RBV dose was associated with a stepwise increase in relapse rate from 11% to 60% (Fig. 3).

Improving the treatment tolerability for genotype 2 or 3 patients has focused on dose reduction of treatment drugs. Weiland *et al.* examined low-dose PEG-IFN-α-2a (135 μg/week) with a weight-based standard dose of RBV (11 mg/kg daily) for genotype 2 and 3 patients.[158] Recently, Inoue *et al.* reported neither PEG-IFN nor RBV drug exposure were critical in reaching rapid virological response and SVR.[159]

Recommendation 23: In genotype 1 patients, PEG-IFN is dose-dependently correlated with c-EVR, independent of RBV dose. The administration over 80% of the scheduled dose of PEG-IFN-α-2a or over 1.2 μg/kg per week of PEG-IFN-α-2b should be chosen as a starting dose: a marked dose reduction of PEG-IFN should not be risked at the start even for patients with disadvantage (e.g. aged patients). (Level 2b/3, Grade B.)

Recommendation 24: In genotype 1 patients, RBV shows a dose-dependent correlation with the relapse after treatment. Maintaining the RBV dose over 80% of the scheduled dose or over 10 mg/kg per day (12 mg/kg per day, if possible) during the complete treatment period can lead to suppression of the relapse in HCV genotype 1 patients responding to PEG-IFN-α-2b plus RBV, especially in c-EVR patients. (Level 2b/3, Grade B.)

Recommendation 25: In genotype 2/3 patients, reducing drug doses of PEG-IFN and RBV (down to 400 mg/day) has no significant effect on virological responses. (Level 2a, Grade B.)

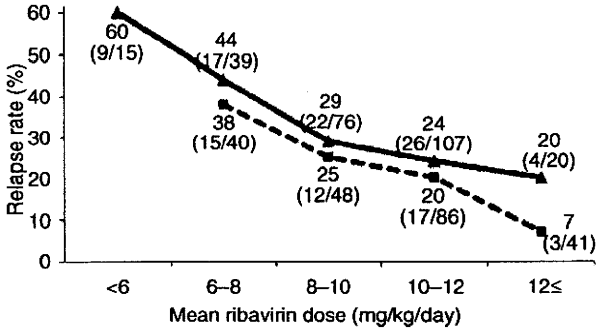

Figure 3 Relapse rate according to pegylated interferon (PEG-IFN)-α-2b and ribavirin doses during treatment of patients who completed treatment, which was stratified with the mean ribavirin doses (-▲-). Group with the mean PEG-IFN dose <1.4 μg kg/week (···■···). Group with the mean PEG-IFN dose ≥1.4 μg kg/week. There was no significant difference between the two PEG-IFN-α-2b-dose groups ($P = 0.17$).

Treatment for patients without elimination of HCV

Tarao *et al.* showed the rate of HCC appearance was significantly higher in HCV-related cirrhotic patients with a high ALT value (≥80 IU/mL) than in those with a lower ALT value (<80 IU/mL).[70] This suggested that suppression of inflammation in the liver with HCV infection is very important to prevent the hepatocarcinogenesis in patients with HCV-related cirrhosis.

Omata *et al.* assessed the effects of oral ursodeoxycholic acid (UDCA) on serum biomarkers. CH-C patients with elevated ALT were assigned randomly to 150 ($n = 199$), 600 ($n = 200$) or 900 mg/day ($n = 197$) UDCA intake for 24 weeks. As a result, the median changes in serum ALT at the end of treatment were shown to be −15.3, −29.2 and −36.2%, respectively, although serum HCV RNA did not change in any group.[160]

A glycyrrhizin product, Stronger Neo-Minophagen C (SNMC; Minophagen Pharmaceutical, Tokyo, Japan), is used widely in Japan and has been reported to improve ALT levels and liver inflammation.[161,162] Furthermore, Ikeda *et al.* reported liver carcinogenesis was suppressed by long-term administration of glycyrrhizin, using a cohort of 1249 patients, and its favorable effect on hepatocellular carcinogenesis in those patients with IFN-resistant CH-C.[163,164]

Repeated phlebotomy has been shown to be effective for the improvement of serum ALT as well as progression of fibrosis,[32] however, it remains controversial whether the effects of IFN improve with extensive phlebotomy.[165–169]

In Japan, Yano *et al.* showed the iron removal by repeated phlebotomy improved serum ALT levels in patients with CH-C.[170]

Recommendation 26: Patients whose HCV RNA was not eradicated by PEG-IFN plus RBV and whose ALT and/or AFP levels were not improved by IFN monotherapy or those without indication for IFN therapy should be treated with the liver-supporting therapy (SNMC, UDCA), and if the effect of this medication is inadequate, phlebotomy can be used in combination. (Level 3/6, Grade B/C.)

Treatment of patients with decompensated cirrhosis

The compensated patients who failed to eradicate HCV by antiviral therapy and decompensated patients should be referred for consideration of liver transplantation and liver supporting therapy should be performed. Long-

term nutritional supplementation with oral branched-chain amino acid (BCAA) has been shown to be useful to prevent progressive hepatic failure and to improve surrogate markers.[171,172] Early interventional with oral BCAA was shown to prolong the liver transplant waiting period by preserving hepatic reserve in cirrhosis.

Recommendation 27: Patients with compensated cirrhosis for the prevention of hepatocellular carcinogenesis, should be treated by not only IFN but also with liver supporting therapy (SNMC, UDCA) and/or phlebotomy and/or BCAA in order to improve the liver inflammation and AFP levels. (Level 3, Grade C.)

Novel antiviral drugs

Telaprevir, a protease inhibitor specific to the HCV nonstructural 3/4A serine protease, reduced HCV RNA levels rapidly in early studies. McHuthison *et al.* reported the improved SVR rate with triple therapy for 12 weeks followed by PEG-IFN-α-2a and RBV for 12 weeks.

Thus, the treatment for CH-C is progressing. Therefore, as a treatment strategy, PEG-IFN plus RBV combination therapy should be performed early for aged patients and the patients with the advanced fibrosis. However, the novel antiviral drugs, such as protease inhibitors and polymerase inhibitors, should be taken into account as a candidate of treatment for the patients who can wait for the oncoming drugs.

Recommendation 28: Novel antiviral drugs, such as a protease inhibitor or a polymerase inhibitor, in combination with PEG-IFN plus RBV, can improve the SVR rates in genotype 1 CH-C patients. (Level 2a, Grade A.)

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BASIC STUDIES

Hepatitis C virus protein and iron overload induce hepatic steatosis through the unfolded protein response in mice

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Keywords
endoplasmic reticulum stress – hepatic steatosis – hepatitis C virus – iron – unfolded protein response

Abbreviations
ATF6, activating transcription factor 6; CHOP, CCAAT/enhancer-binding protein homology protein; CPTI, carnitine palmitoyl transferase I; ER, endoplasmic reticulum; FAS, fatty acid synthetase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IRE1, inositol-requiring enzyme1; NAC, *N*-acetyl cysteine; p-eIF2α, phosphorylated eukaryotic initiation factor-2α; PERK, PKR-like ER kinase; PKR, RNA-activated protein kinase; ROS, reactive oxygen species; SCAP, SREBP cleavage-acting protein; SREBP, sterol-regulatory element-binding protein; XBP-1, X-box DNA-binding protein 1.

