Expression and DNA-Binding Activity of Signal Transducer and Activator of Transcription 3 in Alcoholic Cirrhosis Compared to Normal Liver and Primary Biliary Cirrhosis in Humans

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In rats, activation of the cytokine-inducible transcription factor signal transducer and activator of transcription 3 (Stat3) is impaired in the liver after ethanol administration. The aim was to examine Stat3 expression, localization, and activity in alcoholic liver disease (ALD) in humans. Explanted livers of ALD patients were compared to normal and primary biliary cirrhosis livers. Protein expression, DNA-binding, and subcellular localization of Stat3 was examined by Western blotting, electrophoretic mobility shift assays, and immunohistochemistry; and interleukin-6, Stat3, and suppressor of cytokine signaling (SOCS)-3 mRNA expression by quantitative polymerase chain reaction. Stat3 proteins increased markedly in ALD, mainly in hepatocyte and proliferating biliary epithelial cell nuclei. In contrast to normal and primary biliary cirrhosis livers where Stat3 DNA-binding occurred normally, no Stat3 DNA-binding complexes were observed in ALD, although the tyrosine and serine phosphorylation of Stat3 was not altered. Elevated interleukin-6 mRNA was found in ALD whereas Stat3 and suppressor of cytokine signaling-3 mRNA levels were decreased. Although end-stage ALD is characterized by up-regulation of Stat3 proteins, this transcription factor appears to be functionally inactive. Furthermore, decreased transcription of the Stat3 gene in ALD might also affect cytoplasmic reserves of inactivated Stat3 in the long term. Impaired activation and restoration of Stat3 might thus contribute to the development of cell damage leading to liver cirrhosis in ALD. (*Am J Pathol* 2003, 162:587–596)

Since their discovery more than a decade ago, the transcription factors nuclear factor kappa B (NF- κ B) and signal transducer and activator of transcription 3 (Stat3) have received considerable attention. They are involved

in intracellular signal transduction pathways of several proinflammatory cytokines including tumor necrosis factor (TNF)- α and interleukin-6 (IL-6).^{1–5} Studies in animal models of liver injury and growth emphasize the role of these cytokines in liver damage and repair mechanisms.^{6–8} Therefore, disruption of TNF- α - and IL-6-dependent intracellular signal transduction pathways involving NF- κ B and Stat3 might lead to disturbed liver regeneration and repair.^{9,10} Furthermore, the balance between cytokines and cytokine-inducible transcription factors appears to be disturbed in experimental ALD in rats, despite evidence that chronic ethanol consumption leads, in fact, to induction of TNF- α and IL-6.^{11,12}

Acute and chronic alcoholic liver disease (ALD) in humans is also characterized by raised serum and liver levels of several proinflammatory cytokines including TNF- α and IL-6.^{13–19} These molecules are of special interest given the protective effect of pentoxifylline, an inhibitor of TNF- α and TNF- α -inducible cytokines, on disease severity in patients with acute alcoholic hepatitis.²⁰ Studies in animal models of ALD and *in vitro* data have led to the hypothesis that deficient expression of transcription factors in ALD induces a disturbed balance between hepatotoxic and hepatoprotective mechanisms ultimately favoring pathways leading to definite liver damage.^{21,22} However, to date, the suggestion that cytokine-inducible transcription factors are implicated in the pathogenesis in ALD is exclusively based on this experimental work. Because the involvement of cytokine-inducible transcription factors and especially Stat3 in ALD in human tissue has not been described, in this study we have examined the expression, immunolocalization, and DNA-binding activity of Stat3 in livers of patients transplanted for end-stage ALD compared to normal livers and livers of patients transplanted for end-stage primary biliary cirrhosis (PBC).

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Materials and Methods

Patients and Liver Samples

Tissue (0.5- to 1-g cubes) was removed from explanted livers of ALD and PBC patients at transplantation and were immediately frozen in liquid nitrogen and stored at -80°C until use. Normal liver was from surplus tissue of size-reduced liver grafts.

