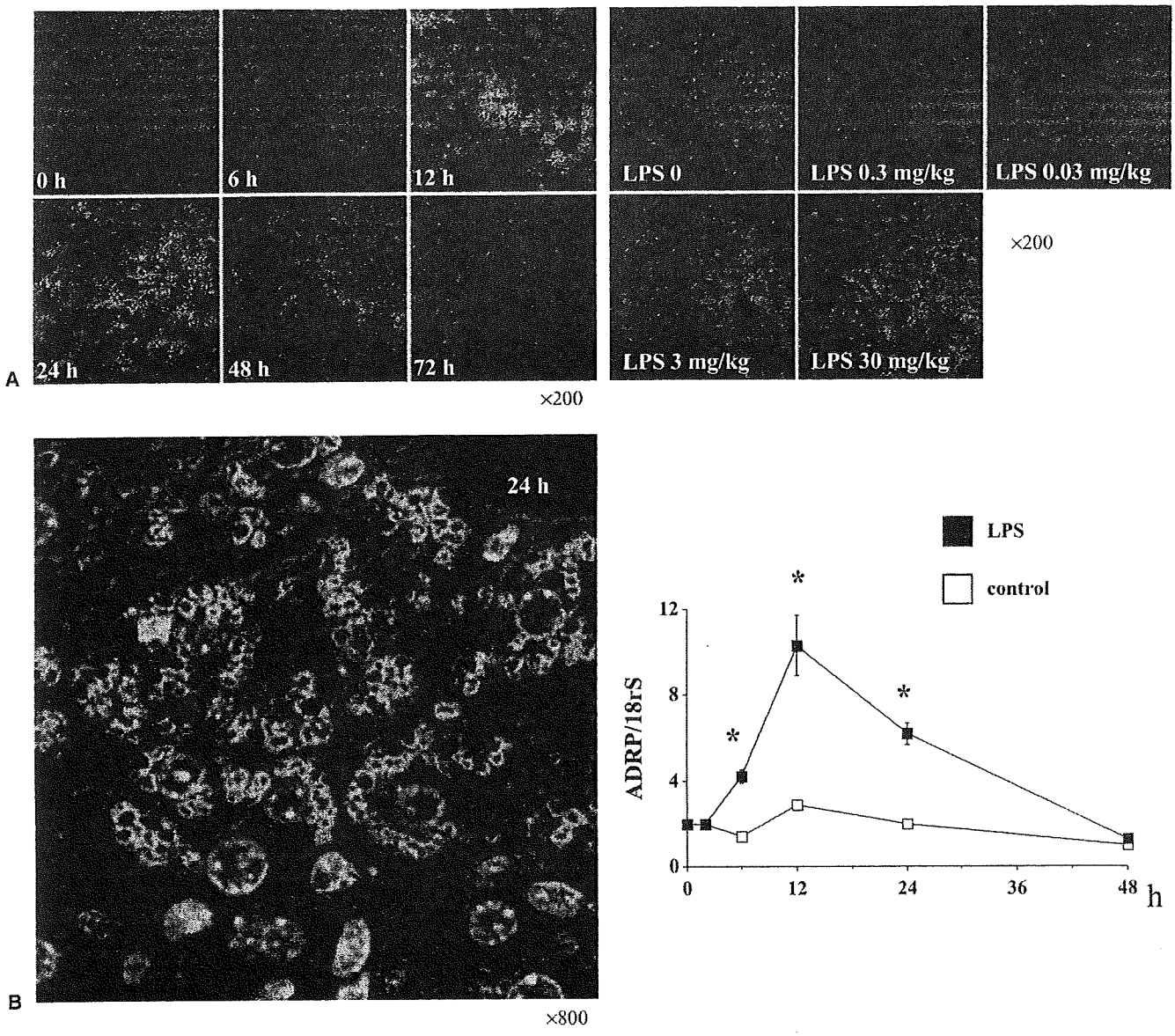

Fig. 5A–D. Representative adipose differentiation-related protein (*ADRP*) immunostaining of liver tissue in mice injected with LPS intraperitoneally. Mice were injected with several doses of LPS intraperitoneally and then killed at several time points. Liver tissues were stained with ADRP antibody. Time-course (**A**) and dose–response (**C**) effects of LPS on ADRP protein expression on the liver are shown. **B** High-power view of liver tissue (ADRP immunostaining) 24 h after LPS (30 mg/kg) injection. **D** Time-course effect of LPS on ADRP mRNA expression in the mouse liver. Mice were injected with a 30 mg/kg dose of LPS intraperitoneally and then killed at several time points. ADRP mRNA expression in the liver was examined by real-time polymerase chain reaction (PCR). Each data point represents the mean ± SEM of five animals. $^*P < 0.01$, compared with control

lipids, we recently demonstrated increased expression of ADRP in fatty liver in patients as well as in *ob/ob* and normal mice fed a high fat diet.[7] In addition, it has been very recently reported that an ADRP antisense oligonucleotide reduced liver steatosis in *ob/ob* and diet-induced obese mice,[18] and protection against fatty liver was observed in mice lacking ADRP,[19] strongly suggesting that ADRP plays a key role in the development of liver steatosis. The present study showed that LPS induced liver steatosis that was accompanied by increased expression of ADRP, indicating that upregulation of ADRP expression is a common molecular event during lipid accumulation in the liver, whatever its cause or mechanism.

Next, we investigated the mechanism by which LPS induces lipid accumulation in the liver in mice. Recent

studies have established a role for hepatic PPARγ in the development and maintenance of steatosis in the liver.[6,20–24] Upregulation of the PPARγ gene in the liver should result in steatosis, whereas lipid accumulation should be prevented by downregulation of the PPARγ gene in hepatocytes.[25–27] We therefore tested the hypothesis that PPARγ is involved in LPS-induced lipid accumulation in the liver. PPARγ mRNA expression in the liver was potently inhibited by LPS. Beigneux et al.[28] have shown that PPARγ mRNA in the liver is drastically reduced by LPS in hamster,[28] in agreement with the present data. These results led us to conclude that PPARγ is not implicated in the formation of lipid droplets in the liver by LPS.

PPARα also plays a key role in liver steatosis.[8,29] PPARα is abundantly expressed in the liver, where it functions as a lipid sensor and recognizes and responds to the influx of fatty acids by stimulating the transcription of PPARα-regulated genes.[30,31] These include genes encoding for mitochondrial, peroxisomal, and microsomal oxidation systems.[29] In conditions of increased demand for fatty acid oxidation, PPARα-null mice fail to upregulate fatty acid oxidation systems in liver with which to oxidize influxed fatty acids, and they develop liver steatosis.[32–34] Administration of PPARα agonists to rats not only prevents the development of methionine- and choline-deficient diet-induced steatohepatitis but also reverses steatohepatitis.[35,36] PPARα expression in the liver was rapidly and potently suppressed by LPS in

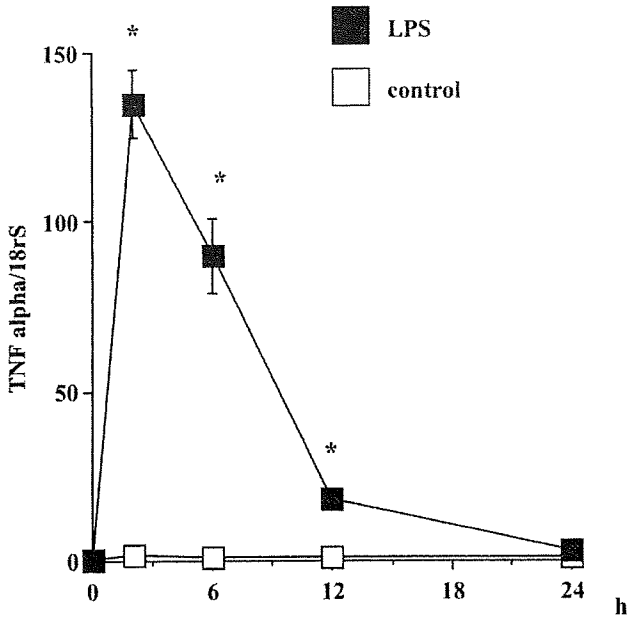

Fig. 6. Tumor necrosis factor (*TNF*) α mRNA expression in the liver was examined by real-time PCR 2, 6, or 12 h after administration of 30 mg/kg LPS to mice. Each data point represents the mean ± SEM of five animals. *$P < 0.01$, compared with control

Table 1. Time-course effect of LPS on SREBP-1 mRNA expression in the mouse liver

	SREBP-1/18rS	
Time (h)	control	LPS
2	1.75 ± 0.12	1.68 ± 0.08
6	2.12 ± 0.14	2.10 ± 0.11
12	2.01 ± 0.13	1.70 ± 0.15

Values are means ± SEM ($n = 5$)
LPS, lipopolysaccharide; SREBP-1, sterol regulatory element binding protein 1

