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Effects of adiponectin transgenic expression in liver of nonalcoholic steatohepatitis model mice

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Abstract

We have previously reported that transgenic mice expressing nuclear sterol regulatory element–binding protein 1c (nSREBP-1c) in adipose tissue under the control of aP2 promoter, an inherited lipodystrophic model with insulin resistance and fatty liver, developed with age liver lesions similar to those of human nonalcoholic steatohepatitis (NASH). Because the spontaneous NASH model mice had marked hypoadiponectinemia, here we assessed the effect of adiponectin transgenically expressed in the liver of nSREBP-1c transgenic mice. The nSREBP-1c/adiponectin double-transgenic mice showed hepatic adiponectin production and restored circulating adiponectin levels. Both subtypes of adiponectin receptors proved to be expressed normally in the liver. Peroxisome proliferator–activated receptor–α was up-regulated in the double-transgenic mice. Histologic findings similar to those observed in the liver specimens of patients with NASH were observed in the livers from nSREBP-1c transgenic mice at the age of 30 weeks. In contrast, the NASH-like hepatic lesions were obviously attenuated in age-matched double-transgenic mice. Immunoreactivity of 8-hydroxy-2′-deoxyguanosine and proliferating cell nuclear antigen–positive cells were increased in nSREBP-1c transgenic mice, but not in the double-transgenic mice. Postload plasma glucose levels were significantly lower in the double-transgenic mice compared with nSREBP-1c transgenic mice, whereas serum leptin levels did not differ significantly in the 2 groups. These observations suggest that hypoadiponectinemia plays a key role in the pathogenesis of NASH associated with insulin resistance and may provide a clue to the novel therapy for human NASH.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease that occurs in subjects without significant alcohol consumption [1-4]. It is often associated with obesity, metabolic syndrome, and insulin resistance. Some patients with NAFLD develop necroinflammatory changes in the liver called *nonalcoholic steatohepatitis* (NASH), and a fraction of those will develop fibrosis that may progress to cirrhosis [5-8]. It may account for some of the cases previously diagnosed as cryptogenic cirrhosis. However, no established treatment exists for this potentially serious disorder.

Histologic analysis of murine models for human NASH may be useful to explore the pathogenesis of the chronic disease and to generate preclinical data for therapeutic interventions. We have previously reported that transgenic mice expressing nuclear sterol regulatory element–binding protein 1c (nSREBP-1c) in adipose tissue under the control of aP2 promoter, an inherited lipodystrophic model with insulin resistance and fatty liver [9], spontaneously developed steatohepatitis [10]. Despite lack of obesity, the nSREBP-1c transgenic mice exhibited insulin resistance, hypertriglyceridemia, elevated levels of transaminases, and liver histology similar to human NASH.

Adipokines have been implicated in the pathogenesis of NAFLD including NASH through their metabolic and pro-/anti-inflammatory activities [11-13]. Most subjects with NASH had hypoadiponectinemia, and serum adiponectin levels were correlated with insulin resistance and the severity

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of hepatic steatosis [11,13]. Transcript expression of adipoR2, a subtype of adiponectin receptor, was significantly reduced in liver biopsy specimens of patients with NASH compared with simple steatosis [12]. However, direct evidence of the role of adiponectin in spontaneously occurring NASH is lacking. Recently, we have generated and characterized transgenic mice expressing adiponectin in the liver [14]. The hyperadiponectinemic mice showed attenuated oxidative DNA damage and extended lifespan on both regular chow and high-fat/high-sucrose diet. In this study, we examined the effects of adiponectin transgenically expressed in the liver on the pathophysiology of NASH model mice.