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Abstract

Background/Aim: Hepatic iron overload and steatosis play critical roles in the progression of hepatitis C virus (HCV)-associated chronic liver disease. However, how these two pathophysiological features affect each other remains unknown. The aim of this study was to investigate how hepatic iron overload contributes to the development of hepatic steatosis in the presence of HCV proteins. *Methods:* Male C57BL/6 transgenic mice expressing the HCV polyprotein and nontransgenic littermates were fed an excess-iron diet or a control diet. Mice in each group were assessed for the molecules responsible for fat accumulation in the liver. *Results:* Hepatic iron levels were positively correlated with triglyceride concentrations in the liver for all mice. As compared with the livers of nontransgenic mice fed the control diet, the livers of transgenic mice fed the excess-iron diet showed a lower expression of carnitine palmitoyl transferase I, a higher expression of sterol-regulatory element-binding protein 1 and fatty acid synthetase and an activated unfolded protein response indicated by a higher expression of unspliced and spliced X-box DNA-binding protein 1 (XBP-1), phosphorylated eukaryotic initiation factor-2α (p-eIF2α), CCAAT/enhancer-binding protein homology protein (CHOP) and abundant autophagosomes concomitant with increased production of reactive oxygen species. Six-month treatment with the anti-oxidant N-acetyl cysteine dramatically reduced hepatic steatosis in transgenic mice fed the excess-iron diet through decreased expression of unspliced and spliced XBP-1, p-eIF2α, and CHOP. *Conclusions:* The iron-induced unfolded protein response appears to be one of the mechanisms responsible for fat accumulation in the liver in transgenic mice expressing the HCV polyprotein.

Hepatic steatosis and iron overload are not only the pathophysiological features of hepatitis C virus (HCV)-associated chronic liver disease (1, 2) but also risk factors for the development of hepatocellular carcinoma (HCC) (3, 4). Thus, these pathophysiological features appear to play critical roles in the pathogenesis of HCV-associated chronic liver disease. The mechanisms underlying HCV-related steatosis are diverse. HCV core protein has been demonstrated to inhibit microsomal transfer protein activity and very low-density lipoprotein secretion (5) and to upregulate the promoter activity of sterol-regulatory element-binding protein (SREBP) 1c, a transcription factor involved in lipid synthesis (6). Persistent activation of peroxisome proliferator-activated receptor α has also been reported to be essential for the development of hepatic steatosis in transgenic mice expressing the HCV core protein (7). As for hepatic iron overload, we have shown that HCV-induced reactive oxygen species (ROS) increase the hepatic iron concentration by reducing hepcidin transcription in transgenic mice expressing the HCV polyprotein (8), and that even modest iron supplementation results in the development of liver tumours,

including HCC, through mitochondrial injury in these transgenic mice (9). However, it remains unknown how these two pathophysiological features affect each other in the progression of HCV-associated chronic liver disease. In our previous study, marked hepatic steatosis was observed at 6 months after commencement of iron overloading in transgenic mice, which was followed by the development of liver tumours. These results clearly indicated that hepatic iron overload was involved in the development of hepatic steatosis in the presence of HCV proteins. The aim of this study was to investigate how hepatic iron overload contributes to the development of hepatic steatosis in transgenic mice expressing the HCV polyprotein. In the present study, we report that iron-induced ROS-activated unfolded protein response may be postulated as one of the possible mechanisms of HCV-related fat accumulation in the liver.

Materials and methods

Animals

The transgene pAlbSVPA-HCV, containing the full-length polyprotein-coding region under the control of the murine albumin promoter/enhancer, was described in detail (10, 11). HCV polyprotein has been demonstrated to be processed into individual proteins in the liver and to be expressed at a biologically relevant level in which transcripts of RNA encoding the complete viral polyprotein are detectable only by a reverse-transcription polymerase chain reaction (11). Of the four transgenic lineages with evidence of RNA transcription of the full-length HCV-N open reading frame (FL-N), the FL-N/35 lineage proved capable of breeding in large numbers. There is no inflammation in the transgenic liver (11). Male FL-N/35 transgenic mice and age-matched C57BL/6 mice (control mice) were fed a normal rodent diet including carbonyl iron (45 mg/kg diet, control diet) or an excess-iron diet (carbonyl iron 225 mg/kg diet) at the age of 8 weeks, bred, maintained and killed by an intraperitoneal injection of 10% pentbarbital sodium preceded by 12-h fasting at 12 months after initiation of feeding according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. As another experiment, six FL-N/35 transgenic mice were fed the control diet for 6 months, and then they were divided into two groups: three fed the excess-iron diet for 6 months with administration of *N*-acetyl cysteine (NAC) and those without NAC. NAC was contained in drinking water (1 g/L).

Hepatic iron and triglyceride content

Iron concentrations in the livers were measured by atomic absorption spectrometry (Hitachi Z-6100, Hitachi Ltd., Tokyo, Japan), as described previously (9), and expressed as μg Fe/g of tissue (wet weight). Lipids were extracted from the homogenized liver tissue by the method of Bligh and Dyer (12). The triglyceride levels were measured with a TGE-test Wako kit (Wako Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions. The protein concentrations in the liver were determined by the method of Lowry *et al.* (13), using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting

Lysates of liver were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), blocked overnight at 4 °C with 5% skim milk and 0.1% Tween 20 in Tris-buffered saline and subsequently incubated for 1 h at room temperature with an anti-human ferritin antibody (Dako, Glostrup, Denmark), anti-rabbit carnitine palmitoyl transferase I (CPT I) antibody, anti-rabbit CPT II antibody (Alpha Diagnostic International, San Antonio, TX, USA), anti-rabbit SREBP1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-rabbit fatty acid synthetase (FAS) antibody (Cell Signaling Technology Inc., Boston, MA, USA), anti-mouse X-box DNA-binding protein 1 (XBP-1) antibody (Santa Cruz Biotechnology Inc.) or anti-bacterially expressed, mouse CCAAT/enhancer-binding protein homology protein (CHOP) fusion protein antibody (Abcam, Cambridge, England). Exceptionally, the proteins were blocked for 1 h at room temperature and subsequently incubated overnight at 4 °C with an anti-rabbit phosphorylated eukaryotic initiation factor-2α (p-eIF2α) antibody (Cell Signaling Technology Inc.).

Histological staining

A portion of liver was immediately snap frozen in liquid nitrogen for determination of hepatic triglyceride and iron concentrations. The remaining liver tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin for histological analysis. Liver sections were stained with haematoxylin and eosin.