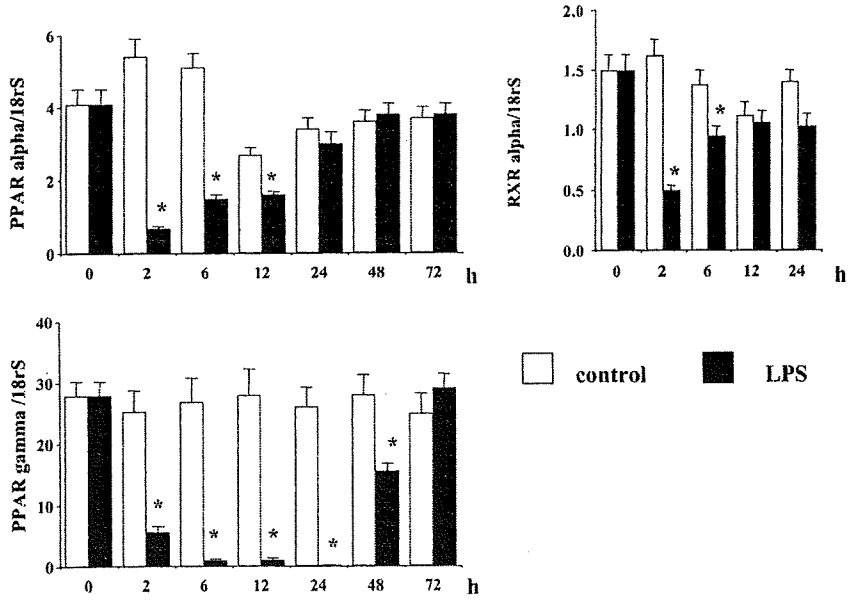

Fig. 7. Time-course change of mRNA expression of peroxisome proliferator-activated receptor (*PPAR*) α, PPARγ, and retinoid X receptor (*RXR*) α which are known to be transcription factors involved in lipid accumulation. Mice were injected with 30 mg/kg LPS intraperitoneally and then killed at several time points. mRNA expression of PPARα, PPARγ, and RXRα in the liver was examined by real-time PCR. Each data point represents the mean ± SEM of five animals. *$P < 0.01$, compared with control

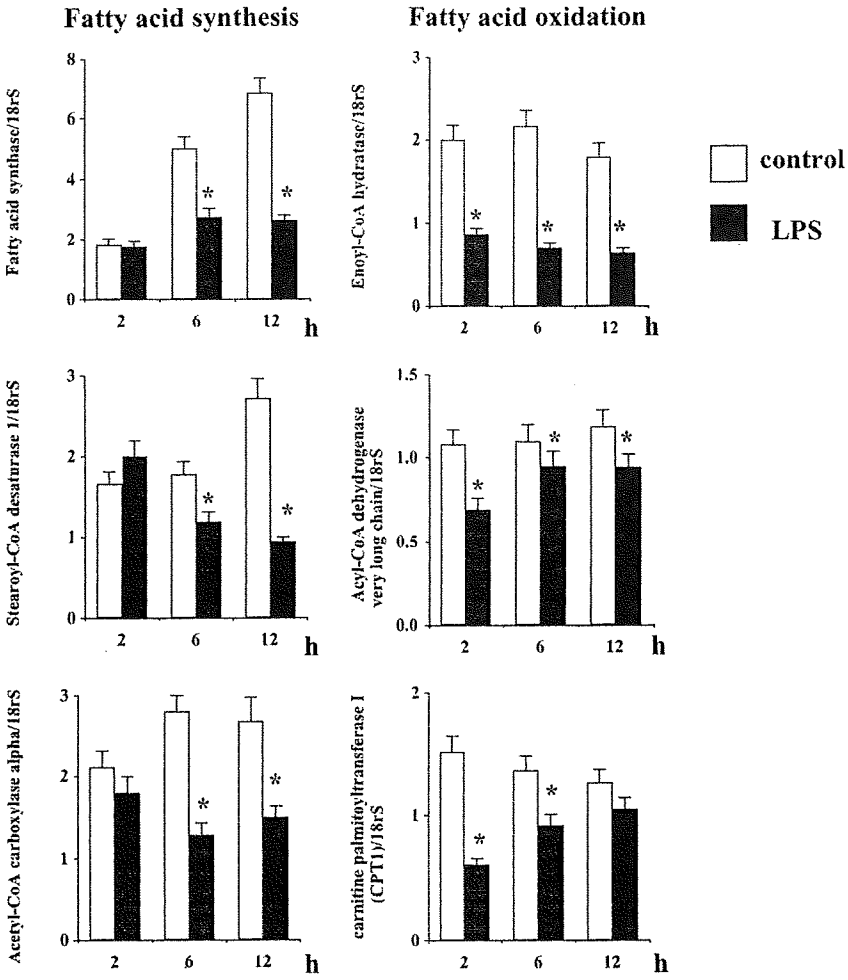

Fig. 8. Time-course change of mRNA expression of several enzymes that play a role in fatty acid synthesis or lipid oxidation in the liver: fatty acid synthase, stearoyl-CoA desaturase, acetyl-CoA carboxylase, enoyl-CoA hydratase, acyl-CoA dehydrogenase, and carnitine palmitoyl transferase-1. Mice were injected with 30 mg/kg LPS intraperitoneally and then killed at several time points. mRNA expression of the above enzymes in the liver was examined by real-time PCR. Each data point represents the mean ± SEM of five animals. $^*P < 0.01$, compared with control

this study, as previously shown by Tai et al.[37] Considering this evidence, the present results suggest that reduced expression of PPARα in the liver may play a role in lipid accumulation caused by LPS.

LPS also inhibited the hepatic expression of RXRα, another nuclear hormone receptor. Because heterodimerization with RXRα is crucial for the action of PPARα,[38,39] the reduction of hepatic RXRα expression alone or in association with PPARα may be a mechanism for inhibiting the expression of multiple genes for lipid oxidation. The fact that decreased expression of transcription factors such as PPARα and RXRα occurred within 2 h after the LPS challenge supports the hypothesis that repression of those transcription factors plays a vital role in the accumulation of lipid in the hepatocytes caused by LPS. Because lipid accumulation was observed 12 h after LPS injection, LPS may suppress PPARα and its heterodimers, causing lipid accumulation in the liver through inhibition of PPARα-targeted genes for fatty acid oxidation, as described below.

Accumulation of lipids in the liver can result from either stimulation of lipid synthesis or inhibition of lipid oxidation. With regard to lipogenesis, de novo fatty acid synthesis in the liver is regulated by transcription factors such as SREBP-1c and PPARγ.[8] In this study, LPS inhibited expression of both these transcription factors in the liver, strongly suggesting that LPS-induced liver steatosis did not result from increased lipogenesis. In fact, our results showed that genes responsible for lipogenesis in the liver were not upregulated by LPS, further indicating that increased lipogenesis is not involved in LPS-induced liver steatosis.

Disruption of fatty acid oxidation can also account for excess lipid storage in the liver.[1] Some of the key enzymes of fatty acid oxidation systems in liver are regulated by PPARα.[29,31] In the present study, LPS potently inhibited expression of PPARα and its target genes, including enoyl-CoA hydratase, acyl-CoA dehydrogenase, and carnitine palmitoyl transferase-1, which are involved in fatty acid oxidation in the liver, indicating that reduced expression of PPARα plays a vital role

in LPS-induced lipid accumulation in the liver through inhibition of fatty acid oxidation.

Wolfe et al.[40] have demonstrated that sepsis and endotoxins increase hepatic very low density lipoprotein (VLDL) production. Several reports have demonstrated that an increase in hepatic VLDL production by sepsis or endotoxins may be caused by an increase in the reesterification of plasma fatty acids derived from the stimulation of lipolysis.[40–42] These findings suggest that an influx of fatty acid into hepatocytes might be involved in LPS-induced lipid accumulation. As clearly shown in the present study, LPS potently increased expression of TNFα mRNA in the liver. The increase in TNFα expression and reduced adipose tissue weight observed in this study after LPS administration suggest that lipolysis followed by an influx of fatty acids into hepatocytes might be involved at least in part in the observed hepatic fatty accumulation. Moreover, it has been reported previously that increased expression of TNFα induces lipolysis and hepatic lipid accumulation through downregulation of PPARα expression in the liver.[43,44]

Fatty acids undergo beta-oxidation in the mitochondria.[1] Some investigators have demonstrated abnormal mitochondrial function after endotoxic shock.[45–47] Therefore, we speculate that LPS may disrupt mitochondrial function, and that this disruption might play a role in the lipid accumulation in the liver, possibly through the inhibition of lipid oxidation. Further studies should be performed to address this possibility.

In summary, our results suggest that LPS induces transient lipid accumulation and expression of ADRP, a lipid droplet surface protein, in the liver through inhibition of fatty acid oxidation by downregulation of PPARα-related genes.

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Iron overload and cofactors with special reference to alcohol, hepatitis C virus infection and steatosis/insulin resistance

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Abstract

There are several cofactors which affect body iron metabolism and accelerate iron overload. Alcohol and hepatic viral infections are the most typical examples for clarifying the role of cofactors in iron overload. In these conditions, iron is deposited in hepatocytes and Kupffer cells and reactive oxygen species (ROS) produced through Fenton reaction have key role to facilitate cellular uptake of transferrin-bound iron. Furthermore, hepcidin, antimicrobial peptide produced mainly in the liver is also responsible for intestinal iron absorption and reticuloendothelial iron release. In patients with ceruloplasmin deficiency, anemia and secondary iron overload in liver and neurodegeneration are reported. Furthermore, there is accumulating evidence that fatty acid accumulation without alcohol and obesity itself modifies iron overload states. Ineffective erythropoiesis is also an important factor to accelerate iron overload, which is associated with diseases such as thalassemia and myelodysplastic syndrome. When this condition persists, the dietary iron absorption is increased due to the increment of bone marrow erythropoiesis and tissue iron overload will thereafter occurs. In porphyria cutanea tarda, iron is secondarily accumulated in the liver.

