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p53 Involvement in the Pathogenesis of Fatty Liver Disease*

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Obesity is a major health problem in industrialized societies, and fatty liver disease (hepatic steatosis) is common in obese individuals. Oxidative stress originating from increased intracellular levels of fatty acids has been implicated as a cause of hepatocellular injury in steatosis, although the precise mechanisms remain to be elucidated. p53, widely known as a tumor suppressor, has been shown often to be activated in stressed cells, inducing cell cycle arrest or death. Here we demonstrate that p53 is involved in the molecular mechanisms of hepatocellular injury associated with steatosis. We found that p53 in the nucleus is induced in the liver from two mouse models of fatty liver disease, *ob/ob* and a transgenic mouse model that overexpresses an active form of sterol regulatory element-binding protein-1 in the liver (TgSREBP-1), the one with obesity and the other without obesity. This activation of the p53 pathway leads to the elevation of p21 mRNA expression, which can be considered an indicator of p53 activity, because *ob/ob* mice lacking p53 generated by targeting gene disruption exhibited the complete restoration of the p21 elevation to wild type levels. Consistent with these results, the amelioration of hepatic steatosis caused by *Srebp-1* gene disruption in *ob/ob* mice lowered the p21 expression in a triglyceride content-dependent manner. Moreover, p53 deficiency in *ob/ob* mice resulted in a marked improvement of plasma alanine aminotransferase levels, demonstrating that p53 is involved in the mechanisms of hepatocellular injury. In conclusion, we revealed that p53 plays an important role in the pathogenesis of fatty liver disease.

Obesity is frequently associated with a number of diverse diseases, including type II diabetes, hyperlipidemia, and cardiovascular disease, and is a major health problem in industrialized societies (1, 2). Epidemiologic evidence suggests that liver disease might be a major contributor to overall obesity-related morbidity and mortality, affecting 70% of obese individuals (reviewed in Refs. 3 and 4).

The genetically obese *ob/ob* mice develop obesity, insulin resistance, and glucose intolerance because of an inherited deficiency of the appetite-suppressing hormone, leptin (5–9). The absence of leptin presents the most severe obesity known in both rodents and humans (10) and provides a good model of obesity and its related syndromes, including fatty liver disease. It has been documented that lipogenesis in the liver is increased in *ob/ob* mice (7, 8). We have demonstrated previously that the disruption of sterol regulatory element-binding protein-1 (SREBP-1),[1] the key transcriptional regulator of lipogenesis (reviewed in Refs. 11 and 12), leads to marked amelioration of hepatic steatosis (13, 14).

The pathogenesis of liver disease associated with obesity has remained poorly understood. Although fatty liver is typically benign, it is believed that in a subset of individuals gradual progression from hepatic steatosis to steatohepatitis and eventually to cirrhosis can occur. Since liver cell damage is reversible by caloric restriction at least at the early stages of the disease, it is postulated that nutritional overload plays a pivotal role in the mechanisms of hepatocellular injury. Notably, fatty liver disease also develops without obesity in some mouse models, when SREBP-1 expression and thereby lipogenesis are elevated in the liver (15, 16). Thus, the retention of triglycerides within hepatocytes is a prerequisite for the development of fatty liver disease, exerting toxic effects on hepatocytes. As a mechanism by which overnutrition causes liver cell damage, oxidative stress originating from increased intracellular levels of fatty acids is considered to be involved (3, 4), although these concepts remain largely hypothetical.

The p53 gene was the first tumor-suppressor gene to be identified and has been found to be inactivated in most human cancers (17). The p53 protein prevents division of stressed cells or causes them to undergo programmed cell death (apoptosis). For example, γ-irradiation activates p53 to turn on the transcription of $p21^{Waf1/CIP1}$, which binds to and inhibits cyclin-dependent kinases, thus blocking the G_1-S and G_2-to-mitosis transitions. The stresses that activate p53 are diverse, ranging from DNA damage to oxidative stress, hypoxia, and heat shock (18). Thus, p53 has been thought to be a guardian against cellular stresses and has been extensively studied and well established as a tumor suppressor. However, much less is known about other roles of p53 beyond tumor suppression. Notably recent reports (19–21) document that p53 is involved in the molecular mechanisms of some types of hepatocellular injury.

