isolated from human urine and serum as short cysteine-rich peptide forms[46,47]. Hepcidin is homologous to the members of the defensin family of antimicrobial peptides and has been reported to exhibit antimicrobial activity *in vitro*[46]. It is the studies with transgenic mice that have highlighted the importance of this protein in the regulation of iron metabolism. Namely, hepcidin knockout mice, deficient in the expression of hepcidin, develop severe iron overload, whereas mice overexpressing hepcidin display iron deficiency and anemia[48,49]. Moreover, the synthesis of hepcidin has also been shown to be responsive to the body iron levels. Iron overload state induces hepcidin synthesis whereas iron deficiency and anemia have the opposite effect[50,51].

Liver is the main site of hepcidin synthesis in the body[50]. It is synthesized as an 84 amino acid precursor protein and is subsequently cleaved into the biologically active 25 amino acid peptide form[8,9,50,52]. In the liver, hepcidin is primarily expressed in the hepatocytes (Figure 1). Although to a much lesser extent, other cells of the liver and other organs also display hepcidin expression. The exciting discovery of the interaction between hepcidin and the iron exporter protein ferroportin was pivotal to our understanding of how hepcidin regulates iron metabolism[53,54]. The binding of hepcidin to ferroportin induces the internalization and degradation of the ferroportin protein, which in turn inhibits iron from being transported into the circulation. This is how hepcidin accomplishes the regulation of iron mobilization between distant locations (i.e. duodenum, bone marrow, reticuloendothelial macrophages) in the body (Figure 1)[8].

The synthesis of hepcidin in the liver is modulated by numerous upstream regulators[1,2]. Transferrin receptor 2, the genetic hemochromatosis gene product, Hfe, and the juvenile hemochromatosis gene product, Hjv, are positive regulators of hepcidin expression[55-62]. Mutations in these genes result in an increase in body iron stores (i.e. hemochromatosis) due to the down-regulation of hepcidin expression. Recently, a novel candidate TMPRSS6, a transmembrane serine protease also known as matriptase 2, has been identified. Interestingly, TMPRSS6 is the first identified negative regulator of liver hepcidin expression. In other words, TMPRSS6 keeps hepcidin expression under control in the liver[63,64]. Accordingly, patients expressing TMPRSS6 mutations exhibit iron-refractory iron deficiency anemia due to elevated hepcidin produc-

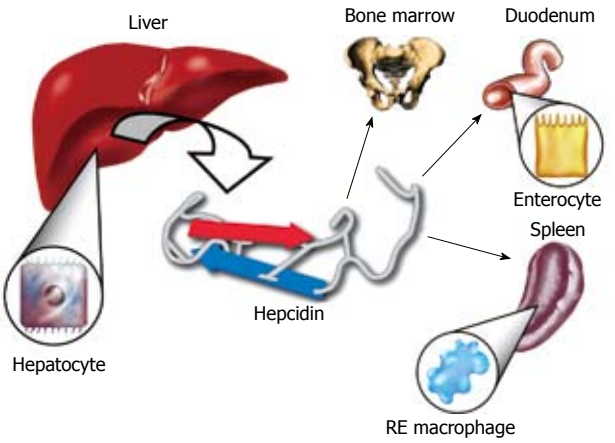

Figure 1 The Iron regulatory hormone hepcidin. Hepcidin is synthesized in the hepatocytes of the liver as an 84 amino acid precursor protein. It is subsequently cleaved into the 25 amino acid biologically active peptide form and is released into the circulation. Hepcidin plays a central role in the regulation of iron metabolism by inhibiting the release of iron from the enterocytes of the duodenum and from reticuloendothelial macrophages. Hepcidin blocks the export of iron from these cells by binding to the iron exporter protein, ferroportin, which induces the internalization and degradation of ferroportin protein. As a soluble mediator, hepcidin establishes the cross-talk between distant organs in the body in order to maintain iron homeostasis.

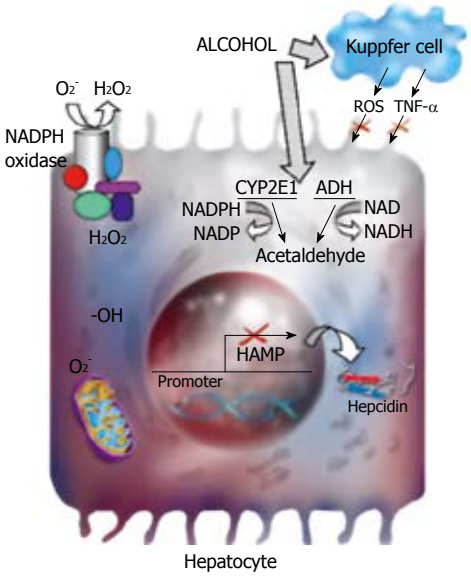

Figure 2 Hepcidin and alcohol. Alcohol is metabolized by alcohol dehydrogenase (ADH) and cytochrome P4502E1 (CYP2E1) in the liver. Alcohol-induced oxidative stress leads to the suppression of hepcidin promoter activity and hepcidin transcription in the liver. The parenchymal, but not the non-parenchymal cells of the liver are involved in the regulation of hepcidin transcription by alcohol-induced oxidative stress. The activation of CYP2E1 or NADPH oxidase and changes in mitochondrial functions are involved in alcohol-induced oxidative stress in hepatocytes. The role of these pathways in the regulation of hepcidin transcription by alcohol requires further investigation.

tion[63]. Moreover, the splicing defects in the TMPRSS6 gene lead to the mask phenotype, a recessive, chemically induced mutant mouse phenotype[64]. This phenotype results from the reduced absorption of dietary iron due to high levels of hepcidin expression[64]. However, the signaling mechanisms involved in these regulatory processes (negative or positive) are as yet unknown.

KUPFFER CELLS, ALCOHOL AND HEPCIDIN

Liver macrophages (Kupffer cells) also play a pivotal role in the progression of ALD. Alcoholics have elevated levels of lipopolysaccharide (LPS) in their circulation, which is believed to prime the macrophages leading to the release of proinflammatory cytokines, particularly TNF-α, and reactive oxygen species[65-67]. The importance of Kupffer cells in ALD has been demonstrated in studies where their depletion or inactivation blocked alcohol-induced symptoms, including inflammation, fatty liver and necrosis, in experimental animal models of chronic alcohol exposure[68,69].

In advanced forms of ALD and in experimental animal models of ALD, besides parenchymal cells, Kupffer cells also exhibit iron accumulation[20,70,71]. Reticuloendothelial (RE) macrophages play a major role in iron trafficking and recycling to meet changes in the body's iron demand[72]. Like the parenchymal cells of the liver, the RE system also stores large amounts of iron bound to the storage protein, ferritin. RE macrophages acquire iron mainly by phagocytosing senescent red blood cells. However, macrophages also express the divalent metal transporter1 (DMT1), natural resistance associated macrophage protein1 (Nramp1), hemoglobin scavenger receptor (CD163) and transferrin receptor1 (TrfR1), all of which are involved in iron uptake and transport[72-75]. The livers from experimental animal models of ALD and Japanese patients with ALD have been reported to exhibit significantly more TrfR1 mRNA or protein expression, respectively, compared to control animals or healthy human subjects[76,77]. Macrophages can take up transferrin-bound iron *in vitro*. However, a significant uptake of transferrin-bound iron by macrophages has not been observed *in vivo* in humans[72,78]. Although an increase in TrfR1 mRNA expression was observed in Kupffer cells of experimental animals, the increase in TrfR1 protein expression in Japanese patients with ALD was observed mainly in the hepatocytes[20,76,77]. It should also be noted that mice with disrupted TrfR1 gene, deficient in the global expression of TrfR1 receptor, display abnormalities only in erythropoiesis and neurologic development[79]. The Kupffer cells of rats exposed to chronic alcohol have also been shown to display an increase in mRNA levels of Hfe, the gene for genetic hemochromatosis[77]. Hfe gene product does not bind iron and is not directly involved in iron uptake. However, it is involved in the regulation of iron metabolism as shown by the fact that mice deficient in Hfe expression develop iron overload[80,81]. Hfe is believed to achieve this by modulating the expression and iron responsiveness of hepcidin in the liver[57,58,82]. On the other hand, the iron release from macrophages is regulated by the iron exporter protein ferroportin[83,84]. The multicopper ferroxidase, ceruloplasmin may also play a role in iron efflux in macrophages[85-87]. The Kupffer cells of animals subjected to alcohol exposure have been shown to display an increase in both ferroportin mRNA and protein,

indicative of increased iron export[77]. Of note, hepcidin binds to ferroportin and induces its internalization and degradation[53,54]. The increase in ferroportin expression in the Kupffer cells of these animals may be due to suppressed hepcidin expression. Alcohol has been shown to down-regulate hepcidin expression both in animal models of ALD and in patients with ALD[11-15]. However, Xiong *et al*[77] have reported no change in plasma prohepcidin levels and a decrease in hepcidin mRNA levels in Kupffer cells in their experimental model.

Iron has been reported to prime Kupffer cells for alcoholic liver injury in rats exposed to chronic intragastric alcohol infusion[70], The proinflammatory cytokine TNF-α is a key player in alcohol-induced liver injury[88-90]. Iron has been shown to activate the transcription factor, NF-κB in hepatic macrophages and induce the synthesis and release of TNF-α[70,91]. Following erythrophagocytosis, Kupffer cells have been shown to display elevated levels of LPS-induced NF-κB activation[70]. Treatment of cultured Kupffer cells *in vitro* and *ex vivo* with iron chelators blocked both NF-κB activation and TNF-α synthesis[70,71]. Proinflammatory cytokines (IL-1, IL-6, TNF-α) have been suggested to increase iron uptake into the monocytes of patients with rheumatoid arthritis[92]. It is therefore possible that alcohol and/or iron-induced release of TNF-α may serve as a feedback loop for further iron uptake into the Kupffer cells. On the other hand, Olynyk *et al*[93] have reported that deposition of iron in Kupffer cells impairs LPS-induced proinflammatory cytokine production in these cells. Xiong *et al*[77] have reported that the intracellular labile non-heme iron pool is involved in peroxynitrite or LPS-mediated activation of NF-κB and TNF-α release in Kupffer cells isolated from experimental animal models of chronic alcohol exposure. The intracellular labile iron pool is a transitory, free (non-ferritin-bound) pool of iron, which is chelatable by commonly used iron chelators. This pool of iron contributes to the generation of reactive oxygen species but its biological relevance is unclear[94]. Transient changes in the dynamics of the labile iron pool is not only affected by increased iron uptake but also by prooxidant chemicals, which induce the reductive release of iron from intracellular stores[95,96]. Xiong *et al*[77] have demonstrated that iron dextran treatment of cultured Kupffer cells *in vitro* does not affect the basal TNF-α release. Interestingly, Kupffer cells isolated from mice two weeks after iron dextran injection, exhibited significant increases in basal TNF-α release (independent of peroxynitrite or LPS stimulation). These findings suggest that either other cells in the liver (besides Kupffer cells) or yet unknown signals contribute to this process *in vivo*. It is also possible that the discrepancy between *in vitro* and *in vivo* iron loading regarding basal TNF-α release from Kupffer cells may not be due to iron (iron loading) *per se* but rather due to the differences in the level of oxidative stress.

The synthesis of hepcidin in the liver is mediated by several stimuli including iron and inflammation. Kupffer cells have been shown not to play a role in the regulation of hepcidin expression by iron *in vivo*[97,98]. The data in the literature regarding the involvement of Kupffer cells in the regulation of liver hepcidin expression by inflammation is contradictory[97-99]. Montosi *et al*[97] have reported a role for Kupffer cells in the regulation of hepcidin expression by inflammation in mice. On the other hand, Lou *et al*[98] and Theurl *et al*[99] have demonstrated that the depletion of Kupffer cells in mice does not abrogate the up-regulation of liver hepcidin expression by LPS.

The role of Kupffer cell activation and TNF-α signaling in the regulation of hepcidin expression by alcohol *in vivo* has also been reported[100]. The inactivation of Kupffer cells by gadolinium chloride in rats pair-fed with alcohol Lieber DeCarli diets for 6 wk or mice fed with ethanol in the drinking water for 1 wk did not reverse the alcohol-induced suppression of hepcidin expression in the liver[100]. Moreover, similar results were obtained when Kupffer cells were depleted by liposomes containing clodronate[100]. When phagocytosed by the Kupffer cells, clodronate released from the liposomes induces apoptosis and thereby depletes the Kupffer cell[101]. When co-cultured, Kupffer cells have been suggested to exert a negative effect on hepcidin synthesis in hepatocytes[99]. However, the depletion or inactivation of the Kupffer cells has been reported to not induce any significant changes in basal hepcidin expression levels in the livers of both control and alcohol-treated animals *in vivo*[100]. Interestingly, one week of ethanol treatment (in the drinking water) was sufficient to induce NF-κB activation and TNF-α and IL-6 release in mice, compared to control mice fed with plain water[100]. The neutralization of TNF-α inhibited the activation of NF-κB[100]. However, neither the neutralization of TNF-α nor the absence of TNF-α receptor I and II expression altered the effect of alcohol on hepcidin expression (i.e. down-regulation)[100]. These findings therefore strongly suggest that the activation of Kupffer cells and TNF-α signaling are not involved in the regulation of hepcidin expression by alcohol *in vivo*. These findings are also in agreement with Lou *et al*[98], who demonstrated that Kupffer cells are not required for the regulation of liver hepcidin expression by LPS-induced inflammation.

The suppression of hepcidin synthesis in the liver by alcohol occurs very early (within days) following alcohol exposure and involves hepatocytes, but not Kupffer cells (Figure 2)[12,100]. It is noteworthy that hepatocytes are the main site of hepcidin synthesis in the liver[50]. Alcohol-induced oxidative stress in hepatocytes is one of the main mechanisms by which hepcidin expression in the liver is down-regulated by alcohol[12]. The decrease in liver hepcidin synthesis leads to an increase in intestinal iron transport and liver iron content[10-12]. Hence, alcohol dysregulates iron homeostasis by suppressing hepcidin expression in the liver. Although Kupffer cells are not involved in the initial stages of this process induced by alcohol, the increase in iron stores (due to low levels of hepcidin) will further activate Kupffer cells, and thereby lead to the release of proinflamatory cytokines. It is therefore possible that unlike LPS, the priming of Kupffer cells by iron[70] occurs in later stages of ALD and exacerbates the inflammatory processes by facilitating further

release of TNF-α, and thereby contributing to liver injury.

ALCOHOL-INDUCED OXIDATIVE STRESS AND HEPCIDIN

Alcohol metabolism generates reactive oxygen species and lipid peroxidation products (malondialdehyde, 4-hydroxynonenal) during the oxidation of ethanol by alcohol dehydrogenase and cytochrome P4502E1 to form acetaldehyde. Serum thioredoxin levels are also an indicator of oxidative stress and they have been reported to be significantly higher in patients with ALD compared to healthy subjects[102]. Due to its capacity to take part in the Fenton reaction as a transition metal, iron itself induces the generation of reactive oxygen species (superoxide, hydroxyl iron), which subsequently damage cellular membranes *via* lipid peroxidation[3,103]. Iron and alcohol act as a deadly cocktail to exacerbate liver injury. Orally effective iron chelators have been reported to attenuate alcohol-induced hepatic lipid peroxidation in rats[104].

Alcohol-induced oxidative stress is involved in the suppression of hepcidin promoter activity and hepcidin transcription in the liver *in vivo* by inhibiting the DNA-binding activity of the transcription factor, C/EBP α[12]. Both Kupffer cells and hepatocytes are involved in alcohol-induced oxidative stress. However, a role for Kupffer cells in the regulation of hepcidin expression by alcohol in the liver has been excluded (Figure 2)[100]. This strongly suggests the involvement of hepatocytes in the regulation of hepcidin expression, and thereby iron metabolism by alcohol. Multiple pathways are involved in alcohol-induced oxidative stress in hepatocytes; redox state changes, the activation of CYP2E1 or NADPH oxidase and changes in mitochondrial functions[105-109]. Alcohol metabolism, which is accompanied by oxidation and reduction reactions, causes an imbalance in the redox state of the cell by generating excess NADH (reduced nicotinamide adenine dinucleotide). CYP2E1 enzyme, present in liver microsomes, has a high redox potential and is also metabolically active in the absence of alcohol[107]. It produces reactive oxygen species such as superoxide and hydrogen peroxide, which is increased by alcohol exposure leading to lipid peroxidation, oxidative stress and tissue injury. NADPH oxidase catalyzes the reduction of molecular oxygen to generate superoxide[110,111]. The liver expresses both phagocytic and non-phagocytic isoforms of NADPH oxidase[112,113]. Alcohol-induced liver pathology was not observed in mice with deficient NADPH oxidase activity suggesting a role for this enzyme in the progression of alcoholic liver disease[109]. Mitochondria play a key role both in iron biogenesis and alcohol metabolism[3,108,114]. Alcohol induces lesions in the proteins of the mitochondrial electron transport chain and decreases the rate of hepatic ATP synthesis[114-116]. This induces the transfer of unpaired electrons to molecular oxygen and elevates the production of reactive oxygen species. The involvement of these changes in the regulation of hepcidin expression in hepatocytes by alcohol needs further investigation (Figure 2).

Several factors including iron, viral infection, endotoxin and reactive oxygen species have been implicated to act as second hit factors in the progression of alcoholic liver disease. Of note, hepcidin expression in the liver is regulated by iron, alcohol, hepatitis C viral proteins, inflammation, hypoxia and oxidative stress[10-12,48,100,117-120]. Nishina *et al*[119] have recently shown that hepatitis C viral protein-mediated oxidative stress suppresses hepcidin transcription by altering the activity of the transcription factor, C/EBP α. These findings are in agreement with our previously published results demonstrating a role for alcohol-mediated oxidative stress in the inhibition of C/EBP α activity and hepcidin transcription[11,12]. Hepcidin may therefore act as a potential second hit factor in the progression of alcoholic liver disease.

CONCLUSION

Patients with alcoholic liver disease frequently display elevated iron stores. Recent reports by several groups strongly suggest that this may be a regulated mechanism rather than iron simply leaking out of injured intestinal cells. Hepcidin, an iron regulatory hormone synthesized in hepatocytes, plays a key role in the regulation of iron metabolism. Alcohol-mediated oxidative stress suppresses hepcidin expression in the liver leading to increased intestinal iron uptake and liver iron storage. Reduced hepcidin expression may be one of the mechanisms leading to elevated iron stores following alcohol exposure. Hepcidin may therefore act as a second hit in the progression of ALD. Both hepatocytes and Kupffer cells play a role in ALD. However, Kupffer cells have been reported to not be involved in the regulation of liver hepcidin expression by both acute and chronic alcohol exposure. Hepatocytes are the main site of alcohol metabolism in the liver leading to the generation of reactive oxygen species and the depletion of antioxidants. Alcohol-induced oxidative stress in hepatocytes is therefore one of the mechanisms leading to the suppression of hepcidin synthesis and thereby to iron overload, which in turn acts as a secondary risk factor in ALD. Accordingly, iron has been shown to prime Kupffer cells leading to the release of TNF-α in experimental animal models of ALD. The distruption of hepcidin synthesis and thereby iron metabolism by alcohol may have clinical relevance. Hepcidin therefore holds promise as an early diagnostic marker and as a candidate for therapeutic strategies for ALD.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Impact of asialoglycoprotein receptor deficiency on the development of liver injury

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Abstract

The asialoglycoprotein (ASGP) receptor is a well-characterized hepatic receptor that is recycled *via* the common cellular process of receptor-mediated endocytosis (RME). The RME process plays an integral part in the proper trafficking and routing of receptors and ligands in the healthy cell. Thus, the mis-sorting or altered transport of proteins during RME is thought to play a role in several diseases associated with hepatocyte and liver dysfunction. Previously, we examined in detail alterations that occur in hepatocellular RME and associated receptor functions as a result of one particular liver injury, alcoholic liver disease (ALD). The studies revealed profound ethanol-mediated impairments to the ASGP receptor and the RME process, indicating the importance of this receptor and the maintenance of proper endocytic events in normal tissue. To further clarify these observations, studies were performed utilizing knockout mice (lacking a functional ASGP receptor) to which were administered several liver toxicants. In addition to alcohol, we examined the effects following administration of anti-Fas (CD95) antibody, carbon tetrachloride (CCl_4) and lipopolysaccharide (LPS)/galactosamine. The results of these studies demonstrated that the knockout mice sustained enhanced liver injury in response to all of the treatments, as shown by increased indices of liver damage, such as enhancement of serum enzyme levels, histopathological scores, as well as hepatocellular death. Overall, the work completed to date suggests a possible link between hepatic receptors and liver injury. In particular, adequate function and content of the ASGP receptor may provide protection against various toxin-mediated liver diseases.

Key words: Asialoglycoprotein receptor; Asialoglycoprotein receptor deficient mice; Receptor-mediated endocytosis; Alcohol; Carbon tetrachloride; Anti-Fas; Lipopolysaccharide/galactosamine; Toxicant-induced liver injury

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THE ASIALOGLYCOPROTEIN RECEPTOR AND ITS POTENTIAL ROLE IN LIVER INJURY

The asialoglycoprotein (ASGP) receptor, also termed the hepatic binding protein or the Ashwell receptor, was discovered nearly four decades ago by Ashwell and Morell, and was described as a hepatocellular surface carbohydrate that binds glycoproteins lacking terminal sialic acid residues (asialoglycoproteins)[1,2]. Subsequently, many studies have contributed to the detailed characterization of the ASGP receptor, describing its functional role in the binding, internalization and transport of a wide range of glycoproteins, which have exposed galactose or N-acetylgalactosamine residues, *via* the process of receptor-mediated endocytosis (RME)[3-6]. However, translating altered ASGP receptor function and its altered clearance of serum glycoproteins to disease states remains a topic of current

research efforts. This ongoing interest is fueled by the knowledge that the ASGP receptor can bind a variety of important plasma proteins that include transport proteins (i.e. transferrin)[7], enzymes such as alkaline phosphatase[8], immunoglobulins including IgA[9], apoptotic hepatocytes[10,11], fibronectin[12] and platelets[13]. Additionally, the expression of the ASGP receptor has been clinically correlated to the level of hepatic function that is lost during liver diseases related to cancer, viral hepatitis, and cirrhosis[14,15]. Overall, the quest to identify and understand the physiological role(s) of the ASGP receptor, and the consequences that may result from alterations in the function and/or expression of this abundant hepatocellular binding protein, continues.

In search of the physiological roles of the ASGP receptor, our lab initially concentrated on characterizing the role of the ASGP receptor and RME events during a serious and common form of liver injury, alcoholic liver disease (ALD). Alcoholism, and resultant ALD, are indeed significant biomedical problems. Specifically, recent data has noted that chronic liver disease and cirrhosis was the 12th leading cause of death in the United States in the year 2005, and that out of those deaths, approximately 47% of them were due to ALD[16]. Therefore, defining potential contributing mechanisms (such as altered protein trafficking and impaired hepatic receptor functions) may aid in the elucidation of potential therapeutic treatments for ALD. In that effort, our laboratory has extensively studied the RME process and parameters of the ASGP receptor following the administration of ethanol to rodents.

The ASGP receptor consists of major and minor subunits, which in the rat were identified as rat hepatic lectin (RHL) 1 and RHL 2/3, that have respective molecular weights of 42, 49 and 54 kDa[17]. The selective binding and uptake of terminal galactosyl bearing proteins requires the formation of hetero-oligomers between these major and minor forms, and that binding activity was calcium and pH dependent[2,5,18]. Also, the subcellular distribution of the receptor revealed that approximately one-third of the total ASGP receptor pool was associated with the plasma membrane located on the basolateral surface of the hepatocyte[19]. Additionally, it was shown that the total ASGP receptor population consisted of two functionally distinct receptor populations, designated State 1 and State 2, which were involved in the endocytosis and intracellular processing of ligands by different pathways[20-22].

Utilizing these known properties, we studied the effects of ethanol on the ASGP receptor itself, as well as endocytic processes, using isolated hepatocytes, whole liver sections, and perfused livers obtained from rats voluntarily fed an ethanol containing diet over a time course of administration. In summary, differential effects were observed over the time course of treatment in the ability of ethanol and resultant metabolites to affect the ASGP receptor and RME events. Specifically, after early periods of ethanol feeding (1-2 wk), we found that the observed decrease in ligand binding capacity of the ASGP receptor could be attributed to inactivation and redistribution of the receptor[23]. However, after more chronic ethanol administration (5-8 wk), the functional alterations of the receptor were found to be reflective of reductions in the content, synthesis, and mRNA expression of the receptor[23]. Also, it was determined that ethanol treatment caused equal inactivation of both State 1 and State 2 receptors, suggesting that ethanol may be unique compared to other agents (e.g. monensin, vanadate, and chloroquine) that are known to inflict post-translational modifications, such as acylation, selectively to just the State 2 population[24]. In other studies, it was revealed that the ASGP receptor was hyperphosphorylated over the time course of treatment, which could contribute to the aberrant activity of the receptor by disrupting the phosphorylation/dephosphorylation state associated with normal recycling of the receptor[25]. We were also able to demonstrate that the ASGP receptor is involved in the recognition and uptake of apoptotic cells and that this process was significantly altered in hepatocytes obtained from ethanol fed rats[11]. Overall, the results from these studies revealed that ethanol administration impairs multiple aspects of RME by the hepatic ASGP receptor, such that binding, internalization and degradation of ligands internalized by the receptor were found to be significantly altered. Additionally, it was shown that these defects are associated with alterations in the ASGP receptor's physiologically relevant role of clearing apoptotic cells. Taken together, our findings have important implications for the pathogenesis of alcoholic liver injury and potentially for other forms of liver diseases in which RME is profoundly affected. In more recent studies, a mouse model lacking the ASGP receptor was used to gain a better understanding of the associations that may exist between alterations in receptor function and the generation of pathological liver injury.

THE ASGP RECEPTOR-DEFICIENT MOUSE MODEL

The ASGP receptor in mice is a hetero-oligomeric receptor composed of 2 subunits that are both required for its function. These subunits have been named murine hepatic lectin (MHL), with the major subunit called MHL-1 and the minor subunit called MHL-2[26]. ASGP receptor-deficient (RD) mice have a complete lack of the MHL-2 protein and were generated by homologous recombination with a gene replacement vector in embryonic stem cells[27]. MHL-2 appears to be required for the post-translational stability of MHL-1, as these mice have substantially reduced protein content of MHL-1, even though MHL-1 mRNA expression remains the same[27]. Although the MHL-1 protein is still detected in low levels in the RD mice, these levels are unable to induce a measurable clearance of 125I-labeled asialo-orosomucoid[27]. Despite lacking functional ASGP receptors, these knockout mice remain viable and fertile, and appear to have a normal lifespan. In addition, these mice do not display any obvious phenotypic abnormalities[27,28].

As previously mentioned, we have found that chronic alcohol administration markedly decreased mRNA expression and content of the ASGP receptor

in rats prior to the appearance of pathology such as fibrosis[23,29]. Thus, it was felt that the RD mice might provide a powerful tool to examine the role of the ASGP receptor and help delineate pathways by which liver injury occurs in general, as well as during alcoholic liver injury. Currently, we are utilizing the knockout mouse model to examine the link between ASGP receptor function and liver injury, in the context of various models of toxic liver injury such as alcohol, anti-Fas, carbon tetrachloride (CCl_4) and lipopolysaccharide (LPS)/galactosamine. In this report, we present a brief overview of our findings to date.

MODELS OF LIVER INJURY AND THEIR EFFECTS ON ASGP RECEPTOR-DEFICIENT MICE

Alcohol

Alcohol-induced liver injury has previously been found to be related to several events, including ethanol metabolism (*via* alcohol dehydrogenase[30-33]), generation of reactive oxygen species (*via* cytochrome isoforms such as CYP2E1[34-36]), interaction of other liver products (such as cytokines[37,38]) and the induction of apoptosis through the Fas death receptor system[39]. In the search for cellular signaling and mechanisms resulting as a consequence of these events, studies were performed that examined the effects of ethanol on hepatocellular protein trafficking, particularly the process of RME utilizing the hepatic ASGP receptor.

As mentioned previously, we examined the effects of ethanol administration using a rat model exclusively; the rats showed decreased ligand binding, internalization and degradation of several ligands including asialo-orosomucoid, which are processed by RME[4,23,40-43]. In order to assess the effect of ethanol administration on RME by the ASGP receptor using a mouse model, we obtained wild-type (WT) mice possessing abundant ASGP receptor activity and ASGP receptor-deficient (RD) mice lacking MHL-2 from the Jackson Laboratories (Bar Harbor, ME). The mice were fed a Lieber de-Carli liquid diet (with or without 5% by volume ethanol) for ten days[44]. When hepatocytes from these mice were incubated with 125I-ASOR (a representative ligand for the ASGP receptor), WT mice showed ethanol-induced alterations that were consistent with our observations for rats, with an approximately 50% decrease in ligand binding, internalization and degradation in isolated hepatocytes[4,23,44]. However, binding, internalization and degradation of the ligand by RD hepatocytes was negligible, regardless of diet[44]. In addition, the presence of apoptotic bodies was found to be approximately three-fold higher in the livers of RD mice compared to WT mice, irrespective of diet[44]. As a result of this work, it is hypothesized that a potential consequence of altered ASGP receptor function is impaired clearance of ethanol-generated apoptotic cells, resulting in the observed accumulation of apoptotic bodies. Furthermore, other work has shown that these bodies have the potential to promote a variety of responses within the liver, such as the activation of Kupffer cells and the subsequent release of proinflammatory and profibrogenic substances, leading to the enhanced susceptibility to hepatocellular damage that is observed following ethanol administration[11,45].

Anti-Fas

Anti-Fas is an antibody that specifically recognizes and works as an agonist of the Fas antigen[46]. Fas is a member of the TNF receptor superfamily and is a key mediator of apoptosis[47]. This receptor is found in hepatocytes, cholangiocytes, sinusoidal endothelial cells, stellate cells and Kupffer cells[48]. Ligation of Fas results in the recruitment of adaptor proteins, such as Fas-associated death domain (FADD) and procaspase 8, to form the death-inducing signaling complex (DISC)[47]. Caspase 8 can then either directly or indirectly cleave procaspase 3 to mediate apoptosis[47]. A variety of studies have shown that the injection of anti-Fas into mice causes widespread apoptosis and ultimately results in focal hemorrhage and hepatocyte necrosis, making Fas injection a model for fulminant hepatic failure[46,49,50].

From studies related to Fas-mediated cell death in our laboratory, we have shown that the metabolism of ethanol in WIF-B cells (hepatoma hybrid cells) was involved in enhanced Fas protein localization to the membrane, leading to increased activity of the upstream initiator caspases (caspase 2 and caspase 8) and the subsequent downstream activation of caspase 3[39]. As an extension to these studies, aimed to characterize the role of Fas-mediated death in injured hepatocytes, anti-Fas (0.1 or 0.2 μg/g body weight) was injected intraperitoneally into WT and RD mice, which were monitored for up to 48 h[51]. Receptor-deficient mice showed an enhancement of liver injury with higher aspartate transaminase (AST) and alanine transaminase (ALT) activities in the serum compared to the enzyme levels detected in WT mice[51]. Similarly, pathology showed that the RD mice had increased steatosis, inflammation and necrosis compared to the WT mice[51]. As expected, caspase 3 activities were found to be increased 5- to 6-fold in WT mice at 2 h and 16 h after anti-Fas injection, with caspase activities returning to baseline levels by 24 h[51]. However, the activity of caspase 3 remained elevated in the RD livers at all times following treatment and was significantly enhanced over the WT livers at 24 h and 48 h post anti-Fas injection[51]. Overall, the livers of the RD mice were found to be more susceptible than the livers of the WT mice to anti-Fas injection; showing greater apoptosis and increased ECM deposition of collagen and fibronectin[51].

Carbon tetrachloride

Another agent used to study liver injury is carbon tetrachloride (CCl_4), which can cause liver damage through a number of mechanisms. Carbon tetrachloride is metabolized through the action of the mixed function cytochrome P450 system of the endoplasmic reticulum to form the trichloromethyl free radical (CCl_3•), which can subsequently be converted to the trichloromethyl peroxy radical (CCl_3OO•) in the presence of oxygen[52,53].

These free radicals are highly reactive and can bind covalently to cellular macromolecules forming nucleic acid, protein and lipid adducts. When these radicals attack the polyunsaturated fatty acids of the cellular membranes, the fatty acid free radicals generated initiate autocatalytic lipid peroxidation, ultimately resulting in the loss of membrane integrity[52,53]. Carbon tetrachloride can also induce cellular hypomethylation, leading to inhibition of protein synthesis (possibly through ribosomal RNA hypomethylation) and defects in lipid and lipoprotein metabolism[53]. Finally, CCl_4 also affects hepatocellular calcium homeostasis, either by disrupting membrane integrity or by opening certain membrane calcium channels. High levels of Ca^{2+} in the cell can then activate Ca^{2+}-responsive enzymes such as proteases, endonucleases and phospholipases and lead to cell death *via* apoptosis and necrosis[52,53]. The consequences of CCl_4 toxicity include centrilobular steatosis, inflammation, apoptosis and necrosis[52-54].

In our studies, WT and RD mice were injected with CCl_4 (1 mL/kg body weight) and monitored up to a week after injection[55]. Carbon tetrachloride injection caused greater liver injury in the RD mice, as evidenced by the RD mice having increased AST and ALT activities in the serum, compared to the WT mice 48 h post CCl_4 injection[55]. Histologically, centrilobular liver damage was observed in WT mice by 48 h after injection[55]. At this time point, RD mice had more severe damage, showing a greater number of neutrophilic inflammatory infiltrates[55]. In order to elucidate the mechanisms by which this damage is caused, malondia dehyde (MDA), deposition of α-smooth muscle actin (α-SMA) and the percentage of TUNEL-positive hepatocytes was measured[55]. Levels of MDA in the WT mice were not significantly increased throughout the course of the experiment[55]. In contrast, RD mice showed increased contents of MDA as early as 24 h post CCl_4 injection and these levels were maintained up to 48 h[55]. α-SMA was increased significantly in both the WT and RD mice, with the RD mice having a more prolonged increase (between 48 h to 72 h) than the WT mice (only at 72 h)[55]. In addition, α-SMA content was approximately 2-fold of that in the WT liver at 48 h, 96 h and 7 d following injection[55]. Finally, RD mice had significantly more TUNEL-positive hepatocytes than the WT mice at 48 h post injection (2.4-fold more)[55]. This suggests that the absence of functional ASGP receptor resulted in increased lipid peroxidation, perturbations in ECM turnover and increased apoptosis[55].

LPS/galactosamine

Lipopolysaccharide (endotoxin) and galactosamine can be used either alone or in combination with each other to cause liver injury in mice. The metabolism of galactosamine leads to hepatotoxicity by depleting uridine nucleotides and UDP-hexoses and concurrently increasing UDP-hexosamines, primarily in hepatocytes[56,57]. The depletion of uridine nucleotides (as mentioned above) results in an inhibition of RNA and protein synthesis[58]. It has also been suggested that metabolism of galactosamine results in an impaired biosynthesis of macromolecular cell constituents[56]. This impairment leads to plasma membrane injury, which results in an influx of calcium ions and a commitment to cell death[58]. In rats, galactosamine leads to an inflammatory infiltrate of polymorphonuclear leukocytes and lymphocytes and foci of hepatocellular necrosis, resembling the effects of human viral hepatitis[57,59].

Mice and rats are relatively resistant to the lethal effects of LPS[60]. Thus, LPS is injected in concert with galactosamine for a model of fulminant hepatitis[61]. It is thought that galactosamine-induced suppression of RNA synthesis leads to an increased tumor necrosis factor (TNF) production by macrophages, resulting in an increased susceptibility to LPS[60-62]. Tumor necrosis factor was proposed as the agent responsible for the lethality, because lethality was retained by substituting LPS with TNF and was inhibited by anti-TNF antibody[63,64]. Thus, in LPS/galactosamine injection, apoptosis induced by TNF occurs initially and is followed subsequently by necrosis[65].

In our studies, a sub-lethal dose of LPS (50 μg/kg body weight) combined with galactosamine (350 mg/kg body weight) was injected into WT and RD mice *via* the intraperitoneal route and the mice were monitored up to 4.5 h[66,67]. After LPS/galactosamine injection, WT mice maintained normal liver lobular architecture[66]. However, RD mice showed considerable liver injury with areas of portal inflammation, hepatocellular necrosis, increased inflammatory cell infiltration and hemorrhage[66]. These histological observations were further corroborated by the RD mice having increased serum AST and ALT activities at 4.5 h after LPS/galactosamine injection, which were not observed in the WT mice[66]. Also, RD mice showed increased apoptosis, having significantly enhanced caspase 3 activities and TUNEL-positive cells at 4.5 h post injection, whilst there were no changes measured in WT mice[66]. Additionally, the serum content of the pro-inflammatory cytokine interleukin 6 (IL-6) was increased in RD mice compared to WT mice at 3 and 4.5 h after LPS/galactosamine injection[67]. Overall, the results demonstrate that the RD mice are more susceptible to the development fulminant liver injury as a result of sub-lethal treatment with LPS/galactosamine[66,67]. Given that the induction of apoptosis is a consequence of LPS/galactosamine treatment, the enhanced susceptibility to liver damage observed in the RD mice may be related to the inability of hepatocytes to phagocytose and clear the dying apoptotic cells *via* the ASGP receptor.

THE LINK BETWEEN FUNCTIONAL ASGP RECEPTOR AND LIVER INJURY

After the administration of the four toxicants mentioned above (alcohol, anti-Fas, CCl_4 and LPS/galactosamine), RD mice consistently sustained greater liver injury than WT mice, as evidenced by increased indices of liver damage (serum AST and ALT activities) and worse pathology (light microscopy). These four agents of liver injury cause damage through different biochemical pathways or signaling cascades. Therefore, it appears that proper functioning of the ASGP receptor may provide

universal protection against liver injury from these toxicants and possibly others. Although it is not known exactly how an adequately functioning receptor can protect the hepatocyte, the use of the knockout mice treated with these four toxicants highlight some of the possible mechanisms.

It appears that with all four toxicants, caspase 3 or TUNEL-positive cells are increased. Apoptosis is a highly regulated mode of cell death that helps to maintain tissue homeostasis in a healthy organ[68]. However, it appears that when apoptotic death factors are inappropriately expressed due to the introduction of pathological stimuli, such as the four toxicants, apoptosis becomes one of the common pathways by which liver injury is caused. Increased accumulation of apoptotic cells has been shown to occur during alcohol-induced liver injury in a variety of species, including humans, and is thought to play an important role in the progression of liver injury[69-75]. During the process of forming apoptotic cells, glycoconjugates on the cell surface losing their sialic acid masks the increase[10,76]. Since the ASGP receptor binds to desialylated proteins, the receptor recognizes and binds the altered glycans on apoptotic bodies, resulting in phagocytosis and efficient removal of the dying cells[11]. Thus, we speculate that the ASGP receptor exerts protection by removing apoptotic bodies in a timely fashion.

