STAT-3 Overexpression and p21 Up-Regulation Accompany Impaired Regeneration of Fatty Livers

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Fatty liver is an important cause of morbidity in humans and is linked to impaired liver regeneration after liver injury, but the mechanisms for impaired liver regeneration remain unknown. In the normal liver, the interleukin (IL)-6/STAT-3 pathway is thought to play a central role in regeneration because this pathway is disrupted in IL-6-deficient mice that exhibit impaired liver regeneration after 70% partial hepatectomy (PH). To determine whether inhibition of STAT-3 is involved in fatty liver-related mitoinhibition, regenerative induction of STAT-3 was compared in normal mice and leptin-deficient ob/ob mice that have fatty livers and markedly impaired liver regeneration after PH. In both groups, two waves of STAT-3 activation were observed, the first in endothelia and the second in hepatocytes. Before PH, a significantly higher percentage of ob/ob endothelial and hepatocyte nuclei expressed phosphorylated (activated) STAT-3. After PH, phospho-STAT-3 accumulated in liver nuclei of lean mice and this response was markedly exaggerated in ob/ob mice. Moreover, a striking inverse correlation was noted between hepatocyte nuclear accumulation of phospho-STAT-3 and DNA synthesis (as assessed by bromodeoxyuridine labeling), as well as cyclin D1 mRNA induction and protein expression. In contrast, STAT-3 activation was positively correlated with p21 protein expression in both groups of mice. Because these results link exaggerated STAT-3 activation with impaired hepatocyte proliferation, STAT-3 inhibition cannot be a growth-arrest mechanism in ob/ob fatty livers. Rather, hyperinduction of this factor may promote mitoinhibition by up-regulating mechanisms that impede cell cycle progression. (*Am J Pathol* 2002, 161:155-161)

In humans, nonalcoholic fatty liver disease is most frequently associated with obesity^{1,2} and diabetes mellitus,¹⁻³ and less commonly with drug effects⁴ or malab-

sorption/malnutrition syndromes.⁵⁻⁷ Impaired liver regeneration can be an important clinical complication of steatosis, manifesting as increased morbidity and mortality after partial hepatic resection⁸ and as delayed graft function and graft failure in liver allografts.⁹⁻¹¹ The mechanism(s) of impaired liver regeneration remain unclear but investigations in rodent models of fatty liver disease have begun to elucidate abnormalities in cell cycling that may explain nonalcoholic fatty liver disease. In the ob/ob mouse model of nonalcoholic fatty liver disease, leptin-deficient mice spontaneously develop steatosis and hepatomegaly. Interestingly, ob/ob mice have increased basal rates of hepatocyte proliferation¹² and have up-regulated anti-apoptotic pathways,¹³ but despite this, have impaired liver regeneration in response to LPS injury.¹³ In the genetically obese fa/fa Zucker rat, impaired liver regeneration was found to be associated with interruption in the normal IL-6 signaling pathway, a critical pathway in normal hepatocyte proliferation.¹⁴

IL-6 signaling activates members of the STAT family of transcription factors. In addition to IL-6, a number of other growth factors and cytokines can activate the STAT family of transcription factors, which play important roles in a wide variety of cellular processes ranging from cell growth to apoptosis. In normal liver regeneration after partial hepatectomy (PH), STAT-3 is rapidly activated in hepatocytes¹⁵ after IL-6 signaling through the gp130 receptor complex, which leads to phosphorylation and activation of STAT-3. STAT-3 can also be activated by other cytokines such as G-CSF¹⁶ and leptin,¹⁷ but in the PH model, STAT-3 is activated almost exclusively by IL-6.¹⁸ STAT-3 can, in turn, activate at least three separate pathways, leading to cell cycle progression, growth arrest/differentiation, or anti-apoptosis.¹⁹

Given the central role of STAT-3 in the IL-6 signaling pathway and preliminary results from this laboratory associating increased IL-6 and STAT-3 expression with steatosis in the ob/ob mouse model of nonalcoholic fatty liver disease,²⁰ we hypothesized that STAT-3 signaling may contribute to impaired regeneration of fatty livers by aberrant up-regulation of growth arrest and differentiation pathways. To further explore this hypothesis, we evalu-

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ated the activation of STAT-3 by measuring the hepatic content of total and phosphorylated STAT-3 and determined the cellular localization of phosphorylated (ie, activated) STAT-3 expression after PH in ob/ob and lean, control mice. These results were correlated with markers for cell cycle progression to S phase, ie, bromodeoxyuridine (BrdU) labeling and cyclin D1 mRNA and protein levels, and with induction of inhibitors of the G₁/S phase transition, p21 and p27.