We have reported previously (22) that p53 is activated in adipocytes of *ob/ob* mice, being involved in the mechanisms of dysregulated gene expression in adipose tissue of obese animals. These mice have largely excessive fat deposition in adi-

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[1] The abbreviations used are: SREBP-1, sterol regulatory element-binding protein-1; ALT, alanine aminotransferase; TG, triglyceride; WT, wild type.

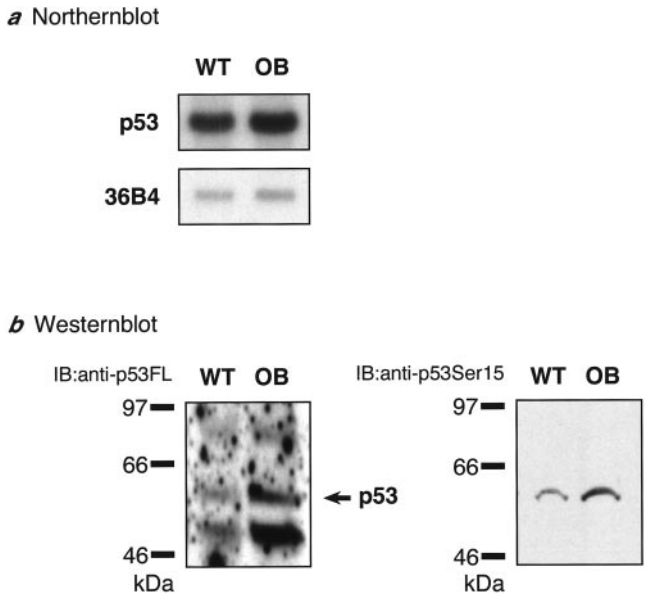

FIG. 1. **p53 activation in the nucleus of *ob/ob* mouse liver.** *a*, Northern blot; *b*, Western blot analysis of p53 in WT and *ob/ob* (*OB*) mouse liver. *a*, total RNA (10 μg) pooled equally from three male mice for each group was subjected to Northern blotting, followed by hybridization with the indicated cDNA probes. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. *b*, aliquots of nuclear protein (20 μg) extracted from livers equally pooled from three male mice for each group were subjected to SDS-PAGE. Immunoblotting (*IB*) using antibody against full-length p53 protein (*left panel*) and against phosphorylated Ser-15 (*right panel*) is shown. Series of experiments were performed twice for the same number of animals, and the data from a representative experiment are shown.

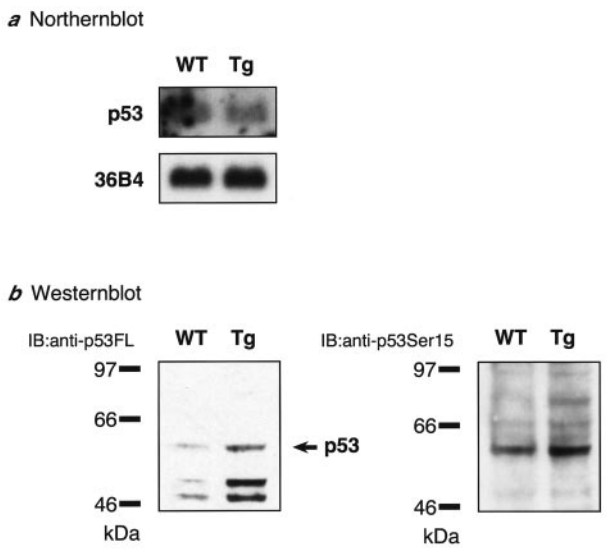

FIG. 2. **p53 activation in the nucleus of TgSREBP-1 mouse liver.** *a*, Northern blot; *b*, Western blot analysis of p53 in WT and TgSREBP-1 (*Tg*) mouse liver (three male mice each). *a*, total RNA (10 μg) was subjected to Northern blotting, followed by hybridization with the indicated cDNA probes. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. *b*, aliquots of liver nuclear protein (20 μg) were subjected to SDS-PAGE. Immunoblotting (*IB*) using antibody against full-length p53 protein (*left panel*) and against phosphorylated Ser-15 (*right panel*) is shown. Series of experiments were performed twice for the same number of animals, and the data from a representative experiment are shown.