Key words: Iron overload; Cofactors; Alcohol; Chronic hepatic C; Non-alcoholic steatohepatitis; Insulin resistance; Hepatocellular carcinoma

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INTRODUCTION

In hereditary hemochromatosis, patients having HFE trait are more susceptible to iron overload when cofactors such as alcohol, hepatitis viruses, and abnormal porphyrin metabolism are present. Even in the absence of hereditary hemochromatosis, there are several conditions associated with secondary iron overload in which iron deposition is rather mild[1]. For example, in alcoholics and patients with chronic hepatitis C, intrahepatic iron is increased and liver injury is accelerated, followed by development of fibrosis, cirrhosis and hepatocellular carcinoma (HCC). In addition, abnormal copper metabolism and several causes for iron-loaded anemia are also important cofactors which influence the background iron overload. Furthermore, there is accumulating evidences that fatty acid accumulation without alcohol and obesity itself modifies insulin resistance through iron[2] and fibrogenesis of the liver[3]. In this review, the role of cofactors on iron overload will be discussed in three categories such as alcohol, hepatitis C virus infection and steatosis with obesity, the most common cofactors in liver iron overload.

COFACTORS AFFECTING BODY IRON METABOLISM AND IRON OVERLOAD

There are several factors which affect body iron metabolism and accelerates iron overload. Table 1 lists cofactors and disease conditions which are known to accelerate hepatic iron accumulation independent from responsible genes for hereditary hemochromatosis. Alcoholic and hepatic viral infections are the most typical examples for clarifying the role of cofactors in iron overload. In addition, abnormal copper metabolism and several causes for iron-loaded anemia such as thalassemia and myelodysplastic syndrome are also important factors which influence the background iron overload. When this condition persists, the dietary iron absorption is increased due to the increment of bone marrow erythropoiesis[4] and tissue iron overload will occur thereafter. These patients are usually anemic in spite of increased body iron stores (iron-

Table 1 Cofactors of iron overload

1	Alcohol (Alcoholic liver disease)
2	Infection (Hepatitis C virus infection, *etc*)
3	Obesity and insulin resistance (Nonalcoholic steatohepatitis)
4	Copper (Ceruloplasmin deficiency)
5	Porphyrin (Porphyria)
6	Ineffective erythropoiesis (Thalassemia, myelodysplastic syndrome)
7	Others

loaded anemia), and require frequent blood transfusions, which further exaggerate secondary iron overload, in which conditions of new oral iron chelators are effective[5]. In patients with ceruloplasmin deficiency, anemia and secondary iron overload in liver and neurodegeneration are reported[6]. Furthermore, there are accumulating evidences that fatty acid accumulation without alcohol and obesity itself modifies iron overload states. Ineffective erythropoiesis is also an important factor to accelerate iron overload. This condition is associated with diseases such as thalassemia, aplastic anemia, and myelodysplastic syndrome. In porphyria cutanea tarda, iron is secondarily accumulated in the liver and phlebotomy and oral iron chelators are effective as well as in hemochromatosis.

ALCOHOL

Alcohol is one of the most important cofactors to modify or enhance iron accumulation in the liver. Excess intake of alcohol induces alcoholic liver diseases (ALD) such as fatty liver, fibrosis, hepatitis, and cirrhosis, in which iron overload is frequently associated[7]. By Perls' iron stain, excess iron accumulation was found in hepatic tissues with ALD, but not in any normal hepatic tissues[8]. In ALD, iron is deposited in both hepatocytes and reticuloendothelial (Kupffer) cells. In advanced cases of ALD, which is also called as "alcoholic siderosis", the reticuloendothelial iron deposition is dominant. In earlier stages of ALD such as fatty liver and fibrosis, iron deposition is mild and is preferentially present in hepatocytes rather than in Kupffer cells, which finding is more frequently observed in Japanese patients who have mild clinical phenotype comparing with those in US[9].

The reactive oxygen species (ROS) produced play an important role in the development of ALD[10]. The expression of 4-hydroxy-2-nonenal (HNE)-protein adducts, which is a lipid peroxidative product is increased in oxidized hepatocytes[11]. Chronic alcohol ingestion in experimental animals is associated with oxidative stress as reflected by increased hepatic levels of lipid peroxidation products such as malondialdehyde and HNE, both of which have been implicated in hepatic fibrogenesis in the intragastric ethanol infusion model[12]. Furthermore, lipid peroxidation products induce gene expression of procollagen α-1 (I) and increase collagen production by several folds in cultured hepatic stellate cell[13]. In human ALD, there is a positive correlation between iron deposition and histological intensity of HNE-protein adduct[14]. As shown in Figure 1, the distribution of HNE-protein adducts and iron granules appeared to be

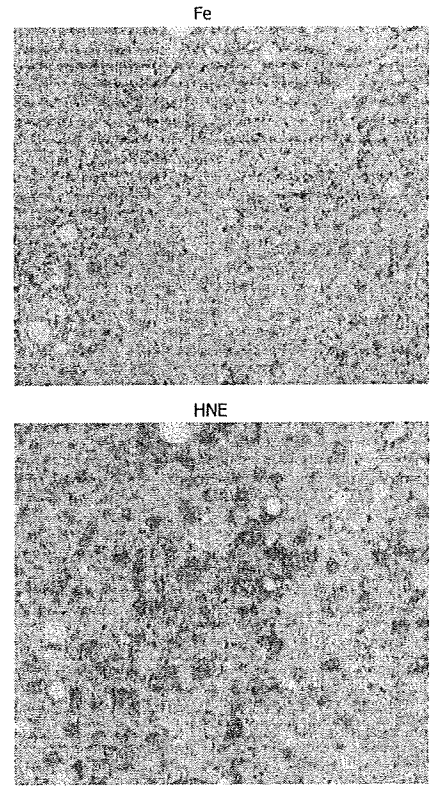

Figure 1 Iron staining and immunohistochemical staining of 4-hydroxy-2-nonenal-modified protein (HNE-protein) adducts in human alcoholic liver disease. The localization of HNE-protein adducts and iron in hepatocytes appeared to be identical (from ref. 14 with some modifications).

identical, suggesting that iron may be associated with the production of HNE-protein adduct. As hepatic iron is visualized by Perls' reaction as an insoluble protein-bound iron such as hemosiderin, this form of iron may be inactive for the production of ROS. But, the free iron responsible for Fenton reaction should be present close to the protein-bound iron, and may be involved in the production of HNE-protein adducts. There are two pathways to generate ROS through ethanol metabolism. Oxidation of ethanol by alcohol dehydrogenase to form acetaldehyde, which is subsequently oxidized to acetate and ultimately carbon dioxide and water. During the oxidation process of acetaldehyde involving aldehyde oxidase and xanthine oxidase, superoxide (O_2^-) is produced[15]. In addition, cytochrome P450 is involved in the metabolism of ethanol, in which ROS are also generated in microsomes[16]. Among ROS, hydroxy radical (OH^-) is most potent, which is produced via Fenton reaction in the presence of free iron and the resulted OH^- can easily cause cell damage by oxidizing lipid, proteins, and nucleic acids. In an intragastric infusion mouse model of ALD, supplementation of carbonyl iron advanced peri-venular fibrosis to bridging fibrosis and cirrhosis[17]. Oxidative stress arising from hepatocytes and macrophage activates hepatic stellate cells by increasing the production of cytokines such as transforming growth factor-β (TGFβ), directly or indirectly. The dietary iron supplementation was associated with increased NF-κB activation[18], and the up

regulation of NF-κB responsive proinflammatory genes such as IL-1β, TNFα, and MIP-1[19].

In advanced cases of ALD, iron is accumulated more prominently in Kupffer cells than in hepatocytes, mainly due to repeated endotoxemia and hyper-cytokinemia of TNFα and IL-1β[20]. These cytokines induced hepatic uptake of transferrin iron *in vitro*[21] and *in vivo*[22]. In mild cases of ALD, iron is preferentially stained in hepatocytes, rather than in Kupffer cells, suggesting that hepatocyte is the main site of early iron storage in the liver. However, it is not clear why iron is accumulated in liver parenchymal cells of alcoholics in such conditions. Two possibilities can be drawn: one is the increased uptake of iron in hepatocytes, and another is the increased iron absorption through hepcidin, which is a newly found antimicrobial peptide, and is a negative regulator of iron absorption and reticuloendothelial iron releases[23]. Hepatocytes have several pathways for iron uptake. Iron in serum is usually bound to transferrin and iron-bound transferrin is taken up via transferrin receptor (TfR) with high affinity or via other unknown mechanism with greater capacity, but low affinity independent of high affinity receptor[24]. There are two molecules of transferrin receptor: transferrin receptor 1 (TfR1) and transferrin receptor 2 (TfR2). TfR1 has a high affinity to serum transferrin and considered to be functional, while the function of TfR2 is not clear yet, even though the TfR2 gene is responsible for genetic hemochromatosis[25]. In normal hepatocytes, TfR2 is constitutively expressed. But, TfR1 is down-regulated, suggesting that TfR1 does not contribute to the steady state hepatic iron uptake. Recently, Wallace *et al* reported that homozygous TfR2 knockout mice had no TfR2 associated with typical iron overload, and there was no upregulation of hepcidin mRNA, suggesting that TfR2 is required to iron regulated expression and is involved in a pathway to HFE and hemojuvelin[26]. In addition, DMT1 may be involved when serum iron concentration exceeds transferrin iron binding capacity[27]. It is noteworthy that TfR1 is regulated by cellular iron levels or oxidative stresses post-transcriptionally and it is possible that ethanol may augment TfR1 expression by producing oxidative stresses. According to immunohistochemical investigation, TfR1 expression was increased in hepatocytes in 80% of hepatic tissues with ALD, but was not detected in any normal hepatic tissues[28]. It is noteworthy that the mean duration of abstinence of patients who demonstrated positive TfR1 expression in hepatocytes was significantly shorter than that of patients who demonstrated negative TfR1 expression.

