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Hepatology Research

Hepatology Research 2011; **41**: 375–385 doi: 10.1111/j.1872-034X.2010.00769.x

Original Article

Pitavastatin inhibits hepatic steatosis and fibrosis in non-alcoholic steatohepatitis model rats

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Aim: Non-alcoholic steatohepatitis (NASH) may progress to liver cirrhosis, and NASH patients with liver cirrhosis are at risk of developing hepatocellular carcinoma. Statins, 3-hydroxy-3-methyglutaryl-coenzyme A reductase inhibitors, are well known to reduce low-density lipoprotein cholesterol and reduce the incidence of coronary heart disease and other major vascular events by anti-inflammatory and antifibrotic effects, and antiproliferative properties in colorectal cancers have also been reported. Recently, statins have been reported to improve hepatic steatosis; however, the effect on fibrosis is controversial.

Methods: The effects of pitavastatin (one of the strongest statins) were examined using a choline-deficient L-amino acid-defined (CDAA) diet liver fibrosis model.

Results: Pitavastatin significantly attenuated increases in serum aspartate aminotransferase, alanine aminotransferase, hepatic steatosis, oxidative stress, pre-neoplastic lesions (glutathione S-transferase placental form-positive lesions), expression of cytokines, such as tumor necrosis factor-α and transforming growth factor-β1, and the expression of tissue inhibitor of metalloproteinase-1, tissue inhibitor of metalloproteinase-2 and type I procollagen genes followed by attenuating fibrosis of the liver of CDAA-fed rats.

Conclusion: These results indicate that pitavastatin may inhibit steatosis, hepatic fibrosis and carcinogenesis in rat model of NASH.

Key words: NASH, statin, steatosis, fibrosis

INTRODUCTION

NON-ALCOHOLIC STEATOHEPATITIS (NASH) is a common liver disease that may progress to cirrhosis, liver failure and liver cancer. The histological findings of NASH are characterized by steatosis, hepatic inflammation and injury to liver cells. In patients with highly progressive fibrosis in NASH, the 5-year cumulative incidence of hepatocellular carcinoma (HCC) was 20%[1] and HCC seemed to occur in advanced fibrotic stages in the liver, but the natural history of NASH has not yet been revealed.

Various factors are involved in the transition from non-alcoholic fatty liver disease (NAFLD) to NASH. The two-hit theory postulating the existence of an additional factor (second hit) after steatosis (first hit) has been generally proposed,[2,3] and oxidative stress and insulin resistance in its background are considered to be important. Because the causes of NASH are diverse, no definite therapy has been established. Recently, several reports have revealed the improvement of NASH, such as by the antioxidant vitamin E (α-tocopherol),[4] angiotensin II type 1 receptor blocker (ARB),[5,6] insulin resistance-improving agents[7] and peroxisome proliferator-activated receptor (PPAR)-γ ligand in NASH patients[8] and NASH model rats.[9] On the other hand, some controversial papers have been reported.[10]

Statins, 3-hydroxy-3-methyglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are well known to reduce low-density lipoprotein (LDL) cholesterol concentrations and to reduce the incidence of coronary heart disease and other major vascular events.[11] Some experimental reports have revealed that statins can reduce liver triglyceride[12] and ameliorate severe hepatic steatosis,[13] and are therefore capable of improving NASH. On the other hand, clinically, the study of statin treatment for NASH is not adequate.[14–16] It has been

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Received 9 April 2010; revised 27 November 2010; accepted 18 December 2010.

reported that statins improved liver steatosis or the non-alcoholic fatty liver disease activity score but their efficacy for fibrosis is controversial. It is well known that statins have many non-cholesterol-dependent effects as well as cholesterol-lowering effects[17] and some antifibrotic effects have been reported.[18] We therefore hypothesized that statins had inhibiting effects not only on triacylglycerol (TG) consumption and inflammation but also on fibrogenesis and carcinogenesis of the liver. A study of the effects on statins on all these effects in NASH has not been reported. The aim of this study was to investigate the experimental inhibiting effects on steatosis, fibrosis and carcinogenesis of statins in NASH using a rat model and to reveal the clinically useful potential of statins for NASH.

METHODS

Animals

SIX-WEEK-OLD male Wistar rats were purchased from CLEA Japan Inc. (Tokyo, Japan), housed under controlled lighting (12:12-h light : dark cycle), and food and water were freely accessible throughout the study period. Pitavastatin was purchased from Kowa Pharmaceutical (Tokyo, Japan). The choline-deficient L-amino acid-defined (CDAA) diet and choline-supplemented L-amino acid-defined (CSAA) diet were obtained in powder form (Dyets, Bethlehem, PA, USA), as described in previous reports.[19,20] Pitavastatin powder was mixed uniformly into the CDAA and CSAA diets at concentrations of 0 and 5 (mg/kg per day). Based on the national regulations and guidelines, all experimental procedures were reviewed by the Institutional Laboratory Animal Care and Use Committee (IACUC) of Nagoya City University, and were finally approved by the President of the University (no. H19-04).

Experimental protocol

The study periods were 2 and 10 weeks. In the 2-week experiment, two groups of eight rats each received a CSAA diet containing 0 and 5 mg/kg per day pitavastatin, and two groups of eight rats each received a CDAA diet containing 0 and 5 mg/kg per day pitavastatin. In the 10-week experiment, similarly, two groups of eight rats each received a CSAA diet or a CDAA diet containing 0 and 5 mg/kg per day pitavastatin. We measured the amount of food that the rats ate every day to determine the amount of pitavastatin necessary to establish a daily dose of 5 mg/kg of rat weight. As food consumption among the rats was essentially equally, we considered that 5 mg/kg per day pitavastatin was consumed during the experimental periods.

Biochemical parameters

In all experiments, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and TG were measured.

Histopathological and immunohistochemical examinations

Five-micrometer-thick sections of the right lobe of all rat livers, fixed in 10% formalin for 24 h and embedded in paraffin, were processed for hematoxylin–eosin and Masson trichrome staining. Glutathione S-transferase placental form (GST-P)-positive lesions (as suitable markers for pre-neoplastic lesions in rat hepatocarcinogenesis)[21] and nitrotyrosine-positive lesions (as oxidant stress)[22] (#06–286 upstate) were immunohistochemically assessed employing the avidin–biotin–peroxidase complex method. Fat drops, fibrosis, nitrotyrosine and GST-P-positive areas in the liver were quantified using a Provis microscope (Olympus, Tokyo, Japan) equipped with a charge-coupled device camera (Sony, Tokyo, Japan), and subjected to computer-assisted analysis with IPAP-WIN software (Sumitomo Techino Service, Hyogo, Japan). Ten different randomly selected areas per specimen were analyzed. The areas of fat drops, fibrosis and nitrotyrosine-positive cells were expressed as a percentage of the total area of the specimen. GST-P-positive lesions were expressed as a percentage of the total area, and the number of GST-P-positive lesions was counted in 10 specimens.

Real-time polymerase chain reaction (PCR) for quantitative assessment of mRNA expression

Tissue inhibitor of metalloproteinase-1 (TIMP-1), tissue inhibitor of metalloproteinase-2 (TIMP-2), matrix metalloproteinase-2 (MMP-2) and tumor necrosis factor-α (TNF-α) have been implicated in fibrosis in NASH, and transforming growth factor-β1 (TGF-β1) is also a key cytokine causing fibrinogenesis in NASH. We therefore examined their expressions.

Total RNA was extracted using Trizol reagent according to the manufacturer's recommended protocol (Life Technologies, Grand Island, NY, USA). RNA extracts were reverse-transcribed with random hexamers and avian myeloblastosis virus reverse transcriptase using a commercial kit (Takara, Kyoto, Japan). Expressions of TIMP-1, TIMP-2, MMP-2, TNF-α, and type I procollagen were evaluated by real-time PCR using an ABI

prism 7000 Sequence Detection system (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol. Probes and primers for TIMP-1 (ID: Rn00587558_m1), TIMP-2 (ID: Rn00573232_m1), MMP-2 (ID: Rn02532334_s1), TNF-α (ID: Rn99999017_m1), type I procollagen α1 (ID: Rn00901649_g1) and TGF-β1 (ID: Rn00572010_A1) were purchased from Applied Biosystems. The relative target was glyceraldehyde-2-phosphate dehydrogenase (GAPDH; ID: 4352338E) mRNA in an identical cDNA sample using the standard curve method recommended by the manufacturer.

Western blot analysis

For the analysis of protein expression, liver specimens were disrupted in lysis buffer containing (final concentrations) 25 mmol/L HEPES (pH 7.4), 120 mmol/L NaCl, 5 mmol/L ethylene glycol tetraacetic acid, 10% glycerol, 1% Triton X-100, 50 mmol/L NaF, 100 μM Na-o-vanadate, 5 mmol/L Na-pyrophosphate and protease inhibitor cocktail. After 10 min on ice, lysates were centrifuged on ice for 5 min and either used immediately or stored at −70°C until use. The lysates were dissolved in Laemmli buffer, and then placed in a bath of boiling water for 5 min. Subsequently, each sample was separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with 1% bovine serum albumin in Tris-buffered saline Tween-20 (TBST) (20 mmol/L Tris [pH 8.0], 150 mmol/L NaCl and 0.01% Triton X-100) for 1 h at room temperature, the samples were probed with anti-TGF-β1 (IC.5559–100; Biovision Inc., San Francisco, CA, USA), anti-α-smooth muscle actin (α-SMA) antibody (Dako Japan, Tokyo, Japan), and anti-PPAR-γ antibody (sc-7196 rabbit polyclonal antibody), followed by goat anti-mouse or antirabbit antibody conjugated with horseradish peroxidase. After extensive washing with TBST, membranes were treated with enhanced chemiluminescence reagent (Amersham Pharmacia Biotech Inc., Schenctady, NY, USA). Bands were quantified densitometrically. Where required, the density of each band was normalized by comparison to the β-actin (using ab6276-100 antibody; ABcam, Cambridge, MA, USA) protein bands measured in the same membrane.

Serum TGF-β1 concentration determined by enzyme-linked immunosorbent assay and quantification of TG in liver

The TGF-β1 concentration in serum was measured using an Immunoassay Kit (KAC1688; BioSource). TG was measured using a Triglyceride Quantification Kit (Bio-Vision) according to the instructions.

Human hepatic stellate cell line LX-2

Human hepatic stellate cell line LX-2, which was kindly donated by Dr Scott L Friedman, was used to analyze pitavastatin efficacy for the inactivation of human hepatic stellate cells (HSC).[23] The LX-2 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) with 10% fetal bovine serum. Cells were seeded at a density of 5.0×10^5 cell/mL in monolayer culture on uncoated 60-mm plastic dishes. All cultures were incubated at 37°C in a humidified atmosphere of 5% air. Medium with or without pitavastatin was changed and, after 24 h, the culture cells were harvested with lysis buffer.