Preparation of Cellular Extracts

Liver Homogenates

Liver tissue was cut into small pieces and then homogenized at 4°C with a Teflon homogenizer in a buffer containing: 50 mmol/L Hepes, pH 7.5, 100 mmol/L KCl, 3 mmol/L MgCl_2 , 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 5 mmol/L dithiothreitol, 0.1 mmol/L phenylmethyl sulfonyl fluoride, 10 mmol/L NaF, 1 mmol/L Na_3VO_4 , 10% glycerol, 0.1% Tween-20, 1 $\mu\text{g}/\text{ml}$ aprotinin, and 1 $\mu\text{g}/\text{ml}$ leupeptin. The homogenate was spun in a microfuge at 10,000 rpm for 10 minutes at 4°C . The supernatant was carefully aspirated, divided into several aliquots, frozen in liquid nitrogen, and stored at -80°C until use.

Liver Nuclear Extracts

Liver nuclear extracts were prepared as described by Greenbaum and colleagues²³ with slight modifications. The homogenization buffer contained: 10 mmol/L Hepes pH 7.6, 25 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethyl sulfonyl fluoride, 1 mmol/L NaF, 1 mmol/L Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 2 mol/L sucrose, and 10% glycerol. Dialysis step was omitted at the end of the procedure. Aliquots from each sample were prepared, immediately frozen in liquid nitrogen, and stored at -80°C until use.

All manipulations were performed at 4°C . All nuclear extracts and homogenates were handled in the same way and stored for only a few days to avoid degradation. Protein concentration was determined using a BCA protein assay with serum albumin as a standard (Pierce Chemical, Rockford, IL). All reagents and chemicals were purchased from Sigma-Aldrich Ltd., Poole, Dorset, UK.

RNA Isolation

Total RNA was prepared from frozen liver tissue using the guanidine thiocyanate and cesium chloride method.²⁴

Immunoblotting

Between 100 and 150 μg of protein was resolved on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane (Hybond C-extra; Amersham International plc, Little Chalfont, UK). The blotted membrane was blocked (1 hour at room temperature) in Tris-buffered saline (TBS) containing 5% of nonfat dry milk. All of the following incubations were

performed at room temperature (except where stated otherwise) in TBS containing 1% of nonfat dry milk. The incubation steps were followed by three washing steps of 5 minutes with TBS containing 0.1% Tween 20. Primary and secondary antibodies used were as follows: Stat3 rabbit polyclonal (1:2500, 1 hour; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Stat3 Y705 and Ser 727 rabbit polyclonal (1:1500, 2 hours; Upstate Biotechnology, Lake Placid, NY) antibodies, and peroxidase-conjugated mouse anti-rabbit IgG (1:20,000 to 1:50,000; 1 hour; Jackson ImmunoResearch, West Grove, PA). The antigen-antibody reaction was visualized using the Amersham chemiluminescent detection system followed by exposure of the membranes to Hyperfilm-ECL (Amersham) for 1 to 3 minutes.

Three separate Western blots of each sample were done and blots checked for equal protein load and complete transfer by staining gels and membranes with Coomassie blue.

Protein expression was quantified by densitometry using the Gel Doc 2000 system and software (Bio-Rad Laboratories, Nazareth, Belgium). Results are expressed as arbitrary optical density units.

Electrophoretic Mobility Shift Assays (EMSA)