Electron microscopy

Liver specimens were fixed in 2.1% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol and propylene dioxide and embedded in Epok. Thick sections (1 μm in width) were stained with toluidine blue to identify steatosis by light microscopy. Thin sections were stained with uranyl acetate and lead citrate, and examined using a Hitachi-7000 transmission electron microscope (Hitachi Ltd.).

In situ detection of reactive oxygen species

In situ ROS production in the liver was assessed by staining with dihydroethidium, as described previously (8). In the presence of ROS, dihydroethidium (Invitrogen Corp., Carlsbad, CA, USA) is oxidized to ethidium bromide and stains nuclei bright red by intercalating with the DNA (14). Fluorescence intensity was quantified using NIH image analysis software for three randomly selected areas of digital images in each mouse.

Statistical analysis

Quantitative values are expressed as mean ± SD. Two groups among multiple groups were compared by the rank-based, Kruskal–Wallis ANOVA test, followed by the Scheffé test. Data between two groups were compared by Student's *t*-test. The statistical significance of correlation was determined by the use of a simple regression analysis. A P value of < 0.05 was considered to be significant.

Results

Correlation between iron and triglyceride contents in the liver

Dietary intake and body weight were measured every 4 weeks until 12 months after commencement of iron loading, and these parameters did not differ significantly among any of the 4 groups. The hepatic iron content (267 ± 94 μg/g liver weight) of FL-N/35 transgenic mice fed the excess-iron diet was significantly greater than that of nontransgenic and FL-N/35 transgenic mice fed the control diet at 12 months after commencement of iron loading (Fig. 1A), and was comparable to that of a large number of patients with chronic hepatitis C in extensive studies (15, 16). The hepatic ferritin level of FL-N/35 transgenic mice fed the excess-iron diet was significantly greater than that of nontransgenic mice fed the control diet (Fig. 1B). The hepatic iron content was positively correlated with the hepatic triglyceride concentration when both parameters were compared for all mice ($r = 0.63$, $P = 0.002$, Fig. 1C). These results were consistent with our previous observation that FL-N/35 transgenic mice fed the excess-iron diet demonstrated the most severe steatosis in the liver among the four groups (9).

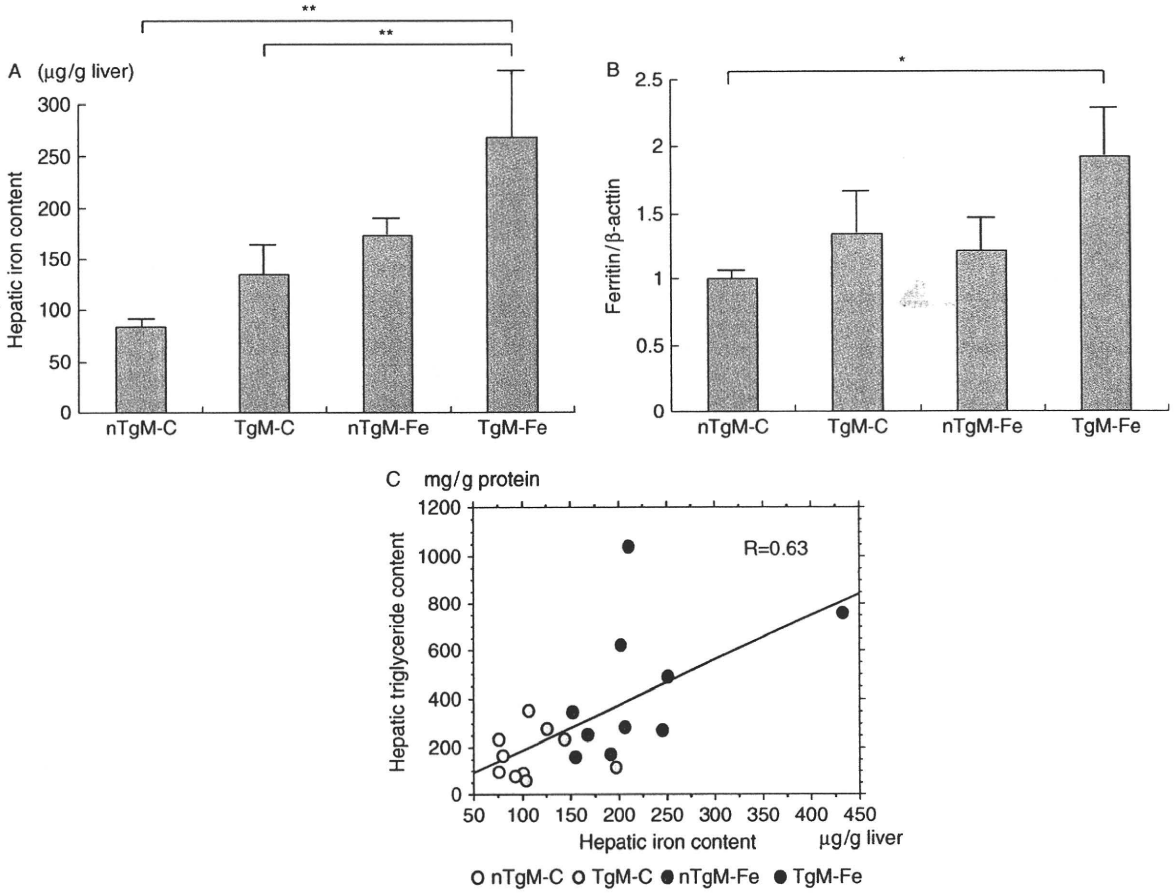


Fig. 1. Hepatic iron contents and ferritin levels, and correlation between iron and triglyceride contents in the liver. (A) The hepatic iron content was measured by atomic absorption spectrometry in five mice in each group at 12 months after initiation of iron loading. (B) Immunoblots for ferritin were performed using liver lysates obtained from four mice in each group. The protein expression was normalized with β-actin. (C) The correlation between hepatic iron and triglyceride levels was determined in 20 mice from four groups. nTgM-C: nontransgenic mice fed the control diet, nTgM-Fe: nontransgenic mice fed the excess-iron diet, TgM-C: FL-N/35 transgenic mice fed the control diet, TgM-Fe: FL-N/35 transgenic mice fed the excess-iron diet. $^{*}P < 0.05$, $^{**}P < 0.01$.