Materials and Methods

Animals

Eight-week-old ob/ob C57BL-J6 mice and their lean, heterozygote littermates (Jackson Laboratories, Bar Harbor, ME) were maintained in a temperature- and light-controlled animal facility and were permitted *ad libitum* access to water and standard pellet chow for 2 weeks. After this time interval, ob/ob and lean mice were injected with 50 μ g of BrdU per gram body weight and underwent 70% PH according to the technique of Higgins and Anderson.²¹ Mice were sacrificed by cervical dislocation at 0, 1, 6, 24, and 36 hours after surgery. The liver was immediately dissected from the carcass and a 5-mm cross-section was fixed in 10% buffered formalin, with the remainder snap-frozen in liquid nitrogen and stored at -80°C for subsequent processing. Results were compared to ob/ob and lean mice that were given BrdU without PH.

Evaluation of Hepatocyte-Proliferating Activity

Previous work in our laboratory has shown that in ob/ob mice, BrdU labeling closely parallels proliferating cell nuclear antigen labeling, with a peak in both labeling indices at 24 hours.²⁰ Thus, proliferating activity was evaluated with BrdU labeling and experiments were performed to 36 hours. For each liver, 10 $\times$ 400 fields were examined and the number of BrdU-positive hepatocyte nuclei were counted. The results were then grouped to obtain the mean and SEM for each time interval.

Evaluation of Total and Phosphorylated STAT-3, Cyclin D1, p21, and p27 Protein Expression

Liver nuclear proteins were extracted as previously described according to the techniques of Lavery and Schibler.²² Proteins concentrations were demonstrated by a dye-binding assay according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). For immunoblot assays, liver proteins (40 μ g/lane) in Lamelli buffer were separated by polyacrylamide gel electrophoresis and transferred to nylon membranes. A brief incubation in 5% low-fat milk was used to block nonspecific bindings and membranes were incubated overnight at 4°C , washed, and exposed to secondary antiserum. Signal intensity was quantified with a densitometer and samples were normalized to values in lean time 0. Primary

antibodies were used according to the manufacturer's directions for total and phosphorylated STAT-3 (1:1000; Cell-Signaling Technology, Beverly, MA), p21 (1:400; Santa Cruz Diagnostics, San Diego, CA), p27 (1:400; Santa Cruz Diagnostics), and cyclin D1 (1:400; Santa Cruz Diagnostics).

Isolation of RNA and RNase Protection Assay

Total RNA was isolated from the liver of each mouse as previously described.²⁰ RNA was quantified by spectroscopy and the quality evaluated on gel electrophoresis. To detect cyclin D1 mRNA, an RNase protection assay was used according to the manufacturer's instructions (PharMingen, San Diego, CA). GAPDH or 18S RNA expression were concurrently evaluated as loading controls. Ten μ g samples of mRNA were used and samples from two to three mice were evaluated for each time point.

Immunohistochemistry

Histological sections were obtained from formalin-fixed, paraffin-embedded tissues. Five- μ m sections were stained with phospho-STAT3 antibody (Tyr705, no. 9131; Cell Signaling Technology), which recognizes only phosphorylated STAT-3 (pSTAT-3), at a 1:50 dilution after heat antigen retrieval. A biotin-free immunostaining system was used to visualize the antibody (EnVision; DAKO, Carpinteria, CA). A hepatocyte-labeling index (percentage of positive nuclei) was determined by counting 500 hepatocyte nuclei in sequential high-powered fields ($\times$ 400). In addition, 100 portal vein and 100 central vein endothelial cells were counted. The results were grouped to obtain the mean and SE of mean for each time interval.

Results

STAT-3 Activation and Localization during Liver Regeneration

By Western blot analysis, pSTAT-3 protein expression was higher in ob/ob mice than lean littermates at time 0 and at all subsequent time points after PH (Figure 1). Immunohistochemistry was then used to determine cellular localization of pSTAT-3 expression. This was detected predominately in the nuclei of hepatocytes and endothelial cells, but was also present in occasional biliary epithelial cells, inflammatory cells, and Kupffer cells. pSTAT-3 nuclear accumulation was seen in substantially more hepatocytes and endothelia in ob/ob mice than lean mice at all time points (Figure 2).