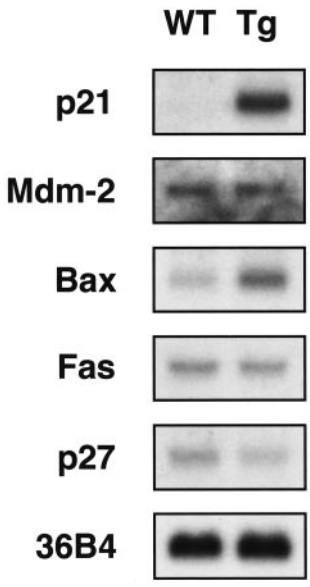

FIG. 3. **p53 activation in TgSREBP-1 mouse liver causes elevation of p53-regulated genes.** Northern blot analysis of p53 downstream genes in livers from WT and TgSREBP-1 (*Tg*) mice (three male mice each) is shown. Total RNA (10 μg) was subjected to Northern blotting, followed by hybridization with the indicated cDNA probes. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. Series of experiments were performed twice for the same number of animals, and the data from a representative experiment are shown.

pocytes and hepatocytes. If overloaded fat stresses adipocytes thereby activating the p53 pathway, the same mechanism could be provoked in the steatotic hepatocytes of obese animals, presumably leading to hepatocellular injury.

Based on these considerations, we hypothesized that steatotic hepatocytes are under various stresses that induce p53, which gives rise to liver cell damage and plays an important role in the development of fatty liver disease. In our present study, we demonstrate that nuclear p53 is induced in the liver from two mouse models of fatty liver disease, one with obesity and the other without obesity, elevating p21 mRNA expression as an indicator of its activity and that, as we expect, p53 is involved in the mechanisms of hepatocellular injury accompanied by steatosis.

EXPERIMENTAL PROCEDURES

Animals—$Lep^{+/ob}$-C57BL/6J and $p53^{+/-}$-C57BL/6J (23) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice deficient in SREBP-1 were established as described previously (24) and were backcrossed 6 times onto the C57BL/6J background. These mice were intercrossed to produce *ob/ob* mice lacking p53 ($ob/ob \times p53^{-/-}$) or SREBP-1 ($ob/ob \times Srebp\text{-}1^{-/-}$) as described previously (13, 22). Genotypes at the p53 loci were determined by PCR (22); those at the leptin loci were by a PCR-based restricted fragment length polymorphism analysis (13), and those at the SREBP-1 loci were by Southern blot analysis with BamHI digestion (24). SREBP-1a transgenic mice overexpressing amino acids 1–460 of human SREBP-1a under control of the rat phosphoenolpyruvate carboxykinase promoter (TgSREBP-1) were made as described previously (15). Genotypes at the transgenic loci were determined by PCR, and heterozygous mice were used. Mice were housed in a temperature-controlled environment with a 12-h light/dark cycle and free access to water and a standard chow diet (Oriental MF, Oriental Yeast, Tokyo, Japan). All experiments were performed with 12-week-old male mice. All animals were sacrificed in an early phase of the light cycle.

Nuclear Protein Extraction and Western Blotting—Nuclear extracts from mice livers were prepared as described previously (25). Aliquots of nuclear protein (20 μg) were subjected to SDS-PAGE. p53 was detected using a 1:500 dilution of anti-p53FL (sc-6243, Santa Cruz Biotechnology). The phosphorylation at Ser-15 of p53 was detected using a 1:1000 dilution of anti-p53 Ser-15 (9284S, New England Biolabs). Bound antibodies were detected with a horseradish peroxidase-coupled anti-rabbit IgG secondary antibody (Amersham Biosciences) and visualized using SuperSignal West Dura chemiluminescent substrates (Pierce).

