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Inhibition of Signal Transducer and Activator Transcription Factor 3 in Rats with Acute Hepatic Failure

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In fulminant hepatic failure, survival is not possible without recovery of sufficient hepatocyte mass. Remarkably, only a few studies exist that provide insight into the mechanisms that control proliferation of residual hepatocytes after extensive hepatocyte loss. In this regard, the role of growth-regulatory factors, including pro-inflammatory cytokines such as interleukin-6 (IL-6), is not well understood. In the present study we show that in rats with critically low (10%) hepatocyte mass, whether with or without ongoing liver cell necrosis, inhibition of liver regeneration is associated with early and sustained increase in blood IL-6 levels. Under these conditions, the signal transducer and activator of transcription (Stat3) DNA binding activity was lowered at the time of G1/S cell-cycle transition. We further demonstrate that the protein inhibitor of activated Stat3 (PIAS3) and the suppressor of cytokine signaling (SOCS-1) were up-regulated early after induction of liver failure (6–12 h). *In vitro,* IL-6 induced PIAS3 expression in HGF stimulated rat hepatocytes. These findings suggest that after massive hepatocyte loss, an early and rapid rise in blood IL-6 levels may weaken the hepatic regenerative response through up-regulation of Stat3 inhibitors PIAS3 and SOCS-1. © 2000 Academic Press

Key Words: **hepatocyte; hepatic failure; hepatic regeneration; cell growth; signal transduction; transcription factors; hepatectomy.**

The ability of mammalian liver to restore partial loss has long been recognized. The most thoroughly investigated example of this process is the rat model of two-thirds hepatectomy (PH) (1–4). In rats, the liver loss of such magnitude induces the maximal hepatocyte proliferative response. Resections greater than standard PH have been shown to inhibit liver regeneration (5, 6), but the underlying mechanisms are poorly understood. Recent studies have suggested that after massive hepatocyte loss, impairment of liver regeneration may be related to decreased hepatocyte growth factor (HGF) and HGF receptor *c-met* expression (5, 6), and increase in blood levels of a potent growth suppressor transforming growth factor β1 (TGF-β1) (7).

Liver regeneration is a tightly regulated process involving different liver cell populations and a finely orchestrated interplay between growth factors, cytokines, hormones, extracellular matrix components and other regulators (1, 2). Evidence obtained in "knock out" mice suggested that IL-6/Stat3 signaling may play a pivotal role in the initiation of hepatocyte proliferation by helping hepatocytes enter a state of replicative competence ("priming") so that they can respond to growth-stimulatory factors (such as: HGF, EGF, and TGF-α) (8–11). Recently, it has been suggested that Stat3 is also involved in the G1 to S cell-cycle transition through the up-regulation of cyclins D2, D3, A, and cdc-25A, and concomitant down-regulation of cdk inhibitors (p21, p27) (12). It is well established that the plasma levels of HGF and many other cytokines, including IL-6, IL-1 and TNF-α, are elevated in acute and chronic hepatic failure of various etiologies (13–15), yet liver regeneration is attenuated (5). We hypothesized that after massive hepatocyte loss, IL-6 dependent signal transduction pathway is inhibited. We herein report that in rats with surgically-induced hepatic failure and critically low (10%) hepatocyte mass, despite early and sustained increase in blood IL-6, Stat3 was not activated at 12 h postoperatively and translocation of Stat3 into the cell nuclei was suppressed. We further present evidence indicating that inhibition of IL-6/Stat3 signaling was preceded by the induction of potent Stat3 inhibitors: (1) suppressor of cytokine signaling (SOCS-1) and (2) protein inhibitor of activated Stat3 (PIAS3).