2. Materials and methods

2.1. Generation of double-transgenic mice

Transgenic mice (C57BL/6 background) expressing nSREBP-1c in adipose tissue [9] were purchased from Jackson, Bar Harbor, ME, and bred in our laboratory mating to wild-type C57BL/6 mice (Nippon CLEA, Shizuoka, Japan). Generation of transgenic mice expressing full-length human adiponectin in the liver in the genetic background of C57BL/6 was previously described [14]. Female mice that expressed nSREBP-1c in adipose tissue were bred with adiponectin-expressing male mice to produce a double-transgenic line. The double-transgenic mice were identified by polymerase chain reaction of tail DNA using nSREBP-1c–specific primers (5′-CTACATTCGCTTTCTGCAAC-3′ and 5′-ATAGAAGGACACCTAGTCAG-3′) and human adiponectin transgene-specific primers (5′-TGAATTCGGGCTCAGGATGCTGTTGCT-3′ and 5′-AGGATCCTGATCAGTTGGTGTCATGGTA-3′). Male mice heterozygous for both nSREBP-1c and human adiponectin were used in the following experiments. All mice were fed standard mouse chow (347 kcal/100g, protein 24.9 g/100g, fat 4.6 g/100g; Nippon CLEA) and water ad libitum. The weight of epididymal fat pad was measured at the age of 20 weeks. All procedures were approved by the Ethics Review Committee for Animal Experimentation of Kurume University School of Medicine.

2.2. Biochemical assays

Glucose tolerance was assessed using intraperitoneal glucose tolerance test (IPGTT). The IPGTT was performed by injecting glucose (1 g/kg in 10% solution) intraperitoneally in overnight-fasted mice. Glucose levels in blood obtained from the tail veins were measured by the glucose dehydrogenase method using Free Style (Nipro, Osaka, Japan) at 0, 30, 60, and 120 minutes after glucose injection. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride, and total cholesterol levels were determined by spectrophotometric enzyme assays using peroxidase, lipoprotein lipase, and cholesterol oxidase, respectively (Wako, Osaka, Japan). Serum levels of mouse leptin and adiponectin were measured with enzyme-linked immunosorbent assay kits from R&D (Oxon, United Kingdom) and AdipoGen (Seoul, Korea), respectively.

2.3. Northern blot analysis

Northern blot analysis of adiponectin receptor 1 (AdipoR1) and receptor 2 (AdipoR2) was performed using total RNA of the liver from male mice aged 20 weeks. Total RNA of 20 μg each was electrophoresed in a formaldehyde-agarose gel, transferred to a nitrocellulose membrane (GE Healthcare Bio-Sciences, Piscataway, NJ), and immobilized by ultraviolet light. Labeling of an adiponectin-specific probe [14], prehybridization, hybridization, washing, and signal detection were carried out using an enhanced chemiluminescence system (GE Healthcare Bio-Sciences) according to the manufacturer's recommended protocol.

2.4. Western blot analysis

Total protein extracts were loaded onto a 7.5% polyacrylamide gel, and separated proteins were transferred onto Hybond nitrocellulose membrane (GE Healthcare Bio-Sciences). The membranes were blocked in 5% skim milk for 60 minutes with gentle shaking, washed in Tris-buffered saline–Tween 20, and incubated with rabbit peroxisome proliferator–activated receptor (PPAR)–α antibody (Abcam, Cambridge, United Kingdom) at 1:2000 dilution overnight. After incubation, the membranes were extensively washed 5 times and then incubated for 60 minutes with peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (Wako, Tokyo, Japan). Visualization was performed using an ECL kit according to the manufacturer's protocol (GE Healthcare Bio-Sciences).

2.5. Histologic studies

Liver tissues were fixed in neutral formalin and embedded in paraffin, and sections underwent either hematoxylin and eosin (HE) staining for standard microscopy or Azan-Mallory staining to observe the location of extracellular matrix. Each specimen was classified into one of the following histologic subtypes for the purpose of comparative analysis: type 1—fatty liver that is predominantly macrovesicular, more than 33% of the lobules alone; type 2—fat accumulation and lobular inflammation; type 3—fat accumulation and ballooning hepatocytes; and type 4—fat accumulation, ballooning hepatocytes, and either Mallory hyaline or fibrosis. We dealt with types 3 and 4 as NASH, according to a report by Matteoni et al [8]. Immunoreactivity of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage [15], in the liver was examined as described previously [16]. Briefly, deparaffinized sections were incubated with 5 μg/mL of mouse monoclonal anti–8-OHdG (Japan Institute for the Control of Aging, Fukuroi, Japan) overnight, followed by incubation with alkaline phosphatase-labeled horse anti-mouse IgG (Vector, Burlingame, CA), and