Another way that the ASGP receptor might be protective is through its role in regulating turnover of ECM. Anti-Fas and CCl_4 treatments lead to increased deposition of collagen, fibronectin or α-SMA in the RD mice in comparison to the WT mice. The ASGP receptor has a direct link to cellular fibronectin clearance because cellular fibronectin displays terminal galactose residues, making it a ligand of the ASGP receptor[12]. Cellular fibronectin is one of the first ECM proteins that accumulates during fibrosis[77-79] and thus impairments of ASGP receptor function could lead to increased ECM deposition and hence lead to fibrosis and cirrhosis.

Two additional ways that liver injury could be mediated is through increased lipid peroxidation (MDA content) or by increased contents of pro-inflammatory cytokines, such as IL-6. At present, it is not known how ASGP receptor function is related to perturbations in levels of these two substances. In addition, there may be dysregulation of other asialoglycoproteins not examined at this point. The total absence of ASGP receptor function does not result in a measurable increase in the steady state concentrations of galactose-terminating glycoproteins in the plasma of knockout mice[28]. Thus, the ASGP receptor is unlikely to be involved in the normal turnover of serum glycoproteins. However, in toxicant-induced injuries, acute surges of asialoglycoproteins may overwhelm the alternative galactose recognition systems[28]. Thus, the ASGP receptor might function to prevent an acute increase in potentially harmful asialoglycoproteins. Altogether, the ASGP receptor knockout mouse model provides an excellent tool to elucidate the relationship between ASGP receptor function and liver injury.

Table 1 Changes in ALT activity, TUNEL positive cells, AST activity, caspase 3 activity, collagen content, IL-6 content, MDA content and α-SMA content in RD mice compared to WT mice

Challenge	RD mice compared to WT mice
Alcohol	Liver TUNEL-positive hepatocytes
Anti-Fas	Liver TUNEL-positive hepatocytes
	Serum ALT
	Serum AST
	Liver caspase 3 activity
	Collagen deposition in extracellular matrix
	Fibronectin deposition in the extracellular matrix
CCl_4	Liver TUNEL-positive hepatocytes
	Serum ALT
	Serum AST
	Liver MDA content
	Liver α-SMA content
LPS/galactosamine	Liver TUNEL-positive hepatocytes
	Serum ALT
	Serum AST
	Liver caspase 3 activity
	Serum IL-6 content

ALT: Alanine transaminase; AST: Aspartate transaminase; IL-6: Interleukin 6; MDA: Malondialdehyde; α-SMA: α-smooth muscle actin; RD: Receptor-deficient; WT: Wild-type. The various conditions the mice were challenged with were alcohol (Lieber de-Carli ethanol diet for 7 d), anti-Fas (0.2 μg/g body weight), CCl_4 (1 mL/kg body weight) and LPS/galactosamine (50 μg LPS/kg body weight and 350 mg galactosamine/kg body weight).

CONCLUSION

The ASGP receptor is an abundant hepatic receptor that recognizes desialylated ligands. After binding to its ligand, the receptor internalizes and facilitates transport of specific ligands by the process of receptor-mediated endocytosis. Previously, studies have shown that ASGP receptor function is impaired in disease states such as alcoholic liver disease. This gave us the impetus to examine if proper ASGP receptor function offers protection against liver injury or if defects in function occurred as a result of liver damage. To examine this, we utilized a knockout mouse model, which lacked functional ASGP receptor in comparison to wild-type animals in the context of various toxic challenges (alcohol, anti-Fas, CCl_4 and LPS/galactosamine). After all four challenges, the receptor-deficient mice consistently showed more liver injury than the wild-type animals (Table 1), proving that ASGP receptor function is protective. Thus, these studies highlight receptor-mediated endocytosis as a novel mechanism that may be involved in the induction of toxin-induced liver injury. At present, the precise nature of the specific ligands involved or the pathways that lead to further injury have not been determined. However, in the studies reviewed here, it is likely that impaired clearance of apoptotic bodies, perturbations in extracellular matrix deposition, oxidative stress, and cytokine dysregulation may play roles in the progression of disease. In the future, further clarification of the pathways by which liver injury occurs (including altered ASGP receptor-mediated endocytosis) will provide new therapeutic leads.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Hepatitis C virus and ethanol alter antigen presentation in liver cells

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Abstract

Alcoholic patients have a high incidence of hepatitis C virus (HCV) infection. Alcohol consumption enhances the severity of the HCV disease course and worsens the outcome of chronic hepatitis C. The accumulation of virally infected cells in the liver is related to the HCV-induced inability of the immune system to recognize infected cells and to develop the immune responses. This review covers the effects of HCV proteins and ethanol on major histocompatibility complex (MHC) class Ⅰ- and class Ⅱ-restricted antigen presentation. Here, we discuss the liver which functions as an immune privilege organ; factors, which affect cleavage and loading of antigenic peptides onto MHC class Ⅰ and class Ⅱ in hepatocytes and dendritic cells, and the modulating effects of ethanol and HCV on antigen presentation by liver cells. Altered antigen presentation in the liver limits the ability of the immune system to clear HCV and infected cells and contributes to disease progression. HCV by itself affects dendritic cell function, switching their cytokine profile to the suppressive phenotype of interleukin-10 (IL-10) and transforming growth factor beta (TGFβ) predominance, preventing cell maturation and allostimulation capacity. The synergistic action of ethanol with HCV results in the suppression of MHC class Ⅱ-restricted antigen presentation. In addition, ethanol metabolism and HCV proteins reduce proteasome function and interferon signaling, thereby suppressing the generation of peptides for MHC class Ⅰ-restricted antigen presentation. Collectively, ethanol exposure further impairs antigen presentation in HCV-infected liver cells, which may provide a partial explanation for exacerbations and the poor outcome of HCV infection in alcoholics.

Key words: Alcohol; Antigen presentation; Hepatitis C Virus; Interferon alpha and gamma; Liver; Major histocompatibility complex (MHC) class Ⅰ; MHC class Ⅱ

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INTRODUCTION

In non-cytolytic viral infections, the immune system (and mainly T-lymphocytes) is necessary to clear the infected cells. The most specialized and effective homeostatic control is provided by cytotoxic T lymphocytes (CTLs) (CD8+ T-lymphocytes). For clonal expansion, CTLs need activating "help" from CD4+ T-lymphocytes, which, in turn, require recognition of viral antigens on professional antigen-presenting cells (APC), including dendritic cells (DC), macrophages and B-lymphocytes.

Recognition of antigens by T-lymphocytes depends upon the expression of the major histocompatibility complex (MHC) on the surface of APC. MHC molecules involved in antigen presentation include MHC class Ⅰ, MHC class Ⅱ and MHC-like CD1 molecules[1]. MHC class Ⅰ molecules are expressed on the nuclear cells and load protease-generated peptides to CD8+ T-lymphocytes. MHC class Ⅱ molecules are expressed on the surface of specialized APC and load peptides generated in the endocytic compartment to CD4+ T cells. CD1 molecules bind acyl chains, therefore allowing T cells to recognize fatty acids, glycolipids and lipopeptide antigens (self or foreign)[2].

Hepatitis C virus (HCV) is an example of an intra-

cellularly persistent virus, which targets liver cells and is eliminated by immune cells[3,4]. In acute HCV infection, antigen presentation plays a pivotal role in the activation of immune response, while HCV protein-induced defects in antigen presentation lead to insufficient activation of the immune system, which controls the killing of infected hepatocytes[5]. Accumulation and persistence of virally infected cells provide a basis for the chronic course of HCV infection. The reduced clearance of virus and increased viral load usually observed in immunodeficient and in alcohol-consuming patients are associated with a high risk of hepatocarcinoma development[6,7].

LIVER AS AN IMMUNE PRIVILEGE ORGAN

Liver is considered an immunological organ[8]. Liver cells are involved in the clearance of foreign antigens, which come from the gastrointestinal tract. The physiological role of the liver is to face antigenic flow from the gastrointestinal tract and to respond to this intervention by activating the immune response. To escape immune activation, liver cells use a mechanism of immune tolerance, which prevents unnecessary immune-mediated damage of hepatocytes.

Some liver cells, such as liver dendritic, Kupffer, endothelial and stellate cells serve as antigen presenting cells[9]. Kupffer cells (KC) are the resident macrophage population, which act as incompetent antigen-presenters[10]. They produce IL-10, which down-regulates CD4+ T-cell activation[11,12]. Reactive oxygen species (ROS) production is an essential trigger of antigen presentation in KC, accompanied by induction of MHC class Ⅱ and co-stimulatory molecule expression[13]. In contrast to KC, liver endothelial cells (LSEC) are known as potent APC that present antigens in a MHC class Ⅱ-restricted manner to CD4+ T-cells[9]. The efficacy of their antigen presentation is comparable to DC[8]; however, IL-10 and TGFβ secreted by KC and LSEC as well as lipopolysaccharide (LPS), which comes with blood from the gastrointestinal tract, can down-regulate their antigen-presenting properties[14,15]. Both KC and LSEC express MHC antigens, costimulatory and adhesion molecules and produce IL-1 and IFNγ, suggesting that these are relatively mature cells[16]. In contrast, liver DC are immature cells, which express MHC antigens on their surface, but express a relatively small amount of co-stimulatory molecules, which does not allow them to efficiently stimulate naïve T-cells[17]. Hepatic DC play an important role in the induction and regulation of immune responses, by interacting with CD4+, CD8+ lymphocytes and natural killers (NK) cells. These cells typically reside only around portal triads and like other DC, they capture, process and transport antigens to regional lymphoid tissues[16]. Two subpopulations of DC, myeloid and plasmocitoid cells are found in liver at a low density. Exposure of progenitor DC to IL-10 and TGFβ generates suppressive and tolerogenic effects[18]. In the liver, they are resistant to DC maturation stimuli, such as IFNγ and TNFα, while matrix proteins, such as collagen type 1, induces maturation[19]. However, even being tolerogenic in the liver, hepatic DC are the main professional liver APC, which can further migrate to lymphoid tissue to undergo maturation[20]. In addition to other liver cells, stellate cells have also recently been characterized as having antigen presentation properties[21].

Almost 60% of liver cells are hepatocytes. Due to specific liver architecture, hepatocytes are exposed to a mixture of portal venous and hepatic arterial blood. Liver parenchyma is actively involved in immune response by expressing a variety of receptors. Because liver is a site of apoptotic CD8+ T-lymphocyte accumulation[22], it raises the question whether liver cells, including hepatocytes, induce apoptosis in activated CD8+ T-lymphocytes, thereby promoting a tolerogenic response or they specifically attract apoptotic T-lymphocytes without causing T-lymphocyte death. Resting hepatocytes act as APC for MHC class I -restricted T-cells, while MHC class Ⅱ and CD1 are not constitutively expressed on hepatocytes[23]. However, the role of hepatocytes as potent APC activating intrahepatic lymphocytes is contradictory. While some studies indicate that activation of naïve CD8+ T-lymphocytes does not happen in case the primary activation occurs within the liver, other studies suggest that the presentation of antigens in the liver showed no immunosuppressive effect on the activation of CD8+ T-cells and that hepatocytes are an excellent priming site for naïve CD8+ T-cells[24-26].

ANTIGEN PROCESSING AND PRESENTATION IN THE CONTEXT OF MHC CLASS Ⅰ

Proteolysis is the first step in the antigen processing and presentation pathway. Peptide products are transported by transporters associated with antigen processing (TAP) to endoplasmic reticulum (ER), where they assemble in a trimolecular complex with β2-microglobulin and the heavy chain of MHC. Assembly is facilitated by TAP and a number of chaperones and allows class I molecule to bind the peptide to achieve optimal MHC class I loading. These complexes are transported to the plasma membrane, where they interact with the T-cell receptor (TCR) of a T-lymphocyte. TAP has sequence preference and peptide-size limitation, which is matched to MHC class I . To be presented in a context of MHC class I , antigenic proteins undergo the processing to peptides and then the peptides are further cleaved, to fit into the MHC class I groove. Certain proteases participate in the cleavage of peptides for antigen presentation, and the major protease is proteasome.

For activation, CD8+ cells require presentation of MHC class I -peptide complexes on professional APC cells. Peptides can be generated from viral proteins sensitized by infected APC or from proteins originally synthesized by other infected "donor" cells (cross-priming, or cross presentation). Cross-priming can be carried by mo-

lecular chaperones and come from apoptotic cells[27]. In addition, it is suggested that these peptides are captured by the lysosomal compartment, with further trafficking to cytosol followed by proteasomal processing for MHC class I loading[28]. However, other authors question the role of proteasomal processing in the cross-presentation mechanism[29].

PROTEASOME, AMINOPEPTIDASES AND INTERFERON GAMMA-INDUCED GENERATION OF PEPTIDES FOR ANTIGEN PRESENTATION

Degradation of intracellular proteins into oligopeptides for antigen presentation is catalyzed by the proteasome[30]. This multicatalytic enzyme exists as a 26S particle, which is ATP-dependent and recognizes ubiquitylated polypeptides. Another form, the 20S proteasome, can degrade substrate proteins in an ubiquitin-independent manner. Both the 26S and 20S proteasome particles degrade antigenic proteins that may later be presented as peptides, depending on the properties of the protein. The chymotrypsin-like and the trypsin-like activities of the proteasome are related to antigen presentation due to their ability to cleave peptide bonds after hydrophobic and basic amino acids, respectively[30]. In cells treated with IFNγ, proteasome particles acquire two novel MHC-encoded low molecular weight subunits, known as LMP-2 and LMP-7, and a third subunit, MECL-1, encoded by genes outside the MHC locus[31]. The acquisition of these three subunits converts the constitutive proteasome to the immunoproteasome and alters its peptidase activities for the generation of uniform sized 8-10-mer peptides[32]. The importance of immunoproteasome induction for antigen presentation has been confirmed using LMP-2 and LMP-7-knockout mice, which demonstrated impaired antigen presentation, reduced expression of MHC class I and poor CTL response to viral epitopes[33,34]. The immunoproteasome is responsible for cleavage of peptides at their C-termini[35]. However, some antigenic peptides have N-terminal extensions that are unable to bind to the MHC class I groove. Aminopeptidases, namely, cytosolic leucine aminopeptidase (LAP), finally trims the epitopes to 8-9 amino acid residues necessary to form the complexes with MHC class I [36]. This enzyme is also IFNγ-inducible[37]. Trimmed peptides are transported to the endoplasmic reticulum (ER) by TAP. In the ER lumen, the peptide can be further trimmed by downstream endoplasmic reticulum aminopeptidase I (ERAP1)[38] to generate a stable complex with MHC class I heavy and light chains (β2-microglobulin). These complexes, with the help of molecular chaperones, traffic to the cell surface to be recognized by CD8+ T-cells[39,40]. The immune system relies heavily on intracellular protein degradation to recognize a complex of surface MHC class I molecules with short peptide fragments of eight to nine amino acids[41]. Induction of CTLs is programmed by the spectrum of presented MHC class I -peptide complexes on the cell surface[42].

HCV, MHC CLASS Ⅰ-RESTRICTED ANTIGEN PRESENTATION AND EFFECTS OF ETHANOL

The effects of HCV proteins on MHC class I -restricted antigen presentation are not widely studied and most of the findings are related to MHC class Ⅱ-restricted antigen presentation. In HCV infected patients, associations between the human MHC and sustained virological response have been found, indicating that the various immunogenetic backgrounds of chronic hepatitis C patients are related to the differences in the course of the disease[43].

Processing of CTL epitopes in HCV infected cells may be altered due to mutations generated by HCV, which interfere with the ability of the peptide to be cleaved by the proteasome[44]. Nevertheless, interferon alpha-mediated induction of immunoproteasome or aminopeptidase expression has been shown as a necessary step for CD8+ T-cell activation in acute HCV infected chimpanzees[45,46]. In addition to HCV-restricted mutations of viral proteins, proteasome activity is also modulated by HCV. Thus, proteasome activity in the nuclear compartment is up-regulated by PA28γ activator which forms a complex with HCV core protein[47], while non-structural HCV protein, NS3, interacts with LMP-7, an immunoproteasome subunit, and reduces its activity, potentially providing a negative effect on the generation of peptides for antigen presentation[48]. Our previous studies on HCV core protein expressing Huh7 cells, which also express CYP2E1, revealed that core protein slightly enhances 20S proteasome activity, by 20S proteasome-core protein direct interactions and by induction of low CYP2E1-dependent oxidative stress[49]. However, this proteasome activation is reversed after ethanol exposure, where ethanol treatment considerably reduces proteasome function due to induction of high oxidative stress[49]. Indeed, the dependence of proteasome on the level of oxidative stress has been previously demonstrated[50,51], and ethanol is known to suppress proteasome function in liver cells[52-55]. Ethanol-elicited suppression of proteasome activity in the liver ultimately results in reduced generation of antigenic peptides[56] and reduced MHC class I -restricted antigen presentation on hepatocytes (Osna *et al*, Hepatology, in press). Presentation of peptide-MHC class I complexes on virally infected hepatocytes (HCV, HBV infections, *etc*) as target cells is crucial for CTLs because when clonal expansion of CTLs is established, the next important restriction for elimination of infected cell is the availability of peptide-MHC class I complexes on the surface of target cells (hepatocytes), which are recognized by CTLs. Thus, even if CD8+ T-cells are activated by the presentation of HCV peptides on professional APC, the recognition of the peptide-MHC class I complexes on hepatocytes

(which are target cells for CTLs) may be limited under ethanol-induced oxidative stress.

MHC CLASS Ⅱ-RESTRICTED ANTIGEN PRESENTATION

Loading of MHC class Ⅱ molecules with antigenic peptides requires the involvement of specialized APC, such as DC, B-lymphocytes and macrophages. Phagocytosis is the main way to capture antigenic proteins by DC or macrophages, while B-lymphocytes use the antigen specific B-cell receptors[57]. Antigens are digested into peptides (which are longer than those required for MHC class I -restricted antigen presentation) by proteases at endosome compartments. Cathepsins, intracellular acidic proteases, play a pivotal role in the processing of internalized antigens into class Ⅱ-presentable T cell epitopes[58]. These peptidases play a dual role by generating the peptides or by destroying them. The coupling of peptides with MHC class Ⅱ is controlled by chaperones and takes place in late endocytic vesicules[44].

When pathogen-associated molecular patterns (PAMPs) on macrophages are recognized by innate immunity receptors (such as Toll-like (TLR)-, mannose-, Fc- and complement- receptors), they induce production of pro-inflammatory cytokines, namely, IFNγ and colony-stimulating factor (GM-CSF). These cytokines increase the expression of MHC class Ⅱ and co-stimulatory molecules in the cells. Chronic TLR signaling induced by LPS may impair MHC class Ⅱ-restricted antigen presentation[59]. A large number of class Ⅱ-bound proteins are derived from cytosolic proteins. Because the autophagy inhibitor has been shown to block the presentation of cytosolic peptides by MHC class Ⅱ molecules, some authors discuss the role of autophagy in delivery of these cytosolic peptides to the endocytic route[60].

After immature DC engulf pathogens, they undergo biochemical changes, such as secretion of TNFα, IL-6, IL-12, IL-10 and IFNα and expression of co-stimulatory molecules, including CD80, CD86 and CD40. These cells traffic to the nearest lymph node for presentation to T-lymphocytes. However, only mature DC can efficiently prime naïve T-lymphocytes, because only these cells generate MHC class Ⅱ molecules, by redistribution of class Ⅱ molecules and cathepsins to peptide-loading compartments and by enhancement of lysosomal acidification[61]. It is still unclear how the peptide-MHC class Ⅱ complex is recruited to the cell surface from the endocytic compartment in DC. Human DC are subdivided into two big categories: myeloid DC and plasmocytoid DC. In addition to phenotypic differences, they express different TLRs and secrete a different spectrum of cytokines. Thus, myeloid DC express TLR3 and upon stimulation, produce IL-12p70, thereby promoting Th1 response, while plasmocytoid DC express TLR7 and TLR9 and secrete IFNα[62]. Therefore, myeloid DC cells are considered classic antigen presenters, while plasmocytoid DC have a limited ability to capture, process and load antigen onto MHC molecules[63]. Activation of DC is positively regulated by IFNγ and additional CD40 ligation; anti-inflammatory cytokines, such as IL-10, inhibit both IL-12 and IFNα expression, which results in pathogen survival[64].

MHC CLASS Ⅱ-RESTRICTED ANTIGEN PRESENTATION, HCV AND ETHANOL

HCV infection affects DC function in many ways. Firstly, there is a decrease in the frequency of peripheral myeloid or plasmocytoid DC in chronically infected patients[65]. This may be, in part, related to an accumulation of intrahepatic DC in HCV infection, due to altered DC trafficking[66]. However, another study suggested that HCV directly targets mature cells because some HCV proteins (core, NS3 and NS5) induce apoptosis in DC[67]. Secondly, myeloid DC from chronic HCV patients have a decreased capacity to stimulate allogenic T-lymphocytes[68]. Furthermore, HCV core and E1 genes introduced in DC obtained from uninfected donors, lower their capacity to stimulate allogenic T-cell response[69]. In addition, core, NS3 and NS4 viral proteins were reported to influence the differentiation of DC from monocytes, due to IL-10 secretion[70]. The function of myeloid DC obtained from HCV patients is also decreased due to a reduced level of co-stimulatory molecules and down-regulated HLA-DR expression[5,68]. In addition, in chronic HCV patients, the production of IL-12 by DC is suppressed by HCV proteins, but can be restored by successful antiviral therapy[71]. Thirdly, the impairment of plasmocytoid DC function has been reported in HCV patients and reduced IFNα production upon DC stimulation with TLR ligands has also been observed[72]. This decrease in IFNα production can be attributed to monocyte-derived TNFα and IL-10[73]. Interestingly, the other studies did not demonstrate defective abilities in plasmocytoid DC to initiate immune response[74], thereby questioning the altered presentation of HCV proteins by these cells to CD4+ cells.

The proposed mechanisms of ethanol-HCV interactions were summarized elsewhere[75]. Ethanol drastically enhances the negative effects of HCV on DC function. A possible reason for this is that alcohol stimulates HCV expression at HCV RNA and proteins levels, which is, in part, related to up-regulation of the Cox2 pathway[76]. Chronic ethanol feeding suppresses CD4+ T cell proliferation in response to antigens as well as CTL activity after DNA-based vaccine immunization with NS5 and HCV-expressing plasmids[77-79]. This CD4+T-cell response was restored by co-administration of IL-2–expressing plasmid, while the restoration of CTL activity required administration of GM-CSF[77,78]. Alcohol doubles the effects of HCV by decreasing expression of co-stimulatory molecule, B7, and IL-12 production, increasing IL-10 production and lowering allostimulatory capacity[80-82]. Furthermore, human studies demonstrated that ethanol affects the allostimulatory activity of resting and activated DC generated from peripheral blood mononuclear cells in the presence of cytokines[81],

indicating that HCV infection impairs DC function, which is further exacerbated by ethanol. Recent studies performed on alcohol-fed mice, immunized with HCV DNA-based vaccine that induces immune response to NS5 HCV protein, demonstrated that ethanol exposure reduced the number of splenic DCs without altering endocytosis capacity. It also reduced the lymphoid DC population, as well as expression of co-stimulatory molecules, CD40 and CD86, altered cytokine profile (enhanced production of IL-1 and IL-10 and decreased secretion of IL-12, IFNγ and IL-6)[83]. Finally, CTL response to NS5 was shown to be impaired, but was corrected by syngeneic transfer of control DC[83].

HCV, IFN SIGNALING AND ETHANOL

Antigen presentation is so tightly regulated by IFNs that it should be analyzed in the context of IFN signaling. The reported effects of HCV proteins on IFN signaling are quite controversial. Most studies revealed down-regulation of STAT1 phosphorylation in response to IFNα[84-86] attributed to SOCS3 activation by HCV core or NS5A proteins. In addition to that, NS3 protein plays a role in dissociation of the adaptor molecule, MAVS that disrupts the early activation of IFNβ signaling by the virus[87,88]. Subsequently, the disruption of STAT1 phosphorylation by the NS3/NS4 complex was documented[89]. In some studies, suppressed STAT1 phosphorylation was linked to either the ability of the core protein to specifically interact with the SH2 domain, preventing STAT1 hetero- or homodimerization[90] or to direct targeting of STAT1 with core protein and subjecting activated STAT1 to degradation by the proteasome[91]. The latter hypothesis is consistent with our recent findings that core protein activates proteasome function[49]. Core protein also inhibits the IFN-induced nuclear import of STAT1 and STAT2 and transcription of antiviral genes[92,93]. HCV proteins interfere with IFNα signaling related to the attachment of activated STAT1 to DNA. Methylation on arginine residues, which prevents the formation in STAT1-PIAS1 complexes, is altered in chronic hepatitis C patients[94,95]. Protective effects of the methylation regulators, S-adenosyl methionine and betaine, were shown to correct HCV-induced inhibition of IFNα signaling *in vitro*[96]. Interestingly, in other studies, core protein has been shown to activate interferon stimulated response element (ISRE) and gamma activated site (GAS) sequence promoters in the presence or absence of IFNα and γ and to stimulate INOS promoter, INOS protein induction as well as activation of IFN-inducible 2'5'-oligoadenylate synthetase (2'5'-OAS)[97,98]. Less is known about the interference of HCV with IFNγ-induced signaling, which is up-regulated by core protein, due to enhanced IFNγR2 expression[99]. In a cell culture system, prolonged treatment with IFNγ attenuated IFNα-signaling[100]. Acute ethanol treatment of hepatoma cells inhibited anti-viral actions of IFN against HCV replicon, induced serine STAT1 phosphorylation, but blocked tyrosine phosphorylation[101]. Recently, it has been shown that alcohol metabolism induces increased replication of HCV and attenuates the antiviral effects of IFN[102]. However, the number of studies regarding the combined effects of ethanol and HCV proteins on IFN signaling is very limited.

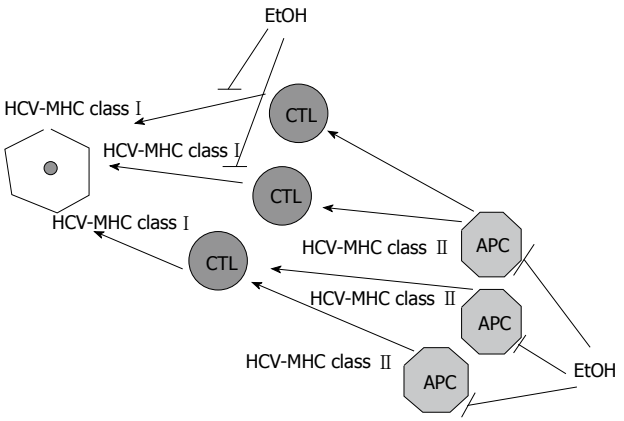

Figure 1 Alcohol (ethanol) reduces antigen presentation on HCV-infected liver cells. HCV interferes with the capacity of professional antigen presenting cells (APC) to prime the immune response. Ethanol (EtOH) further dysregulates antigen presentation, suppressing both MHC class Ⅰ-restricted antigen presentation on infected hepatocytes and MHC class Ⅱ-restricted antigen presentation on APC.

CONCLUSION

HCV alters antigen-presentation capacities of professional APC, thereby contributing to the persistence of virally infected cells. The most prominent effects were observed on MHC class Ⅱ-restricted antigen presentation and DC functions. Ethanol potentiates the effects of HCV, providing further suppression of both MHC class Ⅰ- and class Ⅱ-restricted antigen presentation on DC, hepatocytes and others hepatic APC, which diminish CTL response. IFNs which support antigen presentation by activating APC and peptidases lose this property in the presence of HCV and alcohol, because HCV and alcohol, separately or in combination, reduce IFN signaling. This, in part, explains why alcohol consumption exacerbates the course, worsens the outcome and reduces the responsiveness to interferon alpha treatment in chronic HCV infection. The effects of alcohol (ethanol) on HCV-restricted antigen presentation are summarized in Figure 1.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Alcohol metabolites and lipopolysaccharide: Roles in the development and/or progression of alcoholic liver disease

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Abstract

The onset of alcoholic liver disease (ALD) is initiated by different cell types in the liver and a number of different factors including: products derived from ethanol-induced inflammation, ethanol metabolites, and the indirect reactions from those metabolites. Ethanol oxidation results in the production of metabolites that have been shown to bind and form protein adducts, and to increase inflammatory, fibrotic and cirrhotic responses. Lipopolysaccharide (LPS) has many deleterious effects and plays a significant role in a number of disease processes by increasing inflammatory cytokine release. In ALD, LPS is thought to be derived from a breakdown in the intestinal wall enabling LPS from resident gut bacterial cell walls to leak into the blood stream. The ability of adducts and LPS to independently stimulate the various cells of the liver provides for a two-hit mechanism by which various biological responses are induced and result in liver injury. Therefore, the purpose of this article is to evaluate the effects of a two-hit combination of ethanol metabolites and LPS on the cells of the liver to increase inflammation and fibrosis, and play a role in the development and/or progression of ALD.

Key words: Alcoholic liver disease; Inflammation; Fibrosis; Sinusoidal liver endothelial cells; Kupffer cells; Hepatocyte; Stellate cells; Precision cut liver slices

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INTRODUCTION

It has become increasingly clear that alcohol alone is not solely responsible for the initiation and/or progression of alcoholic liver disease (ALD). While alcohol consumption does increase fatty liver, lipid peroxidation, and reactive oxygen species (ROS), these insults are typically not enough to induce the onset of more severe forms of liver disease[1]. The "two-hit" proposal of ALD is of interest because inflammation caused by the metabolites of ethanol [ROS, aldehyde modified proteins or lipopolysaccharide (LPS)] increase the levels of cytokines/chemokines resulting in a deleterious positive feedback loop that propagates liver inflammation, infiltration of inflammatory cells[2,3], and fibrosis. To support this, aldehdye modified proteins[4,5] and endotoxin (LPS)[6,7] have been detected in the serum and/or livers of patients with ALD. These substances have been shown to increase the release of TNF-α, interleukin-1β, and prostaglandin by Kupffer cells, sinusoidal endothelial cells and stellate cells. This release in turn promotes an influx of

inflammatory cells leading to an increase in cellular damage promoting the development of necrosis and eventually liver failure[8-11]. The purpose of this review was to more closely examine how a "two-hit" model of ethanol metabolism and LPS interaction affects resident liver cells in the progression and/or development of ALD. Many excellent reviews[12-14] exist concerning inflammatory cell activation and recruitment to the damaged liver during the development of ALD and therefore will not be discussed here[15-17].

ANIMAL MODELS OF ETHANOL AND LPS-INDUCED LIVER INJURY

Current animal models of ALD have provided many valuable findings[18]. However, in general, these models do not produce the type of end-stage liver failure observed in patients with ALD. To counter this, models have been developed that combine ethanol treatment strategies with an additional injury cofactor. One way of developing more overt injury (e.g. steatohepatitis and fibrosis) in order to mimic the human disease is to combine ethanol and LPS treatment. LPS is a component from the cell walls of bacteria found in the gut as normal flora. Typically, when gram-negative bacteria break down, LPS is released and removed by endothelial cells lining the blood vessels or Kupffer cells (KCs) in the liver. If the normal activity of the gut epithelium is disrupted, as has been shown to happen with acute or chronic ethanol ingestion, the LPS released from degrading bacteria can cross into the bloodstream[6,7,19]. Even though the exact mechanism of this is unknown, it is suggested that with chronic ethanol consumption, ethanol can damage the cells lining the interior of the intestine and increase the amount of LPS entering the blood stream. In addition, ethanol impairs KCs and prevents them from clearing LPS from the bloodstream[11,20]. When LPS enters into the bloodstream and moves to the liver, it activates KCs by interacting with the CD14 and Toll-like receptor 4 (TLR-4) molecules on the surface of the cell. This interaction causes a cascade which results in production of ROS and release of inflammatory cytokines (i.e. TNF-α, IL-1β, IL-6, IL-10), which in turn activates signaling cascades and causes injury to the primary liver cells, the hepatocytes[8,21].

There have been a number of different approaches utilized to investigate the effects of ethanol consumption and LPS on ALD. The first hit from ethanol metabolism results in the production of ethanol metabolites (i.e. acetaldehyde and acetate). These metabolites increase redox state, steatosis, production of ROS, and lipid peroxidation. This in turn, increases other reactive aldehydes like malondialdehyde and 4-hydroxynonenal. These aldehydes can react with or adduct proteins to alter normal liver functions, induce cell death, and/or liver inflammation. This makes the liver more susceptible to a second hit, probably by LPS. The second hit perpetuates liver injury and fibrosis as a result of LPS-induced oxidative stress, cytokine release, and subsequent infiltration of immune cells[2].

Two-hit animal models

In a two-hit model of ALD described by Koteish *et al*[22], C57BL-6 mice were maintained on diets containing 4 mL/L ethanol for five weeks. Pair-fed control mice were fed an identical volume of ethanol-free diet. At the end of the five weeks, the mice received one 10 μg injection of LPS and sacrificed at 0.5, 1.5 and 6 h post-injection. Histological results showed that the livers from control pair-fed mice had features of apoptosis, but no inflammatory cell infiltrates or hemorrhage. In contrast, hepatocytes in the ethanol-fed mice showed fat accumulation, inflammatory cell infiltration, and hemorrhage.

Procaspase-3, procaspase-8, Jun N-terminal kinase (JNK), TNF-α and TNFR-1 are mediators of LPS-induced hepatotoxicity. Apoptosis in this model was evaluated by determining the levels of procaspase-3 and procaspase-8. Immunoblot analysis to examine procaspase-3 levels showed that the ethanol-fed mice exhibited a decreased content of procaspase-3 relative to the pair-fed mice before and after LPS injections. Evaluation of procaspase-8 showed that it was elevated in the ethanol-fed mice compared to the pair-fed mice.

Examination of JNK activity demonstrated an eight-fold increase 1.5 h after LPS exposure alone. In contrast, LPS had no effect on JNK activity in ethanol-fed mice. TNF-α can interact with TNFR-1 and initiate a signaling cascade that activates procaspase-3 and inducing apoptosis. The expressions of TNF-α and TNFR-1 were found to be steady between the ethanol-fed and control pair-fed mice. Cytokines (IL-10, IL-15 and IL-6) that inhibit TNF-α activity have been shown to prevent liver damage from LPS. These cytokines were up-regulated in both groups but to a greater extent in the ethanol-fed mice.

Thus, the Koteish mouse animal model provides an excellent way to set up a long-term study showing the interaction of ethanol and LPS. This model is beneficial as there is no surgery to perform and only one injection of LPS is administered at the end of the study.

In the von Montfort *et al*[23] model, four groups of C57BL-6J mice were given different treatments with epinephrine and ethanol. In the first group, 2 mg/kg per day of epinephrine was administered *via* an osmotic pump implanted in the dorsal area for five days. In previous studies this epinephrine dose had been shown to effectively mimic the effects of ethanol. The second group (controls) underwent sham surgery to mimic the implantation of the osmotic pump. The third group was injected with ethanol (6 g/kg per day) for three days. The last group was given a maltose/dextrin injection that was comparable calorifically to the ethanol injection. An injection of 10 mg/kg LPS was administered after the 5-d period and 24 h after the last ethanol dose, the mice were sacrificed at one, eight and 24 h later.

Histological results showed normal hepatocytes in the control animals and in the animals with the epinephrine pump/no LPS injection. Mice injected with LPS alone showed mild inflammation, whereas the mice receiving the epinephrine pump/LPS showed enhanced

liver damage. Importantly, the mice that were given ethanol/LPS showed dramatic liver damage. Plasma AST levels showed that the mice infused with epinephrine alone had no significant increase in plasma AST levels as compared with control mice. Animals treated with LPS only showed a progressive increase in AST with values approximately two-fold higher than the controls. Exposure of animals to epinephrine and ethanol with LPS exposure showed a significant increase in AST levels after LPS exposure by a factor of approximately four and six, respectively. The expression of pro-inflammatory genes, TNF-α, IL-6, and PAI-1 (plasminogen activator inhibitor 1) were analyzed by RT-PCR. In this part of the study, LPS alone stimulated all three genes as early as one hour after injection. Epinephrine pretreatment alone did not affect the expression of these genes, but paired with LPS showed an increased expression of all three genes. The induction of PAI-1 caused by LPS was significantly greater (two-fold) in the presence of epinephrine. The peak expression of TNF-α and IL-6, one hour after LPS exposure was not enhanced by epinephrine pre-exposure. However, these levels were significantly elevated when compared with the LPS alone group at the 8 h time point. Twenty-four hours after LPS injection, mRNA levels of all three returned to basal levels with no difference between the LPS and epinephrine/LPS groups.

Inflammation by LPS is not only enhanced by increased production of pro-inflammatory cytokines, but by impairing the expression of anti-inflammatory genes. Key anti-inflammatory genes such as IL-10, SOCS-1 and SOCS-3 were not significantly changed following epinephrine infusion alone, or in the sham-treated mice.

Curcumin (CMN) has been shown to exhibit anti-inflammatory, anti-bacterial, anti-oxidant properties, and reducing oxidative stress caused by ethanol. This animal model effectively shows how CMN pretreatment protects from LPS-induced liver damage. Kaur *et al*[24] used CMN in a model where six groups of male Wistar rats were administered different treatments in conjunction with CMN. In these studies CMN was administered at different doses (5, 30, 60 mg/kg) in the presence or absence of LPS. Rats were sacrificed six hours after an LPS injection on the last day.

Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), and alkaline phosphatase (AP) were analyzed in all rats which showed a marked rise in serum levels, following LPS injection causing increased liver damage. LPS also caused a rise in serum bilirubin and a decrease in serum total protein compared to control rats. Treatment with CMN significantly decreased the elevated levels of AST, ALT, AP, and bilirubin in response to LPS challenge. Serum nitrite levels and lipid peroxidation were decreased, whereas hepatic GSH, reduced glutathione, and superoxide dismutase (SOD), were increased. Additionally, TNF-α and IL-6 cytokines increased in LPS-administered rats compared to normal rats. CMN administration for seven days effectively blocked the rise of TNF-α and IL-6 cytokines in the LPS-challenged rats. Histology results did not reveal any morphological alterations in the control group but did show marked morphological disruption in LPS-administered rats. Treatment with CMN attenuated these morphological changes.

In the Gustot *et al*[25] animal model, C57BL-6J mice were fed a liquid ethanol diet adapted from the Lieber-DeCarli diet *ad libitum* for 10 d followed by an injection intraperitoneally with one of the following: 60 or 120 μg lipoteichoic acid (LTA), 60 or 120 μg peptidoglycan (PGN), 60 or 120 μg polyinosine-polycytidylic acid (polyIC), 30 or 60 μg LPS, 60 μg flagellin or 60 or 120 μg 7-allyl-8-oxoguanosine (loxoribine). Control mice were pair-fed with a control diet and injected with the same volume of saline solution after 10 d. Mice were sacrificed eight hours after injection.