Further quantification of endothelial and hepatocyte expression was performed using a labeling index. pSTAT-3 staining in the endothelium from the portal and central veins was similar within each time point (data not shown), so data from these endothelia were combined for subsequent analysis. In the lean mice, the endothelium showed a rapid increase in pSTAT-3 nuclear staining that

Figure 1. STAT-3 activation after PH. Nuclear extracts from four lean and four ob/ob mice were examined for total and phosphorylated STAT-3 with an immunoblot assay using 40 μ g of nuclear proteins. A representative immunoblot is shown.

peaked at 1 hour and then declined somewhat, but remained elevated above baseline for all remaining time points (Figure 3). In the ob/ob mice, the endothelium showed elevated pSTAT-3 staining at baseline and also showed a rapid increase, but the peak was delayed until 24 hours after surgery.

In hepatocytes, basal levels of pSTAT-3 were markedly elevated in the ob/ob mice over that of their lean littermates at time 0 (Figure 3) and remained higher at all subsequent time points. In lean mice, hepatocyte expression of pSTAT-3 peaked at 24 hours with 5% of hepatocytes showing nuclear labeling. In contrast, the peak in the ob/ob mice was at 36 hours with 37% of hepatocytes showing nuclear positivity. The pSTAT-3 staining in the ob/ob hepatocytes was still increasing at 36 hours, so the true peak may be at a later time point, but in both cases the peak hepatocyte staining followed peak endothelial staining by 12 to 24 hours.

Hepatocyte Proliferation after PH

In normal liver regeneration, STAT-3 is known to be induced by factors such as epidermal growth factor and IL-6 that are associated with hepatocyte proliferation. Hence, induction of STAT-3 in the regenerating liver remnant could stimulate DNA synthesis in the remaining hepatocytes. Consistent with this concept, induction of STAT-3 after PH is virtually abolished in IL-6-deficient mice, which have impaired liver regeneration after PH.^{18,23,24} Therefore, enhanced STAT-3 activity in ob/ob mice may accompany increased hepatocyte proliferative activity. To assess this, the incorporation of BrdU by hepatocyte nuclei was compared in lean and ob/ob mice at various times after PH. Although increased baseline labeling was seen in ob/ob livers, BrdU labeling was significantly inhibited in this group after PH (Figure 4).

The inhibited incorporation of BrdU in ob/ob hepatocyte nuclei after PH suggests that the cells are being arrested in the prereplicative period. To evaluate this possibility, induction of cyclin D1 mRNA and protein after PH were compared in lean and ob/ob mice. In ob/ob

mice, cyclin D1 induction is significantly impaired after PH, ie, the mRNA induction is delayed until 36 hours after PH and even at this time point, cyclin D1 protein expression remains significantly reduced (Figure 5).

STAT-3 is not known to regulate cyclin D1 gene expression directly. However, it does function as a transcriptional activator of p21 and p27, genes that encode cyclin-dependent kinase inhibitors (CKI). Immunoblot analysis demonstrates that these CKIs are relatively overexpressed in ob/ob mice compared to lean controls (Figure 6). In lean mice, p27 is expressed constitutively but decreases transiently after PH. In contrast, p21 is not expressed basally but increases transiently during the regenerative response. Ob/ob mice have greater hepatic p27 content basally and, although p27 expression declines somewhat after partial PH, it remains greater in ob/ob mice than controls at all time points. Similarly, the induction of p21 is enhanced in ob/ob mice compared to controls, mirroring the differences in STAT-3 induction between these two groups. Thus, in these studies increased regenerative induction of STAT-3 occurs in ob/ob hepatocytes that exhibit significant inhibition of DNA synthesis after PH. This up-regulation of STAT-3 is accompanied by the enhanced induction of p21, a STAT-3-regulated CKI that is known to halt the G₁/S phase transition in hepatocytes, along with preserved expression of p27 after PH, another STAT-3-regulated CKI that inhibits mitogen-induced DNA synthesis in hepatocytes.²⁵ Taken together, these results do not support the concept that STAT-3 inhibition impairs hepatocyte proliferation in fatty livers. Rather, they suggest that STAT-3 hyperstimulation induces CKIs that inhibit cell cycle progression in fatty hepatocytes, leading to impaired regeneration. Our results do not separate delayed from decreased regeneration.