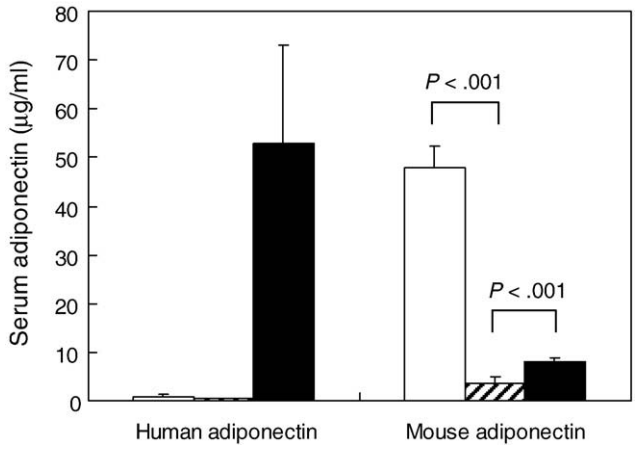

Fig. 1. Serum levels of human and mouse adiponectin at the age of 10 weeks. Open column, wild-type C57BL/6 mice (n = 8); hatched column, nSREBP-1c transgenic mice (n = 8); closed column, nSREBP-1c/adiponectin double-transgenic mice (n = 8). Means and SD.

visualized by 3,3′-diaminobenzidine. To detect proliferating cell nuclear antigen (PCNA), liver sections were incubated with 1:50 diluted rabbit anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight. After washing with phosphate-buffered saline, sections were incubated with biotinylated second antibody solution and visualized with Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA). The number of hepatocytes positive for PCNA was counted under a light microscope in at least 20 fields at ×400.

2.6. Statistical analysis

Numerical data were expressed as means and SD. Unpaired Student *t* test was performed to assess statistical significance between groups. *P* values less than .05 were considered significant.

3. Results

To assess the role of adiponectin in the development of NASH, we generated nSREBP1c/adiponectin double-transgenic mice expressing human adiponectin in the liver. Circulating human adiponectin levels of the double-transgenic mice were 53 ± 20 μg/mL at the age of 20 weeks (Fig. 1). The human adiponectin levels were almost compatible to normal adiponectin levels of age-matched wild-type mice (48 ± 4 μg/mL). Furthermore, endogenous mouse adiponectin levels were slightly but significantly higher in double-transgenic mice than in nSREBP-1c transgenic mice without adiponectin transgene. No difference was observed in expression levels of either subtype of adiponectin receptor, AdipoR1 or AdipoR2, in the liver between the nSREBP-1c transgenic mice without adiponectin transgene and the double-transgenic mice (Fig. 2A, B). Western blot analysis showed that PPAR-α protein was increased in the liver from double-transgenic mice when compared with wild-type and nSREBP-1c transgenic mice (Fig. 2C). As previously reported [9], the nSREBP-1c transgenic mice showed hypoleptinemia, which was attributable to decreased adipose tissue mass. Transgenic expression of human adiponectin in the liver did not affect serum leptin levels of the nSREBP-1c transgenic mice (Fig. 3A). No significant difference was obtained in the weight of epididymal fat pad between nSREBP-1c transgenic mice and the double-transgenic mice (Fig. 3B).

At the age of 20 weeks, the serum levels of AST and ALT were elevated in the nSREBP-1c transgenic mice when compared with wild-type mice (Fig. 4). The AST levels were significantly reduced by the production of human adiponectin in the liver. The double-transgenic mice had normal ALT levels, although the reduction was not statistically significant. No significant difference was observed in total cholesterol or triglyceride levels between nSREBP-1c transgenic mice and the double-transgenic mice (Fig. 4). The nSREBP-1c transgenic mice had impaired glucose tolerance [9]. The IPGTT performed at the age of 20 weeks showed significantly lower plasma glucose levels in the double-transgenic mice at 30 and 60 minutes after glucose load than those in the nSREBP-1c transgenic mice (Fig. 5).