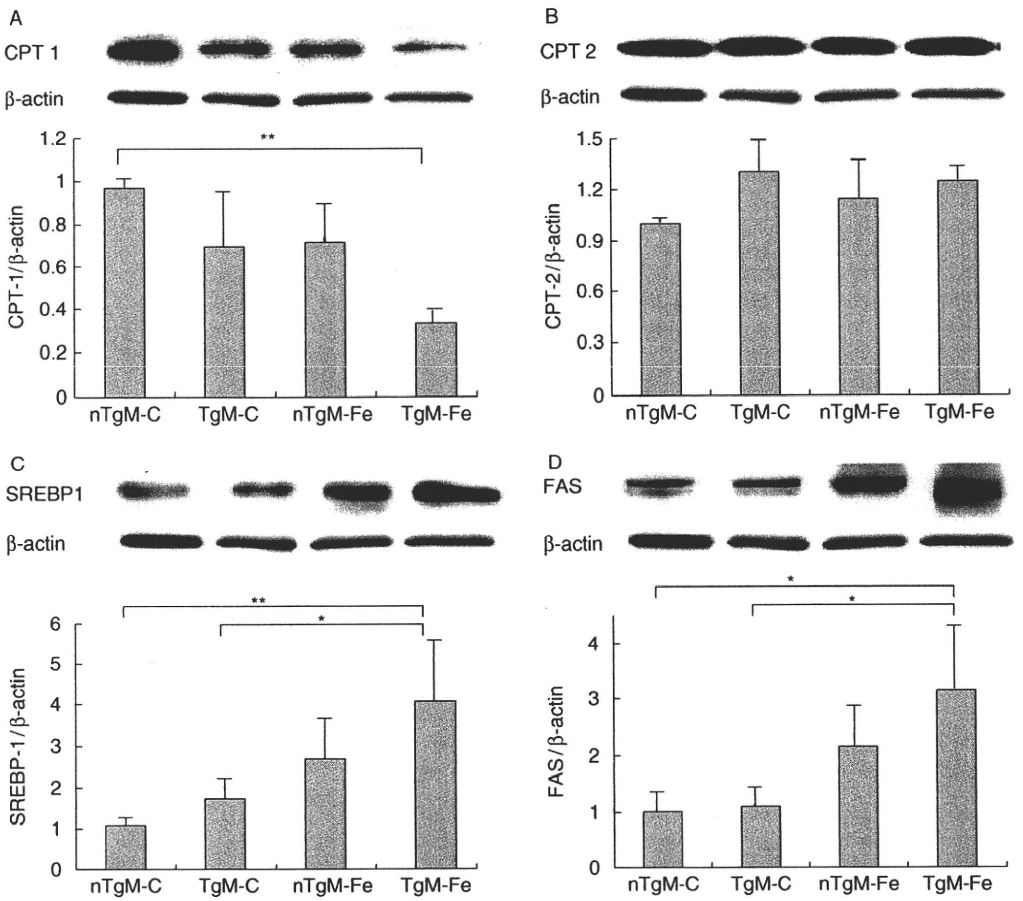


Fig. 2. Expression of carnitine palmitoyl transferase I (CPT1), carnitine palmitoyl transferase I (CPT2), sterol-regulatory element-binding protein I (SREBP1) and fatty acid synthetase (FAS) in the liver. Immunoblots for CPT1 (A), CPT2 (B), SREBP1 (C) and FAS (D) were performed using liver lysates obtained from four mice in each group at 12 months after initiation of iron loading. The protein expression was normalized with β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$. nTgM-C, TgM-C, nTgM-Fe and TgM-Fe; see legend for Figure 1.

Decreased expression of carnitine palmitoyl transferase I and increased expression of sterol-regulatory element-binding protein 1

As we previously reported reduced oxidation activity of fatty acid in iron-overloaded transgenic mice (9), we first examined the expression levels of CPT1 and CPT2, which regulate oxidation of long-chain fatty acids in the mitochondria. The expression of CPT1, but not CPT2, was significantly reduced in FL-N/35 transgenic mice fed the excess-iron diet compared with the nontransgenic mice fed the control diet ($P = 0.0003$, Fig. 2A and B). We next examined the expression level of SREBP1, a transcription factor that activates the genes required for lipogenesis (17), and FAS, a target gene of SREBP1. As shown in Figures 2C and D, the expression of SREBP1 and FAS was significantly greater in FL-N/35 transgenic mice fed the excess-iron diet than in nontransgenic and FL-N/35 transgenic mice fed the control diet, suggesting the involvement of activated lipogenesis in hepatic steatosis in FL-N/35 transgenic mice fed the excess-iron diet. It should also be noted that modest iron supplementation significantly activated lipogenesis in FL-N/35 transgenic mice, but not in nontransgenic mice.

Activated unfolded protein response

Upon endoplasmic reticulum (ER) stress, the SREBP–SREBP cleavage-acting protein (SCAP) complex dissociates from the ER retention protein and subsequently translocates to the Golgi apparatus, where SREBP is cleaved and activated (18, 19). We therefore investigated whether increased expression of SREBP1 was related to ER stress. The unfolded protein response-signalling cascades are initiated by three ER-resident sensors: inositol-requiring enzyme 1 (IRE1), RNA-activated protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (20). IRE1 activation splices unspliced XBP-1 (uXBP-1) to form spliced XBP-1 (sXBP-1) mRNA (21),