Discussion

PH leads to activation of a number of cytokine-dependent and cytokine-independent pathways required for normal liver regeneration.²⁶ One critical pathway is the IL-6 cytokine signaling pathway that primes the hepatocytes to respond to mitogenic signals: IL-6 knockout mice have impaired liver regeneration after PH, but can be rescued by exogenous IL-6 administration.^{18,23,24} The IL-6 signal is transduced through the GP130 signaling complex, which leads to activation of JAK family of kinases, which in turn activates STAT-3 by phosphorylation (pSTAT-3).^{27,28} pSTAT-3 then translocates to the nucleus where it activates transcription of a number of downstream targets.^{27,28} STAT-3 activation does not require *de novo* protein synthesis, indicating that the cytoplasm contains a reserve pool of STAT-3.¹⁵ In this study, we used an antibody that recognizes only the phosphorylated form of STAT-3 to discriminate from the inactive pool of STAT-3. The nuclear (as opposed to cytoplasmic) localization by immunohistochemistry also strongly supports the conclusion that the active, phosphorylated form of STAT-3 was reliably identified.

Figure 3. Both hepatocytes (**A**) and endothelial cells (**B**) in ob/ob mice showed increased pSTAT-3 nuclear labeling at baseline. In response to PH, the lean mice showed a modest increase in pSTAT-3 labeling that peaked at 24 hours, whereas the ob/ob mice showed a delayed, but much greater increase in nuclear staining that had not peaked by 36 hours (**A**). Similarly, the endothelial cells in the ob/ob mice showed increased pSTAT-3 labeling and had a delayed peak compared to the lean mice. Five hundred hepatocytes and 200 endothelial cells (100 portal vein, 100 central vein) were scored in each liver at every time point. Each time point represents two to three mice. Results are shown as mean $\pm$ SEM.

Although STAT-3 signaling has been reported in hematopoietic,²⁹ neural,³⁰ and epithelial tissues,^{31,32} and very recently in endothelium,³³ STAT-3 signaling in the liver has been primarily ascribed to hepatocytes, despite

Figure 4. Hepatocyte nuclear incorporation of BrdU was impaired in ob/ob mice after PH. Mice had BrdU injected into the peritoneal cavity 2 hours before sacrifice. Results are for two to three mice per group (mean $\pm$ SEM).

(to our knowledge) no reported data on specific cellular localization. Our results indicate the presence of at least two separate waves of STAT-3 activation after PH: the first in endothelial cells and the second in hepatocytes. Endothelial expression of pSTAT-3 increased in both the lean and ob/ob mice within an hour after PH, with labeling peaking at 1 hour in lean mice and a delayed peak at 24 hours in the ob/ob mice. The first wave of pSTAT-3 endothelial expression is likely to be IL-6-independent, because increased IL-6 is first detected in the liver 6 hours after PH in the ob/ob mouse.²⁰ In contrast, the time course of pSTAT-3 accumulation in hepatocyte nuclei is compatible with IL-6 induction of STAT-3 in these cells after PH.

Levels of activated STAT-3 were higher in ob/ob mice than lean controls at baseline and at all subsequent time points after PH. The ob/ob mice also had decreased BrdU labeling in the hepatocytes at 24 to 36 hours and increased expression of p21 and p27, two CKIs. Therefore, after PH, increased pSTAT-3 expression in ob/ob hepatocytes is associated with signaling for growth arrest/differentiation, rather than with proliferation. In contrast, constitutive STAT-3 expression apparently does not preclude hepatocyte proliferation, as there is increased BrdU labeling at baseline in ob/ob livers.

This inconsistent relationship between nuclear pSTAT-3 and hepatocyte proliferative activity may reflect differences in cyclin D1 expression. In ob/ob livers, cyclin D1 protein expression is increased at baseline but does not increase after PH. Whereas, in control mice, cyclin D1 protein is barely detected until 24 to 36 hours after PH. Because cyclin D1 binds to and sequesters p21 to form a complex that lacks CKI activity, increases in cyclin D1 may neutralize any increases in p21 that result from enhanced STAT-3 activity. Cyclin D1 also complexes with STAT-3 itself, limiting both the nuclear accumulation and transcriptional activity of STAT-3.³⁴ Thus, the ultimate

Figure 2. ob/ob mice showed significant steatosis at time 0 hours (**A**). Nuclear staining for pSTAT-3 is detected at time 0 hours in an ob/ob mouse liver (**B**). Twenty-four hours after PH, the ob/ob mice showed a similar degree of steatosis (**C**) and significantly more hepatocyte nuclear labeling for pSTAT-3 (**D**). Endothelial nuclear labeling can also be seen in a central vein (**D**). In contrast, livers from lean littermates showed no steatosis at time 0 hours (**E**) and were generally negative for nuclear pSTAT-3 staining (**F**). After PH, there were no morphological changes (**G**) and only occasional scattered hepatocyte nuclei were positive for pSTAT-3 (**H**).