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MATERIALS AND METHODS

Collagenase (type IV), ethylenediaminetetraacetic acid (EDTA), reagents for culture media, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Human recombinant HGF, IL-6, and IL-6 enzyme linked immunosorbent assay (ELISA) kit were from R&D Systems (Minneapolis, MN), tissue culture media (Ham F12, Williams E) were from Gibco (Gaithersburg, MD), fetal bovine serum from Hyclone Laboratories, Inc. (Logan, UT), and Percoll was from Pharmacia (Piscataway, NJ). Permanox dishes (Nunc, Inc., Naperville, IL) were coated with rat tail type I collagen purchased from Collaborative Research (Bedford, MA). BCA protein assay kit was from Pierce Chemicals (Rockford, IL), Trizol reagent was from Gibco BRL (Grand Island, NY), T4 polynucleotide kinase, α-^{32}P dCTP, Klenow DNA Polymerase I, and protease inhibitors were from Boehringer (Mannheim, FRG), [^{32}P]-γATP was from NEN Products (Boston, MA), ECL Western blotting detection system and nitrocellulose and nylon Hybond membranes were from Amersham (Buckinghamshire, UK), IL-6R, gp130, Janus kinas 2 (JAK2) and Stat3 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), proteinase inhibitors (phenylmethylsulfonyl-fluorid, leupen, aprotinine, pepstatin) were from Sigma Chemical Co. (St. Louis, MO). The membranes were hybridized to cDNA probes labeled with [^{32}P]cCTP by random priming DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Rapid-Hyb buffer was from Amersham (Buckinghamshire, UK). As cytokine signal inhibitors, the 2 kb-mouse PIAS3 isolated from the pCMV-flag in between *Xho*I and *Hin*dIII restriction site was a kind gift from Dr. B.K. Shuai (UCLA, Los Angeles, CA), and the 700 bp SOCS-1 isolated from pEF-flag in Xba-1 restriction site was a kind gift from Dr. S. Cory (University of Melbourne, Australia).

Surgical Animal Models

Male Sprague-Dawley rats weighing 250–350 g were housed in a climate-controlled (21°C) room under a 12 h light-dark cycle and were given tap water and standard laboratory rat chow (Rodent Chow 5001, Ralston Purina, St. Louis, MO) *ad libitum*. The animals received humane care according to the guidelines prepared by the National Institute of Health, USA. All operations were performed between 9:00 a.m. and noon under general (methoxyflurane) anesthesia using sterile surgical technique. At the completion of surgery, rats were injected subcutaneously with a 5% dextrose solution (8 ml/100 g body weight).

Partial (2/3) hepatectomy (PH). The two anterior liver lobes (68% liver) were removed following the standard method of Higgins & Anderson (4).

Extensive (90%) hepatectomy (EH). Following removal of the two anterior liver lobes, the portal triad in a common pedicle to the right liver lobes was ligated and both lobes were resected. The two omental liver lobes were left intact.

Fulminant hepatic failure (FHF) model. Following removal of the two anterior liver lobes, the portal triad in a common pedicle to the right liver lobes was ligated, as described previously (5). The two omental liver lobes (8% of the liver) together with a small (1–2% of the liver) amount of liver tissue attached to the front and posterior aspect of the vena cava were left intact. The suggested contributions of the affected liver lobes to the total liver mass are based on earlier data derived from Sprague-Dawley rats subjected to selective portal branch ligation and hepatic resection (16). We have previously shown that in FHF rats, changes in blood chemistry and neurological status (coma, brain swelling) reflect rapid development of hepatic failure and that residual hepatocytes show no signs of proliferation, as judged by negative bromo-deoxyuridine, proliferation cell nuclear antigen, and mitotic index measurements (5).

Sham operation (SO). It consisted of laparotomy and mobilization of the liver.

Isolation of Hepatocytes

Hepatocytes were harvested from livers of Sprague-Dawley rats (200–250 g) by a two-step portal vein perfusion using EDTA and collagenase, as described earlier (17). After enrichment through a Percoll density gradient, viability of the cells was over 90%, as determined by trypan blue exclusion test.

Hepatocyte Cultures

Purified hepatocytes were seeded at 15,000 cells/cm^2 onto 60 mm dishes coated with rat tail type I collagen. Cells were plated in 3 ml of a 1:1 mixture of Ham F12 and Williams E medium supplemented as recommended by Isom *et al.* (18), except that the dexamethasone concentration was reduced to 10^{-7} mol/L and insulin (0.6 mg/L) and proline (32.25 mg/L) were present (19). During the first 4 h in culture, 5 % fetal bovine serum was added to promote cell attachment. The medium was then changed to serum-free medium. Treatment with human recombinant HGF (10 ng/ml) and IL-6 (5 ng/ml) was initiated on day 2, and 24 h later treated cells were harvested. Each cell culture experiment was performed in duplicate and repeated three times.

Blood IL-6 Levels

Levels were measured using ELISA.