RNA Isolation and Northern Blotting—Total RNA from mouse liver was extracted using Trizol reagent (Invitrogen), and 10 μg of RNA samples equally pooled from each group (n = 3–6) were run on a 1%

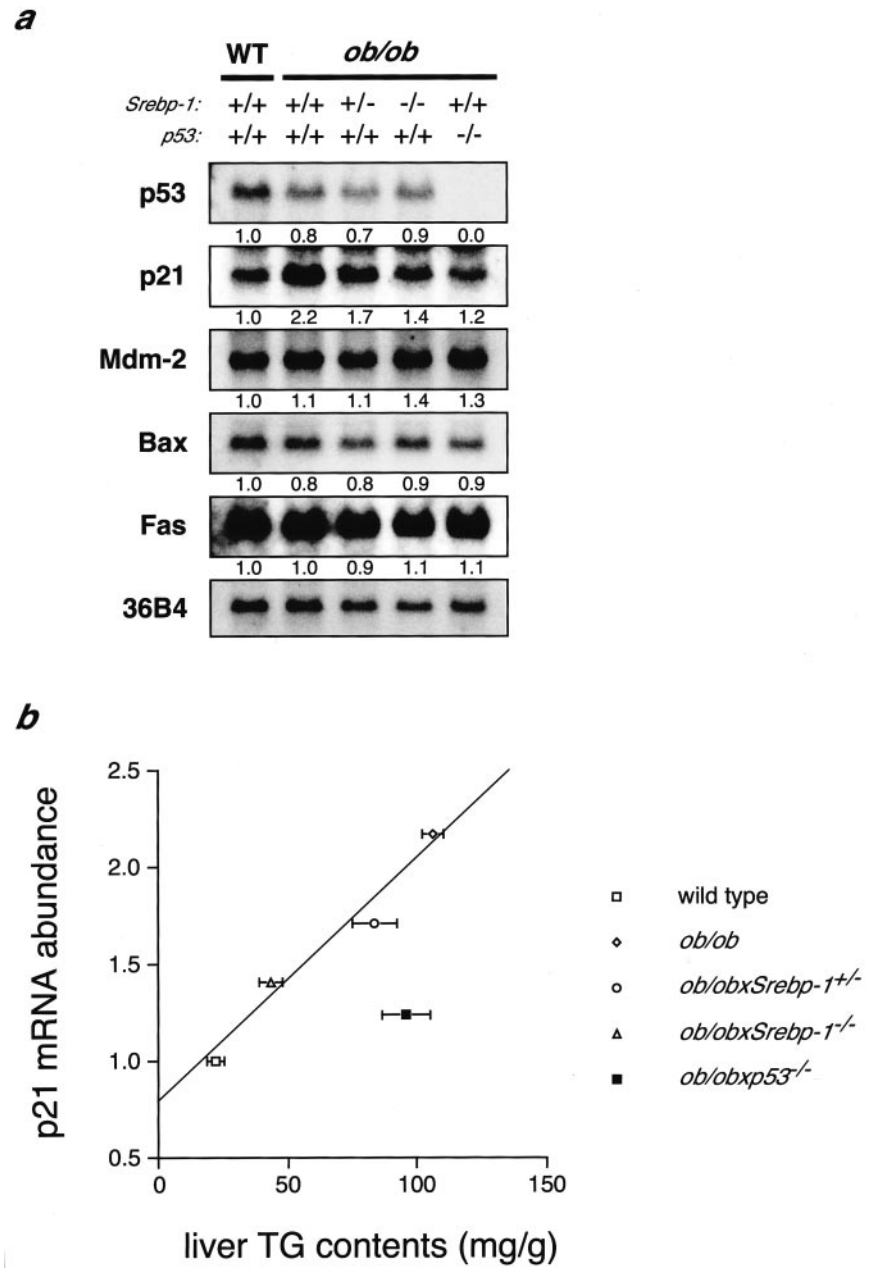

FIG. 4. **p21 mRNA expression as an indicator of p53 activity parallels liver triglyceride contents.** *a*, Northern blot analysis of p53-regulated genes in livers from WT and *ob/ob* mice (six male mice each) lacking SREBP-1 or p53, whose genotypes are indicated in the panel. Total RNA (10 μg) was subjected to Northern blotting, followed by hybridization with the indicated cDNA probes. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. The quantification results obtained with BAS2000 system were normalized to the signal generated from 36B4 (acidic ribosomal phosphoprotein P0) mRNA, and the fold changes against control are displayed *below* each blot. *b*, relationship between liver triglyceride (*TG*) contents (on *horizontal axis*) and p21 mRNA expression level (on *vertical axis*; the same data as shown above) is plotted. Genotypes are indicated in the panel. The regression line among the points for wild type, *ob/ob*, *ob/ob*×*Srebp-1*$^{+/-}$, and *ob/ob* × *Srebp-1*$^{-/-}$ is inserted. *Bars*, S.E.