The ethanol diet led to an increased liver weight compared to the control mice. Histology showed nearly 75% of hepatocytes in ethanol-fed mice exhibited steatosis. Compared to the control-fed mice, the ethanol-fed mice showed significant increase in serum (ALT) levels, and TNF-α mRNA expression. After 10 d, the expression of Toll-like receptors (TLR) 1, 2, 4, 6, 7, 8 and 9 was measured and found to have significant increases over control-fed mice. However, TLR3 and TLR5 were not statistically different in the two groups. To look at the role that endotoxin plays in liver damage in ethanol-fed mice, antibiotics were administered before the experiment began. Cultures from the ethanol-fed mice treated with antibiotics showed a clear decrease in gram-negative bacteria from the mice not treated with antibiotics prior to the start of the experiment. Addition of antibiotics reduced the severity of fatty liver, as shown by decreases in liver weight, serum ALT levels, and steatosis. An increase of lipid peroxidation products (e.g. malondialdehyde, 4-hydroxyaldenals) and a decrease of antioxidant levels (e.g. glutathione) demonstrated hepatic oxidative stress in the livers of ethanol-fed mice.

This animal model is effective because it provides a way to study ethanol consumption and LPS in a way that shows the importance of LPS in the liver for damage to occur. It is important to note that clinically, antibiotics have demonstrated some success in the treatment of ALD patients[26-28].

The studies above used different approaches, but have shown very similar results when comparing the short-term and long-term effects of ethanol. Overall the short-term affects show that ethanol provides a protective affect from LPS by altering the expression of endotoxin receptors and intracellular signaling molecules[20]. In contrast, the long-term effect shows an increased sensitivity to LPS and ethanol resulting in liver damage. Clearly, while these models have helped in gaining new insights into the mechanisms of ALD, the development of new and innovative animal models are needed to better elucidate the development and/or progression of ALD.

EFFECTS OF ETHANOL, METABOLITES, AND LPS *IN VITRO*

Liver sinusoidal endothelial cells (SECs)

SECs are flattened, highly fenestrated, and lack a basement membrane. This leads to their common characterization as "sieve-like". Scanning electron microscopy has shown healthy fenestrae are approximately 150 nm in diameter and make up 6%-8% of the sinusoidal surface. These cells are active filters, selectively moving liquids and solutes from the portal blood into the Space of Disse where they are exposed to parenchymal hepatocytes and lipid storage cells. SECs are also highly endocytotic using scavenger receptors (SRs) to clear the blood of many molecular waste products. They have much higher permeability than other capillaries, and there is constant bidirectional exchange between hepatic parenchyma and blood *via* SECs. The size of the molecules that can pass through SECs is only constrained by fenestral diameter[29]. These cells are the first line of defense against harmful molecules that could potentially damage the liver. There are many immunological functions of SECs. They have been shown to efficiently remove small (< 200 nm) molecules from the blood using innate immune mechanisms such as scavenger and mannose receptors[29,30]. They have also been shown to express MHC class II, and co-stimulatory molecules (CD40, CD80, and CD86). They also express intercellular adhesion molecules including CD54 and CD106, which attract immune cells during inflammation. This suggests that they are involved in antigen processing and presentation as well as leukocyte recruitment[30].

Capillarization of the SECs occurs when they form basement membranes that have been shown to precede fibrosis[31]. Also, hepatic stellate cells (HSCs), when activated to their collagen producing form, induce fibrosis[32]. It has been shown *in vitro* that quiescent rat HSCs maintain their inactivity and active HSCs revert to quiescence when grown in co-culture with healthy, nitric oxide (NO) producing SECs in the presence of vascular endothelial growth factor (VEGF). However, this effect is not observed when HSCs are co-cultured with capillarized SECs or those not producing NO[33].

There is also evidence that malondialdehyde and acetaldehyde-two ethanol metabolites-form an adduct (MAA) which modifies proteins such that they increase the expression of fibrotic molecules by SECs. One such molecule is fibronectin. Fibronectin is expressed following insult to the liver and is known to activate HSCs and induce fibrosis. After stimulation with MAA-modified bovine serum albumin (MAA-Alb), isolated rat SECs show significant increases over negative controls in expression of soluble fibronectin, cellular fibronectin, and of the EIIIA fibronectin variant (the form most closely associated with activation of HSCs)[34]. Incubation of MAA-Alb with isolated rat SECs for four hours has been shown to elicit increases in pro-inflammatory cytokines/chemokines: an eight-fold increase in TNF-α; a two-fold increase in MCP-1; and a four-fold increase in MIP-2[8]. This increase in fibrotic molecules and pro-inflammatory cytokines provides a potential mechanism by which damage to the liver is mediated by metabolites of ethanol adducted proteins.

Due to their participation in the clearance of LPS from the blood, SECs have mechanisms for the control of inflammatory, leukocyte-mediated response to LPS, while maintaining the population of SRs necessary for toxin and waste clearance. Upon initial contact with LPS, cultures of mouse hepatic SECs release and activate IL-6. This occurs without regard to the presence of TNF-α and requires functional TLR4. SEC responsiveness is decreased following repeated stimulation by LPS. This occurs *via* reduced nuclear translocation of transcriptionally active nuclear factor $\kappa\beta$ (NF$\kappa\beta$). Importantly, repeated exposure to LPS diminishes SEC scavenger function but does not eliminate it. Initial exposure to LPS results in an increased expression of adhesion molecules on leukocytes including CD54, CD106, MCP-1, and IFN-inducible protein 10. None of the above are up-regulated in cultured SECs with previous LPS exposure, and in the case of CD54, this was shown to occur *via* reduced gene expression[35].

Exposure to LPS *in vivo* has been shown to increase both the quantity of small (20 nm) latex beads and the maximum size (from 100 nm to 500 nm) of latex beads that can be ingested by mouse SECs. Increases were not seen in the rate of ingestion of 100 nm beads, which were the most aggressively ingested regardless of LPS stimulation. LPS was also shown to increase the uptake of both BSA (soluble protein) and dextran (soluble carbohydrate). These increases are attributed to LPS-induced actin remodeling by protein kinase C (PKC) and phosphoinositide 3-kinase (PI3-K) as indicated by increased expression of *src*-suppressed C kinase substrate (SSeCKS) on the endothelial cells[36].

Two-hit model effects on SECs

Lining the portal veins of the liver, the SECs are the first line of defense against LPS derived from the gut[30]. Most of the attention in LPS clearance has been attributed to the KCs. However, it has been shown that SECs contribute to the regulation of LPS in the liver[30,35,36]. This regulation of LPS under normal physiological conditions is kept in check by both KC and SECs, which keep the amount of inflammation to a minimum. If LPS levels are increased due to other factors (i.e. alcohol or aldehyde modified proteins), then the potential for disruption of normal homeostasis exists. The fact that alcohol increases both gut permeability (increasing the LPS)[7] and aldehyde modified liver proteins[4], provides a possible two-hit mechanism by which the liver could become damaged.

Chronic exposure to ethanol metabolites in the form of MAA-adducted albumin has been shown to alter the SEC response to LPS. In hepatic SECs isolated from rats, LPS-induced secretions of TNF-α, MCP-1, and MIP-2 all show at least two-fold increases in the presence of MAA modified albumin, but no increase in the presence of unmodified albumin. The TNF-α

response is decreased by chronic ethanol consumption, but MCP-1 and MIP-2 responses are not[8]. The MAA-adduct has also been shown to bind to, and be degraded by, SECs *via* SRs on their surface[37]. It has also been shown that SECs have CD14 and TLR receptors, which are involved in the uptake of LPS[38,39]. The potential exists for both aldehdye modified proteins and LPS to bind their receptors simultaneously, increasing the normal release of pro-inflammatory factors that promote inflammation of immune cells to the liver.

While chronic ethanol consumption and LPS stimulation independently increase apoptosis of SECs (as measured by caspase-3 activity) in pre-ALD rat livers, the combination elicits no additional increases. However, data generated from this study did demonstrate that LPS treatment of animals increased the amount of malondialdehyde (MDA) in the hepatocytes. The increase in MDA provides additional substrate for the potential formation of MAA adducts. Therefore an increase in AA and MDA from alcohol metabolism and an increase in MDA from inflammation-induced cell damage could lead to increased MAA-adducted self cellular material and the subsequent initiation of an autoimmune disease[40].

KCs

KCs are the resident macrophages found in the liver. It is believed that KCs play an important role in the development of ALD[41]. When stimulated with LPS they become activated and release pro-inflammatory and fibrotic cytokines, along with ROS, which can contribute to liver injury[11]. Interestingly, it has been shown that circulating LPS concentrations are increased in the blood of alcoholics, and in rats fed alcohol intragastrically, due to the effects ethanol on increasing the permeability on the intestinal mucosa[42,43]. The circulating LPS derived from intestinal bacteria in turn activate KCs, which initiate their pro-fibrotic and pro-inflammatory effects. KCs can also be activated by interactions with proteins modified by reactive aldehydes associated with ethanol metabolism increasing oxidative stress due to [acetaldehyde (AA) and malondialdehyde (MDA)]. These modified proteins have been associated with ALD[44-47]. LPS may further sensitize KC interactions with aldehyde-modified proteins[8] and may actually be involved in their formation[48].

Chronic ethanol does more than provide the KCs with LPS; it also directly affects their sensitivity to LPS. This section will examine the role that LPS activation of KCs plays in the development of ALD and whether ethanol directly affects KC responses to LPS. The role(s) of aldehyde modified proteins in KC activation and how LPS may affect KC sensitivity to stimulation by these modified proteins will also be examined.

Alcohol causes both tolerance and sensitization to KC activation by LPS[49-51]. Studies have shown that acute ethanol administration inactivates KCs probably due to ethanol's effects on calcium channels and their requirement for TNF-α release[52]. KCs isolated from rats two hours after ethanol treatment indeed lacked increased intracellular calcium normally observed when KCs are treated with LPS. However, when KCs were isolated 24 h after the rats were treated with ethanol, the cells displayed their normal TNF-α production and histological changes upon LPS stimulation. These cells also displayed higher levels of the LPS binding receptor, CD14, which may explain their increased sensitivity to LPS. Treating the rats with antibiotics, which sterilizes the gut removing portal LPS, lowered CD14 expression on KCs isolated 24 h after the administration of ethanol, suggesting that gut derived LPS was the cause of the increased CD14 expression and resultant sensitivity to LPS[53]. From these studies it can be concluded that a single dose of ethanol can either sensitize KCs or induce tolerance to LPS based on timing.

Two-hit model effects on KCs

As discussed above, chronic ethanol exposure seems to increase the sensitivity of KCs to LPS stimulation. One of the major cytokines released by KCs exposed to LPS is TNF-α, which plays a role in the development of ALD[41]. Use of anti TNF-α antibody has been shown to protect against ALD in certain animal models[54]. The role of TNF-α in ALD was also confirmed in studies using TNF-α receptor 1 knockout mouse. In these mice, the pathological changes associated with ethanol treatment were greatly diminished[55]. KCs may become extra sensitive to LPS and as a result increase their production of TNF-α when chronically exposed to ethanol. This increases CD14 expression and changes the signaling cascade molecules involved in LPS stimulation induced by ROS. These events change transcription factor binding to DNA and increase stability of mRNA involved in TNF-α production.

KCs stimulated by LPS also have effects on HSCs. Hepatic fibrosis is characterized by an over deposition of extracellular matrix components. HSCs are involved with the production of extracellular matrix (ECM) in the liver and during fibrogenesis they undergo a process of activation and proliferation leading to excess collagen synthesis[56]. LPS activated KCs have been shown to be capable of activating HSC *in vitro*, and levels of ECM production have been directly correlated to increased HSC proliferation[57].

The *in vitro* effects that LPS-stimulated KCs have on HSCs may be similar to what is seen during *in vivo* fibrogenesis. Higher levels of LPS in the blood of chronic alcoholics may serve as the catalyst for KC activation and therefore may promote HSC activation and fibrogenesis. The production of TGF-β1 by LPS-stimulated KCs is one of the most significant steps in the activation of HSCs[57].

Studies have indicated that the detection of acetaldehyde (AA), malondialdhyde (MDA), and AA MDA hybrid (MAA) modified proteins adducts, correlate with increased liver enzymes and liver damage[45,47,58]. Experiments have also demonstrated that administering gadolinium chloride with ethanol results in the decreased accumulation of MDA and especially AA protein adducts in the livers of rats chronically fed alcohol indicating a role for KCs in AA protein adduct formation[48].

LPS stimulation of KCs and their ability to release

pro-inflammatory cytokines might also promote immune system recruitment and surveillance, which might help to promote an immune response to these modified proteins. Circulating antibodies to aldehyde-derived epitopes have been identified[59]. KCs might be involved in the actual presentation of these molecules to the immune cells and be stimulated by these adducted proteins to release pro-inflammatory cytokines. SRs found on their surfaces can bind MAA modified proteins and subsequent binding of these proteins leads to increased levels of TNF-α. When KCs are stimulated with low levels of LPS in addition to MAA modified albumin (MAA-Alb), TNF-α secretion increases six to eight fold. The levels of LPS used for co-stimulation were so low that LPS alone did not result in any TNF-α secretion[8].

In summary, KCs are a key component in the development and/or progression of ALD. LPS is a potent activator of KCs, causing them to release cytokines such as TNF-α and TGF-β1, which have been indicated in the development of ALD. Acute administration of ethanol can lead to tolerance or sensitivity to LPS in isolated KCs, while chronic ethanol exposure usually induces a state of ethanol sensitivity marked by increased cytokine production. Cytokines produced by KCs stimulated by LPS can lead to proliferation and activation of HSCs resulting in ECM production. KCs might be involved in the formation of aldehyde protein adducts and these adducts might promote a pro-inflammatory response in KCs, especially in the presence of LPS.

Stellate cells (HSCs)

HSCs undergo activation and proliferation when under the influence of acute and chronic liver injury events. Liver fibrosis and cirrhosis occur in the chronic stages of injury and represent activation of HSCs and secretion of matrix from these cells. Activation of HSCs also occurs in liver injury in acute stages where this damage is known to be able to resolve on its own[60]. Chronic liver injury events influenced by alcohol and LPS are of interest as they are associated with a constant hepatic insult that leads to life threatening complications with liver transplantation as the only viable option[60]. As HSCs are involved in the pathway of the wound healing of the liver, their association with alcohol and LPS is an important relationship to understand.

Metabolism of ethanol by the liver is an extremely oxidative event resulting in the development of acetaldehyde (AA). AA is further metabolized into acetate *via* the mitochondrial enzyme acetaldehyde dehydrogenase (ALDH)[61]. This is a slow reaction that allows for AA buildup over time when alcohol is consumed, and while ADH activity is not greatly influenced, CYP2E1 and other microsomal enzymes are greatly stimulated[61]. This buildup of AA has also been shown to form stable aldehyde adducts on proteins, which stimulate collagen synthesis, activate protein kinase C, and promote the release of chemokines MCP-1 and MIP-2[62-64]. Activation of HSCs increases the release of collagen and matrix proteins, which begin the fibrogenic response of wound healing[32,61].

TGFβ1 is the major profibrotic cytokine in the normal wound healing process. TGFβ1 messenger expression in HSCs is elevated by ethanol and acetaldehyde, whereby collagen type I gene expression is up-regulated[32]. Indeed, mouse α2 (I) collagen promoter was shown to have greater activation in transient transfection experiments when TGFβ1 and acetaldehyde were introduced in tandem, rather than alone, suggesting that TGFβ1 could play a direct part in collagen I gene activation[65].

Two-hit model effects on stellate cells

In rodent models, using ethanol to induce substantial fibrotic liver injury is problematic regardless of the concentration or length of treatment. Therefore two-Hit models have been used in rodents with the hope to better emulate the fibrotic injury found in human disease states, using some secondary factor with ethanol. Degradation of ethanol increases oxidative stress within the hepatic system, generating free radicals, leading to further hepatic events such as endotoxemia. These events increase gut permeability allowing for more LPS release making LPS clearance more difficult[10,11,19].

Karaa *et al*[66] looked for therapeutic agents that slow or possibly prevent ALD progression. S-adenosyl-L-methionine (SAMe) has previously been shown to be a precursor in glutathione (GSH) synthesis in the transsulferation pathway, which is an important hepatic antioxidant. The fact that chronic ethanol consumption greatly limits hepatic SAMe storage, GSH synthesis, and increases gut permeability, allows LPS to act as a secondary agent in the two-hit model.

Karaa *et al*[66] examined how SAMe acted as an antifibrotic agent by looking at liver fibrosis, HSC activation, and collagen deposition. This model used Lieber DiCarli liquid diet containing ethanol (or a calorie matched diet) concurrently with twice weekly LPS injections during an eight week period. Ethanol alone led to steatosis, some immune cell infiltration (mainly neutrophils), HSC activation, and increased hepatic collagen production. When LPS was introduced with ethanol, hepatic infiltration of neutrophils was increased as well as increased activation of HSCs and preferential pericellular collagen deposition. When SAMe was introduced with LPS and chronic ethanol administration, the antioxidative properties of SAMe were apparent. GSH stores depleted by ethanol were replenished and hepatic oxidative stress was reduced.

Quiroz *et al*[67] also looked at the effect of LPS on rat HSC (CFSC-2G) in relation to ethanol and AA. GSH, oxidized GSH (GSSG), IL-6, and collagen secretion were measured. The authors found that lipid peroxidation levels were increased in all experimental conditions versus controls (controls being HSCs with LPS, ethanol, or AA alone). MDA response to ethanol and acetaldehyde exposure did not show a significant change with LPS pretreatment. Experimental cells showed a 2.5 fold increase in GSSG content with LPS and ethanol, and a 5.5 fold increase with LPS and AA, with control values of GSH being much lower. Collagen content was also greatly enhanced with pretreatment of LPS, 120%

greater with ethanol and 209% greater with AA. TGF-β secretion was similar to that of the controls, but IL-6 was greatly up-regulated[3].

Pretreatment of LPS resulted in an increase of intracellular GSSG, leading to the formation of mixed disulfides with protein thiols, thus lowering the ability to fight oxidative stress induced hepatic injury[67]. The authors theorized that with the aforementioned change in GSH and GSSG levels, HSCs pretreated with LPS might also generate additional ROS. They also speculated that IL-6 not only promotes hepatocyte proliferation, but enhances collagen production by these activated HSCs. Therefore, "LPS pretreatment of HSC adds to the damage produced by ethanol and acetaldehyde by diminishing GSH content and increasing GSSG content, collagen, and IL-6 secretion"[67].

Precision cut liver slices (PCLS)

Precision cut liver slices (PCLS) may provide an alternative to other *in vitro* model systems using isolated liver cells to study the combined effects of ethanol and LPS. PCLS are representative of the whole liver and have recently been developed as an *in vitro* model of ethanol induced liver injury[68]. PCLS exhibit significant ethanol-induced damage in as little as 24 h. This model uses PCLS originating from Wistar rats, cultured in the presence or absence of 25 mmol/L ethanol in a roller system under 95% O_2[68]. Over a 96 h time period this model efficiently metabolizes ethanol, produces AA, develops a reduced redox state and fatty liver, and exhibits impaired albumin secretion. All of these phenomena are characteristics of early liver injury. Interestingly, in the presence of 4-methylpyrazole (4-MP), an inhibitor of ethanol metabolism, all of the ethanol-mediated effects are ameliorated or significantly reduced, indicating that the metabolites of ethanol are responsible. In addition, recent studies have shown that 25 mmol/L ethanol induces sustained production of IL-6, depletion of GSH, increased lipid peroxidation, and induction of fibrogenesis (increased expression of smooth muscle actin and deposition of collagen in sinusoidal areas). All these phenomena are inhibited by 4-MP, implicating ethanol metabolites. The production of IL-6, GSH depletion, and lipid peroxidation all precede the induction of fibrogenesis, suggesting that inflammation and production of reactive oxygen species are responsible.

PCLS have also been used to examine the effects of LPS on the liver[69,70]. LPS induced expression of TNF-α, IL-1β, IL-6 and IL-10 within 24 h of incubation. Consistent with other studies indicating TNF-α induces expression of other cytokines, TNF-α expression was maximal by five hours, whereas expression of the other cytokines was maximal by 16-24 h[69]. The authors attributed this to activation of KCs, but activation of SECs cannot be ruled out, as they also respond to LPS. Additionally, production of nitric oxide (NO) gradually increased after LPS treatment, starting at five hours after treatment, and continuing throughout the 24 h treatment period[70]. This increase was paralleled by an increase in inducible nitric oxide synthase (iNOS) expression in the hepatocytes. Inhibition of TNF-α and IL-1β attenuated iNOS expression, indicating a paracrine effect by the cytokines induced by LPS treatment. Production of NO *in vivo* has been shown to have protective effects in inflammation and endotoxemia-induced hepatic injury[71]. Thus, these results might indicate a compensatory response by the PCLS to the inflammatory response induced by LPS treatment.

One previous study examined the combined effects of LPS and ethanol on PCLS cytotoxicity[72]. This study incubated PCLS in the absence or presence of 0.5%-8% ethanol and/or 0.1-100 μg/mL LPS for up to 12 h. Ethanol at 1% or more exhibited a time- and dose-dependent hepatotoxicity by itself, whereas LPS had no appreciable effect. However, when combined, 2% ethanol and increasing concentrations of LPS exhibited additive effects on hepatotoxicity with 100 μg/mL LPS inducing the most injury (70% viable) compared to 2% ethanol alone (90% viable) at 12 h. While these concentrations of both ethanol and LPS are extremely high, they do provide an upper limit for future studies to examine the combined effects of ethanol and LPS. In fact, preliminary data by this laboratory suggests that more physiological levels of ethanol (25 mmol/L) and LPS (10 ng) exhibit additive effects on inflammatory processes and induction of oxidative stress in PCLS.

CONCLUSION

Studies into the pathogenesis of ALD have demonstrated that ethanol, LPS, and the metabolites of ethanol all may have a significant role in the development and/or progression of this disease. However, the data also strongly suggests that while each of these components are necessary, alone they are not sufficient to induce the pathogenesis of ALD. Indeed, it is becoming more apparent that combinations of one or more of these components must occur to induce ALD, which would suggest that at the very least a "two-hit" model is involved in the pathogenesis of ALD. Figure 1 shows a proposed hypothesis of the "two-hit" model of ALD.

Support for this concept can be found in some very simple observations. Acute (down-regulation) versus chronic (no effect) ethanol exposure decreases the response of KCs and SECs to LPS, which is thought to be a major co-factor in ALD. It has also been shown that on some cell types, multiple receptors (Scavenger Receptor A; SRA-1) are present and may bind LPS and degrade this ligand before it binds to TLR4. When the expression of these SRs is altered (ethanol inhibits degradation), LPS is now free to bind to CD14/TRL4 and initiate pro-inflammatory responses. It has also been shown that multiple ligands, ROS, aldehyde-modified proteins, and hyaluronan are involved in the development and/or progression of ALD. Therefore, depending upon which ligands were to bind to their appropriate receptors, then the response would be totally different than if only one of these ligands were to bind. Couple this with the fact that each cell type in the liver expresses different receptors, and it is easy to imagine that the interactions

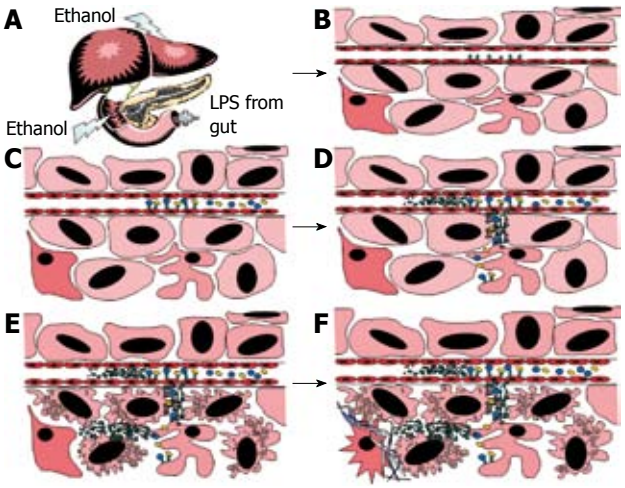

Figure 1 A proposed hypothesis of the two-hit model for the onset and/or progression of alcohol liver disease. A: The prolonged consumption of ethanol has been associated with an increase in gut permeability. Lipopolysaccaride (LPS) may leak out of the gut and into the blood stream, finding its way back to the liver. B: Meanwhile, the breakdown of alcohol and fats in the liver could modify cellular proteins with malondialdehyde and/or acetaldehyde and result in increased levels of these modified proteins. Receptors on endothelial cells (SECs) specific for LPS and aldehyde modified proteins might also be up-regulated. C: The various scavenger receptors bind LPS and/or aldehyde modified proteins circulating in the blood stream. D: Binding of these molecules causes an increased release of pro-inflammatory cytokines, which are dumped into the blood stream and into the liver through the Space of Disse. The release into the liver causes activation of kupffer cells, binding of LPS and modified proteins, and their release of more pro-inflammatory cytokines. E: The cytokine release from SECs and/or kupffer cells signals immune cells infiltrate into the liver and result in damage to the hepatocytes. F: Damage to the hepatocytes increases the amount of TGF-β and other cytokines, causing the stellate cells and SECs to secrete pro-fibrogenic factors. These factors help to rebuild and remodel the liver parenchyma initiating the wound healing responses. Following repair of the liver, there is scaring and some irreparable damage, which can get better if the insults (LPS and modified proteins) are taken away. However, if alcohol consumption remains there becomes a point whenentence, preferably as two shorter sentences, to clarify this?

between the different cell types might change depending upon the receptors expressed. As discussed in this review, it is apparent that these interactions do occur and the development and/or progression of ALD is a complex interaction that may be investigated utilizing a "two hit" or "multiple hit" model.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Alcohol-induced protein hyperacetylation: Mechanisms and consequences

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Abstract

Although the clinical manifestations of alcoholic liver disease are well-described, little is known about the molecular basis of liver injury. Recent studies have indicated that ethanol exposure induces global protein hyperacetylation. This reversible, post-translational modification on the ε-amino groups of lysine residues has been shown to modulate multiple, diverse cellular processes ranging from transcriptional activation to microtubule stability. Thus, alcohol-induced protein hyperacetylation likely leads to major physiological consequences that contribute to alcohol-induced hepatotoxicity. Lysine acetylation is controlled by the activities of two opposing enzymes, histone acetyltransferases and histone deacetylases. Currently, efforts are aimed at determining which enzymes are responsible for the increased acetylation of specific substrates. However, the greater challenge will be to determine the physiological ramifications of protein hyperacetylation and how they might contribute to the progression of liver disease. In this review, we will first list and discuss the proteins known to be hyperacetylated in the presence of ethanol. We will then describe what is known about the mechanisms leading to increased protein acetylation and how hyperacetylation may perturb hepatic function.

INTRODUCTION

The liver is the major site of ethanol metabolism and thus sustains the most injury from chronic alcohol consumption. In the hepatocyte cytosol, alcohol dehydrogenase (ADH) converts ethanol to acetaldehyde, a highly reactive intermediate. The acetaldehyde is further metabolized in mitochondria to acetate by acetaldehyde dehydrogenase (ALDH). Alcohol is also metabolized by the resident ER enzyme, cytochrome P450 2E1 (CYP 2E1). CYP 2E1-mediated ethanol metabolism not only leads to the formation of acetaldehyde, but also to the formation of oxygen and hydroxyethyl radicals that in turn promote the formation of other highly reactive intermediates[1]. All of these reactive metabolites can readily and covalently modify proteins, DNA and lipids[2-7]. More recently, it has become apparent that alcohol exposure induces protein covalent-modifications that are part of the natural repertoire. To date, these post-translational modifications include increased methylation, phosphorylation and acetylation[8-14]. In particular, numerous proteins have been identified that are hyperacetylated upon ethanol exposure, and this list is expanding rapidly. As for adduct formation, it is not clear how increased acetylation is related to the progression of alcohol-induced hepatotoxicity. In this review, we will first list and discuss hepatic proteins known to be hyperacetylated by ethanol exposure. We

will then describe our current understanding of the mechanisms and physiological consequences of protein hyperacetylation.

ETHANOL-INDUCED LYSINE HYPERACETYLATION

For over 40 years it has been recognized that proteins can be acetylated and that the modification comes in two forms[15]. One is the irreversible, co-translational N-terminal acetylation of α-amino groups of mainly serine and alanine, but also of threonine, methionine and glycine. The other form is the reversible, post-translational modification of ε-amino groups on lysine residues located within a polypeptide[15,16]. The reversibility of lysine acetylation and its presence on an ever expanding list of nuclear and nonnuclear proteins have led some to postulate that it might rival phosphorylation in its ability to regulate cellular processes[16]. Thus, alcohol-induced protein hyperacetylation likely results in major physiological consequences that contribute to the progression of hepatotoxicity. In this section, we will first discuss individual hepatic proteins that are known to be hyperacetylated upon ethanol exposure (Table 1). We will then comment on recent work that implicates many more candidates for alcohol-induced lysine acetylation (Table 2).

Table 1 Ethanol-induced hyperacetylated proteins

Protein	EtOH exposure	System	References
Histone H3	Acute and chronic	Hepatocytes, rat liver, stellate cells	[8,17-21]
p53	Chronic	Rat liver, VLA17 cells	[61]
PGC-1α	Chronic	Rat liver, mouse liver	[12,14]
SREPB-1c	Chronic	H4IIEC3 cells	[12]
α-tubulin	Chronic	WIF-B cells, rat liver	[11,39,75]
AceCS2	Chronic	Rat Liver	[13]

Table 2 Newly identified ethanol-induced hyperacetylated proteins

Liver subcellular location[1]	EtOH exposure	Function	No. proteins identified
Cytosol	Chronic	AA metabolism	7
		Carbohydrate metabolism	4
		Other metabolic pathways	3
		Oxidative stress	3
		Other	2
Mitochondria	Chronic	Lipid metabolism	12
		AA metabolism	5
		Oxidative phosphorylation	2

[1]Our unpublished results.

Histone H3

Histones were the first proteins known to be acetylated[15,16], and histone H3 was the first protein known to be hyperacetylated after ethanol treatment. This effect has been observed in isolated hepatocytes and in rat liver after both acute and chronic ethanol exposure[8,17-21]. Although histone H3 encodes at least four acetylated lysines (lys 9, 14, 18 and 23), acetylation of lysine 9 is selectively increased after exposure to physiological ethanol concentrations[8]. Because histone H3 hyperacetylation was prevented by both 4-methylpyrazole (4-MP) and cyanamide, it was concluded that the modification requires alcohol metabolism and is likely mediated by the ethanol metabolite, acetate[8]. Interestingly, lysine 9 is also reversibly methylated, and in ethanol-treated hepatocytes, its methylation is decreased[9]. Furthermore, alcohol also induces hypermethylation of lysine 4 and phosphorylation of neighboring serine residues (ser 10 and ser 28)[10]. It will be important to consider all of these modifications as the functional consequences of histone H3 hyperacetylation are defined (see below).

p53

p53 is a tumor suppressor that is mutated in over half of all human cancers. It is activated by DNA damage and functions to stop cell cycle progression. For over a decade it has been known that p53 is reversibly lysine acetylated, and that this modification promotes enhanced DNA binding[15,16]. Very recently, p53 has been shown to be hyperacetylated upon chronic ethanol exposure in rat liver lysates[14]. Although multiple lysines are known to be acetylated, it is not yet known which residue(s) is hyperacetylated after ethanol consumption. Furthermore, it is not yet known whether hyperacetylation requires ethanol metabolism or whether it alters p53 DNA binding properties.

Sterol response element binding protein-1c (SREBP-1c)

SREBPs are a family of transcription factors that regulate lipid and cholesterol synthesis. SREBP-1c is the major form expressed in liver and is known to activate numerous lipogenic enzymes. In rat hepatoma H4IIEC3 cells, ethanol treatment for 24 h led to a dose-dependent increase in SREBP-1c acetylation[12]. This result was confirmed in livers from ethanol-fed mice indicating it has physiologic importance. It is not yet known whether hyperacetylation requires ethanol metabolism nor is it known which lysine is hyperacetylated. However, more is known about the mechanism by which SREBP-1c is acetylated/deacetylated and how that relates to SREBP-1c function in gene regulation and lipogenesis (see below).

Peroxisome proliferator-activated receptor γ coactivator α (PGC-1α)

The nuclear transcriptional coactivator, PGC-1α, is a key regulator of hepatic glucose homeostasis and lipid metabolism[22]. It has also been implicated in regulating mitochondrial biogenesis and respiration[22]. PGC-1α is known to be acetylated on many lysines,

and in general, acetylation is correlated with decreased transcriptional activity resulting in decreased expression of genes involved in mitochondrial fatty acid oxidation and gluconeogenesis. Thus, it is notable that PGC-1α hyperacetylation is induced by ethanol consumption[12]. Currently, it is not known which lysine(s) is modified or whether PGC-1α hyperacetylation requires ethanol metabolism. However, as for SREBP-1c, there is more known about some likely functional consequences of PGC-1α hyperacetylation (see below).

Acetyl CoA synthetase 2 (AceCS2)

A recent proteomic survey of mammalian cell proteins identified nearly 200 lysine-acetylated nuclear and nonnuclear proteins[23]. Remarkably, this survey further revealed that more than 20% of mitochondrial proteins were lysine-acetylated[23]. Thus, it is not surprising that the acetylation of AceCS2, an enzyme involved in lipid metabolism, is increased upon alcohol exposure, probably on lysine 635[13,24]. Acetylation of this residue correlates with decreased AceCS2 catalytic activity[25], but whether its activity is decreased in ethanol-treated cells is not yet known. Interestingly, alcohol-induced acetylation of mitochondrial proteins, including AceCS2, was not observably altered in livers from CYP 2E1 knockout mice suggesting that the modification does not require CYP 2E1-mediated ethanol metabolism[13]. However, whether ethanol metabolism by ADH and ALDH is required for mitochondrial protein hyperacetylation is not yet known.

Tubulin

Microtubules are one of the three major cytoskeletal systems of the cell. The polymer is made of repeating units of α- and β-tubulin heterodimers that form protofilaments, which in turn assemble into hollow tubes consisting of 13 protofilaments arranged in parallel. Microtubules exist as both dynamic and stable polymers. The latter population is characterized by a longer half-life, resistance to microtubule poisons (e.g. cold and nocodazole) and by specific post-translational modifications on the α-tubulin subunit[26]. These modifications include the removal of a carboxy-terminal tyrosine, polyglutamylation, polyglycylation and acetylation of lysine 40[26]. The functions of these modifications or whether they contribute to microtubule stability are still the subject of debate[27]. Recently, it was determined that chronic ethanol exposure enhanced α-tubulin acetylation at lysine 40 in polarized WIF-B cells and livers from ethanol-fed rats[11]. Increased acetylation correlated to increased stability suggesting that tubulin acetylation might in fact enhance microtubule stability. In WIF-B cells, increased tubulin acetylation and stability displayed both ethanol time- and dose-dependence[11]. Furthermore, tubulin hyperacetylation and stability was prevented by 4-MP and potentiated by cyanamide indicating that ethanol metabolism was required for the effects[11]. Thus, unlike acetate-mediated histone H3 hyperacetylation, tubulin hyperacetylation and increased stability are likely mediated by acetaldehyde. This disparity is likely to be due to the different mechanisms leading to enhanced acetylation of either substrate (see below).

The expanding list

With the growing number of known acetylated proteins and the large number of modifying enzymes, it is likely that numerous hepatic proteins are hyperacetylated in ethanol-treated cells. We initiated a proteomics approach to identify other hyperacetylated proteins from cytosolic and total membrane fractions prepared from livers from control and ethanol-fed rats (manuscript in preparation). So far, about 40 nonnuclear proteins have been identified (but not yet confirmed), half of which were from the cytosolic fraction and half from the total membranes. Remarkably, all the hyperacetylated proteins in the latter fraction were from mitochondria and most were metabolic enzymes (Table 2). Seven of these mitochondrial proteins were also identified in the proteomic survey for acetylated lysines described above[23] partially confirming our result. Also consistent with this finding is a recent study where purified mitochondria from alcohol-fed rats were immunblotted with anti-acetylated lysine antibodies[13]. Numerous immunoreactive species were observed (but not yet identified) suggesting massive mitochondrial protein hyperacetylation after ethanol exposure. Similarly, cytosolic fractions were highly hyperacetylated after ethanol exposure and the proteins identified varied widely in function, ranging from metabolic enzymes to proteins regulating oxidative stress to molecular chaperones. Efforts are currently underway to confirm the acetylation state of these proteins and to determine the functional consequences of their ethanol-induced hyperacetylation.

MECHANISMS OF ETHANOL-INDUCED LYSINE HYPERACETYLATION

Protein acetylation results from the coordinated activities of acetyltransferases and deacetylases[15,16]. Histones were the first proteins known to be acetylated, and accordingly the modifying enzymes were initially named histone acetyltransferases (HATs) and histone deacetylases (HDACs). Although the list of acetylated proteins has since grown to include numerous nonhistone substrates, their names have remained. In this section, we will briefly describe the two classes of enzymes and whether they are expressed in the liver. We will also discuss what is known about how these HATs and HDACs may be responsible for the alcohol-induced increase in lysine acetylation.

HDACs

To date, there are at least 18 known deacetylases that are categorized into four general classes based on sequence homology and cofactor/coenzyme dependence[28] (Table 3). Classes Ⅰ, Ⅱ and Ⅳ are closely related zinc-dependent enzymes whereas the class Ⅲ HDACs are

Table 3 Deacetylases expressed in the liver

Class	Enzyme	In liver? (methods)	Location	Substrates	References
I	HDAC1	Yes (RT-PCR, IB, IHC, activity)	Nucleus	Histones, p53, retinoblastoma, STAT3, androgen receptor, estrogen receptor, Smad7, other transcription factors	[29,32,38,42,89-92]
	HDAC2	Yes (RT-PCR)	Nucleus	Histones, STAT3, other transcription factors	[29,32,38,42]
	HDAC3	Yes (RT-PCR, IB, activity)	Nucleus	Histones, STAT3, Smad7, other transcription factors	[29,32,38,42,91,93]
	HDAC8	Yes (RT-PCR)	Nucleus Cytoplasm	Histones, transcription factors, smooth muscle actin	[29,32,38,94]
II	HDAC4	Yes/No (RT-PCR)	Nucleus Cytoplasm	Transcription factors	[34,38,42]
	HDAC5	Yes (RT-PCR)	Nucleus Cytoplasm	Transcription factors	[34, 38]
	HDAC6	Yes (RT-PCR, IB, IF)	Cytoplasm	Tubulin, cortactin, HSP90	[34,38-42,56,59]
	HDAC7	No	Nucleus Cytoplasm	HIFα, other transcription factors	[34]
	HDAC9	No	Nucleus Cytoplasm	Transcription factors	[34]
	HDAC10	Yes (RT-PCR)	Nucleus Cytoplasm	Phosphatase pp1	[34,95]
III	SirT1	Yes (RT-PCR, IB)	Nucleus	Histones, PGC-1α, LXR, p53, other transcription factors	[12,14,18,42-44,96-99]
	SirT2	Yes/No (RT-PCR, IB)	Cytoplasm	Histone H4, tubulin	[39,42-46]
	SirT3	Yes (RT-PCR, IB)	Mitochondria	Acetyl CoA synthetase 2, glutamine dehydrogenase, ICDH2	[13,43-45,47,48]
	SirT4	Yes (RT-PCR)	Mitochondria	Glutamine dehydrogenase	[43-45,47,48]
	SirT5	Yes (RT-PCR)	Mitochondria	Cytochrome c	[43-45,47,48,61]
	SirT6	Yes (RT-PCR)	Nucleus	DNA polymerase β	[44,45]
	SirT7	Yes (RT-PCR)	Nucleus	RNA polymerase I	[44,45]
IV	HDAC11	Unknown	Nucleus	Unknown	[29]

IB: Immunoblotting; IHC: Immunohistochemistry; IF: Immunofluorescence; HIFα: Hypoxia-inducible factor α; LXR: Liver x receptor; ICHD2: Isocitrate dehydrogenase.

more distantly related, NAD^+-dependent sirtuins[28,29]. Phylogenetic analysis of bacterial HDAC relatives indicates that the evolution of the class I, II and IV family members preceded the evolution of histones[29] suggesting that these HDACs have nonhistone substrates. This conclusion is consistent with the wide-range of mammalian HDAC substrates that have been identified (Table 3).