DNA Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed following Buratowski and Chodosh (20), as described elsewhere (19). The DNA oligonucleotide corresponding to the Stat3 binding site in the α2-macroglobulin promoter was synthesized. The sequence of the top strand of Stat3 binding site was 5′-GATCCTTC TGGGAATTCCTAGAT-3′, that of nuclear factor kappa B (NF-κB) was 5′-TGGTTA ATGATCTACAGTTA-3′, and that of an activator protein 1 (AP-1) was 5′-CGCTTGATGACTCAG-CCGCCGAA-3′. The complementary oligonucleotide was annealed and the double-stranded probe was labeled using α-^{32}P dCTP and Klenow polymerase. The oligonucleotide probe was labeled with [^{32}P]-γATP by using T4 polynucleotide kinase. Thirty micrograms of tissue extract were incubated with the probe in binding buffer (50 mM KCl, 0.5 mM EDTA, 10% glycerol, 25 mM Hepes, 0.5 mM DTT). One hundred times consensus and mutated oligonucleotide unlabeled probes were added for competition assay, and 2 μg anti-Stat3 and 2 μg anti-Stat1 antibody was used in the supershift assay.

Western Blot Analyses

Analyses of whole cell lysates and nuclear protein extracts obtained from residual (viable) liver tissue were performed as described earlier (19). Polyclonal antibodies to IL-6R, gp130, JAK2, and Stat3 were used as primary antibodies.

Northern Blot Analyses

Analyses of total RNA isolated from residual (viable) liver tissue were carried out, as described earlier (19).

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance or by Fisher's exact test, when deemed appropriate. P or α values of $\leq$.05 were considered significant. Data are presented as means $\pm$ SD.

RESULTS AND DISCUSSION

The role of IL-6/Stat3 signaling in liver regeneration has been demonstrated in mice with targeted disruption of the IL-6 gene (9). Treatment of IL-6-deficient mice with a single dose of IL-6 prior to PH recovered Stat3 DNA binding activity, gene (c-*myc*, AP-1, cyclin D1) expression, and normal hepatocyte proliferation. Further, in mice lacking type I TNF receptor (TNFR-I), DNA synthesis after PH is severely impaired and the expected increases in NF-κB and Stat3 activity do not occur (21, 22). Injection of IL-6 in TNFR-I-deficient mice shortly before PH corrected the defect in DNA synthesis and restored Stat3 activity to normal levels. In addition, Stat3 is engaged in the regulation of gp 130-induced G1 to S cell-cycle transition through the induction of cyclin D2, D3, A and cdc25A, and the concomitant down-regulation of cdk inhibitors p21 and p27 (12). These data could suggest that the inhibition of liver regeneration after massive hepatocyte loss is through blocking the IL-6/Stat3 signal transduction pathway. Therefore, we examined Stat3 DNA binding activity in the livers of rats subjected to 90% hepatectomy (EH) and in rats with fulminant hepatic failure (FHF) where similar loss of liver tissue is accompanied by an ongoing liver cell necrosis. We have previously demonstrated that FHF rat model reproduces a number of morphological (e.g., massive hepatocyte loss and apoptosis/necrosis), physiological and biochemical features seen clinically in FHF, including severely impaired ability of the residual liver tissue to regenerate (5). Rats subjected to partial (2/3) hepatectomy (PH) and sham-operation (SO) were used as controls. Rats from each group were euthanized in batches of five at various times after surgery. Whole cell extracts were prepared from viable residual livers and subjected to DNA mobility shift assay using [^{32}P]-labeled oligonucleotides representing the consensus binding sites for Stat3, NF-κB, and AP-1. As shown in Figs. 1 and 2, Stat3 DNA binding activity was markedly increased at 12 h after standard PH; at all other time points studied it was at the level seen in SO controls. In contrast, Stat3 activity in FHF rat livers remained at the level seen in SO rat livers at 2, 6, and 12 h post-induction; after 24 h it was almost undetectable. In EH rats, Stat3 activity was at the level seen in SO rat livers at 2 and 6 h post-hepatectomy, and at 12 and 24 h it was not detected. It is of interest that we failed to demonstrate an increase in Stat3 activity at 2 h after partial (2/3) hepatectomy, even though we repeated EMSA several times and tested additional animals (data not shown), Although our results differ from those by Cressman *et al.* (23), it is worth noting that in this study, the specificity of EMSA was verified by the supershift assay, whereas verification of their data was by the cold competition analysis only. Furthermore, in this study the DNA binding activity of NF-κB and AP-1 in liver failure rat livers was at the level of PH and SO control rats in the early postoperative period (Fig. 3). The latter observation corroborates our recent finding that in FHF rats, the early-immediate gene response (e.g., c-*myc* expression) is preserved (data not shown). Also Panis *et al.* (24) demonstrated that c-*myc* and c-*fos* expression is intact after massive (85%) hepatectomy in rats.