Isolation and culture of HSC and culture HSC

Rat HSC were isolated as described previously with some modifications.[24] In brief, the liver was perfused *in situ* via the portal vein with Ca^{2+}, Mg^{2+}-free Krebs–Ringer (KR) solution followed by 0.1% pronase E (Merck, Darmastadt, Germany) and then 0.032% collagenase (Wako Pure Chemicals, Osaka, Japan) solution at 37°C. The digested liver was minced, and incubated in KR solution containing 0.08% pronase E, 0.04% collagenase and 20 μg/mL DNase (Boehringer-Mannheim, Mannheim, Germany) for 30 min at 37°C (pH 7.3). The resulting suspension was then passed through a nylon mesh. The filtrate was centrifuged at 450 *g* for 8 min. A fraction enriched with HSC was finally obtained by centrifugation in 8.2% Nycodenz (Nycomed Pharma, Oslo, Norway) solution at 1400 *g* for 20 min at 4°C. HSC in the upper white layer were washed by centrifugation at 450 *g* for 8 min, suspended in DMEM containing 10% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia), and supplemented with 100 U/mL penicillin and 100 mU/mL streptomycin (Gibco Laboratories, Life Technologies, Grand Island, NY, USA). Seeding conditions were the same as for cell line LX-2. After incubation for 4 h, non-adherent cells were removed with a pipette and the culture medium was replaced with medium containing pitavastatin or the same concentration of dimethylsulfoxide as a control. Medium with or without pitavastatin was changed every 24 h and cell culture was continued up to 3 days.

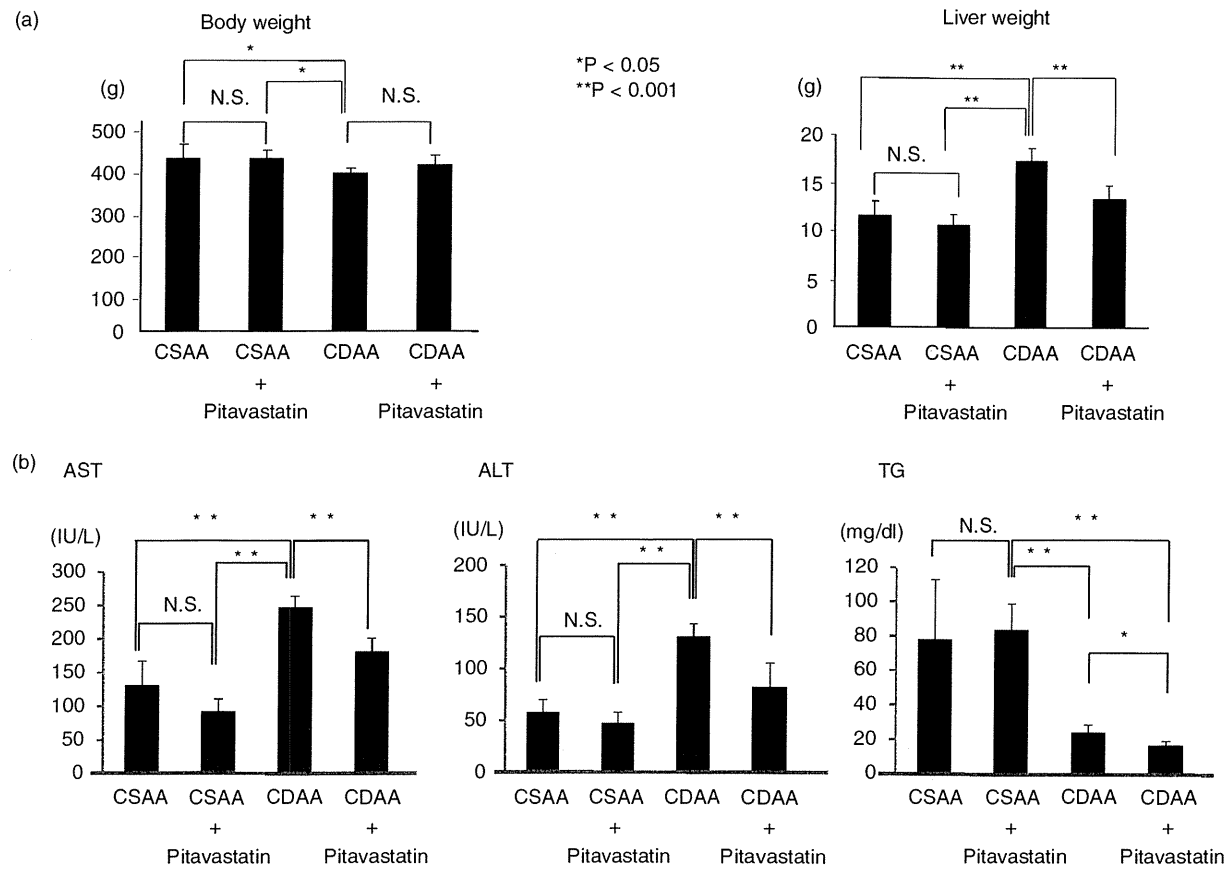

Figure 1 (a) Effect of pitavastatin on the characteristics of rats (10 weeks). Pitavastatin administration did not affect bodyweight changes in choline-supplemented L-amino acid-defined (CSAA)- or choline-deficient L-amino acid-defined (CDAA)-fed rats. The liver weight of CDAA-fed rats significantly increased compared to CSAA-fed rats. The administration of pitavastatin significantly attenuated the liver weight in CDAA-fed rats. (b) Effect of pitavastatin on serum markers (10 weeks). In CDAA-fed rats, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) increased significantly, whereas pitavastatin reduced them. Triacylglycerol (TG) decreased in CDAA-fed rats, and pitavastatin more significantly reduced the level in this model. N.S., not significant.

Statistical methods

Statistical analysis was conducted using ANOVA. The results are expressed as the means ± standard deviation of four or more individual experiments.

RESULTS

Effect of pitavastatin on characteristics of rats

AFTER 10 WEEKS of feeding, the bodyweight of rats fed the CDAA diet was lower than those fed the CSAA diet, and pitavastatin had no influence on bodyweight, whereas rats fed the CDAA diet for 10 weeks showed an increase in the liver weight of 18.5 ± 2.1 g compared with 11.0 ± 0.9 g for rats fed the CSAA diet. Pitavastatin treatment at 5 mg/kg per day in CDAA-fed rats attenuated the increase in liver weight relative to that in CDAA-fed rats that did not receive pitavastatin ($P < 0.001$, Fig. 1a). The serum AST and ALT levels in CDAA-fed rats were significantly higher than in CSAA-fed rats; however, TG was lower in CDAA-fed than in CSAA-fed rats. Treatment of the CDAA-fed rats with pitavastatin decreased both AST and ALT levels (Fig. 1b).

Histological findings of liver steatosis

In this model, after 2 weeks, significant steatosis was observed in CDAA-fed rats, and pitavastatin attenuated

Table 1 Quantitative analysis of liver steatosis in choline-supplemented L-amino acid-defined (CSAA) or choline-deficient L-amino acid-defined (CDAA)-fed rats with or without pitavastatin

Treatment (no. of rats)	After 2 weeks		After 10 weeks	
Fat drop area (mean ± SD) (%)				
CSAA (8)	0	$P < 0.001$	0	$P < 0.001$
CDAA (8)	47.1 ± 5.8		30.7 ± 6.4	
CDAA + pitavastatin (8)	36.8 ± 3.7	$P < 0.001$	20.6 ± 5.0	$P < 0.05$
Quantitative triacylglycerol in the liver of CSAA- or CDAA-fed rats mg/g wet weight (mean ± SD)				
CSAA (8)	2.5 ± 1.9	$P < 0.001$	6.4 ± 2.9	$P < 0.001$
CDAA (8)	557.0 ± 73		201.5 ± 37.8	
CDAA + pitavastatin (8)	420.7 ± 99	$P < 0.001$	144.2 ± 14.6	$P < 0.001$

After 2 and 10 weeks, liver steatosis had progressed in CDAA-fed rats, and pitavastatin (5 mg/kg per day) decreased the development of liver steatosis.
After 2 and 10 weeks, triacylglycerol in the liver tissue had increased significantly in CDAA-fed rats and pitavastatin (5 mg/kg per day) attenuated its accumulation.
SD, standard deviation.

the development of steatosis, and the size of fat drops in hepatocytes of rats fed the CDAA diet with pitavastatin seemed to be smaller than in rats fed the CDAA diet without pitavastatin (data not shown). After 10 weeks, the same tendency was observed but the area of steatosis was reduced due to being occupied by fibrosis, and a lower level of significance was observed between with and without pitavastatin in CDAA-fed rats (Table 1). After 2 and 10 weeks, TG in the liver tissue had increased significantly in CDAA-fed rats and pitavastatin (5 mg/kg per day) attenuated its accumulation (Table 1).

Histological findings of fibrosis

Histological analysis of the livers of CDAA-fed rats at 10 weeks revealed extensive fibrosis and the accumulation of extracellular matrix. In contrast, pitavastatin significantly inhibited the development of liver fibrosis (Fig. 2a) shown with 5 mg/kg per day administration. Image analysis showed that the extent of liver fibrosis in CDAA-fed rats treated with pitavastatin was significantly reduced (Fig. 2ca).

Effect of pitavastatin on oxidative stress and GST-positive lesions in the rat liver (10 weeks)

Nitrotyrosine, a marker of oxidative stress, in liver sections was investigated immunohistochemically. Nitrotyrosine-positive cells were more frequent in the liver sections of CDAA-fed rats, and pitavastatin administration markedly reduced nitrotyrosine-positive cells (Fig. 2b,cb). GST-P-positive lesions consisted mainly of these nodules. It has been reported that GST-P[21] is a suitable marker of pre-neoplastic lesions in rat hepatocarcinogenesis. The results of quantitative analysis were studied. The concomitant administration of 5 mg/kg per day pitavastatin significantly reduced the number and area of GST-P-positive lesions, compared with the livers of rats fed the CDAA diet without pitavastatin (Fig. 3).

Effect of pitavastatin on rat liver fibrogenesis

To investigate the effect of pitavastatin on fibrogenesis and fibrolysis in the rat liver, we assessed mRNA expressions of TIMP-1, TIMP-2, MMP-2 and type I procollagen employing real-time PCR after 10 weeks (Fig. 4). TIMP-1, TIMP-2, MMP-2 and type I procollagen mRNA in the liver significantly increased in CDAA-fed compared to CSAA-fed rats. The addition of 5 mg/kg per day pitavastatin to the CDAA diet significantly reduced the expressions of TIMP-1, TIMP-2, MMP-2 and type I procollagen mRNA in the liver, compared with those of rats fed the CDAA diet without pitavastatin. The addition of 5 mg/kg per day pitavastatin to the CSAA diet caused no changes compared to the CSAA diet without pitavastatin.