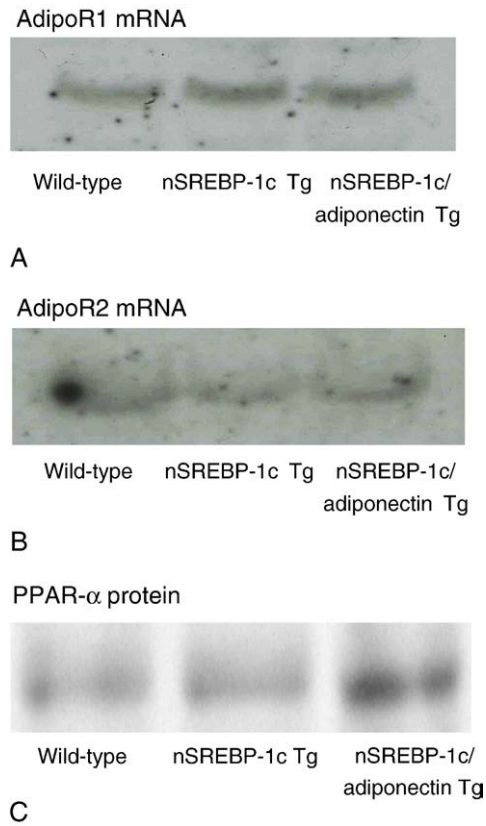

Fig. 2. Expression of adiponectin receptor transcripts and PPAR-α protein in the liver. Messenger RNA of AdipoR1 (A) and AdipoR2 (B) was analyzed by the Northern blot analysis using 20 μg total RNA each from the liver of wild-type, nSREBP-1c transgenic, and double-transgenic mice at the age of 20 weeks. Increased PPAR-α levels were observed in nSREBP-1c/adiponectin double-transgenic mice by the Western blot analysis (C). Western blotting was performed 4 times with essentially the same results.

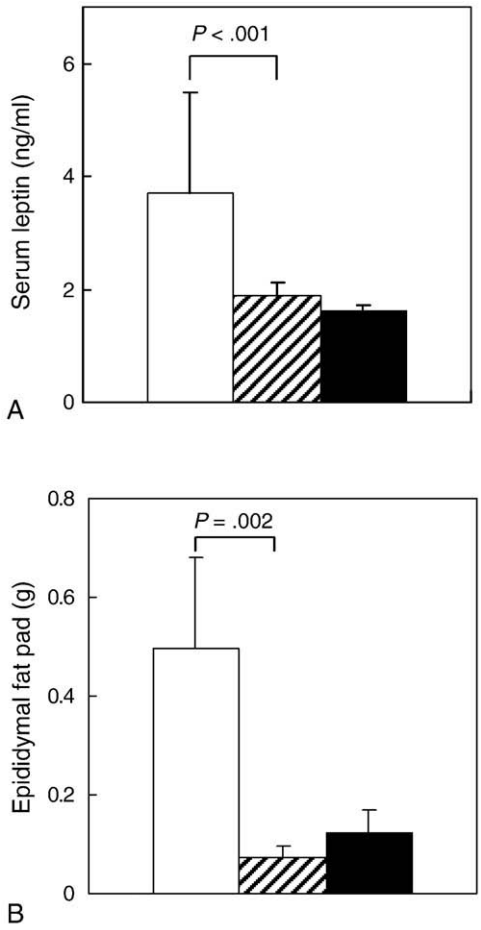

Fig. 3. Serum leptin levels at the age of 30 weeks (A, n = 7 each) and weight of epididymal fat pad at the age of 20 weeks (B; wild type, n = 6; transgenic and double transgenic, n = 4). Open column, wild-type mice; hatched column, nSREBP-1c transgenic mice; closed column, nSREBP-1c/adiponectin double-transgenic mice.