Ten to 20 μg of nuclear proteins were preincubated for 10 minutes at room temperature with 2 μg of poly (dl-dC) in the following binding buffers: NF- κB (distilled water containing 1 mol/L of dithiothreitol at a 1/40 dilution); Stat3 (10 mmol/L Hepes, 50 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol). Double-stranded oligonucleotides were end-labeled with [γ - ^{32}P]-ATP and purified using the QIAquick nucleotide removal kit (Qiagen Ltd., Crawley, West Sussex, UK) following the instructions of the manufacturer. Two μl of the labeled probe were added to the extracts and the mixtures were further incubated for 30 minutes at room temperature and then electrophoresed (200 V, 2 hours) on a 5% polyacrylamide gel (29:1 cross-linking) in a $0.5\times$ TBE buffer (44 mmol/L Tris, 44 mmol/L boric acid, 1 mmol/L EDTA). In cold competition experiments, unlabeled oligonucleotide was incubated with the extracts for 30 minutes at room temperature before the addition of the radiolabeled probe. For antibody supershift assays, 5 μl of the antibody (1 $\mu\text{g}/\mu\text{l}$) were added to the respective samples after 30 minutes of incubation with the labeled probe. The samples were incubated at room temperature for an additional 30 minutes before electrophoresis. NF- κB anti-p50- and p65-specific and anti-Stat3-specific polyclonal antibodies were from Santa Cruz Biotechnology. Anti-Stat1-specific polyclonal antibody was obtained from Transduction Laboratories, Lexington, KY. The following probes were used: NF- κB double-stranded consensus oligonucleotides (Promega Benelux, Leiden, The Netherlands) and preannealed chromatography-purified double-stranded oligonucleotides from the serum-inducible factor-binding element in the c-fos promoter; GATCCTCCAGCATTCCCCGTAAATCCTCCAG (Stat3). Gels were dried and exposed to a Hyperfilm MP (Amersham) for 16 to 24 hours.

Fresh aliquots from identical nuclear extracts were used for both EMSAs to ensure homogeneity of the reactions.

Immunofluorescent Staining

Frozen sections (3.6 μm) were fixed in acetone for 10 minutes, air-dried, foil wrapped, and stored at -20°C until use. Before use, sections were allowed to warm to room temperature while remaining foil wrapped. The primary antibody (Stat3) diluted in phosphate-buffered saline (PBS) (1:25) containing fetal calf serum and 0.1% sodium azide was added and incubation was continued for 60 minutes at room temperature in a moist chamber. The sections were washed for 30 minutes in PBS and then incubated for an additional 30 minutes with a sheep anti-rabbit IgG fluorescein isothiocyanate conjugate (1:25; The Binding Site Ltd., Birmingham, UK) at room temperature. Thereafter, sections were washed for 60 minutes in a PBS bath and then mounted in 90% glycerol containing 2.5% diazabicyclo-octane to retard fading. Specificity of the staining was confirmed by omitting the primary antibody from the reaction, by replacing the primary antibody with an equally diluted nonspecific rabbit immunoglobulin (IgG) (DAKO A/S, Glostrup, Denmark) and by use of blocking peptides (Santa Cruz).

Reverse Transcription and Real-Time Polymerase Chain Reaction (PCR)

Total liver RNA was treated with 10 U of RNasin (Promega Benelux B.V.) and 1 U of DNase (Promega) for 15 minutes at 37°C in an incubation buffer (10 mmol/L Tris-HCl, 50 mmol/L NaCl, 25 mmol/L MgCl_2) to avoid any DNA contamination during PCR. The absence of genomic DNA contamination was assured by submitting samples to PCR amplification in which reverse transcriptase had been omitted from the reaction. After incubation, the purified RNA was extracted using phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma) and chloroform (Sigma) and precipitated by adding 3 mol/L of sodium acetate and 100% ethanol. Five μg of purified RNA were preincubated with random hexamer and water for 10 minutes at 70°C . Four hundred units of M-MLV (Moloney murine leukemia virus) reverse transcriptase (Gibco BRL, Merelbeke, Belgium) were added together with dithiothreitol and dNTPs (deoxynucleoside triphosphate) and the reaction was continued for a further 60 minutes at 37°C .

Quantitative PCR analysis was performed with the GeneAmp 5700 Sequence Detection System and software (Applied Biosystems, Den IJssel, The Netherlands) using Cybergreen fluorogenic probes. Ribosomal protein L19 (RPL19) RNA was chosen as an internal standard. IL-6, Stat3, suppressor of cytokine signaling (SOCS)-3, and RPL19 primers were designed using the Primer Express design software (Applied Biosystems). The following primers were used: IL-6: sense, ctccaggagcccagctatga; anti-sense, cagttgccttctcctcctggg; Stat3: sense, ggaggaggcatcggaag; anti-sense, atctgtgtgacaccaacga; SOCS-3:

sense, ttccagcatctctgtcgaagac; anti-sense, cggcagctgggtgacttt; RPL19: sense, caagcggattctcatggaaca; anti-sense, tggtcagccaggagcttctt. RNA derived from lipopolysaccharide-stimulated human endothelial cells (gift from Dr. S. Moniotte, UCL-Fath, Brussels, Belgium) was used to prepare standard dilution curves for IL-6, Stat3, SOCS-3, and RPL19. PCR reactions were performed according to the standardized thermal profile of the system previously set by the manufacturer. All tissue and standard curve samples were run in duplicate at the same time in a single 96-well reaction plate (MicroAmp Optical, Applied Biosystems) using appropriate primers and probes. PCR products of each sample were compared to their standard dilution curves for quantification. The final result of each sample was normalized to its respective RPL19 value.

Statistical Analysis

Results are expressed as means $\pm$ SEM. The statistical differences between the groups were tested using the Student's *t*-test. Statistical significance was assumed for a *P* value <0.05 .

Results

Immunoblotting of Stat3

Stat3 expression was found in homogenates of ALD and PBC as well as in normal livers (Figure 1, A and B). Analysis of Stat3 protein expression in PBC livers did not show major variations compared to normal liver tissue (Figure 1B). However, a trend toward increased Stat3 amounts was observed in ALD livers compared to normal liver tissue (3.01 ± 0.32 and 2.13 ± 0.88 , respectively) (Figure 1A). Given this slight but not significant increase in overall Stat3 expression in ALD livers, we investigated if there was a difference in protein expression of phosphorylated Stat3 required for dimerization and nuclear translocation. Analysis of tyrosine- and serine-phosphorylated Stat3 molecules at positions 705 and 727, respectively, did show an increased expression of both molecules in livers from ALD patients compared to normal livers (Figure 2A). The difference reached statistical significance for tyrosine-phosphorylated Stat3 (2.92 ± 1.15 and 0.32 ± 0.19 , $P = 0.047$, respectively) whereas the trend toward increased expression of serine-phosphorylated Stat3 in ALD livers was not significant (5.76 ± 0.16 and 3.59 ± 1.75 , $P = 0.177$, respectively). In PBC livers (Figure 2B), tyrosine-phosphorylated Stat3 rose slightly (0.022 ± 0.002 and 0.018 ± 0.002 , $P = 0.292$, respectively) whereas a significant increase in serine-phosphorylated Stat3 was observed when compared to normal livers (0.013 ± 0.003 and 0.003 ± 0.001 , $P = 0.027$, respectively).

In contrast to liver homogenates, Stat3 bands were principally detected in nuclear extracts from ALD and PBC livers (Figure 1, A and B). At equal protein load, a Stat3 band was only seen in one of six samples from normal livers. However, it was possible to reveal faint

Figure 1. Samples from six normal, six ALD, and six PBC livers were screened for Stat3 protein expression. **A:** Representative Western blot of total Stat3 protein expression in liver homogenates (**top**) and nuclear extracts (**bottom**) of patients with ALD (**lanes 5 to 7**) compared to normal livers (**lanes 2 to 4**). A cytosol extract from regenerating rat liver was used as a positive control (C+, **lane 1**). Stat3 was detected in homogenates of normal (**lanes 2 to 4**) and ALD livers (**lanes 5 to 7**). However, Stat3 proteins were only found in nuclear extracts of ALD livers (**bottom, lanes 5 to 7**). **B:** Representative Western blot of total Stat3 protein expression in liver homogenates (**top**) and nuclear extracts (**bottom**) of patients with PBC (**lanes 5 to 6**) compared to normal livers (**lanes 1 to 3**). Stat3 was detected in homogenates of normal (**lanes 1 to 3**) and PBC livers (**lanes 4 to 6**). However, Stat3 proteins were principally found in nuclear extracts of PBC livers (**bottom, lanes 4 to 6**).

Stat3 bands in nuclear extracts from the remaining five normal livers when the gel was loaded with high amounts (up to 300 μ g) of nuclear proteins (not shown).