Effect of pitavastatin on TNF-α, TGF-β1 and α-SMA

The relative expression of TNF-α mRNA was higher in CDAA-fed than in CSAA-fed rats, and pitavastatin significantly attenuated the expression of TNF-α (Fig. 4).

Protein in the serum and mRNA in the liver of TGF-β1 were examined. The protein levels of TGF-β1

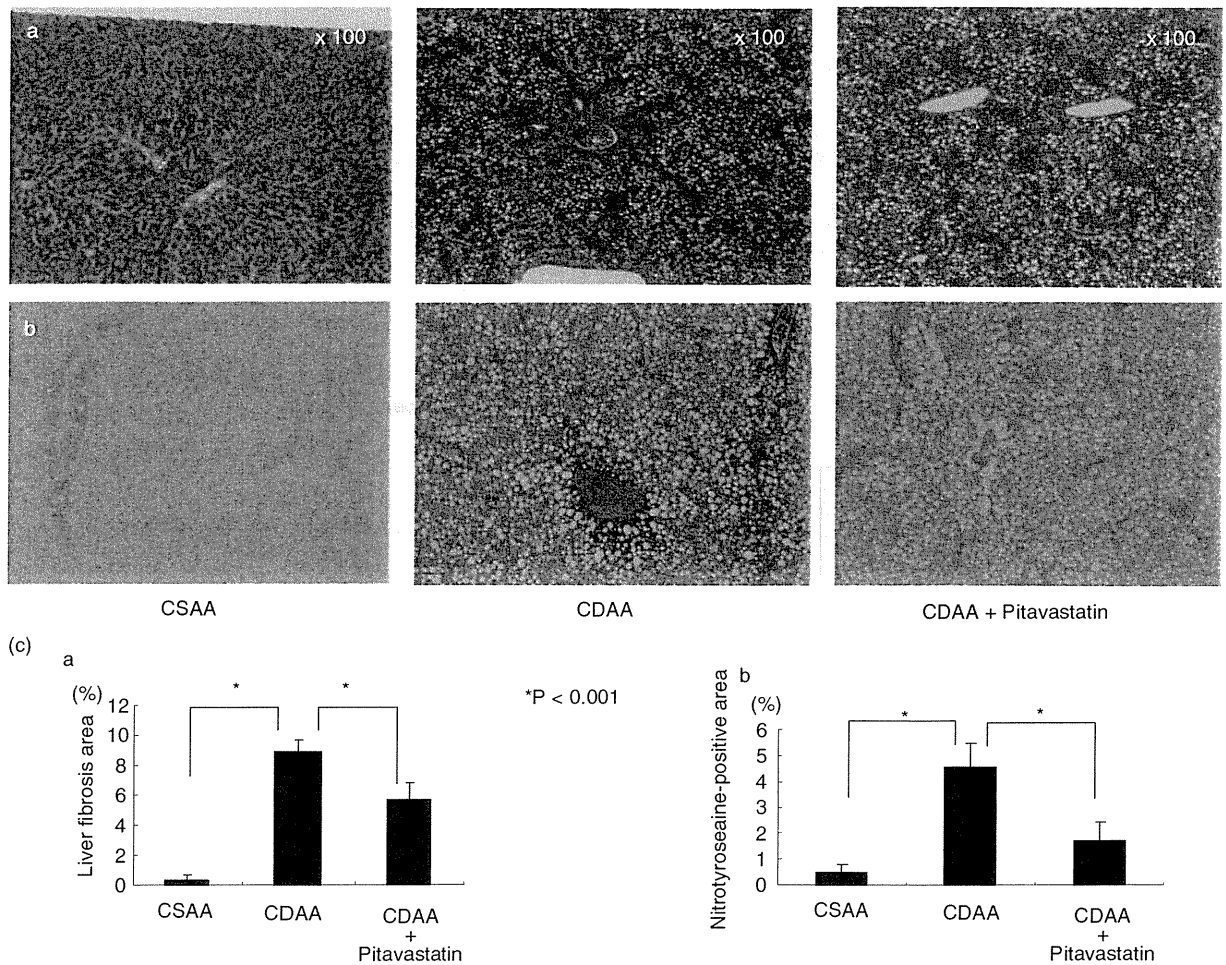

Figure 2 (a) Photomicrographs of liver sections stained with Masson trichrome staining. Choline-supplemented L-amino acid-defined (CSAA)-fed rat, choline-deficient L-amino acid-defined (CDAA)-fed rat and CDAA-fed rat with pitavastatin for 10 weeks. Pitavastatin markedly inhibited liver fibrosis (original magnification ×100). The degree of development of liver fibrosis was quantified using a computerized image analysis system. The liver fibrosis area is shown as a percentage of the microscopic field (ca). Liver histology of CDAA-fed rats showed severe fibrosis. The administration of pitavastatin significantly attenuated the development of fibrosis in CDAA-fed rats. Data are shown as the means ± standard deviations. (b) Photomicrographs of liver sections stained with nitrotyrosine in a CSAA-fed rat, CDAA-fed rat and CDAA-fed rat with pitavastatin for 10 weeks. Pitavastatin markedly inhibited oxidative stress (magnification ×100). The degree of oxidative stress in the liver was quantified using a computerized image analysis system (cb). Nitrotyrosine-positive cells in CDAA-fed rats increased significantly more than in CSAA-fed rats. The administration of pitavastatin significantly attenuated nitrotyrosine-positive cells in CDAA-fed rats. Data are shown as the means ± standard deviations.

in the serum of CDAA-fed rats were significantly higher than in CSAA-fed rats, and pitavastatin significantly attenuated its progression (Fig. 5a). The same effects were observed in the liver tissue. The mRNA expression of TGF-β1 for CDAA-fed rats was higher than that for CSAA-fed rats, and pitavastatin reduced the expression of TGF-β1 (Fig. 5b). As a marker of HSC activation, α-SMA expression was examined in the rat liver. In CDAA-fed rats, α-SMA expression was significantly higher than in CSAA-fed rats, and pitavastatin significantly attenuated its expression (Fig. 5c,d).

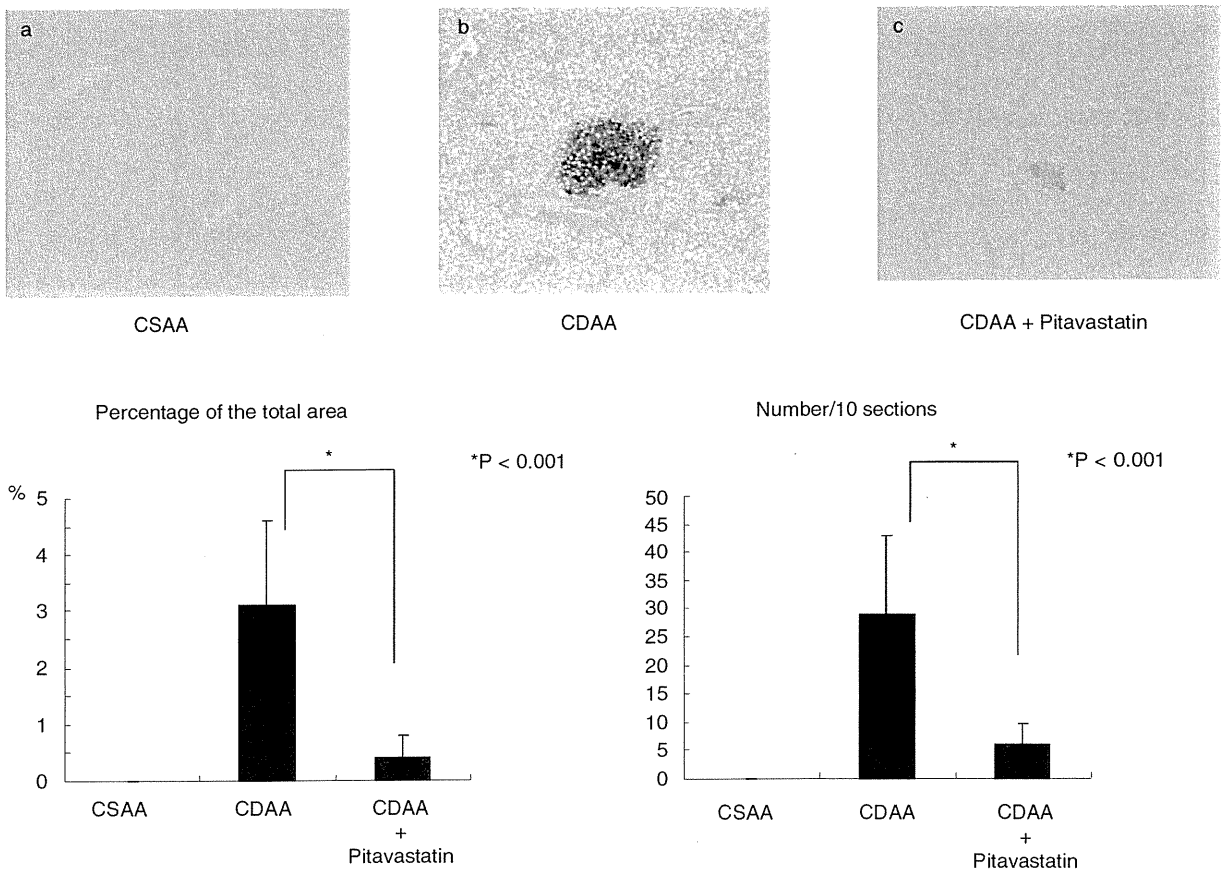

Figure 3 Photomicrographs of liver sections with stained glutathione S-transferase placental form (GST-P)-positive lesions in a choline-supplemented L-amino acid-defined (CSAA)-fed rat (a), choline-deficient L-amino acid-defined (CDAA)-fed rat (b) and CDAA-fed rat with pitavastatin (c) for 10 weeks (magnification ×100). The GST-P-positive percentage of the total area and numbers in 10 sections were measured and quantified using a computerized image analysis system. The GST-P-positive lesions in CDAA-fed rats increased, and the administration of pitavastatin significantly attenuated both the percentage of the total area and number in the 10 sections. Data are shown as the means ± standard deviations.