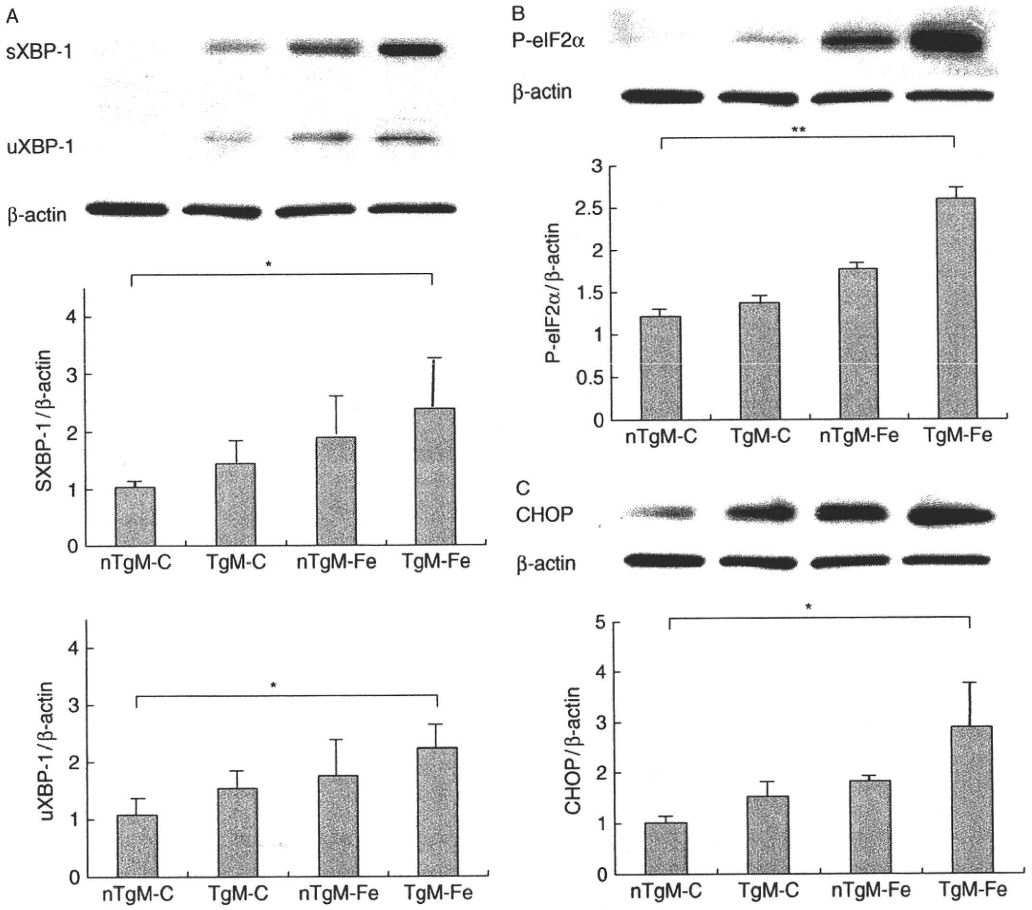


Fig. 3. Expression of spliced X-box DNA-binding protein 1 (sXBP-1), unspliced X-box DNA-binding protein 1 (uXBP-1), phosphorylated eukaryotic initiation factor-2α (p-eIF2α) and CCAAT/enhancer-binding protein homology protein (CHOP) in the liver. Immunoblots for sXBP-1 and uXBP-1 (A), p-eIF2α (B) and CHOP (C) were performed using liver lysates obtained from seven full-length HCV-N open reading frame (FL-N/35) transgenic mice fed the excess-iron diet and four mice in the three other groups at 12 months after initiation of iron loading. The protein expression was normalized with β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$. nTgM-C, TgM-C, nTgM-Fe, and TgM-Fe; see legend for Figure 1.

and was assessed with the sXBP-1 protein level (22). PERK activation was evaluated by measurement of p-eIF2α and CHOP (23). ATF6 activation was assessed with uXBP-1 expression (24). The expression of uXBP-1, sXBP1, p-eIF2α and CHOP was significantly greater in FL-N/35 transgenic mice fed the excess-iron diet than that in nontransgenic mice fed the control diet (Fig. 3).

Autophagy

We next examined the formation of autophagosomes at the ultrastructural level to confirm the activation of the unfolded protein response, because autophagy has been shown to be induced by the unfolded protein response (25–27). As shown in Figure 4, autophagosomes (indicated by arrows) were abundantly found in the liver in FL-N/35 transgenic mice fed the excess-iron diet. In contrast, autophagosomes were not present in the liver of nontransgenic mice fed the excess-iron diet. These results were compatible with the increased expression of uXBP1, sXBP1, p-eIF-2α and CHOP in FL-N/35 transgenic mice fed the excess-iron diet, suggesting activation of the unfolded protein response. Thus, activation of the unfolded protein response appeared to be involved in the development of hepatic steatosis in FL-N/35 transgenic mice fed the excess-iron diet.

Reactive oxygen species generation and endoplasmic reticulum stress

Superoxide has been reported to be selectively involved in ER stress-mediated apoptosis (28). It is also reported that anti-oxidants reduce ER stress and improve protein secretion (29). These results suggest that ROS production induces ER stress. We evaluated *in situ* ROS production in the liver by staining with dihydroethidium and assessed whether treatment with an anti-oxidant reduced hepatic steatosis through inhibition of the unfolded protein response. ROS production was significantly higher in FL-N/35

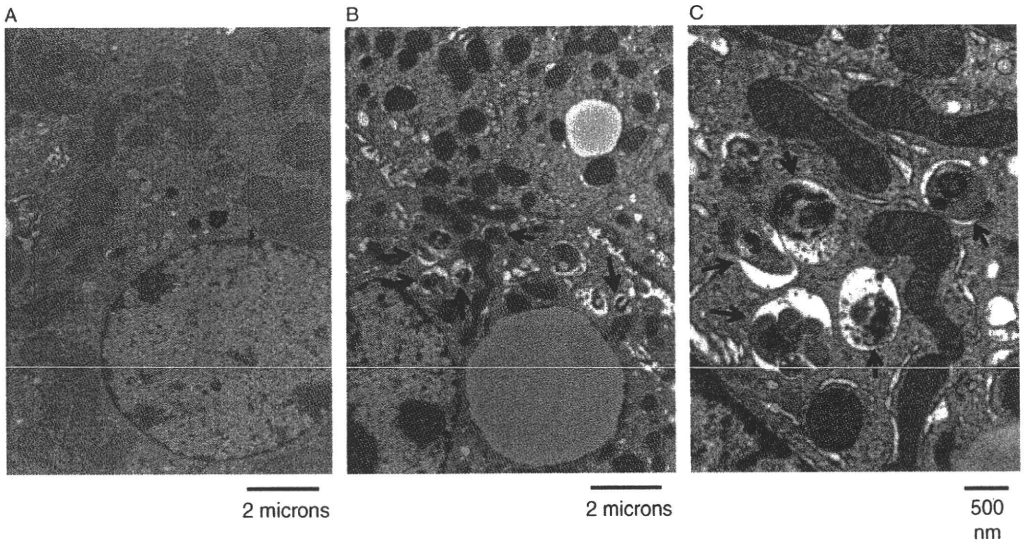


Fig. 4. Electron microscopy of the liver of an FL-N/35 transgenic mouse and a nontransgenic mouse, both of which were fed the excess-iron diet for 12 months. (A) Nontransgenic mouse, (B) full-length HCV-N open reading frame (FL-N/35) transgenic mouse and (C) Magnified picture of B. Autophagosomes (indicated by arrows) are abundantly found in the liver of the FL-N/35 transgenic mouse fed the excess-iron diet. Magnification scales are indicated below each picture.