Ethanol exposure in the presence of iron to the primary cultured-hepatocytes demonstrated an increase of TfR expression, and this augmentation was suppressed by the inhibitor of alcohol dehydrogenase, 4-methyo pyrazole, but enhanced by a inhibitor of acetaldehyde dehydrogenase, cyanamide, suggesting that ethanol metabolite acetaldehyde itself is involved for the induction of TfR1 by ethanol[29]. By functional uptake assay using ^{59}Fe-transferrin, the additional ethanol exposure increased transferrin-iron uptake into hepatocytes, while non-transferrin-bound iron (NTBI) uptake[30] was not increased. It has been reported that TfR1 expression was up-regulated both transcriptionally[31] and posttranscriptio nally[32]. This regulation is induced either by iron deficiency state or oxidative stress such as H_2O_2 and nitric oxide via iron regulatory protein, IRP[33]. In addition to the direct cell toxicity, acetaldehyde produces free radicals[34] and free radicals modify IRP activity[35,36].

Table 2 Speculated effects of iron on HCV

1	Immunological modification (Immunological escape of HCV)
	Decrease of Th1 activity
	Impaired function of macrophage and Kupffer cells
	Decrease of innate immunity (Natural resistance macrophage protein 2)
2	Increase of liver toxicity by iron-mediated radical formation
	Reactive oxygen production through fenton reaction
	Induction of apoptosis
	Acceleration of fibrinogenesis
	DNA damage and carcinogenesis
3	Effect on cell signalling
	Decrease of interferon responsiveness by NFκB activation
4	HCV proliferation
	Activation of translation initiation factor 3 (eIF3)
	Suppression of HCV RNA polymerase (NS5B) activity

Body iron homeostasis is strictly regulated by a balance between the processes such as dietary iron absorption in enterocytes, iron transport by transferrin in circulation, iron utilization and storage in bone marrow and liver. The increase of intestinal iron absorption was one of the mechanisms of the hepatic iron deposition in alcoholics[37]. In patients with hereditary hemochromatosis, serum pro-hepcidin is lower than that of normal controls, suggesting that iron absorption is increased in spite of high iron storage[38]. It is speculated that down-regulation of hepcidin might be one of important factors for pathogenesis of iron overload in ALD[39]. Serum pro-hepcidin concentration in ALD was significantly lower than that in healthy subjects, and pro-hepcidin/ferritin ratios in ALD were lower than healthy subjects[40]. In the ethanol-loaded mouse model which has a mild steatotic change, the hepcidin mRNA and protein expression were significantly lower than that of control. In addition, alcohol-loading might disrupt the sensing signal of inflammatory cytokines, and then down-regulate hepcidin expression, following the increased iron absorption from small intestine. Recently, the mechanism of hepcidin downregulation by alcohol has been elucidated: a decreased hepcidin expression in mouse liver is accompanied with an increase of DMT1 and ferroportin1, and a decrease of hepcidin promoter activity and DNA-binding activity of CCAAT/enhancer-binding, protein α (C/EBPα)[41].

HEPATITIS C VIRUS INFECTION

Hepatitis C virus infection is one of the most common disorders in liver diseases involving chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Table 2 summarizes the effect of iron on hepatitis C virus infection. In the Third National Health and Nutrition Examination Survey, HCV infection is significantly associated with higher serum levels of ferritin and iron in the US population[42]. The mean serum levels of

ferritin and iron were significantly higher among subjects with HCV infection than among subjects without liver disease[43]. In addition, serum ferritin levels were directly and significantly correlated with serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase, whereas platelet counts were inversely correlated with serum ferritin. It is also found that lipid peroxidative products such as malondialdehyde are increased in hepatic tissues with CH-C[44]. In 1994, an initial report was published that phlebotomy was effective in improving the serum ALT level in patients with CH-C[45] and a national prospective study confirmed the results[46]. Since then, it was reported that hepatic iron accumulation in CH-C predict a response to interferon (IFN) therapy[47], and phlebotomy before and during IFN therapy improved virological and histological response to short-term IFN therapy evaluated at the end-of-treatment[48]. This observation is reasonable considering the finding that oxidative stress impairs interferon alpha signal by blocking JAK-STAT pathway[49]. The standard therapy for hepatitis C is now a combined therapy of interferon-α and ribavirin, in which patients with viral response to treatment seemed to develop higher soluble transferrin receptor levels[50] with decline in serum iron and ferritin than non-responders, revealing intracellular reduction of iron store depending on the result of treatment including hemolytic reaction by ribavirin[51]. This is an interesting observation that decrease of iron status may be an additional effect of the combination therapy with interferon and ribavirin. Moreover, HFE mutations are also associated with increased sustained virologic responses by antiviral long-term treatment, while it is well known that HFE mutations are associated with increased iron loading[52]. However, some reports suggest that iron depletion was unable to trigger interferon response, so that there are conflicting data. It should be further investigated whether hepatic iron content modify the response to interferon[53,54]

From these observations, iron and related molecules seem to be key factors in the hepatocytes to influence the disease condition of CH-C, and also development of cirrhosis and maybe hepatocellular carcinoma. Clinical data on phlebotomy on CH-C generally indicates that phlebotomy does not influence the viral load in vivo. On the other hand, in vitro study on HCV replication is controversial: iron promotes HCV translation by up-regulating expression of the translation initiation factor eIF3 by reporter assay[55], whereas iron suppresses HCV replication by inactivating the RNA polymerase NS5B[56].

As previously described, hepatocytes have two iron uptake systems, transferrin-mediated and nontransferrin-bound iron-mediated pathway. Transferrin and TfR1 are molecules involved in the classical pathway of cellular iron uptake, but are faintly expressed in normal hepatocytes, and is down-regulated in iron-loaded hepatic tissues with hemochromatosis. Concerning the post-transcriptional regulation of TfR1, two mechanisms are postulated through the activity change of IRP which is already mentioned. In CH-C, TfR1 expression was up-regulated and DMT1 expression was down-regulated in the condition of hepatic excess iron accumulation, suggesting that regulation of DMT1 expression is iron-dependent, but that of TfR1 expression is iron-independent in CH-C[57]. In patients with CH-C, serum values of inflammatory cytokines such as IL-1β, IL-6, and TNFα have been reported to be high in comparison with those in normal controls. In addition, TfR1 was up-regulated by IL-1β, IL-6, and TNFα in HepG2. Administration of IL-6 augments hepatic uptake of transferrin-bound iron (^{59}Fe), and this is mainly mediated through hepatocytes, but not through Kupffer cells. These results suggest that the up-regulation of TfR1 expression in CH-C might be caused by increase of inflammatory cytokines that proceeded from HCV infection, although there is a possibility that the components of HCV themselves may induce TfR1 expression directly or indirectly.

Like wise, the up-regulated TfR1 might act as a key molecule for hepatic excess iron accumulation in CH-C; however, there are several candidate molecules which cause this condition. For instance, each mutant of HFE, TfR2, hepcidin, hemojuvelin and ferroportin1 (also known as Ireg1 or MTP1) with substitution of amino acid causes the similar phenotype of hemochromatosis. That is, these facts indicate that at least 5 molecules are involved in the familiar hemochromatosis[58]. In hepatocytes, TfR2 predominantly expresses in the normal condition[59] and the disruption of TfR2 gene caused the hepatic iron overload, a phenotype of hemochromatosis, suggesting that TfR2 should also have important role in hepatic iron metabolism[60]. This receptor might act as a sensor of iron status because hepatic TfR2 protein level was increased in iron loaded rats and was decreased in iron deficient rats. Recently, Takeo et al reported that in CH-C TfR2 protein expression is increased parallel with ferroportin1[61], although the meaning of this TfR2 elevation is still to be elucidated[62].

In addition, there was a significant correlation of hepcidin mRNA expression in the liver with hepatic iron concentration and serum ferritin, but did not correlate with ALT, AST, HAI, or viral load. In inflammatory conditions, hepcidin is regulated transcriptionally by IL-6[63] and IL-1β[64] independent of liver iron content. It is noteworthy that, in contrast to other inflammatory states, hepcidin mRNA expression in the liver was independent of markers of inflammation in hepatitis C, suggesting that iron stores in patients with hepatitis C regulate hepcidin expression, and that iron loading in chronic hepatitis C is not due to inappropriate hepcidin expression[65]. However, there is still a controversial result concerning the hepcidin metabolism in chronic hepatitis C that serum pro-hepcidin is down-regulated[66]. The role of hepcidin in chronic hepatits C seems to need further consideration.

The role of iron on the hepatocellular carcinoma (HCC) development in patients with chronic hepatitis C is another major concern. In primary hemochromatosis, iron could be involved in the development of HCC in associated with cirrhosis, suggesting a strong link between heavy iron overload and HCC development. In cases of chronic hepatitis C, it is also known that HCC are developed 20 to 30 years after the infection of hepatitis C virus through the progression of the disease from chronic hepatitis and cirrhosis. In Long-Evans Cinnamon (LEC) rat, an animal model of human

Wilson disease which spontaneously developed hepatitis and liver fibrosis, HCC is frequently developed after the rats have recovered from initial fulminant hepatitis and subsequent liver fibrosis. This is considered to relate to progressive iron accumulation in the animal[67], and iron depletion prevents their development of hepatic cancer[68]. Even though the iron deposition in chronic hepatitis C is mild compared with that in hemochromatosis, iron may be an independent factor on the risk of HCC. It is reported that liver fibrosis is a favorable environment of proliferation of cancer cells by releasing transforming growth factor β, and there is a strong link between liver fibrosis and liver iron deposition. In clinical trials of phlebotomy, the hepatic content of 8-OH deoxyguanosine is decreased and fibrotic score is improved. An important issue in hepatocaricinogenesis in chronic hepatitis C is the closely related sustained production of ROS during inflammation and fibrosis. Moriya *et al* reported that HCC developed in HCV core transgenic mice after the age of 16 mo, and showed high hepatic lipid peroxidation levels in old (more than 16 mo) core transgenic mice, than in control[69]. However, the association of HCV transgenic mice, and HCC development disappeared with advanced passaging of animals, suggesting that HCC development in HCV transgenic mice cannot be simply explained by HCV infection, but requires additional cofactors. A recent study by Furutani *et al* clearly showed that hepatic iron overload induces HCC in transgenic mice expressing HCV polyprotein[70]. Transgenic animal carrying full length polyprotein-coding region (core to NS5B, nts 342-9378) by using pAlb promoter/enhancer was fed with excess iron diet. After 6 mo feeding, the transgenic mice showed marked steatosis and increased 8 hydroxy-2'deoxyguanosine content in association with the hepatic iron accumulation. Twelve months after feeding, 45% of transgenic mice developed hepatic tumors including HCC. It is noteworthy that the steatosis does not accompany with inflammation but a remarkable ultrastructural alteration of mitochondria associated with decreased degradation activity of fatty acids.