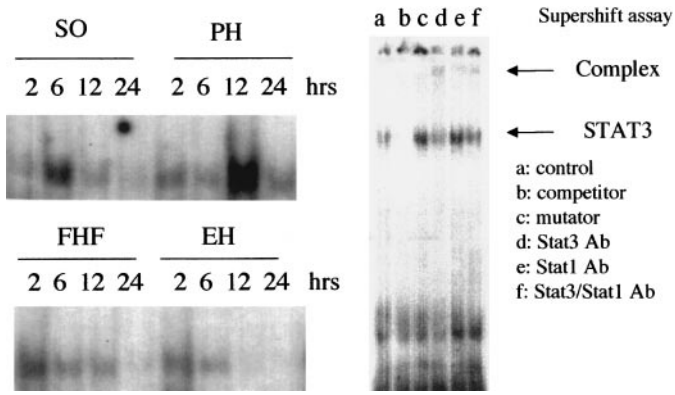

FIG. 1. Stat3 DNA binding activity in the livers of rats subjected to extended (90%) hepatectomy (EH) and after induction of fulminant hepatic failure (FHF), where similar loss of liver tissue is accompanied by ongoing liver cell necrosis. Rats subjected to partial (2/3) hepatectomy (PH) and sham-operation (SO) were used as controls. Rats from each group were euthanized in batches of five at the indicated time intervals. Whole cell extracts were prepared from viable residual livers and Stat3 activation was determined by DNA gel mobility shift assay using radiolabeled oligonucleotide probe containing the Stat3 binding site in the α2-macroglobulin promoter. An autoradiogram representative of 4–5 independent tissue samples per each time point is shown. Competition binding and DNA gel mobility supershift assay identify the protein in the shifted bands as Stat3.

These results demonstrated that after massive hepatocyte loss (EH, FHF), Stat3 activation is blocked at the time which in normal PH control rats is associated with the beginning of G1 to S cell-cycle transition. It is known that activation of Stat3 by IL-6 is through a signal transducing β-chain (gp130) receptor unit and that phosphorylation of quiescent cytoplasmic Stat factors by Janus tyrosine kinases allows Stat3 translocation into the nucleus and binding to the promoters of the respective target genes (12, 25–27). Therefore, whole cell extracts were prepared from remnant livers and subjected to Western blot analyses to measure the protein levels of both IL-6 receptor subunits (IL-6R, gp 130) as well as JAK2. In addition, nuclear extracts were prepared and also subjected to Western blotting to measure the Stat3 content. As shown in Fig. 4, no differences in protein levels of IL-6R, gp130 and JAK2 were found among four groups studied. Significantly, the nuclear Stat3 levels were very low in FHF and EH rat livers when compared to those harvested from PH and SO rats (Fig. 5).

