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Contents

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Contents

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REVIEW

Chemoprevention of hepatocellular carcinoma in patients with hepatitis C virus related cirrhosis

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Abstract

Interferon (IFN) therapy has been reported to decrease the risk of hepatocellular carcinoma (HCC) and improve survival by preventing liver-related deaths in patients with chronic hepatitis C virus (HCV) infection, while the role of IFN therapy on the natural history of hepatitis C related cirrhosis is still under debate. The ideal goal of therapy is to prevent the progression into end-stage disease. The use of IFN in patients with HCV compensated cirrhosis reduces the negative clinical evolution independently of the type of laboratoristic and virological response. In our experience, IFN therapy in HCV compensated cirrhosis is barely useful in prevention of HCC, as cirrhosis itself represents a risk of cancer. Some authors noted that IFN treatment reduces the risk of HCC independently of the virological response. It would probably be interesting to evaluate the efficacy of weekly low-dose pegylated (PEG)-IFN therapy in patients with HCV cirrhosis and to assess potential benefits of long-term PEG-IFN plus Ribavirin treatment.

Key words: Chemoprevention; Cirrhosis; Hepatitis C virus; Hepatocellular carcinoma

Core tip: Interferon (IFN) therapy has been reported to decrease the risk of hepatocellular carcinoma (HCC) and improve survival. The use of IFN in patients with hepatitis C virus (HCV) compensated cirrhosis reduces the negative clinical evolution independently of the type of the laboratoristic and virological response. In our experience, IFN therapy in HCV compensated cirrhosis is barely useful in the prevention of HCC, as cirrhosis itself represents a risk of cancer. It would probably be interesting to evaluate the efficacy of weekly low-dose pegylated (PEG)-IFN therapy in patients with HCV cirrhosis and to assess potential benefits of long-term PEG-IFN plus Ribavirin treatment.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most common cause of cancer death in the world[1]. The most current statistics available estimate 609000 deaths globally from this disease in 2004[2].

The risk of HCC in patients with chronic hepatitis C is highest in patients who have established cirrhosis, in whom the incidence of HCC is between 2%-8% per year[3].

There is a single prospective population-based study of the risk of HCC in hepatitis C virus (HCV) patients. In this study of 12008 men, being anti-HCV-positive conferred a 20-fold increased risk of HCC compared with anti-HCV-negative subjects. Hepatitis C infected patients who do not have cirrhosis have a much lower risk

of developing HCC[3].

NATURAL HISTORY

The overall natural history of HCV infection is somewhat variable and the long-term risks are difficult to estimate due to the extended periods of time involved. Approximately 20% of subjects with post-transfusion chronic hepatitis C developed histological evidence of cirrhosis in the first 10 years after transfusion[4].

Factors that promote progression of chronic hepatitis include alcohol consumption, male sex, age over 40 years at the time of infection, and severe histology at the time of initial diagnosis. Co-infection with human immunodeficiency virus and/or hepatitis B virus (HBV) has also been associated with premature and more severe liver disease[5].

The risk of developing HCC in HCV patients can be estimated from the results of natural history studies of HCV infection and studies on the development of HCC in patients with HCV-related cirrhosis. If 60% of patients exposed to HCV develop a chronic infection and 20% of them develop cirrhosis within 10 years, it means that approximately 12% of all these patients will be at high risk for HCC.

Any chronic inflammatory liver disease has the potential to induce HCC but the pathophysiological process most commonly associated with the disease is cirrhosis, found in up to 80% of cases. However, knowledge of all possible sources is important, considering that 20% of cases are due to non cirrhotic, non viral causes[6].

Viral, environmental, hereditary and dysmetabolic causes of cirrhosis certainly have a strong correlation with HCC. HBV infection is the leading cause of chronic liver disease and HCC around the world. HCV-RNA was found in about 65% of hepatitis B surface antigen-negative patients at diagnosis of HCC. Patients who develop cirrhosis may stay in a compensated state or may decompensate with ascites, jaundice, hepatorenal syndrome, hepatic encephalopathy or variceal bleeding.

Patients with compensated cirrhosis had a 5 year mortality of 50% and a 10 year mortality of 70%. The probability of decompensated cirrhosis was 18% at 5 years and 29% after 10 years of cirrhosis[7].

The prognosis of HCC remains poor for the majority of patients who present with advanced disease. Treatment options depend on the tumor size, the number of lesions and the stage of cancer. Thirty percent of patients are candidates for surgical resection and the recurrence rate is about 50% at 3 years. In 2008, a major breakthrough in the treatment of advanced HCC was announced in the form of sorafenib (multikinase inhibitor) which was shown to increase the median overall survival from 7.9 to 10.7 mo without severe side effects in a randomized, placebo-controlled phase-Ⅲ trial. However, sorafenib did not delay time to symptomatic progression[8,9].

HEPATOCARCINOGENESIS AND HCV

HCV is a noncytopathic virus of the Flaviviridae family. The HCV single-stranded RNA genome encodes nonstructural proteins (NS2, NS3, NS4A, NS4B and NS5B), which associate with endoplasmic reticulum membrane to form the viral replicase and the viral envelope proteins (E1 and E2).

The precise mechanism by which HCV infection results in HCC is not well known. After hepatic injury incurred by HCV, there is necrosis followed by hepatocyte proliferation. Continuous cycles of this destructive-regenerative process foster a chronic liver disease condition that culminates in liver cirrhosis.

Probably cirrhosis itself is responsible for malignant transformation of hepatocytes; in cirrhosis inflammation, in fact, the increased cellular turnover and fibrosis favor mutations in hepatocytes and the eventual development of HCC[10-12].

It has been noted that the HCV core protein has some potential direct carcinogenic effects *in vitro*, including the transformation of rat embryo fibroblasts to the malignant phenotype and the suppression of apoptotic cell death in culture. In addition, HCV-RNA and/or core protein have been suggested to impair dendritic cell functions that are important for T-cell activation[9]. Furthermore, the HCV core protein and the NS5A nonstructural proteins have been implicated in the evasion from immune-mediated cell killing by interacting with various factors involved in this process. In addition, the protease activity of NS3 is enhanced by the NS4A cofactor and the NS3-4A protease activity is involved in blocking the ability of the host cell to mount an innate antiviral response[13,14].

HCV core protein can activate the mitogen-activated protein kinase signaling pathway, modulate cell proliferation, and promote the induction of reactive oxygen species.

Moreover, HCV core-E1-E2 transgenic mice develop significantly larger tumors than transgenic mice expressing core alone or nontransgenic mice. The accelerated tumor phenotype is attributable to suppression of apoptosis[15].

At the molecular level, the interaction between oncogenes, tumor suppressor genes and several growth factors may play an additional role in HCC development.

NS5A has been shown to interact with and inactivate p53 by sequestration to the perinuclear membrane, thereby affecting the p53-regulated pathways that control cell-cycle progression, cellular survival, response to hypoxic and genotypic stresses, and tumor angiogenesis.

A p73 overexpression and nuclear accumulation in HCV-associated HCC has been observed. The *p73* gene activates the transcription of p53-responsive genes and inhibits cell growth by inducing apoptosis. p73 seems to be strongly involved in hepatocarcinogenesis, probably through a protein-protein interaction with the HCV proteins[16].

Loss and/or mutation of p53 and genomic instability also characterize hepatocarcinogenesis. p53 loss and/or mutation is shown to occur during progression to HCC; however, there is some evidence that loss and mutation of p53 might also occur in the initial stages of hepatocarcinogenesis[9,16].

Telomere shortening is a feature of HCV chronic liver disease and cirrhosis and telomerase reactivation has been associated with HCV hepatocarcinogenesis[9].

There is a strong activation of telomerase reverse transcriptase in nearly 90% of human HCCs. Telomerase reactivation has been suggested to promote HCC progression (increased micro-vessel density and HCC recurrence after resection)[9].

Alcohol is an important cofactor in patients with HCV infection and it has been estimated that 30% of patients with alcoholic liver disease are infected with HCV[3]. Subjects with both HCV infection and alcohol addiction have been shown to develop more severe fibrosis and have higher rates of cirrhosis and HCC than non-drinkers. The risk for developing HCC has also been shown to increase as levels of alcohol intake rise. The dominant mechanism for synergism between alcohol and HCV infection appears to be increased oxidative stress[9].

Data linking being overweight/obesity to liver disease and HCC are well established. The risk of incident HCC is 3 times higher in patients with a body mass index of 30 kg/m^2[17].

Finally, genetic polymorphisms are important risk factors for liver disease evolution: genetic variations involved in oxidative stress, controlling hepatic lipid storage, modulating endotoxin inflammation and polymorphic variants of fibrosis associated genes are correlated with fast evolution towards cirrhosis in patients with chronic HCV[11].

ROLE OF INTERFERON TREATMENT IN COMPENSATED CIRRHOSIS

Tremendous efforts have improved the understanding of the pathogenesis and treatments of HCC but relatively little effort has been made to develop effective chemoprevention of HCC[8].

Chemoprevention is defined as the use of natural or synthetic agents to reverse, suppress or prevent premalignant conditions from progressing to invasive cancer. Chemoprevention may be classified into 3 categories: primary (preventing cancer in healthy subjects), secondary (preventing cancer in subjects with premalignant conditions, for example, the presence of cirrhosis) and tertiary (preventing recurrence)[18].

The ideal goal of secondary chemoprevention is to eradicate HCV at the beginning of the disease and to prevent progression into end-stage disease and HCC. Interferon (IFN) therapy has been reported to decrease the risk of HCC and improve survival by preventing liver-related deaths in patients with chronic HCV infection (mainly in those with a complete and sustained response), while the role of IFN therapy on the natural history of HCV related cirrhosis and on chemoprevention of HCC is still under debate.

In our experience[19], we conducted an experimental research involving patients with compensated HCV-related cirrhosis (Child-Pugh A) histologically confirmed, abnormal alanine-aminotransferase (ALT) values and serum HCV-RNA positivity. IFN therapy *vs* no therapy have been compared. Qualitative and quantitative detection of HCV-RNA was performed with the Cobas Amplicor HCV Test, version 2.0 and the Cobas Amplicor HCV Monitor, version 2.0 (Roche Diagnostics, Branchburg, NJ, United States). The qualitative assay is able to detect HCV-RNA at a concentration of 50 IU/mL, with a positive rate of 95% or greater. The Cobas Amplicor HCV Monitor, version 2.0 has a dynamic range between 600 and 8.5 × 10 IU/mL. HCV genotyping was performed by sequencing of the 5'-untranslated regions (5'UTR) (Visible Genetics Tru-Gene Hepatitis Assay, Toronto, Canada). The phylogenetic analysis of the 5'UTR nucleoside sequence, together with appropriate homologous references of the main subtype sequences, permitted genotyping[19-21].

In both groups we investigated the incidence of the following negative events: cirrhosis worsening (passage from Child A to Child B or C), HCC onset, and death or orthotopic liver transplantation (OLT).

A cohort of 122 patients, prospectively enrolled, was retrospectively analyzed to assess the effect of IFN therapy (mean follow-up: 96 ± 18.3 mo). Only patients who had received a blood transfusion before 1980 were selected. Fifty-nine patients (mean age: 55.3 ± 7 years) (mean follow-up 96.5 ± 18 mo) received IFN (3 MU three times a week for 12 mo), 8 stopped therapy for side effects; 71 patients did not receive IFN (mean age: 56.8 ± 8 years) (mean follow-up 95.4 ± 17.8 mo).

Baseline patient characteristics were similar, including age, sex, HCV-RNA genotype, liver function tests, alpha-fetoprotein levels, leukocyte and platelet counts, and alcohol consumption.

Response to therapy was assessed as follows: a sustained virological response (SVR) was defined as the absence of serum HCV-RNA for at least 6 mo after interferon-alpha therapy; a sustained biochemical response was defined as a decrease in serum ALT activity to within the reference range but with persistently detectable serum HCV-RNA. Non-response was defined as persistence/relapse of HCV-RNA and no decrease/relapse in ALT activity at the end of the therapy[19].

All patients were subjected to ultrasound follow-up investigations, performed at 4-6 mo intervals. Laboratoristic exams were carried out at all clinical visits: every 15-30 d (in the group of treated patients) or every 4-6 mo (in the group of untreated patients). If necessary, a spiral CT, angiography and endoscopic examination were performed and in suspected HCC, fine needle biopsy under sonographic guidance was carried out.

Final results showed that treated patients exhibited

Table 1 Worst evolution (negative events altogether evaluated) and hepatocellular carcinoma incidence in interferon treated patients *n* (%)

	Worst evolution	HCC
NR	13/22 (59.0)	7/22 (31.8)
SR	3/11 (27.2)[b]	3/11 (27.2)
RR	7/18 (38.8)[b]	5/18 (27.7)
Not treated	63/71 (88.7)[b]	24/71 (33.8)

[b]$P < 0.01$ *vs* non responder (NR). SR: Sustained responder; RR: Relapse responder; HCC: Hepatocellular carcinoma.

negative events in 45% of the cases (23/51) and untreated patients in 88.73% (63/71) ($P < 0.0001$).

In the IFN treated group, 6 patients (11.76%) showed worsening cirrhosis according to Child's classification, 15 patients (29.4%) developed HCC, 1 patient died, and 1 patient underwent liver transplantation. In the IFN untreated group, 27 patients (38%) showed worsening cirrhosis (*vs* treated patients $P = 0.003$), 24 developed HCC (*vs* treated patients $P = 0.752$) and 12 died or underwent OLT (16.9% *vs* treated patients $P = 0.054$) (9 died and 3 underwent OLT).

A complete and sustained response (SR) to IFN was observed in 11 of the 51 patients treated (21.5%), a relapse (RR) was observed in 18 cases (35.2%) and no response (NR) in 22 cases (43.1%).

Data confirm that, in relationship to the type of response to therapy, a worse evolution occurred (negative events altogether evaluated) in NR (13/22, 59%) rather than in SR (3/11, 27.2%) and RR (7/18, 38.8%) ($P < 0.01$). It is important to underline that untreated patients developed more negative events (63/71, 88.7%) than NR patients (13/22, 59%) ($P < 0.01$) (Table 1).

No particular differences have been noticed in relationship to HCC onset (SR 3/11, 27.2%; RR 5/18, 27.7%; NR 7/22, 31.8%; no treated patients 24/71, 33.8%) ($P > 0.05$) (Table 1).

Afterwards, we evidenced that the cumulative probabilities of developing HCC were not significantly higher in untreated patients compared with IFN treated cases when assessed by the Kaplan-Meier and log-rank test ($P > 0.05$)[22].

DISCUSSION

There is evidence that the use of IFN as a therapy for chronic hepatitis may slacken the natural history of the pathology, particularly in patients who have a long-term sustained response[19,23-26].

Meta-analysis demonstrated a decrease of HCC incidence in treated patients in comparison with those untreated and other studies evidenced a lower risk of cancer in subjects with virus clearance in comparison with non-responders[27,28].

The use of IFN in viral cirrhosis is controversial with respect to its cost effectiveness[18,29]. A remarkable heterogeneity among the study was found and the magnitude of the overall effect is low[19,22,30-40] (Table 2).

From our data, it seems that the administration of IFN in HCV compensated cirrhosis is barely useful in the prevention of HCC when cirrhotic structural alterations arise as cirrhosis itself represents a risk factor for HCC.

In 1995, a small randomized clinical trial showed a decrease in the incidence of HCC in subjects with HCV cirrhosis treated with IFN-alpha compared with untreated controls[30,31]. In the wake of this study, several controlled trials were performed[30,41]. Three meta-analyses evaluated whether IFN reduces the incidence of HCC in patients with HCV-related cirrhosis. The most recent review shows that IFN seemingly decreased the HCC rate in all but one of the 20 studies included in the meta-analysis. The rate difference between IFN-treated patients and controls of each trial ranged from -33.3% to +3.9%. The pooled estimate of the treatment effect was significantly in favor of a preventive effectiveness of IFN ($P < 0.00001$). A remarkable heterogeneity among the studies ($P > 0.0001$) was found. The most prominent heterogeneity was in the difference of magnitude of the treatment effect on the risk of cancer[30].

However, the magnitude of the overall effect is low and the observed benefit might be due to spurious associations. The preventive effect is stronger among sustained responders to IFN, which intrinsically represents a small proportion of all cirrhotic patients[30].

Thereafter, three observational cohort studies, 2 prospective studies conducted in Japan[30,42] and Taiwan[30,34] and one retrospective study conducted in Italy[30,35], confirmed that the efficacy of IFN in the chemoprevention of HCC is exclusively linked to the achievement of a SVR, whereas no benefit in reducing HCC development has been observed in non-responder patients[30].

Some authors noted that treatment with IFN reduces the risk of HCC independently of the virological response[31,43,44]. The reason is unclear, although it has been suggested that the anti-proliferative activity or other properties[45] of IFN may be responsible. In accordance with Kowdley[46], it is perplexing that only 6 or 12 mo of therapy can produce this benefit. Moreover, some authors reported some cases of HCC onset after 3-6 years of SVR in patients treated with IFN, with moderate or severe stage of liver fibrosis before IFN treatment[47-49]. Nojiri *et al*[50] retrospectively studied 5 patients who had developed HCC more than 10 years after the termination of IFN therapy. These patients had achieved a long-term SVR. The authors concluded that HCV patients who respond to IFN therapy should undergo long-term follow-up, even after a SVR, especially if they have an advanced histological fibrosis stage or higher serum ALT or other risk factors.

Mazzaferro *et al*[39] reported that IFN does not affect overall prevention of HCC recurrence after resection and Craxì *et al*[18] evidenced that in the setting of secondary chemoprevention, literature data pooling suggests a slight preventive effect of IFN on HCC development in patients with HCV-related cirrhosis. The observed benefit might be due to spurious associations.

Kubo *et al*[51] declared that postoperative IFN-alpha therapy appears to decrease the incidence of recurrence

Table 2 Features of the studies on cirrhotic patients

Type study	Follow-up (mo)	IFN (wk)	Sample size	Mean age (yr)	RR	Ref.
RCT	24-86	6 MU tiw (12-24)	T 45/C45	55	-0.4	[31]
NRCT/P	12-71	3 MU tiw (52)	T193/C91	57	-0.1	[32]
NRCT/P	60-84	6 MU tiw (26)	T82/C81	56	2	[33]
NRCT/P	43	3 MU tiw (65)	T103/C59	57	-0.05	[34]
RCT	60	6 MU tiw (18) 3 MU tiw (18)	T38/C23	57	> 1	[35]
P	55	1 MU tiw (12) 3 MU tiw (12) 6 MU tiw (12) 9 MU tiw (12)	T72/C72	58	-0.2	[36]
R	96	3 MU tiw (12)	T52/C71	56	> 1	[19]
P	60	6 MU/mo (4) 3 MU/mo (44)	T41/C30	56	-0.1	[37]
R	96	3 MU tiw (12)	T52/C71	56	> 1	[22]
P	60	3-6 MU tiw plus Ribavirin (2-48)	T1057/C562	56	-0.05	[38]
RCT	45	3 MU tiw (48) (post-resection)	T76/C74	66	> 1	[39]
P	96	6 MU tiw (24-48)	T82/T81	57	-0.04	[40]

RCT: Randomized controlled trial; NRCT: Non randomized controlled trial; P: Prospective; R: Retrospective; IFN: Interferon; T: Patients treated; C: Controls; RR: Relative risk.

after resection of HCV-related hepatocellular carcinoma, and also in patients with persistently detectable serum HCV-RNA.

It is important to underline how the management of dysmetabolism, diet and exercise therapy can improve BMI, liver histology and, therefore, the response to pegylated (PEG) interferon and Ribavirin, and decreases the incidence of HCC[52,53].

In fact, it is well known how insulin resistance and adipocytokine disorders may be implicated in HCV hepatocarcinogenesis[54,55].

CONCLUSION

According to Cammà *et al*[56], IFN prevents or delays the development of HCC in patients with HCV-related cirrhosis but the magnitude of the overall effect is low and the observed benefit might be due to spurious associations. The preventive effect is stronger among a sustained virological response to IFN. The cost-effectiveness of treatment for chronic hepatitis C is substantially acceptable[57-59]; in particular, Kawaguchi *et al*[60] affirm that the cost-effectiveness of long-term treatment could be lower than that of patients never treated with IFN, according to their long-term follow-up assessment. More recently, extended analysis of the Hepatitis C Antiviral Long-term Treatment against Cirrhosis Trial[61] showed that long-term PEG-IFN therapy does not reduce the incidence of HCC among patients with advanced hepatitis C who did not achieve SVR. Patients with cirrhosis who received PEG-IFN treatment (PEG-IFNα-2a 90 microg/weekly) for 3.5 years and followed up for a median of 6.1 (maximum, 8.7) years had a lower risk of HCC than controls[1].

In our experience, maintenance therapy in non-responder patients slows down the hepatic fibrotic evolution[62]. Probably, in relationship to the ability of IFN to inhibit the growth of pre-neoplastic cells[63-65], it would be interesting to evaluate the efficacy of weekly low-dose PEG-IFN therapy in patients with HCV cirrhosis and to assess potential benefits of long-term PEG-IFN plus Ribavirin treatment.

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REVIEW

Chronic HCV infection and inflammation: Clinical impact on hepatic and extra-hepatic manifestations

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Abstract

The liver has a central role in regulating inflammation by its capacity to secrete a number of proteins that control both local and systemic inflammatory responses. Chronic inflammation or an exaggerated inflammatory response can produce detrimental effects on target organs. Chronic hepatitis C virus (HCV) infection causes liver inflammation by complex and not yet well-understood molecular pathways, including direct viral effects and indirect mechanisms involving cytokine pathways, oxidative stress and steatosis induction. An increasing body of evidence recognizes the inflammatory response in chronic hepatitis C as pathogenically linked to the development of both liver-limited injury (fibrosis, cirrhosis and hepatocellular carcinoma) and extrahepatic HCV-related diseases (lymphoproliferative disease, atherosclerosis, cardiovascular and brain disease). Defining the complex mechanisms of HCV-induced inflammation could be crucial to determine the global impact of infection, to estimate progression of the disease, and to explore novel therapeutic approaches to avert HCV-related diseases. This review focuses on HCV-related clinical conditions as a result of chronic liver and systemic inflammatory states.

Key words: Hepatitis C virus; Inflammation; Cytokines; Oxidative stress; Hepatic disease; Extra-hepatic diseases

Core tip: Chronic hepatitis C virus (HCV) infection causes liver inflammation by complex and not yet well-understood molecular pathways. HCV-induced inflammation has a significant clinical impact on development of both hepatic disease and HCV-associated extrahepatic manifestations. Knowledge of the complex mechanisms underlying HCV-related inflammation and development of disease as well as individuation of relevant markers of inflammation could be of importance for understanding disease progression, predicting prognosis and, possibly, conceiving new therapeutic approaches targeting the different steps of the inflammatory response.

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INTRODUCTION

Inflammation is a crucial physiological pathway in the homeostatic altered response to a number of exogenous distressing stimuli; however, a chronic inflammatory state or an excessive inflammatory response can produce deleterious effects. The liver plays a central role in regulating inflammation by its capacity to secrete a number of proteins that control both local and systemic inflammatory responses. A number of liver cells, including hepatocytes, hepatic stellate cells (HSCs), Kuppfer cells (KCs), bile duct epithelial cells and sinusoidal endothelial cells, are implicated in the synthesis and secretion of, and response to, inflammatory stimuli. Systemic inflammation is mediated by a number of cytokines released by macrophages as well as by adipocytokines secreted from adipose tissue[1]. The hepatic response to inflammation is characterized by the release of resident soluble mediators that also enter the circulation resulting in a systemic response to hepatic injury.

Chronic hepatitis C virus (HCV) infection[2] causes liver inflammation by complex and not yet well-understood molecular pathways. HCV-induced inflammation has a significant clinical impact on development of both hepatic disease and HCV-associated extrahepatic manifestations. Knowledge of the complex mechanisms underlying HCV-related inflammation and development of disease could be of importance for understanding disease progression, predicting prognosis and, possibly, conceiving new therapeutic approaches targeting the different steps of the inflammatory response. In this respect, it is important to underline that HCV clearance by standard of care does not always mean recovery of all associated pathological conditions.

The aim of the present study was to review the current knowledge on the hepatic and systemic clinical impact of chronic HCV infection as a consequence of local and systemic inflammation. Figure 1 schematically depicts HCV-related factors which give rise to inflammation and its associated clinical conditions.

HCV AND INFLAMMATION: ESSENTIAL POINTS

HCV consists of a single-stranded RNA genome encoding a single polyprotein, which is post-translationally processed into single known proteins, 4 structural (C, E1, E2 and p7) and 6 non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B)[3]. Some of these proteins have a role in starting and maintaining chronic inflammation. NS5A, for instance, promotes inappropriate upregulation of cyclooxygenase-2 (COX-2)[4], which is an inducible COX isozyme able to contribute to chronic inflammation and fibrosis through production of various prostaglandins. Suppression of COX-2 protein levels has been reported to be accompanied by suppression of HCV replication[5].

Chronic HCV infection is characterized by the presence and activation of inflammatory cells in the liver, which are responsible for the persistent inflammatory state contributing to liver fibrosis and damage[6,7]. In addition to local inflammation in the liver, a concomitant low-grade systemic inflammation has been supposed in several studies, as suggested by increased pro-inflammatory cytokine serum levels and activation of blood monocytes in individuals with chronic HCV infection[8,9]. Moreover, chronic HCV infection has been associated with oxidative stress (OXS) activation, which may play a role in development of local and systemic inflammation.

Role of cytokines, proteins and oxidative stress

A number of proinflammatory cytokines appears to be activated in chronic HCV infection. The important role of interleukin-1β (IL-1β) has recently been emphasized[10]. Specifically, HCV has been shown to induce IL-1β production and secretion; hepatic macrophages have been found to produce high concentrations of IL-1β within HCV-infected liver; IL-1β has been demonstrated to play a critical role in inducing liver inflammation and disease progression. HCV-activated Nod-like receptor P3 (NLRP3) inflammasome has been found to be able to induce production of IL-1β, which, in turn, stimulates synthesis of pro-inflammatory cytokines and chemokines, other than gene expression linked to HCV disease severity[11,12].

A cross-talk between HSCs and HCV-infected hepatocytes has been described, which appears to be a key point in HCV-related inflammation. HCV-infected hepatocytes seem to be able to ignite inflammation in response to HSCs. Indeed, in *in vitro* co-cultures of HCV-infected hepatocytes and HSCs, IL-1β secreted by HSCs was shown to induce production of several pro-inflammatory cytokines and chemokines, such as IL-6, IL-8, and macrophages inflammatory proteins (MIP-1α and MIP-1β), by hepatocytes[13]. Moreover, HCV-related proteins (NS3, NS4, NS5) have been reported to trigger human KCs to produce inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1β[14].

Hyperproduction of certain cytokines may cause unbalance leading to specific consequences in the short and long term period. For instance, a higher TNF-α/IL-10 ratio has been found in patients with severe liver disease and hepatocellular carcinoma (HCC)[15]. Furthermore, a significant correlation between TNF-α and the degree of hepatic inflammation, expressed as histologic activity index (HAI), has been reported; likewise, TGF-β levels have been found to be significantly correlated with histologic fibrosis score[16]. TNF-α and TGF-β levels have been found to be simultaneously increased according to the severity of inflammation and fibrosis[17].

HCV has also been shown to activate toll-like receptors (TLRs), molecules implicated in the production of proinflammatory cytokines in the cells of innate immunity. Specifically, HCV core protein and NS3 protein have been demonstrated to activate TLR2[18,19]. TLRs activate, in turn, NF-κB with subsequent transcription of

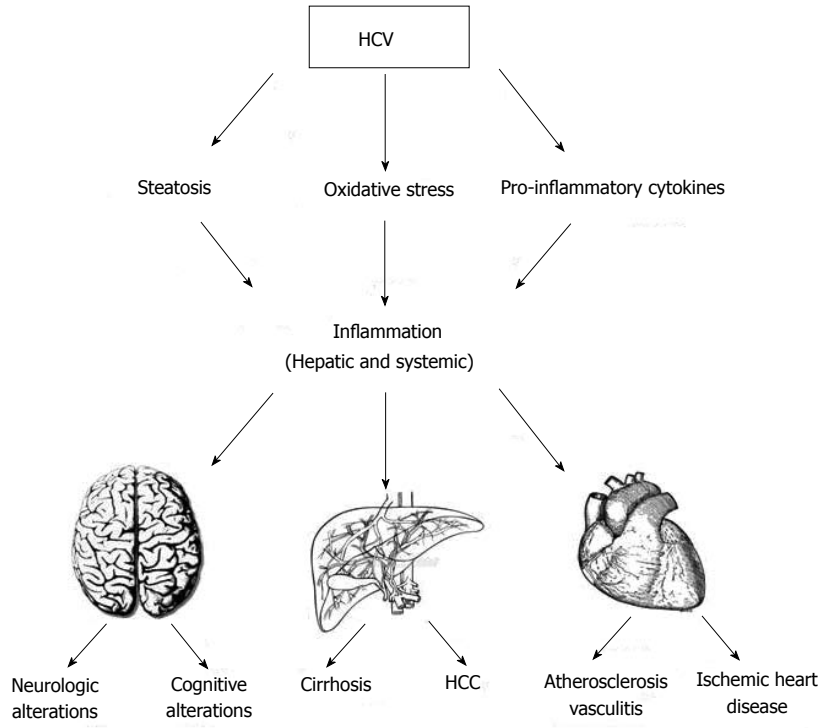

Figure 1 Factors associated with hepatitis C virus infection that contributes to the chronic inflammation and its involvement in hepatic and extrahepatic clinical conditions. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

inflammatory genes. Recent data have revealed increased expression of microRNA-155 and TNF-α production in monocytes, following TLR4 and TLR8 stimulation by HCV core, NS3, and NS5 proteins in chronic HCV infection[20].

In patients with chronic hepatitis C (CHC), intestinal bacterial overgrowth has been reported, which is usually followed by bacterial translocation and elevated blood concentrations of endotoxin (LPS)[21]. LPS induces local and systemic inflammation and is associated with progression to end-stage liver disease. Indeed, HCV-infected patients have been shown to harbor high plasma levels of LPS, intestinal fatty acid binding protein (a marker of enterocyte death), sCD14 (produced by LPS-activated monocytes) and IL-6. Markers of inflammation are remarkably elevated in individuals with severe disease than in those with minimal fibrosis[21]. TNF-α, one of the main cytokines produced following LPS stimulation, seems to be able to induce liver damage by TNF-receptor 1-mediated apoptosis[22]. Moreover, HSCs, which also express functional receptors for both bacterial endotoxin, including TLRs (particularly TLR4), and peptidoglycan recognition proteins, are able to develop a pro-inflammatory phenotype following LPS exposure[23].

CHC infection has been found to be associated with both hepatic and systemic OXS. Increased OXS in hepatitis C has been observed to be significantly linked to chronic inflammation[24,25], although iron overload, liver damage, and proteins encoded by HCV may also play a part. In CHC, OXS results from loss of equilibrium between reactive oxygen species (ROS) and antioxidant defense. ROS and reactive nitrogen species (RNS) are critically involved in the creation of oxidative stimuli required for physiologic hepatocyte homeostasis[26]. OXS-induced damage affects hepatocytes, endothelial cells, KCs, and HSCs through inflammation, ischemia, apoptosis, necrosis and regeneration[27,28]. ROS play an important role in fibrogenesis through increased proliferation of hepatic stellate cells as well as TGF-β and collagen synthesis[29]. Moreover, ROS interfere with repair of damaged DNA, thus making cells more susceptible to spontaneous or mutagen-induced alterations[30]. Increased ROS/RNS levels are associated with decreased antioxidant levels. Therefore, increased production of reactive oxygen and nitrogen species, along with decreased antioxidant defense, favor development and progression of hepatic and extrahepatic complications of HCV infection.

A meaningful role for liver steatosis as "contact point" between OXS and liver damage in CHC patients infected by HCV genotype non-3 has been demonstrated[31]. Hepatic steatosis is a striking feature of HCV infection, having been reported in more than 50% of HCV-infected patients[32]. Both HCV and metabolic conditions, *e.g.*, visceral obesity and insulin resistance (IR), play a role in the development of steatosis[32]. Recently, we have demonstrated that genetic and antropometric host factors may also be implicated in the development of steatosis in CHC patients. Indeed, the patatin-like phospholipase domain-containing 3 gene (*PNPLA3*) p.I148M polymorphism has been reported to influence the development of liver steatosis[33,34]; on the other hand, visceral obesity can increase the association between PNPLA3 p.I148M with liver steatosis[34].

Role of obesity, steatosis, insulin resistance and cannabinoid receptors

Obesity and liver steatosis are commonly observed among patients with CHC and are risk factors for both inflammation and increased hepatic fibrosis. Obesity is associated with a low-grade, chronic inflammatory re-

sponse that may contribute to pathogenesis of obesity-related comorbidities. Obesity and steatosis are associated with increased expression of typical inflammatory markers, such as IL-6 and TNF-α[35]. Hepatic TNF-α has been correlated with increased inflammatory activity, hepatic fibrosis, and liver injury in chronic HCV[35]. In obese-HCV subjects, an enhanced T helper-1 cytokine profile has been associated with hepatocellular injury; accordingly, an increased expression of T cell chemoattractants (IP-10 and MCP-1) in the liver of obese HCV subjects, along with increased inflammatory cell recruitment and CD3 expression, have been reported[36].