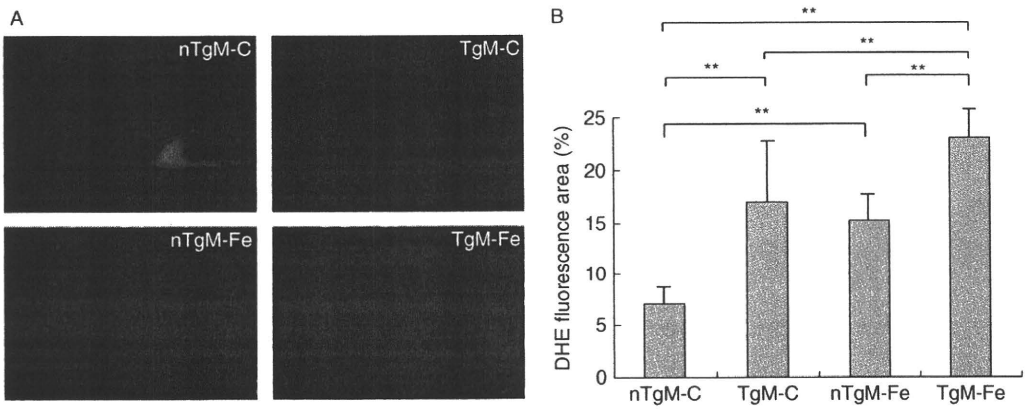
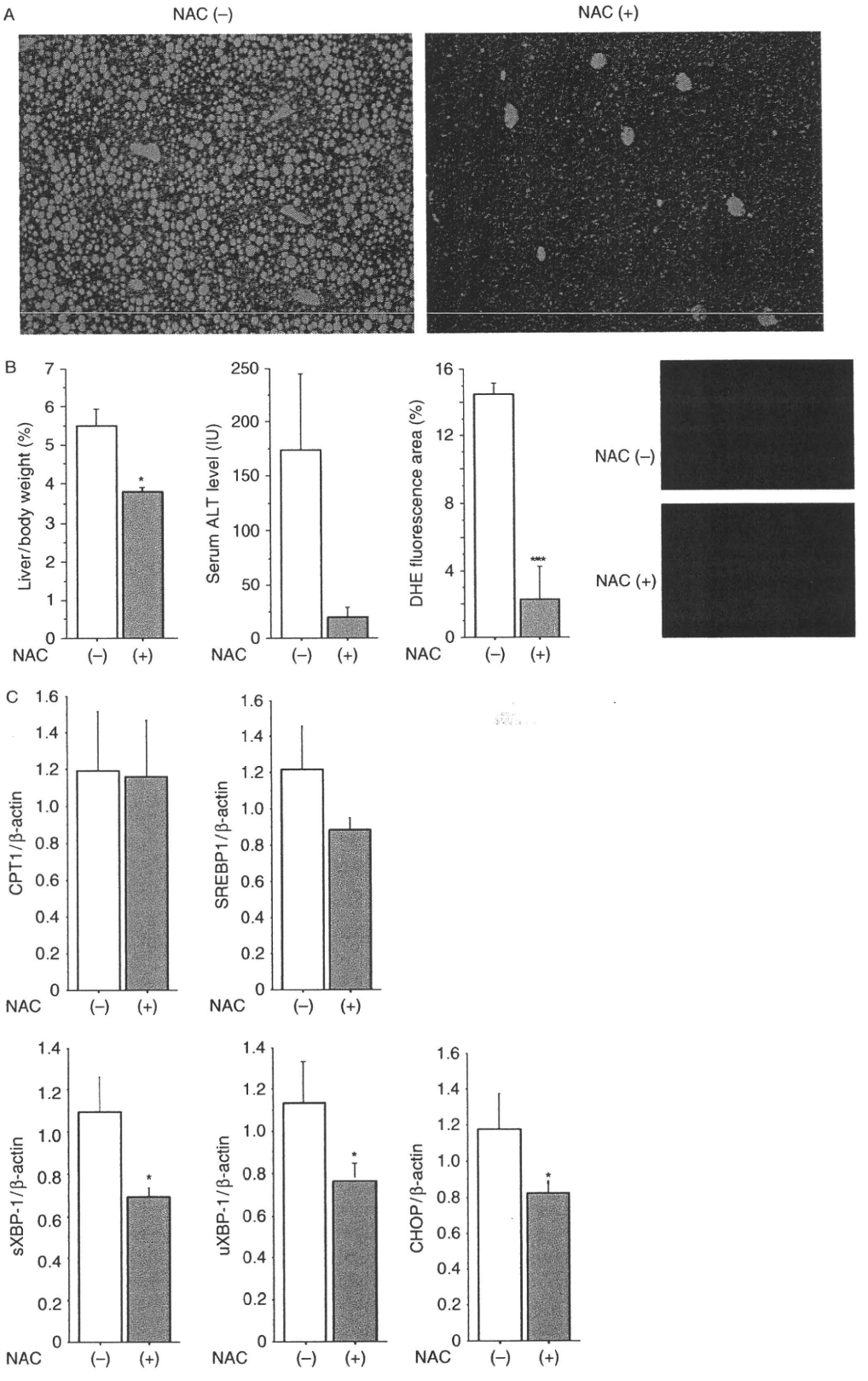


Fig. 5. Reactive oxygen species production in the liver. (A) Frozen liver sections of mice in each group were stained with dihydroethidium. (B) Fluorescence intensity was quantified by NIH image analysis software for three randomly selected areas of digital images for three mice in each group at 12 months after initiation of iron loading. $^{**}P < 0.01$. nTgM-C, TgM-C, nTgM-Fe, and TgM-Fe; see legend for Figure 1.

transgenic mice fed the excess-iron diet than in mice in the three other groups, even though abundant ROS production was found in all mice, except for nontransgenic mice fed the control diet (Fig. 5A and B). ROS production was significantly higher in transgenic mice than in nontransgenic mice irrespective of iron overloading. Iron overloading also significantly increased ROS production irrespective of whether the mice were transgenic or nontransgenic (Fig. 5B). FL-N/35 transgenic mice fed the excess-iron diet had the highest level of ROS production.

A six-month treatment with an anti-oxidant, NAC, dramatically reduced hepatic steatosis in FL-N/35 transgenic mice fed the excess-iron diet (Fig. 6A), together

Fig. 6. Liver histology, and the ratio of liver weight to body weight, serum alanine aminotransferase (ALT) levels, reactive oxygen specie's production and expression of carnitine palmitoyl transferase I (CPT1), sterol-regulatory element-binding protein I (SREBP1), spliced X-box DNA-binding protein 1 (sXBP1), unspliced X-box DNA-binding protein 1 (uXBP1) and CCAAT/enhancer-binding protein homology protein (CHOP) in the liver of full-length HCV-N open reading frame (FL-N/35) transgenic mouse fed the excess-iron diet with/without *N*-acetyl cysteine (NAC) treatment. (A) NAC treatment drastically reduced hepatic steatosis in mice. (B) Frozen liver sections of mice in each group were stained with dihydroethidium. Fluorescence intensity was quantified with the method described in the legend for Figure 5 in three mice in each group. (C)



with a significant reduction in the ratio of liver weight to body weight and ROS production (Fig. 6B). The serum alanine aminotransferase level was also reduced by NAC, but this change was not statistically significant because of the large variance of the data. The expression of uXBP1, sXBP1 and CHOP was significantly reduced after treatment with NAC, suggesting an inhibitory effect of the anti-oxidant on the unfolded protein response (Fig. 6C). The expression of SREBP1 was also reduced by treatment with NAC, but this reduction was not statistically significant ($P = 0.08$). The expression of CPT1, which regulates oxidization of long-chain fatty acids in the mitochondria, did not change after NAC treatment. These results suggested that iron-induced ROS generation induced hepatic steatosis through the activation of the unfolded protein response. It also seemed that the increased lipogenesis through the activated unfolded protein response contributed more to the development of hepatic steatosis than the reduced β-oxidation activity in FL-N/35 transgenic mice fed the excess-iron diet, because the anti-oxidant almost completely inhibited the development of hepatic steatosis without affecting the expression of CPT1.