STEATOSIS AND INSULIN RESISTANCE

Nonalcoholic steatohepatitis (NASH) is a clinical entity characterized by the development of histopathological changes in the liver that are nearly identical to those induced by excessive alcohol intake, but in the absence of alcohol abuse; the presence of macrovesicular steatosis and mixes inflammatory infiltrate associate with varying amounts of Mallory's hyaline, glycogenated nuclei, and focal hepatocyte ballooning degeneration. Clinical features of NASH include obesity, hyperlipidemia, diabetes mellitus, and hypertension. In US population, approximately 25% is obese, and at least 20% of the obese individuals have hepatic steatosis. Thus, non-alcoholic liver disease (NAFLD) is the most common cause of liver dysfunction, and it is believed that NASH becomes a cause of cryptogenic cirrhosis and hepatocellular carcinoma (HCC). In patients with homozygote of HFE-related hemochromatosis, obesity and steatosis affect liver disease progression, and will be cofactors for iron overload. There is one study of Australia that showed that the prevalence of abnormal genotype of HFE in NASH is 31% compared to a normal prevalence of 13% in the general population, sugget that excess iron might be important. A study on North American subjects showed similar results that the prevalence of the *HFE* gene mutation associated with hereditary hemochromatosis are increasing in patients with NASH[71]. In the study dealing Japanese NASH patients, who had no *HFE* gene mutations, a significant staining of liver iron and increased level of thioredoxin, a marker of oxidative stress in addition to the increase of serum ferritin, was observed.

As diabetes and obesity were background conditions of NAFLD, and is thought to be a initial triggering factor, insulin resistance is now considered the fundamental operative mechanism. Insulin resistance is probably the "first step" in NASH, and a close correlation between insulin resistance and iron is speculated. Even though it is not still clear whether secondary iron accumulation increases insulin resistance, or vice versa, oxidative stress may be the elusive "second" hit of possibly multiple steps in the progression of steatosis to fibrosing steatohepatitis[72]. This may be due to the activation of stellate cells[73].

Because hepatic iron promotes oxidative stress, it seems that iron is a contributory cofactor in NASH. This proposal is strengthened by an association with hepatic fibrosis with NASH[74] and was confirmed by measuring serum markers of oxidative stress[75-77]. Excess hepatic iron also occur in insulin resistance-associated iron overload (IRHIO), characterized by hyperferritinemia with normal to mild increases in transferrin saturation. There is an interesting clinical study that venesections and restricted diet are effective in patients with IRHIO[78]. As in IRHIO, restriction of dietary calories, fat and iron improved NAFLD in addition the decrease of levels of serum aminotransferases and ferritin[79]. It seems that the simultaneous disorder of iron and glucose and/or lipid metabolism, in most cases associated with insulin resistance, is responsible for persistent hyperferritinemia and identifies patients at risk for NASH[80]. However, it is still unclear why iron is deposited in IRHIO and NAFLD. There is an interesting report by Bekri *et al* that there is an increase of hepcidin in adipose tissue of the severely obese but of liver, suggesting that severe obesity itself cause hypoferremia due to the overproduction of hepcidin in the adipocytes[81]. This finding may explain the hypoferremia in severe obese patients, but does not show the mechanism of hepatic iron deposition in IRHIO and NASH. Further studies are needed to clarify this issue, including an increase of transferrin iron influx into hepatocytes in NAFLD.

In patients with NASH, increased transferrin saturation correlated positively with the severity of fibrosis in univariate analysis, although it became insignificant when age, obesity, diabetes, and AST/ALT ratio were controlled. A recent study showed improvement in insulin sensitivity with the use of venesection in 11 patients with NASH. Biweekly phlebotomy until serum ferritin concentration became lower than or equal to 30 ng/mL reduced mean serum ALT activity without a significant change of

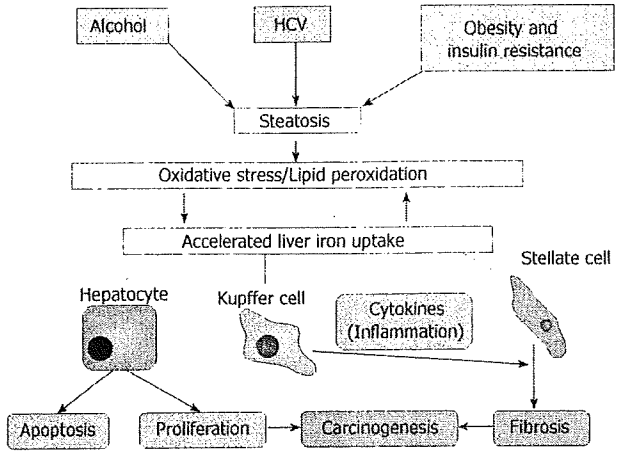

Figure 2 Postulated schema of liver damage occurred by alcohol, HCV infection, obesity and insulin resistant. A common pathway through steatosis/oxidative stress may be responsible for the development of liver fibrosis and carcinogenesis by iron.

body weight, suggesting that iron reduction therapy by phlebotomy will be one of the promising therapies for NASH[82], although this approach cannot be implemented without extensive review.

The natural history of NASH is still unclear, but some patients follow advanced liver fibrosis progressing to cirrhosis and sometimes HCC[83]. It is also known that diabetes increases the risk of hepatocellular carcinoma in US[84]. Further studies are needed to clarify this issue, especially the relation between hepatocarcinogenesis from mild iron accumulation in NASH.

As shown in Figure 2, a common pathway through steatosis/oxidative stress may be present for the development of liver fibrosis and carcinogenesis by iron.

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Hepcidin Is Down-Regulated in Alcohol Loading

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Background: It is common for alcoholic patients to have excess iron accumulation in the liver, which may contribute to the development of alcoholic liver disease (ALD). However, the mechanism of hepatic iron uptake in ALD is still obscure. Recently, a novel iron-regulatory hormone hepcidin was found that suppresses the absorption of iron from the small intestine and the release of iron from macrophages. To elucidate the contribution of hepcidin toward the hepatic excess iron accumulation in ALD, we examined whether alcohol loading affects hepcidin expression both in ALD patients and in an ethanol-fed mouse model.

Methods: Serum prohepcidin concentration was quantified by enzyme-linked immunosorbent assay. Hepatic hepcidin-1 and hepcidin-2 mRNA expressions in mouse liver were evaluated by quantitative real-time reverse-transcriptase polymerase chain reaction method. The protein expression of prohepcidin in mouse liver was examined immunohistochemically by rabbit antimouse prohepcidin antibody.

Results: Serum prohepcidin concentration in ALD was significantly lower than that in healthy subjects ($p<0.001$). Especially, serum prohepcidin concentrations were decreased in the patients whose serum ferritin value was high. In the ethanol-fed mouse model, hepatic hepcidin-1 mRNA expression was significantly lower than that in control ($p = 0.04$). Prohepcidin was expressed in the cytoplasm of hepatocytes of mice liver tissue sections, and its expression was decreased after ethanol loading.

Conclusion: Alcohol loading down-regulates hepatic hepcidin expression and leads to the increase of iron absorption from the intestine.

Key Words: Ethanol, Hepcidin, Iron, Alcoholic Liver Disease.

ALCOHOLIC LIVER DISEASES (ALD) are characterized by fatty liver, fibrosis, and hepatitis, and excess iron is frequently accumulated in the liver tissue (Brissot et al., 1981). In a rat model, a prolonged ethanol loading induced hepatic iron overload (Valerio et al., 1996). Concerning the pathogenesis of ALD, the production of reactive oxygen species (ROS) through ethanol oxidation is considered to play an important role. Among ROS, hydroxy radical (OH^-) is the most potent oxidant, which is produced via the Fenton reaction in the presence of free iron. The resulting hydroxy radicals can easily cause damage to cells by oxidizing lipid, proteins, and nucleic acids. In an intragastric infusion model of ALD, supplementation of carbonyl iron advanced perivenular fibrosis to bridging fibrosis and cirrhosis (Tsukamoto et al., 1995). The dietary iron supplementation was associated with increased nucleating factor-κB (NF-κB) activation (Xiong et al., 2003), and the up-regulation of NF-κB-responsive proinflammatory genes such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (French, 2001). Furthermore, oxidative stress arising from hepatocytes and macrophage may activate hepatic stellate cells by increasing the production of cytokines such as transforming growth factor-β (TGF-β), directly or indirectly (Tsukamoto et al., 1995). We also reported that the localization of 4-hydroxy-2-nonenal (HNE)-protein adducts, which are produced by free radical, and iron in hepatocytes appeared to be identical to ALD (Ohhira et al., 1998).