Effect of pitavastatin on the expression of α-SMA and PPAR-γ in HSC

To confirm the effect of pitavastatin on HSC, we measured the expression of α-SMA and PPAR-γ, an important inducer of TNF-α production, using human HSC lines (LX-2) (Fig. 6a) and isolated HSC from normal rats (Fig. 6b). α-SMA expression was decreased by adding pitavastatin dose-dependently and PPAR-γ expressions increased gradually in a dose-dependent manner. The same experiment was performed using freshly isolated HSC from normal rats. Three days after adding pitavastatin, α-SMA and PPAR-γ protein expressions were examined by western blotting. α-SMA decreased and PPAR-γ increased gradually by adding pitavastatin in a dose-dependent manner.

DISCUSSION

RECENTLY, VARIOUS CLINICAL statin treatments for NAFLD and NASH have been reported.[14–16,25–27] Almost all of these reports, except Nelson *et al.*,[27] describe the reduction of serum aminotransferase or reduction of steatosis of the liver. Nelson *et al.* reported that there was no statistically significant improvement in serum aminotransferase or hepatic steatosis, and the serum low-density lipoprotein of their patients receiving

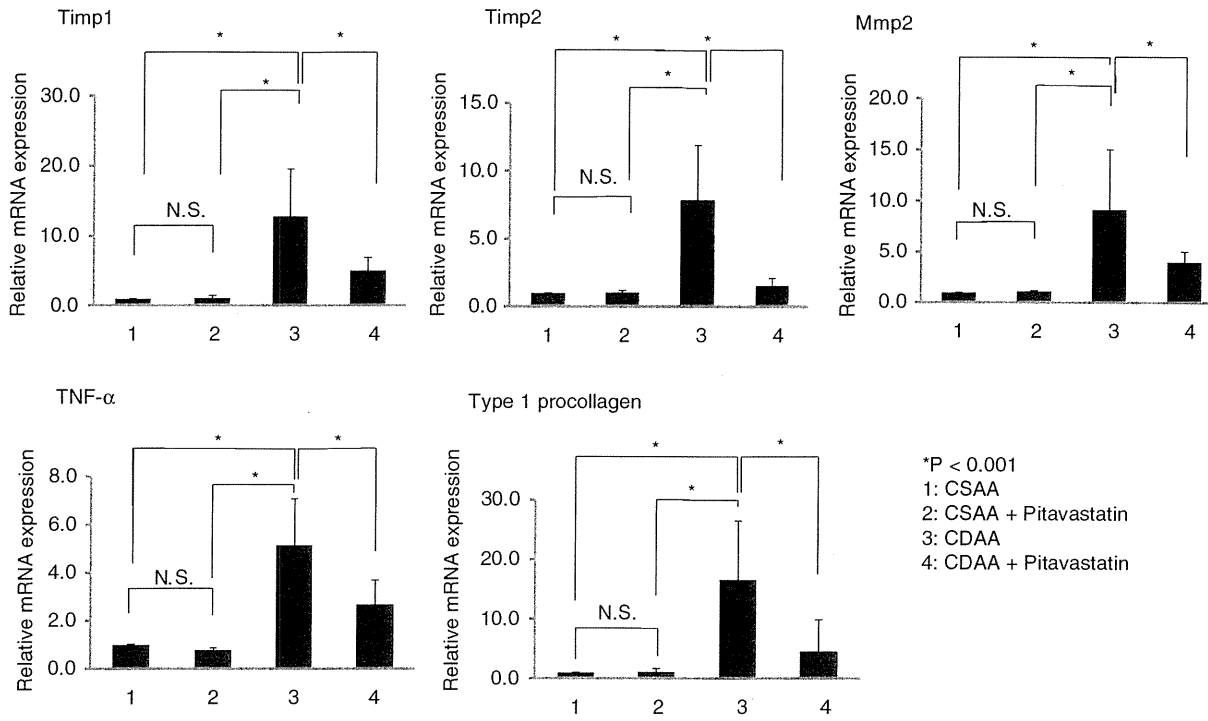

Figure 4 mRNA expressions of tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, matrix metalloproteinase (MMP)-2, tumor necrosis factor (TNF)-α, and type I procollagen. All gene expressions increased in choline-deficient L-amino acid-defined (CDAA)-fed rats, and were significantly attenuated with pitavastatin. Pitavastatin itself had no effect on these expressions in choline-supplemented L-amino acid-defined (CSAA)-fed rats. Data are shown as the means ± standard deviations. N.S., not significant.

simvastatin treatment was not reduced significantly, so one explanation for the different results is the insufficient efficacy of statins. It has been revealed that statins reduce TG in the liver by increasing PPAR-α expression and FA β-oxidation.[12,13] In this study, the serum aminotransferase, steatosis and TG in the liver were significantly reduced by pitavastatin in CDAA-diet rats. These findings are in agreement with the results of numerous clinical and experimental studies. On the other hand, it is important to prevent secondary factors when treating NASH to inhibit liver fibrosis. In clinical trials, the efficacy of statins for liver fibrosis is controversial. Hyogo's data reported that atorvastatin did not reduce liver fibrosis in some cases,[14] whereas Ekstedt *et al.* reported that statins prevented the progression of liver fibrosis despite a high-risk profile and they recommended prescribing statins for patients with elevated liver enzymes because of NAFLD.[26] There have been no studies of the long-term effects of statins, so more clinical research, such as controlled trials, is needed.

Statins have been shown to exhibit pleiotropic effects, including anti-inflammatory, plaque-stabilizing, antithrombotic, antifibrotic and antiproliferative properties,[17,28] and they are also considered to be effective for anti-steatosis and anti-tumorigenesis. Many investigations have reported that statins inhibit fibrosis in various organs[18,29,30] but there are no reports about the efficacy of statins for fibrosis, including liver steatosis and carcinogenesis in NASH, using animal models. Although the CDAA-diet rat does not wholly reflect the human form of this disease, it is a well-established, widely recognized and accepted animal model of NASH.[9,31] At the cellular and molecular levels, liver fibrosis is mainly characterized by cellular activation of HSC and is highly associated with the expression of collagen gene expression, mediators such as TGF-β1, TNF-α, PPAR-γ and oxidative stress.[32–35] In the present experiment, nitrotyrosine was used as an indicator of oxidative stress[22] and after 10-week CDAA diets, the expressions of TGF-β1, TNF-α and oxidative stress were increased, and pitavastatin significantly reduced their expressions and then reduced TIMP-1 and -2 mRNA, which play important roles in the progression of liver fibrosis.[35]

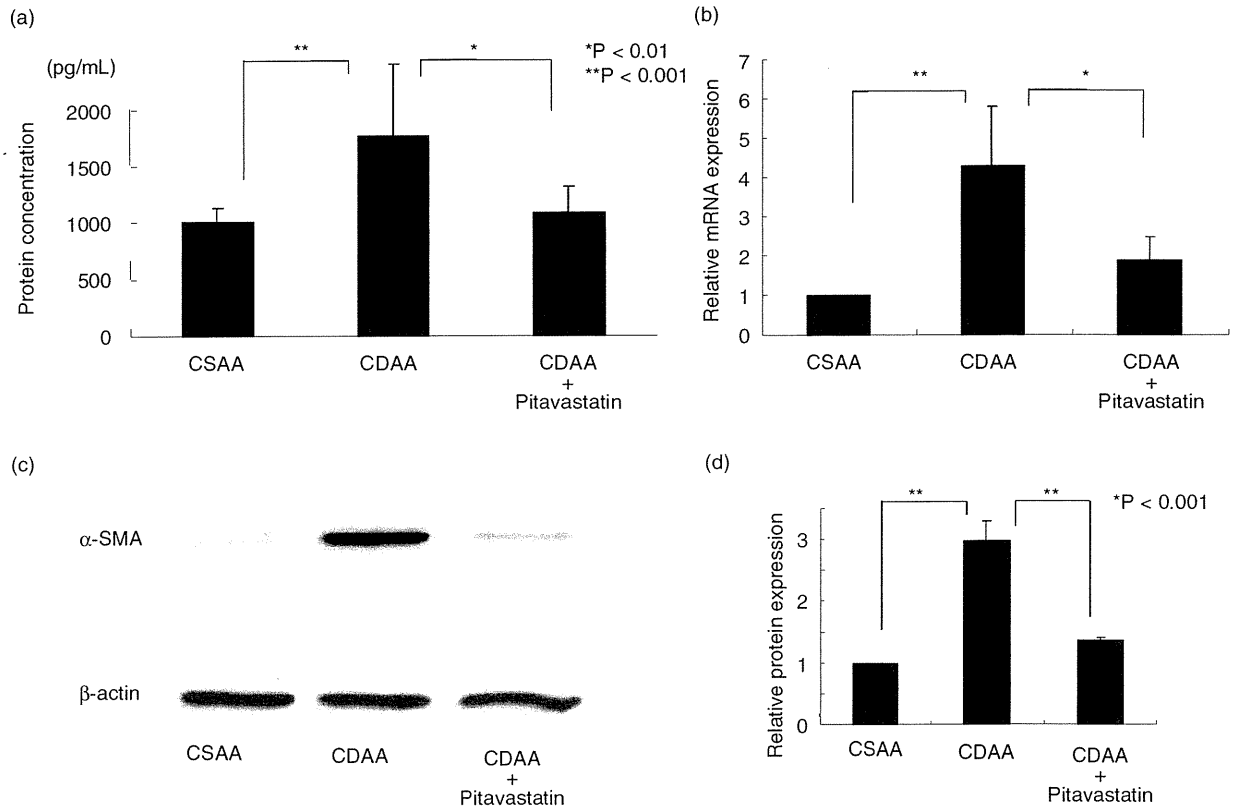

Figure 5 (a) Transforming growth factor (TGF)-β1 protein expression in serum was measured by enzyme-linked immunosorbent assay. TGF-β1 expression increased in choline-deficient L-amino acid-defined (CDAA)-fed rats, and was significantly attenuated by pitavastatin. Data are shown as the means ± standard deviations. (b) TGF-β1 mRNA expression in the liver was increased in CDAA-fed rats and significantly attenuated by pitavastatin. Data are shown as the means ± standard deviations. (c) Western blotting of α-smooth muscle actin (α-SMA) expressions in the liver of rats. (d) Quantitative analysis relative to choline-supplemented L-amino acid-defined (CSAA)-fed rats. α-SMA protein expressions increased in CDAA-fed rats, and pitavastatin administration significantly attenuated them. Data are shown as the means ± standard deviations.

Pitavastatin also inhibited the expression of MMP-2 in this study. Because MMP-2 has been reported to increase when HSC are activated,[36] MMP-2 depression may reveal the inhibition of stellate cell activation by pitavastatin. PPAR-γ is reported as the key molecule of stellate cell activity[37] and several ligands of PPAR-γ prevent the activation of stellate cells and fibrogenesis.[38,39] In this study, the expression of PPAR-γ in HSC decreased and HSC were activated after culture for several days. Pitavastatin increased PPAR-γ and prevented fibrogenesis by inhibiting the activation of HSC, like other PPAR-γ ligands.[9] In our experimental study, pitavastatin significantly improved these liver functions and reduced fibrosis in rat liver.

