Effects of chronic ethanol consumption on cytokine regulation of liver regeneration

SHI QI YANG,1 HUI ZHI LIN,1 MING YIN,1 JEFFREY H. ALBRECHT,2 AND ANNA MAE DIEHL1
1 Johns Hopkins University, Baltimore, Maryland 21205; and 2 Hennepin County Medical Center, Minneapolis, Minnesota 55415

Yang, Shi Qi, Hui Zhi Lin, Ming Yin, Jeffrey H. Albrecht, and Anna Mae Diehl. Effects of chronic ethanol consumption on cytokine regulation of liver regeneration. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G696–G704, 1998.—Ethanol ingestion may interrupt the prereplicative signal transduction that is initiated by injury-related cytokines such as tumor necrosis factor (TNF)-α and TNF-α-inducible cytokines including interleukin (IL)-6. To test this theory, liver regeneration, TNF-α, and IL-6 expression, and cytokine-regulated prereplicative events were compared in ethanol-fed rats and isocalorically fed controls after 70% partial hepatectomy (PH). Ethanol feeding inhibits hepatocyte replication and recovery of liver mass after PH but generally promotes induction of both cytokines in the liver and extrahepatic tissues (i.e., white adipose tissue). Cytokine-regulated events that occur early in the prereplicative period are influenced differentially. TNF-α-dependent increases in hepatic nuclear factor-κB (NF-κB) p50 and p65 expression and DNA binding activity are prevented, whereas IL-6-dependent inductions of hepatic Stat-3 phosphorylation and DNA binding activity occur normally. In contrast, events (e.g., induction of cyclin D1, cdk-1, cyclin D3, and p53 mRNA) that occur at the end of the prereplicative period are uniformly inhibited. These findings indicate that chronic ethanol ingestion arrests the regenerative process during the prereplicative period and demonstrate that increased TNF-α, IL-6, and Stat-3 are not sufficient to assure hepatocyte proliferation after PH.

The injury-related, proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 promote liver regeneration. The importance of TNF-α and IL-6 as hepatotrophic factors is demonstrated by evidence that induction of hepatocyte DNA synthesis after 70% (partial) hepatectomy (PH) is severely inhibited in TNF-α receptor type 1 null mice (44) and IL-6 null mice (8) and in normal rats that have been pretreated with anti-TNF-α antibodies (1). On the other hand, TNF-α and TNF-α-inducible cytokines are thought to mediate the liver injury produced by several hepatotoxins, including ethanol (reviewed in Ref. 31). Circulating levels of TNF-α and IL-6 are increased in patients hospitalized with alcoholic liver disease and correlate with the severity of liver injury and mortality in these patients (4, 17, 20, 23). Similarly, increased production of TNF-α and IL-6 has been noted in rats that develop liver damage while receiving chronic intragastric infusions of ethanol (22). Interestingly, ethanol is one of the few causes of liver injury that is also known to inhibit hepatocyte proliferation both in vitro (5) and in vivo (10, 16, 18, 25, 33, 40). This suggests that ethanol may selectively inhibit prereplicative signaling by TNF-α and/or IL-6. Recent work by several different groups is beginning to outline the intracellular events that appear to be involved in TNF-α- and IL-6-mediated hepatocyte proliferation. There is good evidence that TNF-α is responsible for the induction of NF-κB DNA binding activity that occurs within the first hour after PH (8, 9, 44). This may help the liver to reconstitute its mass after PH, because one of the many functions of NF-κB is to prevent apoptosis (39, 41, 43). NF-κB is also known to activate the transcription of TNF-α-inducible genes, including IL-6 (30, 37, 48). Indeed, antibody neutralization studies (1) and experiments with TNF-α receptor type 1 null mice (44) indicate that TNF-α is predominately responsible for inducing IL-6 after PH. IL-6, in turn, has been shown to play a pivotal role in the activation of Stat-3, which occurs later during the prereplicative period after PH (7, 8). Some have postulated that activated Stat-3 then promotes the progression of hepatocytes through the G1 phase of the cell cycle and into S phase (8, 44), a process that requires induction of cyclin D1 (2, 24, 32). Thus several cytokine-inducible molecules have been identified that, if inactivated by chronic ethanol exposure, could abort the hepatocyte proliferative response that is normally triggered by PH.