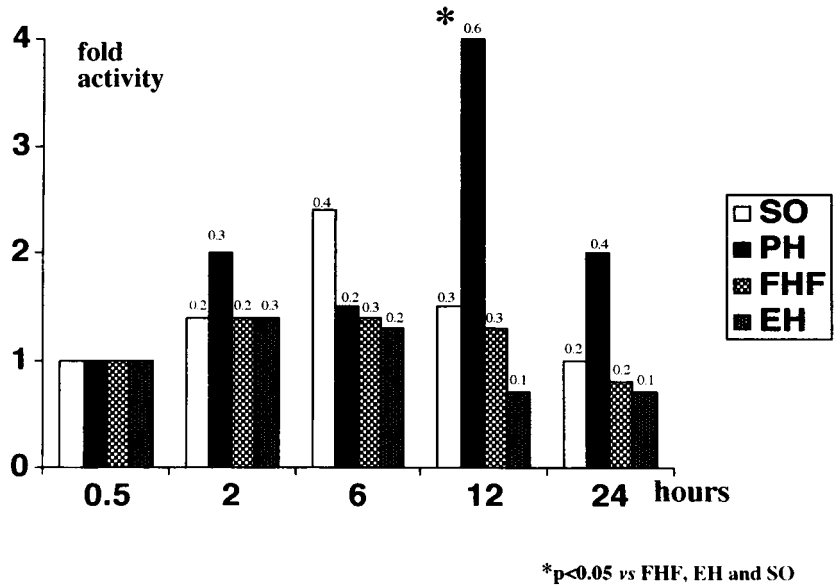

FIG. 2. Stat3 DNA binding activity in the livers of rats subjected to partial (2/3) hepatectomy (PH), extended (90%) hepatectomy (EH), induction of fulminant hepatic failure (FHF), and sham operation (SO), as judged by densitometry of the Stat3 bands obtained during DNA gel mobility shift assays (n = 4–5 per each group and time point). Densitometric measurements were performed using NIH image 1.61 system (Wayne Rasband, NIH, Bethesda, MD) and data derived from five animals per each group and time point are expressed in arbitrary optical density units. The ratios between each band density and that of the background were calculated and the corresponding means ± standard deviation (S.D.) plotted.

The above findings could suggest that the inhibition of Stat3 DNA binding in residual livers of FHF and EH rats is through either blocking the phosphorylation of Stat3 or inhibition of DNA binding, or both. Recently, it has been demonstrated that these effects can be mediated by the SOCS-1 (24, 25) and PIAS3 (26), respec-

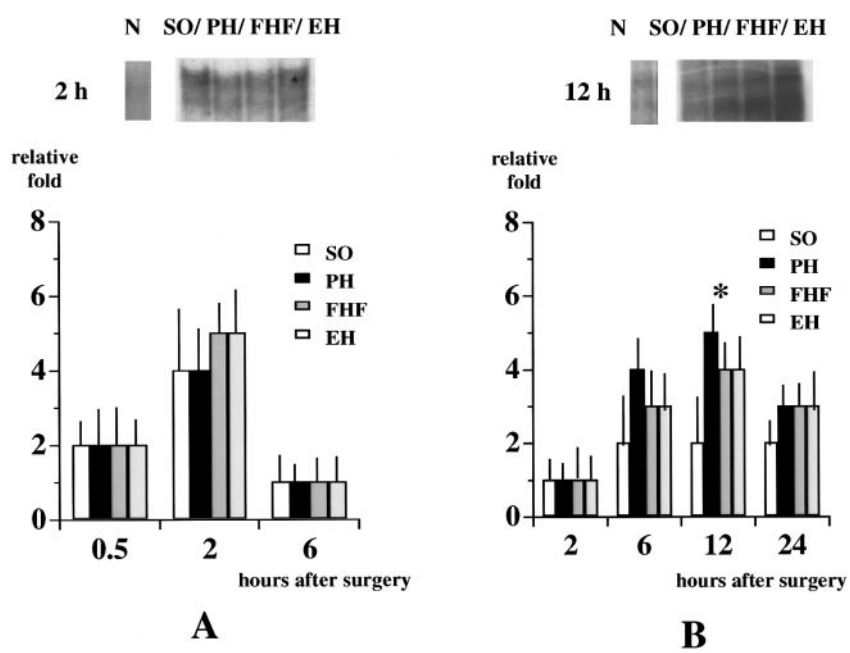

FIG. 3. NF-κB (A) and AP-1 (B) DNA binding activity in the livers of rats subjected to partial (2/3) hepatectomy (PH), extended (90%) hepatectomy (EH), induction of fulminant hepatic failure (FHF), and sham operation (SO), as judged by densitometry of the NF-κB and AP-1 bands obtained during DNA gel mobility shift assays. Densitometric measurements were performed as described in Fig. 2. In the rat livers harvested after 12 and 24 h, no NF-κB activity was detected (A). In contrast, AP-1 activity was present at all time points studied and at 12 h post-PH, it was significantly higher than in SO control rats (B) ($P < 0.05$).