Because the CDAA-fed rats used in the present study are predisposed to HCC, they were also used for the study of carcinogenesis inhibition. In the present experiment, pitavastatin significantly inhibited the development of GST-P-positive lesions, a precancerous state followed by possibility of inhibiting HCC. Statins reduced the risk of several kinds of cancers[40] and the effects of statins, such as the inhibition of cell proliferation, promotion of apoptosis, and inhibition of angiogenesis and metastasis, have been reported.[41] Our data suggest that statins are capable of inhibiting carcinogenesis in NASH patients.

Thus, the present study confirmed the diverse effects of statins, such as anti-inflammatory, antifibrotic and anticarcinogenic, in a rat model of NASH; therefore, not only currently recognized therapeutic agents, such as vitamin E, thiazolidinedione and ARB, but also statins may be useful preventive agents for NASH.

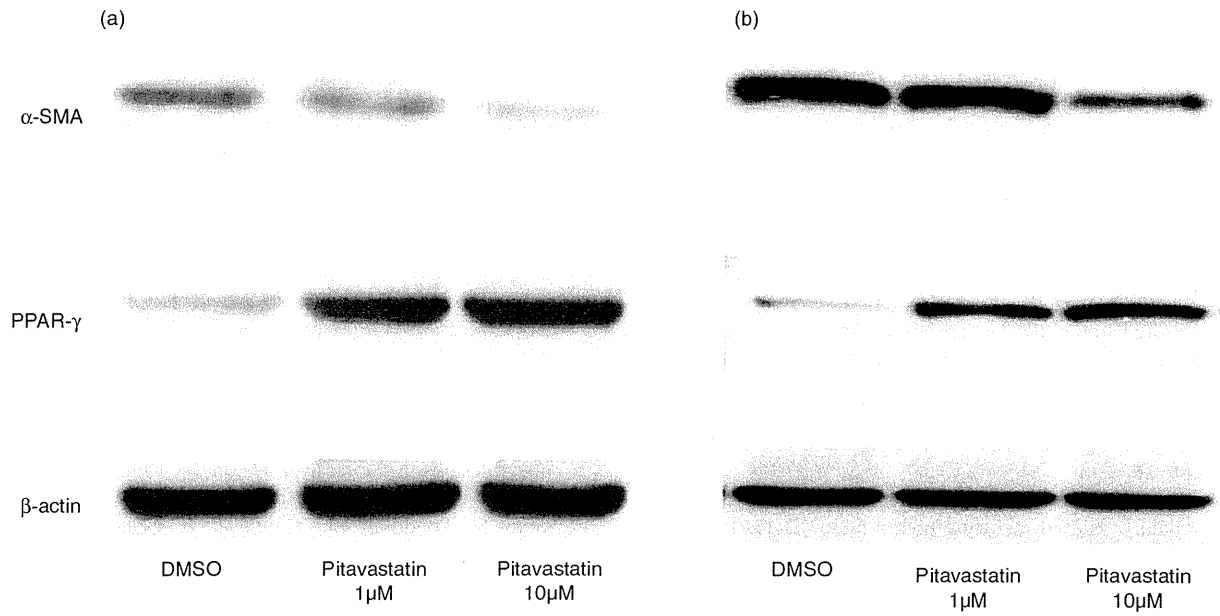

Figure 6 α-Smooth muscle actin (α-SMA) and peroxisome proliferator-activated receptor-γ (PPAR-γ) protein expression on stellate cells. (a) Human hepatic stellate cell (HSC) line LX-2 was incubated with Dulbecco's modified Eagle's medium (DMEM) for 24 h and medium with various concentrations of pitavastatin, and harvested after 24 h. (b) HSC were isolated from the rats and incubated with DMEM for 4 h, and then the culture medium was replaced with medium containing pitavastatin or the same concentration of dimethylsulfoxide (DMSO) as a control, and were harvested after 3 days. In both experiments, α-SMA decreased and PPAR-γ increased gradually after adding pitavastatin in a dose-dependent manner.

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GASTROENTEROLOGY 2009;137:1459–1466

Negligible Contribution of Bone Marrow-Derived Cells to Collagen Production During Hepatic Fibrogenesis in Mice

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See editorial on page 1218.

BACKGROUND & AIMS: Recent studies have reported that bone marrow (BM)-derived cells migrating into fibrotic liver tissue exhibit a myofibroblast-like phenotype and may participate in the progression of liver fibrosis. However, their contribution to collagen production has not been fully verified yet. We revisited this issue by using 2 mechanistically distinct liver fibrosis models introduced into transgenic collagen reporter mice and their BM recipients. **METHODS:** BM of wild-type mice was replaced by cells obtained from transgenic animals harboring tissue-specific enhancer/promoter sequences of α2(I) collagen gene (*COL1A2*) linked to *enhanced green fluorescent protein* (EGFP) or firefly *luciferase* (LUC) gene. Liver fibrosis was introduced into those mice by repeated carbon tetrachloride injections or ligation of the common bile duct. Activation of *COL1A2* promoter was assessed by confocal microscopic examination detecting EGFP signals and luciferase assays of liver homogenates. **RESULTS:** The tissue-specific *COL1A2* enhancer/promoter was activated in hepatic stellate cells following a single carbon tetrachloride injection or during primary culture on plastic. A large number of EGFP-positive collagen-expressing cells were observed in liver tissue of transgenic COL1A2/EGFP mice in both liver fibrosis models. In contrast, there were few EGFP-positive BM-derived collagen-producing cells detected in fibrotic liver tissue of COL1A2/EGFP recipients. Luciferase assays of liver tissues from COL1A2/LUC-recipient mice further indicated that BM-derived cells produced little collagen in response to fibrogenic stimuli. **CONCLUSIONS: By using a specific and sensitive experimental system, which detects exclusively BM-derived collagen-producing cells, we conclude an unexpectedly limited role of BM-derived cells in collagen production during hepatic fibrogenesis.**

Collagen contents in tissue are under control of a dynamic balance between its production and degradation, and a disruption of this equilibrium results in either organ fibrosis or impaired tissue integrity. Irrespective of the etiologies of hepatic injury, liver fibrosis is caused commonly by a chronic and uncontrolled inflammatory/repair process leading to excessive deposition of collagen and other components of extracellular matrix in the liver. Hepatic stellate cells (HSC) are considered to be the main producers of both type I collagen[1] and matrix metalloproteinase (MMP)-13,[2] the major interstitial collagenase degrading type I collagen in rodents. In addition, it has been reported recently that bone marrow (BM)-derived cells participate in both the progression and regression of liver fibrosis by expressing collagen and MMPs, respectively.

We have shown that BM-derived stem/progenitor cells express MMP-13 and MMP-9 and contribute to the spontaneous regression of experimental liver fibrosis induced by repeated carbon tetrachloride (CCl_4) injections.[3] In addition, enhanced mobilization and homing of BM-derived cells by a combination of granulocyte colony-stimulating factor and hepatocyte growth factor stimulated MMP-9 expression in the fibrotic liver tissue and accelerated the recovery from liver fibrosis.[3] Indeed, there have been an increasing number of clinical trials of autologous BM cell infusion therapy to treat patients with critical liver diseases including advanced cirrhosis.[4–8] On the other hand, several experimental and human studies using BM transplantation with sex-mismatched cells[9,10] or genetically marked cells with enhanced green fluorescent protein (EGFP)[11–13] have reported that BM-derived cells migrating into fibrotic liver tissue exhibit the features of collagen-producing cells such as HSC, myofibro-

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; BM, bone marrow; CBD, common bile duct; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell-sorter scanner; HSC, hepatic stellate cell(s); MMP, matrix metalloproteinase.

0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.07.006

blasts, and fibrocytes and may participate in the progression of liver fibrosis.

The present study was designed to reexamine the possible contribution of BM-derived cells to type I collagen production during the progression of liver fibrosis. For this purpose, we utilized 2 mechanistically distinct liver fibrosis models, which were introduced into transgenic collagen reporter mice and their BM recipients. With careful consideration for the experimental design and the qualified methods with high specificity and sensitivity, we conclude that BM-derived cells play an unexpectedly limited role in collagen production during hepatic fibrogenesis. The present study gives a caution to the current growing feeling that overestimates the participation of BM-derived cells in the progression of liver fibrosis.

Materials and Methods

Mice

All animals used in the present study received humane care, and the experiments were approved by the Animal Experiment Committee of Tokai University. C57BL/6 mice were purchased from CLEA Japan Inc. (Tokyo, Japan). A transgenic mouse strain (COL/LUC) that contains the −17,000 to +54 region of the mouse upstream sequence of α2(I) collagen gene (*COL1A2*) linked to a firefly *luciferase* gene was previously described.[14] The −17,000 to −15,450 *COL1A2* sequence exhibits a strong enhancer activity that directs tissue-specific gene expression during embryonic development as well as in adult mouse organs.[14,15] This tissue-specific enhancer and the −350 to +54 minimal *COL1A2* promoter sequences with appropriate linker fragments were cloned into a *Bam*HI site of an EGFP expression plasmid: pEGFP-1 (Clontech Laboratories, Palo Alto, CA). The chimeric DNA fragment was excised from the plasmid to generate transgenic mice (COL/EGFP) that express EGFP exclusively in type I collagen-producing cells. Injection of the purified DNA fragment into fertilized eggs and screening of mice for the presence of transgene were performed as previously described.[14] We established 2 strains of transgenic COL/EGFP mice, which contained approximately 10 and 5 copies of transgene, respectively. Both strains of mice exhibited essentially the same results in the experiments shown in the present study except for the difference in the intensities of EGFP signals. Mice of F3 to F5 generation were used in all of the experiments. Transgenic mice that ubiquitously express EGFP by the cytomegalovirus enhancer and the chicken β-actin promoter (CAG/EGFP) were reported previously.[16]

BM Transplantation

A combination of donor and recipient mice in BM transplantation experiments is illustrated in Figure 1. Transgenic CAG/EGFP, COL/EGFP, and COL//LUC mice were used as BM donors, whereas C57BL/6 wild-type animals were used as recipients. Transplantation of unfractionated whole BM cells including hematopoietic

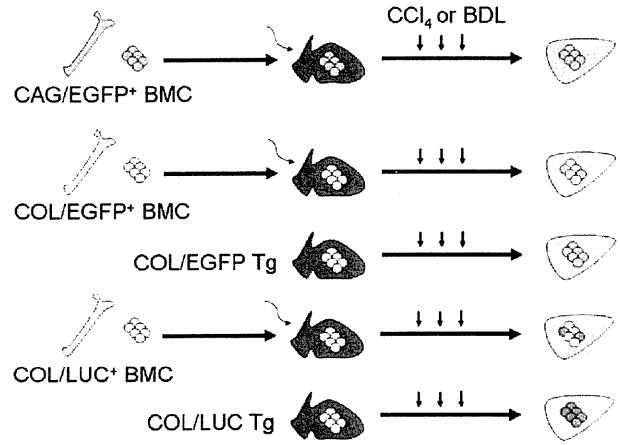

Figure 1. Schematic representation of a combination of donor and recipient mice used in bone marrow transplantation experiments. Whole bone marrow cells (*BMC*) obtained from transgenic CAG/EGFP, COL/EGFP, or COL/LUC mice were injected into the irradiated C57BL/6 wild-type animals. Those recipient mice received subcutaneous injections of 1 mL/kg body weight of carbon tetrachloride (CCl_4) every 3 days for a total of 30 times or underwent ligation of the common bile duct (CBD). Transgenic COL/EGFP and COL/LUC mice (*Tg*) were also included as controls.

stem cells to the irradiated recipient mice was performed as previously described.[3] Engraftment of donor cells was confirmed 6 weeks after BM transplantation by fluorescence-activated cell-sorter scanner (FACS) analyses of EGFP-expressing cells in the peripheral blood of CAG/EGFP recipient mouse or by polymerase chain reaction (PCR) detection of *EGFP* and *luciferase* transgenes in the spleen tissue of COL/EGFP and COL/LUC recipients (Supplementary Figure 1), respectively.