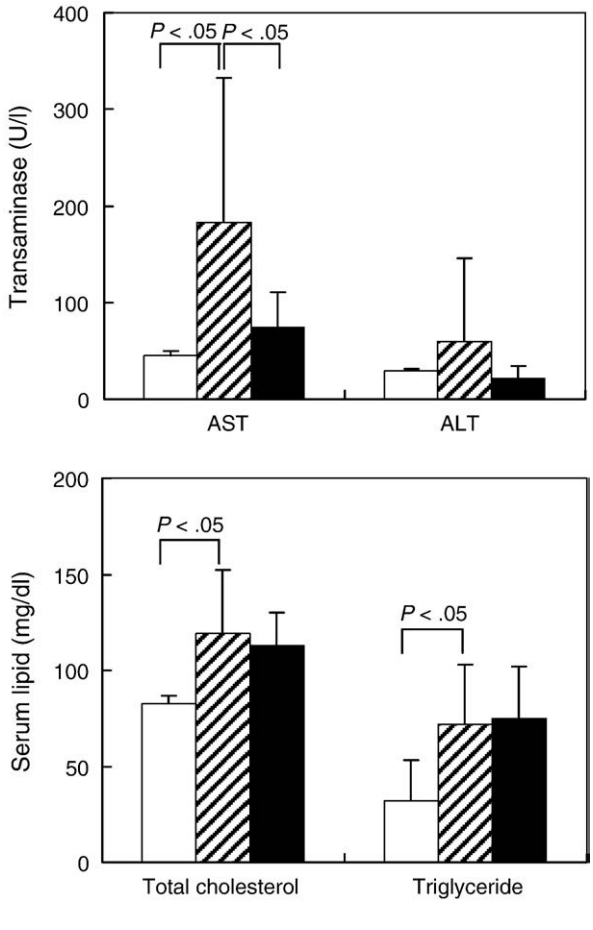

Fig. 4. Serum levels of transaminases (upper panel) and lipids (lower panel) in male mice aged 20 weeks. Open column, wild-type mice (n = 6); hatched column, nSREBP-1c transgenic mice (n = 8); closed column, nSREBP-1c/adiponectin double-transgenic mice (n = 7).

At the age of 30 weeks, liver steatosis, predominantly macrovesicular, occupying more than 33% of the area, was observed in half of the nSREBP-1c transgenic mice. The rate of liver steatosis tended to be lower in age-matched double-transgenic mice (10%). Histologic findings similar to those observed in the liver specimens of the patients with NASH, that is, inflammatory mononuclear cell infiltration, pericellular fibrosis, ballooning degeneration, and Mallory hyaline body formation, were seen in the livers from 13 of 14 nSREBP-1c transgenic mice aged 30 weeks. The frequencies of inflammatory infiltration, ballooning, Mallory hyaline, and fibrosis in the liver were significantly lower in the double-transgenic mice (Fig. 6); mild NASH-like pathology was seen in only 1 of 10 double-transgenic mice. Representative HE-stained liver histology of the nSREBP-1c transgenic mice and the double-transgenic mice at the age of 30 weeks is shown in Fig. 7 (A, D). Azan-Mallory staining revealed perivenular and pericellular fibrosis in 30-week–old nSREBP-1c transgenic mice but not in nSREBP-1c/adiponectin double-transgenic mice (Fig. 7B, E). Immunostaining of 8-OHdG was augmented in the nuclei of hepatocytes and infiltrating mononuclear cells in the liver from the nSREBP-1c transgenic mice (Fig. 7C). In contrast, no immunoreactive 8-OHdG was detected in the liver from age-

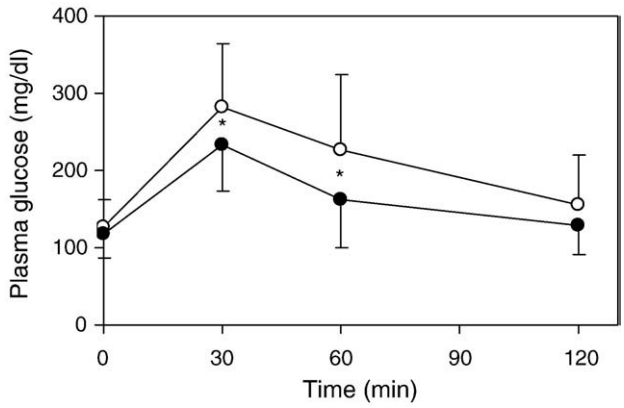

Fig. 5. Intraperitoneal glucose tolerance test of nSREBP-1c transgenic mice (open circle, n = 28) and nSREBP-1c/adiponectin double-transgenic mice (closed circle, n = 18) at the age of 20 weeks. *P less than .05