Concerning the mechanism of hepatic iron accumulation in ALD, we have reported the up-regulation of transferrin receptor 1 expression in hepatocytes by habitual alcohol drinking (Suzuki et al., 2002). This clinical finding was supported by our experimental model showing the induction of transferrin receptor 1 by ethanol in rat primary hepatocyte culture (Suzuki et al., 2004). Thus, the transferrin-mediated uptake of iron is one of the key factors related to excess iron accumulation in hepatocytes (Kohgo et al., 2005). Another important mechanism is the increase of iron absorption from the intestine in chronic alcoholic individuals, but this mechanism is still unknown (Duane et al., 1992). Recently, iron regulatory hormone, hepcidin, which is originally found as a circulating

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antimicrobial peptide produced by hepatocytes and whose expression is regulated by inflammatory stimuli and iron loading, was found to act as a suppressor of iron absorption in the small intestine (Ganz, 2003; Krause et al., 2000; Park et al., 2001). Hepcidin-deficient mouse has a phenotype similar to human hereditary hemochromatosis (Nicolas et al., 2001). This peptide expression was down-regulated in hereditary hemochromatosis that is mainly caused by the increase of iron absorption (Bridle et al., 2003). Therefore, it is possible that the down-regulated hepcidin is one of the important factors in hepatic iron overloads including ALD. However, the level of hepcidin expression in ALD is still unknown. In this study, we firstly evaluated serum prohepcidin level in patients with ALD. We then investigated the expression of both hepatic hepcidin mRNA and prohepcidin protein in an ethanol-loaded mice model.

MATERIALS AND METHODS

Subjects

Serum samples were obtained from 9 healthy individuals and 47 patients with ALD. Alcoholic liver disease consisted of 11 fatty liver, 8 hepatitis, 16 liver fibrosis, and 8 liver cirrhosis in Asahikawa Medical College Hospital and Furano Hospital of Hokkaido Social Service Association. The diagnosis of ALD was according to the Diagnosis Criteria for Alcoholic liver injury, established by the Takada Group in Japanese Ministry of Education (Takada et al., 1993).

Informed consent for this clinical research was obtained from all healthy individuals and patients. We ensured that patients had no infection of hepatitis B and C virus.

Measurement of Serum Prohepcidin Concentration

The serum prohepcidin concentration of each sample was quantified by a sensitive enzyme-linked immunosorbent assay (ELISA; DRG instruments GmbH, Marburg, Germany) (Kulaksiz et al., 2004; Taes et al., 2004).

Acute Ethanol-Loaded Mouse Model

Studies were approved by the Animal Experiment Committee, Asahikawa Medical College. Care of mice, animal experiments, and all reports were carried out in accordance with the rules and guidelines of the Animal Experiment Committee, Asahikawa Medical College. Twelve-week-old male C57BL/6 mice (Charles River Japan Inc., Yokohama, Japan) were housed in a 12-hour light/dark cycle (light on 7 AM), temperature 22 °C, and allowed ad libitum access to diet and water in the Animal experiment unit, Asahikawa Medical College. Mice were loaded with 25% ethanol every 12 hours orally using a sonde and a 1 mL syringe. The amount of loaded ethanol was increased gradually from 5 to 10 g/kg every day. Mice were killed 24 hours after final ethanol loading on day 4. Mice were anesthetized with diethyl ether. Blood was collected by a cardiac puncture to measure serum alanine aminotransferase (ALT). Liver samples were obtained for analyses for histology and quantitative real-time PCR study. Samples for RNA isolation were immediately stabilized in RNA*later* RNA Stabilization Reagent (QIAGEN, Hilden, Germany) for storage.

Biochemical Analysis

Mouse serum ALT was measured by the GOT GPT CII test kit (Wako, Osaka, Japan).

Histopathologic Evaluation

Liver tissue samples were fixed in 10% formalin buffer, embedded in paraffin, and cut. Tissue sections were stained with hematoxylin and eosin (H&E).

RNA Isolation and First-Strand cDNA Synthesis

Total hepatic RNA was isolated from small pieces of liver (20–30 mg) using the QIAGEN RNeasy Mini Kit (QIAGEN). RNA was reverse transcribed using the RETROscript (Ambion Inc., Austin, TX). From each mouse, 1 μg of total RNA was mixed with 2 μL of Random decamers and nuclease-free water in a total volume of 12 μL and heated at 80 °C for 3 minutes. The mixture was then chilled on ice and incubated with 2 μL 10× reverse transcriptase (RT) buffer, 4 μL dNTP mix, 1 μL RNase inhibitor, and 1 μL RT, at 44 °C for 60 minutes. The reaction mixtures were further incubated for 10 minutes at 92 °C. The cDNA was stored at −30 °C until used for real-time PCR.

Quantitative Real-Time PCR

Mouse hepatic hepcidin-1 and hepcidin-2 mRNA expressions and albumin mRNA expression were evaluated in an ethanol-loaded mouse model by the quantitative real-time RT-PCR method. The sequences of primer sets were Hepcidin 1: sense was 5′-CCTATCTC CATCAACAGATG-3′, antisense was 5′-AACAGATACCACACT GGGAA-3′, Hepcidin 2: sense was 5′-CCTATCTCCAGCAAC AGATG-3′, antisense was 5′-AACAGATACCACAGGAGGGT-3′, and albumin: sense was 5′-CTCAGGTGTCAACCCCAA-3′, antisense was 5′-TCCACACAAGGCAGTCTC-3′ (Mazur et al., 2003; Nicolas et al., 2001). For internal control, 18s ribosomal RNA of primer sets (Ambion) was used.

A LightCycler system (Roche Diagnostics GmbH, Penzberg, Germany) was used for quantitative PCR. The PCR profile for Hepcidin consisted of the following steps: 95 °C for 10 minutes to activate FastStart Taq DNA polymerase; 45 cycles each consisting of 95 °C for 10 seconds, 55 °C for 5 seconds, and 80 °C for 10 seconds. For data analysis, the comparative Ct method was used.

Immunohistochemical Study

Frozen tissue sections from mice liver were fixed in 4% paraformaldehyde/PBS and endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in methanol. Sections were then blocked with 2% nonimmune goat serum (DakoCytomation, Carpinteria, CA) in 3% BSA/PBS followed by incubation with 1:100 diluted affinity purified rabbit antimouse prohepcidin antibody (Alpha Diagnostic Intl. Inc., San Antonio, TX) in 3% BSA/PBS, overnight at 4 °C. After washing, sections were incubated with 1:200 diluted biotinylated goat antirabbit antibody in 2% goat serum, and the 3% BSA/PBS ABC-horseradish peroxidase method (Vectastain® Elite ABC kit, Vector Laboratories, Burlingame, CA) and 3,3-diaminobenzidine substrate (DAB Substrate kit, Funakoshi, Japan) were used for detection of antibody according to the manufacturer's instructions. Sections were counterstained with hematoxylin QS (Vector Laboratories). Antibody specificity was confirmed using rabbit nonimmune IgG (DakoCytomation).

Statistical Analysis

The results are expressed as means ± SD. Student's *t*-test or Welch's test was used for statistical analyses of serum biochemical analyses, serum prohepcidin concentration, mouse hepcidin-1, hepcidin-2, and albumin mRNA expressions. Differences in values were considered to be significant when P value < 0.05.

RESULTS

Measurement of Serum Prohepcidin Concentration in ALD

Biochemical analyses of sera in all clinical subjects were performed. Values of serum ALT, γ-glutamyltranspeptidase (GGT), and ferritin of subjects with ALD were higher than those of healthy subjects (respectively, $p = 0.003$, $p < 0.001$, $p < 0.001$ for each comparison) (Figs. 1A–1C). From these data, we confirmed that liver dysfunction and iron overload existed in subjects with ALD.

Serum prohepcidin concentration was measured by using an ELISA system. We performed measurement of serum prohepcidin concentration 3 times using this system and confirmed that the results were reproducible. The serum prohepcidin concentration of healthy subjects and ALD were $1,570 \pm 260$ and 710 ± 540 ng/mL, respectively, showing that the serum prohepcidin concentration in ALD was significantly lower than that in healthy subjects ($p < 0.001$; Fig. 2A). No significant correlation was found in our samples of ALD between prohepcidin and ferritin (Fig. 2B). Prohepcidin/ferritin ratios of healthy subjects and ALD were 13.4 ± 7.5 and 4.8 ± 5.8, respectively, showing that the prohepcidin/ferritin ratio in ALD was significantly lower than healthy subjects ($p < 0.005$; Fig. 2C).

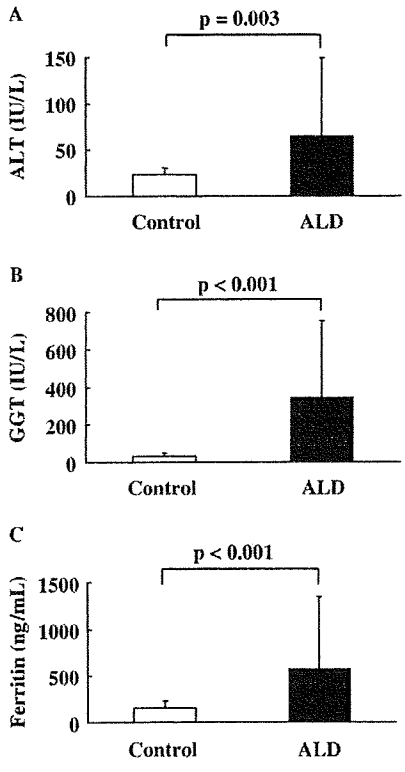

Fig. 1. Biochemical examination of clinical subjects with alcoholic liver disease (ALD). The values of serum alanine aminotransferase (ALT) (**A**), γ-glutamyltranspeptidase (GGT) (**B**), and ferritin (**C**) of subjects with ALD were higher than those of healthy subjects (control). Respectively, $p = 0.003$, $p < 0.001$, $p < 0.001$ for each comparison.