Induction of Liver Fibrosis

Eight weeks after transplantation, BM-recipient mice, as well as transgenic CAG/EGFP, COL/EGFP, and COL/LUC animals, started to be injected subcutaneously with 1 mL/kg body weight of CCl_4 mixed with olive oil every 3 days for a total of 30 times[17] or underwent ligation of the common bile duct (CBD).[18] Three to 4 mice in each group of transgenic mice and their BM recipients were killed 48 hours after the last CCl_4 injection or 14 days after CBD ligation.

Isolation of HSC and FACS Analysis

Murine HSC were isolated by using the collagenase-pronase perfusion method as previously described[19] and subjected to FACS analyses. Presence of EGFP-positive cells in the freshly isolated HSC fraction, peripheral blood, and BM was analyzed by using FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

Confocal Microscopic Examination

Migration of EGFP-expressing cells into fibrotic liver was viewed and analyzed by a confocal laser-scanning microscope: LSM 510 META (Carl Zeiss, Jena, Ger-

many). The emission fingerprinting method[20] was utilized, which distinguished specific fluorescent signals from the background autofluorescence as previously described.[3] EGFP-expressing cells observed in the liver of CAG/EGFP-recipient mice indicate the BM origin irrespective of their phenotypes, whereas those detected in COL/EGFP recipients represent exclusively BM-derived collagen-producing cells migrating into fibrotic liver. Transgenic COL/EGFP mice were also used as a control, showing collagen-expressing cells in the liver irrespective of their origins. Immunohistochemical or immunofluorescence staining was performed as previously described[3] with antibodies against type I collagen (Calbiochem, San Diego, CA), α-smooth muscle actin (α-SMA) (Sigma Chemical Co, St. Louis, MO), and F4/80 (Serotec, Raleigh, NC).

Luciferase Assay

Liver samples obtained from COL/LUC-recipient mice were subjected to luciferase assays to evaluate activation of *COL1A2* promoter in BM-derived cells migrating into fibrotic liver. Transgenic COL/LUC mice were also used as a control to quantify *COL1A2* promoter activity in the liver before and after fibrogenic stimuli. Luciferase assays of liver tissue were performed as previously described,[17,21] and the enzyme activity was normalized against the protein concentration of tissue homogenates.

Statistical Analysis

Values were expressed as mean ± SD. The Mann–Whitney *U* test was used to evaluate the statistical differences between groups: a *P* value of less than .05 was considered statistically significant.

Results

BM-Derived Cells Migrating Into Fibrotic Liver Seldom Differentiate Into α-SMA-Positive Cells in Both Experimental Fibrosis Models

We first examined migration of BM cells into liver tissue in 2 mechanistically distinct fibrosis models introduced into CAG/EGFP-recipient mice. As previously reported,[3] bridging fibrosis connecting the neighboring portal areas and central veins was formed, but complete cirrhosis was not established after 30 times of repeated CCl_4 injections (Figure 2*A*). A large number of EGFP-expressing BM-derived cells migrated into the fibrotic liver 2 days after the last CCl_4 injection, at peak fibrosis (Figure 2*B*). On the other hand, CBD ligation resulted in the accumulation of collagen fibers underneath the dilated and proliferating bile duct epithelium 14 days after the operation (Figure 2*C*). EGFP-expressing BM-derived cells were observed mainly in those fibrotic areas around the dilated bile ducts as well as within the liver parenchyma (Figure 2*D*). However, immunostaining of type I collagen failed to determine precisely whether the EGFP-positive cells present in the fibrous tissue certainly produce collagen or whether they were merely surrounded by collagen fibers (Figure 3*A*). In addition, although EGFP-expressing BM-derived cells and α-SMA-positive cells exhibited the similar distribution following repeated CCl_4 injections (Figure 3*B*) and CBD ligation (Figure 3*C*), they seldom overlapped each other (Figure 3*D*). Three-dimensional confocal microscopic analyses estimated that the mean number of BM-derived α-SMA-positive cells was less than a single cell per each portal area (Figure 3*E*).

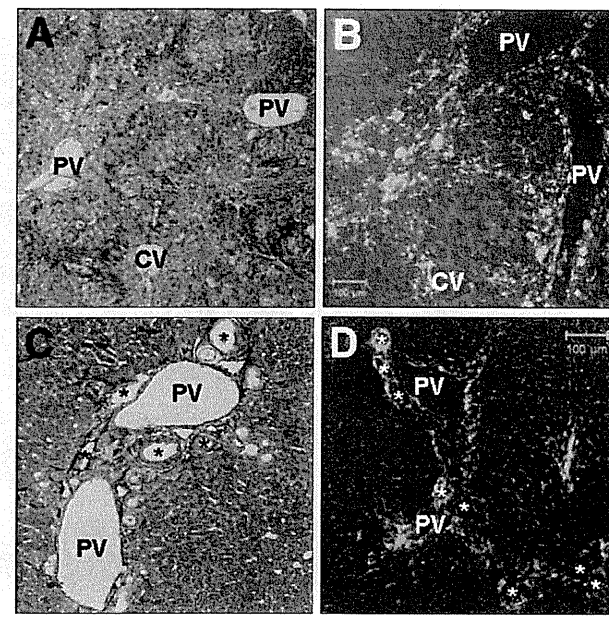

Figure 2. Migration of BM-derived cells into fibrotic liver. Liver specimens were obtained from CAG/EGFP recipient mice following 30 times of repeated CCl_4 injections (*A* and *B*) or 14 days after common bile duct (CBD) ligation (*C* and *D*). The sections were subjected to Azan-Mallory staining (*A* and *C*) or confocal laser-scanning microscopic examination detecting EGFP-positive cells (*green* in *B* and *D*). Representative pictures are shown from 3 to 4 mice in each group. Note that the bile duct epithelial cells do not express EGFP. *Asterisks* indicate dilated bile ducts around the portal vein. *PV*, portal vein; *CV*, central vein. Original magnification, 40× in *A* or 100× in *C*. *Scale bars*, 100 μm in *B* and *D*.

EGFP Expression Driven by the Tissue-Specific COL1A2 *Enhancer Is Stimulated Following Fibrogenic Stimuli in Vivo and During Primary Culture in Vitro*

Next, we examined cell type-specific expression of EGFP driven by the *COL1A2* enhancer. For this purpose, transgenic COL/EGFP mice were first treated with a single dose of CCl_4. Our previous study using transgenic mice that harbor the same tissue-specific *COL1A2* enhancer/promoter sequence linked to a firefly *luciferase* gene (COL/LUC) indicated that *COL1A2* promoter was activated more than 10-fold 72 hours after a single CCl_4 injection.[21] Confocal microscopic examination of excised liver tissue showed a significant number of EGFP-expressing cells present in the centrilobular necrotic areas 48 hours after CCl_4 administration (compare Figure 4*A*

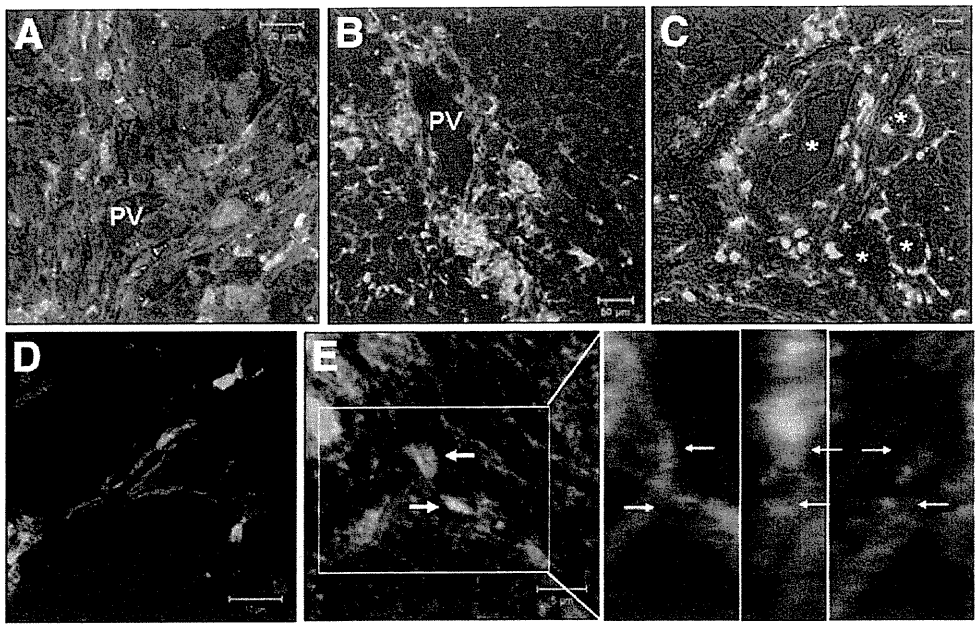

Figure 3. Expression of type I collagen and α-SMA in BM-derived *cells* migrating into fibrotic liver. Liver specimens were obtained from CAG/EGFP-recipient mice following 30 times of repeated CCl_4 injections (*A*, *B*, *D*, and *E*) or 14 days after CBD ligation (*C*). The sections were stained with specific antibodies recognizing type I collagen (*red* in *A*) or α-SMA (*red* in *B–E*). In *panel E* is shown a 3-dimensional rotation analysis of 2 EGFP-positive cells (*arrows*) that coexpress α-SMA. Representative pictures are shown from 3 to 4 mice in each group. *Asterisks* indicate dilated bile ducts. *PV*, portal vein. *Scale bars* are shown in each *panel*.

and *B*). They exhibited a mesenchymal morphology, and none of the parenchymal hepatocytes expressed EGFP. Immunofluorescence studies indicated that approximately half of EGFP-expressing cells were positive for α-SMA, a marker of activated HSC (Figures 4*C* and *D*). In contrast, none of the EGFP-expressing cells were positive for F4/80, a marker of macrophage/Kupffer cells (Figures 4*E* and *F*). FACS analyses indicated that 28.7% ± 1.8% of HSC isolated from CCl_4-treated transgenic COL/EGFP mice were positive for EGFP. Activation of *COL1A2* promoter was also examined in primary culture of HSC obtained from untreated transgenic COL/EGFP mice. Whereas quiescent HSC at day 2 after plating on plastic showed no EGFP fluorescence (data not shown), strong EGFP fluorescence was observed in activated cells at day 7, which was coexpressed with endogenous type I collagen (Figure 5*A*) and α-SMA (Figure 5*B*).