Mechanistically, classes I, II and IV all share a conserved catalytic core consisting of open α/β folds and a tubular active site pocket in which a zinc ion sits. The poised zinc mediates the nucleophilic attack of water on the acetylated lysine substrate resulting in the formation of a tetrahedral oxyanion intermediate. The nitrogen on the intermediate is then primed to accept a proton resulting in a charge relay system between histidine, aspartate, and tyrosine that results in the formation of acetate and the deacetylated lysine[29-31]. This catalytic mechanism was deduced with the help of HDAC inhibitors that displace the zinc ion thereby disrupting the charge relay system. Many of these inhibitors are proving to be promising anti-cancer therapies. The most potent inhibitor, trichostatin A (TSA), is a near perfect fit for the HDAC active site and is often used in studies to confirm the affect of protein acetylation on function (see below)[32].

The class I family members include HDACs 1, 2, 3 and 8 that are closely related to the yeast deacetylase, reduced potassium dependency 3 (RPD3)[32] (Table 3). They are all ubiquitously expressed (including in liver, Table 3), are found almost exclusively in the nucleus, are roughly the same length (370-480 aa) and encode one deacetylase domain[29,30,32,33]. These four HDACs have all been shown to deacetylate histones as well as a variety of transcription factors and other nuclear proteins including STATs and Smads[29,30,32,33] (Table 3). Recent reports suggest that class I enzymes may function together in multiprotein complexes that bind and deacetylate transcription factors[29,30]. Future work is needed to understand how the deacetylase activities are specifically coordinated leading to changes in gene expression.

Class II enzymes are much more variable in size (700-1200 aa) and exhibit more tissue-specific expression patterns with only HDACs 4, 5, 6 and 10 identified in the liver (Table 3). Like for class I enzymes, known class II substrates are mainly nuclear transcription factors[28-30,32-34]. However, the class II deacetylases encode both nuclear import and export signals allowing them to shuttle between the nucleus and cytoplasm where they bind to location-specific substrates[30] (Figure 1, bottom panel). Although interactions between these enzymes and their substrates are thought to regulate their activity and location[29,30], little is known how this process is regulated. The class II HDACs catalyze lysine deacetylation in much the same way as the class I enzymes but their active sites encode a glutamate instead of a glycine[29]. This larger side chain decreases active site size restricting substrate access thereby increasing enzyme specificity. Interestingly, HDAC6 and HDAC10 encode two deacetylase domains in tandem[28,34]. Whether the two sites deacetylate different substrates or whether both are needed for function is not yet clear[35-37]. Of particular interest to this review is HDAC6, the only exclusively cytosolic HDAC found in liver[38,39]. One

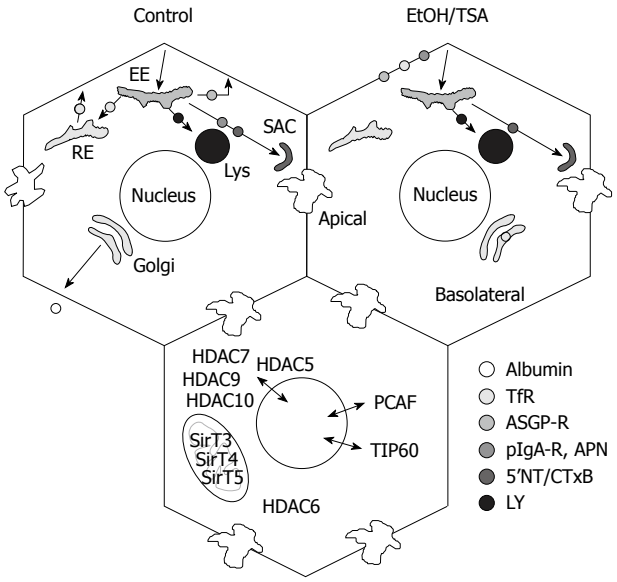

Figure 1 Alcohol-induced defects in protein trafficking can be explained by increased microtubule acetylation and stability. Cargo internalized from the basolateral plasma membrane is delivered to the early endosome (EE) and sorted into at least four different pathways. They are recycled directly back to the plasma membrane (e.g. ASGP-R), delivered to the recycling endosome (RE) before recycling back to the plasma membrane (e.g. Tf-R), delivered to lysosomes (lys) (e.g. LY) or to the apical plasma membrane via the sub-apical compartment (SAC) (e.g. APN, pIgA-R and 5'NT). Albumin secretion is also indicated. In ethanol (EtOH) or TSA treated cells, albumin secretion and the internalization of APN, pIgA-R, Tf-R and ASGP-R is impaired whereas the fluid-phase delivery of LY to lysosomes or the raft/caveolae-mediated internalization of 5'NT and CTxB are not changed. In the lower hepatocyte, the various deacetylases and acetyltransferases that may play a role in the acetylation of nonnuclear proteins are indicated.

of its known substrates is α-tubulin, one of the best characterized, nonnuclear, hyperacetylated proteins in ethanol exposed cells[11,39-42].

Currently there is only one known class IV deacetylase, the recently identified HDAC11. As a result of its high sequence similarity to class I enzymes, HDAC11 is often considered a class I HDAC[30]. At present, little is known about HDAC11's substrates, expression patterns or subcellular distributions. HDAC11 has been shown to act in a complex with other HDACs, most notably HDAC6, suggesting that it may not be an independent lysine deacetylase[29]. Clearly, more work is needed on this class Ⅳ enzyme before we can understand its role in protein acetylation.

Unlike the three classes of zinc-dependent HDACs, class Ⅲ deacetylases are referred to collectively as sirtuins. Sirtuin activity requires NAD^+, and thus each family member encodes a highly conserved, elongated catalytic core containing a Rossman fold characteristic of NAD^+/NADH-binding proteins. Both the NAD^+ and the acetylated lysine substrate bind to the "C pocket" which is located in the cleft between the Rossman fold and a smaller, more variable domain containing a zinc ion. This binding results in a nucleophilic substitution reaction where the NAD^+ and acetylated lysine substrate are converted to nicotinamide, a deacetylated lysine and a novel metabolite, 2'-O-acetyl-adenosine diphosphate ribose[31]. In total, there are seven known sirtuins that all exhibit interesting expression patterns. SirT1 is a widely expressed, well characterized nuclear sirtuin whose long list of substrates includes transcription factors, histones, p53, and PGC-1α[42-44] (Table 3). SirT6 and 7 are two other nuclear sirtuins, and all three have been found in the liver by RT-PCR[44,45]. SirT2 is the lone cytoplasmic sirtuin, and like its counterpart, HDAC6, its main substrate is α-tubulin[46]. Although SirT2 was detected in liver homogenates by RT-PCR, the protein has not been detected at any level in liver lysates or WIF-B cells[39]. SirT3, 4 and 5 are all mitochondrial-specific sirtuins that are ubiquitously expressed (Figure 1, bottom panel). So far, only SirT3 and 5 have confirmed deacetylase activity[47,48]. Interestingly, recent reports suggest that SirT4 (and nuclear SirT6) are instead ADP ribosyltransferases that preferentially ADP-ribosylate acetylated substrates[49,50]. Further studies are needed to examine the effects of ethanol on this activity.

HATs

To date, there are 17 families of HATs that have been grouped according to sequence homology. In general, the N and C terminal domains of the acetyltransferases are structurally and functionally diverse while the catalytic domain is highly conserved. All HAT active sites contain a structurally conserved loop-β strand core domain that binds acetyl Co-A[51]. Also within this core domain is a glutamate that serves as a lysine docking site. The glutamate removes a proton from the docked lysine allowing acetyl transfer from acetyl CoA[51]. From the 17 families, three major groups have emerged: Gcn5/PCAF, p300/CBP and MYST (Table 4). Little is known about the mammalian members of the Gcn5/PCAF family, but in general these HATs have been detected in liver, reside in the nucleus and have mainly nuclear substrates. Only PCAF has been found to distribute to both the nucleus and cytoplasm[52-55] allowing for more varied substrates including histones, p53, and the actin binding protein, cortactin[56-58]. The p300/CBP family consists of p300 and CBP that are nearly structurally identical and are referred to interchangeably[57,58]. They also reside in the nucleus and are expressed in the liver. The least characterized MYST family consists of the mammalian enzymes, TIP60, HBO, MOZ, and MORF[57,58]. Although each of these HATs have been shown to acetylate histones H3 and H4, much still needs to be learned about other possible substrates and whether they function alone or in large multi-HAT complexes.

General roles for HATs and HDACs in ethanol-induced protein acetylation

Changes in both HAT and HDAC expression have been correlated with global changes in protein acetylation. Thus, a simple explanation for ethanol-induced protein acetylation may be altered enzyme levels. The prediction is that either a decrease in HDAC expression or an increase in HAT levels will lead to increased protein acetylation. In addition, changes in enzyme activity or subcellular distribution may lead to hyperacetylation. For example, either a decrease in HDAC activity or a

Table 4 Histone acetyltransferases expressed in the liver

Family	HAT	In liver? (methods)	Location	Substrates	References
Gcn5/PCAF	PCAF	Yes (IB)	Nucleus Cytoplasm	Histones, p53, PGC-1a, other transcription factors, cortactin	[52-58]
p300/CBP	CBP	Yes (IB)	Nucleus	Histones, E1A, p53, SRC-1, TIF2, ACTR, SREBP, other transcription factors	[54, 57, 58]
	p300	Yes (IB)	Nucleus	Histones, E1A, p53, SRC-1,TIF2, TAT, ACTR, SREBP, other transcription factors	[18, 54, 55, 57, 58]
MYST	TIP60	Yes (IB)	Nucleus Cytoplasm	Histones H3 and H4, androgen receptor	[54, 57]
	HBO	Unknown	Nucleus	Histones H3 and H4	[57, 58]
	MOZ	No	Nucleus	Histones H3 and H4	[100]
	MORF	Yes (RT-PCR)	Nucleus	Histones H3 and H4	[101, 102]

E1A: Adenovirus early region 1A; SRC-1: Steroid receptor coactivator; TIF2: Transcriptional intermediary factor 2.

loss of nuclear import could enhance nuclear protein acetylation. Additionally, the sirtuin deacetylases are NAD^+-dependent enzymes. Because ethanol metabolism is NAD^+-depleting, sirtuin activity may be impaired thereby leading to increased protein acetylation. Below, we summarize what is known about how alterations in HAT or HDAC activity/function contribute to ethanol-induced protein hyperacetylation.

Histone H3 hyperacetylation and p300

At present, the mechanisms responsible for ethanol-induced hyperacetylation of histone H3 are not known, but recent studies have provided some clues. In livers of ethanol-fed rats, p300 protein levels were increased about 3 fold, which correlated with increases in overall nuclear HAT activity[17,18,21]. Acetate treatment also led to enhanced nuclear HAT activity and histone H3 hyperacetylation indicating ethanol metabolism is required for this modification[17]. Interestingly, the protein expression of the closely related p300 homologue, CBP, was not altered[18] indicating that ethanol-induced histone H3 acetylation may be specific to p300. These studies further suggest that these two HATs may not be completely interchangeable. To date, it is not known which (if any) HDAC is involved in histone H3 hyperacetylation in ethanol-treated cells. However, total nuclear HDAC activity was significantly decreased in ethanol-treated WIF-B cells, and that decreased activity correlated with increased histone H3 acetylation[39]. In contrast, total nuclear HDAC activity was not altered in ethanol-treated isolated hepatocytes where enhanced histone H3 acetylation was observed[17,21]. The reasons for these disparate results are not known but may be explained by differences in the cell systems used, acute vs. chronic alcohol-treatment, or differences in the deacetylase assay used. Further research is needed to elucidate this further.

SREBP-1c hyperacetylation and SirT1 and p300

As for histone H3, the mechanisms responsible for ethanol-induced hyperacetylation of SREBP-1c are not known, but work has provided some hints. From studies performed in H4IIEC3 cells, overexpressing SirT1 and p300 were found to deacetylate or acetylate SREBP-1c, respectively, confirming that SREBP-1c is a substrate for both enzymes[12]. Increasing ethanol concentrations enhanced p300-mediated SREBP acetylation in these cells while a concomitant decrease in SirT1 protein levels was observed[12,14]. Thus, SREBP-1c hyperacetylation was the result of changes in both types of modifying enzymes. As for SREBP-1c, PGC-1α hyperacetylation was also correlated with decreased SirT1 protein expression[12,14]. At present, it is not known if changes in SirT1 or p300 activity levels correlate with changes in protein levels or whether SREBP-1c and PGC-1α hyperacetylation requires ethanol metabolism. Future work is clearly needed to understand this fully.

Tubulin hyperacetylation and HDAC6

Since the specific microtubule acetyltransferase has not yet been identified, studies thus far have focused on the known hepatic tubulin deacetylase, HDAC6 (Table 3). Although ethanol treatment did not alter HDAC6 subcellular distributions in polarized WIF-B cells, it led to a 25% decrease in HDAC6 protein levels[39]. HDAC6 binding to endogenous microtubules was also found to be significantly impaired by about 70% in ethanol-treated cells and this impairment partially required ethanol metabolism. Measuring HDAC6 tubulin deacetylase activity by two methods further revealed that ethanol did not impair HDAC6's ability to bind or deacetylate exogenous tubulin. This suggests that tubulin from ethanol-treated cells was modified, thereby preventing HDAC6 binding[39].

Although decreased HDAC6 protein levels are a simple explanation for increased tubulin acetylation, it is likely that the impaired microtubule binding has more impact. HDAC6 is abundant in the liver[56,59] such that a 25% decrease in levels may not likely have profound effects on tubulin acetylation. Rather, the 70% impairment in HDAC6 binding to microtubules may have a more dramatic effect; much less of the available enzyme can bind its substrate leading to decreased deacetylation. At present, the nature of the alcohol-induced tubulin modification is not known, but there are some interesting possibilities. Both impaired HDAC6 binding to microtubules and alcohol-induced tubulin hyperacetylation require ethanol metabolism,

and from studies using 4-MP, both events are likely mediated by acetaldehyde[11,39]. Because this highly reactive ethanol metabolite can readily and covalently modify a highly reactive lysine in α-tubulin *in vitro*[60], one provocative possibility is that tubulin acetaldehyde adducts impede HDAC6 binding. Because decreased HDAC6 binding to microtubules was only partially prevented by 4-MP, it is also possible that other reactive ethanol metabolites form detrimental tubulin adducts. Future studies are needed to elucidate this and other details.

Mitochondrial protein hyperacetylation and sirtuins

The ever-expanding list of ethanol-induced hyperacetylated mitochondrial proteins is likely to generate much interest in the mitochondrial-specific sirtuins, but so far, only protein levels of SirT3 and 5 have been examined. Although SirT3 is considered the predominant mitochondrial deacetylase, its expression levels were not changed in livers from ethanol-fed rats[13]. In contrast, SirT5 protein levels were significantly decreased[61]. To date, nothing is known about the subcellular distributions, activities or substrate specificities of these deacetylases in ethanol-treated cells. Furthermore, virtually nothing is known about the HATs required for mitochondrial protein acetylation. Clearly, this is a fertile area of investigation for many researchers.

Cytosolic protein hyperacetylation and nonnuclear HATs and HDACs

Recent proteomics studies have revealed that many cytosolic proteins are hyperacetylated upon ethanol exposure (Table 2). At present, very little is known about the HATs or HDACs responsible for these modifications. However, attention is being turned to those modifying enzymes that are exclusively cytosolic (HDAC6) or those that shuttle between the nucleus and cytoplasm (PCAF, TIP60 and HDACs 5, 7, 9 and 10) (Figure 1, bottom panel). Not only will future studies likely provide a better understanding of alcohol-induced protein acetylation and the progression of hepatotoxicity, they will also undoubtedly identify new substrates for these enzymes. As for studies on the mitochondrial modifying enzymes, this area of research promises to be fruitful.

CONSEQUENCES OF ETHANOL-INDUCED LYSINE HYPERACETYLATION

Although there is an ever-expanding list of proteins that are known to be hyperacetylated upon ethanol exposure, little is known about the functional consequences of this modification. In general, the added acetyl group likely neutralizes the positive charge on lysine while increasing the overall size and hydrophobicity of the side chain. Such changes may result in protein conformational changes that alter function, albeit to a much lesser extent than the addition of a large, highly charged phosphoryl group. Also, lysine acetylation sites have been identified that overlap with nuclear localization signals[23] such that the modification may induce altered protein subcellular distributions. Not only can lysine residues be acetylated, they can also be methylated, sumoylated and ubiquitinylated such that ethanol-induced hyperacetylation may displace other modifications further altering protein function. In fact, p300 acetylation has been shown to prevent its sumoylation thereby repressing its activity[62]. Clearly, mechanistic studies are required to not only understand the functional consequences of acetylation in the normal liver, but also how alcohol-induced hyperacetylation alters hepatic function in the alcoholic liver. In this section, we will describe some recent advances in our understanding of how ethanol-induced acetylation impairs hepatic function. Although it is too early for specific mechanistic details, the results provide an exciting framework for continued investigation.

Histone H3 hyperacetylation alters transcriptional regulation

Alcohol consumption has long been known to lead to changes in gene expression. Many genes are up-regulated including those encoding for enzymes involved in alcohol metabolism, lipogenesis and the regulation of oxidative stress[63,64]. Many genes are also down-regulated, but fewer seem to functionally group together[63,64]. The specific mechanisms responsible for changes in alcohol-induced gene expression are not well defined. Recent studies have been aimed at understanding the role of histone modifications in transcriptional regulation. The amino-termini of histones are characterized by at least six different modifications (acetylation, ubiquitinylation, methylation, phosphorylation, sumoylation and ADP-ribosylation) occurring on lysines, arginines, serines, threonines and histidines[65]. In general, these reversible modifications are thought to change the net negative charge of the amino-terminal domain leading to altered DNA binding and changes in gene expression[66]. Thus, the simple hypothesis is that alcohol-induced lysine 9 hyperacetylation will decrease the amino-terminal net negative charge thereby loosening histone H3 associations with DNA. The relaxed DNA becomes more accessible to the transcriptional machinery, leading to enhanced transcription.

This prediction was tested in ethanol-treated hepatocytes using a series of chromatin immunoprecipitations. In general, histone H3 with acetylated lysine 9 residues was found to be more highly associated with the promoters of genes known to be up-regulated by ethanol exposure (ADH and glutathione S-transferase)[9], consistent with this hypothesis. Also consistent with this hypothesis is that histone H3 with methylated lysine 9 residues (this modification is associated with gene silencing) was more highly associated with promoters of genes known to be down-regulated by ethanol (L-serine dehydratase and CYP 2C11)[9]. Somewhat surprisingly, histone H3 containing methylated lysine 4 residues was found to be highly associated with ADH and glutathione

S-transferase promoters, not with promoters of down-regulated genes[9]. These associations suggest that lysine 4 hyperacetylation and lysine 4 methylation enhance gene expression while lysine 9 methylation represses transcription.

At present, the site-specific differences in promoter associations cannot be fully explained. One implication is that promoter regions are characterized by specific microenvironments that are differentially accessible to histone modifying enzymes and by extension, are differentially affected by ethanol exposure. In order to fully understand these site-specific differences, it will be necessary to fully account for all six possible histone H3 modifications and the modifying enzymes in control and ethanol-treated cells. Also, to determine how each modification or combination of modifications regulates gene expression, assays that directly monitor transcription activation (rather than promoter associations) will need to be developed. Furthermore, recent reports demonstrate that histone hyperacetylation may also lead to nucleosome instability allowing transcription at cryptic promoters resulting in aberrant gene product expression[67,68]. This emerging hypothesis must be considered as the transcriptional consequences of ethanol-induced histone acetylation as elucidated.

Ethanol-induced acetylation of SREBP-1 and PGC-1α leads to altered lipid metabolism

One of the first clinical manifestations of alcohol consumption is the appearance of a fatty liver (steatosis). This is correlated with the up-regulation of many lipogenic enzymes that leads to the alcohol-induced synthesis of hepatic triglycerides and phospholipids[69]. An active area of steatosis research is aimed at identifying members of the transcriptional machinery that regulate gene expression of the enzymes involved in lipid metabolism. It has long been appreciated that SREBP-1 promotes the expression of many genes involved in lipogenesis that are up-regulated after alcohol consumption[69]. However, the exact mechanism by which SREBP-1 leads to enhanced expression is not well-defined. One emerging hypothesis is that SREBP-1 acetylation plays an important role. SREBP-1 is known to be acetylated in its DNA binding domain, and that when acetylated, DNA binding is enhanced. SREBP-1 is also known to be ubiquitinylated on the same lysine residues leading to its proteosomal degradation[70]. Thus, in alcohol-treated hepatocytes the prediction is that hyperacetylation prevents SREBP-1 proteosomal degradation by displacing the ubiquitin while enhancing its DNA binding which leads to increased transcription of lipogenic enzymes.

A similar scenario is emerging for the transcriptional activator, PGC-1α, but it is the deacetylated protein that up-regulates expression of genes regulating fatty acid β-oxidation[22]. Therefore in ethanol-treated hepatocytes, the prediction is that the hyperacetylated PGC-1α will be inactive. Based on this prediction and the one for SREBP-1, a straightforward scenario emerges. Alcohol-induced SREBP-1 hyperacetylation enhances lipid synthesis by activating lipogenic enzyme transcription while PGC-1α hyperacetylation impairs lipid catabolism by inhibiting transcription of enzymes involved in fatty acid oxidation. The altered transcriptional activation in either case leads to hepatic fatty acid accumulation that likely contributes to development of steatosis. Because many other components of the transcriptional machinery are known to be acetylated (Tables 3 and 4), it is likely that alcohol-induced hyperacetylation will have far- reaching effects on hepatic gene expression.

Ethanol-induced microtubule acetylation leads to impaired protein trafficking

Because microtubules are central to multiple cellular processes, changes in their dynamics will likely alter hepatic function. An active area of research has been aimed at understanding the relationship between protein trafficking and alterations in microtubule dynamics. Not only is protein trafficking microtubule-dependent, the trafficking of many hepatic proteins is also impaired by ethanol[71-74]. Two transport pathways appear to be affected: transport of newly-synthesized secretory or membrane proteins from the Golgi to the basolateral membrane and receptor-mediated endocytosis from the sinusoidal surface (Figure 1). One attractive hypothesis is that the alcohol-induced defects in secretion and endocytosis can be explained by increased microtubule acetylation and stability.

To test this hypothesis, recent studies have examined the trafficking of selected proteins in WIF-B cells treated with ethanol or TSA, a potent inhibitor of HDAC6, the major tubulin deacetylase in liver and WIF-B cells[39] (Table 3). Importantly, TSA induces increased microtubule acetylation and stability to the same extent as ethanol[75]. As shown previously *in situ*, the endocytic trafficking of asialoglycoprotein-receptor (ASGP-R) was impaired in ethanol-treated WIF-B cells[75] (Figure 1). This impairment required ethanol metabolism and was likely mediated by acetaldehyde[75]. TSA also impaired ASGP-R endocytic trafficking, but to a lesser extent. Similarly, both ethanol and TSA impaired transcytosis of a single spanning apical resident, aminopeptidase (APN). For both ASGP-R and APN, and for both treatments, the block in trafficking was internalization from the basolateral membrane. Interestingly, no changes in transcytosis of the GPI-anchored protein, 5'nucleotidase (5'NT) (Figure 1), were observed suggesting that increased microtubule acetylation and stability differentially regulate internalization. It was further determined that albumin secretion was impaired in both ethanol- and TSA-treated cells[75] indicating that increased microtubule acetylation and stability also disrupt this transport step. Thus, increased microtubule acetylation and stability explain, in part, the alcohol-induced defects in membrane trafficking.

There is evidence that suggests that different microtubule populations (and/or their modifications) support specific protein transport steps[76]. Of particular interest are studies performed in WIF-B cells that used a novel microtubule depolymerizing drug, 201-F[77]. This

drug specifically depolymerizes dynamic microtubules leaving only stable, acetylated polymers behind. In 201-F-treated cells, both secretion and transcytosis were impaired[77]. Although the specific impaired transcytotic step was not identified, increased basolateral labeling of the apical proteins was observed. These results are remarkably consistent with the findings in ethanol or TSA treated cells where increased populations of stable microtubules were observed (presumably at the expense of dynamic microtubules) that correlated with impaired albumin secretion and basolateral internalization.

An unanswered question from these studies is why 5'NT distributions were not altered in treated cells. Furthermore, the internalization of cholera toxin B subunit (CTxB) (a known raft marker) was also not impaired by ethanol exposure (Figure 1). One possibility is that internalization mechanisms were differentially impaired by ethanol metabolism. There are at least three major internalization routes in mammalian cells: clathrin-mediated, caveolae/raft-mediated and non-clathrin/non-raft mediated[78] that are characterized by specific molecular players, cargoes and regulators. In general, the receptors that displayed impaired endocytosis in ethanol-treated hepatocytes *in situ* (ASGP-R, EGF-R, and to a lesser extent, insulin *via* its receptor)[71-73,79,80] and in WIF-B cells (ASGP-R, transferrin receptor (Tf-R) and polymeric IgA receptor (pIgA-R)[75] (Figure 1) are internalized *via* clathrin-mediated pathways. Interestingly, the non-clathrin/non-raft-mediated fluid phase uptake of Lucifer Yellow was not changed in livers of ethanol-fed rats or WIF-B cells suggesting this pathway is not affected by ethanol metabolism[81] (Figure 1). Thus, we propose that the molecular machinery that drives clathrin-mediated endocytosis is more prone to adduction (by acetaldehyde or other reactive metabolites) or covalent modification such that it is selectively impaired by alcohol treatment.

Another unanswered question from these studies is how the acetylation of lysine 40 specifically contributes to enhanced microtubule stability and trafficking defects. Although lysine 40 is thought to reside in the lumen of the microtubule[82], it is possible that its acetylation may lead to altered tubulin conformation such that interactions with microtubule associated proteins and motors are altered. This hypothesis is supported by the findings that kinesin, dynein and dynactin preferentially bound acetylated microtubules in neuronal cells[83-85]. This is further supported by the finding that vesicles recovered from livers of ethanol-fed rats have decreased motility *in vitro*[86]. Clearly, further studies are needed to fully understand these results.

Mitochondrial dysfunction and protein hyperacetylation

Despite the large number of mitochondrial proteins known to be acetylated, little is known about the functional consequences of the modification on mitochondrial function. So far, only glutamate dehydrogenase and AceCS2 activities have been related to their acetylation states, and in both cases, increased acetylation correlated with decreased activity[25]. Thus, the prediction is that in ethanol-treated hepatocytes, the hyperacetylated enzymes would be inactivated leading to altered lipid metabolism that contributes to the development of steatosis as described above. Also, as described above, acetylation may function as an on/off switch for these and other mitochondrial metabolic enzymes such that alcohol-induced changes in this modification may have a large impact on hepatic metabolism.

However, recent results from knockout mice are not consistent with this conclusion[48]. Striking levels of mitochondrial protein acetylation were observed in livers from SirT3 knockout mice whereas mitochondrial acetylation in SirT4 or SirT5 knockout mice was not changed. This suggests that SirT3 is the predominant mitochondrial deacetylase. Despite the high levels of mitochondrial protein acetylation, there was no discernible phenotype in SirT3 knockout mice. Specifically in liver, there was no change in morphology, no change in mitochondrial numbers and metabolism was not altered suggesting hyperacetylation does not affect mitochondrial function[48]. However, it is possible that hyperacetylation is only detrimental under stressed conditions such that in the alcoholic liver, it leads to mitochondrial dysfunction.

This conclusion is consistent with a recent hypothesis that mitochondrial protein acetylation functions as a sensor for the overall cellular energy status[23]. Kim *et al*[23] suggest that acetyl-CoA and NAD^+ levels are the key indicators of energy status. Coincidentally, these two molecules serve as cofactors for HATs (acetyl-CoA) or sirtuins (NAD^+). Furthermore, over 44% of mitochondrial dehydrogenases that require NAD^+ for activity are known to be acetylated. Thus, one possibility is that lysine acetylation serves as a feedback mechanism for the regulation of dehydrogenases. For example, under NAD^+-depleting conditions, sirtuins are less active resulting in higher protein acetylation and dehydrogenase activities. In contrast, when acetyl-CoA levels are limiting, HATs are inactivated leading to decreased protein acetylation and increased dehydrogenase activities. Thus, in the alcoholic liver where NAD^+ is depleting, increased acetylation is predicted to correlate with impaired dehydrogenase activity and by extension, impaired mitochondrial function. Although it has been suggested that NAD^+ levels recover after prolonged ethanol exposure, the finding that hyperacetylation remains long after chronic ethanol withdrawal[13] suggests that this mechanism may have physiologic relevance. Clearly, this exciting hypothesis needs to be rigorously tested.

CONCLUSION

Chronic alcohol consumption leads to the hyperacetylation of numerous hepatic nuclear and nonnuclear proteins. Although many interesting and provocative mechanisms have been proposed that describe how hyperacetylation contributes to alcohol-induced hepatotoxicity, future work is clearly needed to test these hypotheses. New therapeutic strategies for

treating patients with chronic liver disease may be aimed at reducing protein acetylation. Currently, specific SirT1 activators (e.g. resveratrol and SRT-501) are known to be well-tolerated in humans and are in clinical trials for treatment of various metabolic diseases including type 2 diabetes[87]. Furthermore, resveratrol has been shown to attenuate fatty liver in alcohol-exposed mice[88]. An exciting possibility is that this drug and other specific deacetylase activators or acetyltransferase inhibitors will be useful in treating alcoholic liver disease.

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Role of cathepsin B-mediated apoptosis in fulminant hepatic failure in mice

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Abstract

AIM: To investigate the pathogenic role of cathepsin B and the protective effect of a cathepsin B inhibitor (CA-074Me) in fulminant hepatic failure in mice.

METHODS: LPS/D-Gal N was injected into mice of the model group to induce fulminant hepatic failure; the protected group was administered CA-074me for 30 min before LPS/D-Gal N treatment; the normal group was given isochoric physiologic saline. Liver tissue histopathology was determined with HE at 2, 4, 6 and 8 h after Lps/D-Gal injection. Hepatocyte apoptosis was examined by TUNEL method. The expression of cathepsin B in liver tissues was investigated by immunohistochemistry, Western blot and RT-PCR.

RESULTS: Compared with the normal group, massive typical hepatocyte apoptosis occurred in the model group; the number of apoptotic cells reached a maximum 6 h after injection. The apoptosis index (AI) in the protected group was clearly reduced (30.4 ± 2.8 *vs* 18.1 ± 2.0, $P < 0.01$). Cathepsin B activity was markedly increased in drug-treated mice compared with the normal group ($P < 0.01$). Incubation with LPS/D-Gal N at selected time points resulted in a time-dependent increase in cathepsin B activity, and reached a maximum by 8 h. The expression of cathepsin B was significantly decreased in the protected group ($P < 0.01$).

CONCLUSION: Cathepsin B plays an essential role in the pathogenesis of fulminant hepatic failure, and the cathepsin B inhibitor CA-074me can attenuate apoptosis and liver injury.

Key words: Fulminant hepatic failure; Hepatocyte apoptosis; Cathepsin B; CA-074me

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INTRODUCTION

Fulminant hepatic failure is a rare, but severe, complication of acute hepatitis; it is associated with very high mortality. It has been reported that fulminant hepatic failure is an inflammatory process that causes the death of liver cells by necrosis or by triggering apoptosis[1-3]. Cathepsins are a family of proteolytic enzymes, many of which, including cathepsin B, are cysteine proteinases. Recent evidence suggests that cathepsin B contributes to cell apoptosis[4,5]. It is not known if cathepsin B-mediated hepatocyte apoptosis is involved in the pathogenesis of fulminant hepatic failure. The aim of the present study was to determine if fulminant hepatic failure contributes to a change in the expression of cathepsin B protein and mRNA. To ascertain its pathogenic role in hepatic failure, we examined the protective effect of a cathepsin B inhibitor, CA-074Me [N-(L-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline] on fulminant hepatic failure in mice.

MATERIALS AND METHODS

Animals

Kunming mice (male, 18-20 g, 4 wk of age) were used. Animals were provided by the Animal Center of the First Clinical Hospital of Harbin Medical University. This study conformed to Harbin Medical University's guidelines for the care and use of laboratory animals.

Experimental groups

Seventy-two mice were randomized to three groups (normal control, model and protected). Galactosamine (D-Gal N) 800 mg/kg and lipopolysaccharide (LPS) 100 μg/kg were injected into the abdominal cavity of mice of the model group; mice in the protected group were administered CA-074me (10 mg/kg) for 30 min before LPS/D-Gal N treatment; and the normal control group was given isochoric physiologic saline. Six mice in each group were killed 2, 4, 6 and 8 h after injection. The liver was cut into small pieces and snap-frozen in liquid nitrogen and stored at -70℃, or fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4℃.

Materials

Goat anti-mouse cathepsin B polyclonal IgG and P-conjugated secondary antibodies for immunoblotting were purchased from Santa Cruz Biotechnology Inc., USA; D-Gal N, LPS and CA-074me from Sigma (USA); TUNEL reagent kit from Zhong Shan Biotechnical Ltd (Beijing, China); immunohistochemical kit from Zymed (series SP kits); ABC reagent and DAB kits from Wuhan Boster Biological Technology Co, Ltd (Wuhan, China); Trizol kits from Invitrogen (USA); and the reverse transcription-polymerase chain reaction (RT-PCR) kit from Promega (USA). Primers were synthesized by Shanghai Sangon Biological Engineering Technology Co, Ltd (Shanghai, China).

Histology and TUNEL assay

The liver was fixed in 4% paraformaldehyde for 48 h, and embedded in paraffin. Tissue sections were prepared with a microtome and placed on glass slides. Hematoxylin and eosin staining was done by standard methods. TUNEL assay was carried out with a commercially available kit according to the manufacturer's instructions. Hepatocyte apoptosis in liver sections was quantified by counting the number of TUNEL-positive cells in microscopic high-power fields.

Immunohistochemistry

Sections were incubated with goat anti-mice cathepsin B, which was pre-diluted by the manufacturer for staining formalin-fixed paraffin-embedded tissues. After washing the sections exhaustively, they were incubated for 45 min with biotin-conjugated anti-goat IgG antibody, and then with horseradish peroxidase (HRP)-conjugated streptavidin. Negative control slides were incubated with non-immune immunoglobulin under identical conditions. Liver cell endochylema or nucleus containing yellow granulation served as a positive control, followed by semi-quantitative analysis using Image-plus 6.0 software.

RT-PCR

Total RNA was obtained from whole liver using trizol reagent. The RNA sample was reversely transcribed into cDNA according to manufacturer's instructions. The primers for the experiment were as follows: cathepsin B[6], forward 5'-GAAGAAGCTGTGTGGCACTG-3', and reverse 5'-GTTCGGTCAGAAATGGCTTC-3' (yielding a 198-bp product); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-CTGCACCACCAACTGCTTAG-3', and reverse 5'-GTCTGGGATGGAAATT GTGA-3' (660-bp). GAPDH was used as a control for RNA integrity. Thermal cycling conditions were 15 seconds at 96℃, 62℃ for 20 s, and 1 min at 70℃. Amplification was stopped after 34 cycles. Ten microliters of PCR products were obtained in each group, and confirmed by gel electrophoresis (coloration by EB, Ethidium Bromide). Gel electrophoresis photographs were taken; the band of Cathepsin B PCR products on electrophoresis gel were quantified using a DNA sequencer equipped with Quantity-One analysis software.

Western blotting

Samples were centrifuged at 10 000 × *g* for 20 min at 4℃ to remove solid material. Supernatants were centrifuged at 100 000 × *g* for 1 h at 4℃. Cellular protein from each sample (50 μg) was mixed with sample buffer (0.25 mol/L Tris, pH 6.8, 8% SDS, 40% glycerol, 2.5% bromphenol blue, and 2% β-mercaptoethanol), heated for 3 min at 95℃, applied to a 12% acrylamide gel, separated by electrophoresis (SDS-polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h. Blots were incubated in cathepsin B polyclonal antibody at a dilution of 1: 200 for 2 h. After washed three times for 10 min with TBST, blots were incubated with HRP-conjugated anti-goat secondary antibody (1:5000) for 1 h. Following the secondary antibody incubations, blots were developed using an ECL-plus kit. Blots were visualized using the chemiluminescence detection system (Amersham Pharmaceuticals, Amersham, UK).

Statistical analyses

Data represent at least four independent experiments, and were expressed as the mean ± SD (unless otherwise indicated). The difference was determined using two-way analyses of variance (ANOVA) following SNK. Data were analyzed by SPSS software. $P < 0.05$ was considered to be statistically significant.

RESULTS

Histopathology of liver tissue under light microscopy

Liver histopathology was used to assess if different

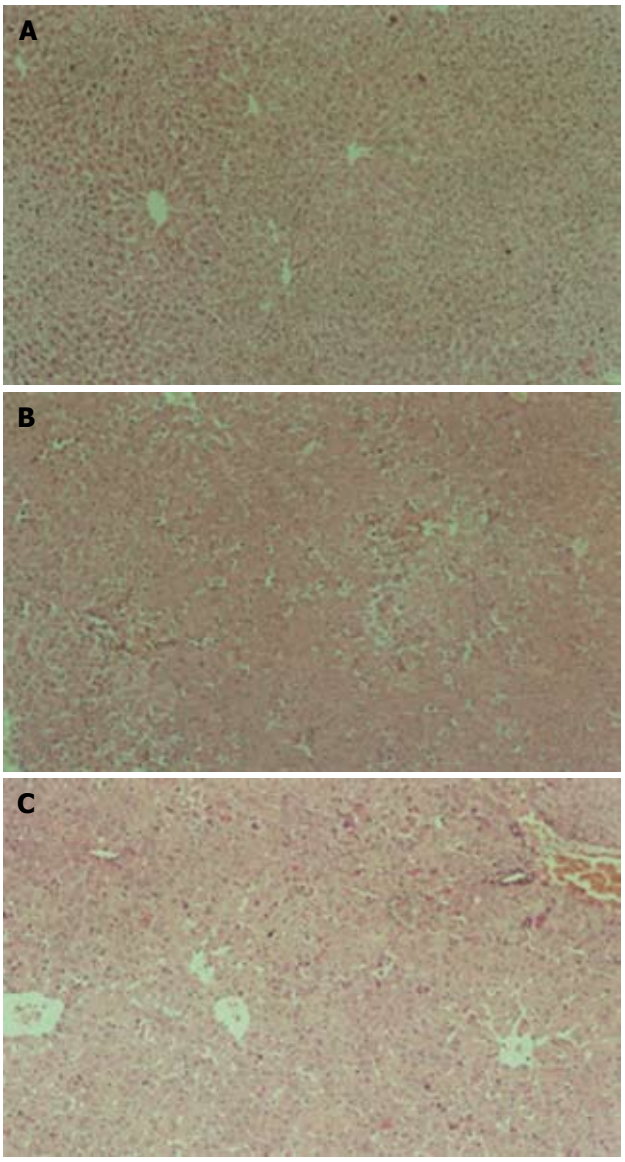

Figure 1 Histopathology of liver tissue at 6 h (HE, × 10). A: Normal group; B: Model group; C: Protected group.