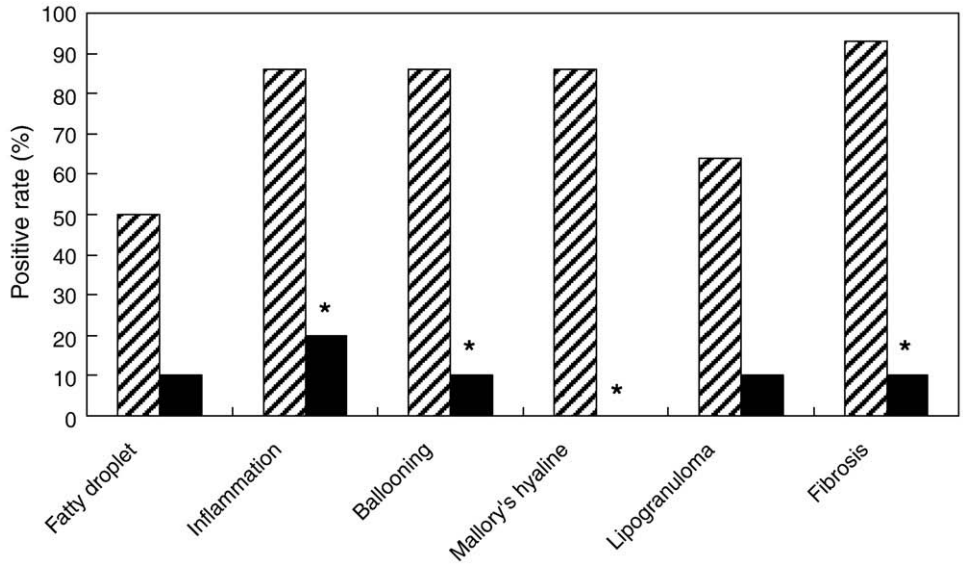

Fig. 6. Histologic findings of the liver from nSREBP-1c transgenic mice (hatched column, n = 14) and nSREBP-1c/adiponectin double-transgenic mice (closed column, n = 10) at the age of 30 weeks. *P less than .01.

matched double-transgenic mice (Fig. 7F). Fig. 7G shows marked fat deposition with macrovesicular steatosis in the liver from an nSREBP-1c transgenic mouse. Proliferating cell nuclear antigen–positive hepatocytes were increased in the liver from nSREBP-1c transgenic mice, whereas the prevalence of PCNA-positive cells was not significantly different between wild-type mice and the double-transgenic mice (Fig. 8).

4. Discussion

Previously, we have reported that the lipodystrophic mice expressing nSREBP-1c in adipose tissue [9] could serve as a model for studying pathogenesis and prevention of human NASH [10], although the transgenic mice lacked obesity and hyperleptinemia. The nSREBP-1c transgenic mice that had insulin resistance, hyperglycemia, hyperlipidemia, and marked fatty liver spontaneously developed intralobular inflammation with ballooning degeneration, focal necrosis, Mallory hyaline bodies, and marked pericellular fibrosis in the intralobular spaces, which are characteristic of NASH. Adiponectin deficiency could be involved in the development of NASH because circulating adiponectin levels were markedly reduced in the nSREBP-1c transgenic mice [10]. Adiponectin treatment was shown to attenuate ethanol-induced liver injury and ameliorate NAFLD in *ob*/*ob* mice [17]. Although hypoadiponectinemia has been implicated in the development of human NAFLD and NASH [11-13], there was no direct evidence that adiponectin has preventive effects on spontaneously occurring NASH. In this study, the double-transgenic mice producing adiponectin in the liver showed normal or slightly elevated serum levels of adiponectin. The development of NASH lesions was prevented in the double-transgenic mice, supporting the hypothesis that hypoadiponectinemia plays a key role in the pathogenesis of NASH.