Visceral obesity plays an important role in the regulation of glucose and lipid metabolism in CHC patients. In obese HCV-infected patients, elevated levels of pro-inflammatory cytokines (namely, TNF-α and IL-6), able to inhibit insulin signalling, and reduced adiponectin levels[37], with consequent development of liver steatosis and IR, have been reported. Obesity has also been associated with increased liver fibrosis and poor response to antiviral treatment[38]. HCV also works by promoting the development of steatosis. Experimental models have demonstrated that expression levels of HCV core protein have profound effects on liver inflammation, steatosis and fibrosis[39]. Generally, IR precedes and has a key role in the development of hepatic steatosis in HCV infection. IR induces steatosis by overflow of substrates to the liver, increased *de novo* lipogenesis, and decreased fatty acid oxidation.

Steatosis has been associated with increased production of reactive oxygen species, which promotes lipid peroxidation and resulting hepatic stellate cell activation. Moreover, steatosis-induced hepatic inflammation has been shown to increase production of several proinflammatory and profibrotic cytokines. In addition, steatosic liver is more sensitive to TNF-α-mediated inflammation, liver injury, and apoptosis[40].

The mechanisms by which HCV-associated steatosis induces fibrosis are complex and not fully understood. OXS, proinflammatory cytokines, IR, and apoptosis seem to play an important role in the development of fibrosis associated with steatosis. Several reports, including a meta-analysis, have demonstrated that increased liver inflammation was associated with accelerated fibrosis in HCV patients with steatosis[41].

HCV infection portends an increased risk to develop type 2 diabetes (DM). HCV-infected patients with DM generally harbor a more severe chronic hepatitis and have a higher risk of developing hepatic cirrhosis and its complications (*i.e.*, hepatic encephalopathy, ascites), a shorter life expectancy, and a higher risk of HCC[42]. During IR, several inflammatory cytokines and lipid metabolites, such as free fatty acids, interfere with normal insulin signaling and promote DM[43]. TNF-α has been identified as the key molecule promoting development of IR and diabetes during HCV infection[44]. In addition, TNF-α is known to increase ROS production; hyperglycemia has also been recognized as a factor leading to OXS; eventually, increased generation of reactive species triggers a signaling cascade capable to alter the activity of insulin receptor substrates, leading to IR[45].

Cannabinoid receptors (CB) are found in high concentration in many organs, including the liver. There are two G protein-coupled CB, CB1 and CB2[46]. CB1 is found in high concentration in the brain, but is also present in many peripheral tissues such as the liver, adipose tissue, and gut. CB2 is found primarily in the immune system, but is also expressed in peripheral tissues including the liver[47]. Recent studies have suggested involvement of the endocannabinoid system in liver diseases. Specifically, CB1 seems to be upregulated in patients with CHC[48], while CB2 seems to have hepatoprotective properties in alcoholic liver disease[49] and in obese children[50]. Recently, we have evaluated the role of a functional polymorphism of the cannabinoid receptor type 2 in a cohort of CHC patients[51]. Our data demonstrated that HCV patients carrying the CB2-63 QQ variant polymorphism had advanced liver disease, higher serum ALT values, and higher necroinflammatory activity than those with the CB2-63 RR and QR variants. Moreover, the CB2-63 QQ variant and fibrosis score were identified as independent factors associated with higher scores of necroinflammatory activity (HAI > 8). Thus, the data suggest that the CB2-63 QQ variant is associated with more severe hepatic necroinflammation in anti-HCV-positive patients, thus confirming a role for the CB2 receptor in HCV-associated inflammation and cellular proliferation as well[51].

HCV, INFLAMMATION AND LIVER DAMAGE

Fibrosis progression

When inflammation fails to resolve an acute infection and chronic inflammation ensues, this process can lead to accumulation of fibrotic tissue. Fibrosis can be considered the result of unbalanced extracellular matrix production and degradation; its generation involves complex mechanisms including fibrogenesis, proliferation, contractility, chemotaxis, matrix degradation, and cytokine release[52].

The majority of the studies carried out in CHC patients have identified hepatic inflammation as the key pathological substrate driving fibrosis development[53]. On the other hand, therapeutic amelioration of hepatic inflammation in CHC has been associated with decreased fibrosis progression[54].

Mechanisms involved in the interrelationship between inflammation and fibrosis are complex and not completely understood. Cytokines play an important role in inflammation, regeneration, and fibrosis during chronic HCV infection; availability of cytokine patterns reflecting different stages of liver disease would be extremely useful in the clinic. In this regard, hepatocyte growth factor is a specific marker of liver cirrhosis[55]. Other proinflammatory small molecules, like ROS and other insoluble

mediators, such as the hepatic neomatrix during wound healing, appear to play a role in the development of liver fibrosis[29].

Hepatic inflammation mediates fibrogenesis also in patients with liver steatosis and CHC. In a meta-analysis including large and geographically different groups of CHC patients, steatosis was confirmed to be significantly and independently associated with progression of hepatic fibrosis through liver inflammation[41].

The role of miR-122 in CHC progression has been recently described. Circulating miR-122 serum levels appeared to be elevated at early stages of disease with high inflammatory activity and low fibrosis levels, while they decreased in the presence of severe fibrosis, probably due to loss of liver cells[56].

Genetic factors have been hypothesized to favor development and progression of fibrosis. In chronic HCV infection, a higher frequency of gene polymorphisms for key inflammatory mediators has been found in association with advanced disease[57]; specifically, definite polymorphisms in CCR5, RANTES, and MCP-1 alleles may predispose CHC patients to a higher degree of liver inflammation and advanced fibrosis[58,59]. COX-2 has too been found involved in inflammation; its -1195GG genotype has been recognized as a genetic marker for liver disease progression in Japanese patients with CHC[60].

HCC development

Chronic HCV infection is a major risk factor for HCC development worldwide. CHC can progress to HCC through fibrosis and cirrhosis. HCC is the 3rd leading cause of cancer death worldwide; it typically arises in patients with chronic inflammation and cirrhosis in 90% of cases[61].

At present, a large body of data links HCV infection, inflammation, free radical production, and carcinogenesis in CHC patients[62-66].

Although HCC pathogenesis in the setting of HCV infection has been subjected to extensive investigations, no conclusive data are available. However, both HCV-induced chronic inflammation and cytokines, involved in fibrosis development and liver cell proliferation, are considered as major pathogenic mechanisms. In contrast to HBV, HCV does not integrate into the host genome and does not have a reverse transcriptase. In infected subjects, both viruses are known to trigger an immune-mediated inflammatory response which either clears infection or slowly destroys the liver[67]. Thus, HCV can be carcinogenic by damaging liver tissue through chronic inflammation. This latter condition predisposes susceptible cells to neoplastic transformation.

During inflammation, a variety of proinflammatory cytokines, chemokines, growth factors and inflammatory enzymes are released[68]. Inflammation may induce cellular DNA mutations through oxidative/nitrosative stress[30]. Free radical production and oxidative genomic injury trigger the cascade of epigenetic (altered DNA methylation), genomic (mutations), and post-genomic (protein oxidation and cytokine synthesis) events leading to HCC[69]. Initially, ROS interact directly with DNA, damaging specific genes controlling cell growth and differentiation, cell cycle, apoptosis, lipid peroxidation and DNA damage repair[70,71]. Moreover, HCV-infected patients have been shown to display increased lipid peroxidation levels[72]. An increased oxidative stress gene response in patients with HCV-related fibrosis and cirrhosis has also been demonstrated by microarray and proteomics studies[73]. Thus, during chronic HCV infection, increased ROS, in part due to inflammation, may impair repair of damaged DNA, resulting in higher cell susceptibility to genetic alterations; this promotes the development and progression of HCC[69,74]. Inflammatory cells have been reported to release cytokines, chemokines, nitric oxide, particularly, an inducible isoform of nitric oxide synthase (iNOS), and NO-derived RNS, which too can cause DNA damage and cellular proliferation[75-77].

Chief elements linking inflammation to cancer through oxidative/nitrosative stress appear to be prostaglandins and cytokines. Capone *et al*[78] evaluated the serum levels of 50 different cytokines, chemokines, and growth factors in 26 patients affected by HCC superimposed on chronic HCV hepatitis and liver cirrhosis. A number of proinflammatory molecules (IL-1α, IL-6, IL-8, IL-12p40, GM-CSF, CCL27, CXCL1, CXCL9, CXCL10, CXCL12, β-NGF) were found to be significantly increased in HCC patients compared to healthy controls. Interestingly, IL-8 and IL-6 concentrations were demonstrated to significantly correlate with a larger tumor burden[78].

HCV, INFLAMMATION AND SYSTEMIC CLINICAL CONDITIONS

HCV and lymphoproliferative diseases

HCV tropism for lymphatic tissue has been conclusively demonstrated. Since infected lymphocytes constitute the main reservoir of the virus, this could explain the development of several lymphoproliferative disorders. Mixed cryoglobulinemia (MC), a B-cell lymphoproliferative disease, has been commonly reported in association with chronic HCV infection; it may progress to overt lymphoma in some subjects.

MC is featured by the presence of serum cryoglobulins, *i.e.*, immunoglobulins (Igs) capable to reversibly precipitate at low temperatures and to form immune complexes consisting of complement, monoclonal and polyclonal Igs (type Ⅱ MC), or polyclonal Igs (type Ⅲ MC), with specificity against HCV antigens. Monoclonal IgM with rheumatoid factor (RF) activity is typically detected in type Ⅱ MC. Viral antigens and HCV-RNA have been consistently isolated from immune complexes, thus confirming the role of the virus in MC pathogenesis[79-81].

Cryoglobulinemia may cause vasculitis due to precipitation of cryoglobulin-containing immune complexes in small- and medium-sized blood vessels. Plugging and thrombosis of small vessels and a systemic inflammatory syndrome are responsible for the many clinical signs and

symptoms of MC, including livedo, purpura (particularly on dependent areas), ulcers, arthralgia, arthritis, *etc*. Vasculitis may also involve the kidneys, the gastrointestinal tract, the peripheral nervous system, and/or other body compartments.

Cryoglobulins are detectable in about 50% of CHC patients; symptomatic disease is usually limited to about 15% of cases. However, symptomatic MC has been found to be associated with a poor prognosis[82]; about 10% of patients are prone to develop B-cell malignancies, especially B-cell non-Hodgkin lymphoma[83-85].

Mechanisms responsible for progression of HCV infection to cryoglobulinemia and other lymphoproliferative disorders are still unknown. Cryoglobulins result from a complex interaction between the virus and the host. In this regard, HCV core protein has been hypothesized to play an important role, having been found within the cryoprecipitate[79,86]. Indeed, HCV core protein has been reported to interact with the globular domain of C1q complement receptor, suggesting a role in complement activation[87,88]. Specifically, HCV core protein may be hypothesized to boost inflammation by enhancing complement activation. Finally, certain motifs on HCV/E2 glycoprotein and HCV/NS3 region have been proposed to act as molecular mimickers of specific Ig portions[89,90].

HCV, atherosclerosis, and cardiovascular diseases

An association between chronic HCV infection and atherosclerosis has been frequently reported in the literature[91-94]. Chronic HCV infection has also been reported in association with coronary artery disease[94-96] and stroke[97].

As previously stated, HCV infection is known to promote immune stimulation, cytokine production, and chronic inflammation[98,99]. HCV has been described to be able to create an inbalance between Th1 and Th2 cytokines, thus altering the equilibrium between cellular immunity, promoted and maintained by IL-2, TNF-α and interferon γ (IFN-γ), and humoral immunity, sustained by IL-4, IL-5, IL-6 and IL-10[100]. HCV-driven prevalence of inflammatory cytokines can be supposed to contribute to development of cardiovascular disease due to several effector mechanisms, including enhancement of intracellular adhesion molecules, expression of anti-endothelium antibodies, and generation of OXS and IR[91].

Recently, the interrelationships between HCV infection, atherosclerosis, and immune response have been carefully examined. In the Heart and Soul Study[101], patients with HCV infection were demonstrated to have higher TNF-α levels and a higher risk of cardiac failure and death than patients without HCV infection. Mostafa *et al*[102] found a more pronounced intima-media thickness in HCV-positive patients than controls; Olivera *et al*[103] reported an intermediate cardiovascular risk, as measured by the Framingham score, a higher levels of pro-inflammatory cytokines (IL-6 and TNF-α), and a higher ratio of proinflammatory/anti-inflammatory cytokines (TNF-α/IL10 and IL-6/IL-10) in non-obese, non diabetic, HCV-infected patients with respect to controls[103].

Cytokine imbalance has also been associated with development of IR in HCV-infected patients. A high TNF-α/adiponectin ratio has been found correlated with IR and atherosclerosis development[104]. Moreover, elevated levels of inflammatory biomarkers, such as matrix metalloproteinase-9, intercellular adhesion molecule-1, and oxidized low-density lipoproteins, have been observed in patients with CHC. Besides the role of HCV in the development of chronic inflammation involving the arteries, HCV RNA sequences and intermediate replicative forms have been detected within carotid plaques, thus suggesting *in situ* viral replication and consequent local pro-atherogenic action[105,106].

A histological feature of CHC is liver steatosis[32]. As for nonalcoholic fatty liver disease (NAFLD)[107-109], HCV-related steatosis can be considered a cardiometabolic risk factor[94]. Patients with chronic HCV infection have been shown to have a higher prevalence of carotid atherosclerosis than both healthy controls and NAFLD patients; moreover, it may detect at a younger age, particularly in the presence of liver steatosis. Indeed, like steatosis, HCV viral load has been found to be independently associated with carotid atherosclerosis[94]. In HCV patients, atherosclerosis was shown to be independently associated with elevated systemic inflammatory markers, such as C reactive protein and fibrinogen[94]. HCV and HCV-related steatosis have been hypothesized to favor the development of atherosclerosis independently of known risk factors such as hypercholesterolemia, smoking and hypertension, through multiple cardiometabolic risk factors, including inflammatory cytokines, hyperhomocysteinemia, hypoadiponectinemia, IR, and features of the metabolic syndrome[94]. Indeed, HCV patients have been reported to have high levels of hepatic and systemic markers of inflammation, particularly ESR, CRP, fibrinogen, and N-terminal pro-brain natriuretic peptide[110]. Overall, available data point to chronic HCV infection as a factor capable to increase the risk of atherosclerosis and vascular disease through systemic inflammation[110-113].

HCV and brain diseases

About 50% of patients with HCV infection suffer from neuropsychiatric symptoms, "brain fog", weakness, fatigue, along with some degree of quality of life impairment, regardless of liver disease severity[114] and HCV replication[115]. A direct effect of HCV on the brain or neurotoxic effects of HCV-related systemic inflammation have been hypothesized.

HCV neuroinvasion has been described[116,117], which could be the cause of neurological disturbances in HCV patients. Evidence suggests that the brain, but not the peripheral nerves or skeletal muscles, is a permissive site for viral replication, as inferred by the detection of replicative intermediate forms of HCV RNA and viral proteins within the central nervous system[118]. Additional mechanisms, contributing to neurological dysfunction,

are possibly related to the effects of circulating inflammatory cytokines and chemokines reaching the brain through an altered blood-brain barrier[119]. Evidence for a direct role for peripheral proinflammatory cytokines in determining impairment of neurocognitive function has been obtained from animal models. Specifically, increased peripheral IL-1 and IL-6 have been found to correlate with increased levels of the same cytokines in the prefrontal cortex and hyppocampus[120]. HCV within the brain seems to be able to also induce a local inflammatory response, since macrophages infected *in vitro* with HCV are able to produce TNF-α and IL-8[121-123].

Neurologic alterations during HCV infection include a broad spectrum of manifestations, ranging from cerebrovascular events to autoimmune syndromes. In HCV infected patients, acute cerebrovascular events, such as ischemic stroke, transient ischemic attacks, lacunar syndromes have been reported, particularly in cryoglobulinemic patients[124-127].

A consistent proportion of HCV-infected patients complain of chronic depression and anxiety. Moreover, about 15% of patients suffer from recurrent depression[128]. At present, definitive conclusions regarding the pathogenesis of cognitive dysfunction, fatigue, and depression in chronic HCV infection cannot be drawn. Many studies seem to suggest a possible direct role for HCV, due to its ability to replicate within the brain[118]. In addition, brain microvascular endothelial cells have been recently demonstrated to support HCV tropism and replication. HCV has been shown to induce apoptosis in these cells, with consequent alterations in the blood-brain barrier, microglia activation, and, eventually, diffusion of inflammatory cytokines and chemokines into the brain[129].

HCV AND INFLAMMATION: THERAPEUTIC PERSPECTIVES

Because of the role of inflammation in the progression of CHC and its systemic manifestations, an anti-inflammatory approach to control and eventually arrest disease would result theoretically useful. For instance, interruption of COX-2 signaling seems to be a possible approach for controlling HCV replication and associated diseases[6,130]. In this regard, non-toxic concentrations of aqueous extracts of the edible seaweed *Gracilaria tenuistipitata* have been successfully tried *in vitro*[130].

Considering the effects of LPS on circulating monocytes and resident KCs, attenuation of microbial translocation and its inflammatory consequences may be deemed advantageous to improve clinical outcome in HCV infection[21].

Therapy for cryoglobulinemia should encompass three objectives: elimination of HCV infection, control of B lymphocyte proliferation, and symptomatic treatment of immune-complex disease. Standard of care for HCV treatment with pegylated interferon plus ribavirin should be considered as the first-line therapeutic option in patients with mild to moderate HCV-related MC; a prolonged treatment (up to 72 wk) may be considered in the case of virological non-responders showing clinical and laboratory improvements. Rituximab (RTX), a chimeric moAb specific for CD20 antigen used for treatment of autoimmune and lymphoproliferative disorders, should be considered in patients with severe vasculitis and/or skin ulcers, peripheral neuropathy, or glomerulonephritis[131]. In a recent study carried out in patients with HCV-related cryoglobulinemia, triple therapy with pegylated IFN-α, ribavirin, and RTX was shown to yield a a complete response in 54.5% of patients, as opposed to 33.3% in those who received only pegylated IFN-α and ribavirin ($P < 0.05$)[132].

Several studies investigating the effects of selenium, glycyrrhizin, polyprenoic acid, vitamin E, and vitamin C have demonstrated that comsumption of higher amounts of antioxidants was not associated with a decreased risk of developing HCC. At present, the best strategy to prevent HCC still lies in antiviral treatment of chronic HCV infection[133]. In the future, novel therapeutic strategies targeting molecules involved in HCV-induced OXS may be expected to enter clinical practice as soon as these mechanisms will be progressively unraveled.

IL-1β is strictly involved in inflammation[134]. Drugs blocking IL-1β seem to be able to control inflammation irrespective of the molecular pathways activated by this cytokine. Canakinumab, a human monoclonal antibody selectively specific for IL-1β, has been shown to reduce the levels of many inflammatory biomarkers. Canakinumab is generally well tolerated and could be theoretically conceived for use in the secondary prevention of cardiovascular disease[134,135].

Pentoxyfilline (PTX), a nonspecific phosphodiesterase inhibitor with anti-inflammatory and anti-fibrogenic properties, has been successfully used in different models of liver disease, including non-alcoholic steatohepatitis[136], inflammation[137], fibrosis/cirrhosis[138], alcoholic liver disease[139] and endotoxinemia[140]. Its beneficial effects have been associated with downregulation of TNF-α, IL-1, IL-6, TGF-β, IFN-γ along with downregulation of stellate cell activation, procollagen I mRNA expression[141], cell proliferation, and extracellular matrix synthesis[142]. PTX has been shown to affect the expression of several pro-inflammatory cytokines in both liver and peripheral blood mononuclear cell of patients with hepatitis C, particularly TNF-α and IL-1β production; thus, PXT could also be considered for inhibition of inflammation in patients with CHC[143].

The endocannabinoid system can be considered a promising target for treatment of CHC, for the reasons stated above[51]. Indeed, the efficacy of peripherally restricted CB1 antagonists on fibrosis has been already validated in preclinical models of NAFLD. Similarly, CB2 receptor is currently considered as a promising anti-inflammatory and antifibrogenic target, although clinical development of CB2 agonists is still awaited[144].

CONCLUSION

A growing body of evidence supports the essential role

of CHC-related inflammation in the pathogenesis of hepatic and extrahepatic HCV-related disease. Defining the mechanisms of HCV-induced hepatic inflammation is paramount for devising attractive approaches to avert HCV-related liver disease. The way to go is still long and difficult, however, understanding the pathogenesis of HCV-related inflammation may help discover novel elements to be targeted for more effective treatment of CHC and its complications. In this regard, current knowledge suggests exploring therapeutic options targeting "inflammation" in CHC in future clinical trials, particularly in nonresponders to standard antiviral treatment.

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REVIEW

Cytoprotective role of heme oxygenase-1 and heme degradation derived end products in liver injury

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Abstract

The activation of heme oxygenase-1 (HO-1) appears to be an endogenous defensive mechanism used by cells to reduce inflammation and tissue damage in a number of injury models. HO-1, a stress-responsive enzyme that catabolizes heme into carbon monoxide (CO), biliverdin and iron, has previously been shown to protect grafts from ischemia/reperfusion and rejection. In addition, the products of the HO-catalyzed reaction, particularly CO and biliverdin/bilirubin, have been shown to exert protective effects in the liver against a number of stimuli, as in chronic hepatitis C and in transplanted liver grafts. Furthermore, the induction of HO-1 expression can protect the liver against damage caused by a number of chemical compounds. More specifically, the CO derived from HO-1-mediated heme catabolism has been shown to be involved in the regulation of inflammation; furthermore, administration of low concentrations of exogenous CO has a protective effect against inflammation. Both murine and human HO-1 deficiencies have systemic manifestations associated with iron metabolism, such as hepatic overload (with signs of a chronic hepatitis) and iron deficiency anemia (with paradoxical increased levels of ferritin). Hypoxia induces HO-1 expression in multiple rodent, bovine and monkey cell lines, but interestingly, hypoxia represses expression of the human *HO-1* gene in a variety of human cell types (endothelial cells, epithelial cells, T cells). These data suggest that HO-1 and CO are promising novel therapeutic molecules for patients with inflammatory diseases. In this review, we present what is currently known regarding the role of HO-1 in liver injuries and in particular, we focus on the implications of targeted induction of HO-1 as a potential therapeutic strategy to protect the liver against chemically induced injury.

Key words: Heme oxygenases; Bilirubin; Hepatitis C; Kupffer cells; Polymorphisms; Immunoregulatory; Hypoxia; Liver ischemia

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HEME OXYGENASES

Heme oxygenases (HOs) are ubiquitous and essential enzymes for all eukaryotic organisms that depend on aerobic oxidation and electron transport *via* heme-containing proteins[1,2]. HOs were first recognized as catalyzing the rate-limiting step in the principal degradative mechanism

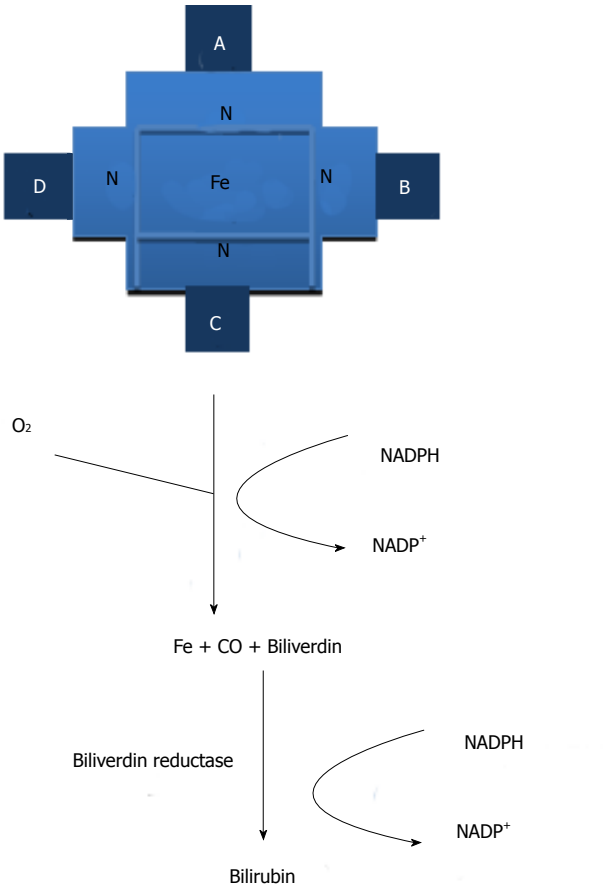

Figure 1 The heme oxygenase enzyme reaction. Heme is enzymatically degraded to yield carbon monoxide, iron and biliverdin (which is converted into bilirubin in a coupled reaction). CO: Carbon monoxide; NADP: Nicotinamide adenine dinucleotide phosphate; NADPH: β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt.

of heme (iron protoporphyrin IX)[2,3] catabolism. In a reaction that requires oxygen and nicotinamide adenine dinucleotide phosphate (NADP), the heme ring is cleaved by HO to yield biliverdin, along with the concomitant release of iron and the emission of carbon monoxide (CO) (in equimolar quantities). Biliverdin (BV) is then reduced to bilirubin (BR) by biliverdin reductase[4] (Figure 1).

Two distinct isoforms of HO (the products of different genes) have been identified. HO-1 is a single transmembrane inducible protein found in endoplasmic reticulum, caveola, nuclei and mitochondria. It is ubiquitously present in mammalian tissues such as liver, spleen, pancreas, intestine, kidney, heart, retina, prostate, lung, skin, brain, spinal cord, vascular smooth muscle cells and endothelial cells. Its expression is relatively low under physiological conditions, except in the spleen where the action of HO-1 is critical to the recycling of iron from senescent erythrocytes[5]. HO-2 shares 40% amino acid homology with HO-1; it is constitutively expressed and may provide an additional temporary buffering function against pro-oxidant heme by means of sequestration (*via* additional heme binding sites located on the enzyme). It is localized to the mitochondria where it likely regulates a variety of cellular functions. The existence of a third distinct isoform of HO encoded (HO-3) has been postulated but it is now clear that this is a non-coding pseudogene[1]. Both isoforms, HO-1 and HO-2, of this enzyme catalyze the same enzymatic reaction, resulting in the degradation of heme[6].

The role that HO-1 plays in the modulation of the immune response has been increasingly studied within the field of immunology and it is now recognized that HO-1 may act as a molecular brake on the activation, recruitment and amplification of immune responses[7]. Over-expression of HO-1 results in reduced expression of endothelial-leukocyte adhesion molecules and reduced activity of the nuclear factor-κB (NF-κB) pathway; conversely, HO-1-deficient animals exhibit increased levels of monocyte chemo-attractant protein-1. In humans, HO-1 deficiency is associated with susceptibility to oxidative stress and an increased pro-inflammatory state correlated with severe endothelial damage[8]. Mice lacking HO-1 develop progressive inflammatory diseases[9] and show enhanced sensitivity to lipopolysaccharide (LPS)-induced toxemia. HO-1 deficiency shows partial embryonic lethality. HO-1 knockout mice display a progressive chronic inflammatory disease characterized by enlarged spleens and hepatic inflammatory lesions. Additionally, the protective properties of HO-1 have been studied in a variety of inflammatory models[6].

EXPRESSION AND TRANSCRIPTIONAL REGULATION OF HO-1

The human *HO-1* gene is located on chromosome 22q12; it is approximately 14 kb long and contains 5 exons[8]. Control of HO-1 transcription is complex and tightly regulated, with differences in expression found between tissues, as well as between species[9]. A wide variety of stimuli have been shown to induce HO-1 expression, including heme, heavy metals, hydrogen peroxide, oxidized low density lipoproteins, hyperoxia, hypoxia, endotoxins, nitric oxide (and nitric oxide donors), cytokines, angiotensin Ⅱ, shear stress and ultraviolet radiation[3] (Figure 2). Its biological function is to provide a specific regulatory mechanism for control of the level of many heme proteins. Multiple regulatory elements control human *HO-1* gene transcription. These elements contain numerous transcription factor consensus binding sites in both the proximal and distal 5' promoter sequences, as well as in an internal enhancer region. *HO-1* gene expression can be up-regulated through its multiple stress response and cadmium response elements, as well as by numerous important transcription factors, including Jun B, activator proteins 1 and 2, and NF-κB[10]. Conversely, the transcription factors Bach1 and Jun D act as negative regulators of human *HO-1* gene expression[2,11].

Hypoxia induces HO-1 expression in multiple rodent, bovine and monkey cell lines, but interestingly, hypoxia represses expression of the human *HO-1* gene in a variety of human cell types (endothelial cells, epithelial cells,

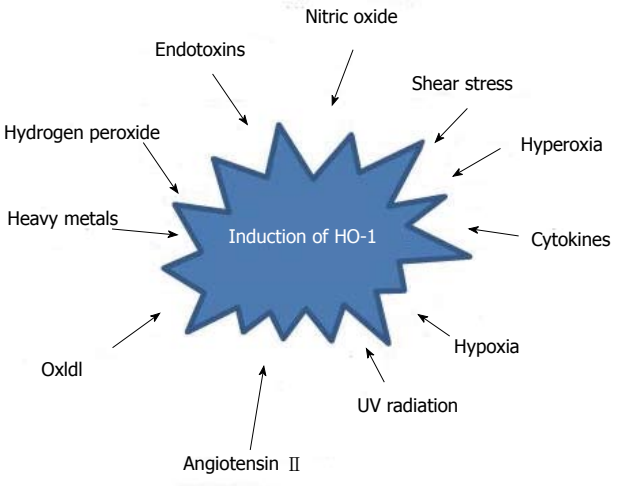

Figure 2 A variety of stimuli induce heme oxygenase-1 expression, a wide array of hepatotoxic chemicals and conditions conferring an adaptive cytoprotective response. HO-1: Heme oxygenase-1; UV: Ultraviolet.

T cells)[5]. The short guanosine/thymine (GT)n[12] repeat in the *HO-1* gene promoter may provide protective effects against carotid atherosclerosis in individuals with a high level of arsenic exposure. Therefore, the translation of HO-1 research from "bench to bedside" must be carried out carefully, with the appreciation that the response of *HO-1* gene expression to certain stimuli may be different in humans and rodent models[9].

HEME OXYGENASE-1 PROMOTER POLYMORPHISMS

There are 3 possible polymorphisms in the 5' flanking region: a (GT)n dinucleotide length polymorphism and two single nucleotide polymorphisms (SNP). Functional polymorphic dinucleotide guanosine/thymine (GT) repeat regions are present in the proximal promoter region of HO-1 approximately encompassing nucleotides -248 to -198, relative to the transcription start repeats regions in the HO promoter. The reported number of GT-repeats range from 12 to 40, although a bimodal distribution exists in most populations, with the main alleles being composed of 23 or 30 repeats. HO-1 mRNA expression and enzyme expression are greater when GT-repeat lengths are short and lesser when they are long[9]. There is some evidence that links H_2O_2 or other HO-1 transcriptional activators and the number of GT repetitions and it has been postulated that conformational changes account for the modulation of transcriptional activity. For instance, a left-handed double-helix structure (Z-DNA conformation) may be formed by the alternating purine-pyrimidine sequences, an arrangement that has been correlated with diminished transcriptional activity in other genes[3]. Indeed, Yamada *et al*[13] observed that GT-dinucleotide repeat polymorphisms in the promoter region of the *HO-1* gene were frequently associated with increased HO-1 responses to exogenous stimuli.

The inducibility of HO-1 is affected by (GT)n polymorphisms[14], as well as the SNP A(-413)T, in the promoter of HO-1[8]. Both short (GT)n alleles and the A-allele have been associated with increased HO-1 promoter activity[4]. For instance, in 308 liver transplantations, HO-1 patient genotypes were correlated with outcome variables[15]. With respect to their (GT)n genotype, liver samples were divided into two classes - those containing short alleles (less than 25 repeats; class S) and those containing long alleles (greater than or equal to 25 repeats; class L). Haplotype analyses confirmed the dominance of the A (-413)T SNP over (GT)n polymorphism[16].

The results of transient transfection assays using luciferase promoter constructs were consistent with *in vitro* assays carried out in lymphoblastoid cell lines containing HO-1 GT repeats of various lengths[9]. Cells homozygous for short GT-stretches had significantly greater HO-1 expression and resistance to oxidant-induced apoptosis compared with cells homozygous for long GT-stretches, demonstrating that polymorphisms in GT-stretch length are functionally significant[9].

Hyperbilirubinemia is associated with multiple repeats of TA nucleotides, as well as with the 211 G mutation, which are polymorphisms found in the *UGT1A1* gene promoter[15] and coding regions, respectively[1]. However, it is not clear how these polymorphisms influence overall bilirubin levels and whether polymorphisms in other genes involved in the bilirubin metabolism pathway also influence overall bilirubin levels[3]. Although the short repeats found in certain HO alleles (< 25 repeats) have been found to have beneficial effects on the serum bilirubin levels and lipid profiles of patients with coronary artery disease (CAD), few studies have examined the effects of these repeats in healthy patients with normal bilirubin levels or patients who carry short GT-repeats (within in the HO-1 promoter) who are at a high risk for developing unconjugated hyperbilirubinemia[17]. Considering the antioxidant and anti-atherogenic properties of bilirubin, the beneficial effects of the S allele on serum bilirubin levels in carriers might be sufficient to exert a protective effect against the development and clinical severity of CAD[3].