Figure 5. Cyclin D1 mRNA (A) and protein levels (B) were lower in ob/ob mice than lean mice after PH. **A:** Changes in the mRNA levels were measured using an RNase protection assay. 18S RNA was concurrently evaluated to ensure lane-to-lane equivalency of RNA loading and transfer and a representative autoradiograph is shown. The graph summarizes the data with two to three mice per time point. **B:** Cyclin D1 protein levels were evaluated with an immunoblot assay using 40 μ g per lane. A representative blot is shown. Results are for two to three mice per group (mean $\pm$ SEM), except for ob/ob at 36 hours, which is a single mouse.

effect of pSTAT-3 is modulated by other cell cycle regulators, some of which are STAT-3-dependent (eg, p21) and others of which (eg, cyclin D1) are not. When cyclin D1 is deficient, as it is from 24 to 36 hours after PH in ob/ob mice, the nuclear accumulation of pSTAT-3 is enhanced, favoring both the production and biological activity of the CKI, p21. This interpretation is consistent with reports that STAT-3 is hyperphosphorylated when cell cycle progression is inhibited in a number of tissues, including the liver.³⁵

Other groups have already demonstrated that p21 is a key inhibitor of G₁ to S phase progression in hepatocytes. For example, it is known that liver regeneration is impaired in p21 transgenic mice.³⁶ p27, another inhibitor of G₁ cyclins, is also increased in ob/ob livers before and after PH. Cells, such as adult hepatocytes, that are in replicative quiescence are known to express p27 and this CKI is normally down-regulated before hepatocytes enter

S phase after PH.³⁷ Therefore, our studies have identified at least two mechanisms that help to explain the reduced hepatocyte BrdU incorporation in fatty hepatocytes after PH. Coupled with the aforementioned STAT-3 data, these findings indicate that liver regeneration is inhibited in ob/ob livers after PH, despite exaggerated activation of STAT-3. Therefore, although the findings in IL-6-deficient mice suggest that STAT-3 activation is necessary for hepatocyte proliferation after PH,¹⁵ the present results demonstrate that pSTAT-3 is not sufficient to induce cell cycle progression in fatty hepatocytes. Hence, the mitoinhibitory mechanisms that inhibit liver regeneration in ob/ob fatty livers do not involve a failure of STAT-3 activation.

On the other hand, the enhanced accumulation of pSTAT-3 might help to protect ob/ob hepatocytes from cytokine-mediated lethality after PH, because in many cell lines, inhibition of STAT-3 potentiates cell killing.^{25,38–40} These cytoprotective actions of pSTAT-3 are thought to involve p21 induction, because strategies that block p21 accumulation generally increase cellular cytotoxicity.^{41,42} Moreover, IL-6 induces p21 in some cell types⁴³ and IL-6 signaling clearly protects hepatocytes in many experimental models of liver injury that increase hepatic exposure to tumor necrosis factor- α .⁴⁴ Tumor necrosis factor- α increases after PH in ob/ob mice,²⁰ and fatty livers are known to be vulnerable to tumor necrosis factor- α toxicity.¹³ Thus, growth inhibition may be an inadvertent consequence of cytoprotective signals that promote sustained STAT-3 activation to protect ob/ob hepatocytes from tumor necrosis factor- α lethality after PH. Viewed in this context, inhibited hepatocyte proliferation may actually be the lesser of two evils for fatty livers.

Figure 6. CKIs p21 and p27 levels were higher at baseline and all subsequent time points in ob/ob mice compared to lean littermates. p21 levels increased in both ob/ob and lean mice, whereas p27 levels showed an initial decrease after PH followed by a modest increase. p21 and p27 protein levels were evaluated with an immunoblot assay using 40 μ g per lane. A representative blot is shown. The graph summarizes the data with two to three mice per time point. Results are for two to three mice per group (mean $\pm$ SEM).

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