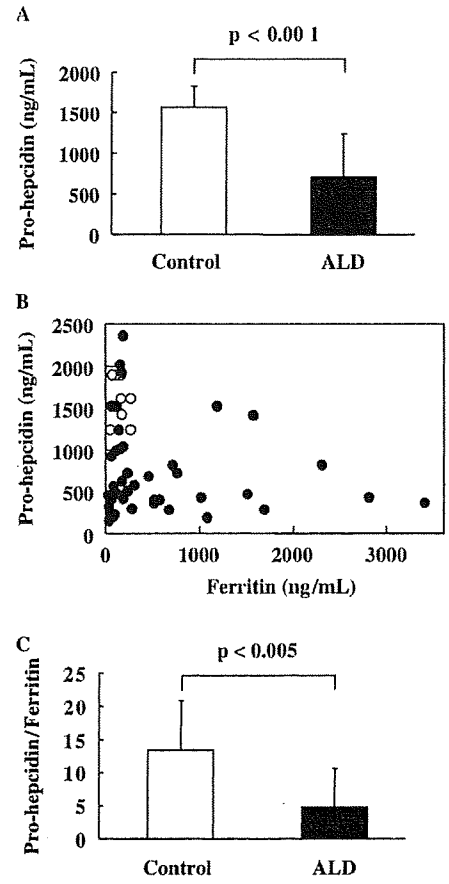

Fig. 2. Evaluation of serum prohepcidin concentration in alcoholic liver disease (ALD). Serum prohepcidin concentration in ALD was significantly lower than that in healthy subjects ($p < 0.001$) (**A**). No remarkable correlation between serum prohepcidin levels and serum ferritin levels was observed in our samples of ALD. Empty circles indicate healthy subjects; filled circles indicate ALD (**B**). The prohepcidin/ferritin ratio in ALD was significantly lower than that in healthy subjects ($p < 0.005$) (**C**).

Hepcidin Expression in the Ethanol-Loaded Mice Model

In the ethanol-loaded mouse model, there was a mild steatotic change in hepatocytes of ethanol-loaded mice with H&E staining. However, there was no inflammatory cell infiltration and necrosis in both lobular and portal regions. (Fig. 3) There was no visible iron deposit in liver tissue by Prussian blue staining under this condition (data not shown). There was no difference in serum ALT level between control and ethanol-loaded mice. Hepatic albumin mRNA expression of ethanol-loaded mice was found to be half of the control using the quantitative real-time PCR method ($p = 0.045$; Fig. 4). In this mouse model, there is a significant metabolic change of lipids and proteins, although there was no significant cytotoxicity for hepatocytes.

Mouse hepcidin has 2 homolog named as hepcidin-1 and hepcidin-2, but only hepcidin-1 can act as iron-regulatory hormone. Mouse hepcidin-1 mRNA expression in liver tissue of ethanol-loaded mice was significantly lower than that of control ($p = 0.04$). Hepcidin-2 mRNA expression

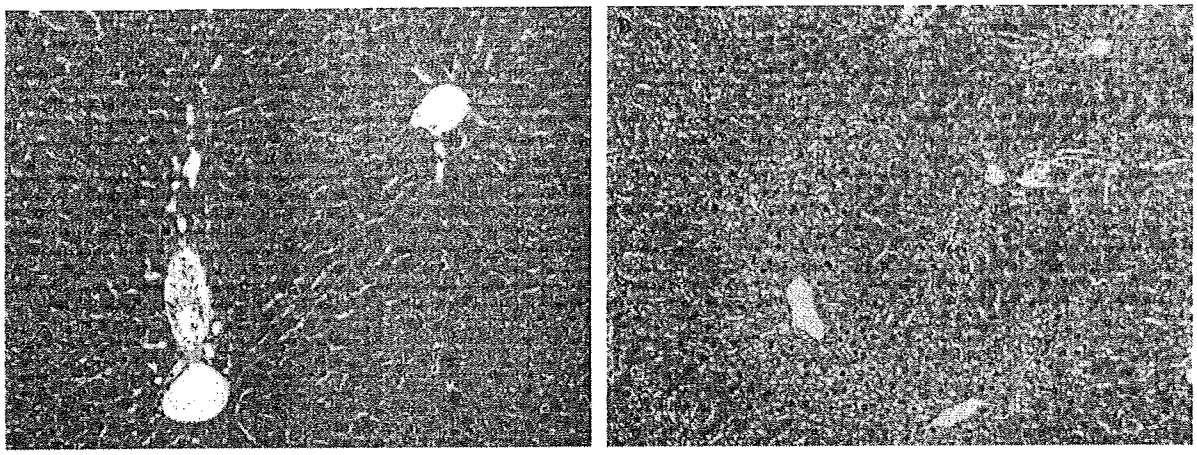

Fig. 3. Histological findings of mouse liver on alcohol loading. There was a mild steatotic change in hepatocytes of alcohol-loaded mice with hematoxylin and eosinstaining. However, there were no inflammatory cell infiltration and necrosis in both lobular and portal regions **(B)**. **(A)** Liver tissue of control mouse. Magnification: ×200.

in liver tissues of alcohol-loaded mice was also lower than that of control ($p = 0.07$; Fig. 5). It is noteworthy that the down-regulation of hepcidin mRNA was more significant than that of albumin mRNA, suggesting that the down-regulated hepcidin mRNA expression was not a result of only the overall decrease of protein synthesis as shown in that of albumin. Next, we investigated the protein expression of prohepcidin by an immunohistochemical study. Hepatocytes of control mice show prohepcidin expression in the cytoplasm as reported previously (Wallace et al., 2005). Prohepcidin was expressed in the cytoplasm of whole intralobular hepatocytes of mice liver tissue sections. Compared with the control, the prohepcidin stain in hepatocytes of ethanol-loaded mice was decreased (Fig. 6). Consequently, the expression of hepcidin in both mRNA and protein level was down-regulated by ethanol loading.

DISCUSSION

In ALD, hepatic iron overload is a well-known phenomenon (Brissot et al., 1981). Actually, in this study, serum ferritin values of patients with ALD, which reflects the body storage iron, were significantly higher than those of healthy subjects in addition to the elevations of serum ALT and GGT. However, the mechanism of hepatic iron overload in ALD has not been elucidated well. Concerning the mechanisms of hepatic iron overload, we must consider both hepatic and intestinal factors in iron metabolism. We have already reported the implication of hepatic up-regulation of transferrin receptor 1 expression in hepatic iron overload of ALD as a hepatic factor (Suzuki et al., 2002, 2004). As another important factor, the increase of iron absorption in the small intestine of chronic alcoholic patients has been reported, but this mechanism is unknown (Duane et al., 1992).

Iron regulatory hormone, hepcidin, which is encoded in the *HAMP* gene and is produced in hepatocytes, suppresses the absorption of iron from the small intestine and the release of iron from macrophage. Moreover, hepcidin is regulated by inflammatory stimuli and iron loading to hepatocytes (Ganz, 2003). The detailed mechanism of the function of hepcidin is still unclear. An inverse relationship has been observed between hepcidin levels and ferroportin levels, which is an iron exporter present on the surface of

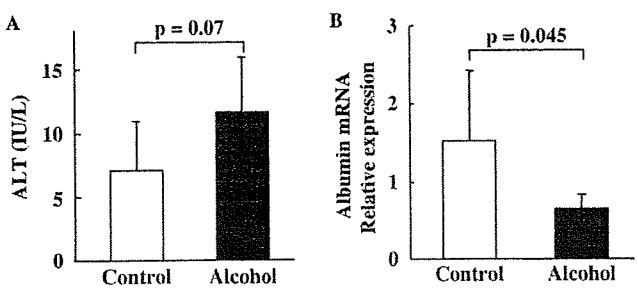

Fig. 4. In an alcohol-loaded mouse model, there was no difference in serum alanine aminotransferase between control and alcohol-loaded mice **(A)**. However, hepatic albumin mRNA expression of alcohol-loaded mice was half that of control ($p = 0.045$) **(B)**.

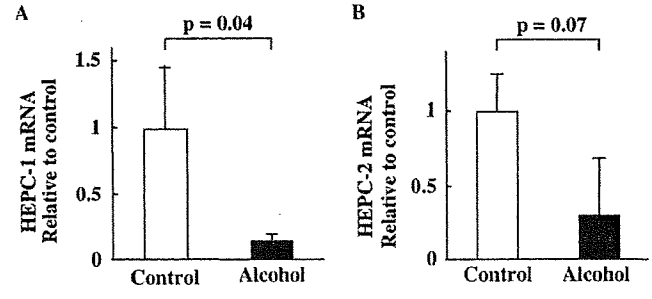

Fig. 5. Mouse hepcidin-1 mRNA expression in liver tissue of alcohol-loaded mice was significantly lower than that of control ($p = 0.04$) **(A)**. Hepcidin-2 mRNA expression in liver tissues of alcohol-loaded mice was lower than that of control ($p = 0.07$) **(B)**.