The double-transgenic mice normally expressed both subtypes of adiponectin receptors, that is, AdipoR1 and AdipoR2, in the liver. Thus, it can be assumed that adiponectin expressed in the liver stimulates adenosine monophosphate–activated protein kinase activation and PPAR-α signaling pathways through AdipoR1 and AdipoR2, respectively. Activation of these pathways may result in increased fatty acid oxidation and reduction of triglyceride content [18,19]. We assessed PPAR-α levels in the liver by the Western blot analysis and found that PPAR-α was increased in the liver from the double-transgenic mice, suggesting the involvement of AdipoR2-mediated signals in the prevention of steatohepatitis. Adiponectin may also exert the protective effect through its anti-inflammatory action [20,21]. It is well known that inflammation is a key mechanism in the progression of fatty liver to steatohepatitis and cirrhosis [22]. In this study, we found that immunolocalization of 8-OHdG, a product of oxidative DNA damage [15], observed in the liver from the nSREBP-1c transgenic mice, was markedly suppressed when adiponectin was produced by hepatocytes. Oxidative stress due to the generation of reactive oxygen species or decreased antioxidant defenses has been implicated in the pathogenesis of NASH [23-25]. Thus, the attenuation of oxidative DNA damage may be a mechanism by which adiponectin prevented the development of NASH. The nSREBP-1c transgenic mice exhibited more hepatocytes positive for PCNA, a marker of cell proliferation. It was reported that PCNA-positive hepatocytes were increased in inflammatory