Discussion

The hepatic iron content of FL-N/35 transgenic mice fed the excess-iron diet was comparable to that of a large number of patients with chronic hepatitis C in extensive studies (15, 16). The positive correlation between the iron level and the triglyceride concentration in the liver was consistent with our previous observation that even modest iron supplementation enhanced hepatic steatosis in FL-N/35 transgenic mice (9), suggesting a potential role of iron in the development of HCV-related steatosis. Although previous studies revealed a direct contribution of HCV core protein to the development of hepatic steatosis (5–7), how iron overload, which is one of pathological features in chronic hepatitis C, affects hepatic steatosis remains unknown. The decreased expression of CPT I suggested reduced β-oxidation activity, because this transmembrane enzyme of the mitochondrial outer membrane has been shown to be the rate-limiting step in the β-oxidation of long-chain fatty acids (30). This result was consistent with our previous observation that the degradation activity of fatty acids *in vivo* was reduced in iron-overloaded transgenic mice. The decreased expression of CPT I may be related to the association of HCV core protein with the mitochondrial outer membrane (31). However, the decreased expression of CPT I seemed to reflect the rather increased synthesis of fatty acids because CPT I is negatively regulated by malonyl-CoA, an intermediate product in fatty acid synthesis, at the transcriptional level (30). In fact, the expression of FAS was significantly increased in FL-N/35 transgenic mice fed the excess-iron diet, which was presumably driven by upregulation of a transcription factor, SREBP1. We could not differentiate SREBP1c from SREBP1a at the protein level because of the lack of an adequate antibody; nevertheless, the expression of SREBP1 was assumed to mainly reflect that of SREBP1c, because the SREBP1c transcript extremely predominates over the SREBP1a transcript in the mouse liver (32).

The regulation of SREBP activation occurs at two levels: transcriptional and post-transcriptional (17). Upregulation of SREBP1c promoter activity has been reported in HCV core gene-transgenic mice (6), but we did not find a significant difference in SREBP1 expression between transgenic and nontransgenic mice without iron overloading. This contradiction may have arisen from a difference in the transgenic mice used in the two studies. In addition, a recent report found no significant difference in the hepatic expression of SREBP1c mRNA between subjects with HCV infection and those with a histologically normal liver (33). HCV has been demonstrated to induce proteolytic cleavage of SREBP1 and 2 in HCV replicon cells (34). As described previously, modest iron supplementation restored a major phenotype of FL-N/35 transgenic mice marked by hepatic steatosis and liver tumour development (9). Thus, the present animal model was useful for understanding the critical role of iron overloading in the development of HCV-related hepatic steatosis. We therefore focused on the post-transcriptional regulation of SREBP1 by iron in the presence of HCV proteins. Upon ER stress, the SREBP–SCAP complex dissociates from the ER retention protein and subsequently translocates to the Golgi apparatus, where SREBP is cleaved and activated (18, 19). FL-N/35 transgenic mice fed the excess-iron diet showed the activated unfolded protein response, assessed by the increased expression of uXBP-1, sXBP-1, p-eIF2α and CHOP, suggesting that the unfolded protein response was activated by iron overloading in the presence of HCV proteins. On the other hand, it is demonstrated that the trans-activating activity of XBP-1 is suppressed, but ATF6 functions properly in HCV replicon cells (35), which is in part contradictory to the present results. Methodological differences (*in vivo* or *in vitro*, iron overload or not, etc.) in two studies may account for this contradiction. Thus, the role of the unfolded protein response in HCV infection alone is still a matter of debate.

To confirm activation of the unfolded protein response in FL-N/35 transgenic mice fed the excess-iron diet, we wanted to assess not only the activation of ER-resident sensors but also the morphological change induced by the unfolded protein response. Autophagy has been shown to play important roles in cell survival after ER stress (25–27). A double-membrane structure, which is called the autophagosome or the autophagic vacuole, is formed *de novo* to sequester cytoplasm. Then the vacuole membrane fuses with the lysosomal membrane to deliver the contents into the autolysosome, where they are degraded and the resulting macromolecules are recycled. Some studies demonstrated a critical role of IRE1 in inducing autophagy under ER stress (25, 36), whereas another study reported the involvement of the PERK-eIF2α signalling pathway, not IRE1, in autophagy induction by ER stress (37). Thus, it is still controversial as to

which transducer is utilized for ER stress-induced autophagy in mammalian cells. The abundant presence of autophagosomes was consistent with the activation of both ER-resident sensors, IRE1 and PERK, in the liver in FL-N/35 transgenic mice fed the excess-iron diet. Although there is no direct link between induction of autophagy and hepatic steatosis in FL-N/35 transgenic mice fed the excess-iron diet, induction of autophagy seemed to support the ER stress-related hepatic steatosis because autophagy is one of the morphological changes under ER stress (25–27).

Iron overload is potentially one of multiple sources of ROS production, as represented in the iron-catalysed Fenton reaction (38). FL-N/35 transgenic mice fed the excess-iron diet had a significantly higher level of ROS production than mice in the three other groups, suggesting a cooperative role of HCV proteins and iron in inducing oxidative stress. ROS have been demonstrated to be involved upstream of the unfolded protein response (28). Anti-oxidants have also been shown to reduce the unfolded protein response and improve protein secretion (29). The present findings that the expression of uXBP1, sXBP1 and CHOP, but not CPT1, was significantly reduced with NAC treatment were consistent with these previous observations, suggesting that iron-induced ROS activated the unfolded protein response in the presence of HCV proteins. How then does ER stress activate SREBP1? There are several lines of evidence suggesting that one mechanism by which ER stress leads to activation of SREBPs is related to downregulation of insulin-induced genes. Downregulation of insulin-induced genes is associated with less retention of SREBPs in the ER, which leads to increased SREBP activation (39–41). As another mechanism, it has been shown that overexpression of glucose-regulated protein 78, one of the ER resident chaperone proteins, inhibits SREBP activation (42). Irrespective of how ER stress activates SREBP, the predominant role of SREBP1 in the ER stress-related hepatic steatosis in FL-N/35 transgenic mice fed the excess-iron diet was similar to that observed in a murine intragastric ethanol-feeding model (43). In conclusion, considering the complexity of the argument and the limited number of evaluated mechanisms, iron-induced ROS-activated unfolded protein response may be postulated as one of the possible mechanisms of HCV-related fat accumulation in the liver.

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IMMUNOLOGY & MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Molecular typing of *Bartonella henselae* DNA extracted from human clinical specimens and cat isolates in Japan

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Keywords
Bartonella henselae; cat scratch disease; multilocus sequence typing (MLST); 16S–23S tRNA-Ala/tRNA-Ile intergenic spacer.

Abstract

Bartonella henselae is the causative agent of cat scratch disease (CSD). To clarify the population structure and relationship between human and cat strains of *B. henselae*, 55 specimens isolated in Japan, including 24 *B. henselae* DNA-positive clinical samples from CSD patients and 31 *B. henselae* isolates from domestic cats, were characterized by multilocus sequence typing (MLST) and the 16S–23S tRNA-Ala/tRNA-Ile intergenic spacer (S1) sequence, which were used previously for strain typing of *B. henselae*. Three different sequence types (STs) were identified by MLST, one of which was novel. Fifty-two strains (94.5%), including all strains detected in CSD patients, were assigned to ST-1. Eight S1 genotypes were observed, three of which were novel. The 52 ST-1 strains were classified into seven S1 genotypes, two of which were predominant in both human and cat strains. In addition, 5.5% of the strains (3/55) contained two different intergenic spacer S1 copies. These results indicate that the predominant *B. henselae* MLST ST-1 in Japan is a significantly genetically diverse population on the basis of the sequence diversity of intergenic spacer S1, and that highly prevalent S1 genotypes among cats are often involved in human infections.