EMSA

Stat3 DNA Binding

A Stat3 DNA-binding complex was detected in normal livers. Cold competition studies and supershift experiments with a Stat3 antibody confirmed that the band was Stat3-specific. Supershift studies using a Stat1 antibody did not modify the band that further sustained the Stat3

Figure 2. Representative Western blot of phosphorylated Stat3 protein expression in liver homogenates. **A:** Analysis of tyrosine (Tyr705)- and serine (Ser727)-phosphorylated Stat3 revealed an increased expression of both phosphorylated Stat3 forms in ALD livers (**lanes 5 to 7**) compared to normal ones (**lanes 2 to 4**). A cytosol extract from regenerating rat liver was used as a positive control (C+, **lane 1**). **B:** Expression of both phosphorylated Stat3 forms was also increased in PBC livers (**lanes 4 to 6**) compared to normal liver samples (**lanes 1 to 3**).

specificity of this DNA-binding complex (Figure 3A). In contrast to normal livers, no DNA-binding complex was found in any of the nuclear extracts from ALD livers ($n = 6$) even if the gels were loaded with up to 20 μ g of nuclear proteins (Figure 3A).

Nuclear extracts from PBC livers were also subjected to EMSA to verify if the absence of Stat3 DNA-binding complexes was specifically linked to ALD. Indeed, Stat3 DNA-binding complexes could be detected in four of six PBC samples. Cold competition studies and supershift experiments with a Stat3 antibody were used to confirm that the band was Stat3-specific (Figure 3B).

NF- κ B DNA Binding

NF- κ B EMSAs were mainly performed as control experiments designed to show the functional integrity of the ALD nuclear extracts. A NF- κ B DNA-binding complex was observed in both normal and ALD livers (Figure 4). This complex was abolished by the unlabeled probe in cold competition experiments. The p50 antibody completely supershifted the band that was competed by the

Figure 3. EMSA of Stat3 in normal, ALD, and PBC livers. **A:** Representative EMSA of Stat3 in normal (lanes 1 to 5) and ALD livers (lanes 6 to 8). A Stat3-specific complex that was competed by a 50-fold excess of the nonlabeled probe (lane 3) was detected in normal (lanes 1 and 2) but not in ALD livers (lanes 6 to 8). Supershift experiments with Stat3 (lane 4)- and Stat1 (lane 5)-specific antibodies further confirmed the Stat3 specificity of the complex detected in normal livers. **B:** Representative EMSA of Stat3 in PBC livers (lanes 1 to 4). Cold competition studies (lanes 5 and 6) as well as supershift experiments with a Stat3-specific antibody (lane 7) confirmed the Stat3-specific nature of the detected DNA-binding complex.

cold probe and a partial shift was also observed with a p65 antibody confirming the specific nature of this complex (Figure 4).

Real-Time PCR

IL-6 mRNA Expression

Given the absence of Stat3 DNA-binding activity in ALD, we investigated whether this observation could be explained by deficient expression of IL-6, a potent in-

Figure 4. Representative EMSA of NF-κB in normal (lanes 1 to 6) and ALD livers (lanes 7 to 11). A NF-κB-specific complex that was competed by a 50-fold excess of the nonlabeled probe (lane 4) was detected in normal (lanes 1 to 3) and ALD livers (lanes 7 to 9). Supershift studies with p50 and p65 antibodies revealed that the specific complex is mainly composed of p50 subunits whereas p65 subunits are only a minor, if any, component of this complex (lanes 5, 6, 10, and 11).

ducer of Stat3 DNA binding. Low amounts of IL-6 mRNA were found in normal livers. In ALD livers, IL-6 mRNA expression was significantly increased ($P = 0.021$) compared to normal livers. IL-6 mRNA was also up-regulated in PBC livers without, however, reaching statistical significance (Figure 5A).

Stat3 mRNA Expression

In view of increased Stat3 proteins detected in nuclear extracts, we also investigated whether transcription of the Stat3 gene was modified in ALD and PBC livers. Surprisingly, Stat3 mRNA levels were significantly decreased by >60% in ALD ($P = 0.009$) and PBC ($P = 0.019$) livers compared to normal liver tissue (Figure 5B).