agarose gel containing formaldehyde and transferred to a nylon membrane. The cDNA probes were cloned as described previously (15, 22, 26). The probes were labeled with [α-^{32}P]dCTP using Megaprime DNA Labeling System kit (Amersham Biosciences). The membranes were hybridized with the radiolabeled probe in Rapid-hyb Buffer (Amersham Biosciences) at 65 °C with the exception of p53, for which ULTRAhyb hybridization buffer (Ambion) was used at 42 °C. The membranes were washed in 0.1× SSC, 0.1% SDS at 65 °C. Blots were exposed to Kodak XAR-5 film.

Blood Chemistries and Liver Lipid Analyses—Plasma alanine aminotransferase (ALT) activities were measured on an automated analyzer at a clinical chemistry laboratory (Special Reference Laboratories, Tokyo, Japan). The contents of triglycerides in liver were measured as described previously (27). Enzymatic assay kit was used for the determination of triglycerides (LH, Wako Pure Chemicals, Tokyo, Japan).

RESULTS

p53 Activation in Two Models of Fatty Livers, ob/ob and TgSREBP-1 Mice—In our first series of experiments, we examined the p53 expression both at mRNA and protein levels in the nucleus from livers of *ob/ob* mice. These mice present a marked increase in hepatic triglyceride contents, *i.e.* fatty livers (hepatic steatosis), and are a good model for fatty liver disease associated with obesity. We extracted nuclear proteins from control and *ob/ob* mice livers, and we analyzed them by Western blotting with anti-p53 and anti-phosphorylated p53 at Ser-15 antibodies. As shown in Fig. 1, we demonstrated that *ob/ob* mouse liver had higher levels of p53 protein in the nucleus than wild type. Consistently, an active form of p53, phosphorylated at Ser-15, was also up-regulated in *ob/ob* livers (Fig. 1). In contrast, p53 mRNA level was not elevated in *ob/ob* livers.

We also performed these analyses of p53 expression in SREBP-1 transgenic (TgSREBP-1) mice. This is another model of fatty liver but does not display systemic obesity. Similarly to *ob/ob* mice, TgSREBP-1 mice showed elevated expression of p53 protein with no increase in mRNA expression levels (Fig. 2). Phosphorylation of p53 at Ser-15 was also increased in parallel.

p53 Activation in Fatty Liver Causes Elevation of p53-regulated Genes—To explore the effects of p53 activation in the nucleus from fatty livers, we further examined the expression profile of p53 downstream genes such as p21, Mdm-2, and Bax

α in these animals. As shown in Fig. 3, these p53-regulated genes were elevated in the livers of TgSREBP-1 mice. In contrast, only p21 was elevated in *ob/ob* livers, whereas Mdm-2 and Bax α were not altered in mRNA expression levels (Fig. 4*a*). The mRNA expression of Fas, another p53 downstream gene, was not increased in either mouse model. These data suggest that p53 activation causes the up-regulation of p21 as its target gene in livers of *ob/ob* and TgSREBP-1 mice, and that the mRNA expression level of p21 is a good indicator of p53 activity in fatty livers.

p21 mRNA Expression as an Excellent Indicator of p53 Activity—To validate that the elevation of p21 in fatty liver is really caused by p53, we assessed the effects of p53 deficiency in *ob/ob* mice. For this purpose, we intercrossed *ob/ob* and p53-null mice, and we obtained six male mice deficient in both leptin and p53 ($ob/ob \times p53^{-/-}$) in the C57BL/6J background. These doubly homozygous mice were equally obese compared with *ob/ob* mice as we reported previously (22). The Northern blot analysis on livers from these mice revealed that the disruption of p53 completely abolished the up-regulation of p21 mRNA expression observed in *ob/ob* mice to the same levels as in wild type (Fig. 4, *a* and *b*). Meanwhile, the mRNA expression levels of Mdm-2, Bax α, and Fas were not influenced by p53 disruption. These results established that p53 activation caused the elevated expression of p21 in *ob/ob* livers and that p21 mRNA level is a good indicator of p53 activity.