To test the theory that ethanol may inhibit liver regeneration by interrupting cytokine-initiated signal transduction, we compared liver regeneration, cytokine expression, and cytokine-regulated prereplicative events in rats that were fed an ethanol-containing diet and control rats that were given isocaloric volumes of a similar diet without ethanol. The results confirm previous evidence that chronic ingestion of ethanol inhibits liver regeneration after PH and demonstrate that this occurs despite normal or enhanced induction of TNF-α, IL-6, and Stat-3, an IL-6-regulated transcription factor. An ethanol-related block in TNF-α-initiated signaling has also been identified. Specifically, TNF-α-dependent, early prereplicative events, i.e., induction of NF-κB p50 and p65 expression and DNA binding activities, are inhibited in the ethanol-fed group. This early inhibition of NF-κB may be related to concomitant superinduction of the TNF-α inhibitor IL-10 and is associated with the inhibition of many of the subsequent events (including the induction of cyclin D1,
cdk-1, cyclin D3, and p53) that normally occur as hepatocytes exit G1 and enter S phase.

METHODS

Materials

Adult Sprague-Dawley rats (mean initial wt 175 g) were purchased from Charles River (Charles River, NJ). The 1982 formulations of the Lieber-deCarli ethanol and control diets were purchased from Bio-Serv (Frenchtown, NJ). In each liter of control diet, fat contributes 350 kcal, protein 180 kcal, and carbohydrate 470 kcal. The ethanol diet is identical, except that ethanol is substituted for 355 of the carbohydrate kilocalories. Chemicals were purchased from Sigma Chemical (St. Louis, MO) with some exceptions. Phenol was purchased from Fluka (Ronkonkoma, NY). Taq polymerase was obtained from Boehringer Mannheim (Indianapolis, IN). Moloney murine leukemia virus reverse transcriptase came from GIBCO (Grand Island, NY). The enhanced chemiluminescence detection system and Hybond N+ nylon membranes came from Amersham (Arlington Heights, IL). GeneScreen nylon membranes were from NEN Research Products (Boston, MA). \(^{[\gamma-32P]}ATP\) was purchased from Amersham. ELISA kits for TNF-\(\alpha\), IL-6, IL-10, and the respective recombinant rat cytokine standards were purchased from Biosource Intl. (Camirillo, CA). Antibodies to p50 and p65 NF-\(\kappa\)B and the oligonucleotide probes used in the NF-\(\kappa\)B and Stat-3 DNA binding assays were obtained from Santa Cruz (Santa Cruz, CA). The cDNA for cyclin D1, cyclin D3, cdk-1, and p53 were provided by Dr. J. Albrecht (University of Minnesota, Minneapolis, MN); oligonucleotide primers and probes for rat TNF-\(\alpha\), IL-6, IL-10, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized as previously described (34, 35).

Methods

Animal feeding and PH experiments. Rats were housed under a 12-h light-dark cycle and were permitted ad libitum consumption of standard rat pellet chow. After a 1-wk equilibration period, the animals were fed either a Lieber-DeCarli control diet or an isocaloric diet with ethanol providing 36% of total dietary kilocalories. Each day, the previous day's intake was measured and the control (pair-fed) rats were fed the average intake of the ethanol-fed rats (29). Food was provided daily at 6 PM. After 5 wk the animals were subjected to 70% PH while under light ether anesthesia, in the early morning (19). PH has been shown to substantially increase the proportion of hepatocytes in S phase and is a standard technique used to assess response to various effectors of regeneration. Several groups including our own (12, 16, 33, 40) showed that rats fed ethanol by this protocol have inhibited DNA synthesis and liver regeneration after PH, but to validate this response some rats were injected with the S-phase marker 5-bromo-2'-deoxyuridine (BrdU) 2 h before death (8). At various times after PH, rats were killed and serum, liver, and epididymal fat were harvested. All experiments were performed in adherence to National Institutes of Health guidelines on the use of experimental animals. Approval of the Animal Use Committee of the Johns Hopkins University was obtained before the experiments were initiated.