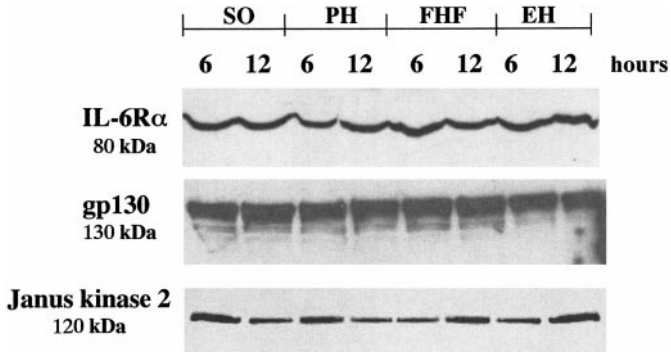

FIG. 4. Expression of IL-6R, gp130, and JAK2 proteins in the livers of rats subjected to partial (2/3) hepatectomy (PH), extended (90%) hepatectomy (EH), induction of fulminant hepatic failure (FHF), and sham operation (SO). Whole cell lysates were prepared from residual (viable) livers harvested after indicated periods of time and analyzed using Western blotting as described under Materials and Methods. An autoradiogram representative of 4–5 independent tissue samples per each time point is shown.

tively. We wondered whether any of these two Stat3 inhibitors become induced in FHF and EH rat livers during the initial cell cycle. Therefore, total RNA was extracted from residual rat livers harvested at various time points after FHF, EH, PH, and SO and subjected to Northern analysis of SOCS-1 and PIAS3 expression. As shown in Fig. 6, in SO rat livers, PIAS3 mRNA was barely detectable, whereas in PH rats it was up-regulated at 12 and 24 h post-hepatectomy. In contrast, FHF and EH rat livers showed early (6 h) and marked PIAS3 up-regulation. Moreover, SOCS-1 expression was similar in SO and PH rats, whereas in FHF and EH rats it was markedly increased at 12 and 24 h postoperatively.

These results suggest that after massive hepatocyte loss, whether with (FHF) or without (EH) ongoing liver cell necrosis, inhibition of Stat3 activation results from

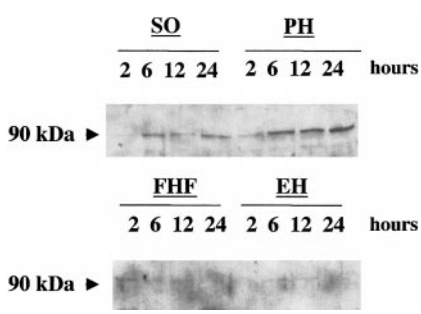

FIG. 5. Expression of Stat3 protein in the livers of rats subjected to partial two-thirds hepatectomy (PH), extended (90%) hepatectomy (EH), induction of fulminant hepatic failure (FHF), and sham operation (SO). The nuclear cell extracts were prepared from residual (viable) livers harvested after indicated periods of time and analyzed using Western blotting as described under Materials and Methods An autoradiogram representative of 4–5 independent tissue samples per each time point is shown.

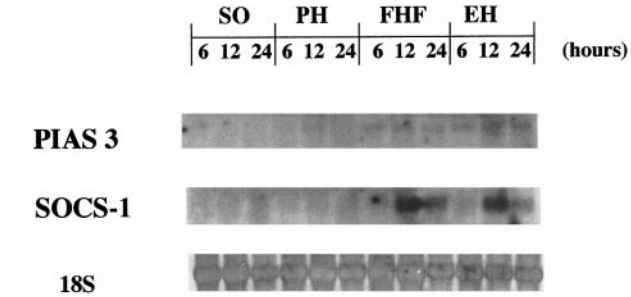

FIG. 6. Expression of Stat3 inhibitors SOCS-1 and PIAS3 in the livers of rats after partial (2/3) hepatectomy (PH), extended (90%) hepatectomy (EH), induction of fulminant hepatic failure (FHF), and sham operation (SO). Total RNA was extracted from residual (viable) rat livers harvested at various time points after surgery and subjected to Northern analysis. An autoradiogram representative of 4–5 independent tissue samples per each time point is shown.

early PIAS3 up-regulation. It is known that IL-6 induces SOCS-1 expression in cultured hepatocytes (25). Therefore, it could be that this cytokine can have a similar effect on PIAS3. To address this question, primary hepatocytes were harvested from rat livers and cultured in hormonally and chemically defined media enriched with HGF (10 ng/ml). Optimal cell density, growth factor concentration and duration of treatments for maximal hepatocyte proliferative response have been determined earlier (28). Treatment with IL-6 (5 ng/ml) was initiated on day 2, and 48 or 72 h later treated cells were harvested and analyzed for PIAS3 expression using Northern blot. As shown in Fig. 7, IL-6 induced PIAS3 mRNA expression in rat hepatocyte cultures. The significance of this finding would be strengthened if FHF and EH rats showed early and marked elevation in blood IL-6 levels. There-