Taha *et al*[18] have shown that pentoxifylline (PTX, a drug used to improve blood flow in patients with circulation problems) in fibrosarcoma L292 cells strongly induces the expression of HO-1. The potential involvement of HO-1 in the regulation of PTX signaling is of particular importance, especially with respect to human HO-1 promoter polymorphisms[19]. The presence of alleles with lower activity (resulting in lower HO-1 expression) was demonstrated to be a contributing factor to increased risk of cardiovascular complications, at least in some populations of patients[5]. The cytoprotective effects of PTX treatment were reproduced by incubation with hemin (an HO-1 activator)[5] or with CORM (a compound that causes the release of CO)[15]. These effects were blocked by treatment with ZnPPIX (zinc protoporphyrin-IX)[20], an HO-1 inhibitor[15]. Thus, both PTX and HO-1 appear to have anti-inflammatory properties, as exemplified by

the inhibition of interleukin (IL)-1β, IL-6, IL-8, TNF-α and granulocyte-macrophage colony-stimulating factor expression, and the induction of IL-10 expression[15,21]. Furthermore, the expression of both genes has been shown to reduce proliferation, migration and adhesion of leukocytes to endothelial cells, through inhibition of CD25 and ICAM-1[6].

Thus, one can expect that the efficacy of PTX treatment is likely to be influenced by these polymorphisms and the efficacy of this treatment is likely to be low in patients with less active HO-1 alleles[15].

The *HO-1* genotype is associated with liver transplantation outcome and these findings suggest that HO-1 mediates graft survival following liver transplantation[1,5]. Therefore, the expression of the *HO-1* gene may be altered by the number of (GT)n repeats and could play an important role in modulating vascular tone under different pathological situations[3].

MODULATORY AND CYTOPROTECTIVE EFFECTS OF HO-1

Induction of HO-1 and its metabolites has a protective effect in a large number of seemingly unrelated pathologies, including sepsis, malaria[20], endotoxic shock, ischemic reperfusion injury (IRI)[4], organ transplant rejection[22], induction of tolerance myocardial infarction, type 2 diabetes[15] and obesity[23]. This wide spectrum of protective effects has been attributed to multilevel mechanisms of cytoprotection and inflammatory modulation. Three catabolic products, BV, BR and CO[22], and ferritin (which is induced by free iron[2]) have been implicated in HO-1 control of apoptosis and cell proliferation. However, the effects of BV/BR and CO do not overlap in terms of their effects on cell signaling and target molecules. The beneficial effects of the three products generated by HO-1 differ not only in their inherent molecular mechanisms, but also in their downstream cellular targets. To date, this is the only enzymatic system known to exhibit such characteristics; a good example of this is the manner in which HO-1 regulates the cell cycle - it increases endothelial cell (EC) proliferation but simultaneously decreases airway and vascular smooth muscle cell cycle progression[4]. A number of intracellular signaling molecules have been identified as being involved in regulating the induction of HO-1; among which upstream signaling kinases, extracellular signal-regulated protein kinase (ERK), c-Jun N terminal kinase and p38 mitogen activated protein kinase (MAPK) have been considered to play major roles in controlling up-regulation of HO-1[24]. Activation of either one or more of these MAPKs by external stimuli triggers *HO-1* gene expression. ERK1/2 and p38 MAPK are involved in the induction of *HO-1* gene transcription by sodium arsenite in the hepatoma cell line.

CO acts through a variety of pathways, including increased cycling of guanosine monophosphate through the activation of guanylate cyclase[15], modulation of inducible nitric oxide synthase[5] and regulation of protein kinases C (which have effects on vascular smooth muscle cells)[6]. Exogenous CO can be delivered in both an *in vivo* model and *in vitro* model, as either a gaseous molecule or in the form of innovative CO-releasing molecules (CORM) that are currently being developed by Hemo-CORM and Alfama Inc[3,19].

HO-1 expression protective effects are linked to a combination of factors, such as removal of the reactive substrate heme, the biological effects of the reaction products, the suppressive effects on monocyte chemoattractant protein 1, and modulation of cell-cycle regulators[7]. It has been suggested that activated Kupffer cells play an important role in the pathogenesis of fulminant hepatic failure, as reflected by the activation of both pro and anti-inflammatory cascades in the innate immune system[8]. Recent studies have identified serum HO-1 levels as a novel diagnostic marker for macrophage activation under conditions including sepsis and hemophagocytic syndrome, which is useful for predicting the severity and prognosis of acute liver injury[1,5].

Kawakami *et al*[22] have confirmed that T helper type 17 (Th17) cells IL-17-producing $CD4^+$ T cells[25] have a fundamental role in the immunopathogenesis of experimental autoimmune encephalomyelitis (EAE). Hammerich *et al*[26] demonstrated that myeloid HO-1 deficiency exacerbated EAE in mice and enhanced infiltration of activated macrophages and Th17 cells to the central nervous system, thereby establishing HO-1 as a critical early mediator of the innate immune response in EAE[3].

The liver is a primary source of the IL-7 response to Toll-like receptor (TLR) stimulation, although IL-7 expression in the liver is limited under steady state conditions[27]. TLR-mediated systemic cytokine expression alters protein expression[9,15]. Profiles in hepatocytes, which produce a number of acute phase response proteins, show markers of systemic inflammation, including ferritin[28], plasminogen and complement components[9] in ischemia-reperfusion injury (IRI)[5]. IL-7 is an important survival factor for T cells and is expressed in the liver as an acute phase reactant in response to inflammation, which allows the immune system to respond to pathogens[9]. IL-7 expression is increased in the liver and kidney tissues only following TLR stimulation, whereas it decreased in the spleen and lymph nodes[27,29]. These results suggest that systemic hepatocyte-mediated IL-7 expression (but not expression mediated by the lymphoid organs) plays an important role in IL-7 mediated T cell homeostasis in response to infection[9].

In recent years, the immunomodulatory capacity of the adaptive responses by HO-1 has received more attention. It has been reported that HO-1 can promote graft tolerance by activation/production of $CD4^+/CD25^+$ regulatory T cells (Tregs) or by promoting the activation-induced cell death of T cells[8]. $CD4^+/CD25^+/Foxp3^+$ Tregs are a functionally distinct subset of mature T cells with broad suppressive activity[9]. Tregs play a key role in the maintenance of peripheral tolerance and Treg defi-

ciencies can lead to progressive autoimmune disorders[5]. Similarly, increased Treg function can prevent allograft rejection and suppress tumor immunity[15] during allogeneic hematopoietic stem cell transplantation (HSCT)[9]. Tregs have been shown to play an important role in establishing tolerance between recipient tissues and donor-derived immunity. This was initially demonstrated in murine studies in which the depletion of Tregs from stem cell grafts resulted in increased graft-versus-host disease (GVHD) and increased Treg levels resulted in the suppression of GVHD following transplant[5]. Patients with active chronic GVHD have lower Treg levels when compared with patients without chronic GVHD[15].

Recent studies have suggested that IL-7 and IL-15 are the primary homeostatic cytokines that drive $CD4^+$ T cell proliferation[25]. Previous analyses found that plasma IL-7 levels are consistently higher following allogenic HSCT, but IL-7 levels do not correlate with the degree of $CD4^+$ T-cell recovery. Thus, IL-7 may promote T-cell expansion following transplants, but high plasma levels of IL-7 persist after lymphopenia has resolved[15]. Unbalanced Th1/Th2 T-cell responses in the liver are a characteristic of hepatic inflammation and subsequent liver fibrosis. The recently discovered Th17 cells, a subtype of CD4(+) T-helper cells mainly producing IL-17 and IL-22, have initially been linked to host defense against infections and to autoimmunity.

HO-1 is not essential for TLR4/MyD88-induced NF-κB/MAPK activation and the subsequent pro-inflammatory cytokine production, but it is required for TLR4/TLR3/IRF3-induced production of IFN-γ[30], as well as the expression of primary IRF3-target genes in macrophages. IRF3 activation and subsequent IFN-γ production is severely impaired in HO-1 knockout macrophages infected with Sendai virus (SeV) used to down regulate HO-1 expression[31]. In the presence of polyI:C, myeloid HO-1 knockout mice infected with *Listeria monocytogenes* (an infection model showing enhanced severity that is dependent on IFN-γ production) showed increased levels of bacterial clearance, whereas control mice succumbed to infection[1,30].

Lee and Bai *et al*[12] identified a potential interplay between IL-10 and HO-1 in the inhibition of LPS-induced inflammatory responses in macrophages. They also provided evidence that HO-1 mediates the anti-inflammatory function of IL-10, both *in vivo*, and that IL-10 and HO-1 activate a positive feedback circuit to amplify the anti-inflammatory response[15]. IL-10 and HO-1 were up-regulated in patients receiving norfloxacin and correlated with norfloxacin in a concentration-dependent manner, whereas proinflammatory inducible nitric oxide synthase, cyclooxygenase-2 and NF-κB behaved inversely. Higher IL-10 levels correlated with lower white blood cell count and higher mean arterial pressure. No correlations were found between IL-10 and disease clinical scores or liver function markers in blood[9].

THE PHYSIOLOGICAL ROLE OF THE HO-1 GENE IN THE LIVER

The liver is the largest gland in the body and is situated slightly below the diaphragm and anterior to the stomach. It consists of two lobes that are wedge-shaped. Two blood vessels enter the liver, namely the hepatic portal vein, with dissolved food substances from the small intestine, and the hepatic artery, with oxygenated blood from the lungs[8,32]. Two ducts originate in the liver and these unite to form the common hepatic duct which opens with the pancreatic duct in the hollow side of the duodenum (the first section of the small intestine). The gall bladder lies inside the liver and is the storage place for bile, which is formed by the liver cells[8].

The hepatic microvasculature is a unique and well-organized system of microvessels, which are composed of parenchymal hepatocytes and a variety of non-parenchymal cells, such as sinusoidal endothelial cells, Kupffer cells[33] and Ito cells. Cytochrome P450[34] is a major contributor to production of bilirubin derived from non-hemoglobin sources in the liver[1].

HO-1 is expressed at low to undetectable levels in hepatocytes and is expressed mainly in Kupffer cells under basal conditions[33]. However, HO-1 undergoes a rapid transcriptional activation in both Kupffer cells and hepatocytes in response to noxious stimuli[35]. A physiological role for *HO-1* gene expression has also been demonstrated in HO-1 deficient mice and in one case of human genetic HO-1 deficiency. Both murine and human HO-1 deficiencies have systemic manifestations associated with iron metabolism, such as hepatic overload (with signs of a chronic hepatitis) and iron-deficiency anemia (with paradoxical increased levels of ferritin)[7]. Determining the role that HO-1 plays in such regulatory mechanisms has become increasingly relevant in recent years because its induction has been shown to prevent ethanol-induced inflammation in the intestine[36] and liver[4], as well as in the prevention of oxidative damage to hepatocytes[5].

MECHANISMS OF ACTION OF HMOX-1 IN THE LIVER

Hepatic ischemia and IR are the leading causes of clinical liver damage and occur[5] during major trauma concomitant with blood loss, extensive liver resection during tumor removal and liver transplantation[37]. Steatosis is a major risk factor for liver surgery because steatotic livers respond poorly to IRI. The predominant form of hepatocyte death is caused by apoptosis in ischemic non-steatotic livers, whereas steatotic livers develop massive necrosis following ischemic injury[1]. Mitochondria are the main cellular source of reactive oxygen species (ROS), which are observed to be dramatically increased in steatotic livers during the reperfusion period[38]. Increased ROS generation results in increased hepatic lipid peroxidation and oxidative stress, as well as decreased adenosine

triphosphate generation, which may partially explain the poor tolerance of steatotic livers to IRI. Increased neutrophil accumulation causes significant sinusoidal endothelial cell injury through the release of free radicals and proteases, which may further aggravate hepatic microcirculation *via* the dysregulation of vasoactive mediators[1]. Kupffer cells activation is also reported to be involved in the poor outcomes of patients with steatotic livers[4]. Oxidative injury leads to hepatocyte damage followed by activation of Kupffer cells and results in the production of a number of inflammatory cytokines, including TNF-α and IL-6. These results suggest that the HO system has a potent protective effect on acute liver injury induced by carbon tetrachloride, CCl_4. In rats, CXCL14 expression was increased in the liver injury phase and returned to normal after[2] liver regeneration, suggesting its involvement in the liver injury or regeneration regulation[5,9]. Inhibition of HO-1 has been shown to protect against tissue injury in livers exposed to CCl_4[11,39]. Animals pretreated with hemin and then exposed to CCl_4 showed a reduced leukocyte infiltration. Elevated HO activity has also been observed in damaged liver tissue, most likely due to interference with the HO pathway by tetrachloride-dependent metabolism triggered by heme overload-associated toxicity. Hepatocyte apoptosis and liver injury were both attenuated in rats pretreated with hemin[15]. Basal HO-1 levels appear to be more critical than the ability to up-regulate HO-1 in response to IRI and they may also be able to predict the success of pharmacologically induced cytoprotection. Consistent with this, cobalt protoporphyrin (CoPP)[40]-induced HO-1 up-regulation suppresses the type-1 interferon pathway downstream of the Toll-like receptor (TLR) 4 system in hepatic IRI models[8,17].

Orthotopic liver transplantation (OLT) is the best available treatment for patients with end-stage liver failure, during which the liver is exposed to some stressful stimuli, such as ischemia and reperfusion injury. HO-1 has been shown to provide cytoprotection during liver ischemia and reperfusion[41].

HO-1 expression has been suggested to have an immune-modulatory effect[2] and up-regulation of HO-1 has been shown to protect livers from IRI and improve graft survival[1]. All of the products formed during this process have potential beneficial effects in transplant settings. CO has vasodilatory effects, which help to maintain microvascular hepatic blood flow[3], BV and BR, and possess potent antioxidant properties[1]. Free iron is itself highly reactive; however, the cellular Fe^{2+} released during heme degradation up-regulates the expression of the Fe^{2+} sequestering protein ferritin, as well as an Fe^{2+} pump, thereby limiting the amount of free iron and preventing the generation of reactive oxygen species[8]. HO-1 participates in iron homeostasis. Javanmard *et al*[42] generated mice with targeted HO-1 null mutations and analyzed parameters of iron metabolism and they discovered that adult HO-1-deficient animals develop both serum iron deficiency and pathological iron-loading, indicating that HO-1 is crucial for the expulsion of iron from tissue stores.

HO-1 expression protects transplanted organs from IRI and immune rejection[8]. This review has demonstrated that persistent over-expression of HO-1 in donor livers can improve survival by expanding T regulatory cells in a model of OLT[43]. The possible mechanisms by which CO protects the liver against cold ischemia-reperfusion do not appear to be associated with down-regulation of the NF-κB-signaling pathway. ROS generation following re-oxygenation causes tissue damage and initiates a cellular cascade leading to inflammation, cell death and, ultimately, organ failure. A growing body of evidence suggests that Kupffer cells and T cells mediate the activation of neutrophil inflammatory responses[33]. The activation of TLRs in Kupffer cells may provide the triggering signal for pro-inflammatory responses in the IRI sequence[8]. Dissecting the signaling pathways that link HO-1 and TLR activation may be important in devising novel therapeutic strategies for combating IRI[4]. The evolutionarily conserved sentinel TLR belong to the IL-1R family and recognize bacterial/viral-specific pathogen-associated molecular patterns[5]. TLRs trigger host inflammatory responses that are mediated by macrophages, neutrophils and complement[44]. The induced cytokine mediators may then activate systemic responses to recruit leukocytes to sites of inflammation. The cellular and molecular mechanisms by which hepatic microvascular flow is regulated during normal and disease conditions are being actively investigated[2].

Intraportal delivery of an adeno-associated virus (AAV) expression vector encoding rat HO-1 (AAV-HO-1) resulted in persistent expression of HO-1 in hepatocytes (and increased HO-1 activity)[45] in transplanted livers, leading to prolonged survival in recipients[15]. Over-expression of HO-1 reduced the Banff rejection activity index, measured by ELISA, by inhibiting the production of IL-2 and TNF-α, decreasing infiltration of $CD4^+$ and $CD8^+$ cells, and increased infiltration of Treg cells into donor livers[5,46].

The spleens of recipients expressed higher levels of Foxp3, TGF-β and IL-10 compared with control rats and the transplanted livers expressed higher levels of Foxp3 and TGF-β[47]. Splenocytes from tolerant recipients had higher levels of Treg cells and responded poorly to allogeneic donor splenocytes. Persistent expression of HO-1 in donor livers by intraportal delivery of AAV-HO-1 improves survival by expanding the number of Treg cells. The HO-1-based therapies described here are promising new strategies for the prevention of liver transplant rejections[48].

During maturation, dendritic cells (DCs) inhabit a variety of microenvironments that contain different levels of chemokines and chemokine receptors. Immature DCs express CCR1, CCR2, CCR5 and CXCR1, while mature DCs express high levels of CCR7, CCL22, CCL5, CCL2 and CXCL10[39]. In CoPPIX-treated mice, CCR7 was observed to be markedly down-regulated in liver DCs[49]. CoPPIX treatment reduced the expression of CCL2

and CCL22, while the expression levels of CXCL10 and CCL5 were unchanged[50]. These results indicate that HO-1 inhibits the phenotypic and functional maturation of DCs, and thus reduces the ability of DCs to stimulate $CD4^+$ T cells[46], and the modulation of Th2 cytokine production by donor-derived DC may contribute to the comparative immune privilege of hepatic allografts[49,51].

HO-1 AS A POTENTIAL THERAPEUTIC TARGET IN LIVER DISEASES

Hepatitis C virus (HCV) represents a major public health burden in both industrialized and developing countries. HO-1 over-expression has been observed to decrease HCV replication, reduce pro-oxidant production in replicon cells, and increase resistance to oxidative injury[52]. These findings strongly suggest potential therapeutic roles for HO-1[53]. Nevertheless, iron was recently shown to inhibit the activity of HCV RNA-dependent RNA polymerase, as well as inhibiting replication in non-structural replicons[54]. However, a potential antiviral role for iron must be considered in the context of clinical data showing that mild increases in iron storage correlate with progressive HCV liver disease and hepatic decompensation. Therefore, a potential role for biliverdin and carbon monoxide as inhibitors of viral replication (*via* their anti-oxidative effects) requires further study[53].

Recent advances in molecular biology have provided new approaches to reducing hepatic IRI with gene therapy[55]. Liver transplantation[56] (a commonly employed treatment for hepatic failure) is still a limited means of therapy and has many drawbacks, including high monetary costs for the patient and donor shortages[8]. Presently, there are a limited number of strategies for protecting liver cells from damage and degeneration[57]. Keeping in mind the increasing amount of experimental evidence demonstrating the antioxidant and anti-inflammatory effects of HO-1 products, induction of this enzyme (or its catalytic activity) by either natural or synthetic compounds may represent an effective strategy to intervene in liver carcinogenesis and other hepatic disorders[11]. A wide variety of chemopreventive agents that elicit cytoprotective, anti-inflammatory and/or antioxidant effects by the induction of HO-1 expression have been recognized[58]. While continuing efforts should be geared towards the search for novel hepatoprotective[59] agents targeting HO-1, certain synthetic or naturally occurring substances already identified appear to induce HO-1 expression as part of their chemoprevention/chemoprotective effects against hepatocarcinogenesis[37] and should be further investigated.

Constitutive HO-1 expression by the Kupffer cells of the liver has been shown to be beneficial in hepatic IRI treatment. The consequences of HO-1 production have been shown to have antioxidant, anti-inflammatory, anti-proliferative, anti-apoptotic, immunomodulatory and vasorelaxant effects[60].

CONCLUSION

The HO-1 system may play an important role in various pathophysiological conditions. Thus, pharmacological modulation of the HO-1 system may represent an effective and cooperative strategy to mitigate liver injury in HCV, although the exact effects are likely to differ depending on the type of disease. Therefore, down-regulating the HO-1 system by pharmacological or genetic means may represent a new therapeutic approach for the management of liver injury. A comprehensive understanding of the mechanisms underlying the observed effects of HO-1 and its products will be needed before their use can be evaluated in clinical applications for the prevention and/or treatment of human diseases, such as HCV.

There are wide-ranging biological implications for HO-1 expression, extending far beyond its initial identified role as the rate-limiting enzyme in heme degradation. The tight regulation of *HO-1* gene transcription and the lack of high levels of constitutive expression reflect the great potential of this "protective" gene for inducing injury under certain conditions. Each HO-1 reaction product can be harmful and (in sufficient quantities) cause tissue injury, with resulting enhanced susceptibility to oxidative stress. Thus, HO-1 is not exclusively cytoprotective or cytotoxic, but it is involved in a complex equilibrium of inflammatory and reparative cellular processes.

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MINIREVIEWS

Lipotoxicity in the liver

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Abstract

Obesity due to excessive food intake and the lack of physical activity is becoming one of the most serious public health problems of the 21st century. With the increasing prevalence of obesity, non-alcoholic fatty liver disease is also emerging as a pandemic. While previously this pathophysiological condition was mainly attributed to triglyceride accumulation in hepatocytes, recent data show that the development of oxidative stress, lipid peroxidation, cell death, inflammation and fibrosis are mostly due to accumulation of fatty acids, and the altered composition of membrane phospholipids. In fact, triglyceride accumulation might play a protective role, and the higher toxicity of saturated or trans fatty acids seems to be the consequence of a blockade in triglyceride synthesis. Increased membrane saturation can profoundly disturb cellular homeostasis by impairing the function of membrane receptors, channels and transporters. However, it also induces endoplasmic reticulum stress *via* novel sensing mechanisms of the organelle's stress receptors. The triggered signaling pathways in turn largely contribute to the development of insulin resistance and apoptosis. These findings have substantiated the lipotoxic liver injury hypothesis for the pathomechanism of hepatosteatosis. This minireview focuses on the metabolic and redox aspects of lipotoxicity and lipoapoptosis, with special regards on the involvement of endoplasmic reticulum stress responses.

Key words: Saturated fatty acid; Lipotoxicity; Steatosis; Lipoapoptosis; Endoplasmic reticulum stress

Core tip: Surplus of free fatty acids contributes to hepatic injuries in obesity and type 2 diabetes. Intracellular accumulation of fatty acyl-CoA causes oxidative and endoplasmic reticulum (ER) stress, which lead to cell death, inflammation and fibrosis. Steatohepatosis is the consequence of an intensive fat synthesis, aiming to reduce the metabolic burden. The higher toxicity of saturated *vs* unsaturated fatty acids is partly due to a limited capacity of the liver cells to insert them into triglycerides. Moreover, increased membrane saturation triggers the ER stress response though a unique mechanism, which aggravates the metabolic derangements and liver injuries.

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INTRODUCTION

Special metabolic features make hepatocytes play a central role in the metabolism of nutrients as well as endo- and xenobiotics. Liver can consume or produce various nutrients with a great compliance and adapts quickly to

changing circumstances. However, the challenge raised by substantial alterations in modern life style and diet or the abuse of certain foods or drinks pushes the limits of adjustability, which leads to liver damage and metabolic disorders. The increasing prevalence of obesity, the metabolic syndrome and type 2 diabetes brings the pathological role of fatty acids into the focus of interest. This review is focused on the metabolic and redox bases of the fatty acid-induced cell injuries (lipotoxicity) and cell death (lipoapoptosis) in hepatocytes, with special regards to the contribution of oxidative and endoplasmic reticulum (ER) stress.

FATTY ACID METABOLISM IN THE LIVER

Liver plays a prominent role in maintaining a balanced nutrient supply in blood throughout the ever changing nutritional and metabolic conditions. Therefore, hepatocytes can shift from intensive fatty acid synthesis (in fed state) to rapid fatty acid breakdown (in starvation). Fatty acids, the most efficiently and economically storable fuel molecules are synthesized from the excess of carbohydrates and amino acids during the absorptive phase (Figure 1A). However, they are not released into the blood plasma as non-esterified or free fatty acids (NEFA or FFA) but incorporated into complex lipids such as triglycerides, phosphoglycerolipids or cholesterylesters in the ER membrane[1]. Microsomal triglyceride transfer protein (MTP) mediates the association of triglycerides with ApoB100 in the ER lumen and the assembled very low density lipoprotein (VLDL) lipoprotein particles finally leave the cell through exocytosis[2] (Figure 1A). Fatty acid components of ingested lipids are absorbed predominantly as reconstructed complex lipids packed in chylomicrons. These lipoproteins reach the systemic circulation through lymph vessels rather than portal veins and deliver triglycerides in the adipose tissue for storage.

FFAs normally derive from triglyceride breakdown in the adipocytes, and hence their abundance occurs in starvation when store mobilization is stimulated by hormones (*e.g.,* glucagon, adrenalin and glucocorticoids)[3]. FFAs are utilized as energy source in the liver cells. After a protein-mediated uptake across the plasma membrane[4], they are activated to acyl-CoA, transported into the mitochondrial matrix *via* carnitine shuttle and catabolized through β-oxidation and citrate cycle[5]. Long term starvation can deprive citrate cycle of intermediates and make it unable to keep pace with acetyl-CoA production. Accumulating acetyl-CoA is diverted toward the synthesis of β-ketobutyrate (acetoacetate) and β-hydroxybutyrate[3]. These ketone bodies are secreted from the hepatocytes into the blood plasma and serve as alternative fuels to most aerobic tissues including the brain (Figure 1B).

Fatty acids, either endogenous or exogenous, are handled in an activated form, *i.e.,* attached to CoA through a thioester bond. Carnitine shuttle leading to degradation as well as acyl transferase enzymes incorporating fatty acids in complex lipids require the formation acyl-CoA in

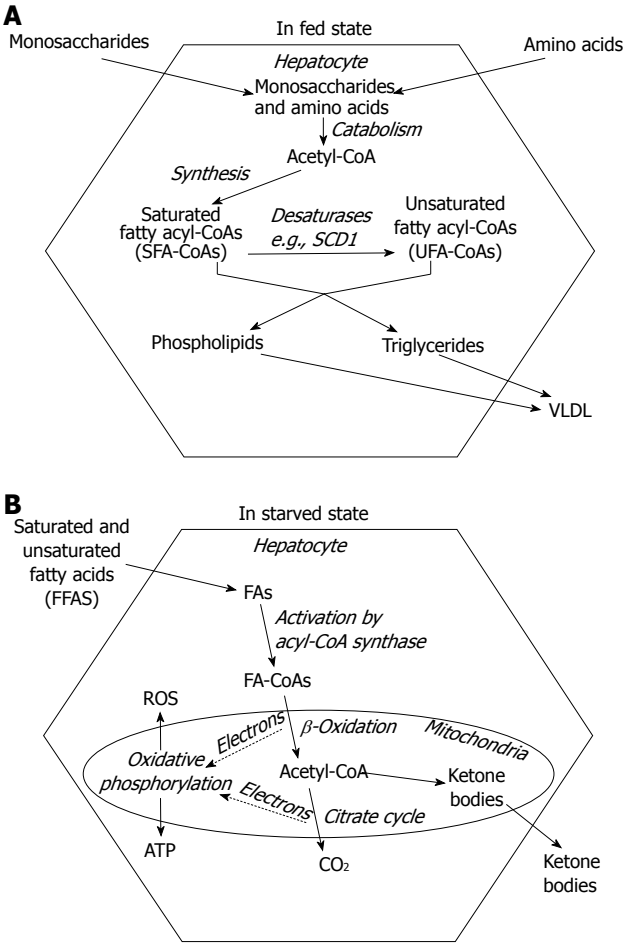

Figure 1 Fatty acid metabolism in the liver. A: In fed state, monosaccharides and amino acids are taken up from the portal blood and converted to fatty acyl-CoAs through acetyl-CoA intermediate; The primary endogenous fatty acids (*e.g.,* palmitate) are saturated. Formation of mono- or polyunsaturated fatty acids involves modification(s) by desaturase enzymes; Newly synthesized fatty acids are inserted into complex (saponifiable) lipids, which in turn are packed in very low density lipoprotein (VLDL) particles and so secreted from the hepatocytes; B: In starvation, free fatty acids (FFAs) derive from triglyceride mobilization in the adipose tissue. After a protein-mediated uptake across the plasma membrane, they are activated to acyl-CoA, and catabolized in the mitochondria through β-oxidation and citrate cycle. These metabolic pathways provide electrons to oxidative phosphorylation, the major source of ATP and ROS in aerobic cells. Shortage of citrate cycle intermediates leads to accumulation of acetyl-CoA, and thence enhances the synthesis of ketone bodies.

the cytosolic compartment[6]. Fatty acyl-CoA molecules can also be modified by ER-associated enzymes to reach the species-specific composition of triglycerides and phospholipids. These modifications include chain elongation and desaturation (Figure 1A). Monounsaturated fatty acids typically contain a cis double bond at the Δ9 position, which is inserted into fatty acyl-CoAs by stearyl-CoA desaturase 1 (SCD1) in humans. SCD1 converts palmitoyl (C16:0)-CoA into palmitoleoyl (C16:1)-CoA and stearoyl (C18:0)-CoA to oleoyl (C18:1)-CoA. It is an iron-containing enzyme that receives electrons from NAD(P)H through cytochrome b5 reductase and cytochrome b5 in the ER membrane[7]. Ncb5or, a novel soluble oxidoreductase contains both cytochrome b5 reductase like and cytochrome b5 like domains and hence it is also proposed

to participate in fatty acid desaturation[8].

Membrane lipid saturation is regulated through the activity of various lysophospolipid acyltransferase enzymes exhibiting different acyl-CoA specificities. For example, among the enzymes involved in phosphatidylcholine remodeling, lysophosphatidylcholine acyltransferase 1 (LPCAT1) preferentially incorporates saturated fatty acids (*e.g.,* palmitate)[9] while LPCAT3 favors polyunsaturated fatty acids (*e.g.,* arachidonic acid)[10], which allows modulation of phospholipid fatty acid composition through altered expression of these isoenzymes[11].

HEPATOSTEATOSIS

Triglyceride deposition within the hepatocytes is the hallmark of both alcoholic and nonalcoholic fatty liver diseases (AFLD and NAFLD)[12]. It occurs when VLDL assembly and secretion cannot keep pace with lipid synthesis. This hepatosteatosis can be caused by various conditions including stimulated hepatic fatty acid synthesis due to overfeeding and high fructose consumption[13] and inhibition of MTP[14,15]. Ethanol, one of the best known inducers of fatty liver both enhances fatty acid synthesis and hinders VLDL secretion[16]. It has been revealed that elevated FFA level should also be considered as a major cause of hepatosteatosis. Combination of genetic predisposition with certain environmental factors, such as sedentary life style, overfeeding and obesity can lead to a surplus of FFA due to deregulation of fatty acid storage and mobilization. The metabolic stress caused by excessive supply of nutrients in various tissues comprises oxidative and organelle stress components. The consequent stress response and inflammatory reaction can lead to insulin resistance, which favors triglyceride mobilization and decreases FFA uptake in the adipocytes[17]. Untrained skeletal muscle of low metabolic rate and of small relative mass has a limited contribution to fuel consumption, which further increases both insulin resistance and the strain on the adipose tissue. Moreover, the increased adipocyte mass is associated with an elevated FFA release. Enlargement of visceral adipose tissue is especially important because it secretes FFA into the portal circulation, *i.e.,* directly to the liver. In addition to the mounting FFA supply, the compensatory hyperinsulinemia stimulates fatty acid synthesis and inhibits fatty acid catabolism in the hepatocytes. When fatty acid input overcomes the capacity of β-oxidation, accumulating acyl-CoA is drained by triglyceride synthesis, which leads to steatosis in the liver (Figure 2A).

Accumulation of fatty acids or fatty acyl-CoAs, however, may be more harmful to hepatocytes than deposition of triglycerides. It has been demonstrated in HepG2 and Huh7 human liver cell lines that lipotoxic effect of the saturated stearate is initiated by interruption of triacylglycerol synthesis. Retained capacity to synthesize and accumulate triglycerides in the presence of unsaturated oleate, in fact, turned out to be protective[18].

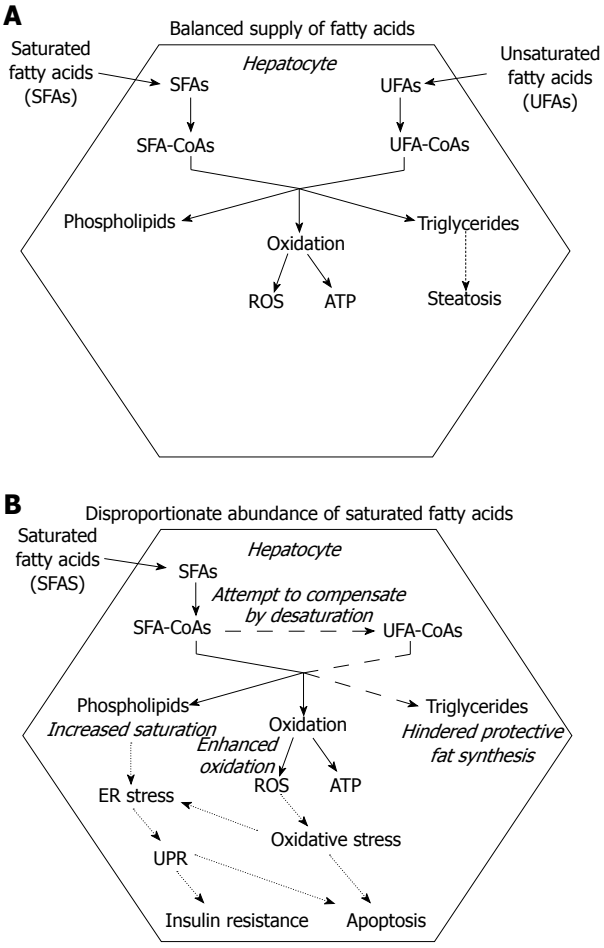

Figure 2 Fatty acid toxicity in the liver. A: Balanced (over) supply of saturated and unsaturated fatty acids allows the hepatocyte to enhance triglyceride synthesis and deposition. Storage of excess fatty acids prevents superfluous fatty acid oxidation and thereby protects against oxidative stress; B: Disproportionate abundance of saturated fatty acids might not be compensated for by fatty acid desaturation (dashed lines). Hindrance of protective fat synthesis favors fatty acid oxidation and leads to excessive ROS generation; The emerging oxidative stress and the increased saturation of membrane lipids trigger the endoplasmic reticulum (ER) stress response, which contributes to insulin resistance and further enhances cell death (dotted lines).

