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 p21WAF1/CIP1, which binds to and inhibits cyclin-dependent kinases, thus blocking the G1-S and G2-M 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.
macromolecules in the liver of rats 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 (22); those at the SREBP-1 loci were by Southern 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 SDDS-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.

EXPERIMENTAL PROCEDURES

Animals—Lep-o/o, 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 × p53−/−) or SREBP-1 (ob/ob × Srebp-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) using a 1:500 dilution of anti-p53FL (sc-6243, Santa Cruz Biotechnology). 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%
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 $^{32}$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 M SSC, 0.1% SDS at 65°C. Blots were exposed to Kodak XAR-5 film.

**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 Involvement in Fatty Liver Disease**—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.

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**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.
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. 4a). 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 × 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 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 × Srebp-1−/−; n = 6) was decreased to near wild type levels, and heterozygotes (ob/ob × Srebp-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 × Srebp-1−/−) showed decreased levels of ALT, and heterozygotes (ob/ob × Srebp-1−/+ ) were at an intermediate position (Fig. 5a). 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 × p53−/−), whose hepatic triglyceride contents were equal to those of ob/ob mice as shown in Fig. 4b, 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.

**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 tissues and exert toxic effects.

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REFERENCES