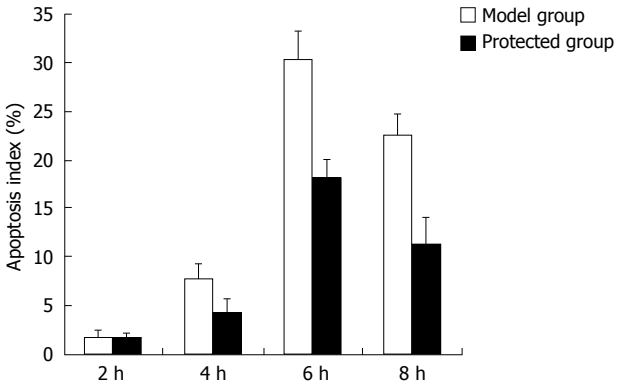

Figure 2 Apoptosis in the model group and protected group measured by TUNEL assay.

groups had different effects on liver injury (Figure 1). Liver histology was normal in the control group. After treatment with D-Gal N and LPS for 6 h, massive

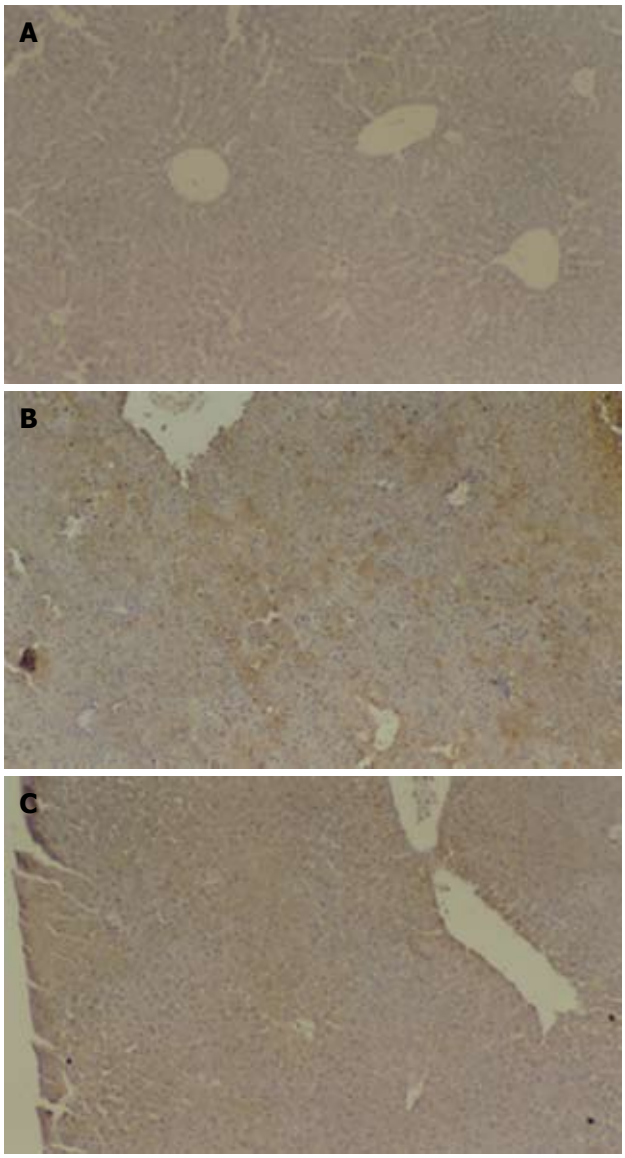

Figure 3 Immunohistochemical analysis of cathepsin B at 8 h (HE, × 10). A: Normal group; B: Model group; C: Protected group.

hepatocyte apoptosis was detected in the model group; 8 h after injection, hepatocyte necrosis with massive infiltrates of neutrophils was widely spread. The protected group had far less apoptosis and necrosis, and little evidence of neutrophil accumulation, especially 6 and 8 h after injection.

TUNEL assay

Hepatocyte apoptosis was quantified using TUNEL assay (see Materials and Methods). None or few TUNEL-positive cells were observed in the normal group. In the model group, massive hepatocyte apoptosis occurred, and the number of apoptotic cells increased with time (Figure 2). The apoptosis index (AI) 2 h after injection of D-Gal N/LPS was (1.8 ± 0.7)%, (7.8 ± 1.5)% at 4 h, and reached a climax at 6 h (30.4 ± 2.8)%. After 8-h treatment, AI was lower than that of 6 h, and reached (22.6 ± 2.2)% at 8 h. Compared with the model group, the apoptotic cells in the protected group were

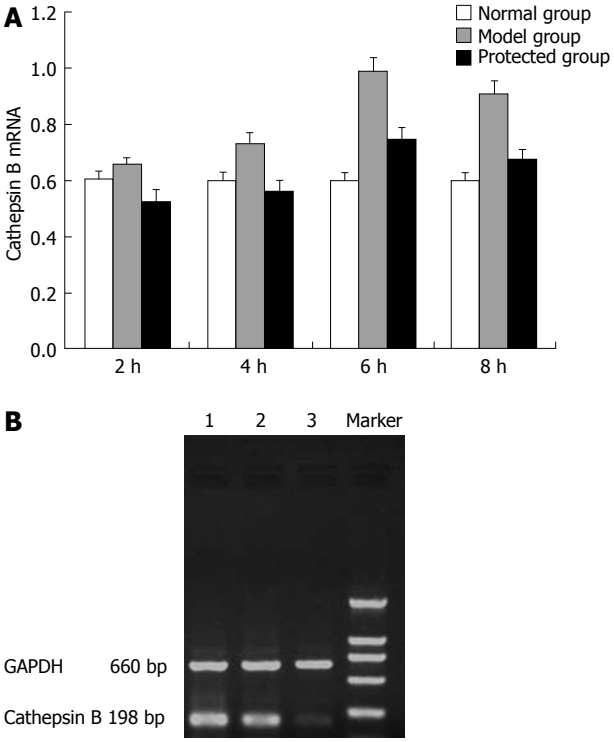

Figure 4 RT-PCR analysis of expression of cathepsin B mRNA in the normal, model and protected groups. A: Expression of cathepsin B mRNA in different groups; B: Images of agarose gel electrophoresis at 6 h; 1: Model group; 2: Protected group; 3: Normal group.

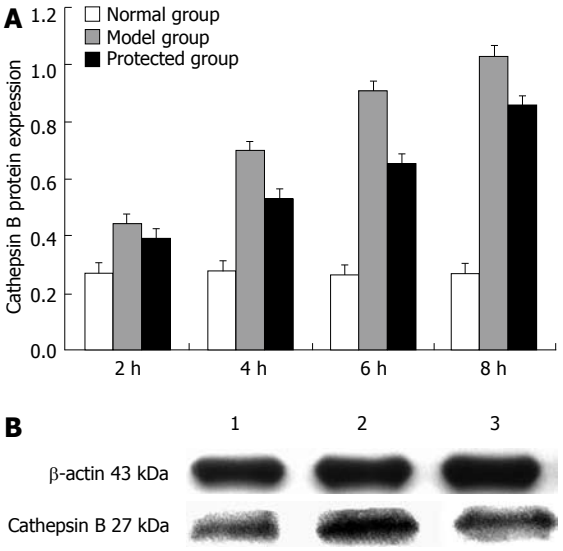

Figure 5 Western blot analysis of expression of cathepsin B protein in the normal, model and protected groups. A: Expression of cathepsin B Protein in different groups; B: Expression of cathepsin B protein at 8 h; 1: Normal group; 2: Model group; 3: Protected group.

obviously reduced at the same time points, particularly at 6 h (18.1 ± 2.0)% and 8 h (11.4 ± 2.6)% ($P < 0.01$).

Immunohistochemical analysis of cathepsin B

Immunohistochemistry for cathepsin B was done to confirm apoptosis (Figure 3). Strongly positive immunoreactivity for active cathepsin B was detected in mice injected with LPS/D-Gal N compared with the normal group. After 6-h and 8-h treatment with LPS/D-Gal N, more expression of cathepsin B was found (0.251 ± 0.010 and 0.280 ± 0.011, respectively). At the same time points, cathepsin B was found to be significantly less expressed in mice of the protected group (0.202 ± 0.008 and 0.241 ± 0.011, respectively, $P < 0.01$).

Expression of cathepsin B mRNA

Semi-quantitative RT-PCR was used to assess the expression of cathepsin B mRNA. Using densitometric analysis in comparison with the housekeeping gene GADPH, we found that the expression of cathepsin B mRNA significantly increased after treatment of LPS/D-Gal N compared with the normal group ($P < 0.01$). The expression of cathepsin B mRNA gradually increased 6 h and 8 h after injection with LPS/D-Gal N and reached a climax at 6 h. At the same time points, expression of cathepsin B mRNA in the protected group was significantly reduced compared with the model group, particularly at 6 h and 8 h (Figure 4).

Western blot analysis

Western blot analysis of cathepsin B protein expression is shown in Figure 5. A prominent band at a molecular size of about 27 kDa was detected, which represented the single-chain form of cathepsin B. Incubation with LPS/D-Gal N at selected time points resulted in a time-dependent increase in cathepsin B protein. The cathepsin B protein began to increase 2 h after treatment with LPS/D-Gal N and increased to maximum activity at 8 h compared with the normal group ($P < 0.01$). Expression of cathepsin B was significantly decreased in the protected group, particularly at 8 h.

DISCUSSION

The traditional view is that hepatocyte necrosis is the main feature of fulminant hepatic failure, but increasing evidence implicates a dominant role for hepatocyte apoptosis in this pathogenesis[1,7]. The major objective of our study was to evaluate apoptosis-mediated fulminant hepatic failure in LPS /D-Gal N-induced liver injury.

Cathepsin B, a lysosomal cysteine protease, is a candidate for an apoptotic mediator originating from acidic vesicles. Cathepsin B is synthesized as a 38-kDa procathepsin B that undergoes sequential processing steps within lysosomes. First, a 30-kDa mature active form is generated by proteolytic cleavage[8]. Further processing involves removal of the NH2-terminal propeptide, cleavage of six residues from the COOH-terminus, and internal excision of residues 127 and 128 to generate a two-chain (a 27-kDa heavy chain and a 4-5-kDa light chain) form of the enzyme with inter-chain disulfide bonds. Pharmacologic inhibition of cathepsin B has been reported to block apoptosis induced by p53 and cytotoxic agents[9]. Recent evidence suggests that cathepsin B contributes to tumor necrosis factor- (TNF-α)-induced apoptosis *in vitro* and *in vivo*[10,11]. In cell culture systems, activation of caspase 8

is associated with the release of cathepsin B from acidic vesicles into the cytosol. In the cytosol, cathepsin B was found to induce mitochondrial release of cytochrome C[12,13] and activate caspase 9 and 3[14]. The importance of this pathway in TNF-α-mediated apoptosis *in vitro* was shown by demonstrating that hepatocytes isolated from cathepsin B knockout mice are resistant to TNF-α-induced apoptosis. More recently, some colleagues have demonstrated a dominant role for cathepsin B in TNF-α-mediated apoptosis. In a murine tumor cell line, caspase inhibition accentuated TNF-α-induced apoptosis by a cathepsin-B pathway. Inactivation of cathepsin B attenuates hepatocyte apoptosis and liver damage in liver reperfusion injury[15,16] and cholestasis injury[17]. CA-074me is a selective inhibitor of cathepsin B[18]; it is highly cell-permeant and can decrease the expression or activity of cathepsin B. During the early stages of pancreatitis, trypsinogen activation in the pancreas is mediated by cathepsin B, indicating that pharmacological interventions that inhibit cathepsin B may prove useful in preventing acute pancreatitis or reducing its severity[19].

In summary, our studies suggested a role for cathepsin B in fulminant hepatic failure. Compared with the normal group, massive hepatocyte apoptosis occurred in the model group, and the number of apoptotic cells increased to a maximum at 6 h. Incubation for longer periods did not lead to further increase in apoptosis and instead resulted in an increase of cell necrosis. The apoptosis index in the protected group was obviously reduced. Consistent with these data, the activity of cathepsin B was markedly increased in drug-treated mice compared with the normal group. Incubation with LPS/D-Gal N for selected time points resulted in a time-dependent increase in cathepsin B activity, and reached a maximum at 8 h. Cathepsin B expression was significantly decreased in the protected group. Collectively, these data suggest that LPS/D-Gal N-mediated cathepsin B expression initiates hepatocyte apoptosis in fulminant hepatic failure. Inhibition of cathepsin B attenuates apoptosis and liver injury, supporting a link between cathepsin B and fulminant hepatic failure. Inhibition of hepatocyte apoptosis with CA-074me seems to be a feasible therapeutic option for fulminant hepatic failure.

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COMMENTS

Background

Fulminant hepatic failure is a rare, but severe, complication of acute hepatitis; it is associated with very high mortality. The traditional view is that hepatocyte necrosis is the main feature of fulminant hepatic failure, but increasing evidence implicates a dominant role for hepatocyte apoptosis in this pathogenesis. Recent evidence suggests that cathepsin B contributes to cell apoptosis.

Research frontiers

It is not known if cathepsin B-mediated hepatocyte apoptosis is involved in the pathogenesis of fulminant hepatic failure.

Innovations and breakthroughs

The current study demonstrated that cathepsin B has an essential role in the pathogenesis of fulminant hepatic failure, and the cathepsin B inhibitor CA-074me could attenuate apoptosis and liver injury.

Applications

Inhibition of cathepsin B attenuates apoptosis and liver injury, and CA-074me seems to be a viable therapeutic option for fulminant hepatic failure.

Terminology

Cathepsin B, a lysosomal cysteine protease, is a candidate for an apoptotic mediator originating from acidic vesicles. CA-074me is a specific inhibitor of cathepsin B; it is highly cell-permeant and can decrease the expression or activity of cathepsin B.

Peer review

This is a well-designed, most part well-written paper, with important new data on the pathogenesis of fulminant hepatic failure. Authors have shown that apoptosis is important in the development and that the process can be partially reversed by specific cathepsin-B inhibitor.

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Clinical significance of "anti-HBc alone" in human immunodeficiency virus-positive patients

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Abstract

AIM: To determine the prevalence and clinical relevance of isolated antibodies to hepatitis B core antigen as the only marker of infection ("anti-HBc alone") among human immunodeficiency virus (HIV) type-1 infected patients. Occult hepatitis B infection frequency was also evaluated.

METHODS: Three hundred and forty eight histories from 2388 HIV-positive patients were randomly reviewed. Patients with serological markers of hepatitis B virus (HBV) infection were classified into three groups: past hepatitis, "anti-HBc alone" and chronic hepatitis. Determination of DNA from HBV, and RNA and genotype from hepatitis C virus (HCV) were performed on "anti-HBc alone" patients.

RESULTS: One hundred and eighty seven (53.7%) HIV-positive patients had markers of HBV infection: 118 past infection (63.1%), 14 chronic hepatitis (7.5%) and 55 "anti-HBc alone" (29.4%). Younger age [2.3-fold higher per every 10 years younger; 95% confidence intervals (CI) 1.33-4.00] and antibodies to HCV infection [odds ratio (OR) 2.87; 95% CI 1.10-7.48] were factors independently associated with the "anti-HBc alone" pattern. No differences in liver disease frequency were detected between both groups. Serum levels of anti-HBs were not associated with HCV infection (nor viral replication or HCV genotype), or with HIV replication or CD4 level. No "anti-HBc alone" patient tested positive for HBV DNA.

CONCLUSION: "Anti-HBc alone" prevalence in HIV-positive patients was similar to previously reported data and was associated with a younger age and with antibodies to HCV infection. In clinical practice, HBV DNA determination should be performed only in those patients with clinical or analytical signs of liver injury.

Key words: Human immunodeficiency virus; "Anti-HBc alone"; Occult hepatitis; Hepatitis B virus DNA; Liver disease

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INTRODUCTION

Infections by hepatitis B (HBV) and C (HCV) viruses are common in human immunodeficiency virus (HIV) infected patients, since nearly all these viruses share the same routes of transmission. HBV infection is present in between 49.2%-68% of HIV-positive patients[1-3] and there is evidence that co-infection can modify the natural history of HBV[4], involves potential consequences on morbidity and mortality and has implications in management of both infections[5]. In fact, nowadays

HBV status is systematically and regularly assessed and systematic HBV vaccination is proposed in those patients without HBV markers.

Over recent years, numerous studies have been published about HBV-HIV co-infection, though some issues still remain unclear, such as clinical relevance and management of those patients with an isolated positive test for antibodies against hepatitis B core antigen ("anti-HBc alone" or defective immunological response)[6,7]. This serological pattern is the second most frequent serological profile of HBV infection, occurring in about 30% of HBV infected patients[1-3].

One of the most controversial questions is about the frequency of occult hepatitis, i.e. HBV DNA positive markers without HBV surface antigen (HBsAg), among patient with a defective immunological response. Some authors consider the "anti-HBc alone" pattern to be a marker of occult HBV infection[1,3,8,9], whereas others have not been able to demonstrate HBV DNA in the sera of these patients[10-13].

This work was carried out to establish the prevalence and clinical significance of the "anti-HBc alone" pattern among HIV-positive patients. The frequency of HBV occult infection, determined by standard assays commonly used in routine clinical practice, was also determined.

MATERIALS AND METHODS

Xeral-Cíes University Hospital is a 700 bed Vigo University-affiliated Hospital which serves an urban population of about 400 000 inhabitants. From 2388 HIV-positive patients, who were followed-up in a specialised clinic of the hospital, 348 clinical histories were consecutively reviewed. Patients with some HBV markers were classified into three groups: chronic hepatitis [positive HBsAg and IgG anti-HBc, negative anti-HBs and IgM anti-HBc and positive or negative "e" antigen (HBeAg)]; past hepatitis (positive anti-HBs and IgG anti-HBc, negative HBsAg and IgM anti-HBc); and "anti-HBc alone" (positive IgG anti-HBc and negative HBsAg, anti-HBs, IgM anti-HBc and anti-HBe).

"Anti-HBc alone" patients were included only when a confirmatory test, performed two weeks later, showed a concurrent result. Patients that had been vaccinated against HBV were excluded.

In every "anti-HBc alone" patient and in those with past hepatitis epidemiologic characteristics (age, sex, risks conduct), co-morbidities (active infections, renal failure, diabetes, cancer, *etc*), antibodies against HCV, immunoglobulin levels and liver function tests were gathered. HCV RNA, HCV genotype and HIV viral load were tested and CD4 level was determined by flow cytometry. Also, abdominal imaging (ultrasound, CT, magnetic resonance) was performed to evaluate the presence of chronic liver disease (inhomogeneous hepatic texture or surface, rarefied hepatic central vein, an enlarged caudate lobe, splenomegaly or collateral veins)[14]. In every case, intake of antiretroviral therapy active against HBV, such as lamivudine or tenofovir, was recorded. Changes in the serological response after introduction of antiretroviral therapy or after an immunological improvement were evaluated. When it was possible, serum was extracted to determine HBV DNA.

Laboratory tests

HBs Ag, anti-HBe and anti-HBc were determined with 3rd generation microparticle enzyme immunoassays (MEIA) for qualitative detection of surface antigen, antibodies against "e antigen" and antibodies against "core antigen" of HBV [AxSYM HBsAg (V2) System, anti-HBe System y Core System. Abbott Laboratories, Chicago, IL, USA]. Anti-HBs was determined by 3rd generation MEIA for quantitative assessment of HBV surface antibodies (Abbott Laboratories, Chicago, IL, USA). Detection limits of the assay were 10-1000 IU/L. Serum HBV DNA levels were determined by an automated quantitative technique of molecular hybridation with genomic amplification; owned primers against the core region of the genome contained in a National "Reference Laboratory" were used. The lower detection limit of this assay was 10^4 copies/mL.

Antibodies against HCV were measured with 3rd generation MEIA to qualitatively determine this antibody (AxSYM HCV version 3.0 System. Abbott Laboratories, Chicago, IL, USA). Serum HCV RNA was quantified by molecular hybridation using a branched DNA technique [Versant HCV-RNA 3.0 (bDNA) - Bayer Diagnostics]. This assay has a lower detection limit of 3200 copies/mL. HCV genotype was determined by automatic sequencing with a fluorescent marker.

Antibodies against HIV-type 1, HIV-type 2 and p24 antigen were determined at the same time by 3rd generation MEIA (AxSYM HIV Ag/Ab Combo System. Abbott Laboratories, Chicago, IL, USA). Positive test results were confirmed by Western-Blot (NEW LAV-BLOT I Bio-Rad. France). HIV viral load was quantified by the Amplicor HIV-1 Monitor (Roche Diagnostics). The lower detection limit of this assay was 40 copies/mL.

The study was reviewed and approved by the Research Ethical Committee of Vigo Hospitality University Complex. All patients gave informed consent to participate in the study.

Statistical analyses

Results were expressed as absolute values (percentage) and median (interquantile range, IQR) as appropriate. Baseline characteristics were compared by using χ^2 or Fisher exact test for categorical data and Mann-Whitney *U* test for continuous data.

When variables were significantly associated ($P < 0.05$) with a defective pattern in the univariate analysis, a backward logistic regression analysis was conducted to identify those factors independently associated with "anti-HBc alone".

Table 1 Differences between HIV-positive patients with "anti-HBc alone" and past hepatitis B

	"anti-HBc alone" pattern (*n* = 55)	Past hepatitis B (*n* = 55)	*P*
Age, yr	38 (33-43)	45 (37-49)	0.001
Male, *n* (%)	38 (69.1)	38 (69.1)	1.000
Transmission mode *n* (%)			0.021
IDU	44 (80)	34 (61.8)	
Homosexual	4 (7.3)	15 (27.3)	
Heterosexual	7 (7.7)	6 (10.9)	
AST (IU/mL)	43 (23-65)	31 (22-47)	0.092
ALT (IU/mL)	39 (19-73)	29 (20-45)	0.301
Albumin (g/L)	42 (38.8-45.6)	43 (40.8-45.9)	0.261
Anti-HCV positive, *n* (%)	45 (81.8)	34 (61.8)	0.020
CDC C stage, *n* (%)	15 (27.3)	19 (34.5)	0.409
CD4 (cells/mm^3)	434 (325-714)	459 (303-636)	0.740
HIV DNA < 50 copies/mL	25 (47.2)	32 (59.3)	0.210

IDU: Intravenous drug user; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

RESULTS

From 348 clinical histories, 187 (53.7%) patients had positive tests against HBV; 118 past hepatitis (33.9%), 14 chronic hepatitis (4%), and 55 "anti-HBc alone" (15.8%). Among patients who had been infected by HBV, 29.4% developed an "anti-HBc alone" pattern and 63% showed a cured past infection. Fifty five patients with past hepatitis were randomly selected to be compared with "anti-HBc alone" patients. Epidemiologic characteristics of both groups are shown in Table 1.

The factors independently associated with a defective pattern were younger age [2.3-fold higher per every 10 years younger; 95% confidence intervals (CI) 1.33-4.00] and antibodies to HCV infection [odds ratio (OR) 2.87; 95% CI 1.10-7.48]. Intravenous drug users (IDU) were significantly more frequent in the "anti-HBc alone" group (80% *vs* 61.8%, $P = 0.021$).

Liver function tests, CD4 levels, HIV viral load or AIDS stages were not significantly different between the two groups. Ultrasound signs of chronic liver disease were only present in HCV co-infected patients ($P < 0.05$). Serum levels of anti-HBs were not associated with HCV infection (nor with viral replication or HCV genotype), and were not associated with HIV replication or CD4 level.

Serum HBV DNA was tested in 30 "anti-HBc alone" patients and no-one was positive. However, 10 patients were taking lamivudine or tenofovir when the tests were performed.

DISCUSSION

In the present study, as in other studies[1-3], a high prevalence of HBV infection (53.7%) among HIV infected patients was found. Although diverse frequencies in the "anti-HBc alone" pattern have been reported according to different geographic areas or selected populations[15,16], the frequency data among HIV patients (24.5-37.8%[1-3]) are fairly similar to data reported in this study (29.4%).

One of the independent factors related to the defective serological pattern was a younger age. This event has been previously reported in only one study[17], in which a higher frequency of "anti-HBc alone" status was also found among women. Nevertheless, in our study the proportion of women in the "anti-HBc alone" group was the same as that in the past hepatitis group (30.9%).

The presence of HCV infection is another independent factor identified in our work which has been reported before[1,3,9,16,18]. A study showed that "anti-HBc alone" phenotype was significantly more frequent in HCV-viraemic than in HCV-recovered patients[18]. HCV replication could produce a down regulation in HBV replication, and this could be expressed as a defective serological pattern[7,18].

IDU has also been reported previously as significantly more frequent in the "anti-HBc alone" group[19] and it has been related to a higher frequency of HCV, as IDU is one of the strongest risk factors for HCV infection.

This is the first study that evaluates liver disease by abdominal imaging scan and no statistically significant differences were found between "anti-HBc alone" patients and past hepatitis patients. In both groups, signs of liver disease were only demonstrated in patients co-infected with HCV.

Occult hepatitis prevalence data reported in "anti-HBs alone" HIV-positive patients varies greatly from 0% to 89.5%[1-3,9,12,17,20,21]. However, in those studies that have found viral replication, detected viral load was usually very low (< 10^3 copies/mL)[3,20]. Ultrasensitive PCR techniques (50-10^2 copies/mL) were broadly used in those experimental studies, but are not available in daily clinical practice. In our study, no case of occult hepatitis was proved by standard assays commonly employed in routine clinical practice that can detect 10^4 copies/mL. Clinical relevance and management of this low viral replication is unclear because a higher incidence of hepatic damage was not found in these patients[16]. On the other hand, current therapeutic guidelines do not recommend starting treatment if the viral load is lower than 10^4-10^5 copies/mL[6,21-24]. More than 90% of doctors that attend HIV patients follow this practice[22]. However, the latest recommendations in HIV-positive patients with the "anti-HBc alone" pattern advise to test for HBV DNA in every patient[6,10,12,13,20]. We believe that HBV DNA testing should be performed only in those patients with an unexplained high level of alanine aminotransferase (ALT) or signs of liver disease.

The present study has some limitations. HBV DNA was not tested in every "anti-HBc alone" patient but non medical reasons prevented us from getting some serum specimens. Moreover, some tested patients were taking lamivudine or tenofovir. However, a study showed that the mean HBV load was similar among patients whether

or not they were treated with lamivudine, and that this was probably associated with an increasing number of resistance mutations[2]. Furthermore, in another study in which one case of occult hepatitis was demonstrated the patient was on lamivudine[11]. The present study displays a complete evaluation of HIV-positive patients with the "anti-HBc alone" pattern, since clinical, virological and radiological parameters have been considered in these patients.

In conclusion, in our population "anti-HBc alone" prevalence in HIV-positive subjects is similar to previously reported data and is associated with a younger age and with antibodies to HCV infection. After evaluating the results of the present study and others with similar results, HBV DNA determination should not be performed in every patient with the "anti-HBc alone" pattern, but only in those patients with unexplained clinical or analytical signs of liver injury.

COMMENTS

Background

Over recent years, numerous studies have been published about hepatitis B virus (HBV)-human immunodeficiency virus (HIV) co-infection, though some issues remain still unclear, such as clinical relevance and management of those patients with "anti-HBc alone" pattern or the frequency of occult hepatitis among patients with a defective immunological response. Some authors consider the "anti-HBc alone" pattern to be a marker of occult HBV infection, whereas others have not been able to demonstrate HBV DNA in the sera of these patients.

Research frontiers

The authors studied the prevalence and clinical significance of the "anti-HBc alone" pattern among HIV-positive patients and the frequency of HBV occult infection, determined by standard assays commonly used in routine clinical practice.

Innovations and breakthroughs

This is the first study that evaluates liver disease by abdominal imaging scan and no statistically significant differences were found between "anti-HBc alone" patients and past hepatitis patients. In both groups, signs of liver disease were only demonstrated in patients co-infected with hepatitis C virus (HCV). No single case of occult hepatitis was proved by standard assay commonly employed in routine clinical practice that can detect 10^4 copies/mL.

Applications

Although the latest recommendations in HIV-positive patients with the "anti-HBc alone" pattern advise to test for HBV DNA in every patient, this and other studies show that viral load reported in these patients is usually low. Current therapeutic guidelines do not recommend starting treatment when viral load is lower than 10^4-10^5 copies/mL. The authors believe that HBV DNA testing should be performed only in those patients with an unexplained high level of alanine aminotransferase (ALT) or signs of liver disease.

Terminology

"Anti-HBc alone" pattern or defective immunological response: positive hepatitis B core antigen as the only marker of hepatitis B infection. Occult hepatitis B: HBV DNA positive without HBV surface antigen.

Peer review

The "anti-HBc alone" pattern is very common among HIV-positive patients and it is not associated with liver injury. However this serological pattern can be associated with occult hepatitis B, usually with a very low viral load of HBV. The authors recommend testing DNA HBV only in those patients with the "anti-HBc alone" pattern and unexplained high levels of ALT or signs of liver disease.

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BRIEF ARTICLES

Prognostic factors for 5-year survival after local excision of rectal cancer

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Abstract

AIM: To evaluate the prognostic factors for 5-year survival after local excision of rectal cancer, and to examine the therapeutic efficacy and surgical indications for this procedure.

METHODS: Clinical data, obtained from 106 local rectal cancer excisions performed between January 1980 and December 2005, were retrospectively analyzed. Survival analysis was performed using the Kaplan-Meier method, statistical comparisons were performed using the log-rank test, and multivariate analysis was performed using the Cox proportional hazards model.

RESULTS: Transanal, transsacral, and transvaginal excisions were performed in 92, 12, and 2 cases, respectively. The rate of complication, local recurrence, and 5-year survival was 6.6%, 17.0%, and 86.7%, respectively. Univariate analysis showed that T stage, vascular invasion, and local recurrence were related to the prognosis of the cases ($P < 0.05$). Multivariate analysis showed that T stage [$P = 0.011$, 95% confidence interval (CI) = 1.194-3.878] and local recurrence ($P = 0.022$, 95% CI = 1.194-10.160) were the major prognostic factors for 5-year survival of cases after local excision of rectal cancer.

CONCLUSION: Local rectal cancer excision is associated with few complications, and suitable for stages Tis and T1 rectal cancer. Prevention of local recurrence, active postoperative follow-up, and administration of salvage therapy are the effective methods to increase the efficacy of local excision of rectal cancer.

Key words: Rectal cancer; Surgery; Local excision; Recurrence; Prognosis

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INTRODUCTION

In China, unlike in Western countries, rectal cancer accounts for approximately 70% of colorectal cancers. Both abdominoperineal resection and anterior resection of rectal cancer can result in postoperative urinary impairment and sexual dysfunction, and the presence of a permanent colostomy significantly impacts the quality of life. With the advancement of imaging techniques, the accuracy of preoperative rectal cancer staging has increased dramatically, and the preservation of physical function in rectal cancer patients has become a very important aim of research. Local tumor excision preserves anal, urinary, and sexual function in patients with low rectal cancer. Some patients with early tumor have suitable indications for local tumor excision[1-5]. This study was to determine the prognostic factors for survival after local excision of rectal cancer.

MATERIALS AND METHODS

Clinical data

This study included 106 low rectal cancer patients at the age of 26-81 years (60 males, 46 females, with a median age of 60 years) who underwent local tumor excision at our hospital between January 1980 and December 2005. The main symptom on presentation was rectal bleeding

(95 patients). Other symptoms included passage of mucus, altered bowel habits. Tumors were located at the anterior wall in 45 patients, posterior wall in 35 patients, and lateral wall in 21 patients, none located in 5 patients. The diameter of the tumor was ⩽ 3.0 cm in 70.5% (80/106) patients and > 3.0 cm in 17.9% (19/106) patients. Colonoscopy or barium enema was performed to exclude multiple primary tumors, while ultrasound, chest X-ray, or computed tomography (CT) was conducted to exclude distant metastases, thus a pathological diagnosis was made before operation.

Treatment method

Transanal excision (TAE), transsacral excision (TSE), and transvaginal excision (TVE) were performed in 92, 12, and 2 patients, respectively. The margin was excised 1-2 cm from the tumor, and postoperative pathology reports indicated negative margins in all cases.

Four patients received preoperative radiotherapy and 41 patients underwent postoperative radiotherapy at the dose of 14-75 Gy (mean 53.5 Gy).

Follow-up

Of the 106 patients, 104 received follow-up (by outpatient appointments, telephone, or mail), and the follow-up rate was 98.1%. Follow-up was performed between 11 mo and 20 years after operation, and the median follow-up time was 72 mo. During the follow-up period, 14 patients died. Of them, 9 died of tumor metastasis, 4 of other conditions, and 1 of a second primary esophageal cancer, respectively.

Statistical analysis

SPSS 13.0 software was used for statistical processing. Survival analysis was performed using the Kaplan-Meier method, univariate analysis of prognostic factors was performed using the log-rank test, and multivariate analysis was performed using the Cox proportional hazards model. $P < 0.05$ was considered statistically significant.

RESULTS

Complications

The complication rate was 6.6% (7/106). Postoperative bleeding occurred in 1 case and wound breakdown was observed in 1 case after TSE. Four cases had anastomotic leakage after TAE, 1 of them was accompanied with bleeding. One case had rectovaginal fistulation after TVE. No death occurred during surgery.

Local recurrence and related factors

Of the 106 patients, 18 (17%) had local recurrence 4-174 mo (mean 48.3 mo) after operation. The local recurrence rate for Tis-, T1-, and T2-stage tumors was 7.1%, 18.7%, and 20.8%, respectively ($P = 0.331$), while the local recurrence rate for tumors with or without vascular invasion was 66.7% and 15.5%, respectively ($P = 0.02$). Surgical treatment was reattempted for 12 patients after recurrence. Local excision was performed in 5 patients. Of them, 1 died after 93 mo and the other 4 survived. Abdominoperineal resection was performed in 6 patients, and Park's procedure was performed in 1 patient. Of these 7 patients, 2 died after 36 and 40 mo, respectively, the other 5 survived. Distal metastases were present in 9 patients, who were then treated with chemotherapy, radiotherapy, or interventional therapy. Four T3 stage patients received preoperative radiotherapy, and local excision was performed after down staging. Tumors recurred in 2 patients with a recurrence rate of 50%.

Prognosis and influencing factors

The overall 5-year survival rate for our patients was 86.7%. The 5-year survival rate for Tis-, T1-, and T2-stage tumors was 100%, 88.8%, and 67.9%, respectively. Univariate analysis showed that infiltration depth, vascular invasion, and local recurrence were the prognostic factors for tumors ($P < 0.05$, Figure 1). Multivariate analysis showed that T stage [$P = 0.011$, OR = 2.152, 95% confidence interval (CI) = 1.194-3.878] and local recurrence ($P = 0.022$, OR = 3.483, 95% CI = 1.194-10.160) were the major prognostic factors for tumors.

DISCUSSION

Although radical resection is an effective treatment for rectal cancer, it can lead to anal, urinary, and reproductive function impairment as well as surgical complications and death. Morson *et al*[6] have reported a 5-year survival rate of 82% in patients after local excision of early rectal cancer. Subsequent studies demonstrated that local excision of early stage low rectal cancer can produce a good outcome and preserve anal, urinary, and reproductive function[3,7-10], but a higher local recurrence rate of tumor (5%-27%) has limited its wide application. Nevertheless, the 5-year survival rate after local tumor excision reported in the literature is 72%-95%[3,7-10]. In our study, the local recurrence rate was 17.0% and the 5-year survival rate was 86.7%, respectively, which are similar to the reported data[3,7-10].

Risk factors affecting low rectal cancer prognosis after excision include the age and sex of patients, treatment methods, and vascular invasion and tumor stage. In our study, the age and sex of patients were unrelated to the prognosis of low rectal cancer. Vascular invasion and tumor stage affected rectal cancer recurrence and prognosis after local excision. Bouvet *et al*[11] found that T stage is the most important pathological factor for local rectal cancer recurrence after excision. In our patients, excision of Tis, T1, and T2 tumors was associated with the 5-year survival rate ($P < 0.05$). The local recurrence and distant metastasis rates were significantly increased when the tumor was poorly differentiated, macroscopically ulcerative, or larger than 3.0 cm in diameter. However, because the number of patients was small in our study, the differences were not statistically significant. It was reported that rectal cancer patients also have a higher rate of lymphatic spread[12,13], and therefore local excision should be recommended

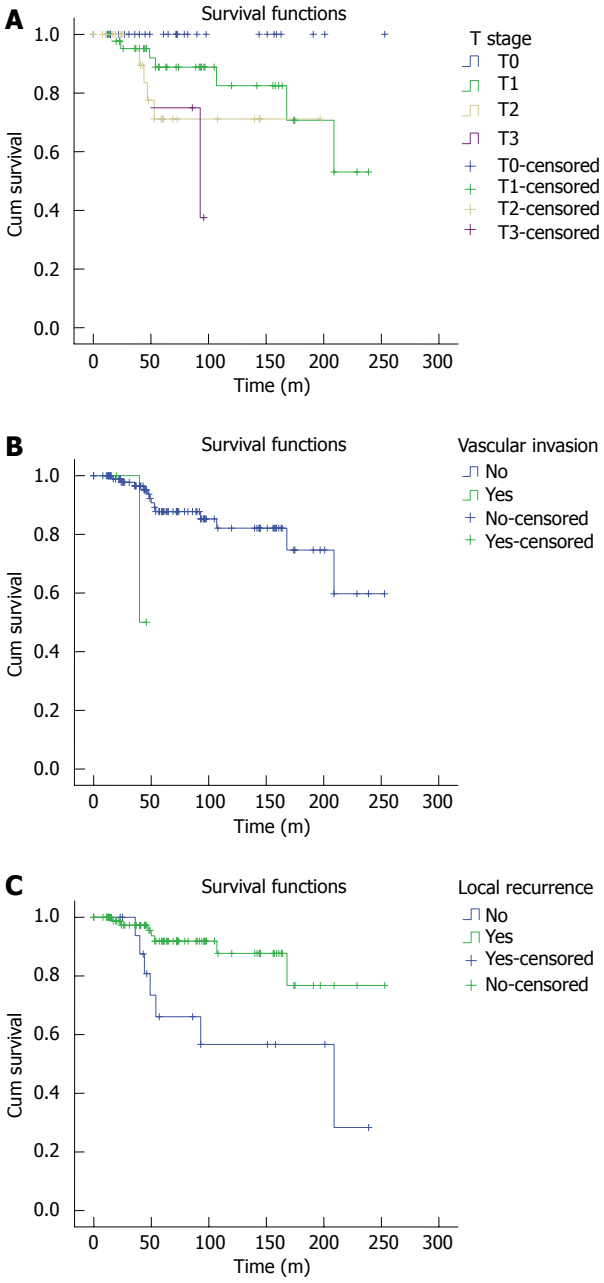

Figure 1 Survival curves for patients with different tumor T stages (A), different vascular invasions (B), and different local recurrence rates (C). The survival rate for the patients with T0 (n = 28), T1 (n = 48), T2 (n = 26), T3 (n = 4) varied significantly (P = 0.022). Patients without vascular invasion (n = 103) had a higher survival rate than those with vascular invasion. (n = 3) (P = 0.01). Patients without local recurrence (n = 88) had a higher survival rate than those with local recurrence (n = 18) (P = 0.005).

with caution for such patients.