SOCS-3 mRNA Expression

Fully activated Stat3 that binds to its DNA-binding site in the promoter of the target gene SOCS-3, leads to an increase in its transcription, as a part of a negative autoregulatory feedback loop. Therefore, the absence of detectable Stat3 DNA-binding complexes in ALD livers could lead to decreased levels of SOCS-3 transcription. Indeed, a reduction by 40% ($P = 0.078$) in SOCS-3 mRNA levels were found in ALD livers in comparison with normal livers. However, SOCS-3 mRNA transcription was also strongly decreased in PBC livers (by 60%, $P = 0.005$) where Stat3 DNA-binding complexes could be demonstrated (Figure 5C).

Figure 5. Quantification of mRNA levels by quantitative PCR. Mean values $\pm$ SEM are represented. Between five (normal, ALD) and nine (PBC) samples adjusted for the respective individual RPL19 level (internal standard) were used to perform the analysis. Significant *P* values in comparison to normal liver are indicated at the top of the columns. **A:** IL-6 mRNA levels are significantly higher in livers of patients with ALD compared to normal liver tissue. A strong trend toward increased IL-6 levels was also observed in PBC livers. **B:** A >60% drop in Stat3 mRNA expression was observed in ALD and PBC livers. **C:** SOCS-3 mRNA levels are decreased in both ALD and PBC livers compared to normal liver samples.

Histology and Immunofluorescence Staining of Stat3

Conventional histological examination of PBC and ALD livers showed an established cirrhosis. The ALD samples did not reveal any histological evidence (steatosis, Mallory bodies, ballooning, inflammatory infiltrate) suggestive of ongoing alcohol consumption. The cut-down liver samples were normal on histology (data not shown).

Western blot results of nuclear extracts from ALD livers suggest the presence of Stat3 on the nuclear level without producing Stat3 DNA binding. To check the Western blot results, control immunofluorescence staining was performed to identify specific cells expressing Stat3 and to investigate whether nuclear translocation does remain intact in ALD livers. In normal livers Stat3 was mainly expressed on hepatocyte nuclei (Figure 6, A and E) and was homogeneously distributed over the entire liver parenchyma. Specific cytoplasmic staining was also observed in biliary epithelial cells of portal tracts (Figure 6, A and C).

In ALD livers, hepatocyte nuclei of regenerating nodules stained positively for Stat3. The density of Stat3-positive hepatocytes was more pronounced in the peripheral layers than in the center of the nodules (Figure 6, B and F). In addition to hepatocytes, biliary epithelial cells of proliferating ducts also showed a positive staining for Stat3. As with normal livers, Stat3 staining could be identified in the cytoplasm of bile ducts in ALD livers. However, and in contrast to normal livers, nuclear staining was also observed in small proliferating bile ducts in ALD (Figure 6D).

No staining in hepatocytes and bile ducts was observed when the primary antibody was omitted from the reaction or when a nonspecific IgG was used. The staining was completely abolished by a Stat3-specific blocking peptide (not shown). However, with these negative controls, a residual staining (autofluorescence) persisted in arterial walls of portal tracts in normal livers suggesting that this is likely to be nonspecific.

Discussion

Ethanol-induced oxidative stress and increased activity of proinflammatory cytokines including TNF- α and IL-6 have been demonstrated in the liver and serum in humans suffering from active ALD.^{13–19,25,26} However, increased production of these cytokines is not sufficient to cause liver injury.¹⁹ Oxidative stress as well as induction of TNF- α and IL-6 leads to enhanced activity of transcription factors such as NF- κ B and Stat3 in various cells including liver parenchymal and nonparenchymal cells in