TOXICITY OF SATURATED FATTY ACIDS AND TRANS FATTY ACIDS

The typical dietary pattern of western populations leads to high intake of saturated fatty acids (SFAs) derived from animal sources. Their metabolic and health effects are similar to those of trans fatty acids (TFAs), *i.e.,* unsaturated fatty acids containing at least one non-conjugated double bond in the trans configuration. TFAs are also found in foods of natural origin because ruminant animals obtain these lipids from bacterial hydrogenation of unsaturated fatty acids in the rumen[19]. In addition, TFAs are also formed during the industrial production of certain foods, and major food sources of dietary TFA in the United States. population were found to be cakes, cookies, pies and pastries[20]. The proinflammatory status favored by both SFAs and TFAs plays an important role in several diseases[21].

The greater hepatotoxicity of saturated *vs* unsaturated fatty acids has been demonstrated in several studies[18,22-25]. Moreover, the unsaturated fatty acids often prove to be protective against SFA-induced toxicity[18,24]. Interruption of triglyceride synthesis, apparently because of the formation of a pool of oversaturated intermediates, seems to be a key event in SFA-induced lipotoxicity. Accumulation of stearoyl-CoA and a decreased capacity of triglyceride production have been found in HepG2 and Huh7 human hepatoma cells exposed to stearate[18]. In accordance with its central role in converting SFAs to monounsaturated fatty acids (MUFAs), SCD1 proved to be protective against SFA-induced hepatocyte lipoapoptosis (Figure 2B). Studies on SCD1 knockout mice lead to the conclusion that prevention of steatohepatitis involves partitioning excess SFAs into MUFAs by hepatic SCD1[26]. Hepatocytes of Ncb5or knockout mice have an increased sensitivity to SFA-induced damage, which can be at least partly attributed to a lower SCD-specific activity in these cells[27]. This observation strongly supports the hypothesized functional link between SCD1 and Ncb5or[8]. Some natural human mutations of NCB5OR have been reported to remarkably decrease the level of active enzyme due to enhanced proteasomal degradation[28]; therefore, the potential role of this gene in human pathology deserves further investigation.

Controversial data have been published regarding the control of autophagy by unsaturated and saturated fatty acids in primary hepatocytes and hepatoma cells[29,30]. Although oleic acid has been found to induce autophagy-mediated apoptosis in mild but not in severe steatosis in HepG2 cells[31], it seems plausible that fatty acid-induced autophagy generally plays a pro-survival role in lipotoxicity[29,30,32]. However, hindrance of the autophagic flux by oxidative stress and/or advanced apoptosis[29,32] is more likely to occur in SFA-induced lipotoxicity. Therefore, the greater toxicity of saturated *vs* unsaturated fatty acids might be also attributed to differential regulation of autophagy.

The health risk raised by the increasing ingestion of TFAs has recently got into the focus of scientific interest. The relationship between dietary fat induced obesity and TFAs is not unequivocally elucidated. Nevertheless, dietary intake of high TFAs increases liver weight and hepatic triglyceride content, and causes deleterious alterations in serum cholesterol levels in rats[33]. TFAs have been shown to be a primary factor responsible for the development of NAFLD in an animal model of American lifestyle induced obesity syndrome either with[34] or without inclusion of high fructose corn syrup[35,36] in the diet. Increased hepatic lipid peroxidation as an indicator of oxidative stress has also been demonstrated in the livers of high TFA-fed rats, and it might underlie the development of NAFLD[37]. Long term ingestion of TFAs has been shown to increase liver triglycerides due to enhanced activity and expression of lipogenic enzymes and elevated expression of sterol regulatory element-binding protein SREBP-1a in mice. However these effects largely depend on the dietary fatty acid composition[38]. The extent and severity of health injuries caused by TFAs as well as the mechanisms of TFA-induced hepatotoxicity remain to be clarified.

LIPOTOXIC OXIDATIVE STRESS

Several observations suggest the role of increased reactive oxygen species (ROS) generation in hepatic lipotoxicity. Growing oxidative challenge is well demonstrated by increased serum levels of ox-LDL and higher serum lipid peroxidation in nonalcoholic steatohepatitis[39]. Palmitate-induced oxidative stress has been reported to contribute to insulin resistance in H4IIEC3 rat hepatocytes[40]. Fatty acid accumulation stimulates ROS generation in the liver presumably due to enhanced β-oxidation and to the consequent electron overflow in the mitochondrial electron transfer chain[41] (Figure 2B). However, a decrease in mitochondrial quinone pool and a related inhibition of mitochondrial oxidative metabolism were also suggested to underlie the increased mitochondrial ROS production in high fat diet[42]. Mitochondrial depolarization, cytochrome c release, and increased ROS production were detected in HepG2 and McNtcp.24 liver cells exposed to saturated FFAs, and the role of lysosomal disruption was also suggested[43]. Increased expression[44] and activity[45,46] of cytochrome P450 2E1 (CYP2E1) monooxygenase likely contributes to the oxidative stress in lipotoxicity. CYP2E1 is an integral membrane protein of the ER and a significant source of oxidative intermediates including free radicals. It is involved in the biotransformation of several endo/xenobiotics, and it carries out omega hydroxylation of fatty acids. CYP2E1 activity was shown to positively correlate with body mass index and with the degree of steatosis[47]. The role of omega hydroxylating CYP4A isoenzymes was also suggested in animal models of nonalcoholic steatohepatitis[48]. CYP4A enzymes are under control of peroxisome proliferator activated receptors, which also induce peroxisomal fatty acid beta oxidation, another prominent ROS generating pathway[49]. Besides the enhancement of oxidative fatty acid metabolism, palmitate-mediated up-regulation of direct ROS production by NADPH oxidase 3 has been also revealed recently in db/db mice and HepG2 cells[50].

Excessive ROS production is directly deleterious by injuring DNA, proteins and lipids. It also favors cell death through activation of certain stress-sensitive signaling pathways, such as nuclear factor κB, p38MAPK, and c-Jun N-terminal kinase (JNK)[51,52]. In addition, a general cellular oxidative stress inevitably affects the intricate redox homeostasis in the ER lumen[53-55]. The controlled maintenance of an oxidized thiol-disulfide redox system in this compartment is the prerequisite of appropriate protein maturation[56]. On the other hand, the sufficiently reduced pyridine nucleotide pool in the ER lumen provides a metabolic basis for prereceptor

glucocorticoid hormone activation[57,58].

LIPOTOXIC ER STRESS

Protein processing, one of the major functions of the ER[56,59], is necessarily affected by any severe dysfunction of the organelle, *i.e.,* the ER stress[60]. Lowered protein folding capacity of the ER renders it unable to keep pace with protein load. Accumulation of immature proteins in the ER lumen triggers a complex primarily adaptive signaling network called the unfolded protein response (UPR) in order to restore ER homeostasis. Unfolded proteins recruit increasing amounts of BiP, ER chaperone, which is thus detached from the luminal domains of three transmembrane ER stress sensor proteins: RNA-dependent protein kinase-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). They initiate the three main branches of the UPR collectively inducing attenuation of protein synthesis, expression of ER chaperones, and degradation of misfolded proteins[61].

Phosphorylation of eukaryotic initiation factor eIF2α by PERK lowers the ER protein load by attenuating general translation, and also enhances the synthesis of ATF4 transcription factor. IRE1-mediated specific mRNA splicing allows the synthesis of X-box-binding protein 1 (XBP1) transcription factor, which enhances the ATF6-dependent adaptive transcriptional alterations (*e.g.,* induction of ER chaperones). When the UPR fails to restore the ER functions, the ER-derived death signals may take effect. Apoptosis is stimulated mostly by induction of CCAAT/enhancer binding protein homologous protein (CHOP) and activation of JNK. It should be noted that JNK activation is also triggered by the oxidative stress in lipotoxicity[50]. In addition to its pro-apoptotic activity, JNK also interferes with insulin signaling by phosphorylating insulin receptor substrate-1 (IRS-1) at serine (307)[62], which represents a key link between ER-stress and insulin resistance.

ER stress contributes to the pathology of several liver diseases associated with steatosis. Activation of the UPR is involved in fatty liver disease either alcoholic or non-alcoholic as well as viral hepatitis. Enhanced apoptosis of liver cells in these diseases may be at least partly due to unresolved ER stress[63]. Mounting evidence support the involvement of ER dysfunctions in the toxicity of fatty acids, especially long chain, saturated ones. The programmed cell death, at least partly due to ER stress, caused by fatty acids is referred to as lipoapoptosis. The role of PERK/ATF4/CHOP signaling pathway has been demonstrated in saturated fatty acid-induced ER stress and lipoapoptosis in L02 and HepG2 human liver cell lines[64]. Elevated exogenous pamitate has been shown to disrupt ER homeostasis by reducing the expression of Bip in HepG2 cells. Overexpression of Bip attenuated ER stress, reduced CHOP expression and protected the cells from palmitate-induced apoptosis[22]. Lipotoxic derangement of ER functions is likely due to the above mentioned oxidative stress, disturbed calcium homeostasis and altered membrane saturation. Membrane bound and luminal oxidase enzymes maintain a thiol oxidizing environment in the ER, a prerequisite of oxidative folding[65]. It is therefore not surprising that the protein processing machinery of the organelle is sensitive to any disturbance of the redox and antioxidant status of the cell[66]. Inhibition of fatty acid oxidation has been shown to protect hepatocytes from ER stress, and this was accompanied by an increased cellular redox potential as judged by an elevated ratio of oxidized to reduced glutathione and enhanced oxidative folding in the ER[67]. Saturated, long chain fatty acids have been shown to reduce thapsigargin-sensitive ER calcium stores and increase biochemical markers of ER stress in H4IIE liver cells and primary hepatocytes[24]. Palmitate- but not linoleate-induced ER stress was prevented by depletion of intracellular calcium flux in the same cell line[68].

It has been demonstrated that insufficient desaturase activity (SCD1 knockdown) or decreased incorporation of unsaturated fatty acids in membrane phospholipids (LPCAT3 knockdown) can synergistically induce the UPR. The effect is likely due to a decrease in membrane phospholipids unsaturation as it is further enhanced by saturated while it is rescued by unsaturated fatty acids[69] (Figure 2B). Recent findings suggest that ER lipid perturbation can trigger the UPR directly and independently of luminal accumulation of unfolded proteins. The mechanisms through which the UPR is activated by accumulating unfolded proteins and membrane lipid saturation turned out to be different as the latter does not involve large protein cluster formation in the ER membrane[70]. Two of the ER stress receptors, IRE1α and PERK have been revealed to respond to increased lipid saturation. Their lipid sensitivity was retained when the luminal unfolded protein sensing domains were removed and was associated to their transmembrane domains[71]. This novel mechanism is in accordance with the greater toxicity of saturated *vs* unsaturated fatty acids as well as with the protective effect of unsaturated fatty acids against saturated fatty acid induced toxicity[18,22-25].

The thapsigargin- and tunicamycin-induced UPR was found to increase the expression of sterol regulatory element-binding protein (SREBP-1c) transcription factor in HepG2 cells, through the cap-independent translation mediated by an internal ribosome entry site[72]. Thapsigargin-induced SREBP-1c activation and the consequent stimulation of fatty acid synthesis were also confirmed in the normal human L02 cell line[73]. This effect might create a positive feed back loop and further aggravate the metabolic disorder in lipotoxic ER stress. On the other hand, several genes related to lipoprotein secretion are controlled by IRE1α, and hence induction of the UPR upon membrane lipid perturbation might contribute to the prevention of hepatic steatosis[74]. Interestingly, mice with hepatocyte-specific IRE1α deletion without ER stress display modest hepatosteatosis, and this is aggravated after induction of ER stress[74]. Therefore, disturbed lipid metabolism can lead to ER stress and trigger the UPR, and the ER stress-dependent

alteration in lipid homeostasis might underlie the hepatic steatosis.

CONCLUSION

The unhealthy combination of modern diet and lifestyle often leads to nutrient surplus and a consequent fat deposition in various non-adipose tissues including the liver. This hepatosteatosis has long been considered as the fundamental cause of hepatic injuries characterizing both alcoholic and non-alcoholic fatty liver diseases, *i.e.* oxidative and ER stress, cell death, inflammation and fibrosis. In light of recent studies, however, fat accumulation is considered rather protective as the hepatocyte damage is mostly caused by fatty acyl-CoA (Figure 1A). Enhanced triglyceride synthesis decreases the oxidative challenge by lowering fatty acid oxidation. The higher toxicity of SFAs *vs* unsaturated fatty acids seems to be at least partly due to an increased tendency of the SFAs to accumulate because of a limited capacity of the liver cells to insert them into triglycerides (Figure 2B). SFA-induced toxicity is indeed enhanced by insufficient activity of the enzymes involved in fatty acid desaturation and triglyceride synthesis while attenuated by simultaneous administration of unsaturated fatty acids. Moreover, growing evidence indicates that increased saturation of phospholipid membranes can trigger the ER stress response though a unique mechanism. The activated signaling pathways lead to insulin resistance and hepatocyte apoptosis, significantly contributing to aggravation of the metabolic derangements and liver injuries caused by SFAs (Figure 2B).

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ORIGINAL ARTICLE

Rapid chromatographic method to decipher distinct alterations in lipid classes in NAFLD/NASH

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Abstract

AIM: To establish a simple method to quantify lipid classes in liver diseases and to decipher the lipid profile in *p62/IMP2-2/IGF2BP2-2* transgenic mice.

METHODS: Liver-specific overexpression of the insulin-like growth factor 2 mRNA binding protein p62/IMP2-2/IGF2BP2-2 was used as a model for steatosis. Steatohepatitis was induced by feeding a methionine-choline deficient diet. Steatosis was assessed histologically. For thin layer chromatographic analysis, lipids were extracted from freeze-dried tissues by hexane/2-propanol, dried, redissolved, and chromatographically separated by a two-solvent system. Dilution series of lipid standards were chromatographed, detected, and quantified. The detection was performed by either 2′,7′-dichlorofluoresceine or a sulfuric acid/ethanol mixture.

RESULTS: Histological analyses confirmed steatosis and steatohepatitis development. The extraction, chromatographic, and detection method showed high inter-assay reproducibility and allowed quantification of the different lipid classes. The analyses confirmed an increase of triglycerides and phosphatidylethanolamine and a decrease in phosphatidylcholine in the methionine-choline deficient diet. The method was used for the first time to asses the lipid classes induced in the p62-overexpressing mouse model and showed a significant increase in all detected lipid species with a prominent increase of triglycerides by 2-fold. Interestingly, the ratio of phosphatidylcholine to phosphatidylethanolamine was decreased, as previously suggested as a marker in the progression from steatosis to steatohepatitis.

CONCLUSION: The thin layer chromatography analysis allows a reliable quantification of lipid classes and provides detailed insight into the lipogenic effect of p62.

Key words: Non alcoholic steatohepatitis; Non alcoholic fatty liver disease; Thin layer chromatography; IMP2/IGF2BP2; p62; Methionine choline deficient diet; Polar lipids; neutral lipids; Phosphatidylcholine/phosphatidylethanolamine ratio; Triglycerides

Core tip: We describe a new method to quantify lipid classes in steatosis/steatohepatitis having advantages over both histology and classical analytical methods. Since lipid classes exert differential pathophysiological actions our method should be of interest for all researchers dealing with mechanisms of steatosis and steatohepatitis. We employ our method to investigate the lipid profile in the steatotic *p62* transgenic mouse

model. p62 was originally identified as an autoantigen overexpressed in hepatocellular carcinoma patients, its expression correlates with poor prognosis, and it induces steatosis. The interesting lipid profile in *p62* transgenic animals suggests that it might advance the step from steatosis towards steatohepatitis.

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INTRODUCTION

The incidence of non alcoholic fatty liver disease (NAFLD) and non alcoholic steatohepatitis (NASH) has dramatically increased in Western countries during the last decades[1-3]. Still, the diagnosis of NAFLD displays a problem since there is a known heterogeneity in the histological staging of lipid accumulation in the liver[4,5]. This problem is equally relevant for research laboratories studying mechanistic and therapeutic aspects of NAFLD and NASH.

A commonly used model for the investigation of NASH is the methionine choline deficient mouse model, which is histologically similar to human NASH regarding steatohepatitis and fibrosis[6]. The methionine choline deficient diet (MCD) model is well characterized regarding its effect on the expression of lipid regulators, such as lipogenic transcription factors and lipogenic enzymes[7,8]. The development of steatosis in the MCD model is attributable in part to impaired very low density lipoprotein (VLDL) secretion due to the deficiency of methionine and choline, which are the precursors for phosphatidylcholine, the main phospholipid coating VLDL particles[9].

An interesting but as yet less characterized steatosis model is the insulin-like growth factor 2 (*Igf2*) mRNA binding protein *p62*/IMP2-2/IGF2BP2-2 transgenic mouse model[10]. p62 was originally identified as an autoantigen in an HCC patient[11] and its expression correlates with poor prognosis in hepatocellular carcinoma (HCC)[12]. Hepatic p62 overexpression induces a microvesicular fatty liver[10], which is characterized by an absence of inflammatory processes and liver damage[10]. Still p62 overexpression amplifies murine NASH and fibrosis[13].

NAFLD, even in the absence of cirrhosis, can progress to HCC[14]. Increasing knowledge suggests that not only the increase in lipid accumulation itself but rather the hepatic lipid composition plays a dominant role in the development of both simple steatosis and steatohepatitis[15]. Lipid composition has in fact been shown to have a pathophysiological relevance in different metabolic diseases[15-18] as well as in cancer[19]. Accordingly, the pharmacologically reduced production of cholesterol by inhibition of hydroxy-methyl-glutaryl-coenzyme A reductase is discussed as a strategy for the chemoprevention and a slower progression of HCC[20]. The comparison of the lipidome of a murine NASH and HCC model with the human NASH and HCC lipidome found significant changes within several fatty acid signatures between the normal, NASH, and HCC lipidome[21]. Therefore, a more comprehensive characterization and understanding of pathophysiological lipidomic changes in liver diseases and common disease models seems mandatory.

For the investigation of lipid composition liquid chromatography-mass spectrometry (LC-MS) is state-of-the-art. However, due to high costs for the equipment and maintenance, the method is not suitable for routine analyses in clinical and research laboratories. Furthermore, the results obtained by LC-MS contain information in a level of detail too complex for most of the studies, in which rather general alterations in lipid classes are of interest. Thin layer chromatography (TLC) offers some advantages over LC-MS. For example, the possibility to apply many different samples on a single TLC plate is in practice often faster than LC[22]. 3D TLC was developed in the 1960s as a reliable method for lipid separation. However, a major limitation of the technique is the fact that it is possible to test only one sample per plate[23]. Since 3D TLC has a very low inter-plate reproducibility it is only suitable for qualitative measurements.

We herein present a rapid and low-cost quantitative 1D TLC, which can detect major lipid classes and can be used to quantitatively compare up to 12 samples per plate. Furthermore, we provide insight into changes of lipid composition in the *p62* transgenic mouse model for the first time[13].

MATERIALS AND METHODS

Materials

Standard substances 1,3-diolein (D3627), L-α-lysophosphatidylcholine from egg yolk (L4129), cholesterol (C8667), glyceryl trioleate (T7140), 3-*sn*-phosphatidylethanolamine from bovine brain (P7693), L-α-phosphatidylcholine (P3556), 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (P7769), non-hydroxy fatty acid ceramide from bovine brain (C2137), and stearic acid (85679) were purchased from Sigma-Aldrich (Taufkirchen, Germany). The standard substances were dissolved in chloroform/methanol [1:1 (v/v)] at a concentration of 1 mg/ml, aliquoted, and stored at -80 ℃. TLC silica gel 60 F_{254} glass plates were purchased from Merck (105715, Merck, Darmstadt, Germany). All solvents were distilled prior to utilization.

Animal models

All animal procedures were performed in accordance with the local animal welfare committee. Mice were kept under stable conditions regarding temperature, humidity, food delivery, and 12 h day/night rhythm.

Steatosis model

p62 transgenic mice were established as described previously[10]. Mice carrying a liver enriched activator protein promoter under tetracycline transactivator control[24] were crossed with *p62* transgenic mice, in which the human *p62* is under the control of the transrepressive responsive element cytomegaly virus (TRE-CMV_{min}). The double positive offspring expresses p62 liver-specifically. The mice were sacrificed at an age between 2.5 and 5 wk.

Steatohepatitis model

Wild-type mice were fed either a methionine choline deficient (MCD, 960439, MP Biomedicals, Illkirch Cedex, France) or a methionine choline supplemented control diet (co, 960441, MP Biomedicals, Illkirch Cedex, France) for 3 wk.

Histology

For hematoxylin eosin (HE) staining 5 μm paraffin slides were rehydrated in a xylol/alcohol series, incubated for 10 min in hematoxylin, washed for 5 min under running water, and incubated for 2 min in eosin.

Extraction of bovine and murine liver lipids

Bovine liver was bought from a local butchery and directly freeze dried and stored at -80 ℃. Lipids from snap-frozen murine or bovine liver samples were extracted by a modified version of a published method[25]. Briefly, 60 mg liver samples were lyophilized, 15 mg of the freeze-dried tissue was dispersed with 18 volumes of a mixture of hexane/2-propanol [3:2 (v/v)] for 10 min, and centrifuged at 4 ℃ and 10000 *g* for 10 min. The supernatant was transferred to a new vial and dried under a nitrogen stream, redissolved in an appropriate volume of chloroform/methanol [1:1 (v/v)], and applied in equal amounts onto the TLC plates.

1D TLC with two solvent system

The TLC plates were prewashed with a mixture of chloroform/methanol [2:1 (v/v)] to remove any contaminants and afterwards activated at 110 ℃ for 1 h. The samples and standard substances were applied onto the TLC plates and chromatographically separated with the first solvent system containing chloroform/methanol/acetic acid/water [50:30:8:3 (v/v/v/v)][26] to half of the plate. The TLC was dried and subjected to chromatography in a second solvent system consisting of heptane/diethyl ether/acetic acid [70:30:2 (v/v/v)][27] to the top of the plate[28].

Detection and quantification

The TLC plates were dried for 30 min under a nitrogen stream and first sprayed with 0.1% 2',7'-dichlorofluorescein (DCF, 109676, Merck, Darmstadt, Germany) in methanol and afterwards with sulfuric acid/ethanol [1:1 (v/v)] followed by heating at 160 ℃[23]. After drying the plates one UV image at 312 nm for DCF and one white top light image for sulfuric acid/ethanol was captured using the Biostep (Jahnsdorf, Germany) dark hood dh-4050 with transilluminator Biostep bioview (excitation 312 nm, UST-20M-8E) and an stationary fixed olympus digital camera (Hamburg, Germany) in combination with the Biostep argus X1 software (version 4.1.10). The unprocessed images in tiff format were quantified using the ImageJ 1.47i software[29].

Statistical analysis

Results are expressed as means ± SE. The statistical significance was determined by independent two-sample *t*-test and was considered as statistically significant when *P* values were less than 0.05. The Microsoft® Office Excel 2003 software (Microsoft Coperation, Redmond, United States) was used for statistical analyses.

RESULTS

Quantification of lipids on the TLC plate

In order to check the linearity of the method used, lipid standards for triglyceride (TG), free fatty acid (FFA), cholesterol (CH), ceramide (CE), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), lysophosphatidylcholine (LPC), and diglyceride (DG) (2.5, 5, 7.5, 10, 12.5, 15, 20 μg, each) were chromatographed, stained, and quantified according to our newly developed method described in the methods section. The DCF spray reagent was susceptible to all subjected lipids, the sulfuric acid/ethanol spray reagent was susceptible to almost all substances except for the FFA stearic acid and LPC (Figure 1A). As expected the band intensities increased with higher amount of the standard substances (Figure 1A). The quantification revealed a strong correlation with R^2 values close to one for all substances (Figure 1B).

Validation of the lipid extraction procedure

For validation of the reproducibility of the extraction procedure, freeze-dried tissue from bovine liver was extracted in seven independent extraction procedures and subjected to chromatography. The extraction procedure revealed a high reproducibility in all lipid classes investigated (Figure 2). PC, PE, TG and CH were most prominent in bovine liver (Figure 2).

Lipid quantification in different mouse models

Steatohepatitis/MCD mouse model: In order to test whether altered lipid composition can be determined reliably we used a well established murine steatohepatitis model, for which altered lipid classes are known[30]. Livers from control and MCD fed mice were processed, extracted, and lipids were chromatographed and detected as mentioned above. Two independent TLC plates revealed a strong increase in TG with DCF and sulfuric acid/ethanol (Figure 3B, C). As the MCD model is characterized by choline deficiency, the levels of PC were significantly decreased, whereas the levels of PE were

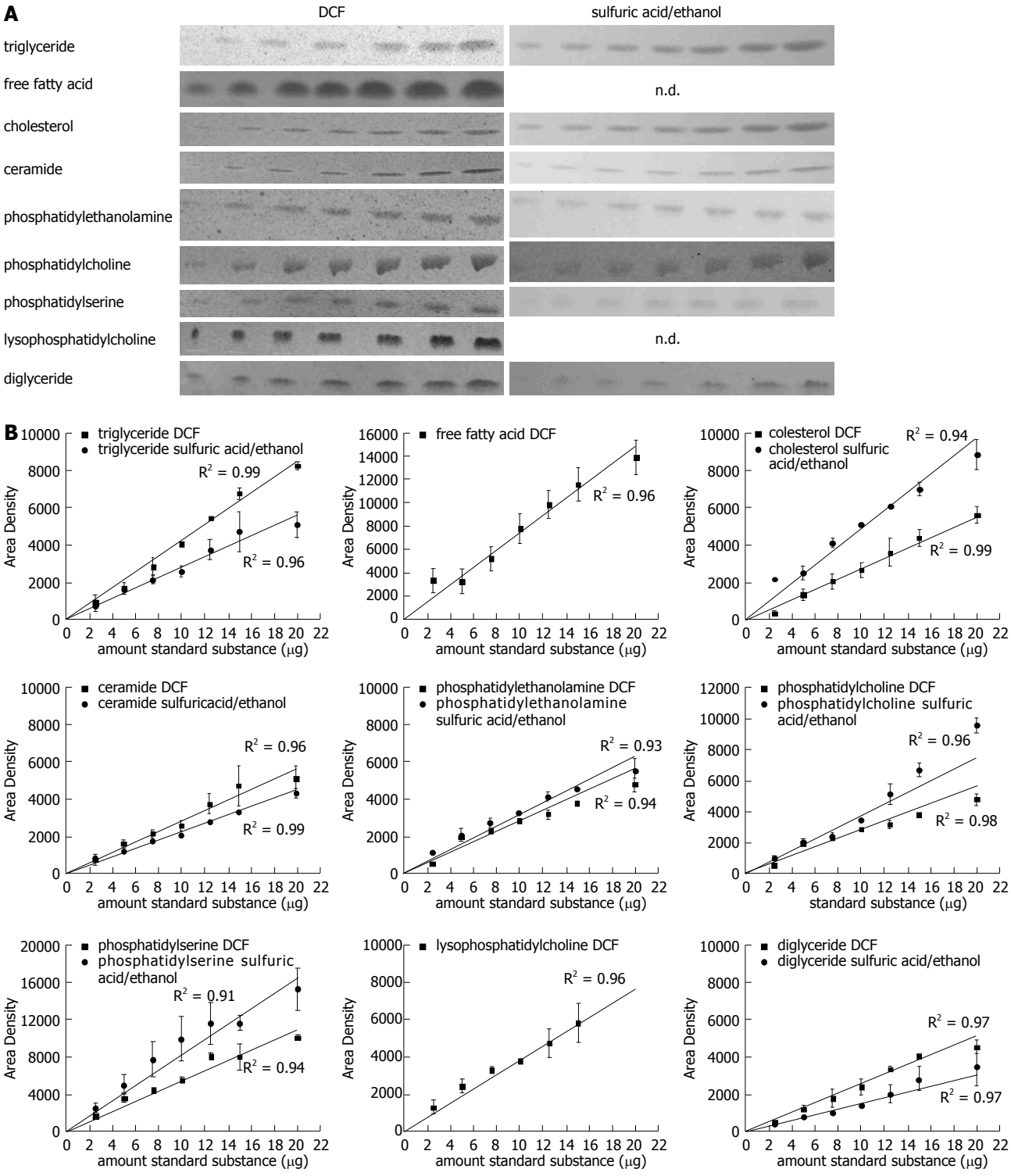

Figure 1 Quantification of lipids on the thin layer chromatography plate. A: Representative lipid dilution series (range: 2.5, 5, 7.5, 10, 12.5, 15, 20 μg) detected with 2',7'-dichlorofluorescein (DCF) or sulfuric acid/ethanol; B: Quantification of the standard dilution series detected with DCF or sulfuric acid/ethanol and quantified with ImageJ. Results represent the mean ± SE from at least two independent thin layer chromatography plates. FFA: Free fatty acids.

significantly increased (Figure 3B, C). We consequently observed a reduced PC/PE ratio by approximately one third ($P = 0.003$) with both detection methods. The other lipid classes investigated were not significantly changed (data not shown). Due to the high amount of TG in this model, the routinely subjected amount of lipid extract had to be reduced by five folds compared to normal tissues. Routinely used amounts led to overloading of the plates (data not shown).

Steatosis/*p62* transgenic mouse model: Since our method confirmed changes in lipid classes in the MCD steatohepatitis mouse model, we used it to characterize changes in lipid classes in the p62 steatosis model. The

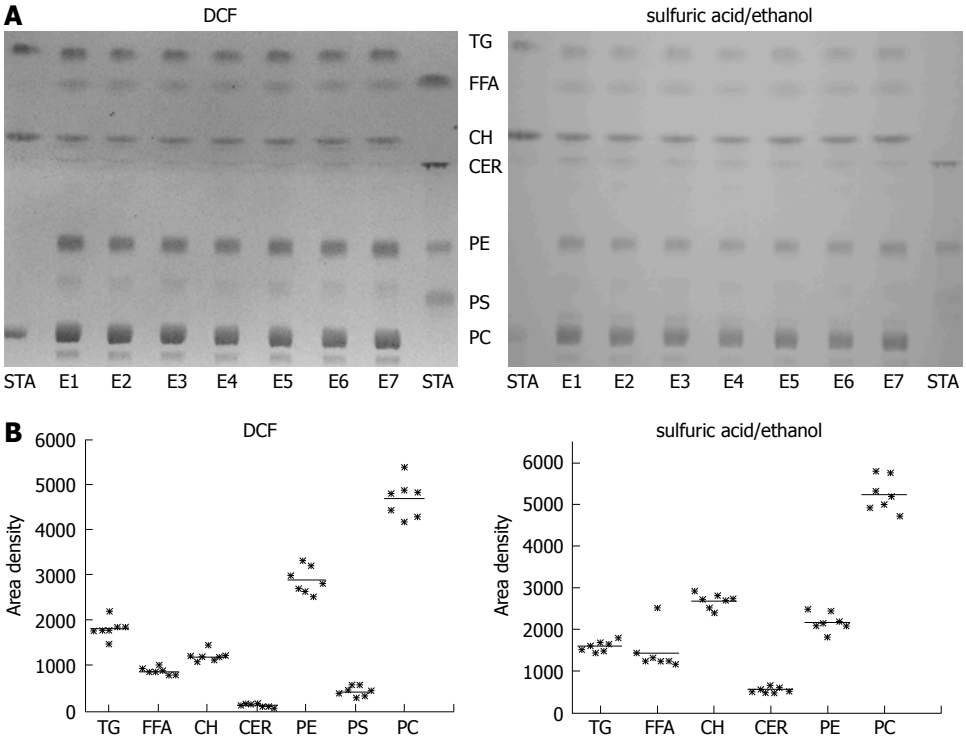

Figure 2 Validation of the lipid extraction procedure. A: Freeze-dried bovine liver was extracted in seven independent extraction procedures (E1-E7). STA: Standard substances co-chromatographed with the samples. left: detection with 2',7'-dichlorofluorescein (DCF); right: detection with sulfuric acid/ethanol; B: Quantification of thin layer chromatography (TLC) with ImageJ detected with DCF (left) or sulfuric acid/ethanol (right).

model shows distinct histologically proven microvesicular lipid incorporation in up to 58% of the animals[10] when specific lipid staining is performed. Accordingly, HE staining revealed a milder extent of steatosis compared to the MCD diet (Figure 3A, D). Two independent TLC plates revealed that all detected lipid classes were significantly increased in the livers of *p62* transgenic animals (Figure 3E, F). FFA, DG and LPC were not detectable (Figure 3E). The strongest effect was observed for TG, which were increased approximately two folds in *p62* trangenic animals compared to wild-type controls (Figure 3F). Interestingly, although the levels of both PC and PE were significantly increased, the PC/PE ratio was significantly decreased by about 10% ($P = 0.05$) with both detection methods. The same was true for the ratio of CH/PC, which was increased by approximately 23%, as validated by the DCF detection ($P = 0.05$).