COL1A2 *Promoter Activation Attributes to Liver Resident Cells but not BM-Derived Cells*

After confirming the cell type-specific activation of *COL1A2* promoter, we next examined whether the

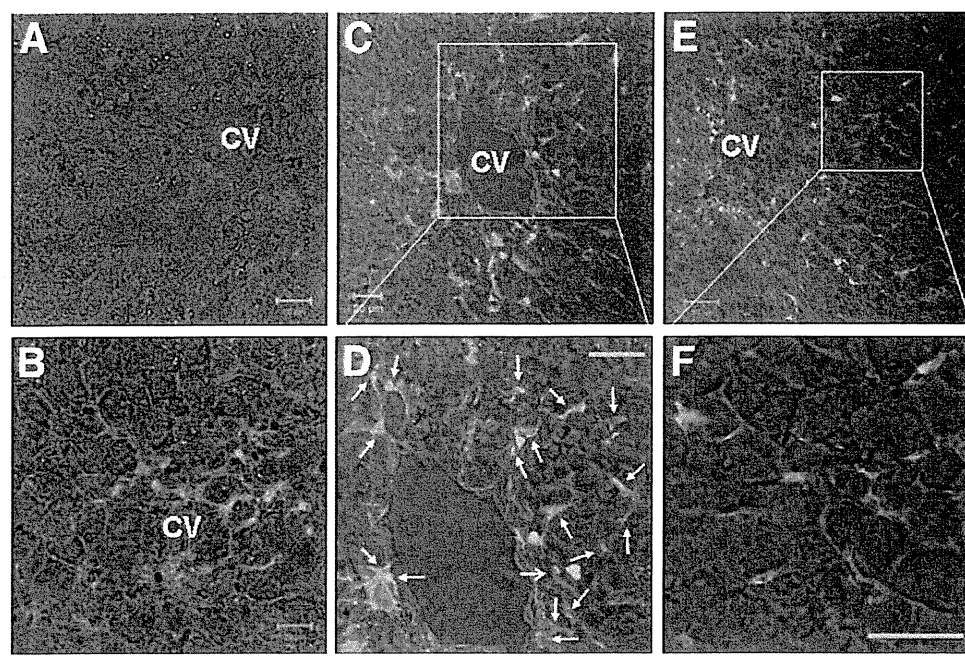

Figure 4. Activation of *COL1A2* promoter following CCl_4 administration. Expression of EGFP (*green*) was examined in liver specimens obtained from wild-type (*A*) or transgenic COL/EGFP mice (*B–F*) 72 hours after a single CCl_4 injection. The sections were stained with specific antibodies recognizing α-SMA (*red* in *C* and *D*) or F4/80 (*red* in *E* and *F*). Representative pictures are shown from 4 mice in each group. *Arrows* indicate EGFP-positive cells that coexpress α-SMA. *CV*, central vein. *Scale bars*, 20 μm in *A–D* or 50 μm in *E* and *F*.

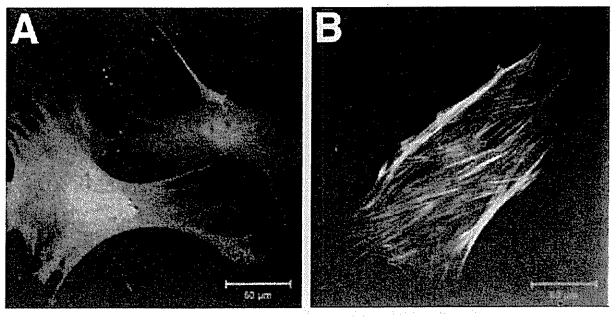

Figure 5. Expression of type I collagen and α-SMA in culture-activated stellate cells. Hepatic stellate cells were obtained from untreated transgenic COL/EGFP mice and subjected to primary culture on plastic for 7 days. Coexpression of EGFP (*green*) and type I collagen (*red* in *A*) or α-SMA (*red* in *B*) was examined by immunofluorescence studies using specific antibodies. Representative pictures are shown from 3 mice. *Scale bars*, 50 μm.

promoter was activated in BM-derived cells or in liver resident cells following fibrogenic stimuli. For this purpose, 2 mechanistically distinct models of liver fibrosis were introduced into transgenic COL/EGFP and COL/LUC mice or their BM-recipient mice. By comparing EGFP expression and luciferase activities between transgenic mice and their BM recipients, we can estimate how each population of BM-derived cells and liver resident cells contributes to collagen production and thus participates in the progression of liver fibrosis. There was no difference between transgenic mice and their BM recipients in the degree of liver fibrosis introduced by repeated CCl_4 injections or CBD ligation (data not shown). A large number of EGFP-positive cells were observed in the portal areas and along the fibrous septa after repeated CCl_4 injections into transgenic COL/EGFP mice, and most of the EGFP-expressing cells were positive for α-SMA staining (Figure 6*A*). On the other hand, coexpression of EGFP and α-SMA was detected in mesenchymal cells present underneath the dilated and proliferating bile duct epithelium after CBD ligation (Figure 6*C*). In contrast, there were few, if any, EGFP-positive BM-derived collagen-expressing cells detected in fibrotic liver tissue of COL/EGFP-recipient mice following either CCl_4 injections (Figure 6*B*) or CBD ligation (Figure 6*D*). Furthermore, as shown in the previous studies,[17,21] luciferase assays of liver tissue revealed that *COL1A2* promoter was activated more than 5-fold following repeated CCl_4 injections into transgenic COL/LUC mice (Figure 7). Similar extent of promoter activation was observed in liver tissue 14 days after CBD ligation (Figure 7). In contrast, *COL1A2* promoter was not activated in liver tissue of COL/LUC-recipient mice. The luciferase enzyme activities in liver tissue of COL/LUC-recipient mice were as low as those in wild-type animals following either CCl_4 injections or CBD ligation (Figure 7). These results clearly indicate that activation of *COL1A2* promoter attributes to liver resident cells, but not BM-derived cells, in both of the 2 mechanistically distinct models of liver fibrosis.

COL1A2 *Promoter Is Not Activated in BM Cells Following Fibrogenic Stimuli*

In the last set of experiments, we examined whether fibrogenic stimuli enhanced *COL1A2* promoter activity in BM cells. FACS analyses indicated that a small population (0.23% ± 0.02%) of BM cells isolated from untreated COL/EGFP reporter mice was positive for EGFP. However, there was no increase in the EGFP-positive ratio of BM cells from transgenic COL/EGFP mice following CCl_4 injections (0.19% ± 0.09%) or BDL (0.20% ± 0.04%). Similarly, there was no difference in the luciferase activities in BM cells isolated from transgenic COL/LUC mice before and after fibrogenic stimuli (data not shown).

Discussion

In the present study, we have revealed an unexpectedly limited role of BM-derived cells in collagen production in 2 mechanistically distinct models of liver fibrosis. Although some of the BM-derived cells exhibited a mesenchymal morphology resembling myofibroblasts, the number of BM-derived α-SMA-positive cells was much smaller than previously reported. More importantly, specific and quantitative analyses of *COL1A2* promoter activation by using a combination of *EGFP* and *luciferase* reporter genes have clearly indicated that BM-derived cells produce little, if any, type I collagen during hepatic fibrogenesis.

There have been a large number of studies showing that BM-derived stem/progenitor cells contribute to the repair of severely injured liver either through transdifferentiation into parenchymal hepatocytes or by cell fusion with liver resident cells. However, the frequency of those phenomena is usually very low.[22] Instead, BM-derived cells often differentiate into a mesenchymal lineage. Several recent studies have indicated that BM-derived cells express MMPs and contribute to the regression of experimental liver fibrosis via mobilization from BM[3] or following a therapeutic cell infusion.[23-25] Based on the results of those experimental studies, there have been an increasing number of clinical trials to treat patients with various liver diseases by infusing either whole or fractionated autologous BM cells.[4-8] On the other hand, several experimental and human studies have reported that BM cells differentiate into HSC,[11,13] myofibroblasts,[9,10] and fibrocytes[12] and may participate in liver fibrosis. These findings are in agreement with the results of a number of studies showing functional contribution of blood-borne collagen-producing cells to tissue repair or fibrosis in various other organs.[26-28] They also give a serious caution that BM-derived cells possess both pro- and anti-fibrotic phenotypes.[29]

However, direct contribution of BM-derived cells to collagen production during hepatic fibrogenesis has not been fully verified for the following critical reasons. First, previous studies employed several different methods to identify the BM origin, such as fluorescence in situ hy-