The excision method of local tumor may affect its prognosis. If R0 tumor excision is achieved, the surgical method used does not affect its local recurrence rate or the survival rate of patients. In our patients, all margins on pathological examination were negative. It was reported that if tumor margin is positive, the recurrence rate of tumor will be high and its prognosis is poor[14]. Therefore, if the margin is positive or borderline positive, the excision margins should be widened, otherwise, local tumor excision should be abandoned. Frozen section examination of margins during surgery ensures margin negativity, which is essential for complete local tumor excision. It was reported that adjuvant chemotherapy and radiotherapy for tumor after local excision can reduce its local recurrence rate[15]. Our data show that radiotherapy was unrelated to local recurrence and prognosis of tumor, which may be due to the different indications for radiotherapy, and the standardized treatment modalities. It is generally believed that rectal cancer patients should undergo adjuvant chemotherapy and radiotherapy after local excision of T2 rectal tumor and poorly-differentiated or high-risk T1 rectal tumor[16].

Salvage therapy is of great importance in the treatment of recurrent rectal tumor after local excision and can be performed when rectal wall involvement is significantly greater than extramural or pelvic cavity involvement. Therefore, the frequency of second surgery is higher in patients treated with local excision than in patients treated with radical resection. Mellgren *et al*[3] reported 17 cases (68%) of recurrent rectal cancer in the rectal wall and 8 cases (32%) of recurrent rectal cancer in extramural. Sengupta *et al*[4] found that 40%-100% of recurrent rectal cancer patients could be cured. Friel *et al*[17] used salvage therapy for 29 T1 or T2 tumor patients with recurrence after local excision (90% involving the rectal wall and 10% completely extramural), and found no residual tumor in 17 survivors after a mean follow-up time of 39 mo. Of the 18 patients with rectal tumor recurrence in our study, 12 were retreated surgically. Of the 5 patients treated with local excision, 1 died after 93 mo and 4 survived. In the 6 patients treated with abdominoperineal resection and 1 patient treated with Park's procedure, 2 died after 36 and 40 mo, respectively, and the other 5 survived, suggesting that salvage therapy is effective against early recurrent rectal cancer.

In rectal cancer patients who are unsuitable for or refuse abdominoperineal resection, local excision can be performed after the tumor is down staged[17]. Kim *et al*[18] showed that preoperative adjuvant therapy can effectively down stage T2 and T3 rectal cancer. In their study, 25 patients who were staged before operation by endorectal ultrasound (ERUS) received radiotherapy (45 Gy, 25 cycles) and chemotherapy (5-flurouracil, 300 mg/m^2 per day, 5 d/week). Partial pathological response was achieved in 4 patients and complete response in 22 patients. In the 22 patients with complete-response, no recurrent rectal cancer occurred after local excision. In our 4 patients with preoperative stage T3 rectal cancer treated with local excision after chemotherapy, the cancer recurred in 2 cases with a recurrence rate of 50%. Since T3 and T4 tumors have a higher recurrent rate, they are unsuitable for local excision in patients who cannot tolerate or refuse abdominoperineal resection.

Local rectal cancer can be treated with a variety of methods, including TAE, TSE, TVE, and minimally invasive endoscopic transanal excision[19]. Irrespective of the surgical method, R0 excision is the most important procedure. TAE, a minimally invasive treatment modality, has few complications[20]. In our 92 patients, TAE had a low complication rate of 2.2%. Preoperative pelvic

CT and ultrasound examination can provide important information for surgical planning. Kwok *et al*[21] analyzed data on the accuracy, sensitivity, and specificity of preoperative CT, magnetic resonance imaging (MRI), MRI-endorectal coil (MRI-ERC), and ERUS for rectal cancer staging, and found that the accuracy of CT, MRI, MRI-ERC, and ERUS is 80%, 74%, 81%, and 84%, respectively, with a higher accuracy for T1 tumors. At present, local excision is a generally accepted treatment modality for stage Tis and T1 rectal cancer. However, close follow-up is required. Early detection and salvage therapy can prolong the survival time of rectal cancer patients.

COMMENTS

Background

Both abdominoperineal resection and anterior resection of rectal cancer can result in postoperative urinary impairment and sexual dysfunction, and the presence of a permanent colostomy significantly impacts the quality of life. With the advancement of imaging techniques, the accuracy of preoperative rectal cancer staging has increased dramatically, and the preservation of physical function in rectal cancer patients has become a very important aim of research. Local tumor excision preserves anal, urinary, and sexual function in patients with low rectal cancer. Therapeutic efficacy and surgical indications for this procedure are controversial.

Research frontiers

Some studies have demonstrated that local tumor excision of early stage low rectal cancer can produce a good outcome and preserve anal, urinary, and reproductive function. However, a higher local recurrence rate (5%-27%) of this procedure has limited its use in clinical practice. Nevertheless, the 5-year survival rate after local tumor excision is 72%-95%. Risk factors affecting low rectal cancer prognosis after local excision include age and sex of patients, treatment modalities, and vascular invasion and tumor stage.

Innovations and breakthroughs

In this study, age and sex of the patients were unrelated to their prognosis. Vascular invasion and tumor stage affected rectal cancer recurrence and prognosis after local excision, and local recurrence and distance metastasis rates increased significantly when the tumor was poorly differentiated, macroscopically ulcerative, or larger than 3.0 cm in diameter. Therefore local excision should be recommended with caution for such patients. Frozen section examination of margins during surgery ensures margin negativity, which is essential for complete local tumor excision. Salvage therapy is of great importance in the treatment of recurrent rectal cancer after local excision.

Applications

Local rectal cancer excision has few complications, and is suitable for stages Tis and T1 rectal cancer. Prevention of local recurrence, active postoperative follow-up, and salvage therapy can greatly increase the efficacy of local excision.

Terminology

Local rectal cancer can be treated with a variety of methods including transanal excision (TAE), transsacral excision (TSE), transvaginal excision (TVE), transsphincter local excision, and minimally invasive endoscopic transanal excision

Peer review

The study is well designed and it results have confirmed the efficiency of different methods for rectal cancer and the discussion is reasonable.

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BRIEF ARTICLES

Morphology and ontogeny of dendritic cells in rats at different development periods

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Abstract

AIM: To study the morphology and ontogeny of dendritic cells of Peyer's patches in rats at different development periods.

METHODS: The morphometric and flow cytometric analyses were performed to detect all the parameters of villous-crypts axis and the number of $OX62^+DC$, $OX62^+CD4^+SIRP^+DC$, and $OX62^+CD4^-SIRP^-DC$ in the small intestine in different groups of rats. The relationship between the parameters of villous-axis and the number of DC and DC subtype were analyzed.

RESULTS: All morphometric parameters changed significantly with the development of pups in the different age groups (F = 10.751, 12.374, 16.527, 5.291, 3.486; P = 0.000, 0.000, 0.000, 0.001, 0.015). Villous height levels were unstable and increased from 115.24 μm to 140.43 μm as early as 3 wk postpartum. Villous area increased significantly between 5 and 7 wk postpartum, peeked up to 13817.60 μm^2 at 7 wk postpartum. Villous height and crypt depth ratios were relatively stable and increased significantly from 2.80 ± 1.01 to 4.54 ± 1.56, 9-11 wk postpartum. The expression of $OX62^+DC$ increased from 33.30% ± 5.80% to 80% ± 17.30%, 3-11 wk postpartum (F = 5.536, P = 0.0013). $OX62^+CD4^+SIRP^+DC$ subset levels detected in single-cell suspensions of rat total Peyer's patch dendritic cells (PP-DCs) increased significantly from 30.73% ± 5.16% to 35.50% ± 4.08%, 5-7 wk postpartum and from 34.20% ± 1.35% to 43.60% ± 2.07% 9-11 wk postpartum (F = 7.216, P = 0.005).

CONCLUSION: This study confirms the age-related changes in villous-crypt axis differentiation in the small intestine. Simultaneously, there are also development and maturation in rat PP-DCs phenotypic expression. Furthermore, the morphological changes of intestinal mucosa and the development of immune cells (especially DC) peaked at 9-11 wk postpartum, indicating that the intestinal mucosae reached a relatively mature state at 11 wk postpartum.

Key words: Intestinal mucosa; Dendric cell; Peyer's patches; Intestinal development

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INTRODUCTION

Different development periods have been suggested to play a role in controlling the development of gastrointestinal mucosal immune responses, which induce the intestinal mucosal immunity reflected differently over different development periods in children. As the lymphoid tissue is the primary site for the induction of mucosal immune responses. The morphometry of the villous-crypt axis in the small intestine reflects the function and adaptation of intestinal mucosal barriers. It was therefore of interest to investigate the potentially disparate phenotypic expression of dendritic cells (DCs) and the morphology of intestinal mucosa

found at different periods as a basis for determining the mechanisms that are apparently critical in intestinal mucosal immunity.

DCs were first identified in 1973 by Steinman and Cohn[1,2] and were found in two locations in the intestinal mucosa: the Peyer's patches (PPs) and lamina propria (LP). PP-DCs were isolated by Spalding in 1983[3]. It is now clear that PP-DCs may be unique in their ability to induce the differentiation of T cells that produce important cytokines such as IL-4 and IL-10 and other cytokines, including TGF-β, which is important for B cell differentiation and bystander suppression after oral antigen feeding[4,5]. PP-DCs primarily perform two important tasks: (1) uptake of antigen after its transcytosis across the follicle-associated epithelium (FAE), which is mediated by immature DCs located largely in the subepithelial dome (SED); (2) T and B cell activation by mature DCs, which are found in the SED. T and B cells activated in PPs are "imprinted" and back to the gut due to the unique ability of PP-DCs to induce lymphocytes to regulate immunity[6-9].

DC studies to date have been primarily carried out in mice *in vitro*. Little literature exists about rat DCs and of DCs *in vivo,* as current techniques for DC isolation are likely to induce phenotypic changes in DC. However, rats are of great value as experimental animals, especially in the field of nutrition. Additionally, some important findings about DC have been achieved through animal experiments. We therefore chose the rat model as the experimental object to detect the statement *in vivo*. In previous studies, DCs in mice had been divided into two subsets: CD8αα+ and CD8αα-DC[10]. CD8αα+ and CD8αα-DC preferentially activate T cells toward Th1 and Th2 differentiation, respectively[11]. CD8αα+DC constitutively cross-presents antigens to T cells, while CD8αα-DC does so upon their activation[12]. Kelsall *et al*[13] also identified two distinct subsets of DCs in 6 to 8-wk-old murine PP. One population of DCs was positioned to capture antigens transported by overlying M cells, while the other subset activated native T cells to become effector cells. Recently, four different subsets of DCs have been described in mouse PPs[14,15]. Conversely, progress in the studies of rat DC is slow because of the relative shortage of reagents. CD11C, MHC-class Ⅱ, and αε-intergin (OX62 antigen) expression is routinely detected in rats to define DC in peripheral and lymphoid tissues. αε-intergin expression is the strongest in mucosa-associated DC[16,17]. Additionally, as early as in 1980, rat DCs were subdivided on the basis of differential expression of CD4 and a member of the SIRP (signal inhibitory regulatory protein) family of molecules (detected by OX41)[18,19]. The existence of $CD4^-/SIRP^-$ and $CD4^+/SIRP^+$DC subsets from the small intestine has recently been described[20]. Some reports[20] have shown that 12 to 16-wk-old rat intestinal and hepatic lymph DC is αE_2 $intergin^{hi}$ (OX-62) and includes two subsets: (1) signal regulatory protein α (SIRPα)$^{hi/low}$ and (2) $CD4^{hi/low}$, which most likely represents murine CD8αα$^{-/+}$DC. In both lymph and the spleen, $SIRP^+/CD4^+$ DCs are more potent than $SIRP^-/CD4^-$ DCs in the activation of allogeneic $CD4^+$ and $CD8^+$ T cells, naïve Ag-specific $CD4^+$ T cells *in vivo*, and sensitized Ag-specific $CD4^+$ T cells *in vitro*. To date, few studies have been conducted in rat PP-DCs at different development periods, our work focused on the morphometry of the villous-crypt axis in the small intestine as well as PP-DC differentiation and maturation in rats during ontogeny. Flow cytometry was used with emphasis laid on the implications of PP-DC interactions with intestinal mucosal immunity.

Our current study was to gain a better understanding of the expression and activation of PP-DCs in Sprague-Dawley rats. Single-cell suspension from the PP of the small bowel was isolated via collagenase A digestion. By collecting and manipulating these cells at 4℃, we maintained the cell activity. Using this model, we ascertained two phenotypically distinct subsets of PP-DC (both $OX62^+$) that were distinguished by the existence or nonexistence of CD4 and SIRP coexpression. We also explored whether age-related development of DC ontogeny and morphological change occurred simultaneously. Our results demonstrate that morphological change is associated with DC ontogeny, suggesting that the intestinal mucosal immune system continuously changes as young rats mature.

MATERIALS AND METHODS

Animals

SD rats were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China. Rats were held in plastic cages in temperature- and humidity-controlled animal quarters under a 12 h light/dark cycle and were fed a standard diet (rodent rat chow) *ad libitum* with free access to tap water. All procedures were approved by the Institutional Animal Care Committee.

Reagents

Abs mouse anti-rat OX62:RPE, mouse anti-rat CD172a: FITC (OX41), and mouse anti-rat CD4:APC (Serotec) were purchased from Shanghai Jingmei Corporation.

Tissue samples

SD rats were bred and maintained under specific pathogen-free conditions at Xinhua Hospital Affiliated to the Medical College, Shanghai Jiaotong University, Shanghai, China. Animals, aged 3 wk and weighing 52-58 g, were divided into 5 groups of equal size and approximately equal mean body weight (54 g), namely groups of 3, 5, 7, 9 and 11 wk. Rats were killed after a postpartum period of 21, 35, 49, 63 and 77 d. Abdominal cavities were opened by horizontal incision along the midsection and guts were excised. Central ileum tissue samples (0.5 cm) were taken. All PP tissue samples were taken from the small bowel.

HE staining

Immediately after collection, ileum tissue samples were washed 3 times in cold phosphate buffered saline (PBS)

Table 1 Morphoriogical changes of intestinal mucosa at different development periods in SD rats (mean ± SD)

	3 wk	5 wk	7 wk	9 wk	11 wk	*F*	*P*
Villous height (μm)	115.24 ± 21.82	140.43 ± 22.30	210.71 ± 59.47	145.43 ± 34.21	205.14 ± 51.31	10.751	0.000
Villous width (μm)	34.41 ± 8.67	60.57 ± 20.61	93.06 ± 35.03	66.91 ± 21.28	38.82 ± 8.96	12.374	0.000
Villous areas (μm^2)	3334.46 ± 1134.11	5451.92 ± 2029.10	13817.60 ± 5236.52	7495.52 ± 3059.18	7209.91 ± 2087.77	16.527	0.000
Crypt depth (μm)	30.06 ± 6.61	44.12 ± 9.25	58.76 ± 14.16	58.70 ± 29.04	48.29 ± 12.94	5.291	0.001
Villous height/crypt depth	3.92 ± 0.76	3.30 ± 0.79	3.74 ± 1.22	2.80 ± 1.01	4.54 ± 1.56	3.486	0.015

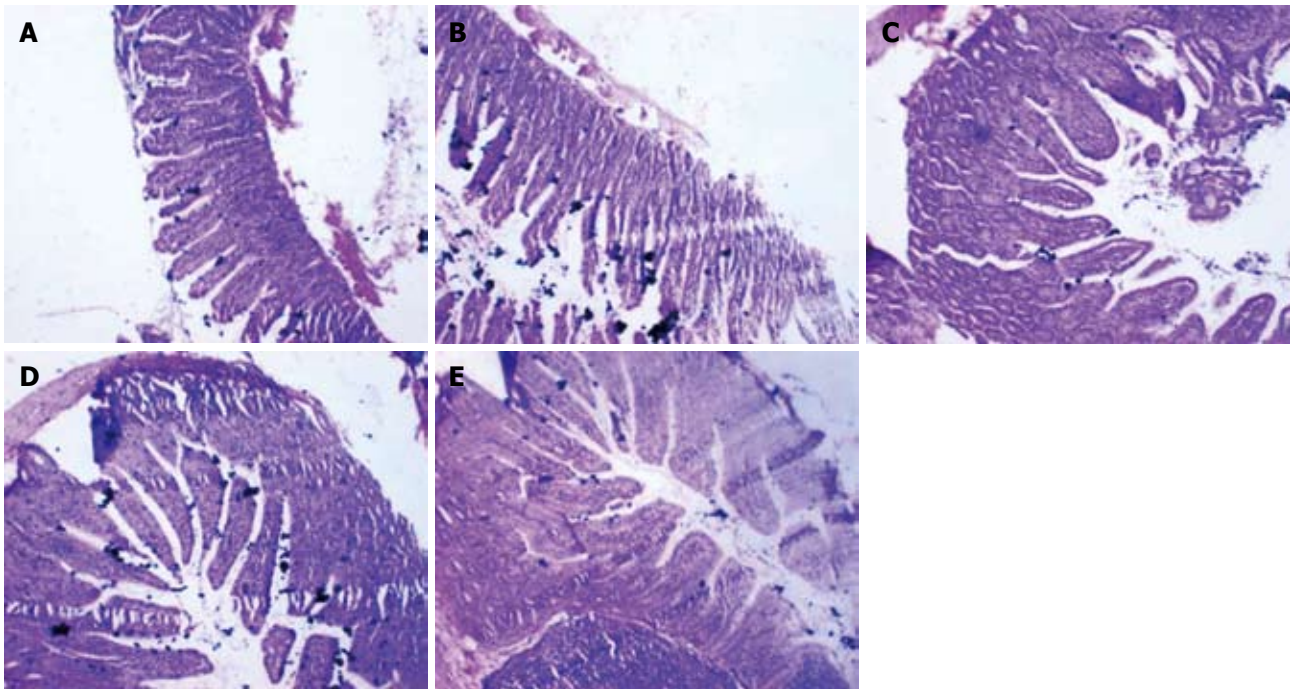

Figure 1 Comparison of morphological changes of small intestinal mucosa (including villous height, villous width, villous area, crypt depth, and ratio of villous height to crypt depth) at different development periods in SD rats (HE, x 100). A: Group of 3 wk; B: Group of 5 wk; C: Group of 7 wk; D: Group of 9 wk; E: group of 11 wk.

and fixed for 48 h in 4% formalin solution. After fixation, specimens were dehydrated and embedded in paraffin. Sections from each sample were cut at a thickness of 4 μm and stained with hematoxylin and eosin (HE).

Determination of staining results

Sections were examined under a light microscope. Villous height, villous width, and crypt depth in all tissues were determined using the image analysis system. Villous height was measured from the top of the villi to the Lamina muscularis mucosae. Villous width was defined as the distance from one crypt-villi junction to the next. The villous area (height × width) was calculated out of these 2 parameters. PP areas in the ileum were imaged and measured in triplicate using a digital camera and software. PP outlines were performed by hand.

Flow cytometry

PP tissue samples were washed extensively 3 times in cold PBS. They were then cut into small segments and placed in cold PBS (4℃). After centrifugation at 800 rpm for 5 min at 4℃, the supernatant was removed and the remaining tissues were digested with 0.75 mg/mL collagenase A for 45 min at 37℃ with periodic agitation. Undigested stromal material was removed by passing over membrane filtration. Single-cell suspension was prepared and cells were incubated with mouse anti-rat OX62:RPE, mouse anti-rat CD172a:FITC (OX41) and mouse anti-rat CD4:APC for 30 min.

Statistical analysis

SPSS 11.0 statistical software was used for data analysis. Results were expressed as mean values with standard deviation (SD). Statistical analysis was completed using one-way ANOVA. Tukey's test was used to determine the significance of the difference between groups when the ANOVA test indicated a significant effect. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Morphological analysis of villous-crypt axis

The morphological parameters of the villous-crypt axis in the small intestine included villous height, villous width, villous areas, crypt depth, and the ratio of villous height to crypt depth. As shown in Table 1, Figure 1 and Figure 2A-E, all morphometric parameters changed significantly with the development of pups in

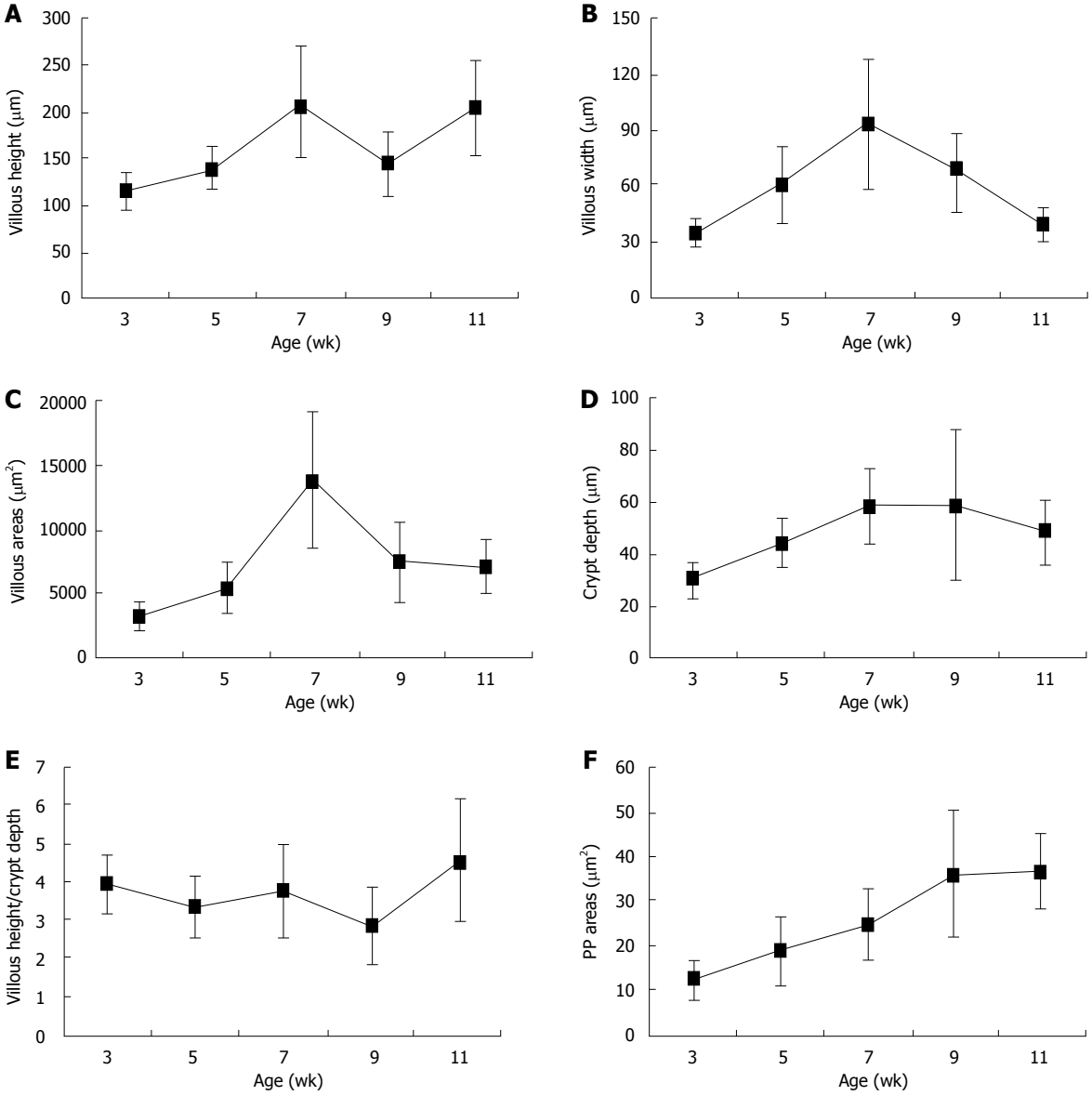

Figure 2 Morphological analysis of villous-crypt axis: All morphological parameters matured as age increased. The results were presented as mean ± SD from 5 rats. A: Villous height increased at 3 wk postpartum, decreased 7 to 9 wk postpartum, and increased again after 9 wk postpartum; B: Villous width increased at 3 wk postpartum, peaked at 7 wk postpartum; C: Villous area increased significantly between 5 and 7 wk postpartum, peaked at 7 wk postpartum; D: Crypt depth increased from 3 to 7 wk postpartum and decreased slightly at 9 wk postpartum; E: Ratio of villous height to crypt depth were relatively stable and increased significantly from 9 to 11 wk postpartum; F: PP increased from 3 to 11 wk postpartum.

the different age groups (F = 10.751, 12.374, 16.527, 5.291, 3.486; $P < 0.05$). Villous height was unstable and increased from 115.24 ± 21.82 μm to 140.43 ± 22.30 μm as early as 3 wk postpartum, decreased from 210.71 ± 59.47 μm to 145.43 ± 34.21 μm at 7-9 wk, and then increased again after 9 wk. Villous width increased from 3 wk postpartum, peaked at 7 wk up to 93.06 ± 35.03 μm, and decreased thereafter. Villous areas increased significantly between 5 wk and 7 wk postpartum from 5451.92 ± 2029.10 μm^2 to 13817.60 ± 5236.52 μm^2, peeked at 7 wk, and decreased from then on, whereas few differences were found between 9 and 11 wk postpartum. Crypt depth increased from 3 wk to 7 wk postpartum, and decreased slightly at 9 wk. Few age-related differences were evident between 7 wk and 9 wk postpartum. Villous height and crypt depth ratio was relatively stable and increased significantly from 2.8 ± 1.01 to 4.54 ± 1.56 at 9-11 wk postpartum.

Morphological analysis of PPs

PPs located in the small intestine showed a highly significant increase in size and number with age ($P < 0.05$), and the degree of increase in different age groups varied from a few to a moderate number (from 12.25 ± 4.69 μm^2 to 37.12 ± 8.20 μm^2 at 3-11 wk postpartum) (Table 2, Figure 2F).

Ontogeny analysis of PP-DCs

Single-cell suspensions of the total PP-DCs in rats were identified by immnolabelling for OX62, which contained two populations distinguished by co-expression of CD4 and SIRPα or not. As shown in Table 3, Figures

Table 2 Changes of the areas of Peyer's patches (PP) at different development periods in SD rats (mean ± SD)

	3 wk	5 wk	7 wk	9 wk	11 wk	*F*	*P*
PP areas (μm^2)	12.25 ± 4.69	18.91 ± 7.73	24.68 ± 7.73	36.13 ± 14.59	37.12 ± 8.20	7.474	0.000

Table 3 Changes in expression of $OX62^+$DC, $OX62^+CD4^+SIRP^+$DC and $OX62^+CD4^-SIRP^-$DC subsets with development of SD rats (mean ± SD)

Age	3 wk	5 wk	7 wk	9 wk	11 wk	*F*	*P*
$OX62^+$DC/total cells in PP (%)	0.333 ± 0.058	0.367 ± 0.115	0.467 ± 0.153	0.533 ± 0.153	0.8 ± 0.173	5.536	0.013
$Ox62^+CD4^+SIRP^+$DC/$OX62^+$DC (%)	31.87 ± 1.99	30.73 ± 5.16	35.50 ± 4.08	34.20 ± 1.35	43.60 ± 2.07	7.216	0.005
$Ox62^+CD4^-SIRP^-$DC/$OX62^+$DC (%)	33.27 ± 5.71	25.33 ± 2.82	24.53 ± 2.64	28.97 ± 1.89	28.43 ± 1.94	3.242	0.060

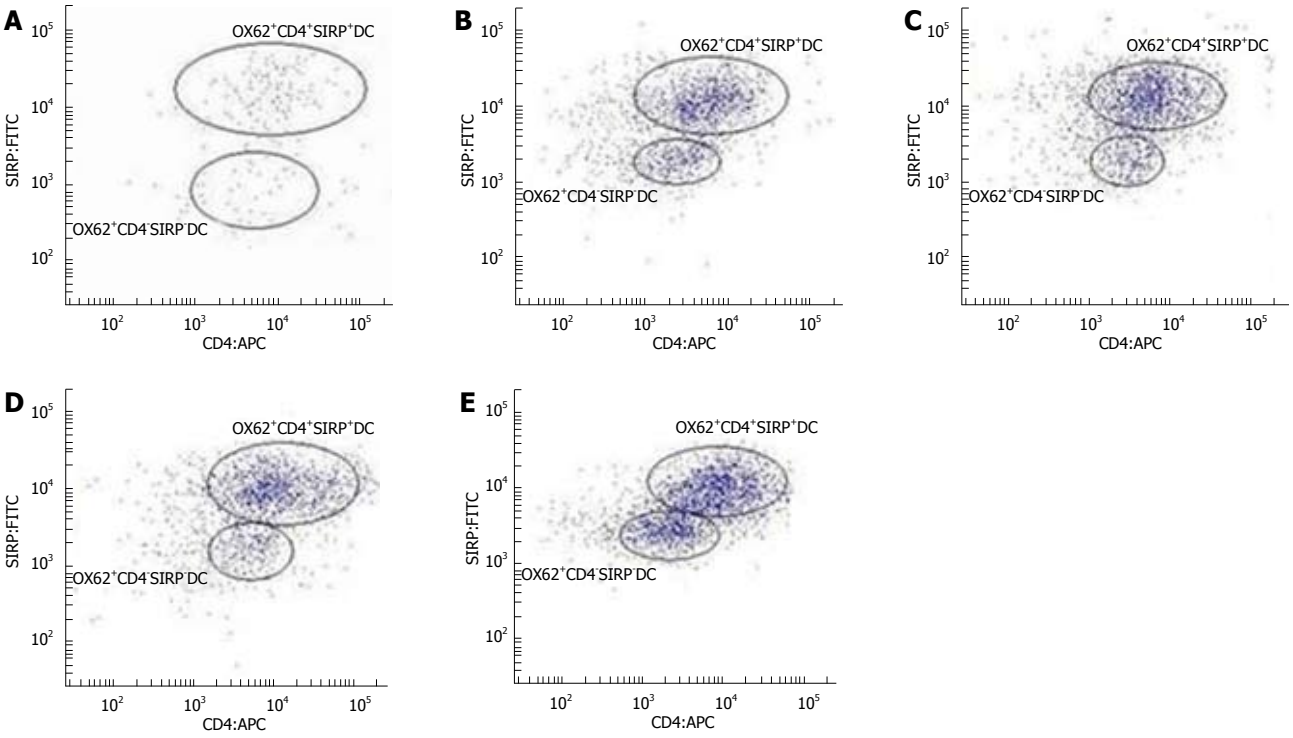

Figure 3 FCM of expression of $OX62^+CD4^+SIRP^+$DC and $OX62^+CD4^-SIRP^-$DC subsets at different development periods in SD rats. A: Group of 3 wk; B: Group of 5 wk; C: Group of 7 wk; D: Group of 9 wk; E: Group of 11 wk.

3 and 4, the expression of $OX62^+$DC (i.e. the total PP-DC) increased from 33.3% ± 5.8% to 80% ± 17.3% at 3-11 wk postpartum. Moreover, age-related changes in total PP-DCs occurred as early as 3-5 wk postpartum and became more pronounced 9-11 wk postpartum. $OX62^+CD4^+SIRP^+$DC subset levels detected in single-cell suspensions of rat total PP-DCs increased significantly from 30.73% ± 5.16% to 35.5% ± 4.08% at 3-5 wk postpartum and from 34.2% ± 1.35% to 43.6% ± 2.07% at 7-9 wk postpartum, whereas few age-related differences were found 9-11 wk postpartum. $OX62^+CD4^-SIRP^-$DC levels varied in different age groups, whose trends were downward on the whole from 33.27% ± 5.71% to 28.43% ± 1.49% at 3-11 wk, although small fluctuations were observed 7-9 wk postpartum.

DISCUSSION

PPs[21] are typical gut-associated lymphoid tissues located along the small intestine wall and serve as the major sites for generation of immunity to intestinal antigens. PPs contain large amounts of immunocytes, of which DCs are the most potent antigen-presenting cell for activation of further immune response. Moreover, intestinal DC results in immune response to the intestinal mucosa, and therefore will not stimulate the immune response system. Thus, intestinal DC[22], one of the best characters representing the maturation and activation of intestinal mucosal immunity, can exist in different levels of maturation and activation that are reflected in different ways, including antigen capture and process, effector cell activation, and cytokine networking.

It is well known that rats at 21-28 d, 28-60 d, and > 60 d postpartum approximate to the human weaning stage, childhood period, and adult period, respectively. Therefore, in our study we selected rats at the ages of 3, 5, 7, 9 and 11 wk postpartum to simulate the different human development periods. We found that DCs

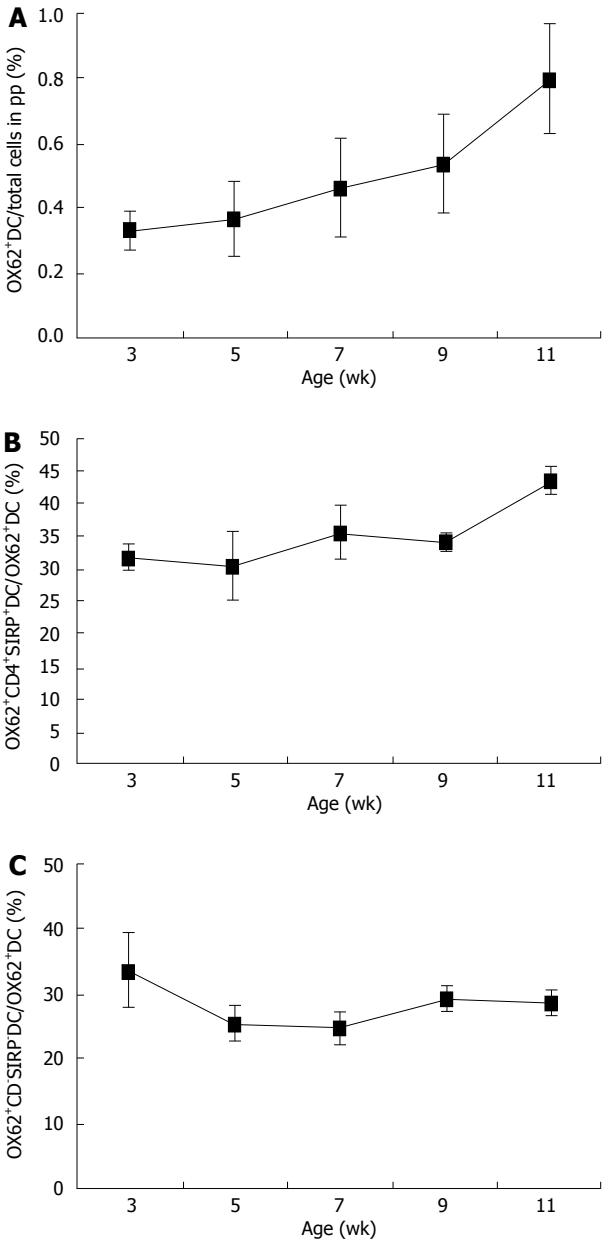

Figure 4 Flow cytometric analyses of $OX62^+CD4^+SIRP^+$ and $OX62^+CD4^-SIRP^-$ PP DCs *in vivo* at 3, 5, 7, 9 and 11 wk postpartum. The results were presented as mean ± SD from 5 rats. A: Significant growth occurred in different age groups for the number of $OX62^+$DCs; B: Levels of $OX62^+CD4^+OX41^+$DC subsets increased significantly at 3-5 wk postpartum and 7-9 wk postpartum; C: $OX62^+CD4^-SIRP^-$DC levels declined on the whole, with small fluctuation from 7 to 9 wk postpartum.

isolated from single-cell suspensions of rat PP revealed OX62 expression and were subdivided based on the differential expression of CD4 and a member of the signal inhibitory regulatory protein (SIRP) family of molecules (detected by the OX41). These results concur with a previous study[17,20]. Trinite *et al*[23] have proposed that CD4 and SIRP expression levels may be related to DC developmental stages or reflect differential induction in response to the nature of the microenvironment and their expression is entirely tissue-specific and related to the function of DC in these tissues. DCs that coexpress CD4 and OX41, a member of the SIRP family, have functional properties typical of mature DCs. In contrast, $CD4^-OX41^-$DCs are weak APCs for specific Ag, and in the allogeneic MLR survive poorly in cultures, contain large cytoplasmic inclusions, and are very strongly nonspecific esterase (NSE).

The current study revealed that development of the rat intestinal mucosa varies among different age groups, and the morphological parameters of the villous-crypt axis in the small intestine reflected proliferation and differentiation with age. Additionally, the total number of PP-DCs as well as the DC subpopulation trends varied significantly. Zaph *et al*[24] have also proposed that immune cells could promote the development of intestinal mucosa, and the mature immune system could promote the development of intestinal morphology. Our results therefore indicate that the development changes consisting of the proliferation and differentiation of intestinal morphology and the activation and maturity of intestinal immune cells (particularly DC) occurred simultaneously and not accidentally. The small intestine morphometric parameters and the total PP-DC increased with age and were more pronounced 9-11 wk postpartum, which indicates that the function of the intestinal mucosal barrier and mucosal immune system of rats gradually matured. The trend of $CD4^+SIRP^+$ DC subpopulations was upward at the same time that $CD4^-SIRP^-$ DC subpopulations were downward, which indicates that the immune system matured with age. The number of PP-DC subpopulations changed notably in the early post-weaning period (3-5 wk postpartum), which may be related to the change in diet from milk to solid feeding as well as intestinal adaptive adjustment induced by an increase in the number of antigens. All morphometric parameters detected in the small intestine increased promptly in rats aged 5-7 wk postpartum compared with rats at other age periods. It has been reported that the phenomenon of villous atrophy in mammals occurs after diet changes; this mechanism is related to a reduction in the rate of cell update and an increase in the rate of cell loss. Moreover, villous atrophy can also result in the intestinal immune function decline; therefore, we speculate that the phenomenon of delayed development of villous parameters may correspond to the diet change in the early post-weaning period (3-5 wk postpartum), which further impacted intestinal mucosal immune function in the late post-weaning period (5-11 wk postpartum). Finally, in the late post-weaning period, the development rate of PP-DCs decreased and the PP-DCs were in a relatively stable state. Additionally, the villous height/crypt depth ratio is a useful criterion for estimating digestive capacity in the small intestine[25]. Although a reduction in villous height occurred 7-11 wk postpartum, maintenance of the villous height/crypt depth ratio 7-11 wk postpartum suggested that a reduction in villous height was less deleterious when it was not accompanied by increased crypt depth. These results coincide with those of Montagne *et al*[25].