DISCUSSION

Within this work we developed a rapid analytical method, which allows to quantify changes in hepatic lipid classes. The newly established method confirmed published findings for the lipid changes in a mouse NASH model and for the first time reports the lipid composition in the *p62* transgenic steatosis model.

TLC method

The one-dimensional TLC with a two-step solvent system and the detection with DCF or sulfuric acid/ethanol was able to separate and to detect the major lipid classes of TG, FFA, CH, CER, PE, PC, PS, LPC, and DG within a time period of 2.5 h (Figure 4). Standard curves revealed a high linearity of the standard substances from 2.5 to 20 μg. The chosen standard substances corresponded with the major lipid classes typically changed in NAFLD/NASH[31]. A lack of reactivity of saturated fatty acids towards a sulfuric acid/ethanol/hexane reagent was reported previously[32] and is in line with our finding that our FFA (the saturated fatty acid stearic acid) and LPC standard (which contains mostly palmitic acid and stearic acid) showed no staining with sulfuric acid/ethanol.

Most publications only investigate an assortment of the most important lipids in liver diseases[18,26,27,31,33]. It is almost impossible to detect all abundant lipid species in tissues within one method, because the physicochemical properties of the broad spectrum of lipid classes are too variable[22]. The lipid class spectrum of the bovine liver extract was quite similar to published studies which showed that PC and PE are the main components of the bovine liver phospholipids[34,35].

The extraction procedure has the advantage to be quick and that it requires relatively low amounts of tissue (approximately 70 mg wet weight tissue) compared to other methods[33]. Additional advantages are a low contamination with non-lipids due to the high apolarity of the solvent mixture, a low toxicity, a low phospholipid degradation, and the possibility to use

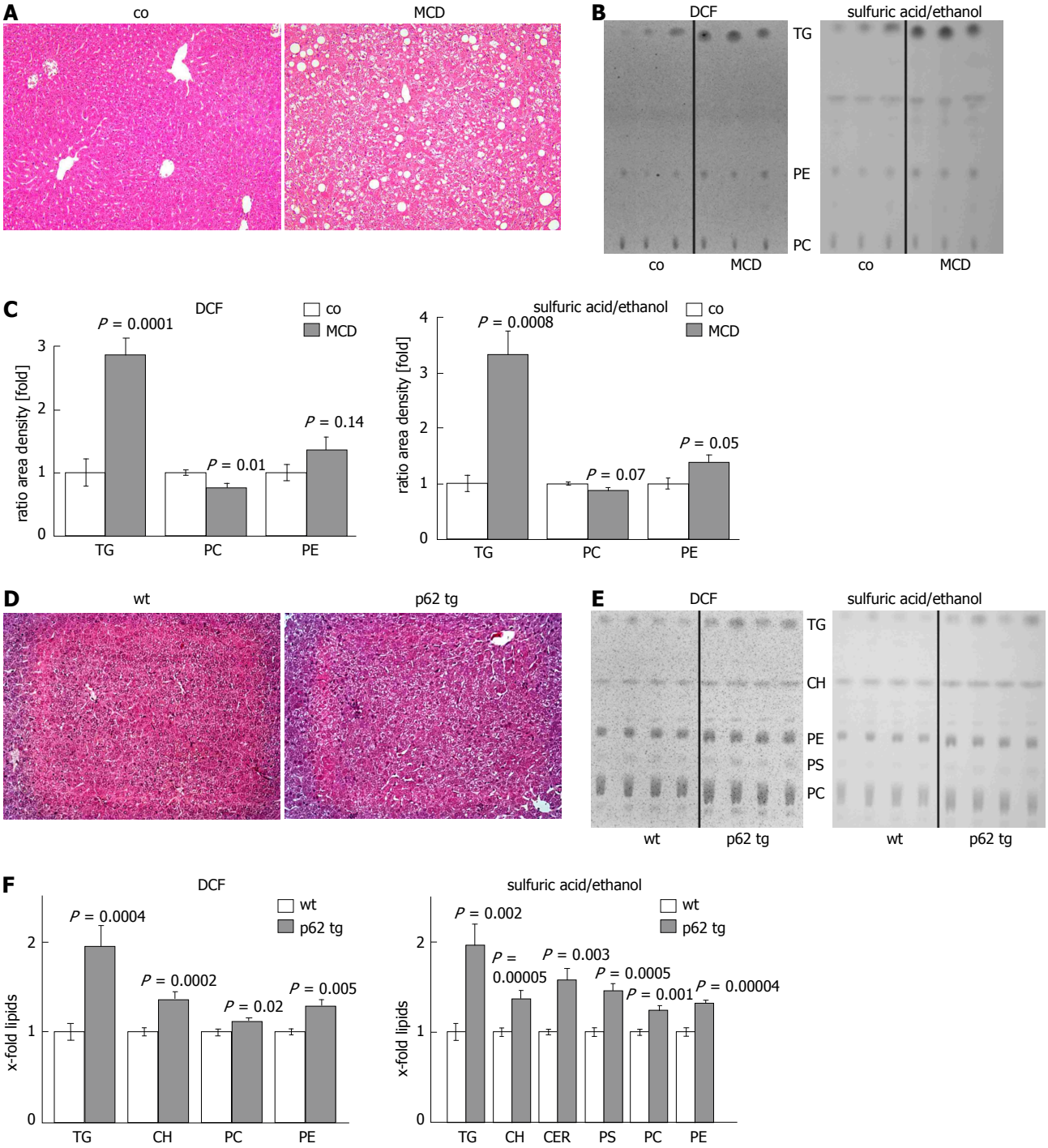

Figure 3 Lipid quantification in different mouse models. A: Representative hematoxylin eosin (HE) staining of control (co) or methionine choline deficient diet (MCD) fed mice (× 200); B: Representative thin layer chromatography (TLC) detected with 2',7'-dichlorofluorescein (DCF) (left) or sulfuric acid/ethanol (right); C: Quantification of TLC with ImageJ, detected with DCF (left) or sulfuric acid/ethanol (right). Results represent the mean ± SE from at least two independent TLC plates with n = 7 in each group; D: representative HE staining of wildtype (wt) and *p62* transgenic mice (p62 tg)(× 200); E: Representative TLC detected with DCF (left) or sulfuric acid/ethanol (right); F: Quantification of TLC with ImageJ detected with DCF (left) or sulfuric acid/ethanol (right). Results represent the mean ± SE from at least two independent TLC plates and n = 4 in each group.

plastic materials[22,36]. Freeze-drying of the liver tissue samples reduces the enzymatic activity of potential lipid degrading enzymes[37]. Taken together, this method is an easy, cheap, and rapid screening method for up to 12 samples in parallel. In addition, it needs only little technical equipment. The TLC method allows detection from the applied crude lipid extracts without the need of additional purification steps.

Confirmation of known changes in lipid classes in the MCD NASH model

After establishing a reliable technique we sought to confirm known alterations in lipid classes in the MCD NASH model. The MCD-induced NASH has the advantage of a histological appearance highly similar to human

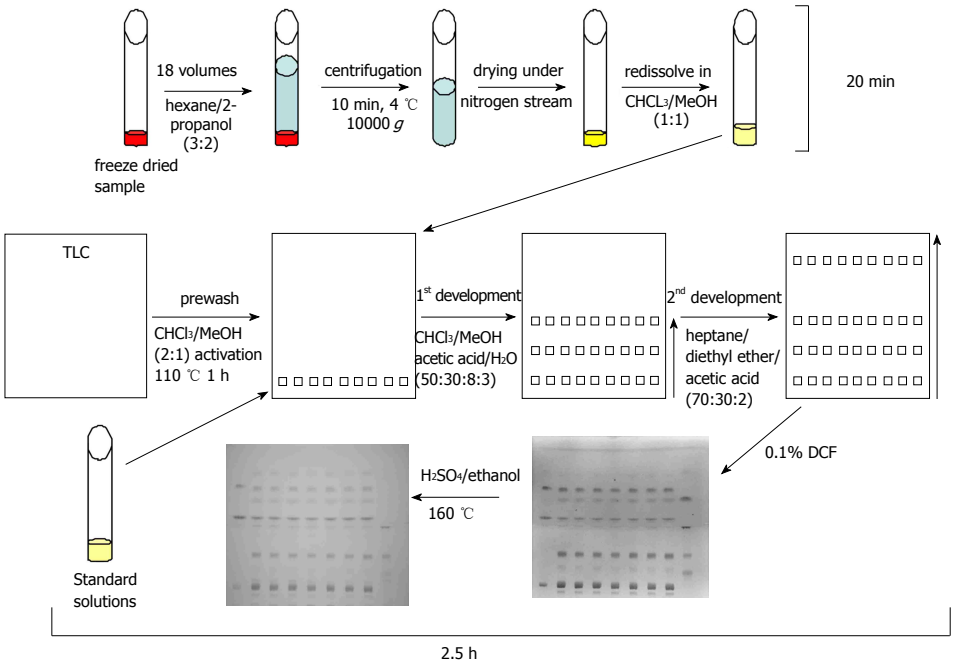

Figure 4 Thin layer chromatography method. Samples were freeze-dried, extracted, centrifuged, and the supernatant was transferred to a new vessel and dried under gaseous nitrogen. In the meantime plates were prewashed and activated. Standard solutions and samples were subjected to the prewashed and activated thin layer chromatography (TLC) plates and developed with the first eluant system to half of the plate. After drying the TLC plate was subjected to a second eluant system in the same direction up to the top of the plate. After drying of the plates, lipids were visualised with 2',7'-dichlorofluorescein (DCF) and afterwards with sulfuric acid/ethanol and heating to 160 ℃.

NASH concerning steatosis, *i.e.,* mixed inflammatory cell infiltrates, hepatocellular necrosis, and eventual pericellular fibrosis mimics[38]. We found strongly increased levels of TG, increased levels of PE, and decreased levels of PC, which led to a significantly decreased PC/PE ratio. Yao *et al*[39] reported decreased PC levels in choline deficient rat hepatocytes. Since PC biosynthesis is partly due to the methylation of PE by S-adenosyl methionine[40], it is not surprising that the lack of methionine in this model resulted in the accumulation of PE. An increase in TG[30] and a decreased mitochondrial PC/PE ratio[41] in the MCD diet was described previously. Therefore, our one-dimensional TLC method could well confirm known alterations in lipid classes in this dietary model of NASH.

Lipid composition in p62-induced steatosis

This is the first study, which clarifies the lipid composition in p62-induced steatosis. The increase in all detected lipid classes might be due to a p62-mediated activation of lipogenic genes induced by the lipogenic growth factor Igf2[42], which is highly overexpressed in *p62* transgenic animals[10]. The p62-induced microvesicular steatosis is difficult to evaluate with simple histological H/E staining (Figure 3D). Still, our TLC method revealed strongly affected lipid accumulation also in histologically normal tissue and allows more reliable and quantitative statements.

Accumulation of TG in hepatocytes is a hallmark of NAFLD[43]. As expected, TG were the lipid class elevated to the highest degree in p62-induced fatty liver. Interestingly, Yetukuri *et al*[44] described a positive correlation between TG and CER in an *ob/ob* steatosis model. The precursor for TG[45], namely DG, were not detectable in our murine models, despite the fact that DG standard series revealed strong signals. A similar observation could be seen for FFA and LPC. Since the age of our investigated transgenic and control animals were 2.5 to 5 wk, the lack of abundance of some lipid species might be explained by the relatively young age. In this context Rappley *et al*[46] reported age-dependent changes in phospholipid levels in mouse brain.

An unaltered content of FFA in human NAFLD was described previously[18]. Since we saw weak signals for FFA, which were not elevated in the *p62* transgenics, no elevation by p62 can be assumed.

One of the most complex investigations of the human NAFLD/NASH lipidome found in the literature reports elevated CH levels, and an increased ratio of CH to PC[18]. Accordingly, these results are in line with the findings in our *p62* steatotic animals. On the other hand, the literature report also found decreased levels of PC and PE, whereas PS remained unaltered[18]. Since increased CH levels are often associated with enhanced PC synthesis[47], increased PC levels in our *p62* transgenic animals might be explainable. Most notably, a decreased PC/PE ratio was observable in *p62* transgenic animals although PC and PE levels were both increased. The distinct manipulation of the PC/PE ratio performed by Li *et al*[48] showed that an elevation of the ratio can in fact reverse steatohepatitis, but not steatosis. This observation strongly suggests that a decreased PC/PE ratio plays a role in the progression from steatosis to steato-

hepatitis. In fact, NASH patients were found to have decreased PC/PE ratios in the same study[48]. The responsible mechanisms are as yet only speculative and might involve the inhibition of the PE N-methyltransferase[48], which converts PE to PC. Among the lipids, which were elevated in the *p62* transgenics, cholesterol[49] and ceramides[50] are highly cytotoxic. Although p62 overexpression induces a benign steatosis in the absence of inflammatory events, we speculate that the increased levels of CH and CER and the decreased PC/PE ratio might finally promote an inflammatory environment in the livers of *p62* transgenic animals. In fact, p62 overexpression can promote the development of NASH and fibrosis[13].

In a conclusion, taken together, we have established a rapid technique to quantify altered lipid classes in experimental models of steatosis and steatohepatitis. The method confirmed known changes in the well-established MCD NASH model and for the first time revealed a distinctly altered lipid composition in the p62 steatosis model. The knowledge of changes in lipid composition might be helpful for the understanding of pathophysiological mechanisms in NAFLD and NASH.

COMMENTS

Background

Non-alcoholic fatty liver disease and non-alcoholic steatohepatitis are mostly of benign appearance, but they are highly discussed as potential risk factors for the development of hepatocellular carcinoma (HCC). Hepatocellular carcinoma is a highly aggressive cancer type with high mortality, which is difficult to detect and to cure. Changes in lipid and fatty acid composition in disease progression to hepatocellular carcinoma are not well characterized, but are suggested to be of major importance. The high impact of these lipidomic changes needs appropriate *in vivo* models, and rapid and reliable methods to quantify the whole spectrum of lipid classes simultaneously.

Research frontiers

A state of the art method used for the identification of lipid classes is lipid chromatography coupled with mass spectrometric detection. This method is highly cost intensive, needs a lot of time for the establishment of the method and requires well-educated and experienced staff. Thin layer chromatography on the other hand represents a well-established technique, which allows a fast establishment in each laboratory within a really short time and allows a highly sensitive detection. As already mentioned, the lipidomic changes in the different states of liver disease progression in diverse *in vivo* mouse models and their potential correlation with human liver diseases are rare.

Innovations and breakthroughs

The thin layer chromatography of lipids is normally limited by the requirement to separately analyze either polar lipids or neutral lipids, each on one plate. Another method is the 3D thin layer chromatography, which allows only one sample per plate. Here the authors describe a fast screening method to chromatograph several samples on one plate, to separate the main polar and neutral lipid classes, to visualize them by two different staining methods, and to quantify them using the freely available ImageJ software within a short time. The authors proved the reliability of the method by comparing the obtained data from a methionine choline deficient non-alcoholic steatohepatitis mouse model to published data. The authors investigated the alterations in lipid classes in the *p62*/IMP2-2/IGF2BP2-2 transgenic mouse model for the first time and found interesting changes, which might indicate the progressive character of this steatosis model.

Applications

The described method can be used by all research laboratories for the lipidomic analyses of liver samples from the whole array of existing and newly developed experimental models for liver diseases. The method is not restricted to steatosis and steatohepatitis, but should also be useful for the analysis of HCC samples. While the gold standard for lipidomic analyses, *i.e.*, lipid chromatography-mass spectrometry is a quite expensive method, the thin layer chromatographic method can also be used in laboratories, which have no access to respective high-end equipment. The *p62* transgenic mouse model might be a potentially interesting model to investigate mechanisms of steatosis and disease progression. Further characterization and correlation to human data might help to understand the role of lipid changes in pathogenesis.

Terminology

Non alcoholic fatty liver disease: non alcoholic fatty liver disease is characterized by a strong accumulation of lipids, especially triglycerides, within hepatocytes; non alcoholic steatohepatitis: non alcoholic steatohepatitis is a steatotic liver, which is characterized by an inflammatory environment and might result in fibrosis; hepatocellular carcinoma: hepatocellular carcinoma is an aggressive form of liver cancer; thin layer chromatography: thin layer chromatography is a chromatographic method, glass or aluminium plates are coated mostly with silica gel and allows separation with different solvent systems.

Peer review

This study seems well done and well written about a subject of increasing relevance and judge that it deserves to be published.

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BRIEF ARTICLE

Presence of disease specific autoantibodies against liver sinusoidal cells in primary biliary cirrhosis

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Abstract

AIM: To investigate the presence of autoantibodies directed against liver sinusoidal cells in primary biliary cirrhosis (PBC).

METHODS: Liver biopsies from 21 PBC patients were studied and compared with 12 liver biopsies from disease controls [3 patients with hepatitis B (HBV) virus, 3 patients with hepatitis C virus (HCV), 3 patients with non-alcoholic steatohepatitis and 3 patients with acute alcoholic hepatitis (AAH)]. As healthy controls, we used tissue specimens adjacent to metastatic liver adenocarcinoma. Normal serum was taken from staff members of the unit. The determination of the cell type targeted by autoantibodies present in the patients sera was performed by indirect immunofluorescence (IIF) analysis using paraffin-embedded liver sections as a substrate. Sera from homologous or heterologous PBC patients or sera from the disease control group were used as primary antibodies. The presence of autoantibodies was identified using confocal microscopy.

RESULTS: In total, 18/21 (85.7%) PBC patients exhibited positive staining in the sinusoidal cells, 10/21 (47.6%) in lymphocytes, 8/21 (38%) in cholangiocytes and 7/21 (33.3%) in hepatocytes, when homologous serum and fluorescein isothiocyanate-conjugated immunoglobulin type G (IgG) secondary antibody were used. PBC sections incubated with heterologous PBC serum showed reduced staining (20% for sinusoidal cells, 20% for lymphocytes, 20% for cholangiocytes and 13.3% for hepatocytes). When IgM immunoglobulin, instead of IgG, was used as secondary antibody, positive staining was observed in 75% of lymphocytes, 62.5% of cholangiocytes, 37.5% of hepatocytes and 50% of the sinusoidal cells with a much stronger staining intensity. No staining was observed when either normal or PBC sera were used as a primary antibody on liver sections from the disease control group. When PBC sera were incubated with healthy control sections, weak positive staining of cholangiocytes was observed in 3/21 (14.3%) PBC serum samples. Steatohepatitis serum on PBC sections gave a positive staining of some hepatocytes and lymphocytes but no staining on viral hepatitis sections. Incubation with HBV sera stained some hepatocytes, cholangiocytes and intra-sinusoidal or portal lymphocytes of PBC, HBV and AAH patients but not HCV patients.

CONCLUSION: In this study, for the first time in diseased liver tissue, we have demonstrated that a large proportion of PBC patients have disease specific autoantibodies against liver sinusoidal cells.

Key words: Primary biliary cirrhosis; Autoantibodies; Sinusoidal cells; Cholangiocytes; Liver tissue

Core tip: In this study, indirect immunofluorescence staining was performed on paraffin-embedded human liver sections of various chronic liver diseases to demonstrate the presence of disease specific autoantibodies targeting sinusoidal cells in patient sera. Liver sections from normal and disease controls were used as the substrate, and sera were the source of primary antibodies. Our findings indicate that disease specific antibodies against liver sinusoidal cells circulate in primary biliary cirrhosis (PBC). Various non disease specific antibodies were also found in PBC and chronic hepatitis B but not in other chronic liver diseases.

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease of unknown etiology that leads to a progressive nonsuppurative destruction of small- and medium-sized intrahepatic bile ducts and eventually to cirrhosis and liver failure[1]. The diagnosis of PBC is based on three criteria: the presence of detectable anti-mitochondrial antibodies (AMAs) in the serum, the elevation of cholestatic enzymes (most commonly, alkaline phosphatase (ALP) for > 6 mo and histological findings in the liver that are compatible with the presence of the disease. A probable diagnosis requires the presence of two criteria, and a definite diagnosis requires all three[2].

The major mitochondrial autoantigens recognized by AMAs are members of the 2-oxoacid dehydrogenase complex (ODC) family[3-5]. Studies based on immunohistochemistry and affinity mass spectrometry have suggested that either PDC-E2 (a member of the ODC family) or a cross-reactive molecule is present in greatly increased amounts at the apical surface of biliary epithelial cells (BECs) from patients with AMA-positive or AMA-negative PBC, but not from normal individuals or patients with other liver diseases[4,5]. Additionally, in other studies, in addition to the intense staining of the apical surface of BECs, positive staining was observed in a subset of macrophages in portal lymph nodes[6] and in hepatocytes[7].

In addition to AMAs, which are the hallmark of PBC, antinuclear antibodies (ANAs) have also been detected in 30% of PBC patients[8-10].

Two PBC-specific ANA immunofluorescence patterns have been identified[11,12]: "multiple nuclear dots", corresponding to the antigens Sp100 and Sp140, promyelocytic leukemia (PML) nuclear body proteins and small ubiquitin-like modifiers (SUMOs)[13,14], and "nuclear membrane" (rim), caused by anti-nuclear envelope antibodies (ANEAs), such as gp210 and nucleoporin p62[15,16]. The anti-gp210 antibodies are highly specific for PBC and are associated with disease activity and severity[17,18].

Nakamura *et al*[19] found that the expression of gp210 is markedly increased on the nuclear envelope of small bile ducts and sometimes at infiltrating mononuclear cells in the portal area and/or periportal hepatocytes in PBC and this expression was positively correlated to disease activity. We also reported that 46.9% of patients with PBC have detectable ANEAs and 21% of them had detectable anti-gp210 antibodies. The presence of these antibodies identifies a subgroup of PBC patients with advanced disease and poor prognosis[20]. So far, no antibodies against liver sinusoidal cells have been reported in diseased liver tissue.

In our previous studies, we hypothesized that the primary initiating event in PBC may be the overproduction of endothelins by liver sinusoidal cells[21,22].

The aim of the present study was therefore to identify the presence of circulating antibodies against liver sinusoidal cells in PBC patients.

MATERIALS AND METHODS

Patients

Liver biopsies from 21 PBC patients were studied and compared with 12 liver biopsies from disease controls [3 patients with hepatitis B virus (HBV), 3 patients with hepatitis C virus (HCV), 3 patients with non-alcoholic steatohepatitis and 3 patients with acute alcoholic hepatitis (AAH)]. As healthy controls, we used tissue specimens adjacent to metastatic liver adenocarcinoma. Normal serum was taken from staff members of the unit. The mean age of the 21 PBC patients was 52.9 ±13.4 years (range: 31-76 years), and the mean Mayo risk score was 4.2 ± 1.0 (range: 2.8-6.5) at diagnosis. Seventeen (81%) patients were stage Ⅰ-Ⅱ, and 4 (19%) were stage Ⅲ-Ⅳ, according to Ludwig[23]. Fifteen patients were positive for ANEAs, and 7 were anti-gp210 positive.

No patients or disease controls had any associated autoimmune diseases.

The study was approved by the Ethics Committee of the University Hospital of Heraklion, and written informed consent was collected from all patients and controls participating in the study.

Indirect immunofluorescence analysis of paraffin-embedded sections

The determination of cell types targeted by autoantibodies present in the patients sera was performed by indirect immunofluorescence (IIF) staining using paraffin-embedded sections as a substrate. As a primary antibody, serum from each patient (homologous), serum from another PBC patient (heterologous) or serum from normal or disease con-

Table 1 Results of the 21 primary biliary cirrhosis patients after indirect immunofluorescence staining with homologous serum and IgG secondary antibody

Patient	Target cells			
	Lymphocytes	Cholangiocytes	Hepatocytes	Sinusoidal cells
1	P	N	N	P
2	N	N	N	P
3	P	P	N	P
4	P	N	N	P
5	N	P	N	P
6	P	N	N	N
7	P	N	P	P
8	N	N	P	P
9	P	P	N	P
10	N	N	N	P
11	P	P	P	P
12	N	P	P	N
13	N	N	N	N
14	N	P	N	P
15	N	N	N	P
16	N	P	P	P
17	N	N	N	P
18	N	N	N	P
19	P	N	N	P
20	P	P	P	P
21	P	N	P	P
Total positive *n* (%)	10 (47.6)	8 (38.0)	7 (33.3)	18 (85.7)

P: Positive staining; N: No staining.

trol was used. Paraffin-embedded liver biopsy sections of 3 μm thickness were deparaffinized in xylene and rehydrated in graded ethanol solutions (100%, 95%, 80% and 70%).

IIF analysis was performed as previously described[24]. Briefly, antigen retrieval was achieved by incubation with citrate buffer (1.8 mmol/L citric acid and 8.2 mmol/L sodium citrate) for 2 h at 37 ℃. After blocking with phosphate-buffered saline (PBS) containing 2 mL/L Triton X-100, 2 mmol/L $MgCl_2$ and 10 mL/L gelatin from cold-water fish skin (Sigma-Aldrich, Germany) for 10 min, the sections were incubated with patient serum for 2 h (dilution 1:50). The sections were washed with blocking buffer and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin type G (IgG) (dilution 1:500, H + L secondary antibody, Chemicon, Millipore, Germany) or immunoglobulin type M (IgM) (dilution 1:100, DAKO, Carpinteria, CA) for 45 min at room temperature. Afterward, nuclei were counterstained by incubating the sections for 5 min with TO-PRO-3 iodide (TO-PRO) (dilution 1:1000 in blocking buffer, Molecular Probes, Inc). Lastly, the sections were rinsed in PBS and mounted with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology, Inc, Germany). Negative controls were generated by omitting the primary antibody. Fluorescence was observed under a Leica SP confocal microscope. In preliminary experiments, the sections were incubated with dilutions of sera up to 1:200. The results were similar, but the staining intensity was weaker, so a dilution of 1:50 was used throughout.

RESULTS

IIF analysis of paraffin-embedded sections

We have observed five types of staining: apical staining of BECs; perinuclear and nuclear staining of lymphocytes, hepatocytes, cholangiocytes and sinusoidal cells, punctuated staining of hepatocytes and cytoplasmic staining of lymphocytes, hepatocytes, cholangiocytes and sinusoidal cells.

PBC sections incubated with PBC serum and FITC-conjugated IgG secondary antibody

Homologous serum: Of the 21 PBC patients 10 (47.6%) yielded positive staining in lymphocytes, 8 (38%) in cholangiocytes, 7 (33.3%), in hepatocytes; and 18 (85.7%) in the sinusoidal cells, (Table 1, Figure 1D-F), although the sinusoidal staining was weak.

Heterologous serum: PBC sections with heterologous PBC serum showed reduced staining [(lymphocytes: 20% of patients, cholangiocytes: 20%, hepatocytes: 13% and sinusoidal cells: 20% (Figure 1G-I)].

PBC sections incubated with PBC serum and FITC-conjugated IgM secondary antibody

Staining with homologous serum was different when IgM was used as a secondary antibody rather than IgG. Positive staining was observed in lymphocytes (75% of patients), cholangiocytes (62%), hepatocytes (38%) and sinusoidal cells (50%), but the staining was very strong (Figure 1J-L).

PBC sections or sections from disease controls incubated with normal serum

No staining was observed (Figure 2A-F).

Sections from disease controls or sections of normal controls incubated with PBC serum

No staining was observed when PBC sera were used on sections from the disease control group (Figure 2G-I). When PBC sera were used as a primary antibody on healthy control sections, weak positive staining of cholangiocytes in 3/21 (14.3%) PBC sera was observed (Figure 2J-L).

PBC sections or sections from disease control group incubated with serum from disease controls

Steatohepatitis serum on PBC sections yielded positive staining of some hepatocytes and lymphocytes (Figure 2M-O) and no staining of viral hepatitis sections (Figure 2P-R).

Incubation with HBV sera yielded positive staining of the hepatocytes, intra-sinusoidal and portal lymphocytes and cholangiocytes of some PBC (Figure 3A-C), HBV

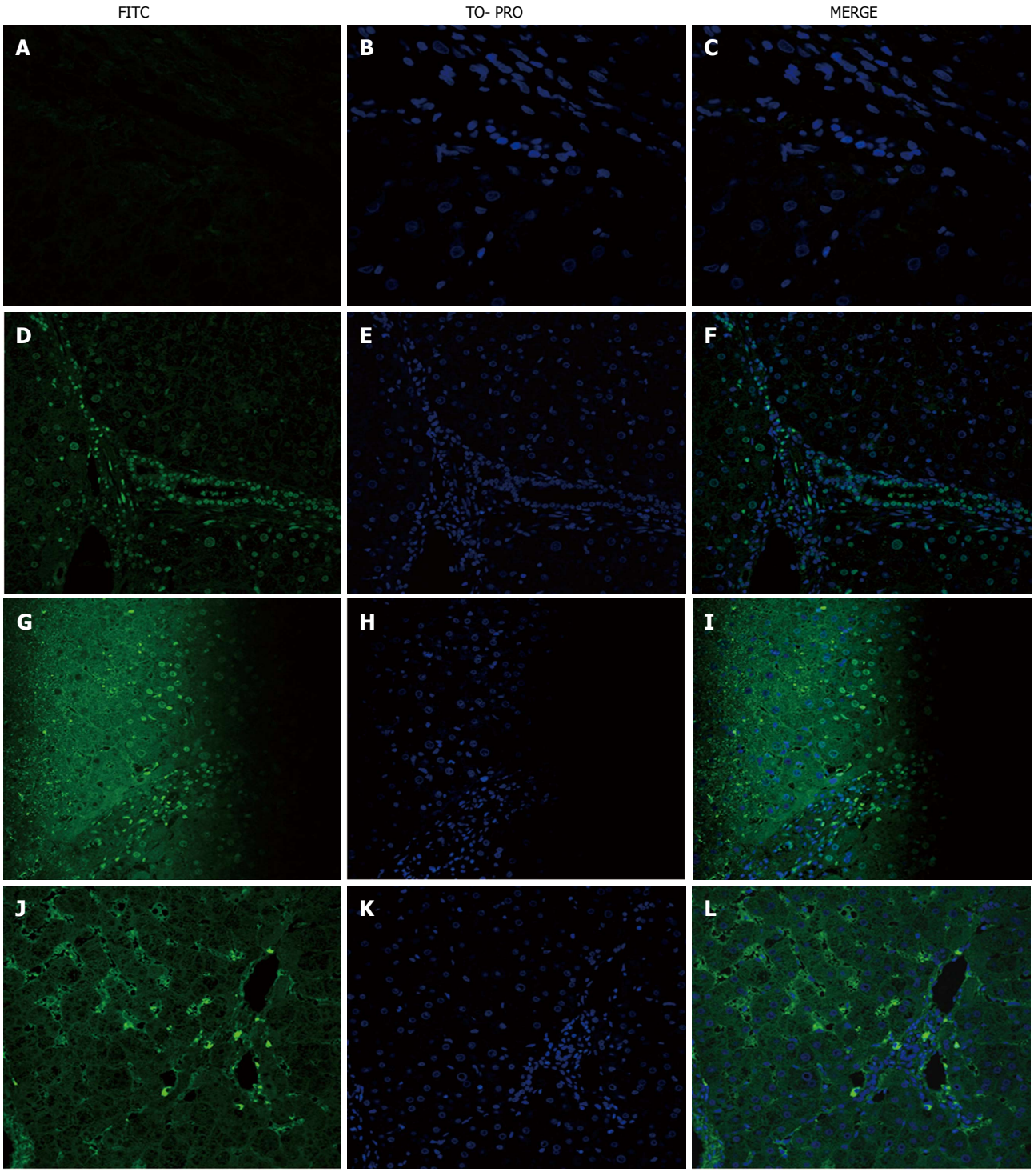

Figure 1 Immunofluorescence of primary biliary cirrhosis sections incubated with primary biliary cirrhosis serum and fluorescein isothiocyanate -conjugated IgG or IgM secondary antibody. A-C: Negative control incubated with IgG secondary antibody; D-F: Primary biliary cirrhosis (PBC) section incubated with homologous PBC serum and IgG secondary antibody, showing staining of cholangiocytes, sinusoidal cells (weak staining) and mononuclear cells, probably lymphocytes; G-I: PBC section incubated with heterologous PBC serum and IgG secondary antibody, showing nuclear and cytoplasmic staining of hepatocytes and lymphocytes; J-L: PBC section incubated with homologous PBC serum and IgM secondary antibody, showing cytoplasmic staining of sinusoidal cells and lymphocytes. Magnification × 200. FITC: Fluorescein isothiocyanate.

(Figure 3D-F) and AAH (Figure 3G-I) patients but not HCV patients (Figure 3J-L).