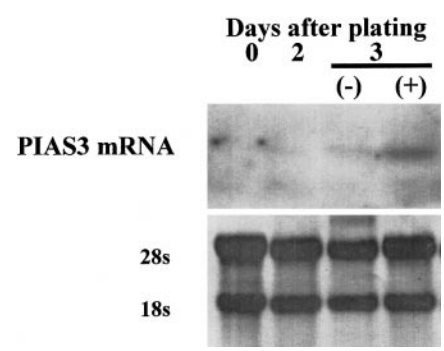

FIG. 7. Influence of IL-6 on the expression of PIAS3 in HGF-stimulated cultures of primary rat hepatocytes. Hepatocytes harvested from livers of Sprague-Dawley rats were seeded at 15,000 cells/cm^2 onto 60 mm collagen-coated dishes and cultured in a hormonally-defined Ham F12 and Williams E (1:1 vol/vol) medium. Treatment with HGF (10 ng/ml) and IL-6 (5 ng/ml) was initiated on day 2, and 24 h later treated cells were harvested, total RNA's were extracted and 20 μg aliquots were analyzed by Northern blot hybridization for RNA encoding PIAS3. Each cell culture experiment was performed in duplicate and repeated three times.

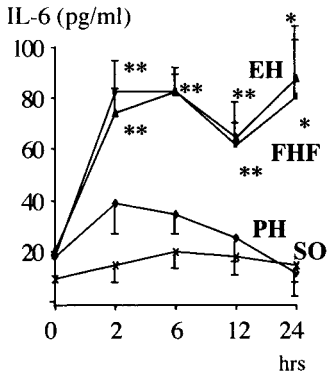

FIG. 8. Blood IL-6 profile in FHF, EH, PH and SO rats. Blood was collected from batches of 5 animals from each group at the indicated period of time and analyzed by ELISA for IL-6 content. Data are shown as means (pg/ml) ± S.D. (*$\alpha < 0.05$; **$\alpha < 0.01$; ANOVA).

fore, we determined the blood profile of IL-6 in both models of severe acute hepatic failure and in partially (68%) hepatectomized and sham-operated rats as controls. As shown in Fig. 8, FHF rats showed early (2 h), significant (eightfold) and sustained increase in blood IL-6 levels. Remarkably, in EH rats in whom there was no ongoing liver cell necrosis, the blood IL-6 profile was identical to that found in FHF rats. The exact cause of early and rapid increases in blood IL-6 levels remains unclear. Although we did not study IL-6 expression in residual livers, they probably did not generate significant amounts of IL-6, a suggestion that is supported by Tovey *et al.* (29) who demonstrated that IL-6 mRNA level was greatly reduced in the livers of patients with severe liver disease, including those with drug induced fulminant hepatitis. Increased production of IL-6 at extrahepatic sites should thus be considered. For example, it is possible that in FHF and EH rats, in which the reticuloendothelial system was severely reduced in size, a rapidly developing endotoxemia stimulated IL-6 production in cells of extrahepatic origin (leukocytes, endothelial cells, epithelial cells). We advance this notion because it has been demonstrated that rats subjected to hepatectomies greater than 70% develop endotoxemia and that lipopolysaccharide is a potent inducer of IL-6 transcription and IL-6 production in polymorphonuclear leukocytes (30, 31). Finally, it is also likely that IL-1β, which in FHF rats is also induced (data not shown), stimulated IL-6 production in epithelial cells, as suggested by the study of Parikh *et al.* (32). In theory, after massive hepatocyte loss, elevated blood insulin levels could have reduced IL-6-inducible Stat3 activity, as suggested by the results of *in vitro* studies by Campos *et al.* (33). However, we previously demonstrated that in FHF and EH rats, blood insulin and glucagon profiles were similar to that found in PH control animals (34).

In summary, the results of this study suggest that after massive hepatocyte loss in the rat, early increase in blood IL-6 levels may weaken the hepatic regenerative response through early up-regulation of Stat3 inhibitors PIAS3 and SOCS-1. As a result, IL-6/Stat3 signaling becomes suppressed at the beginning of the G1 to S cell cycle transition. These observations together with our earlier data on IL-6/TGF-β1 mediated down-regulation of HGF receptor c-*met* expression in FHF and EH rat livers (5, 6, 35) indicate that in acute hepatic failure caused by massive hepatocyte loss, several humoral, cellular, and molecular abnormalities are contributing to the inability of surviving hepatocytes to proliferate.

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