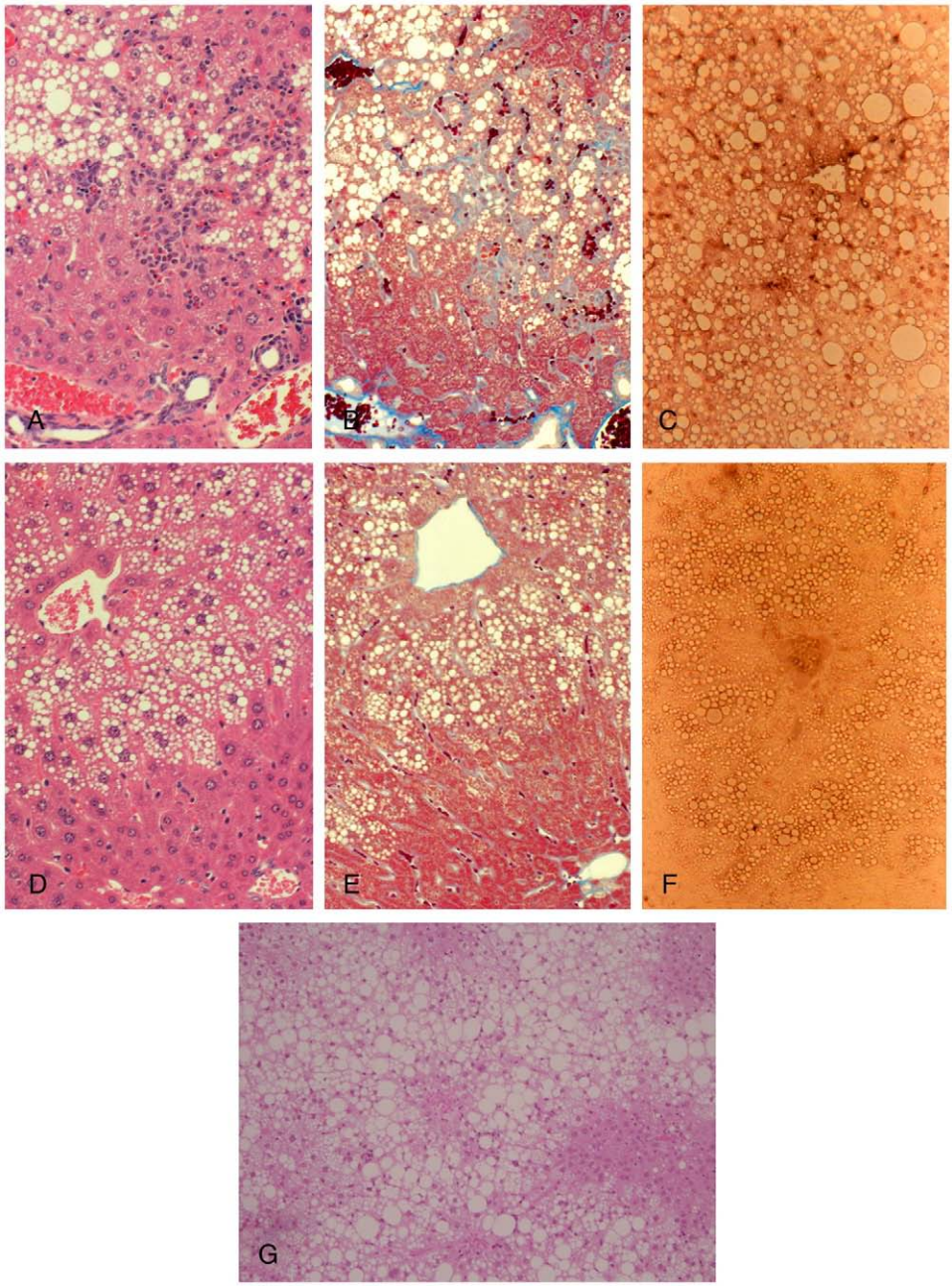

Fig. 7. Representative liver histology of male nSREBP-1c transgenic mice (A, B, C, G) and nSREBP-1c/adiponectin double-transgenic mice (D, E, F) at the age of 30 weeks. Sections were stained with HE for standard microscopy (A, D, G) or Azan-Mallory to observe the location of extracellular matrix (B, E). The 8-OHdG was immunostained using mouse monoclonal anti–8-OHdG and alkaline phosphatase-labeled horse anti-mouse IgG (C, F). Original magnification ×400.

liver diseases including steatohepatitis, chronic hepatitis, and cirrhosis [26]. The suppression of PCNA-positive cells in the double-transgenic mice suggests that adiponectin might prevent the progression of fatty liver disease to cirrhosis and hepatocellular carcinoma by attenuating hepatocyte proliferation.

Recently, it was reported that leptin plays a pivotal role in the development of hepatic fibrosis through up-regulation of transforming growth factor–β in Kupffer cells and stimulation of hepatic stellate cells [27-30]. In this study, however, leptin was unlikely involved in the prevention of NASH because serum leptin concentration was not affected by the transgenic expression of adiponectin. On the other hand, leptin deficiency, as well as hypoadiponectinemia, may play a role in marked insulin resistance associated with lipoatrophy. Yamauchi et al [31] reported that insulin resistance in lipoatrophic mice could be reversed only by the combination of adiponectin and leptin using PPAR-$\gamma^{+/-}$

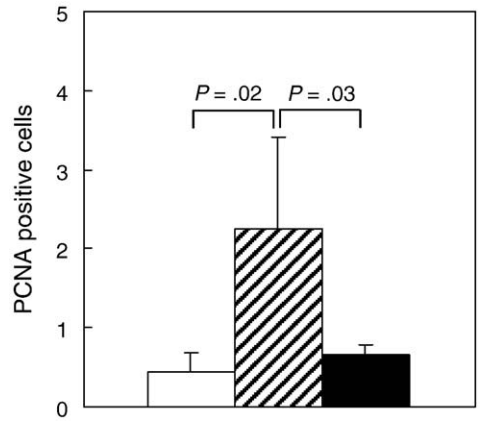

Fig. 8. Ratio of PCNA-positive hepatocytes in liver sections. Open column, wild-type mice; hatched column, nSREBP-1c transgenic mice; closed column, nSREBP-1c/adiponectin double-transgenic mice; n = 4 each. The PCNA-positive hepatocytes were counted under a light microscope in at least 20 fields at a ×400 magnification, and mean number of positive cells per field was calculated.

mice treated with a PPAR-γ/RXR inhibitor. Unlike the model mice in which epididymal fat was absent and serum leptin was undetectable, leptin concentration in nSREBP-1c transgenic mice was approximately one half of that in normal mice. It may be the reason why the NASH-like hepatic lesions were prevented by adiponectin alone.

In conclusion, our study clearly showed the protective effect of adiponectin on liver necroinflammation and fibrosis associated with insulin resistance. Hypoadiponectinemia may be involved in the generation of the second hit, one of which may be reactive oxygen species, in the progression from simple steatosis to NASH.

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