DISCUSSION

In the present study, for the first time in diseased liver tissue, we have identified sinusoidal cells as liver cells targeted by circulating autoantibodies in PBC patients. We previously hypothesized that the primary event in PBC is the excessive production of endothelin 2, possibly by sinusoidal cells[21,22].

The role of certain autoantibodies in PBC is well

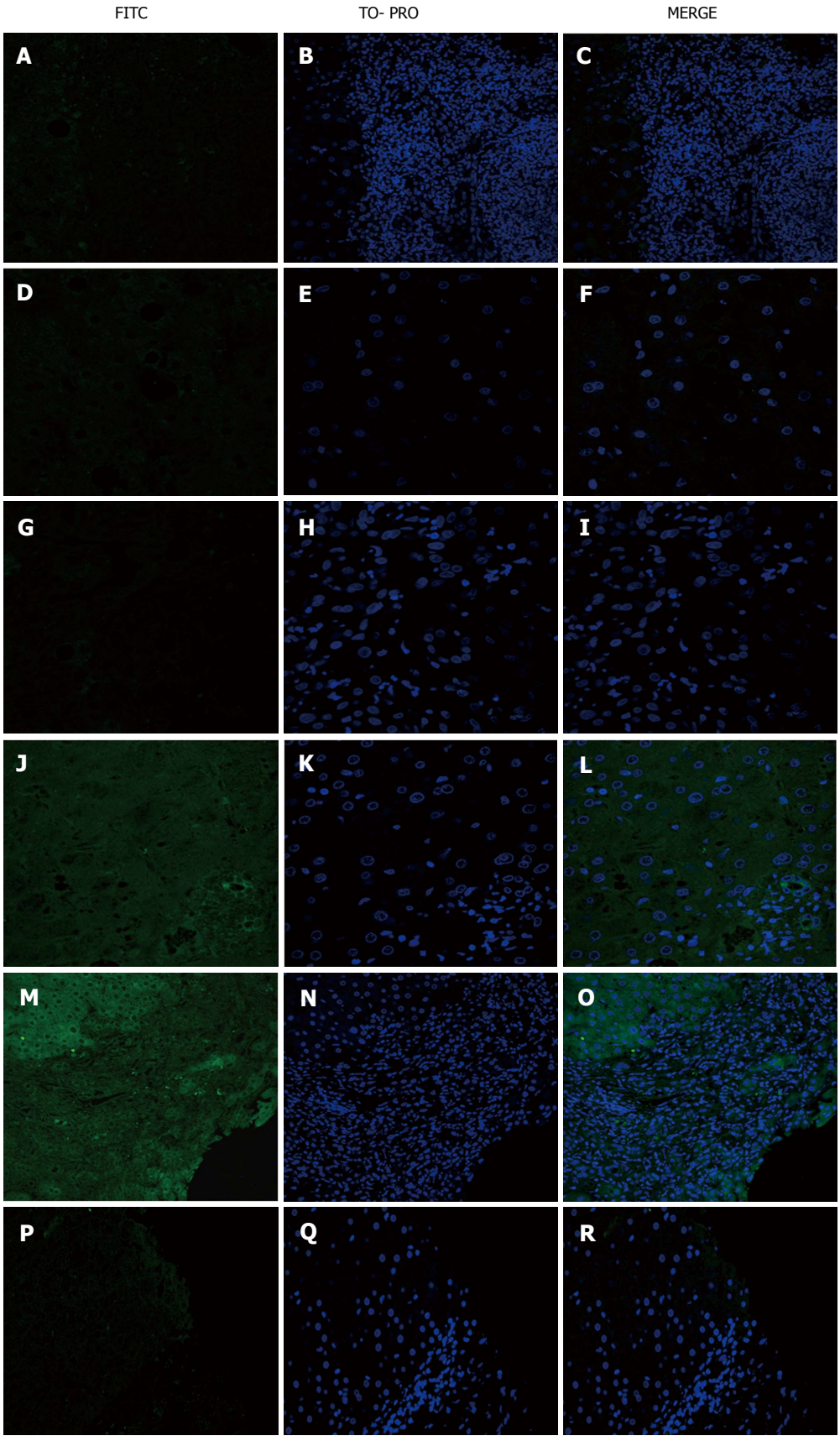

Figure 2 Immunofluorescence of primary biliary cirrhosis, acute alcoholic hepatitis (AAH) or normal sections incubated with primary biliary cirrhosis, disease control or normal serum and fluorescein isothiocyanate -conjugated IgG secondary antibody. A-C: Primary biliary cirrhosis (PBC) section incubated with normal serum; D-F: Acute alcoholic hepatitis section incubated with normal serum; G-I: AAH section incubated with PBC serum; J-L: Normal section incubated with PBC serum, showing weak positive staining of cholangiocytes; M-O: PBC section incubated with steatohepatitis serum, showing positive staining of hepatocytes; P-R: Hepatitis B virus section incubated with steatohepatitis serum. Magnification × 200. FITC: Fluorescein isothiocyanate.

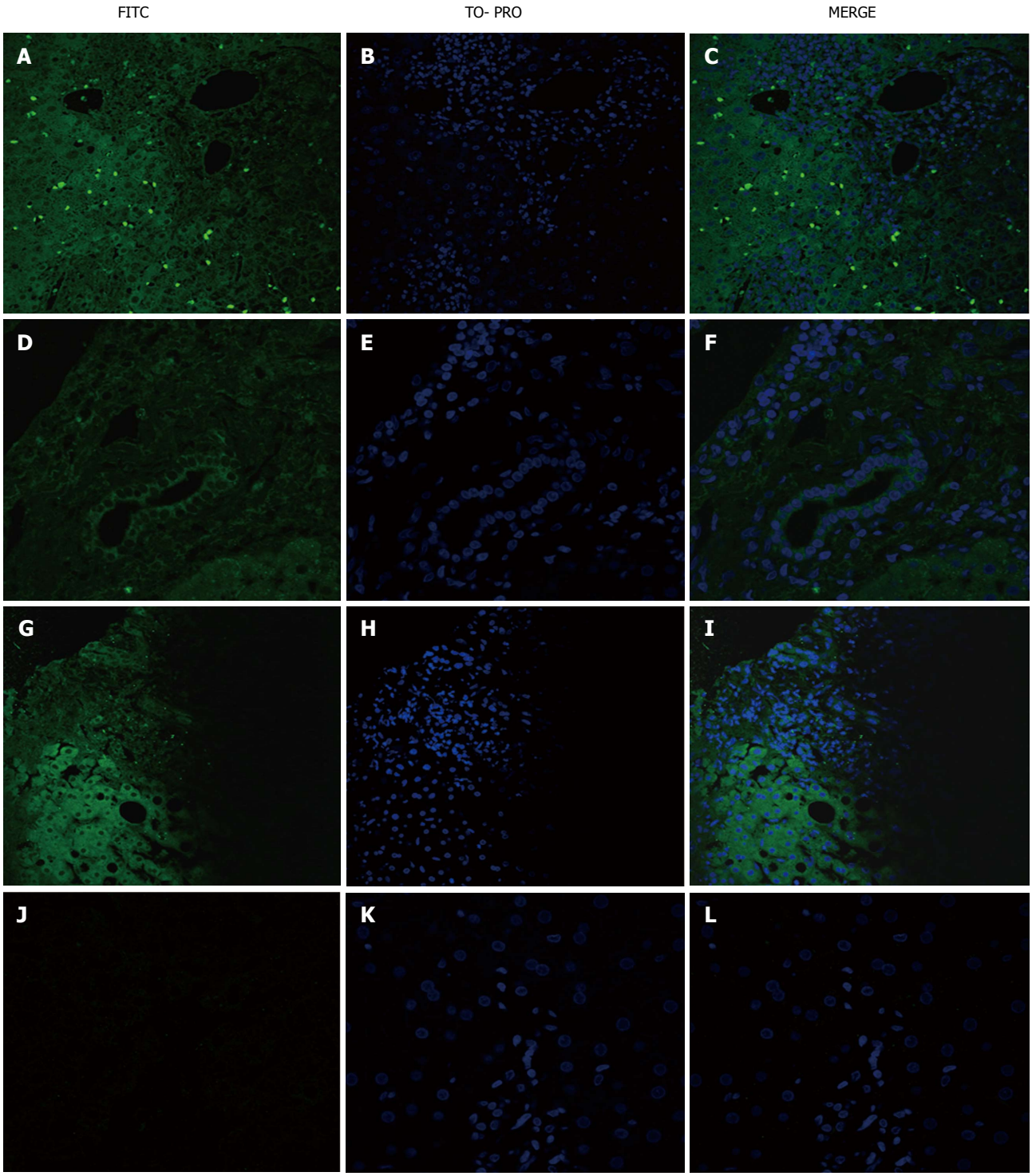

Figure 3 Immunofluorescence of sections from the primary biliary cirrhosis and disease control groups incubated with hepatitis B virus serum and fluorescein isothiocyanate conjugated IgG secondary antibody. A-C: Primary biliary cirrhosis (PBC) section with positive staining of intra-sinusoidal and portal lymphocytes; D-F: Hepatitis C virus (HBV) section with positive staining of cholangiocytes; G-I: AAH section with positive staining of hepatocytes; J- L: Hepatitis C virus section. Magnification × 200. FITC: Fluorescein isothiocyanate.

established from previous studies. Panels of mouse monoclonal antibodies and human combinatorial autoantibodies against specific antigens, such as members of the ODC family[4,6,25-27] or the gp210 antigen[19,28], have been used in paraffin-embedded liver sections, and their significance in diagnosis and prognosis has been verified. Tsuneyama *et al*[4] have demonstrated that both AMA-positive and AMA-negative PBC patients, but not controls, have abnormal expression of either PDC-E2 or a cross-reacting molecule in the apical region of the biliary epithelium.

Using *in situ* nucleic acid hybridization, Harada *et al*[29]. found that there were no increased levels of PDC-E2 mRNA in PBC livers. The researchers suggested that the

increased levels of immunoreactive material either did not arise in BECs or were not derived from material encoded by the *PDC-E2* gene sequence[29]. These data and the recurrence of such abnormal apical staining in liver allografts from PBC but not controls are most easily explained by the suggestion that the molecule at the apical surface of bile ducts in PBC tissue is not PDC-E2, but rather a molecule that bears a cross-reactive epitope. One possible source of such a molecular mimic may be infecting microorganisms, although no specific molecule from such organisms has been identified[7,27].

However, in PBC, as in many other so-called autoimmune diseases, there are many other autoantibodies whose significance is usually unknown[11].

In the present study, we used paraffin-embedded liver sections and homologous and heterologous sera from PBC patients to detect liver cells that are the targets of circulating antibodies. When homologous serum was used along with an IgG secondary antibody, 48% of PBC patients exhibited positive staining of lymphocytes, 38% in bile ducts, 33% in hepatocytes and 86% in sinusoidal cells of the liver. A difference in staining was found when IgM was used rather than IgG to detect autoantibodies in patients with PBC. The staining of sinusoidal cells was weak with IgG but very strong when an IgM secondary antibody was used.

This difference between IgM and IgG antibodies may have a dual explanation. First, patients with PBC express IgM more frequently[30,31] than do patients with other autoimmune hepatic diseases. Alternatively, as we have shown in our previous study, these differences between IgG and IgM depend on the cell type that is being used as a substrate[32]. When heterologous PBC sera were used, similar staining patterns were found, but the percentage was lower, and approximately 20% of the sinusoidal cells of PBC patients were positive. No staining was observed when PBC serum was incubated with liver biopsies from the disease controls.

The staining of the sinusoidal cells seemed to be specific for PBC patients because staining of lymphocytes, biliary epithelial cells and hepatocytes, but not sinusoidal cells, was observed in all other serum-tissue combinations that we used. Thus, sera from the disease control group incubated with either PBC liver or disease control liver showed that in chronic HBV, positive staining was observed in the lymphocytes of 17% of PBC patients, but not in the lymphocytes of patients with other liver diseases. In addition, the hepatocytes of 17% of patients with PBC and 20% of patients with other liver diseases were positive when incubated with chronic hepatitis B serum. The serum of a patient with steatohepatitis yielded very weak positive staining in lymphocytes and hepatocytes from certain patients with PBC but resulted no positive staining in the tissues of patients with other liver diseases.

In two earlier reports, antibodies against liver sinusoidal endothelial cells were described in both autoimmune hepatitis (AIH) and PBC. In the first report[33], sera from patients were incubated with isolated rat liver endothelial cells, and IgG bound to endothelial cells was found in 13% of patients with PBC. In a similar study, patient sera were incubated with isolated human liver sinusoidal endothelial cells, and 59% of PBC patients had reactive antibodies. Moreover, cells incubated with the F(ab)2 fragments of antibodies from either AIH or PBC patients were transformed into a vascular cell phenotype[34]. Since we used sections of liver tissue rather than isolated cells, our findings are not directly comparable with the results of these studies. However, a higher proportion of our patients showed circulating antibodies against sinusoidal cells compared with the proportion in both previous studies. This finding might indicate that these antibodies may recognize other sinusoidal cells, such as Kupffer cells, in addition to endothelial cells.

Our findings indicate that both patient specific and disease specific antibodies circulate in PBC. At the same time, non disease specific antibodies were found in PBC and chronic HBV but not in other chronic liver diseases. However, the findings of the present study do not answer the question of whether antibodies circulating in the serum of patients with chronic hepatitis B recognize the same epitopes as circulating antibodies in PBC; this question needs to be further examined. More detailed studies are also required to further elucidate the specific sinusoidal cell type(s) against which these antibodies are directed.

In conclusion, the present study shows that a large proportion of PBC patients have disease specific autoantibodies against liver sinusoidal cells. To our knowledge, this result has not been previously reported for diseased liver tissue. The target antigens of the sinusoidal cells and the specificity of these antibodies need further study. Moreover, the antibodies clinical significance requires clarification, along with clinical follow-up of a large cohort of patients.

COMMENTS

Background

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease of unknown etiology. The authors previously hypothesized that the primary initiating event in PBC may be the overproduction of endothelins by liver sinusoidal cells. The presence of autoantibodies against isolated rat and human liver endothelial cells have been described in several cases of PBC, but so far, such autoantibodies have not been described in diseased human liver tissue.

Research frontiers

The research objective was to investigate the presence of disease specific autoantibodies against liver sinusoidal cells in PBC serum using human liver tissue as a substrate.

Innovations and breakthroughs

In two previous studies, isolated rat or human liver endothelial cells were used as substrates to detect the presence of specific serum autoantibodies in PBC. In this study, for the first time, indirect immunofluorescence staining was performed using human diseased liver tissues as the substrate and sera from PBC and other chronic liver disease patients as the source of primary antibody.

Applications

The findings of this study indicate the presence of disease specific autoantibodies directed against liver endothelial cells and possibly Kupffer cells in a large proportion of PBC patients. The exact specificity and functional significance of

these antibodies remains to be further elucidated and might be related to the pathogenesis of this disease.

Peer review

The authors explore the presence of autoantibodies directed against liver sinusoidal cells in PBC. For this, they evaluate the liver biopsies of patients with PBC, and from patients with other liver diseases.

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BRIEF ARTICLE

Primary biliary cirrhosis in HBV and HCV patients: Clinical characteristics and outcome

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Author contributions: Rigopoulou EI, Zachou K and Dalekos GN had the original idea for the study, designed the study protocol and wrote the paper; Zachou K and Gatselis NK did the immune serology and virologic work-up and along with Rigopoulou EI; Papadamou G and Dalekos GN assessed the patients from the immunological and virological points of view; Koukoulis GK did the interpretation of the histological data of the patients; Zachou K, Gatselis NK and Dalekos GN collected the whole data, treated the patients in collaboration with Rigopoulou EI and Papadamou G, and performed the follow-up assessment while contributed to the final version of the paper; Koukoulis GK and Dalekos GN wrote the final version of the paper; all authors have seen and approved the final draft of the paper.
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Abstract

AIM: To present the characteristics, management and outcome of patients with hepatitis B virus (HBV) or hepatitis C virus (HCV) infections concurrent with primary biliary cirrhosis (PBC).

METHODS: Since January 2001 to September 2009, we retrospectively evaluated the medical records of all HBV (n = 1493) and HCV patients (n = 526) who are followed in our center for the presence of concurrent PBC. Seventeen patients identified with concurrent viral hepatitis and PBC (8 HCV and PBC; follow-up: 61 ± 37 mo and 9 HBV and PBC; follow-up: 57 ± 38 mo). PBC diagnosis was established if the patients met at least two of the following criteria: positivity for antimitochondrial antibody, elevated cholestatic enzymes and histological lesions of PBC.

RESULTS: HCV or HBV diagnosis preceded that of PBC in most patients by many years. PBC diagnosis was based on the presence of antimitochondrial antibody and elevated cholestatic enzymes in all 17 patients, while one third (5/17; 29.4%) experienced severe pruritus many years before diagnosis. Patients with PBC and HBV were significantly younger at diagnosis of PBC compared to patients with PBC and HCV (56.1 ± 11.2 *vs* 68.5 ± 10.3, respectively, $P < 0.05$). At initial clinical and histological assessment the majority of patients were cirrhotics (10/17; 58.8%) with the group of PBC and HCV carrying the highest frequency (87.5% *vs* 33.3% in PBC and HBV; $P < 0.05$). The patients with HBV and concomitant PBC seem to have better outcome compared to those with HCV and PBC since none of the 6 non-cirrhotics with HBV and PBC developed cirrhosis during follow-up.

CONCLUSION: PBC diagnosis in HBV or HCV patients is very difficult and usually delayed. Therefore, in any case, cholestasis should alert physicians to further search for PBC.

Key words: Antimitochindrial antibodies; Autoimmune liver disease; Hepatitis B virus; Hepatitis C virus; Primary biliary cirrhosis.

Core tip: In hepatitis B virus (HBV) and hepatitis C virus (HCV) patients the possibility of concomitant primary biliary cirrhosis (PBC), is often very difficult to recognize and therefore, a significant delay in PBC diagnosis in this spe-

cific group of patients is usual. In this report, we clearly show that almost 1% of our HBV and HCV cohort had also PBC which had been misdiagnosed or underestimated for many years. The latter could be the reason of unfavorable outcome observed in our patients and in particular, among HCV/PBC patients. Therefore, the existence of cholestasis should prompt physicians to sick for antimitochondrial antibody with various sensitive techniques irrespective of the presence of other liver diseases.

Rigopoulou EI, Zachou K, Gatselis NK, Papadamou G, Koukoulis GK, Dalekos GN. Primary biliary cirrhosis in HBV and HCV patients: Clinical characteristics and outcome. *World J Hepatol* 2013; 5(10): 577-583 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i10/577.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i10.577

INTRODUCTION

Infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) viruses are major public health problems worldwide leading to significant mortality due to hepatic insufficiency and liver cancer[1,2]. The prevalence of HBV infection varies considerably among different parts of the world and depends mainly on the age at which infection occurs. Greece is still considered a country with intermediate prevalence for HBV infection[3,4]. In the region of Thessaly, central Greece, the prevalence of HBV infection has been estimated to be 4.26% in the general population, while clusters of HBV infection have been recently reported in the same area[5]. In contrast, the prevalence of HCV infection is estimated to be low in our area (0.34%), even though clusters of higher HCV prevalence were recently identified by our group[6].

Coexistence, of autoimmune liver diseases like primary biliary cirrhosis (PBC)[7] or autoimmune hepatitis (AIH)[8] with HCV or HBV infections could be a problem especially in endemic areas for viral hepatitis[8], since viruses in general have long been associated in some cases with the induction of autoimmune phenomena up to the development of overt autoimmune diseases[8-13].

In this context, we have recently described the characteristics of 11 patients with concurrent viral HBV or HCV and AIH alongside with clues to diagnosis, patterns of disease progression and outcome as well as difficulties in decision management[14]. Accordingly, the aim of the present study was to explore our data in another autoimmune liver disease namely, PBC by describing the patients' characteristics, management and outcome in patients where HBV and HCV infections concur with PBC since in the current literature there are scarce data to demonstrate the interaction between PBC and viral hepatitis infections.

MATERIALS AND METHODS

From a total of 1493 patients with chronic HBV and 526 with chronic HCV infection, who attended the outpatient clinic from January 2001 to September 2009, we have retrospectively identified 17 patients with concurrent viral hepatitis and PBC and reviewed their medical records. The demographic and clinical characteristics of these 17 patients along with those of 98 and 303 randomly selected patients from our records with HCV and HBV respectively are shown only in a descriptive way in Table 1 since these groups of HCV and HBV infections without concurrent PBC are not appropriate for statistical comparisons to the HCV or HBV patients and concomitant PBC. All patients were followed at the outpatient clinic of the Department of Medicine, Larissa Medical School, University of Thessaly, Larissa, Greece.

Diagnosis of chronic HCV and HBV infections was based on criteria published in our previous reports[5,6,15-18] and according to the consensus statements of the European Association for the Study of the Liver (EASL)[1,2].

Briefly, all HCV patients included in the study met the following criteria: (1) serologic evidence of chronic HCV infection as determined by the detection of antibodies to HCV (anti-HCV) using a second- or third-generation enzyme immunoassay at least twice within 6 mo and (2) active virus replication as defined by the detection of HCV-RNA using a sensitive commercially available quantitative real time PCR kit (COBAS Taqman HCV test; cut-off of detection: 25 IU/mL). All HBV patients included in the study met the following criteria of either chronic hepatitis B such as, (1) serological evidence of chronic HBV infection for at least 6 mo before the entry to the study using commercially available enzyme immunoassays; (2) active virus replication as defined by the detection of HBV-DNA (> 2000 IU/mL), as all patients were HBeAg negative, using a sensitive commercially available quantitative real time PCR kit (COBAS Taqman HBV test; cut-off of detection: 6 IU/mL); (3) persistently or intermittently elevated levels of alanine aminotransferase (ALT) for at least 6 mo before the entry to the study; and (4) histologically proven chronic hepatitis B, or inactive carrier state of HBV infection (HBV positive serology for at least 6 mo but repeatedly undetectable HBV-DNA and normal ALT levels).

According to internationally accepted criteria and to previous publications from our group[12,19-21], patients with PBC met at least two of the following criteria: (1) positivity for antimitochondrial antibody (AMA; positive titre $\geq$ 1/40) either by IIF on in-house rodent tissue substrates confirmed by Western blot using in-house mitochondrial subfraction of rat livers, or by enhanced performance M2 ELISA (M2 EP (MIT3) ELISA, Quanta Lite, INOVA Diagnostics, San Diego, CA), which was shown to have higher sensitivity compared to the conventional anti-M2[20,22]; (2) elevated cholestatic enzymes and (3) histological lesions of PBC (when a liver biopsy had been performed). The presence of other liver diseases and in particular of AIH has been appropriately excluded[8,23].

Response to antiviral treatment in HBV- and HCV-treated patients was assessed according to internationally

Table 1 Demographic, clinical, laboratory and histological characteristics of hepatitis C or hepatitis B virus patients with concurrent primary biliary cirrhosis or without primary biliary cirrhosis

	PBC+HCV (n = 8)	PBC+HBV (n = 9)	P value[2]	HCV (n = 98)	HBV (n = 303)
Sex (male/female)	3/5	2/7	NS	62/36	183/120
Age at diagnosis of viral infection (yr)	59.8 ± 13.9	49.9 ± 13.7	NS	44 ± 17	44.4±15.6
Age at diagnosis of primary biliary cirrhosis (yr)	68.5 ± 10.3	56.1 ± 11.2	< 0.05	NA	NA
Age at last follow up (yr)	71 ± 10	60 ± 12	< 0.10	50 ± 12	53 ± 18.3
Diagnosis of viral hepatitis before primary biliary cirrhosis (no/yes)	0/8	2/7	NS	NA	NA
Interval between diagnosis of viral and primary biliary cirrhosis (mo)[3]	106 ± 90	106 ± 89	NS	NA	NA
Source of viral infection	6 unknown, 1 transfusion, 1 *i.v.* drug use	5 unknown, 2 transfusion, 2 vertical	NS	33 unknown, 22 drug abuse, 32 transfusion, 4 multiple hospitalizations, 3 multiple partners, 1 occupational exposure	9 unknown, 46 sexual, 9 transfusion, 103 vertical, 98 intrafamilial, 38 folk remedies
Active viral infection at diagnosis (no/yes)	0/8	5/4	NS	0/98	187/116
HCV genotype (13 patients with HCV infection)	1b in 3, 3 in 1, undefined in 4	NA	NA	1a/1b in 50, 2a/c in 6, 3a in 27, 4 in 9, 6 undefined	NA
Histology at initial diagnosis (no/yes)	3/5 (4 cirrhosis, 1 moderate fibrosis)	2/7 (1 cirrhosis, 1 pre-cirrhotic, 5 mild fibrosis)	NS	25/73 (18 cirrhosis, 53 mild/ moderate fibrosis, 2 severe fibrosis)	202/101 (23 cirrhosis, 78 mild or moderate fibrosis)
Clinically and/or histologically established cirrhosis at initial diagnosis (no/yes)	1/7	6/3	< 0.05	80/18	265/38
Development of cirrhosis during follow up in non-cirrhotic patients (no/yes/NA)	0/1/7	6/0/3	NA	78/2	260/5
Antiviral treatment (no/yes)	4/4	3/6 (2 pre-emptive due to immunosuppression)	NS	0/98	187/116
Type of antiviral treatment	Peg-IFNa plus ribavirin	Nucleos(t)ide analogues	NA	67 IFNa or Peg-IFNa plus ribavirin, 31 IFNa or Peg-IFNa monotherapy	50 IFNa or Peg-IFNa, 56 Nucleos(t)ide analogues
Response to antiviral treatment (no/yes/NA)	2/2/4	0/6/3	NA	39/59/0	45/71/0
Treatment of primary biliary cirrhosis (no/yes)	2/6	5/4	NS	NA	NA
Duration of therapy (mo)	35.8 ± 24.3	73 ± 68	NS	NA	NA
Type of therapy	UDCA	UDCA[1]	NA	NA	NA
Response to therapy at last follow up (no/yes/NA)	(2/4/2)	(2/2/5)	NA	NA	NA
Total follow-up (mo)	61 ± 37	57 ± 38	NS	51 ± 28	75 ± 23
Liver related death (no/yes)	6/2	8/1	NS	98/0	279/24

Results are expressed as mean ± SD, unless otherwise stated. [1]One of them received during the course of the disease also budenofalk and later combination of prednisolone with methotrexate due to concomitant CREST syndrome and cryoglobulinemia; [2]Means comparison between primary biliary cirrhosis (PBC) + hepatitis C virus (HCV) and PBC + hepatitis B virus (HBV) groups since comparisons between HCV with (n = 8) and without PBC (n = 98) as well as between HBV with (n = 9) and without PBC (n = 303) are not appropriate; [3]Interval between diagnosis of viral and primary biliary cirrhosis was estimated in 15/17 patients, in whom the diagnosis of viral preceded that of autoimmune liver disease (excluded are 2 patients with PBC and HBV infection). NA: Not applicable; NS: Not significant; IFN: interferon; UDCA: Ursodeoxycholic acid (dose: 15 mg/kg per day in two divided doses).

accepted guidelines for the management of HBV and HCV infections[1,2]. Response to UDCA treatment in PBC patients was considered according to the criteria reported by Parés *et al*[24].

Thirteen out of 17 patients (76.5%) consented for determination of the human leukocyte antigens (HLA) by polymerase chain reaction-sequence-specific oligonucleotides (PCR-SSO). All liver histology specimens were reviewed by an expert liver immunopathologist (GKK) who was unaware from the status of liver disease of the patients. All subjects consented to participate in the study at the time of the interview. The ethical committee of the Thessaly University, Medical School approved the study protocol.

Statistical analysis

Data are presented as mean ± SD or median (range) as appropriate. Data were analyzed by t test, the Mann Whitney U test, χ^2 test (two by two with Yates' correction), or Fisher's exact test, where appropriate using the SPSS 17.0 statistical program. Two sided $P < 0.05$ were considered statistically significant.

Table 2 Laboratory values at initial and last follow up in the individual groups of patients with viral hepatitis and primary biliary cirrhosis (mean ± SD)

Laboratory tests	PBC + HCV (n = 8)	PBC + HBV (n = 9)
Bilirubin initial (mg/dL)	1.0 ± 0.4	1.8 ± 1.7
Bilirubin last	4.3 ± 6.8	2.8 ± 5.8
Albumin initial(gr/dL)	3.8 ± 1.0	4.2 ± 0.7
Albumin last	3.5 ± 0.8	4.1 ± 0.7
PT initial (s)	14.5 ± 4.1	13.6 ± 1.8
PT last	14.8 ± 5.2	12.3 ± 1.4
ALT initial (IU/L)	88 ± 91	82 ± 131
ALT last	48 ± 37	27 ± 12
AST initial (IU/L)	74 ± 64	77 ± 98
AST last	53 ± 29	42 ± 38
γ GT initial (IU/L)	92 ± 139	76 ± 95
γ GT last	47 ± 49	29 ± 16
ALP initial (IU/L)	100 ± 29	106 ± 90
ALP last	93 ± 35	90 ± 33
IgG initial (mg/dL)	1764 ± 470	1815 ± 762
IgG last	2133 ± 612	1420 ± 392
IgM initial (mg/dL)	161 ± 69	281 ± 312
IgM last	264 ± 334	251 ± 186

PBC: Primary biliary cirrhosis; PT: Prothrombine time; ALT: Alanine amonitransferase; AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyltranspeptidase; ALP: Alkaline phosphatase; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

RESULTS

Demographic and clinical characteristics of patients

Eight patients had chronic HCV infection and PBC (follow-up: 61 ± 37 mo) and the remaining 9 had chronic HBV infection and PBC (follow-up: 57 ± 38 mo).

Viral infection had been established in 14 out of 17 patients before their first visit to our clinic. On the contrary, the diagnosis of PBC was established in all but two patients after attending our department. Laboratory data at first and at last follow up visit in our outpatient clinic are presented in Table 2.

The diagnosis of PBC was based on the presence of AMA and elevated cholestatic enzymes in all 17 patients (5 males/12 females), while 5 experienced severe pruritus many years before the diagnosis. A liver biopsy was performed in 12 of 17 patients and had PBC features in 3 of them. In terms of AMA positivity, 14 patients were AMA positive by IIF confirmed by immunoblot, while 3 patients being persistently AMA negative by IIF were found to be repeatedly anti-M2 positive tested by the enhanced performance MIT3-based ELISA and immunoblot[20,22]. Two out of 17 patients had also ANA with reactivity against multiple nuclear dots (MND), which is considered specific for PBC[12,25].

Patients with PBC and HBV infection (n = 9)

Age at first visit to our outpatient clinic was 55 ± 11 years. At the time of diagnosis of HBV infection, the disease was active in 4 patients (range of HBV-DNA: 11.689-773.694 IU/mL). In 7 patients (Table 1), HBV infection was diagnosed before their first visit to our clinic (median: 67 mo; range 1-168). In these 7 HBV patients, the diagnosis of PBC followed that of HBV (23.5 ± 41.5 mo after their first assessment in the clinic and 106 ± 89 mo after HBV diagnosis), while in the remaining 2 patients, HBV was diagnosed simultaneously in one and 72 mo after the diagnosis of PBC in the other case. Mean Mayo risk score at diagnosis of PBC was 5.7 ± 1.9, while at last follow-up 5.6 ± 2 (mean follow-up: 57 ± 38 mo). HLA typing was available in 7 patients (6 A24 positive, 4 B35 positive, 2 DR1*14 positive, 1 DRB1*0401 and 1 DRB1*0301).

Liver biopsy was performed in 4 patients close to the time of PBC diagnosis (mean time from PBC diagnosis: 1.8 mo), in 2 at the time of PBC diagnosis and in 1 between HBV and PBC diagnosis (biopsy was not done in 2 patients due to coagulation abnormalities; Table 1).

Antiviral treatment with nucleos(t)ide analogues received all patients with active HBV infection (n = 4) with virological response after 1 year of treatment (2 patients received initially lamivudine, while adefovir was added in 1 of them due to virological breakthrough during follow-up; 1 received adefovir and 1 tenofovir). Two out of the remaining 5 patients with inactive disease received also antiviral treatment as preemptive therapy due to chemotherapy and immunosuppression for breast cancer and CREST syndrome accompanied by cryoglobulinemia, respectively.

None of the 6 non-cirrhotic patients developed cirrhosis during the follow-up period. One patient died of liver related causes during the follow-up (decompensated cirrhosis with development of hepatocellular carcinoma).

Patients with PBC and HCV infection (n = 8)

Age at first visit to the clinic was 66 ± 11 years. HCV infection was active at the time of diagnosis of viral hepatitis in all patients (range of HCV-RNA: 82.894 to more than 850.000 IU/mL). The diagnosis of HCV infection had been established in all but one patient before their first visit to our clinic (median: 114 mo; range: 3-192). HCV genotype was identified in 4 patients (3 patients with 1b and 1 with genotype 3), while in the remaining 4 patients HCV-genotyping revealed undefined results (Table 1). The diagnosis of PBC followed that of HCV in all patients (30 ± 32.1 mo after their first assessment in the clinic and 106 ± 90 mo after HCV diagnosis). Mean Mayo risk score at the time of PBC diagnosis was 6 ± 1.2, while at last follow-up 8.1 ± 3 (mean follow-up: 61 ± 37 mo). HLA typing was available in 6 patients (3 A2 positive, 4 B35 positive, 2 DRB1*14 positive and 2 DRB1*13 positive).