In conclusion, results from the current study indicate that the intestinal mucosal immune system is continuously changing as young rats mature. This

study confirmed the age-related changes in villous-crypt axis proliferation and differentiation in the small intestine. Simultaneously, there are also developments and maturations in rat PP-DCs phenotypic expression. Furthermore, the morphological changes of intestinal mucosa and the development of immune cells (especially DC) peaked at 9-11 wk postpartum, indicating that the intestinal mucosae reached a relatively mature state at 11 wk postpartum. In the early weaning period, the number and the phenotypic expression of PP-DCs as well as the morphology changed rapidly due to ingestion of solid food, however, its mechanism requires further studies.

COMMENTS

Background

Recent evidences indicated that the different development period plays a role in controlling the development of gastrointestinal mucosal immunity. Peyer's patches (PPs) were the primary sites for the induction of mucosal immune responses. Dendritic cell (DC) populations play a major role in regulating gastrointestinal mucosal immune responses. The morphometry of the villous-crypt axis in the small intestine reflects the function and adaptation of intestinal mucosal barriers. It is therefore of interest to investigate the potentially disparate phenotypic expression of DC and the morphology of intestinal mucosa found in different periods.

Research frontiers

PPs are typical gut-associated lymphoid tissues, which contain large amounts of immunocytes. The PP-DC can result in immune response to the intestinal mucosa, and therefore will not stimulate the immune response system. However, the study on DC was most thorough *in vitro* or in serum, because the techniques for DC isolation from mucosal lymphoid issue such as PP are relatively rare. Therefore, this research investigated the PP-DCs and the morphology of intestinal mucosa at different periods as a basis for determining the mechanism by which it may initiate regulatory events that are apparently critical in intestinal mucosal immunity.

Innovations and breakthrough

The authors evaluated, perhaps for the first time, the morphological and ontogeny of dendritic cells at different development periods. The analysis was carried out in 5 groups As a result, all morphometric parameters (including villous height, villous width, villous areas, crypt depth, and villous height and crypt depth ratios) changed significantly with the development of pups at the different age groups (F = 10.751, 12.374, 16.527, 5.291, 3.486; P < 0.05). The $OX62^+CD4^+SIRP^+$DC subset detected in single-cell suspensions of the rat total PP-DCs increased significantly at 3-11 wk postpartum, while the $OX62^+CD4^-SIRP^-$DC levels varied in different age groups.

Applications

This analysis of morphometric parameters and ontogeny of dendritic cells at different development periods may help further study the gastrointestinal mucosal immunity.

Peer review

The measurement of morphometric parameters and ontogeny of immunity cells in gastrointestinal mucosa is useful for studies of gastrointestinal mucosal immunity. Although further studies are required, this study indicates the novel possibility for investigating the mucosal immunity cells in gastrointestine.

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BRIEF ARTICLES

Meta-analysis of capsule endoscopy in patients diagnosed or suspected with esophageal varices

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Abstract

AIM: To review the literature on capsule endoscopy (CE) for detecting esophageal varices using conventional esophagogastroduodenoscopy (EGD) as the standard.

METHODS: A strict literature search of studies comparing the yield of CE and EGD in patients diagnosed or suspected as having esophageal varices was conducted by both computer search and manual search. Data were extracted to estimate the pooled diagnostic sensitivity and specificity.

RESULTS: There were seven studies appropriate for meta-analysis in our study, involving 446 patients. The pooled sensitivity and specificity of CE for detecting esophageal varices were 85.8% and 80.5%, respectively. In subgroup analysis, the pooled sensitivity and specificity were 82.7% and 54.8% in screened patients, and 87.3% and 84.7% in the screened/patients under surveillance, respectively.

CONCLUSION: CE appears to have acceptable sensitivity and specificity in detecting esophageal varices. However, data are insufficient to determine the accurate diagnostic value of CE in the screen/surveillance of patients alone.

Key words: Meta-analysis; Esophageal varices; Esophageal capsule endoscopy; Sensitivity; Specificity

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INTRODUCTION

Variceal bleeding is a significant contributor to the morbidity and mortality associated with cirrhosis and portal hypertension. It has been estimated that up to 90% of patients with cirrhosis will ultimately develop esophageal varices[1]. Once the varices develop, the occurrence of subsequent variceal bleeding in the next 24 mo is 25%-35%, and the risk of the patient dying as a result of the index hemorrhage is up to 50%[2,3]. Esophagogastroduodenoscopy (EGD) is the most effective method of evaluating patients with portal hypertension and cirrhosis[4]. However, the procedure is unpleasant, and still associated with a small but potential risk of complications[5]. In addition, it is often carried out with the patients under sedation, which may bring additional complications in patients with cirrhosis[6].

The PillCam ESO (Given Imaging, Israel) (esophageal capsule endoscopy, ECE) is a novel, wireless endoscope, similar in size to that of the standard PillCam small-bowel capsule endoscope (SBCE), which acquires video-images from both ends of the device during passage through the esophagus, and it has been approved by the United States Food and Drug Administration[7]. ECE provides a potentially less invasive diagnostic alternative in evaluating diseases of the esophagus such as esophageal varices, Barrett's esophagus, and gastroesophageal reflux disease (GERD)/erosive esophagitis[8-11]. Furthermore, Ramirez *et al*[12] attached a string to SBCE in the middle of the device which can allow the capsule to move up and down in the esophagus by swallowing the capsule and pulling the string. This

improvement can extend the retention time of CE in the esophagus and provide adequate information about esophageal details.

Recent years, a number of studies have been performed for evaluating CE in diagnosing esophageal disease, especially for Barrett's esophagus and esophageal varices, and the results varied[8-10]. If CE is a definitive diagnostic tool for esophageal varices, it is necessary to evaluate whether CE is sufficiently accurate for this purpose. In order to estimate the sensitivity and specificity of CE in the diagnosis of esophageal varices, a systematic review and meta-analysis of studies using CE for detecting esophageal varices with EGD as the standard was conducted.

MATERIALS AND METHODS

Study selection

A search in MEDLINE, EMBASE and ENBASE reviews (CDSR, ACP Journal Club, DARE, CCTR, CLCMR, CLHTA and CLEED) was conducted in May 2008. We did not confine our search to English language publications. Thorough literature retrieval for studies comparing CE and EGD for detecting esophageal varices in patients diagnosed or suspected as having esophageal varices was conducted along with a search of reference lists.

An additional search of abstracts presented at the proceedings of Digestive Disease Week (DDW) from 2004 to 2008 and international conference on CE through 2004 to 2007 was performed. If multiple updates of the same data were found, we selected only the most recent version or the most complete data for analysis. The search strategy employed is shown in Figure 1.

Data extraction

Predefined criteria had to be met for the studies to be included. Studies comparing CE diagnostic accuracy in patients diagnosed or suspected as having esophageal varices with EGD as standard were included. CE frames were assessed by an investigator who was blinded to patient's EGD findings. The studies which met the following one or more criteria were excluded: (1) esophageal varices were detected by CE but were performed in obscure gastrointestinal bleeding patients or for assessment of small bowel diseases or in miscellaneous patients with esophageal diseases; (2) string CE was used to detect esophageal varices; and (3) studies with patient number less than 10.

The study parameters were extracted first independently and subsequently in consensus if there was a disagreement between the reviewers (Liao Z, Gao R) concerning the numeric value of a parameter. Data were extracted based on the previous reported standards. The absolute number of true-positive, false-positive, true-negative and false-negative was retrieved or calculated with Bayes theorem if values for sensitivity and specificity and predictive values were reported. These were also performed separately by the two reviewers and subsequently checked for an agreement. The full text of papers (if available) of all relevant studies were obtained.

Statistical analysis

Meta-analysis was undertaken using MetaDiSc statistical software (Meta-DiSc, version 1.4, Madrid, Spain) to estimate the overall sensitivity and specificity. χ^2 test was then performed to test for heterogeneity between studies, *P* value less than 0.1 was considered significant for heterogeneity. Wherever zero counts occurred for study data, a continuity correction of 0.5 was added to each value for that study. In order to define the calculation of sensitivity and specificity, fixed effect model was used when *P* value was more than 0.1 for heterogeneity test and random effect model used for *P* value was less than 0.1[13].

RESULTS

Eighty-one articles were selected through original searches in MEDLINE, EMBASE and ENBASE reviews (CDSR, ACP Journal Club, DARE, CCTR, CLCMR, CLHTA and CLEED) databases. Fifty-one articles were excluded after review of the titles and abstracts, leaving 30 articles for detailed evaluation by two independent reviewers. Of these, five met our inclusion criteria[10,14-17]. Two studies were identified by hand search in DDW 2008 meeting abstracts and reference lists respectively[18,19]. In total, seven studies, involving 446 patients were appropriate for meta-analysis. In the seven studies, CE type was PillCam ESO without string and they were all published in English. One study was performed in western Europe[14], one in Australia[15], two were international multi-center studies[10,17] and three in the USA[16,18,19]. The patient inclusion/exclusion criteria were all reported in these studies. All patients were for clinically indicated screening (suspected) or surveillance (diagnosed) of esophageal varices. EGD was performed after CE in the same day for most patients, and all patients in these studies served as their own controls. All endoscopists who assessed the CE images were blinded to the EGD diagnoses. CE transit time was variable from 134.5 s (median) to 251 s (Table 1).

Forrest plots for sensitivity and specificity are shown in Figures 2 and 3. Reported sensitivity ranged from 68.4% to 100% in the individual studies, while specificity ranged from 8.3% to 100%. The pooled estimate of sensitivity was 85.8% (95% CI: 80.5%-90.1%) and the pooled estimate of specificity was 80.5% (95% CI: 74.7%-85.5%). However, both estimates were subject to significant heterogeneity ($P = 0.010$ and $P < 0.001$, respectively). In the presence of such heterogeneity, pooled estimates should be interpreted with caution.

Studies included in our analysis were further classified into two subgroups: screening group and screening/surveillance group. There were four studies including 106 patients in the screening group, no heterogeneity ($P = 0.166$) was found for the analysis of sensitivity, and the pooled sensitivity was 82.7% (95% CI: 72.2%-90.4%),

Table 1 Summary of studies included for meta-analysis

Author and year of publication	Patients (screening)	Country	Article type	Study design	Setting	Patients inclusion/ exclusion criteria reported	Two methods performed time	Transit time in esophagus
Lapalus, 2006	20 (20)	France	O	P, B	S	Yes	EGD after ECE on the same day	213 s
Eisen, 2006	32 (10)	International	O	P, B	3 centers	Yes	EGD after ECE on the same day	134.5 s (median)
Smith, 2007	15 (15)	Australia	A	NR, B	S	Yes	EGD within 1-2 h after ECE	NR
Groce, 2007	21 (21)	USA	A	P, B	S	Yes	EGD immediately after ECE	251 s
Pena, 2008	20 (8)	USA	O	P, B	S	Yes	EGD within 1h after ECE	NR
Jensen, 2008	50 (50)	USA	O	P, B	S	Yes	NR	NR
de Franchis, 2008	288 (195)	International	O	P, B	11 centers	Yes	EGD within 48 h after ECE	NR

O: Original paper; A: Abstract; P: Prospective; B: Investigator who reviewed ECE results was blinded to EGD results; NR: Not reported; S: Single center.

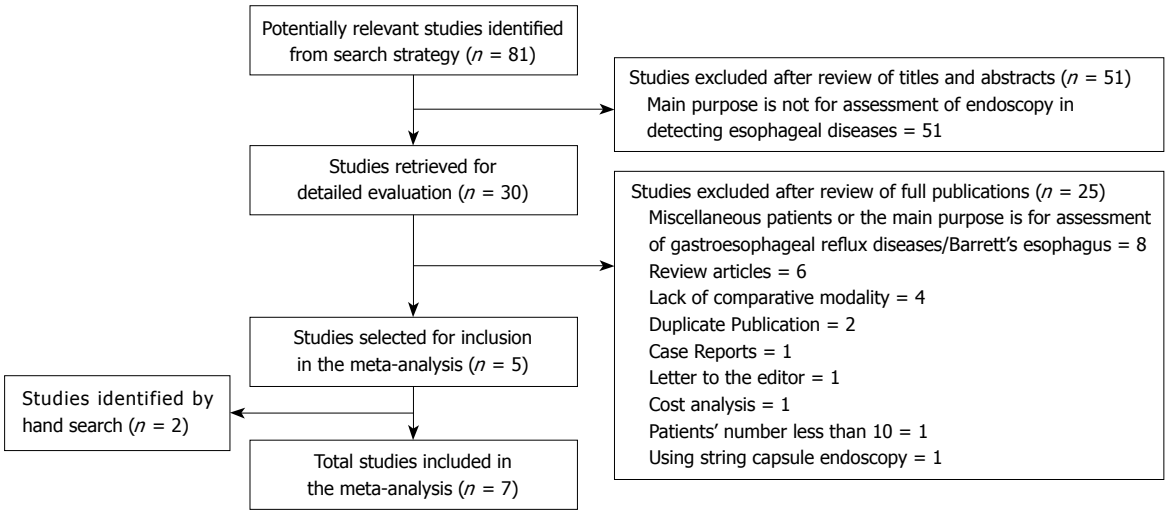

Figure 1 Search flow for Meta-analysis.

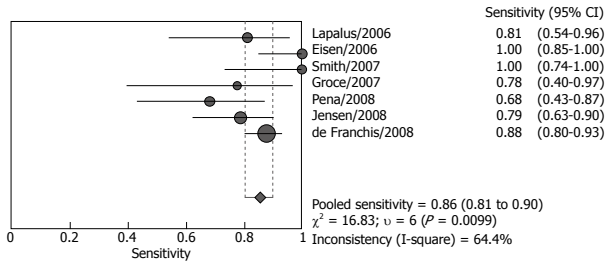

Figure 2 Pooled sensitivity of total studies included for meta-analysis.

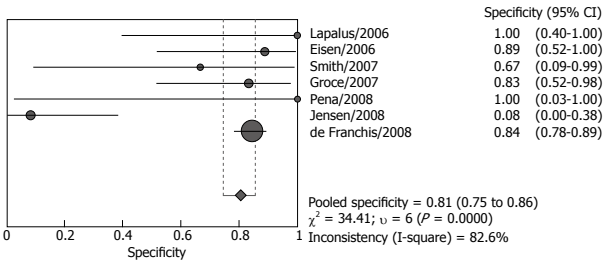

Figure 3 Pooled specificity of total studies included for meta-analysis.

however, heterogeneity was found significant for specificity ($P < 0.001$), the pooled result was 54.8 % (95% CI: 36.0%-72.7%) (Figures 4 and 5). Both screened patients and patients under surveillance were included in the other three studies, involving 340 patients, and the detailed endoscopic results cannot be independently extracted for screen patients or surveillance patients. The pooled sensitivity was 87.3% (95% CI: 80.9%-92.2%) in a random effect model ($P = 0.004$) and pooled specificity was 84.7% (95% CI: 78.8%-89.5%) in a fixed effect model ($P = 0.789$). (Figures 6 and 7)

DISCUSSION

Meta-analysis has been performed for SBCE in detecting small bowel disease comparing with other methods. It is reported SBCE was superior when compared with push enteroscopy and small bowel barium radiography for OGIB ($P < 0.00001$)[20]. In detecting Crohn's disease, SBCE was also superior to small bowel barium radiography ($P < 0.001$), colonoscopy with ileoscopy ($P = 0.02$) and CT ($P = 0.001$)[21]. Furthermore, the yield of SBCE for small bowel diseases was similar to double-balloon enteroscopy in combination with oral and anal approaches[22].

In the largest study performed in 11 centers (288 patients), the sensitivity and specificity were 88.0% and 84.4%, respectively, and ECE had good agreement with EGD[17]. The present meta-analysis indicates that CE appears to have acceptable sensitivity and specificity for esophageal varices with EGD as the standard. However, the limitations of these data need to be appreciated.

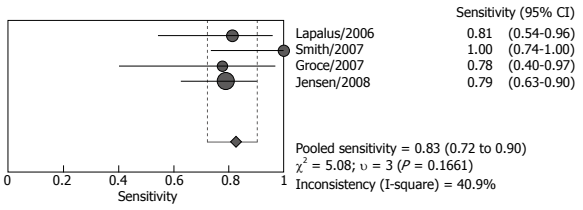

Figure 4 Pooled sensitivity of studies for screening patients.

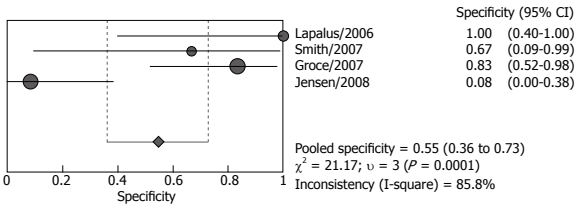

Figure 5 Pooled specificity of studies for screening patients.

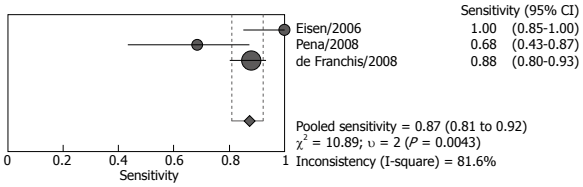

Figure 6 Pooled sensitivity of studies for screening/surveillance patients.

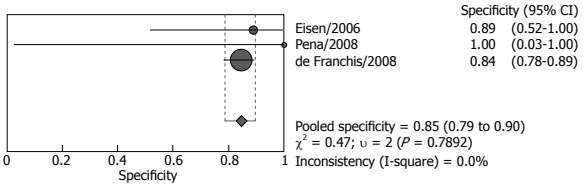

Figure 7 Pooled specificity of studies for screening/surveillance patients.

Firstly, because this new device has not been widely used for detecting esophageal varices, and the number of studies was small. Secondly, sample sizes of different studies were significantly different, ranging from 15 to 288 patients. So we considered the *P* value less than 0.1 as significant for heterogeneity in this analysis. At last, patients underwent CE including both screened patients and patients under surveillance, in 3 studies the detailed endoscopic results can not be extracted for these patients, and the accurate pooled diagnostic value of CE for patients under surveillance was not known.

There was considerable heterogeneity between the studies. In Pena's and Groce's studies, the sensitivity of ECE in detecting esophageal varices was only 68.4% and 77.8% respectively[16,18]. In Eisen's and Smith's studies, the sensitivity was both as high as 100%[10,15]. The diagnostic specificity of these seven studies varied significantly, the specificity was lowest in Jensen's study, only 8.3%, and the accuracy of capsule for detecting esophageal varices was modest[19]. However, in Lapalus's[14] and Pena's[16] studies, the specificity was both 100%. There was no heterogeneity in the analysis of sensitivity and specificity for the screening group and the screening/surveillance group, respectively. Although the sample was too small to undertake meta-regression to identify the cause of the heterogeneity, some potential reasons for heterogeneity may be identified. Except for the small sample size of most studies, the most possible causes for the existence of heterogeneity may be related to the experience of the endoscopists and the number of endoscopists who read the capsule images. Although the ECE investigators were all blinded to EGD findings, their experience was not described in detail. Only in Pena's study, the endoscopists were those with more than 5 years of experience in reading SBCE but no experience in reading ECE[16]. In two studies, ECE images were reviewed by two independent investigators[14,19], and one investigator in an other five studies[10,19].

The pooled sensitivity and specificity of the screening/surveillance group was higher than the screening group. The pooled data of screened patients may be confused by Jensen's study, which had the largest number of patients in the screening studies and with a specificity of 8.3%[19]. In studies with screening/surveillance patients, the detailed results cannot be extracted, so the pooled data of surveillance patients cannot be obtained. One defect in the three studies was the percentages of screening or surveillance patients that were variable (Figure 1).

In summary, CE appears to have acceptable sensitivity and specificity in detecting esophageal varices, however, it seems inaccurate in screening patients based on the present data. There was insufficient data to determine the accurate diagnostic value of CE in patients under surveillance alone. Also, further researches with large numbers of patients are needed.

COMMENTS

Background

Capsule endoscopy (CE) has been reported to play an important role in detecting esophageal diseases, especially for esophageal varices. There are concerns about whether CE is sufficiently accurate for evaluating this desease.

Research frontiers

Data concerning CE in detecting esophageal varices with esophagogastroduo denoscopy (EGD) results as the standard were derived from published original papers or conference abstracts. A meta-analysis was conducted to estimate the sensitivity and specificity of CE in diagnosis of esophageal varices.

Innovations and breakthroughs

The current study demonstrated that CE appears to have acceptable sensitivity and specificity in detecting esophageal varices. However, it seems inaccurate in screening patients.

Applications

The diagnostic accuracy of CE was low in screening patients in this study. Studies for further evaluating CE diagnostic accuracy in screening patients alone were needed.

Terminology

Esophageal capsule endoscope (ECE) is a novel, wireless endoscope, similar in size to small-bowel capsule endoscope, which acquires video-images from both ends of the device during passage through the esophagus.

Peer review

This meta-analysis was performed based on five peer reviewed papers and 2 in abstract form, involving about 500 patients. This study was well done, however, the missing rate of the small-sized varices is high and this tool it suitable for medium- and large-sized varices.

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Antisense expression of PKCα improved sensitivity of SGC7901/VCR cells to doxorubicin

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Abstract

AIM: To explore whether antisense blocking of protein kinase C alpha (PKCα) would reverse multi-drug resistance (MDR) in the vincristine (VCR)-resistant human gastric cancer cell line SGC7901/VCR.

METHODS: SGC7901/VCR cells expressing antisense PKCα, SGC7901/VCR/aPKC, were established by transfection with a recombinant plasmid reversely inserted with PKCα cDNA. Empty vector (PCI-neo)-transfected cell clones, SGC7901/VCR/neo, served as the control. Western blot method was used to detect PKCα content in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells, using PKCα-specific antibody. The sensitivity of SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells to doxorubicin (DOX) *in vitro* was determined by MTT assay. The uptake of DOX in these cells was detected with fluorescence spectrophotometer.

RESULTS: Western blot analysis showed that the PKCα protein level was about 8.7-fold higher in SGC7901/VCR cells than that in SGC7901 cells, whereas the protein expression of PKCα was reduced by 78% in SGC7901/VCR/aPKC cells when compared with the SGC7901/VCR cells. SGC7901/VCR/aPKC cells had a 4.2-fold increase in DOX cytotoxicity, accompanied by a 1.7-fold increase of DOX accumulation in comparison with SGC7901/VCR cells.

CONCLUSION: PKCα positively regulates MDR in SGC7901 cells, and inhibition of PKCα can partially attenuate MDR in human gastric cancer cells.

Key words: Multi-drug resistance; Protein kinase C alpha; SGC7901; Gastric cancer

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INTRODUCTION

Resistance of cancer cells to chemotherapeutic drugs is a major problem in clinical treatment of cancer. Multi-drug resistance (MDR) is the most important form of drug resistance characterized by decreased cellular sensitivity to a broad range of chemotherapeutic drugs, including anthracyclines, vinca alkaloids, epipodophyllotoxins, taxanes, antibiotics and some of the new topo- I inhibitors[1,2]. Classical MDR is mainly caused by overexpression of P-glycoprotein (Pgp), which is coded by the MDR1 gene and functions as an ATP-dependent drug-efflux membrane transporter that rapidly extrudes a variety of hydrophobic anticancer drugs from exerting cytotoxic effect[3,4]. Additionally, studies have shown that MDR is also accompanied by changes in the activity of protein kinase C (PKC).

Activation of PKC results in phosphorylation of Pgp and a decrease in drug accumulation, while inhibition of PKC can partially reverse the MDR phenotype[5,6].

PKC comprises a family of at least 12 distinct serine/threonine kinase isoenzymes which are found in varying ratios in the cytosolic and membrane fractions of cells, depending on the type of issue and its physiological state[6,7]. The family of PKC is composed of three subclasses: classical, novel and atypical PKC, each having different isoforms. The members of the classical PKCs (α, β I , β II and γ) bind phorbol esters and are Ca^{2+} dependent. The novel PKCs (δ, ε and η) do not depend on Ca^{2+} but bind phorbol esters. The third subfamily includes the atypical PKCs (τ, ζ, λ and μ), which do not bind to either Ca^{2+} or phorbol ester. It is likely that each isoform has a specific role in a given cell[5,7]. PKC regulates numerous cell processes including proliferation, apoptosis and differentiation by phosphorylating proteins in response to transmembrane signals from hormones, growth factors, neuro-transmitters and pharmacological agents[5]. Recently accumulated evidence indicated that PKC, especially classical PKC, plays a significant role in the formation of cancer MDR[5,6]. The isoenzymes of PKC possess distinct differences in localization in different cells, and research on distinct function of isoforms in cancer MDR has important significance[5,6].

Among those isoforms of PKC, PKCα is likely to play a decisive role in maintaining MDR phenotypes in some cancer cells, and may therefore represent potential novel targets for the treatment of cancers[6,8-12]. In an effort to see whether down-regulation of a single isoform of PKC could affect drug resistance, vincristine (VCR)-resistant human gastric carcinoma cell line SGC7901/VCR was transfected with an expression vector containing the cDNA for PKCα in the antisense orientation, and their expression of PKCα, doxorubicin (DOX) sensitivity and DOX accumulation were determined.

MATERIALS AND METHODS

Materials

MTT and DOX were purchased from Sigma, VCR from the Twelfth Shanghai Pharmaceutical Product Factory, Lipofectamine 2000 from Invitrogen, nitrocellulose membranes and 3', 3'-diaminobenzidene (DAB) from Sigma, G418 and rabbit polycolonal anti-PKCα from Gibeco-BRL. Plasmid pSP64-PKCα was kindly provided by Dr. PJ Parker (Imperial Cancer Foundation, England). The eukaryotic expression vector, plasmid PCI-neo, was kindly provided by Dr. Jun-Jie Xu (Institute of Microbiology and Epidemiology, Chinese Academy of Military Medical Sciences). The human gastric cancer cell line SGC7901, and its VCR-resistant counterpart SGC7901/VCR selected by stepwise exposure of parental SGC7901 cells to increasing concentrations of VCR, were purchased from Wuhan University Type Culture Collection (Wuhan, China).

Cell culture

Both SGC7901 and SGC7901/VCR cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum at 37℃ in a humidified atmosphere of 5% CO_2. The SGC7901/VCR cells were cultured in the presence of 0.8 μmol/L VCR and grown in drug-free medium 2 wk before the experiments.

Plasmid construction

The full-length cDNA encoding PKCα cDNA (2.3 kb) was recovered from Sal I sites in pSP64-PKCα plasmid and subcloned into Sal I sites of PCI-neo plasmid. The recombinant plasmid with the PKCα cDNA in the antisense orientation was confirmed by BamH1 and/or Sal I restriction digestion, and designated PCI-neo-aPKCα.

Cloning of cells transfected with antisense vector PCI-neo-aPKCα

The process was performed as described by Wang *et al*[13]. Briefly, SGC7901/VCR cells were seeded in six-well plates to 70%-80% confluence. Empty vector PCI-neo and antisense vector PCI-neo-aPKCα were transfected into SGC7901/VCR cells *via* Lipofectamine 2000. The two kinds of transfected cells were named SGC7901/VCR /neo and SGC7901/VCR/aPKC respectively. After two days of transfection, cells were selected by culture medium containing G418 (400 mg/L) for 2 wk. The single clone was picked out using limiting dilution method and was expanded and maintained in medium containing 400 mg/L G418 until 1 wk prior to experiments.

Western blot analysis

The whole cell lysates were extracted with lysis buffer containing 1% Triton-100, 50 mmol/L NaCl, 50 mmol/L sodium fluoride, 20 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium vanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 0.5% NP40. An aliquot was taken for protein determination, and the remainder was mixed (1:1) with 2 × SDS sample buffer and boiled for 5 min. Samples were run on 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. After blocking, the membranes were incubated with rabbit polycolonal antibody against PKCα isoform at a dilution of 1:1000. Blots were washed three times with PBST to block nonspecific binding sites and incubated with secondary antiserum (goat anti-rabbit IgG conjugated to horseradish peroxidase) for 1 h at 37℃, then washed three times with 50 mmol/L Tris-HCl (pH 6.8). Color was developed with DAB as the substrate.

Cytotoxicity assay

The cells in exponential growth were seeded in 96-well plates at a density of 2×10^4 cells per well and 24 h later graded DOX (at the concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10 μmol/L) were added, respectively. The total medium volume of each well was 200 μL. Three days after drug addition, 10 μL MTT (5.0 g/L in PBS) was added. After 4 h of incubation, supernatants were removed and replaced by 150 μL DMSO. After formazan solubilization, the absorbance at 570 nm

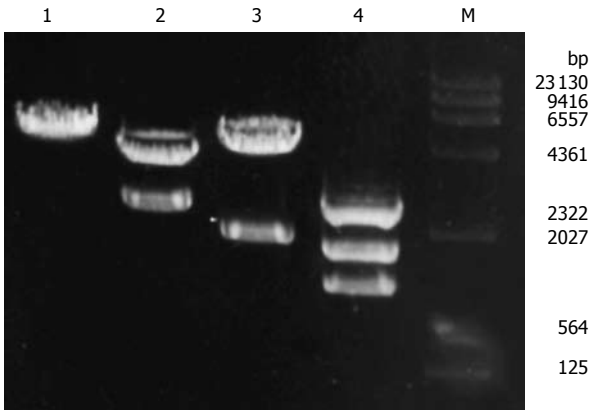

Figure 1 Restriction map of recombinant plasmid PCI-neo-aPKCα. Lane 1: PCI-neo digested with *Sal* I *Eco*R1; Lane 2: PCI-neo-aPKCα digested with BamH1; Lane 3: PCI-neo-aPKCα digested with Sa I; Lane 4: PCI-neo-aPKCα digested with *Bam*H1 + *Sal* I ; Lane M: λ DNA *Hind* Ⅲ Marker.

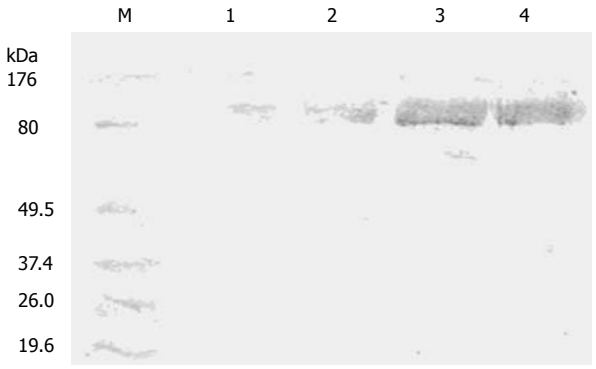

Figure 2 Western blot identification of PKCα protein in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells. Lane M: Benchmark™ Marker; Lane 1: SGC7901 cells; Lane 2: SGC7901/VCR/aPKC cells; Lane 3: SGC7901/VCR/neo cells; Lane 4: SGC7901/VCR cells.

was recorded using an automated microplate reader. IC_{50} (concentration resulting in 50% inhibition of cell growth) values for DOX were calculated as 100% from plotted results using untreated cells.

Intracellular DOX accumulation

The assay was performed as described by our previous report[2]. Briefly, 1.0×10^6 cells were exposed to 4.0 μmol/L DOX for 60 min. Following incubation, the cells were washed twice with ice-cold PBS, then suspended in 6.0 mL of 50% ethanol -0.3 mol/L hydrochloride, extracted for 2 h, centrifugated at $100 \times g$ for 10 min, and the supernatant was collected. DOX-associated mean fluorescence intensity (MFI) was measured by fluorescence spectrophotometer at an excitation wavelength of 488 nm and an emission wavelength of 590 nm. The DOX standard solution ($0.1\text{-}1.0 \times 10^3$ nmol/L) was prepared, and a standard work curve with related quotient was constructed.

Statistical analysis

Data were expressed as mean ± SD. Student's *t* test was used to assess statistical significance of differences. $P < 0.05$ was considered statistically significant.

RESULTS

Establishment of SGC7901/VCR cells expressing antisense PKCα

To construct the PKCα antisense expression plasmid, the full-length cDNA of PKCα (2.3 kb) recovered from Sal I sites in pSP64-PKCα plasmid was subcloned into Sal I sites of the eukaryotic expression vector PCI-neo (5.5 kb). A recombinant plasmid with PKCα cDNA in the antisense orientation was obtained and designated PCI-neo-aPKCα, which contains the neomycin resistance gene neo for drug selection. Two fragments were obtained by digesting PCI-neo-aPKCα with *Sal* I , one was PKCα cDNA fragment about 2.3 kb and the other was PCI-neo cDNA fragment about 5.5 kb (Figure 1). And the inserted fragment was verified by sequencing (data not shown). SGC7901/VCR cells were transfected with either PCI-neo or PCI-neo-aPKCα, respectively, and G418-resistant clones were isolated and amplified after G418 selection. Empty vector-transfected cell clones were named SGC7901/VCR/neo, which together with the parental cell line served as the controls for these experiments. Clones transfected with the PKCα antisense expression plasmid (PCI-neo-aPKCα) were named SGC7901/VCR/aPKC.

Western blot analysis was performed to assess protein abundance of PKCα in SGC7901, SGC7901/VCR, SGC7901/VCR/aPKC and SGC7901/VCR/neo cells. As shown in Figure 2, the PKCα protein level was about 8.7-fold higher in SGC7901/VCR cells than that in SGC7901 cells. Transfection of SGC7901/VCR cells with the control vector did not alter PKCα expression, whereas the protein expression of PKCα was reduced by 78% in SGC7901/VCR/aPKC cells when compared with the SGC7901/VCR cells.

Modulating effect of antisense expression of PkCα on MDR

Using the MTT assay, the *in vitro* cytotoxicity of DOX in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells was examined. As shown in Table 1, SGC7901/VCR cells were 23.5 times more resistant to DOX in comparison with SGC7901 cells. The IC_{50} in SGC7901/VCR/aPKC was 4.2-fold lower than that in SGC7901/VCR cells, indicating that antisense PkCα could partially reverse resistance to DOX in multi-drug resistant SGC7901 cells.

Effect of antisense expression of protein kinase Cα on intracellular accumulation of DOX in multi-drug resistant SGC7901 cells

DOX accumulation in SGC7901, SGC7901/VCR, SGC7901/VCR/neo cells and SGC7901/VCR/aPKC was measured by fluorescence spectrophotometry after incubation of cells with 4.0 μmol/L DOX for 60 min. As shown in Figure 3, SGC7901/VCR cells accumulated 4.3-fold less DOX than SGC7901 cells, whereas SGC7901/VCR/aPKC cells showed 1.7-fold more drug retention than SGC7901/VCR cells.

Table 1 Modulating effect of antisense expression of protein kinase Cα on resistance to DOX in multi-drug resistant SGC7901 cells

Cell lines	IC_{50} of DOX (μmol/L)	Resistance index (RI)
SGC7901	0.076 ± 0.0074	1.0
SGC7901/VCR	2.4 ± 0.14[b]	31.6
SGC7901/VCR/neo	2.3 ± 0.19[b]	30.3
SGC7901/VCR/aPKC	0.58 ± 0.076[b]	7.6

[b]$P < 0.01$ *vs* IC_{50} of SGC7901. The data are presented as mean ± SD.

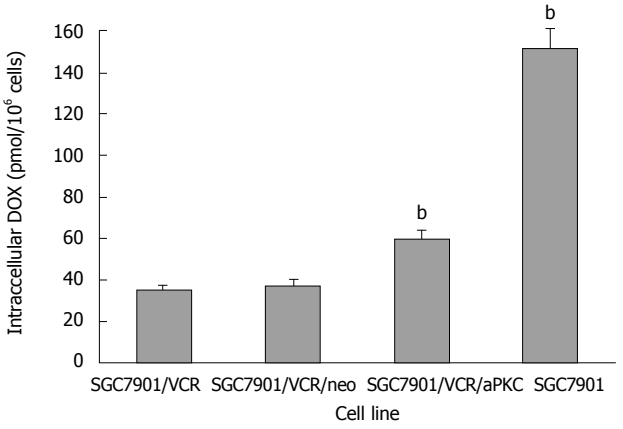

Figure 3 Accumulation of intracellular DOX in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells. Cells were incubated with 4.0 μmol/L DOX for 60 min. Each point represents the mean ± SD from three experiments. [b]$P < 0.01$ *vs* SGC7901/VCR cells.

DISCUSSION

MDR to cancer treatment has been studied for more than 20 years. No useful method of reversing MDR suitable for clinical use has yet emerged from this large quantity of work. The reason could be complicated mechanisms involved. There are several ways for cancer cells to develop MDR[4]. The most investigated mechanisms with known clinical significance are: (1) activation of transmembrane proteins effluxing different chemical substances from the cells, in which Pgp is the best known efflux pump; (2) activation of the enzymes of the glutathione detoxification system; (3) alterations of the genes and the proteins involved in the control of apoptosis (especially p53 and Bcl-2)[10]. Recent studies have indicated a role for PKC in the regulation of the MDR phenotype. A number of studies have demonstrated that PKC activity was elevated in MDR-selected cell lines, and several PKC inhibitors are able to partially reverse MDR and inhibit Pgp phosphorylation[5,6]. Pgp is phosphorylated by PKC and that phosphorylation positively modulates its transport function[14]. The PKC family consists of at least 12 isoforms, and different isoforms of PKC possess distinct differences in expression and function in different MDR cells[12-15]. The elevated PKC activity level of the MDR cancer cells was frequently a result of increased PKCα expression in most cancers[8-12], but in some cancers it was due to enhanced expression of other isoforms, such as PKCβ I , PKCγ or PKCη[15-18].

SGC7901/VCR is an established VCR-resistant cell line selected by stepwise exposure of parental SGC7901 cells to increasing concentrations of VCR. Previous reports have proved that the SGC7901/VCR cells possess the characteristics of classical MDR with overexpression of Pgp, and the SGC7901/VCR cell line has been successfully used as an *in vitro* MDR reversal model by several study groups, including ours[19-23]. The recent report showed that the expression of PKCα was significantly higher in SGC7901/VCR cells than in SGC7901 cells, and there was no significant difference in the expression of PKCβ I , PKCβ II and PKCγ between SGC7901/VCR and SGC7901 cells[10].

In the present study, we observed that the expression of PKCα was significantly higher in SGC7901/VCR cells than in SGC7901 cells, which was consistent with the previous report by Han *et al*[10]. To investigate the possible effect of PKCα on MDR for chemotherapeutic drug in human gastric cancer cells, we established SGC7901/VCR cells stably expressing antisense PKCα. We demonstrated that down-regulation of the predominant PKCα isoform expressed in SGC7901/VCR cells by the expression of antisense PKCα led to greater DOX accumulation and partial reversal of resistance to DOX. These data support the thesis that MDR in this cell line is modulated, in part, by PKCα. These results suggest that a more effective means of reducing PKCα activity, possibly with RNA interference, might be a more efficient way of increasing drug sensitivity in this MDR cell line.