Figure 6. Stat3 immunostaining was performed for all six normal and six ALD liver samples screened by Western blots. A representative fluorescein isothiocyanate immunofluorescence staining of Stat3 in normal (A, C, E) and ALD livers (B, D, F) is shown. In normal liver, Stat3 was homogeneously distributed over the liver parenchyma (A, low magnification). At high magnification (red dotted box in A) it became evident that Stat3 localized mainly to hepatocyte nuclei (E, arrows). Staining in bile ducts was principally restricted to the cytosol sparing the nuclei (C, arrows) at high magnification (yellow dotted box in A). In ALD, regenerating nodules (N) as well as structures within the fibrotic septa separating different nodules showed a positive immunofluorescence staining (B, low magnification). At high magnification (red dotted box in B), the staining observed in the regenerating nodules was confined to hepatocyte nuclei (F, arrows). The positive structures within the septa (yellow dotted box in B) corresponded to proliferating bile ducts (BD) showing weak cytoplasmic and strong nuclear (hot spots indicated by arrows) staining (D, high magnification). BD and PV indicate the lumen of bile ducts and the portal vein, respectively. The yellow dotted lines in C and D indicate the boundaries of the bile ducts.

animals and humans.^{27–29} Indeed, induction of these transcription factors constitutes an important step in the signal transduction pathway used by these cytokines.^{30,31} There is evidence that the cytokine-transcription factor balance might be disturbed in experimental ALD in rats.¹² Alcohol also affects the expression and induction of Stat3 in regenerating rat liver and in cell-culture systems.^{32,33} However, no data are available concerning the short- and long-term impact of chronic ethanol exposure on Stat3 function in human tissue. We therefore studied the expression and activation of Stat3 in patients transplanted for end-stage ALD. Stat3 was found to be up-regulated at protein levels but interestingly it appeared to lack active DNA-binding capacity. This clearly raises a question regarding its biological activity despite up-regulated IL-6 levels and the fact that Stat3 serine and tyrosine phosphorylation required for nuclear translocation and DNA binding remained unaltered.

Stat3 was easily detectable by Western blot analysis in ALD and normal livers. However, the overall subcellular distribution of Stat3 proteins differed between normal and ALD tissue. Analysis of Stat3 expression and activity did show an increased expression of relatively high amounts of Stat3 protein in nuclear extracts of ALD livers. However, no DNA-binding complexes could be detected in the ALD samples whereas nuclear extracts from normal livers contained small amounts of Stat3 proteins very actively binding DNA. It is unlikely that the absence of Stat3 DNA-binding complexes in ALD livers is because of degraded protein extracts because identical extracts have produced a DNA-binding complex in NF- κ B EMSAs. However, we cannot exclude that some transcriptionally active Stat3 complexes might be present in ALD livers that we failed to reveal because of sensitivity limits of the techniques used. Nevertheless, the impact of these barely detectable complexes seems to be insufficient to promote prolonged and efficacious activation of liver repair mechanisms.

In addition, Stat3-positive immunofluorescence staining of hepatocyte and biliary epithelial cell nuclei confirmed the Western blot results and further showed that nuclear translocation, which requires dimer formation of Stat3, was not inhibited in ALD livers. IL-6 is a potent activator of Stat3 by inducing Stat3 phosphorylation and nuclear translocation through the gp130-Jak pathway.³⁴ Our findings of significantly increased IL-6 levels in ALD livers exclude the possibility that decreased or deficient expression of IL-6 might be responsible for incomplete activation of Stat3 unable to bind DNA.

In vitro and *in vivo* data suggest that ethanol inhibits IL-6-induced Stat3 expression in rat hepatocytes.^{32,33} Furthermore, ethanol also inhibits leptin-induced Stat3 activation in cultured human hepatoma cells.³⁵ Under both circumstances, ethanol seems to directly interfere with Stat3 tyrosine and/or serine phosphorylation.^{33,35} Despite this, our results indicate that tyrosine and serine phosphorylation of Stat3 is not affected in end-stage ALD in humans. In contrast, expression of both phosphorylated Stat3 molecules seems to be up-regulated in homogenates of ALD livers compared to normal livers. Taken together, it appears that the IL-6-gp130-Jak path-