Disruption of SREBP-1 Ameliorates Fatty Livers and Thereby Lowers p21 Expression—To clarify further the relationship between fatty livers and the p53-p21 pathway, we analyzed *ob/ob* mice deficient in SREBP-1. These mice were obtained by intercrossing of *ob/ob* and SREBP-1-null mice in the C57BL/6J background (13), and as we reported previously, the double homozygotes exhibit a marked improvement of fatty livers. As expected, p21 mRNA expression in livers of double homozygotes ($ob/ob \times Srebp\text{-}1^{-/-}$; $n = 6$) was decreased to near wild type levels, and heterozygotes ($ob/ob \times Srebp\text{-}1^{+/-}$) were at an intermediate level (Fig. 4, *a* and *b*), so the improvement of hepatic steatosis by *Srebp-1* gene disruption in *ob/ob* mice completely paralleled the decrease in p21 expression. These results demonstrate that amelioration of fatty livers lowers the elevated expression of p21.

p53 Disruption Alleviates Liver Injury of ob/ob Mice—ALT is a liver-specific enzyme that is released into the blood when hepatocytes are damaged. Therefore, plasma ALT levels are widely used as an excellent marker to evaluate liver injury, and it is well known that they are elevated in humans and animals with fatty livers including *ob/ob* mice. To estimate the degree of liver cell damage in *ob/ob* mice lacking SREBP-1, we measured plasma ALT levels in these mice. As expected, *ob/ob* mice without either allele of *Srebp-1* gene ($ob/ob \times Srebp\text{-}1^{-/-}$) showed decreased levels of ALT, and heterozygotes ($ob/ob \times Srebp\text{-}1^{+/-}$) were at an intermediate position (Fig. 5*a*). These values are completely in parallel with the hepatic triglyceride contents, indicating that plasma ALT levels are a good marker for hepatic steatosis accompanied by liver injury. Next we evaluated plasma ALT in *ob/ob* mice deficient in p53. Notably, the double homozygotes ($ob/ob \times p53^{-/-}$), whose hepatic triglyceride contents were equal to those of *ob/ob* mice as shown in Fig. 4*b*, exhibited marked reduction in plasma ALT levels compared with *ob/ob* mice. These findings demonstrate that p53-mediated cytotoxicity plays an important role in the pathogenesis of liver injury concomitant with hepatic steatosis.