Liver biopsy was performed in 2 patients at the time of HCV diagnosis and in 3 after the diagnosis of PBC (mean time from PBC diagnosis 3 mo). However, at the time of the first visit to our clinic, all but one patient had clinically and/or histologically evident cirrhosis (Table 1).

During the follow-up period 4 patients (including 1 with multiple treatment failures in the past) received 48 wk

regiment with Peg-IFN-α and ribavirin with sustained virological response in 2 of them. Reasons for not treating the 4 remaining patients were age older than > 65 years at diagnosis of HCV infection in 3 and psychiatric history in 1 patient.

The only one patient being non-cirrhotic at initial assessment developed histologically proven cirrhosis 168 mo later. Two out of 8 patients died of liver related causes 27 and 57 mo of the follow-up, including 1 patient, who developed hepatocellular carcinoma.

Comparison between groups

Patients with PBC and HBV were younger at first visit in our clinic compared to patients with PBC and HCV (55 ± 11 years *vs* 66 ± 11 years; $P = 0.07$). Patients with PBC and HBV were significantly younger at diagnosis of PBC than patients with PBC and HCV ($P < 0.05$) (Table 1). At first assessment 7 of 8 patients (87.5%) with PBC and HCV and 3 of 9 patients (33.3%) with PBC and HBV had clinical evidence of cirrhosis ($P < 0.05$). There were no significant differences concerning the interval between diagnosis of viral hepatitis and PBC and also the total follow-up of the patients.

DISCUSSION

The present study describes the characteristics of 17 patients with concurrent diagnosis of HBV or HCV infections and PBC alongside with clues to diagnosis, patterns of disease progression, management and outcome. There are several points to be stressed. In the majority of patients the diagnosis of viral hepatitis preceded that of PBC by many years. Several scenarios can be suggested. Autoimmune liver diseases can remain silent for variable period of time before developing symptoms[7,8,12,19,26]. Of relevance, viral infections, xenobiotics, drugs and microbes can operate as triggers for the development of liver autoimmunity in genetically predisposed individuals[7-9,11,12,14,19,25,27,28].

In all but two patients with chronic HBV and HCV infections described in our study PBC was diagnosed during the follow-up period for viral hepatitis. The diagnosis of PBC was based in all patients on the presence of elevated cholestatic enzymes and AMA identified either by IIF and immunoblot or by a sensitive MIT3-based ELISA (IgG and IgA) and immunoblot, in those being AMA negative by IIF[12,19,20,22]. In addition, ANA specific for PBC, identified in two of these patients, could serve not only as diagnostic tools for PBC but also as prognostic markers of the disease[12,25,29]. The detailed work-up for PBC was performed mostly due to persistently elevated cholestatic enzymes during the follow-up period, while intractable pruritus, which is considered a prominent feature of PBC was also evident in almost one third of patients.

These data suggest that existence mostly of biochemical indices of cholestasis should prompt physicians to sick for AMA with various sensitive techniques as well as for PBC-specific ANA irrespective of the presence or not of other liver diseases[12,20,22,25,29]. Since the discovery of AMA, their subsequent inclusion in "routine" autoantibody testing, has led to PBC being more frequently diagnosed at an earlier stage of disease progression being mainly asymptomatic[7,12,19,20,25]. Indeed, up to 60% of PBC patients are reported to be asymptomatic at diagnosis with less severe disease in terms of liver biochemistry and histology compared to symptomatic patients[7,12,19,20,22,26,30].

In accordance with this, the repetitive presence of AMA in our patients, who were mostly asymptomatic, may well signify that PBC was in its early stages. Though, follow-up studies have demonstrated progression of disease in the majority with reduced morbidity and mortality[31]. In cases were both cholestasis and pruritus existed long beforehand, the diagnosis of PBC and initiation of UDCA in early stages of the disease could have resulted in significant benefit[19,24]. Of note, in patients with viral hepatitis in whom a liver biopsy was available, histological features compatible or typical for PBC were present in 11% of them. This highlights again the importance of careful evaluation of liver biopsy, which in conjunction with laboratory and clinical parameters can help to identify other chronic liver diseases with the potential to independently cause significant morbidity and mortality and/or alter the natural history of the "original" disease. Of relevance, the review of biopsies from 1842 patients with HBV and/or HCV infection from Canada revealed features of other diseases in 20.5% of them, including 3 cases with PBC[32].

The identification of AMA in patients with HCV infection was recently shown to be more frequent than previously thought, reaching up to 8% of the HCV population in a multicenter study from Spain and South America[33]. Additionally, in an Italian study, 8% of 170 patients with PBC were found to have HCV infection[34]. In these studies coexistence of both diseases might have accelerated the development of cirrhosis and/or neoplasia[33,34]. In accordance to this observation, patients with PBC and HCV infection were characterized by significantly higher frequency of cirrhosis compared to our patients with HBV and concomitant PBC. In the whole cohort of patients reported here more than half were cirrhotic at first visit to the clinic, while 17% of them died during a 4 year follow-up period. The big proportion of cirrhosis in this cohort could be potentially ascribed to the presence of two chronic liver diseases, their duration being indeterminate in the majority of them. The fact that diagnosis of PBC was established with delay in the majority of these patients depriving them from beneficial treatment regimens might have also contributed to the progression of liver disease in general. On the other hand however, the potential negative impact of HBV and HCV on the clinical course of PBC cannot be excluded though the number of our case series is adequate for a relatively rare disease like PBC.

In conclusion, our data indicates that chronic viral infections concomitant with PBC are often very difficult to recognize given the heterogeneity of liver diseases,

the absence of awareness of this possibility and the shortfall of many centers outside reference centers to use reliable test for the detection of AMA and/or PBC-specific ANA and enough expertise, including collaboration with histopathologists, for the interpretation of the laboratory results. In other words, the existence mostly of biochemical indices of cholestasis should prompt physicians to sick for AMA and PBC-specific ANA with various sensitive techniques irrespective of the presence of other liver diseases in order to achieve a prompt and rapid diagnosis of concurrent PBC. In addition, we showed that the outcome of patients with viral hepatitis seems to be affected negatively by the concurrent PBC. Although the number of patients in this case study is relatively small, the patients with HBV and concomitant PBC seem to have better outcome compared to those with HCV and PBC probably because of the use of nucleos(t)ide analogues which contrary to IFNs-based treatments in HCV, can control HBV replication with no adjacent effect, related to exacerbation of autoimmune phenomena. The latter finding is also in accordance with our recent report on the better outcome of patients with HBV infection and AIH compared to HCV patients with concurrent AIH[14]. Taken together our findings further support the need of close follow-up of at least HCV patients with concomitant autoimmune liver diseases.

COMMENTS

Background

Coexistence, of autoimmune liver diseases like primary biliary cirrhosis (PBC) with hepatitis B virus (HBV) or hepatitis C virus (HCV) infections could be a problem especially in endemic areas for viral hepatitis, since viruses in general have long been associated in some cases with the induction of autoimmune phenomena up to the development of overt autoimmune diseases. Therefore, the discrimination between liver autoimmunity and viral liver disease with autoimmune features is mandatory, since these two conditions involve different disease strategies.

Research frontiers

The characteristics, patterns of disease progression, management and outcome of these patients with HBV and HCV infections concurrent with PBC need to be defined since currently there are scarce data to demonstrate the interaction between PBC and viral hepatitis.

Innovations and breakthroughs

This is one of the larger series of patients with HBV or HCV infections concomitant with PBC (n = 17) ever published. According to the authors' records almost 1% of our HBV and HCV cohort had also PBC which had been misdiagnosed or underestimated for many years. This delay could explain the unfavorable outcome observed in the authors' patients and in particular, among HCV/PBC patients.

Applications

Chronic viral infections concomitant with PBC are often very difficult to recognize given the heterogeneity of liver diseases, the absence of awareness of this possibility and the shortfall of many centers outside reference centers to use reliable test for the detection of antimitochondrial antibody (AMA) and/or PBC-specific ANA and enough expertise, including collaboration with histopathologists, for the interpretation of the laboratory results. Therefore, existence mostly of biochemical indices of cholestasis should prompt physicians to sick for AMA and PBC-specific ANA with various sensitive techniques irrespective of the presence of other liver diseases in order to achieve a prompt and rapid diagnosis of concurrent PBC.

Terminology

Concurrent PBC with HBV and HCV is defined as the presence of cholestasis accompanied by AMA or PBC-specific ANA detection with or without liver biopsy compatible of PBC lesions in patients with well-established chronic HBV or HCV infections.

Peer review

This is an interesting retrospective analysis of 17 patients with PBC who had in addition infections by HBV or HCV. The study cohort was derived from 1493 patients with HBV infections and 526 patients with HCV infections. The details are well described, the conclusion asks for better evaluations of patients with HBV or HCV infections regarding concomitant PBC.

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BRIEF ARTICLE

Risk factors for liver fibrosis among human immunodeficiency virus monoinfected patients using the FIB4 index in Morocco

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Abstract

AIM: To study the prevalence and risk factors of significant hepatic fibrosis in Moroccan human immunodeficiency virus (HIV) monoinfected patients.

METHODS: We conducted a cross-sectional study among HIV monoinfected patients (negative for hepatitis B surface antigen and hepatitis C antibody). Clinical and laboratory data were collected from the data base of the Infectious Diseases Unit in Ibn Rochd Hospital Center [age, gender, duration of HIV infection, CD4 T lymphocyte count, HIV viral load, glycemia and current or prior use of antiretroviral and antiretroviral therapy (ART) duration]. The primary outcome was a FIB4 score > 1.45. Multivariable logistic regression identified independent risk factors for FIB4 > 1.45.

RESULTS: A FIB4 score > 1.45 was identified in 96 among 619 (15.5%). HIV monoinfected patients followed up between September 1990 and September 2012. Multivariate analysis showed that only a viral load > 75 (OR = 2.23, 95%CI: 1.36-3.67), CD4 > 200 cells/mm^3 (OR = 0.39, 95%CI: 0.21-0.72) and age at FIB4 index calculation (OR = 1.10, 95%CI: 1.07-1.13) were independently associated with the occurrence of FIB4 index (> 1.45). Gender, duration of HIV infection, glycemia, use of antiretroviral therapy and ART duration were not associated with significant fibrosis by FIB4.

CONCLUSION: FIB4 score > 1.45 was found in 15.5% of Moroccan HIV monoinfected patients. Age, HIV viremia > 75 copies/mL and CD4 count > 200 cells/mm^3 are associated with liver fibrosis. Further studies are needed to explore mechanisms for fibrosis in HIV monoinfected patients.

Key words: Human immunodeficiency virus; FIB4; Liver; Monoinfected; Risk factors

Core tip: We evaluated, for the first time in Morocco, the prevalence and risk factors of significant hepatic fibrosis in Moroccan human immunodeficiency virus (HIV) monoinfected patients using the FIB4 score which represents a noninvasive, composite index that is a validated measure of hepatic fibrosis. FIB4 score > 1.45 was found in 15.5% of Moroccan HIV monoinfected patients. Age, HIV viremia > 75 copies/mL and CD4 count < 200 cells/mm^3 are associated with liver fibrosis. Gender, duration of HIV infection, glycemia, use of antiretroviral therapy and antiretroviral therapy duration were

not associated with significant fibrosis.

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INTRODUCTION

About 33 million people are infected with human immunodeficiency virus (HIV). Most of them live in low and middle income countries[1]. The liver is a major target of HIV infection and a wide spectrum of liver disease can be seen in patients with HIV infection, ranging from steatosis, steato-hepatitis, non cirrhotic portal hypertension and hepatocellular carcinoma[2]. Recent data shows that HIV *per se* (without viral hepatitis infection) can induce liver fibrosis but few studies[3-5] have identified risk factors involved in liver fibrosis among HIV monoinfected patients.

Liver biopsy is the gold standard technique for diagnosis of liver fibrosis but it is an invasive procedure that can induce non-negligible morbidity and mortality. Thus, liver biopsy is not an acceptable technique for monitoring progression liver fibrosis and for pursuing appropriate epidemiological analysis. Many alternative biochemical markers have been introduced to avoid liver biopsy [fibrotest, actitest, aspartate aminotransferase (AST) to platelet ratio index (APRI) and FIB4]. FIB4 was previously validated as a fibrosis marker in HIV/HVC co-infection[6] and has recently been used to determine advanced fibrosis in HIV monoinfected patients[7].

To date, there is no data in Morocco regarding liver fibrosis in HIV monoinfected patients. To identify risk factors for liver fibrosis in Moroccan HIV monoinfected patients, we examined hypothesized risk factors: CD4 counts[7], hypoglycemia[8,9], antiretroviral therapy (ART) use and HIV viremia[7,10].

MATERIALS AND METHODS

Study design and patients

We conducted a cross sectional study of patients followed in IBN ROCHD University Hospital center. We used the database "NADIS" that was launched in 2005 to describe the demographic, clinical and laboratory characteristics of Moroccan HIV infected patients. All laboratory confirmed HIV patients and Hepatitis B virus surface antigen (HBsAg) negative/anti HVC negative were enrolled in the study.

Study outcome

The major study outcome was significant fibrosis, as determined by FIB4 index > 1.45. FIB4 score was calculated during the last consultation using the following formula: [age (year) ×AST (U/L)]/[platelet (10^9/L) × alanine aminotransferase (ALT) (U/L)1/2]. All participants provided informed consent prior to sample and clinical data collection. HIV RNA levels were qualified using second or third generation Quantiplex branched DNA assays (Chiran). HBsAg was tested with Austria-IL-125 RIA (ABBOTT laboratory). Hepatitis C virus (HCV) status was obtained through HCV enzyme immunoassay 2.0.

Data collection

All data were collected from "NADIS" IBN ROCHD University Center Hospital and included: age, gender, duration of HIV diagnosis, presence of diabetes mellitus (glucose level greater than 126 mg/dL or self reported), CD4 count during the last visit, HIV viral load at first and last visit, AST, ALT and platelet at the last visit, use of ART (receipt of three antiretroviral drugs from at least two different drug classes), ART duration and prior of current use of Zidovudine, Lamivudine, Efavirenz, Nevirapine, Lopinavir, Ritonavir, Emtricitabine, Stavudine, Tenofovir and Didanosine. FIB4 index was used as a categorical variable with two levels < 1.45 and > 1.45 corresponding to the recommended cutoff used to exclude the presence of advanced fibrosis[6].

Statistical analysis

Statistical analysis was performed using SPSS Base 20 statistical software (SPSS, Inc., Chicago, IL). We included only patients in whom in the last visit AST, ALT, RNA HIV level, CD4 level and platelet count were available. Duration of HIV infection was calculated as the difference between the first and the last visit. The results were expressed as mean ± SD. For categorical data, χ^2 test were used to assess differences between patients with and without significant fibrosis by FIB4 score (FIB4 > 1.45). For continuous data, *t* test for continuous variables was used. A two tailed *P* value of less than 0.05 was taken to indicate statistical significance. Univariate analysis was performed to study characteristics associated with FIB4 > 1.45. Variables with *P* values < 0.1 in univariate analysis were used in the multivariate regression analysis with backward variable selection.

RESULTS

Characteristics of the study population

Six hundred and nineteen patients were studied. Three hundred and fifty-four patients were female, with a mean age of 39.85 ± 9.56 years. Five hundred and twenty-three patients had a FIB4 < 1.45. 87 had a FIB4 between 1.45 and 3.25 and only 9 patients (1.45%) had a FIB4 > 3.25. Mean HIV diagnosis was 4.71 ± 3.91. Mean antiretroviral duration was 4.45 ± 3.77 years. Thirty-six patients (5.8%) had diabetes mellitus. Five hundred and seventy were under antiretroviral therapy (92.1%).

At the time of analysis, mean AST was 24.66 ± 20.84

Table 1 Baseline subject characteristics, overall and by significant fibrosis as determined by FIB4 score

	All patients (n = 619)	Patients with FIB4 > 1.45 (n = 96)	Patients with FIB4 < 1.45 (n = 523)	P value
Male gender	42.80%	51.04%	41.30%	0.920
AGE	39.8 ± 9.5	46.5 ± 8.9	38.6 ± 9.1	< 0.001
Duration of HIV infection	4.7 ± 3.9	4.2 ± 4.2	4.7 ± 3.8	0.250
ART TTT	92.08%	90.62%	92.35%	0.540
ART duration	4.4 ± 3.7	4.5 ± 4.12	4.5 ± 3.7	0.340
AST	24.6 ± 20.8	39.6 ± 46.6	21.9 ± 8.3	0.000
ALT	21.2 ± 16.1	25.6 ± 24.9	20.7 ± 13.7	0.670
PQ	256.9 ± 33.8	170.5 ± 61.5	272.7 ± 338.0	0.003
CV	76060 ± 39663	260460 ± 765929	42212 ± 268494	0.007
CD4	602.2 ± 367.6	403.16 ± 266.5	638 ± 372	0.001
CD4 < 200	12.40%	23.95%	10.32%	0.001
CV < 75	57.20%	41.66%	60.03%	0.001
Diabetes	5.80%	8.33%	5.35%	0.240
Zidovudine	87.20%	80.20%	88.52%	0.030
Lamivudine	89.00%	85.41%	89.67%	0.210
Efavirenz	79.30%	77.08%	79.73%	0.580
Nevirapine	11.10%	10.41%	11.28%	1.000
Lopinavir	23.30%	22.91%	23.32%	1.000
Ritonavir	29.70%	30.20%	29.63%	0.900
Emtricitabine	8.10%	11.45%	7.45%	0.210
Stavudine	23.40%	29.16%	22.37%	0.150
Tenofovir	8.90%	12.50%	8.22%	0.170
Didanosine	5.30%	6.25%	5.16%	0.620

HIV: Human immunodeficiency virus; ART: Antiretroviral therapy; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

and mean ALT 21.52 ± 16.10. Mean platelet count was 256.96 ± 313.80. Mean CD4 count was 602.24 ± 367.65. Mean viral load was 76060.4 ± 396639 and 12.4% (77 patients) had a CD4 count below 200 cells/mm^3, 57.62% (354 patients) had viral load below 75 copies/mL.

In univariate analysis, only CD4 count (P = 0.001), viral load > 75 copies/mL (P = 0.007), age (P = 0.000) and prior or current Zidovudine intake (P = 0.03) were associated with a FIB4 over 1.45. In contrast, gender, ART therapy, ART duration, diabetes and prior or current intake of Lamivudine, Efavirenz, Nevirapine, Lopinavir, Ritonavir, Emtricitabine, Stavudine, Tenofovir, Didanosine, Abacavir, Raltegravir, Indinavir and Nelfinavir were not associated with a FIB4 over 1.45.

Multivariate analysis showed that only a viral load > 75 (OR = 2.23, 95%CI: 1.36-3.67), CD4 > 200 cells/mm^3 (OR = 0.39, 95%CI: 0.21-0.72) and age at FIB4 index calculation (OR = 1.10, 95%CI: 1.07-1.13) were independently associated with occurrence of a high FIB4 index (> 1.45).

DISCUSSION

Since antiretroviral therapy has been widely used, liver diseases have become a major cause of morbidity among HIV patients. The presence of viral co-infection B and C are a common cause of liver disease[11-16]. Moreover, recent data suggest a direct role of HIV virus in hepatic fibrosis[3-5]. Studies carried out in HIV monoinfected patients found a mild to moderate increase in liver enzymes[6-17].

Indirect data indicate a possible direct role of HIV virus in liver damage and fibrosis. In HIV-HVC co-infected people, slower fibrosis progression is associated with HIV suppression[18] and liver related mortality is less in patients under ART[19,20]. Thus, in monoinfected HIV patients, without hepatitis B or C co-infection, elevation of AST and ALT is associated with a higher HIV RNA level[21-23].

HIV can induce liver fibrosis through interacting with many kinds of liver cells; Kupffer cells can be stimulated by lipopolysaccharide (LPS) due to increased permeability induced by HIV infection. Once stimulated, Kupffer cells produce pro-inflammatory cytokines, such as tumor necrosis factor-α, transforming growth factor β, interleukin (IL)-6 and IL-12[24]. These cytokines are responsible for chemotaxis of monocytes and T cells to the liver[25]. HIV can also stimulate stellate cells (HSC) though CxCR4 receptors[26]. Activated HSC showed increased fibrogenesis and increased collagen production and alpha smooth muscle[27] in patients with HIV-HVC co-infection.

The first interesting result of our study is the prevalence of advanced fibrosis (FIB4 > 3.25) estimated as 1.45% among Moroccan monoinfected patients. This finding disagrees with previously published studies; using the APRI index in 1845 HIV monoinfected patients, Sulkowski *et al*[5] found 7% significant fibrosis, as defined by APRI > 1.5[5].

Another study, carried out in 432 monoinfected HIV patients, identified significant fibrosis by APRI in 8.3% patients[3]. Moreover, in a recent study, 1.3% of monoinfected HIV women had a FIB4 > 3.25[7]. Interestingly, only 1% of HIV monoinfected had significant fibrosis measured by elastometry[4]. APRI had platelet count in the denominator, which accounts for the relatively higher frequency of liver fibrosis by APRI compared to elastometry which can be explained by the frequency of thrombocytopenia commonly found among HIV patients[28].

Our study found that HIV viremia was a risk factor for significant liver fibrosis among HIV monoinfected patients. Previous studies identified HIV RNA as an independent factor of elevated AST and ALT in HIV monoinfected patients[6,17].

HIV viremia was also associated with a high FIB4 index score in HIV-HVC co-infected patients[18]. Our results are in concordance with previously published studies that used non invasive methods to assess liver fibrosis. Using the APRI index, Piazza found that detectable HIV viremia is a risk factor for liver fibrosis in HIV monoinfected patients[10]. Another recent study found that a 1 log[10] increase in HIV RNA was associated with a median increase in FIB4 of 12%[29].

Another risk factor for significant hepatic fibrosis in our sample was CD4 counts. This result is also in accordance with those of Blackard who reported that CD4 T cell counts are negatively associated with FIB4. Some studies reported that AST/ALT was negatively associated with CD4 T cell counts. Other studies did not find CD4

T cell counts as an independent factor for liver fibrosis[10].

The last risk factor for significant hepatic fibrosis in our study was age at FIB4 index calculation. This result disagrees with studies previously published in HIV monoinfected patients[7,10,29]. This is in concordance with a study carried out by Blanco who found that old age is associated with liver fibrosis by elastometry. Also, age $\geqslant$ 40 was a high factor for liver fibrosis progression in HIV[30]. In HIV-HVC co-infected patients, older age was found to be an independent factor for advanced liver fibrosis measured by elastometry[31].

Another interesting result of our study is that ART use and its duration were not independently associated with liver fibrosis. This result agrees with previously published studies[3,10,29] suggesting that liver fibrosis is not increased by long term use of highly active antiretroviral therapy in HIV monoinfected patients.

Our study has many advantages. Firstly, it is the first study carried out in Moroccan people and the design of the study is distinctive. Secondly, most of the included patients in this study received ART and the prior and current use of the most commonly prescribed HIV drug was included in the univariate analysis. Furthermore, in our sample, the impact of ART duration on liver fibrosis was also assessed, whereas other studies included mostly ART naïve patients or those receiving less than an optimally suppressive ART regime[7,32]. On the other hand, our study has several limitations. Firstly, the cohort was recruited from a university hospital and all patients were in regular HIV care. Secondly, liver fibrosis evaluation was based on the FIB4 index that is not widely assessed in HIV monoinfected patients; FIB4 has been validated as a liver fibrosis index in HVC and HIV-HVC co-infected patients[6,33]. It did not exclude some patients that were misclassified by FIB4. All conditions, decreasing platelet count (idiopathic thrombocytopenia) or increasing ALT or AST (drug hepatotoxicity) can lead to an imprecise FIB4 index. Thirdly, other confounding factors were not studied, such as alcohol use and other hepatotoxic drugs (*e.g.*, antibiotics, acetaminophen). Thus, our study is unable to specify the independent effect of these factors on liver fibrosis.

Our study found that 15.5% of Moroccan HIV monoinfected patients had a FIB4 > 1.45. HIV RNA load, CD4 count and age were independently associated with liver fibrosis. Longitudinal studies are required to examine the exact role of the HIV virus on liver fibrosis progression in monoinfected patients.

COMMENTS

Background

The liver is a major target of human immunodeficiency virus (HIV) infection and a wide spectrum of liver disease can be seen in patients with HIV infection, ranging from steatosis, steato-hepatitis, non cirrhotic portal hypertension and hepatocellular carcinoma. Recent data shows that HIV *per se* (without viral hepatitis infection) can induce liver fibrosis.

Research frontiers

The aim of this study is to identify risk factors involved in liver fibrosis among HIV monoinfected patients in Morocco using the FIB4 index, including the use of antiretroviral therapy.

Innovations and breakthroughs

This study is the first one carried out in Moroccan people. Moreover, most of the included patients in this study received antiretroviral therapy and the prior and current use of the most commonly prescribed HIV drug was included in the univariate analysis.

Applications

The prevalence of advanced fibrosis (FIB4 > 3.25) is estimated to 1.45% among Moroccan HIV monoinfected patients. Thus, advanced liver fibrosis is a rare situation in HIV monoinfected patients in Morocco.

Peer review

A very interesting study with relevant findings in the noninvasive prediction of fibrosis field for monoinfected HIV patients.

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CASE REPORT

A fascinating presentation of hepatic hydrothorax

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Abstract

We report this case of a 43-year-old woman with hepatitis-C cirrhosis who presented with a large right sided pleural effusion complicated by hypoxic respiratory failure and altered mentation necessitating dependence on mechanical ventilation. The pleural effusion spontaneously resolved upon initiation of mechanical positive pressure ventilation and recurred almost immediately after weaning the patient off the ventilator. The pre-ventilation, ventilation and post-ventilation chest X-ray films in chronological order present a striking visual demonstration of fluid dynamics and pathophysiology of hepatic hydrothorax, thereby obviating the need for a dedicated diagnostic test. We also report this case to highlight the treatment strategies for this often intractable complication.

Key words: Hepatic hydrothorax; Hydrothorax; Cirrhotic pleural effusions; Refractory pleural effusions; Pleural effusions in cirrhosis

Core tip: We aim to publish this fascinating presentation of a case of hepatic hydrothorax that clearly provides an insight into pathophysiology of its development. We also presented its natural course and management. Such awareness will prompt institution of timely therapy and prevent long term sequelae of this potentially fatal condition.

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INTRODUCTION

Hepatic hydrothorax is an uncommon manifestation of portal hypertension from cirrhosis which results in extracellular fluid accumulation. Hepatic hydrothorax is defined as a transudative pleural effusion in cirrhotic patients without underlying pulmonary or cardiac disease and is often > 500 mL in volume[1,2]. It is seen in approximately 5%-12% of all patients with cirrhosis[1,3,4]. Accumulation of fluid within the pleural cavity has greater clinical ramifications when compared to ascites, as even lesser volumes (1-2 L) can cause significant dyspnea and hypoxia. Therefore, prompt drainage of pleural fluid along with implementation of salt restriction and initiation of diuresis is often required.

CASE REPORT

A 43-year-old Hispanic woman who was born and raised in the United States, presented to the hospital with acutely altered mental status. The patient was known to have liver cirrhosis (Child-Pugh Class: C) from Hepatitis-C infection. A provisional diagnosis of hepatic encephalopathy was made and the patient was admitted to the critical care unit. Physical examination at the time of admission revealed a tachypneic and hypoxic disoriented woman. Other pertinent positive findings of physical examination included icteric sclera, decreased breath sounds over the right lung fields and bilateral pitting type of pedal edema. The patient was intubated and placed on mechanical ventilation. A chest X-ray taken immediately after intubation

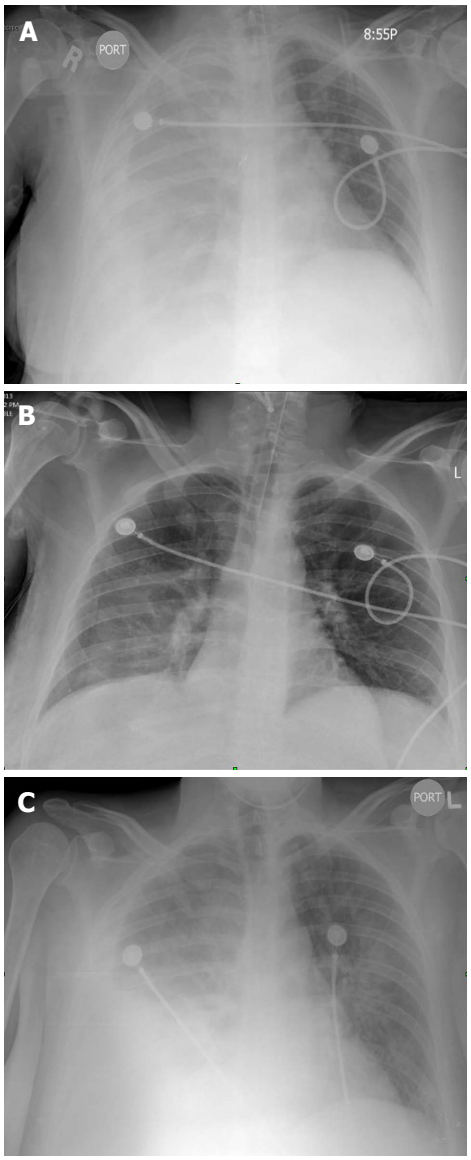

Figure 1 Pre-ventilation, ventilation and post-ventilation chest X-ray films in chronological order. A: Chest X-ray taken immediately after intubations showing complete opacification of right hemithorax from a massive pleural effusion; B: Chest X-ray taken a day after the intubation showing near-complete resolution of the right sided pleural effusion; C: Chest X-ray taken after extubation showing recurrence of the right sided pleural effusion.

showed complete opacification of right hemithorax from a massive pleural effusion (Figure 1A).

Initial set of laboratory studies showed normocytic, normochromic anemia (Hemoglobin of 9.6 g/dL), severe thrombocytopenia (39 K/μL) , abnormal coagulation parameters (PT of 20.6 s and aPTT of 62.2 s) , abnormal liver enzymes (alanine transaminase of 41 IU/L, aspartate transaminase of 98 IU/L, elevated alkaline phosphatase of 193 IU/L) with jaundice (elevated total and direct bilirubin levels of 3.8 and 2.8 mg/dL respectively), profound serum hypoalbuminemia (2.0 g/dL). Serum ammonia level was elevated at 245 μmol/L. Echocardiogram revealed concentric left ventricular hypertrophy with normal ejection fraction of 83%. Thoracentesis was performed, with pleural fluid revealing a total protein of 1.0 gm/dL and a Serum-Pleural fluid Albumin Gradient of > 1.1, alluding to its transudative properties.

Hospital course of the patient was marked by treatment for hepatic encephalopathy with Lactulose and Rifaximin; work-up for a possible underlying precipitating factor. Septic work up including blood and urine cultures were non-contributory. The patient was not started on intravenous diuretics, in-spite of hypervolemia, in view of progressively worsening renal function (serum creatinine had trended up from 1.1 to 1.9 mg/dL) within 24 h of admission. Patient was initiated on intravenous Albumin infusions at a dose of 1 gm/kg. body weight/d. Renal function improved with these colloid infusions thus making Hepatorenal Syndrome unlikely and pre-renal azotemia secondary to third-spacing, more likely. Chest X-ray taken a day after the intubation revealed near-complete resolution of the right sided pleural effusion (Figure 1B). Patient was continued on mechanical ventilation for another day before being extubated upon improvement in her respiratory status. The patient was noted to have progressive improvement in her mentation and stable respiratory function over the next few days. On day-6 of hospitalization, patient was again found to be disoriented and hypoxic with a chest X-ray showing recurrence of the right sided pleural effusion (Figure 1C). The patient was reintubated and placed on mechanical ventilation.

Patient was considered for a tracheostomy in view of prolonged and recurrent intubation. However she could not undergo the procedure in view of her unstable medical condition, severe thrombocytopenia and progressively worsening coagulopathy. Patient was subsequently transferred to hospice care unit for palliative extubation.

DISCUSSION

This patient presented with multiple symptoms that are prototypical of hepatic hydrothorax secondary to decompensated liver cirrhosis, including dyspnea, hypoxia, laterality of pleural effusion (right sided in up to 65%-87% of cases)[4], transudative nature of pleural effusion and intractable nature of the pleural effusion. Even as hepatic hydrothorax is extensively reported in literature and could be found in almost 5%-10% of all patients with cirrhosis, the peculiar and strikingly labile presentation of hepatic hydrothorax in our patient, is noteworthy.

Several mechanisms have been postulated to explain the development of hepatic hydrothorax including decreased colloid osmotic pressure from hypoalbuminemia[5], increased azygos system pressure leading to leakage of plasma into the pleural space[5,6] and transdiaphragmatic migration of peritoneal fluid into the pleural space *via* lymphatic channels[7]. However, the most widely accepted theory is of the leakage of ascitic fluid *via* diaphragmatic defects[8,9]. The development of hepatic hydrothorax parallels other forms of fluid accumulation in cirrhosis. The

basic pathophysiology of accumulation of fluid within the third space in cirrhosis, involves development of portal hypertension[10]; splanchnic vasodilation with subsequent activation of RAAS (renin-angiotensin-aldosterone system), activation of sympathetic nervous system and augmentation of Vasopressin release[11-13].