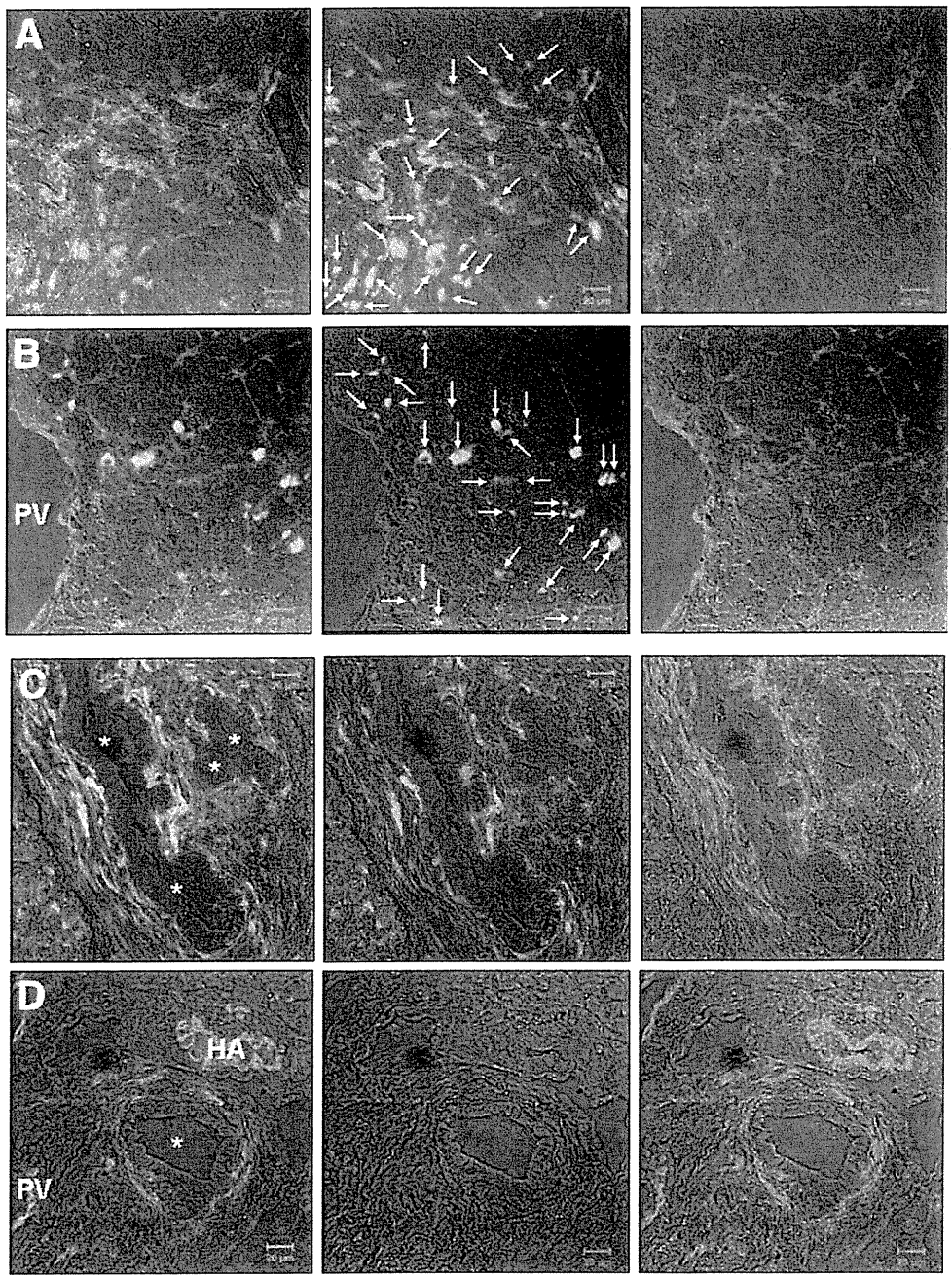

Figure 6. Activation of *COL1A2* promoter in BM-derived cells and liver resident cells. Expression of EGFP (*green*) was examined in liver specimens from transgenic COL/EGFP mice (*A* and *C*) or their BM recipients (*B* and *D*) following 30 times of repeated CCl_4 injections (*A* and *B*) or 14 days after CBD ligation (*C* and *D*). The sections were stained with specific antibodies recognizing α-SMA (*red*). In the *middle* and *right panels* are shown the images of only EGFP and autofluorescence (*middle*) and α-SMA (*right*), respectively. Representative pictures are shown from 3 to 4 mice in each group. Note that the pale signals (*arrows*) observed in *panels A* and *B* represent nonspecific autofluorescence but not the specific EGFP signals. *Asterisks* indicate dilated bile ducts. *PV*, portal vein; *HA*, hepatic artery. *Scale bars*, 20 μm.

bridization technique to detect Y chromosomes[9,10] and immunohistochemical staining of EGFP.[11-13] However, the quality of the employed methods and obtained results was not always enough in terms of both specificity and sensitivity. Second, it is difficult to prove collagen production by BM-derived cells present within the fibrous tissue because of an abundant amount of extracellular collagen accumulated around the cells (Figure 3*A*). Therefore, most of the previous studies failed to demonstrate clearly collagen production by BM-derived cells and relied primarily on the presence of myofibroblast-like cells in the fibrous tissue and their expression of α-SMA.[9-11] Finally, and in relation to the second issue, even if some of the cells exhibit the feature of α-SMA-positive myofibroblasts, it does not necessarily mean that they certainly produce collagen and contribute directly to the progression of liver fibrosis.[30] The cellular entity of BM-derived collagen-producing cells, if present, has not been established yet.

Considering the limitations in the previous studies described above, we tried in the present study to evaluate the direct contribution of BM-derived cells to collagen production by using more specific and quantitative methods. To identify only the specific EGFP fluorescence, we utilized in the previous and present studies the emission fingerprinting method,[3] which successfully elimi-

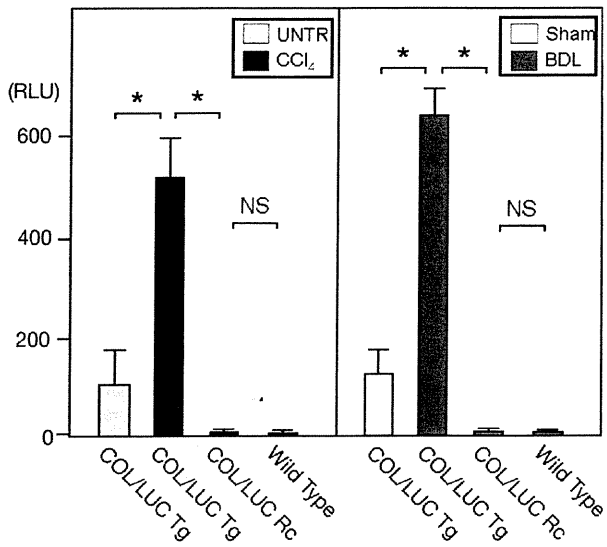

Figure 7. Quantitative analysis of *COL1A2* promoter activation following fibrogenic stimuli. Transgenic COL/LUC mice (*Tg*) and their BM recipients (*Rc*) as well as wild-type animals received repeated CCl_4 injections for a total of 30 times or underwent CBD ligation. They were killed 48 hours after the last CCl_4 injection or 14 days after CBD ligation, and obtained liver tissue was subjected to luciferase assays determining *COL1A2* promoter activity. Liver specimens from untreated (*UNTR*) or sham-operated mice were also analyzed as controls. Luciferase activity was normalized against the protein concentration of tissue homogenates. The values are mean ± SD obtained from 4 mice in each group. The *asterisk* indicates that the difference between the groups is significant. *RLU*, relative luminescence units; *NS*, not significant.

nated the background autofluorescence (Figure 6*A* and *B*). Moreover, we used a firefly *luciferase* gene driven by a tissue-specific *COL1A2* enhancer sequence. Strong tissue specificity of the enhancer that is activated exclusively in collagen-producing cells,[14,15] coupled with the use of sensitive *luciferase* gene as a reporter, engaged a highly specific and quantitative analysis of promoter activation.[21] By using COL/LUC-recipient mice, we demonstrated a complete lack of *COL1A2* promoter activation in BM-derived cells following fibrogenic stimuli.

On the other hand, experiments using transgenic reporter mice also possess several limitations. For example, EGFP is known to be sometimes cytotoxic,[31] and one may argue that BM-derived collagen-producing cells cannot survive for a long time while leaving a large amount of collagen in fibrotic tissue. However, cells with strong EGFP signals were observed in fibrotic liver tissue of CAG/EGFP-recipient mice (Figure 2*B* and *D*) and transgenic COL/EGFP animals (Figure 6*A* and *C*). In addition, the results of parallel experiments using the *luciferase* gene as another reporter (Figure 7) also excluded such a concern. Another limitation of transgenic mouse study is that the expression levels of transgene may vary even by using the same enhancer/promoter sequence. In addition, there is always a chance that the used enhancer/promoter sequence lacks a regulatory element and may not label the entire collagen-expressing populations in the mouse. It should be noted, however, that exactly the same COL/LUC reporter mice and their BM recipients as the present study have been utilized to determine the functional contribution of BM-derived cells to collagen production in various other organs. One of such studies clearly indicated that the luciferase activity in BM-derived cells was dramatically increased in the scar area following experimental myocardial infarction.[28] Our recent study using the same mice also showed a limited but significant amount of collagen produced by BM-derived cells during bleomycin-induced dermal fibrosis (Higashiyama et al, manuscript submitted). On the other hand, an experiment using the same COL/LUC mice failed to demonstrate the contribution of BM-derived cells to collagen production in renal fibrosis induced by a unilateral ureteric obstruction.[32] Collectively, it could be argued that the extent of contribution of BM-derived cells to collagen production may vary depending on the etiologies of tissue injury and/or the sites of affected organs.

The results of the present study do not deny completely the production of type I collagen by BM-derived cells in certain experimental and clinical conditions, depending on the etiologies, severity, and stages of liver fibrosis. However, we excluded their contribution to collagen production in 2 mechanistically distinct models of liver fibrosis. In addition, we also tested at an earlier stage of liver fibrosis induced by 10 times of CCl_4 injections but did not find any evidence for collagen production by BM-derived cells (Supplementary Figure 2).

The localization of BM-derived EGFP-positive cells shown in Figure 2 was almost entirely associated with the fibrotic areas. Although functional properties of the majority of those cells have not been defined yet, our previous study demonstrated that some of them were hematopoietic progenitor cells and inflammatory cells such as neutrophils and macrophages.[3] The present study showed that none of them expressed collagen or α-SMA, but the latter types of cells may participate in the progression of liver fibrosis by secreting several inflammatory cytokines. Further studies are necessary to reveal the functional cross talk between the BM-derived cells and liver resident collagen-producing cells.

In conclusion, this study has clearly revealed a limited role of BM-derived cells in collagen production during hepatic fibrogenesis. The BM origin of cells has to be determined much more carefully, and the results of the present study give a caution to the current growing feeling that overestimates the participation of BM-derived cells to collagen production during the progression of liver fibrosis.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.07.006.

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Received May 10, 2009. Accepted July 7, 2009.

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Acknowledgments

The authors thank Dr Benoit de Crombrugghe for his generous gift of transgenic collagen reporter mice, Dr Masaru Okabe for transgenic mice that constitutively express enhanced green fluorescent protein, and Dr Kiyoshi Higashi for his continuous support and helpful suggestions throughout the work.

Conflicts of interest

The authors disclose no conflicts.

Funding

Supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan; a grant from the Scleroderma Research Committee of the Ministry of Health, Labour and Welfare, Japan; and a research grant from Mitsui Life Social Welfare Foundation.