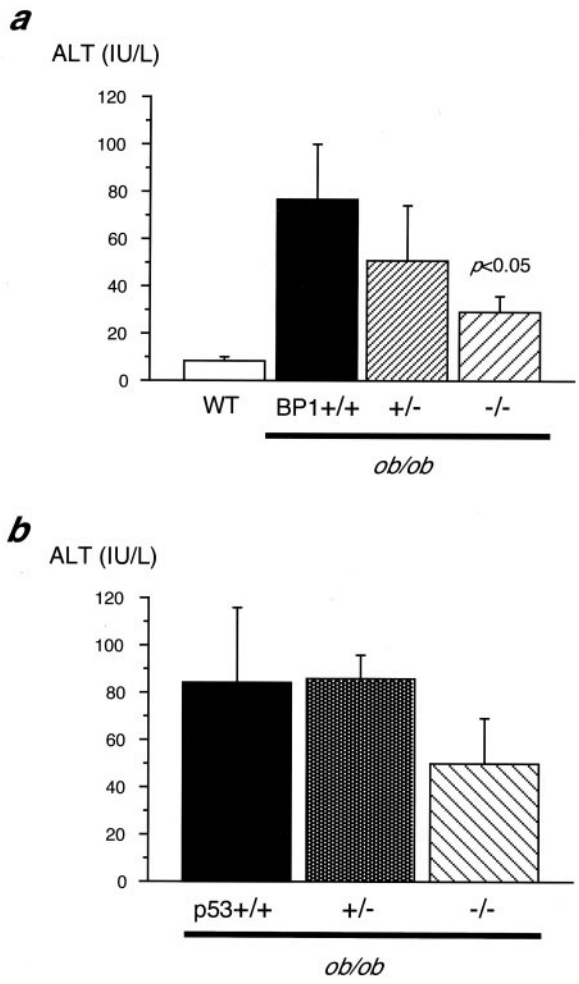

FIG. 5. **p53 disruption alleviates liver injury of *ob/ob* mice.** *a*, plasma ALT activity in WT and *ob/ob* mice with various *Srebp-1* genotype (+/+, +/−, or −/− as indicated). Each group consists of six male mice. *p* value was calculated by Student's *t* test against *ob/ob* with BP1+/+ mice. *b*, plasma ALT levels in *ob/ob* mice lacking p53 allele (six male mice each) are shown. Genotypes are as indicated. Mice were 12-week old. *Bars*, S.E.

DISCUSSION

Our present studies clearly demonstrate that p53 is activated to induce p21 expression in two different models of hepatic steatosis, and that the p53 pathway is involved in the pathogenesis of fatty liver disease. Although others have reported that p53 activation itself coincides with fatty liver in humans (28), this is the first report to describe the role of p53 in the mechanisms of hepatocellular injury accompanied by hepatic steatosis.

The mechanisms of hepatocellular injury associated with steatosis have largely been unknown. It is postulated that excess fat accumulation in the liver predisposes cells to hepatocellular injury, and that this is presumably caused by the cellular toxicity of excess free fatty acids, oxidative stress, and lipid peroxidation (3, 4). All of these can potentially activate the p53 pathway as causes of p53 activation are diverse, ranging from DNA damage to oxidative stress and hypoxia (18). In particular, oxidative stress is currently stated to be a central mechanism of cytotoxicity in steatotic liver, and multiple potential sources of oxidative stress have been identified including cytochrome P4502E1 (CYP2E1), peroxisomal β-oxidation, mitochondrial electron leak, and ceramide production (4, 29). These oxidative stresses might possibly be involved in the mechanisms of p53 activation observed in our current study. Moreover, reactive oxygen species-induced cytokine release (transforming growth factor-β, tumor necrosis factor-α, or interleukin-8) that may contribute to the pathogenesis of liver injury (19, 30) has also been shown to induce p53 activation (21, 31, 32).

We have reported recently (22) that p53 is activated in adipocytes of *ob*/*ob* mice. It is very intriguing if one can assume that some kind of stresses brought about by excess fat accumulation compel this activation and that the same mechanisms could be turned on in case of steatotic hepatocytes. This analogy is also remarkable from the standpoint of the lipotoxicity theory proposed by Unger (29), where it is assumed that in the pathogenesis of obesity triglycerides overaccumulate in various tissues and exert toxic effects.

The molecular mechanism by which p53 causes hepatocellular injury also remains obscure. Bax protein is reported to be implicated in the cytotoxic effects of p53 through transcriptional up-regulation (33) and is demonstrated to be increased in *ob*/*ob* mouse liver (34). However, we detected no elevation of Bax mRNA expression in *ob*/*ob* liver nor reduction in *ob*/*ob* mice lacking p53 (*ob*/*ob* × $p53^{-/-}$), although it was induced in TgSREBP-1 mouse liver. Fas mRNA expression was not increased, which is also reported to be a p53-regulated cell surface protein known to trigger apoptosis (35). These results are not surprising because it is known that the p53 target genes are widely divergent and tissue-dependent (36). As far as we examined, the only gene found to be up-regulated by p53 was p21, but it is not generally considered as a cytotoxic molecule. The actual mediator of the cytotoxic action of p53 needs to be identified in further investigations.

Our findings may have good clinical implication for a therapy against fatty liver disease in that inhibition of p53 might possibly prevent steatotic hepatocytes from being damaged. Remarkably, it was recently demonstrated that inhibition of p53 protects liver tissue against endotoxin-induced hepatocellular injury (20), increased sensitivity to which is reported to be involved in the pathogenesis of fatty liver disease (37).

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