Introduction

Bartonella henselae is the causative agent of cat scratch disease (CSD). Cats represent the major reservoir for *B. henselae*. Infected cats are usually asymptomatic and develop relapsing bacteremia for long periods (Kordick *et al.*, 1995). Human infection usually occurs through scratches or bites by infected cats and presents as CSD, typically with localized lymphadenopathy. Occasionally, the infection may have an atypical presentation due to blood-borne spread, such as bacteremia, endocarditis, encephalopathy, neuroretinitis, or systemic CSD with hepatic and splenic granuloma (Anderson & Neuman, 1997; Murakami *et al.*, 2002; Tsuneoka & Tsukahara, 2006). Disease symptoms depend on the immune status of the host; in immunocompromised hosts, the bacteria are often present in blood and involved in angioproliferative disorders such as bacillary angiomatosis and peliosis hepatis (Welch *et al.*, 1992).

Isolation of *B. henselae* from patients is extremely difficult (La Scola & Raoult, 1999). The diagnosis of CSD relies on clinical manifestations, history of contact with cats, serology, or the detection of bacterial DNA in tissue specimens by PCR (Regnery *et al.*, 1992; Anderson *et al.*, 1994; Murakami *et al.*, 2002; Woestyn *et al.*, 2004; Tsuneoka & Tsukahara, 2006). *Bartonella henselae* strains are divided into two 16S rRNA (*rrs*) genotypes (16S type I/Houston-1 and 16S type II/Marseille), which correspond to two distinct human serotypes (Drancourt *et al.*, 1996; La Scola *et al.*, 2002). Although both genotypes are present worldwide, 16S type II appears to be dominant in the European cat population, whereas 16S type I is more common in Asia, including Japan (Maruyama *et al.*, 2000; Boulouis *et al.*, 2005).

Multilocus sequence typing (MLST) is a nucleotide sequencing-based genotyping method in which variations in approximately 450–500-bp internal fragments of

housekeeping genes (generally seven) are indexed (Maiden, 2000). MLST analysis of 182 feline and human *B. henselae* isolates from Europe, North America, and Australia revealed that sequence type (ST)-1 was most significantly associated with human infection, but that the geographical distribution of STs was not homogenous (Arvand *et al.*, 2007). However, the use of highly conserved housekeeping genes in MLST often fails to detect variability in closely related strains. Compared with housekeeping genes, intergenic spacers are highly variable, thus generating a clearer population structure (Li *et al.*, 2009). In the multispacer typing (MST) scheme for *B. henselae*, the 16S–23S tRNA-Ala/tRNA-Ile intergenic spacer (S1) is the most variable spacer, containing a 15-bp variable number tandem repeat (VNTR) (Li *et al.*, 2006). PCR-based genotyping methods can be applied directly to clinical specimens (Rodrick *et al.*, 2004; Li *et al.*, 2007). However, no data are available regarding the predominant strains causing CSD in Japan.

In this study, we examined 55 human and feline *B. henselae* specimens by MLST and S1 sequence to uncover the genotypic distribution and relationship between human and cat strains of *B. henselae* in Japan. Furthermore, we analyzed the structural diversity of ST-1 using the intergenic spacer S1 sequence to generate a clear population structure.

Materials and methods

Clinical specimens

Twenty-four human clinical specimens consisted of five lymph node specimens and 16 pus specimens from patients with typical CSD, one blood specimen from a patient with bacteremia, one liver specimen from a patient with hepatic granuloma, and one spleen specimen from a patient with splenic granuloma. The specimens were obtained from various regions of western Japan, including Yamaguchi prefecture, from 1997 to 2008.

Bacterial strains

The 31 *B. henselae* isolates were derived from 290 blood samples collected from domestic cats in western Japan, mainly Yamaguchi prefecture, from 2003 to 2004 (Tsuneoka *et al.*, 2004). Primary isolates of *B. henselae* from cat blood samples were grown on chocolate agar plates with 5% defibrinated sheep blood at 35 °C in 5% carbon dioxide (CO_2) for 2 weeks. The strains were stored at − 80 °C until use. Subcultures were performed on chocolate agar plates with 5% defibrinated sheep blood at 35 °C in 5% CO_2 for 5 days. A single colony of each isolate was passaged once on agar before the extraction of bacterial DNA.

DNA extraction

Total genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Identification of *B. henselae*

Bartonella henselae was detected with PCR targeting 414 bp of the *htrA* gene and 172 bp of the *Bartonella* species-specific 16S–23S rRNA internal transcribed spacer region, and confirmed by partial sequencing of the 16S rRNA gene using broad-host-range primer 16SF together with 16SR, as described previously (Anderson *et al.*, 1994; Bergmans *et al.*, 1996; Jensen *et al.*, 2000). No bacterial species other than *B. henselae* was detected in any sample.

PCR amplification and sequencing

For MLST, eight genes (*rrs*, *batR*, *gltA*, *ftsZ*, *groEL*, *nlpD*, *ribC*, and *rpoB*) were amplified and sequenced directly using MLST primers for *B. henselae* as described previously (Iredell *et al.*, 2003). The intergenic spacer S1 was amplified and sequenced directly using S1 forward primer and S1 reverse primer as described previously (Li *et al.*, 2007). When direct sequencing of spacer S1 was unsuccessful because of an atypical number of VNTRs, locus-specific PCR was performed using S1 forward primer with one of two locus-specific primers: BH12700-R (5′-ACGCCAATGT GTTATCCACTT-3′) or BH13810-R (5′-GAAACTTGTCGA TGATCAGGC-3′). The PCR mixture contained 1 × Phusion HF Buffer (Finnzymes, Espoo, Finland), 0.4 U Phusion DNA polymerase (Finnzymes), 200 μM dNTP, 500 nM of each primer, 4–100 ng DNA template, and sterile-distilled water, in a final volume of 20 μL. The reaction conditions were as follows: denaturation at 98 °C for 30 s; 35–50 cycles at 98 °C for 10 s, 56–62 °C for 30 s, and 72 °C for 30–120 s; and a final extension step at 72 °C for 10 min. PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) and then sequenced directly using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on both strands with a 3130 Genetic Analyzer (Applied Biosystems).

Sequencing analysis and phylogenetic analysis

The nucleotide sequences were analyzed with DNA SEQUENCING ANALYSIS software version 5.1 (Applied Biosystems). Alleles, STs, and S1 genotypes were assigned in accordance with published data (Iredell *et al.*, 2003; Li *et al.*, 2006, 2007; Arvand *et al.*, 2007). The novel allele and S1 sequence were carefully confirmed on multiple occasions, and the sequences were deposited in the DNA Data Bank of Japan (DDBJ). New S1 genotypes were deposited in the MST-Rick