The dynamics of third space fluid are governed by the pressures of the cavities in which it exists. The negative intrathoracic pressure during inspiration aids in migration of fluid from peritoneal to the pleural space[3]. If the volume of this fluid exceeds the absorptive capacity of the pleural membranes, hepatic hydrothorax ensues. Our patient clearly exhibited this remarkable physiology of fluid dynamics while on and off mechanical ventilation. Patient had severe intrinsic liver disease resulting in formation of ascites and subsequently hepatic hydrothorax by mechanisms aforementioned. Mechanical ventilation imparted positive pressure that is transmitted to the intrapleural space. This positive pressure pushed the fluid back into the now-less pressurized peritoneal cavity, explaining the complete resolution of the right sided pleural effusion, post-intubation. After weaning off the positive pressure ventilation, the negative intrapleural pressure was quickly reestablished to aid patient inspire. This collaterally led to transmigration of fluid from ascitic cavity into the pleural space, thereby leading to reestablishment of hepatic hydrothorax.

The corner stone of management of hepatic hydrothorax is evaluation for liver transplantation[14]. However, in patients such as ours, who are acutely symptomatic and are not candidates for liver transplantation, the goal is to provide symptomatic relief. These palliative measures include large volume therapeutic thoracentesis[15] without the placement of an indwelling chest tube[16,17], salt restriction and diuresis[1,3]. Patients with hydrothorax refractory to these conservative measures might benefit from TIPS (Transjugular Intrahepatic Portosystemic Shunt) placement. However the success rate of TIPS is not encouraging in patients with advanced liver disease (Child Pugh Class C) such as seen in our patient[18]. The patient was not an ideal candidate for TIPS placement in view of her hepatic encephalopathy as well.

This case was unique, as it clearly demonstrates the fluid movements mirroring the pressure gradients between two different third space compartments, without the use of nuclear isotope studies involving the intraperitoneal administration of 99mTc-sulphur colloid or 99mTc-human serum albumin. This case highlights the challenges posed by refractory hepatic hydrothorax and often poor outcomes in presence of underlying advanced liver disease.

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CASE REPORT

Severe cholestasis due to adalimumab in a Crohn's disease patient

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Abstract

Elevation of liver biochemistry has been reported with anti-tumor necrosis factor agents, but overt liver failure rarely reported. Autoimmune hepatitis has been more commonly reported with infliximab than adalimumab (ADA). Our case, however, describes the first reported case of ADA-associated severe cholestatic injury. A 39-year-old female with Crohn's disease developed severe jaundice after initiation of ADA. All serologic tests and imaging studies were normal. Liver biopsy showed prominent pericentral canalicular cholestasis, without features of steatosis or sclerosing cholangitis, consistent with drug-induced cholestasis. The serum total bilirubin peaked at 280 μmol/L, and improvement was seen after 5 wk with eventual normalization of liver enzymes at 10 wk. Our case describes the first reported case of ADA-associated severe cholestatic liver disease and the first histopathologic examination of this adverse drug effect. Clinicians need to be aware of this potential drug-induced liver injury when prescribing this commonly used biologic medication.

Key words: Crohn's disease; Cholestasis; Adalimumab; Anti-tumor necrosis factor agents; Drug-induced liver injury

Core tip: Anti-tumor necrosis factor agents are commonly used in the treatment of inflammatory bowel disease and other inflammatory conditions. Drug-induced injury to the liver induced by the agents includes autoimmune hepatitis, direct hepatocellular necrosis and cholestasis. Our patient, a 39-year-old female with Crohn's disease, developed severe jaundice after initiation of adalimumab. We present the first report of adalimumab-associated severe cholestatic injury and the first histopathologic examination of this adverse drug effect. Clinicians need to be aware of this potential severe drug-induced liver injury when prescribing this commonly used biologic medication.

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INTRODUCTION

This case represents the first reported case of adalimumab-induced severe cholestatic liver disease, occurring in a patient treated for fistulizing Crohn's disease (CD). All other causes for liver injury were ruled out with extensive investigations, with supporting evidence from a liver biopsy. Liver enzymes abnormalities normalized within 10 wk after drug cessation, with no recurrence. Anti-tumor necrosis factor (TNF) agents have been re-

ported to cause a variety of liver-related abnormalities, but our case describes the first report of adalimumab (ADA)-associated severe cholestatic injury and the first histopathologic examination of this adverse drug effect. Clinicians need to be aware of this potential severe drug-induced liver injury when prescribing this commonly used biologic medication.

CASE REPORT

A 39-year-old female of East Indian descent with a history of CD, was treated with azathioprine (2 mg/kg) due to persistence of draining perianal fistulas and recurrent abscesses. Azathioprine led to marked improvement in her perianal fistulizing disease, without medication side effects or elevation of liver enzymes for over three years. Adalimumab (Humira, Abbott Laboratories Canada, St. Laurent, QC) was started, due to worsening perianal disease. She developed hives and arthralgias after with each injection of adalimumab, treated with antihistamines and Tylenol. Routine bloodwork [including liver biochemistry, international normalized ratio (INR)] performed monthly was consistently normal.

Seven months after commencing adalimumab, she developed worsening fatigue and arthralgias, along with jaundice, dark urine, pruritis and acholic stools. On presentation to hospital, she was found to be deeply jaundiced, without stigmata of chronic liver disease, encephalopathy or ascites. Initial bloodwork was as follows; total bilirubin 167 (normal < 22 μmol/L), direct bilirubin 129, alanine aminotransferase (ALT) 15, aspartate aminotransferase (AST) 16, gamma glutamyl aminotransferase 121, alkaline phosphatase 183, INR 1.8, platelets 276. Subsequent laboratory tests were obtained, with a normal alpha-1-antitrypsin level (2.31), normal ceruloplasmin (312), negative autoimmune serology (anti-mitochondrial antibody (AMA) negative, antinuclear antibody (ANA) negative, anti-smooth-muscle antibody (ASMA) negative, anti-tTG negative, IgA (0.47). Iron profile was normal, with negative serologies for hepatitis B and C.

Concomitant medications included azathioprine 200 mg/d (2 mg/kg), adalimumab 40 mg every 2 wk, hydromorphone contin, gabapentin, spironolactone, levothyroxine, and insulin. Adalimumab was discontinued on admission, with continuation of her other medications, except azathioprine. Further investigations including abdominal ultrasound with doppler and Magnetic resonance cholangiopancreatography did not show any abnormalities. endoscopic retrograde cholangiopancreatography showed no stones, strictures, beading or other features of primary sclerosing cholangitis. A liver biopsy showed marked cannalicular cholestasis, [predominantly in a pericentral (zone 2 and 3) distribution], associated with numerous large intracannicular bile thrombi throughout the parenchyma. This was accompanied by foamy degeneration, hepatocyte rosettes and patchy cytoplasmic condensation and eosinophilia. Large numbers of ceroid laden macrophages were identified on PASD stain in keeping with increased hepatocyte turnover. Only rare foci of lobular inflammation were present and no significant portal tract expansion or edema were identified. Furthermore, there was only minimal portal inflammation, composed predominantly of lymphocytes with very occasional eosinophils; plasma cells were not present, nor was there any evidence of interface hepatitis. Mild ductular reaction with occasional ductules containing intraluminal bile was present. Importantly, features of bile duct injury or loss were not present. There was no significant steatosis (< 2% of hepatocytes contain lipid droplets) and the connective tissue stains show mild patchy periportal fibrosis only, without any evidence of fibrous septa. Stains for iron did not demonstrate an increase in stainable iron and no alpha-1-antitrypsin like globules were detected on PASD stain (Figure 1).

In summary, the biopsy shows marked cholestasis without significant inflammation. From a histological perspective the possible causes included predominantly drugs/medications, total parenteral nutrition, sepsis and benign familial forms of cholestasis, the latter ones were considered to be unlikely in this clinical setting. There were no features to suggest large duct obstruction and recent radiology did not show any dilatation of the biliary tree. As such, it was felt that the biopsy findings represented the histological changes of a drug induced liver injury.

After initial worsening of her jaundice and INR, spontaneous improvement was seen after 5 wk, and eventual normalization of liver enzymes at 10 wk. The patient remains on azathioprine and currently has normal liver biochemistry, over 6 mo after her initial presentation with jaundice.

DISCUSSION

Elevation of liver enzymes has been reported during treatment with anti-TNF antibodies such as infliximab (IFX), but overt liver failure has been rarely reported[1]. However, cases of acute liver injury requiring liver transplantation (LT) have been reported in a review of post-marketing data and one of these cases occurred in a CD patient. Anti-TNF agents can lead to acute liver injury by at least three mechanisms: precipitation of de novo autoimmune hepatitis, cholestasis and direct hepatocellular necrosis[2]. The potential hepatotoxicity of IFX was addressed in a recent consensus statement[3]: IFX therapy may be considered in patients with clinically significant liver disease, but should be avoided in patients with aminotransferases (*i.e.,* serum ALT and AST) over three times normal and that liver biochemistry should be obtained prior to IFX initiation. Autoimmune hepatitis due to anti-TNF agents for inflammatory bowel disease has more commonly been reported with infliximab, and the majority of patients responded to drug cessation and steroid treatment. Normalization of liver enzymes was typically rapid, occurring within 2 mo after infliximab discontinuation[4].

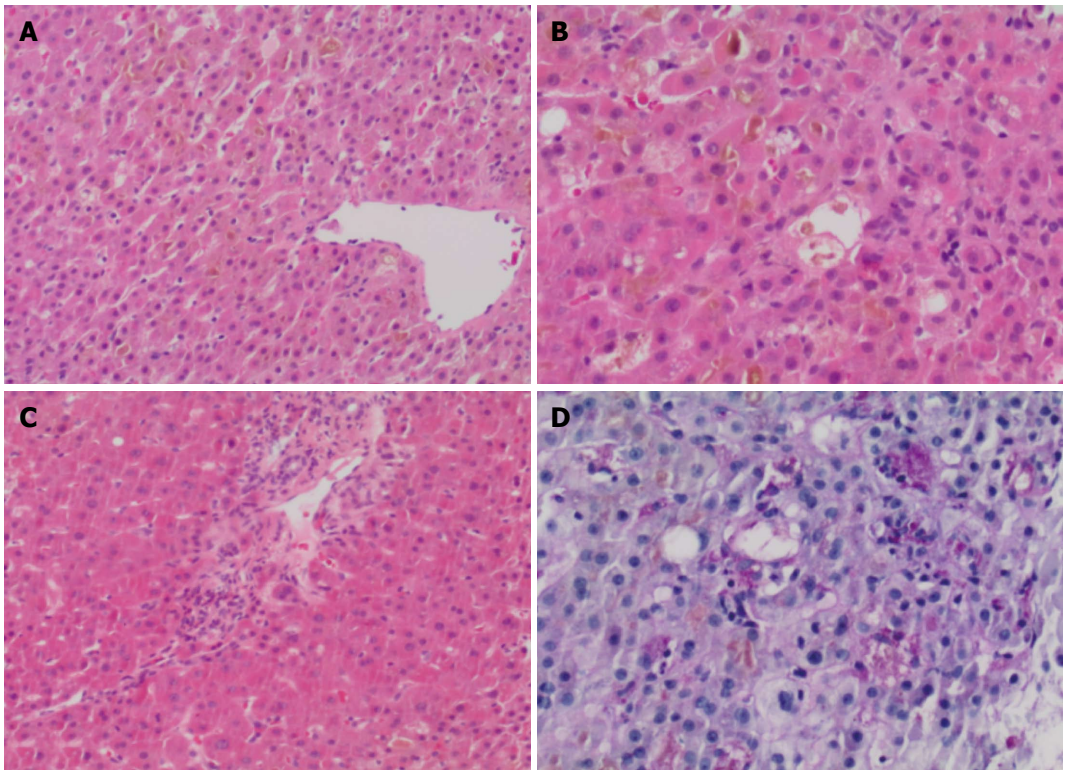

Figure 1 Histopathological changes. A: The liver biopsy showed marked cannalicular cholestasis, predominantly in a pericentral (zone 2 and 3) distribution; B: Numerous large intracannicular bile thrombi were present throughout the hepatic parenchyma, associated with early foamy degeneration, hepatocyte rosettes and patchy cytoplasmic condensation and eosinophilia. Lobular inflammation, as is the norm in cholestatic hepatitis, were minimal within the lobule; C: The portal tracts showed only minimal inflammation, without any evidence of bile duct injury or loss and there was no interface hepatitis present; D: Large numbers of ceroid laden macrophages were identified on PASD stain in keeping with increased hepatocyte turnover. Magnification: (A, C, D) x 40; (B) x 200.

Cholestatic liver disease due to IFX has also been described, albeit much more seldom. One case report described a female patient with rheumatoid arthritis who developed severe cholestatic liver disease with hepatic failure necessitating LT[5]. Cholestatic liver injury has occurred after treatment with IFX for inflammatory bowel disease, with spontaneous resolution 6 wk after drug discontinuation[6]. Other cases of infliximab-related autoimmune hepatitis were subsequently treated with an alternate anti-TNF agent (etanercept, adalimumab), and was well tolerated[7].

The medical literature concerning the hepatotoxicity of ADA, however, is sparse and, unlike IFX, there are very few reports. Our case assumes importance as drug induced liver injury can be specific to the molecular structure of the drug in question. In terms of previously reported ADA liver injury, during controlled studies for rheumatoid arthritis, 1%-4% of ADA-treated patients developed liver enzyme elevation greater than twice the upper limit of normal. However, this was similar to the percentage of liver enzyme abnormalities found in placebo-treated patients. In clinical trials of patients with psoriatic arthritis and ankylosing spondylitis, elevation of ALT and AST in the range of 1.5-3 times the upper limit of normal was more common in patients receiving adalimumab than in controls, both when adalimumab was given as monotherapy and when it was used in combination with other immunosuppressive agents. ADA has been also been associated with development of autoimmune hepatitis in several case reports; one patient with rheumatoid arthritis was successfully switched to abatacept[8]. A case of subacute liver failure during therapy with adalimumab for psoriatic arthritis has been reported[9]. Another patient who had developed acute toxic hepatitis with necrosis due to infliximab for CD was successfully treated with adalimumab with no recurrence of liver enzyme abnormalities. We are unaware of any previous reports of cholestatic liver injury in association with ADA and similarly, the liver histopathology of ADA-associated cholestatic liver injury has not previously been reported.

Hepatotoxicity is a known side effect of azathioprine, occurring at a rate of hepatic abnormalities of 3%-10% after longterm treatment. Hepatic abnormalities are usually limited to abnormal liver function tests and minor change seen on liver biopsy specimens[10]. Azathioprine-induced hepatotoxicity can be grouped into three syndromes: hypersensitivity, idiosyncratic cholestatic reaction, and endothelial cell injury (with resultant raised portal pressures, veno-occlusive disease, or peliosis hepatis). Azathioprine has been associated with a wide variety of hepatic complications, including hepatocellular injury, veno-occlusive disease, peliosis hepatitis, hepatoportal sclerosis, and nodular regenerative hyperplasia[10]. In addition, bland cholestasis and cholestatic hepatitis with bile duct injury have been rarely reported

in case report form. Even fewer cases of severe cholestatic hepatitis have been reported in the literature, developing in patients with inflammatory bowel disease, lupus or rheumatoid arthritis[11]. The latency from the initial exposure to azathioprine to the onset of jaundice ranges from 2 wk to 3 mo in previous reports.

Our patient had been maintained on azathioprine for 3 years until the development of jaundice, without previous elevations in liver enzymes, but developed jaundice within 7 mo after the initiation of adalimumab. Of importance, she is currently maintained on azathioprine (2 mg/kg) without any abnormalities in liver biochemistry which would almost certainly exclude azathioprine as the cause of her severe cholestasis. Our case describes the first reported case of ADA-associated severe cholestatic injury and the first histopathologic examination of this adverse drug effect. Clinicians need to be aware of this potential severe drug-induced liver injury when prescribing this commonly used biologic medication.

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CASE REPORT

Advanced hepatocellular carcinoma responds to MK615, a compound extract from the Japanese apricot "*Prunus mume*"

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Abstract

MK615, a compound extracted from the Japanese apricot "*Prunus mume*" has been reported to have *in vitro* anti-tumor activities against several cancer cell lines, including hepatocellular carcinoma (HCC). However, the clinical effects and feasibility of administering MK615 for patients with HCC were unknown. We experienced a case with advanced HCC for which MK615 was effective against both lymph node and pulmonary metastases. A 60-year-old female underwent surgical resection of a 9 cm HCC in the right lobe. The pathological diagnosis was moderately differentiated HCC with vascular invasion. The HCC recurred in the liver 8 mo after the surgery. Radiofrequency ablation and transarterial infusion chemotherapy were performed, but the recurrence was not controlled. One year after the intrahepatic recurrence, pulmonary and lymph metastasis appeared. Sorafenib was administered, but was not effective. Then, MK615 was administered as a final alternative therapy after informed consent was obtained from the patient. Three months later, her alpha-fetoprotein level decrease and both the lymph node and pulmonary metastases decreased in size. The patient has survived for more than 17 mo after the MK615 administration, and was in good condition. Although further investigations are necessary to clarify its safety and efficacy in humans, MK615 may be useful for the treatment of HCC, without serious adverse effects.

Key words: MK615; Hepatocellular carcinoma; Japanese apricot; *Prunus mume*

Core tip: We experienced a case with advanced hepatocellular carcinoma (HCC) for which MK615, a compound extracted from the Japanese apricot "*Prunus mume*" was effective against both lymph node and pulmonary metastases. MK615 was administered as a final alternative therapy. Three months later, her alpha-fetoprotein level decrease and both the lymph node and pulmonary metastases decreased in size. MK615 has been reported to have *in vitro* anti-tumor activities against several cancer cell lines, including HCC. Although further investigations are necessary to clarify its safety and efficacy in humans, MK615 may be useful for the treatment of HCC, without serious adverse effects.

Hoshino T, Takagi H, Naganuma A, Koitabashi E, Uehara S, Sakamoto N, Kudo T, Sato K, Kakizaki S. Advanced hepatocellular carcinoma responds to MK615, a compound extract from the Japanese apricot "*Prunus mume*". *World J Hepatol* 2013; 5(10): 596-600 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i10/596.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i10.596

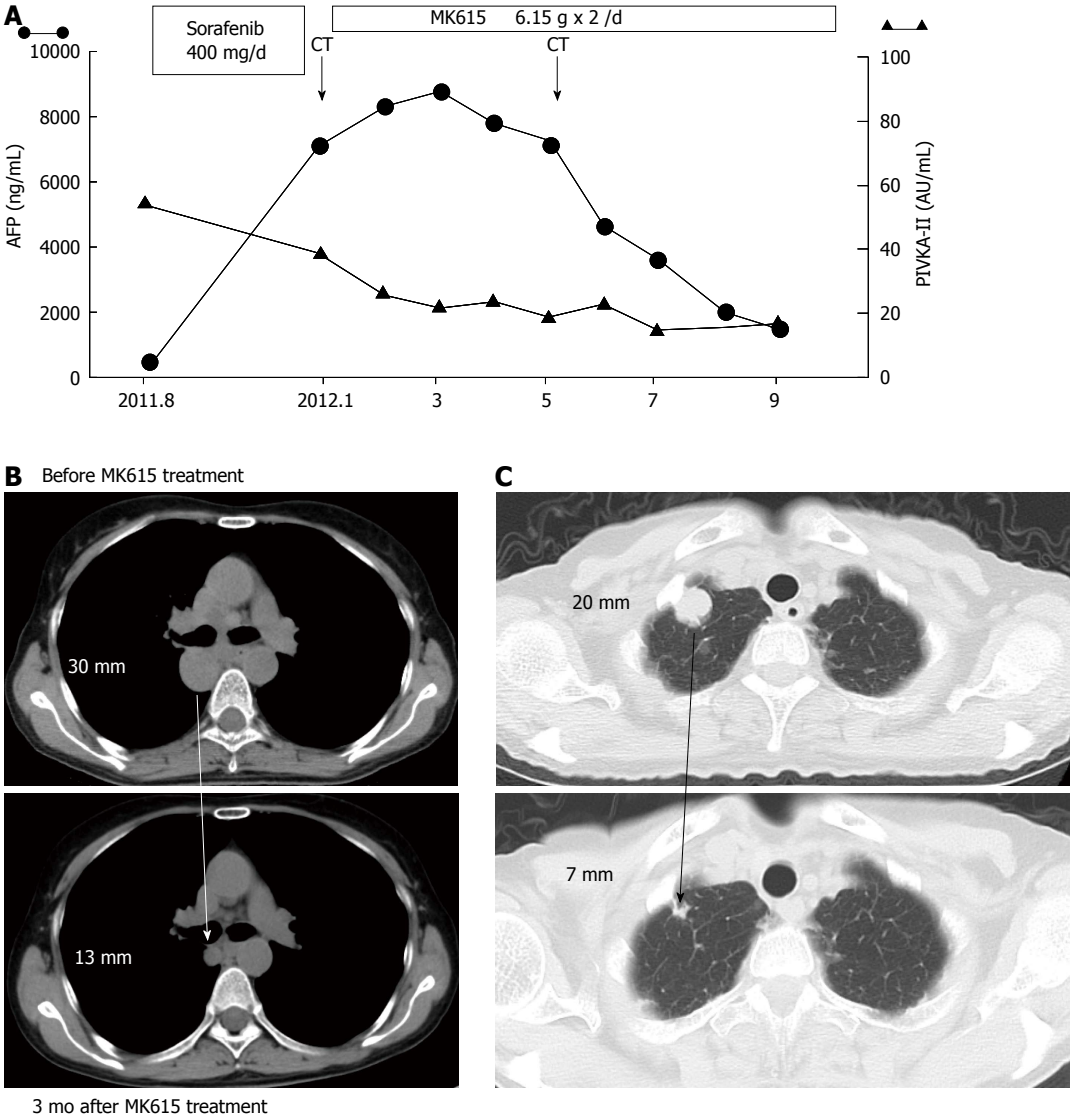

Figure 1 Clinical course of the 60-year-old female patient with stage IVB hepatocellular carcinoma (HCC). A: The change of tumor markers; B: After 3 mo of MK615 treatment, the lymph node metastasis decreased in size from 30-13 mm in diameter; C: Three months after the MK615 treatment, the lung metastasis was decreased in size from 20-7 mm in diameter. AFP: Alpha-fetoprotein.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most intractable cancers, and the clinical outcome is still unsatisfactory despite improvements in the therapeutic strategies for HCC[1-3]. For the patients with advanced HCC with poor liver function, surgical resection, radiofrequency ablation (RFA) or transarterial chemoembolization (TACE) cannot be applied because of their adverse effects, and palliative care is the only recommended treatment for such patients[1-3]. Although these patients might be cured by a liver transplant, such treatment is usually not possible due to the severe donor shortage[4]. For advanced stage HCC patients with preserved liver function, sorafenib is usually indicated, but its side effects, such as cytopenia and liver dysfunction, sometimes require a disruption or discontinuation of the treatment.

MK615 is a compound extracted from the Japanese apricot "*Prunus mume*"[5] and contains several triterpenoids, such as oleanolic acid and ursolic acid[5]. It has been reported that MK615 inhibits cell growth and induces the death of several tumor cell lines[5-7], including gastric cancer[5], promyelocytic leukemia[5], breast cancer[8], pancreatic cancer[9], HCC[10,11], colon cancer[12], esophageal cancer[13], malignant melanoma[14,15] and lung cancer cells[16]. The activities underlying the anti-tumor effects of MK615 have been reported to include the induction of apoptosis[5,8], autophagy[12,16] and the suppression of aurora A kinase[9,11]. Furthermore, some clinical studies have shown promising effects in some cancer patients[5,12]. Recently, hepatoprotective effects of MK615 have been reported for patients with chronic liver diseases[17]. However, the clinical benefit of MK615 for HCC patients has not been evaluated.

We recently experienced a case with advanced HCC in which MK615 was effective for both lymph node and pulmonary metastases. Although further investigations are necessary to clarify the safety and efficacy of the

Table 1 Characteristics of the patients who received MK615 for more than 3 mo

Case	Sex	Age	Stage	CP	Previous treatment	Duration (mo)	Response	Cause of death
1	F	64	IVA	A	5FU + IFN, Sorafenib	3	PD	HCC progression
2	F	85	IVA	C	None	3	SD	HCC progression
3	M	63	IVA	C	None	6.5	SD-PD	HCC progression
4	M	57	IVA	A	TACE	3	PD	HCC progression
5	F	73	IVA	C	None	6	PD	Survived
This case	F	60	IVB	A	Surgery, RFA, TAI	17	PR	Survived

CP: Child-Pugh classification, duration; duration of MK615 treatment; F/M: Female/male; HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization; TAI: Transcatheter arterial infusion chemotherapy.

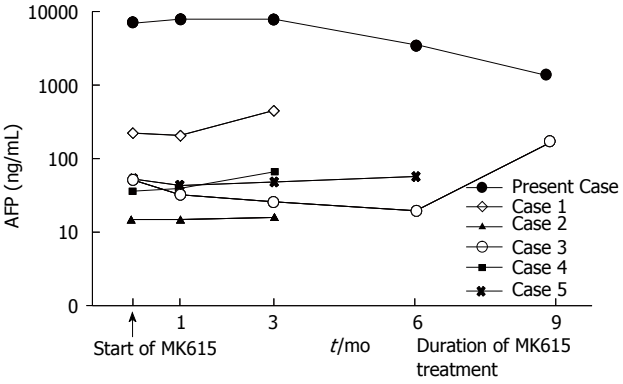

Figure 2 Serial changes in the alpha-fetoprotein levels in the 6 patients during the course of MK615 treatment. AFP: alpha-fetoprotein

treatment in human patients, MK615 may be useful for the treatment of HCC, without the serious adverse effects associated with the current treatments.

CASE REPORT

A 60-year-old underwent surgical resection of a primary HCC lesion in the right lobe that was 9 cm in diameter. The pathological diagnosis was moderately differentiated HCC, ly2, v2, n0 and m0. The HCC recurred in the liver 8 mo later and RFA was performed, but the recurrence was not controlled. Transarterial infusion chemotherapy, including cisplatin and a 5-fluorouracil/cisplatin combination was performed for the intrahepatic recurrence. One year after the recurrence, pulmonary and lymph metastases appeared. Sorafenib was administered, but was not effective.

Then, MK615 was administered as a final alternative therapy after informed consent was obtained from the patient. MK615 (Misatol ME^{R}) was kindly provided by AdaBio Co Ltd. (Takasaki, Japan). A total of 6.5 g of Misatol ME^{R} was administered twice per day. The administration of MK615 was approved by the internal review board of Takasaki General Hospital, and adopted the protocol for clinical research entitled, "The clinical feasibility study of Misatol ME^{R} (MK615) for the patients with advanced stage-hepatocellular carcinoma".

Three months later, the patient's alpha-fetoprotein (AFP) levels decreased (Figure 1A) and both the lymph node (Figure 1B) and pulmonary (Figure 1C) metastases decreased in size. This patient has survived for more than 17 mo and was in good condition at her latest follow-up examination in August 2013.

MK615 administration

We conducted a preliminary clinical trial of MK615 for HCC. Six patients, including this case, received MK615 treatment as alternative therapy. These patients were not able to receive the conventional treatments, including surgical resection, RFA and TACE, because of advanced HCC and/or poor liver function, and so were administered MK615. The modified RECIST[18] was applied for the evaluation of the therapeutic effect of the treatment. The characteristics of the patients who had taken MK615 for more than 3 mo are shown in Table 1. All cases were of stage IV disease. The overall survival was 4.8 mo from the start of MK615 administration. The changes in AFP are shown in Figure 2. Although there was only one PR case, no serious adverse events were observed.

DISCUSSION

MK615 contains *ume*-derived triterpenoids, including oleanolic acid and ursolic acid. Triterpenoids had been reported to suppress the growth of many cancer cell lines[19-22]. Although the activities underlying the antitumor effects have been reported to include the induction of apoptosis[5,8], autophagy[12,16] and the suppression of aurora A kinase[9,11], the exact mechanism(s) of action of MK615 are still being elucidated. A large amount of basic data regarding the effects of MK615 against cancer cells *in vitro* have been published[5-16]. However, there has been little clinical data with regard to the effects of MK615 against cancer. One study with a small number of clinical cases showed that MK615 was useful for malignant melanoma[15], and the clinical efficacy and safety of MK615 has been reported for patients with chronic liver disease[17]. However, the clinical benefit of MK615 for HCC patients has not been evaluated.

We experienced a case of advanced HCC in which MK615 was effective for both lymph node and pulmonary metastases. Concerning the relationship between MK615 and HCC, Sakuraoka *et al*[10] reported that MK615 suppresses the proliferative effects of glyceraldehyde-derived advanced glycation end-products on a

HCC cell line, HuH7, by decreasing the expression of their cellular receptor (RAGE). Another study reported that MK615 inhibited the growth of two HCC cell lines, HuH7 and Hep3B, in a dose-dependent manner[11]. A cell cycle analysis revealed that MK615 increased the population of cells in the G_2/M phase[11] and that MK615 suppressed the expression of Aurora A[11]. These studies demonstrated that MK615 has anti-tumor effects against HCC. Although the mechanism(s) of anti-tumor activity in the present case is unknown, MK615 appears to exert anti-tumor effects on HCC *in vivo*. This case is the first case demonstrating the clinical efficacy of MK615 against HCC.

We also attempted to treat six patients with advanced stage HCC with poor liver function. Our policy is that if the patients had a chance to be treated with conventional anticancer treatments, the patients should be treated using these treatments, and alternative treatments are reserved only for those with no other options. Although the present case was the only PR, none of the subjects experienced adverse effects of the treatment. Therefore, it is considered that the effects of MK615 may be useful for patients with advanced HCC, particularly for patients with poor functional reserve, and that the treatment is not associated with the severe adverse effects associated with the conventional treatments.

Although further studies are required to demonstrate the safety and efficacy of MK615 for HCC patients, the preliminary results are promising. We are planning to conduct a clinical study of combination therapy using MK615 with other anti-cancer agents, and/or a controlled study with a large number of patients with advanced HCC.

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A correction of misinformation regarding Herbalife

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Abstract

The authors of the subject article by Senadhi *et al* have misrepresented the safety and regulatory status of Herbalife's products. While we are very concerned with the unwarranted and unfavorable publicity that the inaccuracies listed could generate for Herbalife, we would welcome any inquiries that these authors may have to better clarify our commitment to the safety and quality of our products as has been demonstrated in part by our ability to establish positive relationships with regulatory authorities worldwide through continued cooperation and compliance. This letter clarifies the misinformation presented about Herbalife in the subject article.

Key words: Hepatitis; Drug-induced liver injury; Herbalife; Herbal; Dietary supplements

Core tip: The authors of the subject article by Senadhi *et al* have misrepresented the safety and regulatory status of Herbalife's products. Most importantly, the authors have misinformed the readership that Food and Drug Administration (FDA) has taken action against Herbalife for its known association with reports of liver injury. FDA has taken no action on the company for this reason or any other reasons to date.

Appelhans K, Najeeullah R, Frankos V. A correction of misinformation regarding Herbalife. *World J Hepatol* 2013; 5(10): 601-602 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i10/601.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i10.601

TO THE EDITOR

In an article published in the August 27, 2012 issue of the *World Journal of Hepatology*[1], the authors described a case of herb-induced hepatitis in a patient with human immunodeficiency virus. In the abstract and introduction of this article, the authors referred to their general opinion that herbal or over-the-counter supplements are unregulated, thus being unsafe and carrying potential risks that some of these products contain ingredients that may be hepatotoxic. To further this point, the authors stated that Food and Drug Administration (FDA) has taken regulatory actions against several companies that have been "known" to be associated with reported cases of liver injury. The authors used Herbalife and Hydroxycut as specific examples of such regulatory action. First of all, we would like to express our concern for the inappropriate reference that these authors have made regarding the safety and regulatory status of our products. FDA has had the opportunity to investigate Herbalife's products with full cooperation from the company, and no regulatory action has been taken against the company to date. This is not the case for Hydroxycut, which the manufacturer agreed to recall and reformulate in 2009 amidst growing safety concerns including FDA's consumer and health professional warning statements to stop the consumption of these products. Therefore, the authors' comparison between our product and Hydroxycut is inappropriate and completely unfounded. Secondly, the authors erroneously stated that Herbalife is a GNC product which is incorrect. Herbalife is a brand and not

a single product, and our products are sold directly to consumers through our distributor network and not purchased through any retail establishments, let alone GNC. Finally, the authors have cited an article[2] to support the aforementioned misinformation regarding Herbalife and this reference does not contain any data to support these statements. Furthermore, Herbalife formally rebutted the reference article[3] and the authors did not cite this information which is a biased representation of the information associated with Herbalife in the literature to date.

While we are very concerned with the unwarranted and unfavorable publicity that the inaccuracies listed above could generate for Herbalife, we would welcome any inquiries that these authors may have to better clarify our commitment to the safety and quality of our products as has been demonstrated in part by our ability to establish positive relationships with regulatory authorities worldwide through continued cooperation and compliance.

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In press

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

Organization as author

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

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

Volume with supplement

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

Issue with no volume

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

No volume or issue

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

Books

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

Chapter in a book (list all authors)

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

Author(s) and editor(s)

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

Conference proceedings

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

Conference paper

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

Electronic journal (list all authors)

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

Patent (list all authors)

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

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