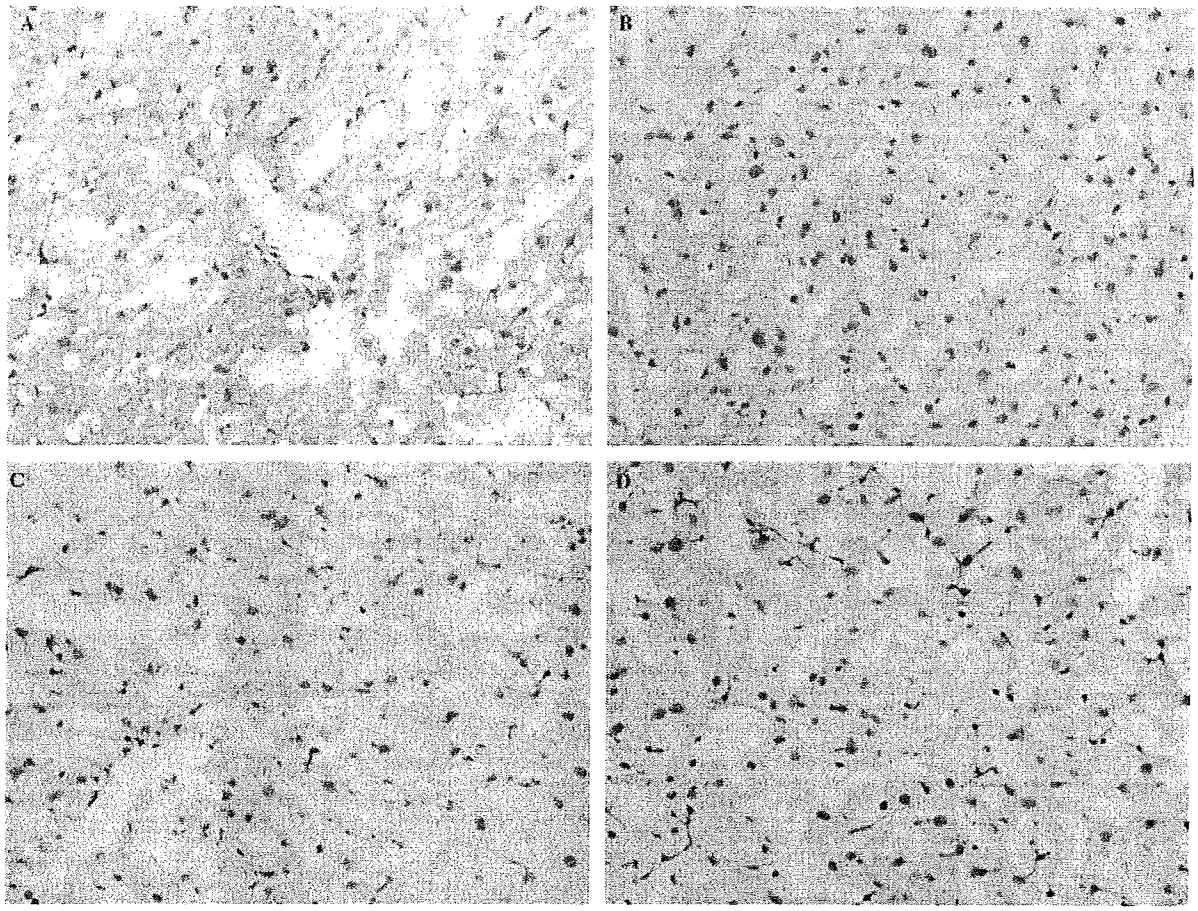

Fig. 6. Immunohistochemical analysis for prohepcidin in the liver with alcohol loading. Whole intralobular hepatocytes of control mice show prohepcidin expression in the cytoplasm (**A**). However, that in hepatocytes of alcohol-loaded mice was decreased compared with control (**C**). (**B**) and (**D**) were incubated in preimmune rabbit IgG as negative control. Magnification: ×400.

enterocytes and macrophages. Nemeth et al. (2004a) demonstrated hepcidin bound to ferroportin in tissue culture cells, resulting in the internalization and degradation of ferroportin, leading to a decrease of export of cellular iron. In brief, hepcidin should be up-regulated in hepatic iron overload to prevent iron overload by decreasing iron absorption. However, in the liver of patients with hereditary hemochromatosis related to the HFE gene or transferrin receptor-2 gene mutation and that in an animal model, hepcidin was down-regulated despite significant hepatic iron overload (Bridle et al., 2003; Kawabata et al., 2005; Nemeth et al., 2005; Nicolas et al., 2003; Pietrangelo et al., 2005; Wallace et al., 2005). In hemochromatosis, iron absorption is increased, and then hyperferremia occurs, and excess iron is accumulated in parenchymal cells of the liver, pancreas, heart, and muscle, etc. As hepcidin is considered to be a key molecule for primary iron overload, there is another possibility that secondary hepatic iron overloads including ALD also may be caused by secondary dysfunction of hepcidin expression.

It is very difficult to quantify the level of hepcidin peptide in a sample, because active hepcidin peptide is 25 amino acid sequence including 8 cysteines that form 4 disulfide bonds, and has a hairpin amphipathic structure (Hunter et al., 2002). There is no report about a convenient method for quantification of an active form of hepcidin peptide, except Western blot analysis (Dallalio et al., 2003; Nemeth et al., 2003). Instead, the ELISA system for quantification of prohepcidin, which is a proregion sequence of an inactive form, has been reported (Kulaksiz et al., 2004). In this study, we used this ELISA system and investigated the concentration of prohepcidin in subjects with ALD as a precursor form. It was reported that urinary hepcidin excretion was positively correlated with serum ferritin, reflecting the increase of hepcidin production in iron-overloaded livers in patients with iron overload, infections, or inflammatory diseases (Nemeth et al., 2003). It is noteworthy in this study that the serum prohepcidin level in ALD was more suppressed than healthy subjects. Furthermore, serum prohepcidin concentrations were especially decreased in some patients, whose serum ferritin value was high, as seen in patients with untreated hereditary hemochromatosis. As a ratio of serum prohepcidin and serum ferritin (prohepcidin/ferritin ratio) has

been shown to have excellent performance in estimating iron metabolism condition in hereditary hemochromatosis, we adapted this formula for ALD. In patients with ALD, our study showed that a prohepcidin/ferritin ratio was also significantly lower than healthy subjects as seen in patients with primary hemochromatosis. Therefore, we speculate that down-regulation of hepcidin might be one of the important factors for pathogenesis of iron overload in ALD.

To confirm these clinical data, we further investigated whether ethanol loading suppressed the expression of hepcidin in liver tissue using a quantitative real-time PCR system. Hepcidin mRNA expression in the liver of an alcohol-loaded mouse was decreased. Concerning the pathological findings of this alcohol-loaded mouse liver tissue, hepatic steatosis was present that is compatible with the early stage of ALD frequently observed in human alcoholic individuals. Accumulation of small lipid droplets in hepatocytes was observed in this acute alcohol-loaded mouse model. However, there was no infiltration of inflammatory cells and necrosis of hepatocytes. As the serum ALT level was also in the normal range, the cytotoxicity to hepatocytes by alcohol loading in this model could be ignored and alcohol-induced liver injury by more than a moderate degree did not occur in this model. It is also noteworthy that there is no visible iron deposit in liver tissue by Prussian blue staining, suggesting that hepcidin down-regulation may be an early event of ALD before a significant iron deposition occurs in the liver. It is noteworthy that the down-regulation of hepcidin mRNA was more significant than that of albumin mRNA, suggesting that the down-regulated hepcidin mRNA expression was not a result of only the overall decrease of protein synthesis as shown in that of albumin. However, we have to be careful while concluding that the down-regulation of hepatic hepcidin mRNA in this alcohol-loaded mouse model is specific for ethanol at this time.

Concerning the localization of prohepcidin in hepatocytes, previous studies have suggested that prohepcidin is expressed in the nucleus (Pigeon et al., 2001). However, hepcidin was detected in the cytoplasm of HepG2 cells, whereas in liver tissue sections hepcidin expression was observed in the basolateral membrane of hepatocytes (Kulaksiz et al., 2004). Recently, Wallace et al. (2005) demonstrated that prohepcidin localized to the secretory pathway, primarily the Golgi apparatus in liver cells and tissues. We could also demonstrate that prohepcidin is expressed in the cytoplasm of whole intralobular hepatocytes of mice liver, confirming Wallace's finding.

Regulation of hepcidin expression is still unclear. Hepcidin expression is up-regulated by inflammatory stimuli (Nicolas et al., 2002) and iron loading (Mazur et al., 2003). Furthermore, proinflammatory cytokines (e.g., IL-6, IL-1; Lee et al., 2005; Nemeth et al., 2004b) were formerly reported as inducers for hepcidin. The mouse genome contains 2 highly similar hepcidin genes: hepcidin 1 and hepcidin 2. Although hepcidin-1 is key molecule of iron metabolism, hepcidin-2 does not act on iron metabolism like hepcidin-1, which may act only as an effector molecule for innate immunity (Lou et al., 2004). In this study, both hepcidin-1 and hepcidin-2 were down-regulated. So change of stimuli of cytokines through alcohol metabolism is considered to be the main mechanism of down-regulation of hepcidin expression by ethanol loading rather than change of stimulus of iron.

Stimulation of IL-6 or IL-1 up-regulates hepcidin expression both in vitro and in vivo, whereas TNF-α down-regulates it (Lee et al., 2005; Nemeth et al., 2004b). We measured the serum IL-6 and TNF-α concentration of ethanol-loaded mice by an ELISA system. However, there was no difference in these cytokines between control and ethanol-loaded mice (data not shown). Kupffer cells derive cytokines in liver tissue to play important paracrine roles in hepatocytes. Actually, it is reported that the mRNA level of cytokines in isolated Kupffer cells from a rat model of ALD have changed and the cytokines have important roles in the pathogenesis of ALD (Kamimura et al., 1995). However, it is noteworthy that in depleted Kupffer cells by liposome-encapsulated clodronate, iron, and inflammation are able to induce hepcidin gene expression independently of Kupffer cells (Lou et al., 2005; Montosi et al., 2005). Therefore, hepatocytes might play a key role in the regulation of hepcidin gene expression by sensing iron and inflammatory signals. Alcohol loading might disrupt the sensing signal of inflammatory cytokines. Then, it might down-regulate hepcidin expression, following the increased iron absorption from the small intestine. Further studies are needed to clarify these issues.

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