COMMENTS

Background

Protein kinase C (PKC) constitutes a family of closely related protein serine/threonine kinase which are sub-grouped into classical (α, β I , β II , and γ), novel (δ, ε, η, and θ), and atypical (ι and ζ) isoforms. The members of the PKC family are involved in the regulation of numerous cell processes including proliferation, apoptosis, and differentiation. It is likely that each isoform has a specific role in a given cell.

Research frontiers

A recent study confirmed that PKCα, PKCβ I , PKCβ II and PKCγ were expressed in both human gastric carcinoma cell line SGC7901 and vincristine (VCR)-resistant human gastric carcinoma cell line SGC7901/VCR, but the expression of PKCα was significantly higher in SGC7901/VCR cells than that in SGC7901 cells. There was no significant difference in the expression of PKC-β I , PKC-β II and PKCγ between SGC7901/VCR cells and SGC7901 cells. In this report, the authors transfected SGC7901/VCR cells with an expression vector containing the cDNA for PKCα in the antisense orientation to see whether down-regulation of PKCα could affect drug resistance in SGC7901/VCR cells.

Innovations and breakthroughs

In the present study the authors observed that down-regulation of the predominant PKCα isoform expressed in SGC7901/VCR cells by the expression of antisense PKCα led to greater doxorubicin accumulation and partial reversal of resistance to doxorubicin. These data support the thesis that MDR in this cell line is modulated in part by PKCα.

Applications

This study provided a new target against MDR in human gastric carcinoma, suggesting that a more effective means of reducing PKCα activity, possibly with RNA interference, might be a more efficient way of increasing drug sensitivity in this MDR cell line.

Peer review

The manuscript entitled "Antisense expression of PKCα improved sensitivity of SGC7901/VCR cells to doxorubicin" by Wu *et al* examines the effects of anti-

sense inhibition of PKCα expression on the MDR phenotype of gastric cancer cells. The authors used antisense technology to establish a derivative of the multi-drug/vincristine-resistant SGC7901-VCR cell line with reduced expression of PKCα. Using this isogenic pair of cell lines, the authors conclude that PKCα deficiency leads to increased intracellular accumulation of DOX and partially restores sensitivity to the drug, thus reversing the MDR phenotype.

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CASE REPORT

Cannabinoid hyperemesis syndrome: Clinical diagnosis of an underrecognised manifestation of chronic cannabis abuse

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Abstract

Cannabis is a common drug of abuse that is associated with various long-term and short-term adverse effects. The nature of its association with vomiting after chronic abuse is obscure and is underrecognised by clinicians. In some patients this vomiting can take on a pattern similar to cyclic vomiting syndrome with a peculiar compulsive hot bathing pattern, which relieves intense feelings of nausea and accompanying symptoms. In this case report, we describe a twenty-two year-old-male with a history of chronic cannabis abuse presenting with recurrent vomiting, intense nausea and abdominal pain. In addition, the patient reported that the hot baths improved his symptoms during these episodes. Abstinence from cannabis led to resolution of the vomiting symptoms and abdominal pain. We conclude that in the setting of chronic cannabis abuse, patients presenting with chronic severe nausea and vomiting that can sometimes be accompanied by abdominal pain and compulsive hot bathing behaviour, in the absence of other obvious causes, a diagnosis of cannabinoid hyperemesis syndrome should be considered.

Key words: Cannabinoid; Cannabis; Cyclic vomiting; Hyperemesis; Marijuana; Vomiting

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INTRODUCTION

Cannabis has been used recreationally for millennia and is the third most commonly used drug after tobacco and alcohol[1,2]. Research into the neurobiology of the compound has led to the discovery of an endogenous cannabinoid system. The therapeutic potential of cannabinoids has been recognized and these compounds are utilized as anti-emetics[3-5]. Recently, a distinct syndrome in chronic cannabis abusers characterized by recurrent vomiting associated with abdominal pain and a tendency to take hot showers has been increasingly recognised. This clinical manifestation is paradoxical to the previously identified therapeutic role of cannabinoids as anti-emetics. We describe the case of a young male seeking repeated emergency room care with recurrent nausea and vomiting.

CASE REPORT

A 22-year male presented with recurrent episodes of nausea, refractory vomiting, and colicky epigastric pain for one week. The symptoms were characterized by treatment-resistant nausea in the morning, continuous vomiting, and colicky epigastric abdominal pain. Each episode lasted 2 to 3 h and increased with food intake. He often had two or more episodes a day during the symptomatic period. He had been treated for the severe nausea and vomiting in the emergency room on two occasions in the preceding two months. He also reported having learned to help himself by taking a hot bath each time the symptoms appeared, which dramatically

improved his symptoms. This habit had become a compulsion for him for symptom relief with each episode of hyperemesis. On physical examination his mucous membranes were dry, his pulse rate was 102/min and blood pressure was 140/100 with positive orthostasis. The remainder of the physical examination was unremarkable. His complete blood count and comprehensive metabolic panel were unremarkable. In addition, serum amylase and lipase levels were within the normal range. His urine drug screen was positive for tetrahydrocannabinol (THC). Abdominal X-ray series and ultrasonography were within normal limits.

Oesophagogastroduodenoscopy revealed Grade 2 distal oesophagitis and hiatal hernia. On further interviewing, he admitted to consistent marijuana abuse for the past 6 years, often smoking cannabis every hour or two on a daily basis. The patient and his mother did not recall any significant past illnesses or recurrent vomiting when he was a child. He was treated with intravenous fluids with steady improvement in symptoms, and metoclopramide, pantoprazole and morphine for the abdominal pain. It was explained that marijuana was the cause of his symptoms and he was advised not to resume marijuana abuse. On subsequent follow-up, he had abstained from cannabis and remained symptom-free.

DISCUSSION

Cannabis is one of the most commonly abused drugs worldwide. Over the past decade, marijuana has remained the most commonly used illicit substance with close to 50% of high school seniors admitting use at some time[1]. It is estimated that each year 2.6 million individuals in the USA become new users and most are younger than 19 years of age[6].

The long-term and short-term toxicity of cannabis abuse is associated with pathological and behavioural effects. However, cannabis has also been suggested to have therapeutic properties with anticonvulsive, analgesic, antianxiety and anti-emetic activities. Cannabis has also been used to treat anorexia in patients with acquired immunodeficiency syndrome[3-5]. The actions of cannabis are mediated by specific cannabinoid receptors. The first of the cannabinoid receptors-CB-1-was identified in 1990 and this finding revolutionized the study of cannabinoid biology. Since then, a multitude of roles for the endogenous cannabinoid system has been proposed. A large number of endogenous cannabinoid neurotransmitters or endocannabinoids have been identified, and the CB-1 and CB-2 cannabinoid receptors have been characterized[7]. The CB-1 receptors exert a neuromodulatory role in the central nervous system and enteric plexus[8]. Cannabinoid type 2 receptors have an immunomodulatory effect and are located on tissues such as microglia[5]. The presence of other receptors, transporters, and enzymes responsible for the synthesis or metabolism of endocannabinoids are being recognised at an extraordinary pace. Cannabinoids have a wide variety of effects on the body systems and physiologic states (Table 1) due to their actions on the receptors as well as direct toxic effects.

Table 1 Harmful effects of cannabinoids on body systems[1,6,7]

Cognitive and mental health
Impaired memory
Impaired attention, organization and integration of complex information
Association with schizophrenia
Increased risk for depression
Pulmonary
Carcinogenic effect
Obstructive lung disease
Increased propensity toward infections
Acute and chronic bronchitis
Behavioural
Weapon possession and physical fighting
Unwanted and unprotected sexual encounters
Unwanted pregnancies
School dropout
Amotivational syndrome
Impairment of driving skill and coordination
Endocrine
Decreased testosterone, sperm motility and production, disruption of ovulatory cycle
Pregnancy
Low birth weight
Problems with attention, memory and higher cognitive function
Cardiovascular
Stroke
Dose-dependent increase in HR
Orthostasis
Decreased exercise tolerance
Precipitation of angina or myocardial infarction

The anti-emetic effect of cannabinoids is largely mediated by CB-1 receptors in the brain and the intestinal tract, although some of their effect may also be receptor-independent. However, in this report, we were presented with the paradoxical effect of hyperemesis in a susceptible chronic cannabis abuser. Such a paradoxical response has previously only been demonstrated following acute toxicity to an intravenous injection of crude marijuana extract[9]. Proposed mechanisms of cannabinoid hyperemesis include toxicity due to marijuana's long half-life, fat solubility, delayed gastric emptying, and thermoregulatory and autonomic disequilibrium *via* the limbic system[10]. Cannabinoids are known to impair peristalsis in a dose-dependent manner[11,12], which can theoretically override the centrally mediated anti-emetic effects, thus leading to hyperemesis. It is not known why the hyperemesis syndrome surfaces after several years of cannabis abuse. The effects of cannabinoids on the functions of the thermoregulatory and autonomic mechanisms of the brain can lead to behavioural changes[10]. Such effects might be the underlying mechanism for the compulsive hot bathing behaviour. There is also a supposition that the syndrome could represent a type of cyclic vomiting. Cyclic vomiting syndrome (CVS) in adults is now very well recognized, and it has been proposed that marijuana contributes to CVS[13]. However, unlike the other forms of CVS, patients with cannabinoid hyperemesis are not likely to have a history of migraine or other psychosocial stressors and the peculiar behaviour of hot showers is

Table 2 Clinical diagnosis of cannabinoid hyperemesis

Essential for diagnosis:
History of regular cannabis use for years
Major clinical features of syndrome
Severe nausea and vomiting
Vomiting that recurs in a cyclic pattern over months
Resolution of symptoms after stopping cannabis use
Supportive features
Compulsive hot baths with symptom relief
Colicky abdominal pain
No evidence of gall bladder or pancreatic inflammation

unique to this syndrome.

Based on the published research and case reports[10,14-16], we propose the set of clinical characteristics for the diagnosis of cannabinoid hyperemesis syndrome shown in Table 2. Allen *et al*[10] first noted this condition in a group of nineteen patients from Australia with chronic cannabis abuse and cyclical vomiting illness. An earlier case report by de Moore *et al*[17] described a chronic cannabis abuser with psychogenic vomiting, which was complicated by spontaneous pneumomediastinum. Subsequent reports have identified similar clinical presentations[7-9,18]. Given the high prevalence of chronic cannabis abuse worldwide and the paucity of reports in the literature, clinicians need to be more attentive to the clinical features of this underrecognised condition.

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CASE REPORT

Liver cell adenoma showing sequential alteration of radiological findings suggestive of well-differentiated hepatocellular carcinoma

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Abstract

A liver tumor 35 mm in diameter was found incidentally in a 40-year-old woman who had no history of liver diseases or the use of oral contraceptives. Radiological diagnostics showed the typical findings of liver cell adenoma (LCA). Dynamic computed tomography revealed that the tumor showed a homogenous enhancement in the arterial phase and almost the same enhancement as the surrounding liver parenchyma in the delayed phase. The tumor was found to contain fat on magnetic resonance imaging. A benign fat containing liver tumor was suggested. However, radiological findings altered, which caused us to suspect that a well-differentiated hepatocellular carcinoma (HCC) containing fat was becoming dedifferentiated. Partial hepatectomy was performed and the pathological findings showed the typical findings of LCA. This case was an extremely rare LCA, which had no background of risk for LCA and developed the sequential alteration of the radiological findings to suspect well-differentiated HCC.

Key words: Liver cell adenoma; Hepatocellular carcinoma; Diagnosis; Hepatectomy

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INTRODUCTION

Liver cell adenoma (LCA) is a benign tumor of the liver parenchyma that is associated with the use of oral contraceptives or with glycogen-storage disease[1]. The occurrence of LCA in patients without such backgrounds is extremely rare[2]. We report a case of LCA found in a 40-year-old woman without a history of oral contraceptive use in which the sequential alteration of the radiological findings suggested well-differentiated hepatocellular carcinoma (HCC).

CASE REPORT

In November 2006, a 40-year-old woman developed lower abdominal pain and was admitted to a hospital. The patient had no history of liver disease, alcohol consumption, oral contraceptive use, nor the use of any other medication. The family history of the patient was not noteworthy. The patient was diagnosed as having a left tubo-ovarian abscess and tubo-ovariectomy was performed. The resected ovary and oviduct showed the findings of endometriosis with bacterial infection, but there was no indication of neoplastic lesion. At this time, a space-occupying lesion (SOL) 35 mm in diameter was found in the liver by abdominal ultrasonography and computed tomography (CT). In the CT, the lesion in

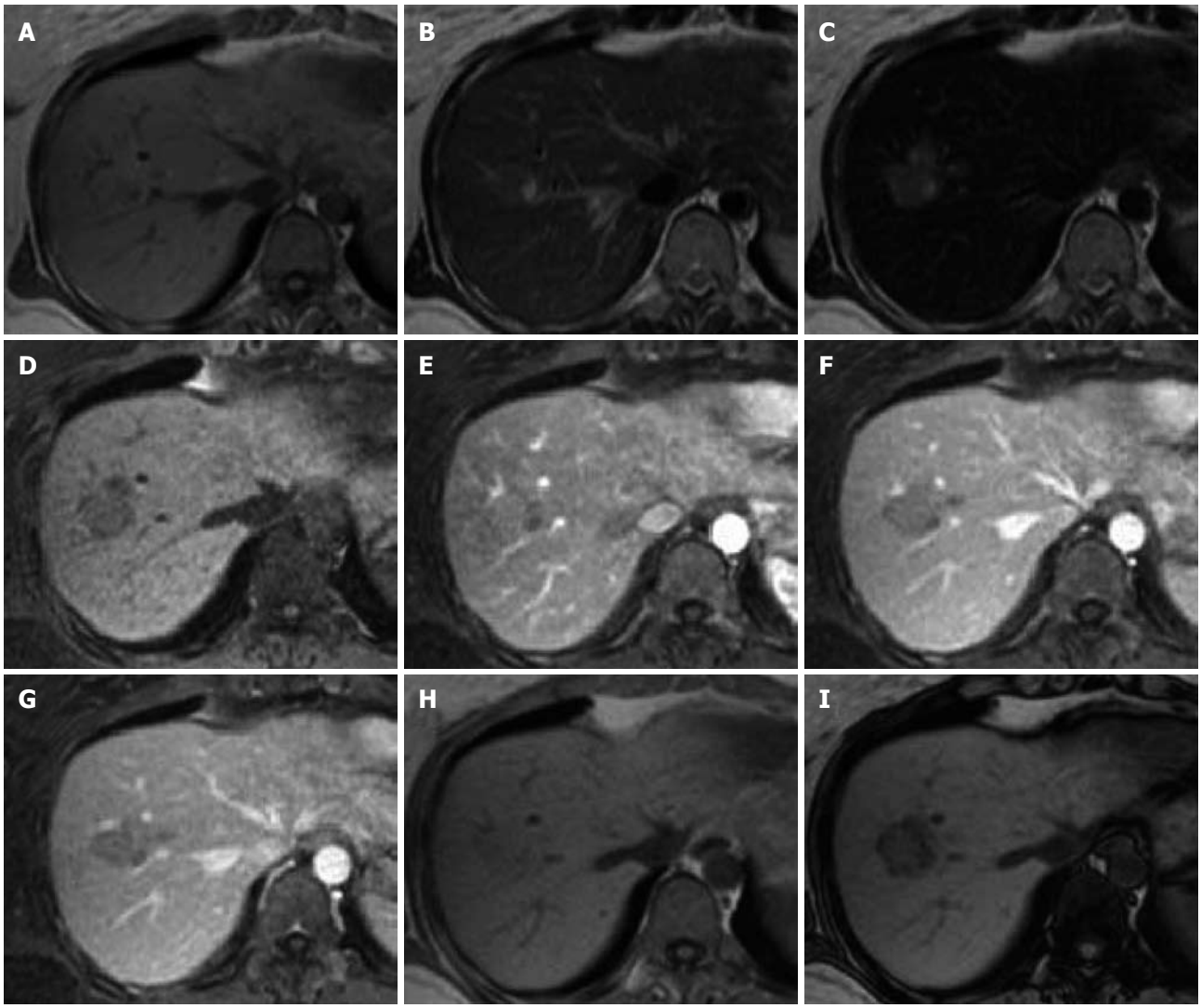

Figure 1 Magnetic resonance imaging (MRI). A: T1; B: T2; C: T2-SPIO; D: Plain; E: Arterial phase; F Portal phase; G: Delayed phase; H: T1 in phase; I: T1 opposed phase.

the liver (segment 8) showed slight low density by plain examination and an enhancing effect by the contrast agent. In the magnetic resonance imaging (MRI), the SOL showed almost isointensity on T1 and slightly high intensity on T2 (Figure 1A-B). Superparamagnetic iron-oxide (SPIO)-MRI indicated that the SOL did not show most of the SPIO uptake (Figure 1C). On the gadolinium-enhanced T1, the SOL indicated a slight homogenous enhancement in the arterial phase, and almost the same enhancement as the surrounding liver parenchyma in the delayed phase (Figure 1D-G). The SOL showed almost isointensity on the in-phase-T1 and slightly low intensity on the opposed-phase-T1, which indicated that the SOL contained a fat component (Figure 1-H, 1-I). A capsule or scars were not detected in the SOL. A benign, fat containing tumor was suggested. The dynamic CT in March 2007 showed that the SOL had not changed in size (Figure 2). The SOL indicated low density by plain examination, showed a slight homogenous enhancing effect in the arterial phase, and, an enhancing effect that was slightly lower than the surrounding liver in the delayed phase (Figure 2). In the dynamic CT after four months, the density of the SOL on plain examination was elevated and the enhancing effect in the arterial phase increased slightly, although the size and the form of the SOL did not show apparent changes (Figure 3). Because of the alteration of the findings in this CT, we suspected that the findings were suggestive of well-differentiated HCC. In August 2007, the patient was referred to our hospital for further examination and treatment. The physical examination of the patient showed no abnormal findings. The blood test indicated no abnormalities including liver related enzymes except slight iron deficiency anemia (Table 1). All the hepatitis virus markers were negative. There were no abnormal findings suggestive of autoimmune liver disease or metabolic liver disease including glycogen-storage disease. Hepatic arteriography was performed and the nodule presented with a slight tumor stain in the arterial phase and the hepatic parenchymal phase, and it did not show an apparent drainage vein (Figure 4). Positron emission tomography showed no difference in accumulation between the nodule and the surrounding hepatic parenchyma and no abnormal accumulation

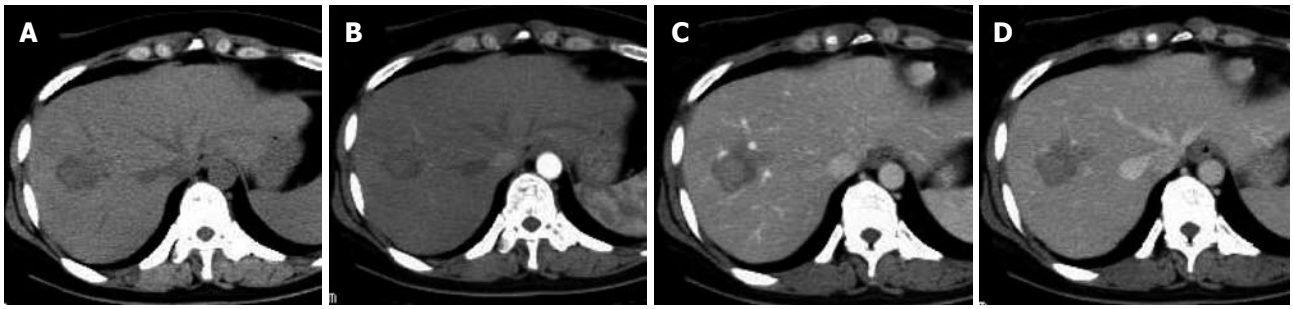

Figure 2 Computed tomography (CT) in March 2007. A: Plain; B: Arterial phase; C: Portal phase; D: Delayed phase.

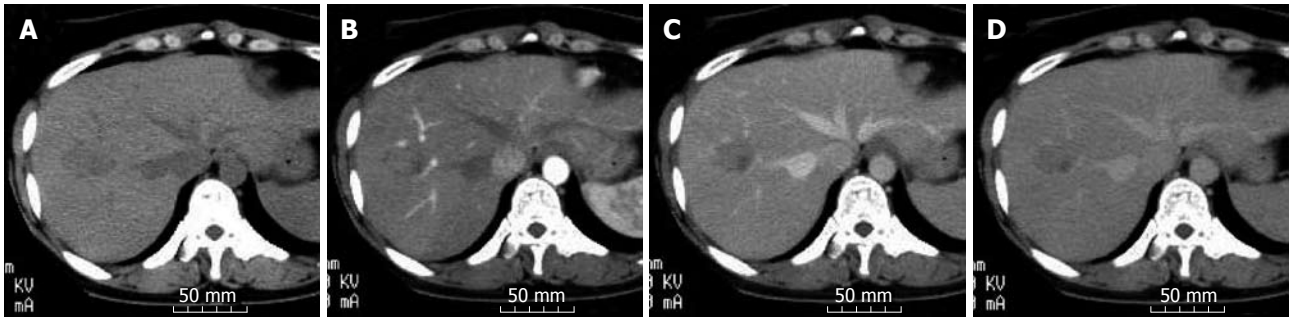

Figure 3 CT in July 2007. A: Plain; B: Arterial phase; C: Portal phase; D: Delayed phase.

Table 1 Laboratory data

Indicators	Value
White blood cell	3980/μL
Hemoglobin	9.8 g/dL
Platelet	232 × 10^3/μL
Prothrombin time	105%
Activated partial thromboplastin time	26.7 s
Aspartate aminotransferase	17 IU/L
Alanine aminotransferase	15 IU/L
Lactate dehydrogenase	146 IU/L
Alkaline phosphatase	175 IU/L
Gamma glutamyl transpeptidase	23 IU/L
Cholinesterase	257 IU/L
Total bilirubin	0.6 mg/dL
Direct bilirubin	0.1 mg/dL
Total protein	6.9 g/dL
Albumin	4.2 g/dL
Blood urea nitrogen	7.5 mg/dL
Creatinine	0.6 mg/dL
Uric acid	4.0 mg/dL
Na	142 mEq/L
K	4.2 mEq/L
Cl	106 mEq/L
Total cholesterol	202 mg/dL
Triglyceride	77 mg/dL
Glucose	101 mg/dL
C-reactive protein	0.03 mg/dL
IgG	1157 mg/dL
IgA	206 mg/dL
IgM	124 mg/dL
HBs-Ag	Negative
HBs-Ab	Negative
HCV-Ab	Negative
α-fetoprotein	5.1 ng/mL
Des-γ-carboxy prothrombin	34 mAU/mL
Anti-nuclear antibody	Negative
Anti-smooth muscle antibody	Negative
Anti-mitochondrial antibody	Negative

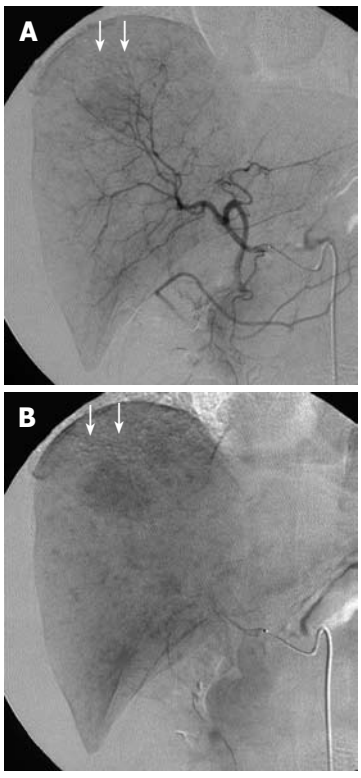

Figure 4 Digital subtraction angiography (DSA). A: Arterial phase; B: Liver parenchymal phase. Arrow: Tumor stain.

suspicious of malignant tumors in other organs. No abnormal findings were found suggesting a primary tumor in other organs in whole-body CT. Digestive tract endoscopy showed no abnormal findings. Taken together, the above-mentioned findings suggested the

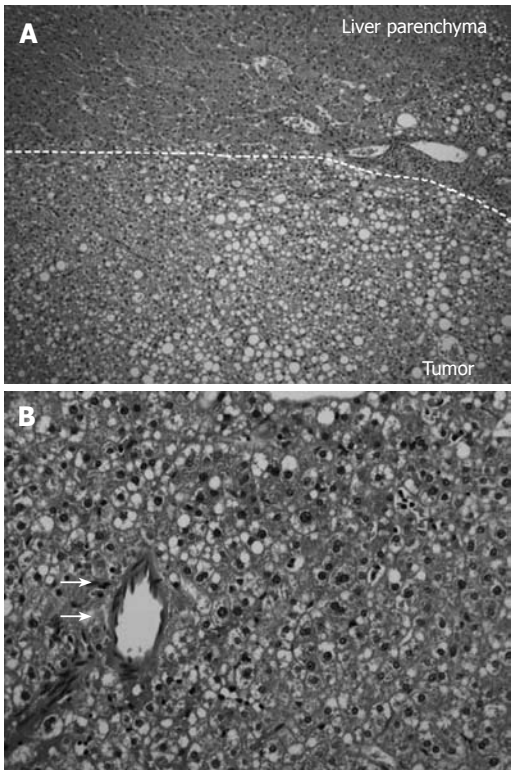

Figure 5 Pathological findings. A: Hematoxylin and eosin staining, original magnification, × 50; B: Hematoxylin and eosin staining, original magnification, × 200. Arrow: Myogenic artery.

possibility that the nodule was a well-differentiated HCC that was dedifferentiating. After receiving informed consent, partial hepatectomy was performed on August 28, 2007.

The pathological finding (Figure 5) revealed the following. Hepatocytes more lucent than that of the surrounding hepatic parenchyma had proliferated thickly in the nodule. Hepatocytes did not show severe cellular atypia. Exclusion of the surrounding hepatic parenchyma was apparent, but direct invasion to the surrounding was not seen. The nodule did not contain the portal tracts and bile ducts. Myogenic arteries, large lipid droplets, hemorrhage and degeneration were seen in the nodule. The surrounding hepatic parenchyma had no abnormal findings except fatty change with large lipid droplets around the nodule. Thus, we diagnosed the nodule as a LCA.

DISCUSSION

LCA is considered a rare benign tumor of the liver and is distinguished from solitary tumors and adenomatosis[3,4]. LCA was a very rare tumor until an association with oral contraceptives was reported. Before 1954, only two cases were detected among 5000 autopsies in 36 years[5]. The association with oral contraceptives was thoroughly described in the 1970s[6,7], and many cases of LCA have been reported since[8-10].

Due to the progress of diagnostic imaging techniques, the typical findings of LCA have been identified[4,11]. A typical LCA shows low density or isodensity on plain CT, presents a homogenous contrasting effect on arterial phase and does not show an apparent wash out on delayed phase. A typical LCA in MRI shows almost the same signal intensity as the surrounding parenchyma on T1 and T2 and shows high intensity on fat suppression T2. LCA may often present difficulties in a differential diagnosis with well-differentiated HCC and focal nodular hyperplasia[12-14]. The pathological findings of a typical LCA include the following[2,10,15-17]: (1) the tumor consists of hepatocytes with almost normal nuclei and cytoplasm presenting a homogenous increase, (2) and does not include the portal area and bile duct, (3) hepatocytes form hepatic cords, but sinusoids are pressed, and hepatic lobule structure is absent, (4) the tumor includes macrovesicular fatty changes, hemorrhage, degeneration, and myogenic arteries.

LCA can be divided into three categories: (1) LCA associated with oral administration of medicines including oral contraceptives and steroids[8-10], (2) LCA developing as a complication of glycogen-storage diseases[18-20], (3) adenomatosis[3,4,15]. Solitary LCAs are most frequently caused by oral contraceptives[7,21]. The occurrence of adenomatosis is unrelated to the use of oral contraceptives and the frequency of development is not related to sex[15]. It is reported that LCA occurs in patients of glycogen-storage disease type I [19]. In addition to oral contraceptives, oral administrations of such medications as clomiphene[22], barbituric acid[23] or androgen[24,25] are reported as risk factors for LCA. Also, it is reported that LCAs associated with oral contraceptives show regression after discontinuing the drug[26,27].

The main complications of LCA include tumor hemorrhage and malignant transformation. It is reported that hemorrhage is found in about one half of LCAs and can result in death[9,16,28]. Malignant transformation of LCA is considered to be rare. However, in several reported cases, LCA associated with glycogen-storage diseases and glucocorticosteroids developed HCC[29-32]. In another reported case, hepatocarcinogenesis occurred several years after discontinuation of oral contraceptives[32].

Although LCA is a benign liver tumor, treatment is often conducted to avoid hemorrhage and malignant transformation[3,4]. Surgical treatments such as lobectomy[21,33], enucleation[34] and liver transplantation[35] are performed for LCA. Percutaneous ethanol injection[20] and transcatheter arterial embolization[33] are established therapies for low invasive treatment of HCC, and were reported to be performed for LCA. However, precise pathological examination is not possible by these treatments. Since a definitive diagnosis of LCA by diagnostic imaging alone or by needle biopsy is difficult[11], tumor resection is often the most suitable approach. Our case was found by chance during treatment of the endometriosis complicated with bacterial infection. The patient did not have a history of using oral contraceptives or glucocorticosteroids which are known risk factors for LCA. Nor did the patient have metabolic liver disease including glycogen-storage

disease. By MRI, the tumor was found to contain fat. In dynamic studies, the tumor showed a homogenous enhancement in the arterial phase and almost the same enhancement as the surrounding liver parenchyma in the delayed phase. These findings were typical for LCA. However, the tumor showed an elevation of the density on plain CT and an increase of the early enhancement in the arterial phase, which caused us to suspect that well-differentiated HCC containing fat was becoming dedifferentiated. Accordingly, hepatic resection was performed.

In a prospective study of 48 LCA cases reported by van der Windt *et al*[27], serial observation was considered appropriate for tumors with the typical image findings of LCA if the diameter was less than 5 cm. Five of the 48 cases of LCA were resected because the radiological findings changed during serial observations and three of the five cases had well-differentiated HCC, while in the remaining two cases, it was difficult to distinguish between well-differentiated HCC and LCA pathologically. The present case showed the typical radiological findings for LCA and was 35 mm in diameter without an increasing tendency. However, diagnosis only by radiological findings is difficult in patients without a background supporting a diagnosis of LCA such as a history of receiving the above mentioned drugs or glycogen-storage disease. Resection of the tumor in the present case was therefore considered appropriate because of the risk of hemorrhage and possible malignant transformation.

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Intraductal papillary mucinous neoplasm in chronic calcifying pancreatitis: Egg or hen?

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Abstract

Intraductal papillary mucinous neoplasm (IPMN) is an increasingly reported entity. Extensive pancreatic calcification is generally thought to be a sign of chronic pancreatitis, but it may occur simultaneously with IPMN leading to diagnostic difficulties. We report a case of a patient initially diagnosed with chronic calcifying pancreatitis who was later shown to have a malignant IPMN. This case illustrates potential pitfalls in the diagnosis of IPMN in the case of extensive pancreatic calcification as well as clues that may lead the clinician to suspecting the diagnosis. The possible mechanisms of the relation between pancreatic calcification and IPMN are also reviewed.

Key words: Intraductal papillary mucinous neoplasm; Endoscopic ultrasound; Calcifying pancreatitis; Carcinoembryonic antigen; Endoscopic retrograde cholangiopancreatography

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TO THE EDITOR

Intraductal papillary mucinous neoplasm (IPMN) is an increasingly reported entity, representing up to one third of all pancreatic cystic neoplasms[1]. IPMNs typically present with cystic dilatation of the pancreatic duct associated with mucin production and variable cellular atypia. Although extensive pancreatic calcification is generally considered to be a pathognomonic sign of chronic pancreatitis, it may also occur simultaneously with IPMN which may lead to diagnostic confusion[2,3]. We report a case of a patient who was initially diagnosed with chronic calcifying pancreatitis and was later shown to have an IPMN.

An 81-year-old man was referred to the gastroenterology clinic due to weight loss, diarrhea, and anemia. He did not drink any alcohol and got quit of smoking pipe 50 years ago. Medical history was unremarkable apart from chronic obstructive pulmonary disease and type 2 diabetes mellitus diagnosed one year prior to presentation. Laboratory tests revealed mild normocytic normochromic anemia with 11.3 g/L hemoglobin (reference 13.0-17.0 g/L) but essentially normal white cell and platelet counts as well as normal electrolytes, creatinine, albumin, liver function tests, amylase, endomysial antibodies, plasma protein electrophoresis, and thyroid function tests.

Esophagogastroduodenoscopy was normal. A CT-colonography showed normal colonic appearances but there was evidence of a grossly atrophic calcific pancreas with a dilated pancreatic duct. No mass lesion was seen. The patient did not have a history of acute pancreatitis, abdominal radiotherapy or trauma to the epigastric region. His serum calcium and triglycerides were normal. Fecal elastase was < 15 μg/g of stool (reference > 500 μg/g of stools). Therefore , exocrine pancreatic insufficiency due to idiopathic chronic calcifying pancreatitis was diagnosed and pancreatic enzyme substitution was started.

Despite some initial improvements, the patient reported further weight loss six months after initial

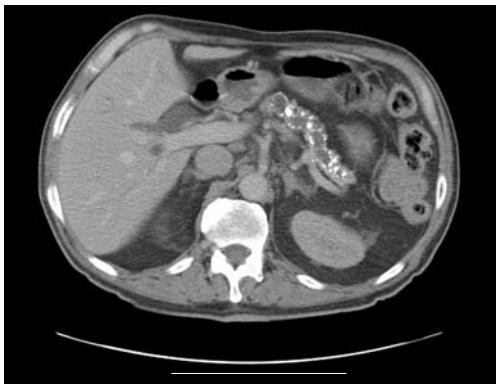

Figure 1 Abdominal computed tomography image showing gross pancreatic duct dilatation and extensive pancreatic calcification as well as bile duct dilatation. No distinct mass was seen.

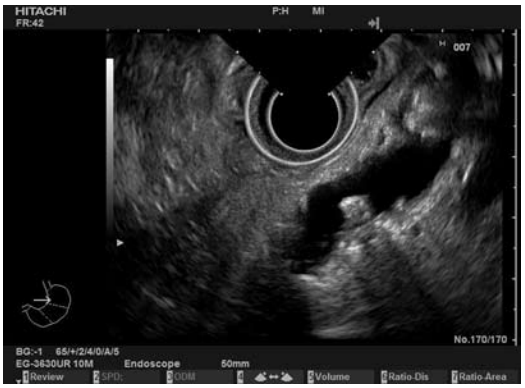

Figure 2 Endoscopic ultrasound revealing a dilated pancreatic duct (> 10 mm in diameter) with mural irregularities, calculi within the pancreatic duct, and an atrophic pancreas.

presentation. Laboratory testing showed that his alkaline phosphatase was 2629 IU/L (reference 95-320 IU/L), bilirubin was 42 μmol/L (reference 3-17 μmol/L), and alanine aminotranferase was 347 IU/L (reference 10-45 IU/L). A pancreatic protocol CT demonstrated gross pancreatic atrophy and calcification, dilated pancreatic as well as intrahepatic and common bile ducts. No mass lesion was seen (Figure 1). Endoscopic ultrasound confirmed these findings and also showed an enlarged heterogeneous pancreatic head with dilated side branches of the pancreatic duct as well as an area of hypoechogeniccity, but did not show any distinct mass. The main pancreatic duct in the head was dilated with its diameter > 10 mm and with wall irregularities (Figure 2). Mucus extrusion from a protrubing gaping major papilla was observed. Endoscopic retrograde cholangiopancreatography was attempted in which opacification of the main pancreatic duct showed multiple filling defects suggestive of intraductal mucin. Common bile duct cannulation failed and biliary drainage was achieved by means of a percutaneous transhepatic cholangiography. Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels in mucin aspirated from the pancreatic duct were 24132 ng/mL and 60840 U/mL, respectively. On the basis of the imaging findings, the fact that mucus extrusion was observed from the papilla, and the high levels of CEA and CA 19-9 in pancreatic duct aspirates, a malignant IPMN was diagnosed. Due to his clinical condition, the patient was judged not to be a candidate for surgery and was offered palliative therapy.

Twelve to sixty percent of patients with IPMN have a history leading to a diagnosis of chronic pancreatitis[4,5] and roughly 2% of all diagnoses of chronic pancreatitis are associated with IPMN[5]. However, few reports of patients with concomitant calcyfing pancreatitis and IPMN are available[2,3]. In a cohort study of 473 patients suffering from chronic pancreatitis, only 6 were found to have IPMN during follow-up but none had calcifications[5]. Of the 16 cases of patients with IPMN and pancreatic calcification published thus far, 8 had diffuse pancreatic calcification which did not involve the tumor itself in most of the cases[2,3]. The pathogenesis of calcification in IPMN is unknown. Apart from the unlikely possibility that the two entities may occur coincidentally, it is possible that IPMN may be a complication of chronic calcifying pancreatitis, but no report of a patient with this condition developing IPMN in the course of the disease is available. It has also been proposed that IPMN may be responsible for pancreatic calcification due to chronic partial ductal obstruction by mucin plugs[2,3]. In any case, the concomitant presence of pancreatic cystic lesions, duct dilatation, and calcification should raise the suspicion of an IPMN. Particular care should be taken when diagnosing chronic pancreatitis in patients who do not present typical characteristics of the disease, namely age over 50 years, moderate alcohol intake, and non-smokers, which may be suggestive of IPMN[5]. In our case, the patient was 81 years of age and did not consume any alcohol as verified by the patient himself and his relatives.

Although CEA and CA 19-9 measurements are frequently performed during the diagnostic work-up of patients with pancreatic cystic lesions, it has been suggested that cyst fluid or pancreatic duct CEA or CA 19-9 is not helpful in the differentiation between benign and malignant IPMNs[6] but published data in the literature are not unanimous[7]. A CEA > 800 ng/mL has been reported to be helpful in differentiating mucinous cystadenocarcinomas from mucinous cystadenomas[8]. Although only 5/16 patients with concomitant calcifying pancreatitis and IPMN previously reported were shown to have a malignant lesion[2,3], in the present case the fact that both CEA and CA 19-9 in the pancreatic duct aspirate were extremely high was felt to be highly suggestive of malignancy. A main pancreatic duct > 6 mm in diameter and protrusion of the papilla of Vater are also considered signs predicting malignancy[9]. Thus, endoscopic ultrasound is not just a technique to obtain fluid from cystic pancreatic lesions for tumor marker analysis, but it also provides invaluable morphological information on mural pancreatic duct irregularities, presence of solid lesions or septa in cysts. Compared to other imaging methods, it achieves the highest detail resolution.

In summary, concomitant presence of pancreatic

cystic lesions, duct dilatation, and calcification should raise the suspicion of an IPMN, especially when patients do not present typical characteristics of chronic pancreatitis, namely age over 50 years, moderate alcohol intake, and non-smokers. Inspection of the ampulla with a duodenoscope and aspiration of fluid from the pancreatic duct or endoscopic ultrasound-guided fine needle aspiration from a cystic pancreatic lesion for analysis of CEA and CA 19-9 may be useful in the diagnosis of such patients.

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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
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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
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Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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Patent (list all authors)

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Write as mean ± SD or mean ± 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 υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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