way leading to Stat3 phosphorylation is preserved in end-stage ALD livers. In addition, Stat3 phosphorylation not only occurs normally in ALD but intracellular redistribution of Stat3 from the cytoplasm to the nucleus also seems to be intact given the positive Stat3 bands in nuclear extracts on Western blots and a clear nuclear pattern of Stat3 staining on immunofluorescence. Taken together, these observations suggest that inhibition of Stat3 occurs at the nuclear level downstream of the activation cascade that involves tyrosine and serine phosphorylation steps thought to be essential for complete Stat3 activation. Control experiments with tissue extracts derived from PBC livers were performed to test if these observations are specifically linked to ALD. PBC livers showed similar results regarding Stat3 protein expression. However, the experiments revealed one striking difference between ALD and PBC livers, ie, the preservation of a Stat3 DNA-binding complex in the majority of the PBC nuclear extracts. This important difference strongly suggests that the inability of Stat3 to bind DNA is specifically linked to ALD and does not represent a general phenomenon in liver cirrhosis. Interestingly, the functional impairment of Stat3 proteins in ALD does not seem to be time-dependent because it persists for at least 6 months after histology-proven alcohol abstinence, which is a prerequisite for liver transplantation to be performed. Further studies examining the role of molecules interfering with Stat3 DNA binding as, for instance, the recently described protein inhibitor of activated STAT (PIAS)^{36,37} might provide further explanation for the discordance between Stat3 protein expression and activity in ALD livers.

SOCS-3 represents a potential functional inhibitor of Stat3. Transcription of the SOCS-3 gene is generally activated by Stat3 itself actively binding DNA as a part of an autoregulatory feedback loop.^{38,39} Differences in SOCS-3 transcription and SOCS-3 mRNA levels could therefore serve as an indicator reflecting the activity of this transcription factor. The finding of reduced SOCS-3 mRNA levels in ALD livers adds an additional piece to the puzzle indicating an impairment in Stat3 function. Nevertheless, SOCS-3 mRNA was even more suppressed in PBC livers despite preservation of their Stat3 DNA-binding activity. Although, the IL-6-gp130-Stat3 pathway is a very potent transcriptional activator of the SOCS-3 gene, other molecules such as interferon gamma, several growth factors, and interleukins also induce SOCS-3 transcription.⁴⁰ Lower IL-6 levels observed in PBC as well as the presence of additional inducers of Stat3 in ALD might explain why SOCS-3 mRNA is still detected in ALD livers and strongly suppressed in PBC livers considering the sensitivity of the technique used here. It is, however, unlikely that differences in SOCS-3 expression account for the absence in Stat3 DNA binding in ALD because SOCS-3 seems to interfere mainly with Stat3 tyrosine phosphorylation³⁵ that was clearly preserved in ALD.

Interestingly, transcription of the Stat3 gene was also reduced by >60% in ALD and PBC livers compared to normal liver tissue. This observation suggests that the modifications in Stat3 protein expression observed in ALD and PBC are rather a consequence of posttransla-

tional mechanisms than because of changes in the transcription rate of the Stat3 gene. Although the drop in Stat3 mRNA levels seems to be a more general phenomenon in liver cirrhosis, the physiological consequences of reduced Stat3 transcription could be of relevance because restoration of the cytoplasmic pool of inactivated Stat3 might be retarded and/or insufficient at long term. Thus, the hepatocyte might not only have to face the problem of inhibition of Stat3 DNA-binding activity of most of the activated Stat3 but it might also be confronted with a progressive depletion of intracellular stores of inactivated Stat3.

In conclusion, our study suggests that end-stage ALD is characterized by increased expression of Stat3 proteins including its phosphorylated forms. However, the Stat3 transcription factor appears to be functionally inactive with inhibition occurring at the level of the transcriptional complex. Control experiments with nuclear extracts derived from PBC livers confirmed that the functional impairment of Stat3 is specifically linked to ALD. A vicious circle of permanent Stat3 activation via the IL-6/gp 130 pathway, degradation of activated Stat3 that fails to bind DNA by the proteasome machinery, and insufficient restoration of Stat3 cellular resources could lead to profound disturbances in hepatic repair mechanisms in advanced ALD in humans. It would be interesting to determine whether impaired activation of Stat3-dependent hepatoprotective and hepatotrophic pathways at earlier stages of the disease contribute to the development of permanent cell damage finally leading to alcoholic liver cirrhosis.

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