Evaluation of liver regeneration. As others (8, 44) have done, we used three parameters (hepatocyte incorporation of BrdU, mitotic index, and liver weight) to assess the regenerative response at 24 and 48 h after PH. Formalin-fixed liver sections from BrdU-injected rats were treated with peroxidase-conjugated antibodies to BrdU. Two different investigators counted the number of darkly stained (BrdU positive) hepatocytes.
cytokeratin in 10 different ×400 fields on coded sections from four different rats per treatment group. The average number of BrdU-labeled hepatocytes was calculated for each animal and used to derive the mean (±SE) number of BrdU-labeled hepatocytes per treatment group. The number of hepatocytes with mitotic bodies was counted on parallel hematoxylin and eosin-stained liver sections to derive the mean (±SE) mitotic index for each group. The extent to which the liver mass had been reconstituted was also evaluated. Resected liver weight was normalized to the weight of the rat’s entire liver at the time of death. For each group, the weight of the remnant liver was normalized to the weight of the initial liver weight according to the following formula: (wt of liver remnant + initial liver wt) × 100. Data from four rats per treatment group were used to calculate the mean (±SE) at each time point. Results of all three parameters were evaluated by ANOVA using computer statistical software.

RNA evaluation. Total liver RNA was isolated according to the protocol of Chomczynski and Sacchi (6) and quantified by measurement of ultraviolet absorption at 260 nm. Representative aliquots of RNA from each rat (4 rats per feeding group per time point) were separated by electrophoresis on denaturing agarose gels, followed by ethidium bromide staining to confirm RNA concentration and quality. Twenty micrograms of RNA samples per lane were fractionated on denaturing agarose gels and transferred to GeneScreen membranes. Membranes were stained with 0.04% methylene blue to confirm the lane-lane equivalency of RNA loading/transfer, and then hybridized with cDNA probes for cyclin D1, cdk-1, cyclin D3, or p53 as described previously (45). After being washed under stringent conditions, membranes were exposed to Kodak X-AR film with intensifying screens. Autoradiograms were analyzed by scanning laser densitometry (Molecular Dynamics, Sunnyvale, CA).

Cytokine transcript levels of TNF-α, IL-6, and IL-10 in liver and adipose tissue of rats were measured using a semiquantitative reverse transcriptase (RT)-PCR technique described previously (34, 35). Three micrograms of total RNA were reverse transcribed, and diluted product was used for specific cytokine amplification in a Perkin Elmer thermal cycler using Taq DNA polymerase and cytokine-specific oligonucleotides. PCR reaction conditions were strictly defined for each cytokine such that a log-linear relationship was obtained between the amount of specific cytokine mRNA and the signal intensity of the probed PCR product in the detection system throughout the range of cytokine mRNA levels in the samples evaluated. To assure equivalency of input RNA for each PCR reaction, transcripts for GAPDH, a constitutively expressed RNA, were also evaluated in each sample. PCR products were separated on 1.2% agarose gels, transferred to nylon membranes using standard blotting techniques, and visualized using enhanced chemiluminescence detection systems.

Input RNA from three to four different rats per group per time point were evaluated. In total, 32 different RNA samples were reverse transcribed and analyzed by PCR. To minimize potential interassay variability and to permit valid comparison between the two feeding groups across a range of time points, expression levels of TNF-α were evaluated in the same PCR assay at each particular time point were always evaluated in the same PCR assay. Thus, for each cytokine that was evaluated, three different Southern blots (each of which contained samples from both feeding groups at every time point) were generated and analyzed by laser scanning densitometry. To evaluate reproducibility of the data collected at each time point,
results on each blot were normalized to the time-zero pair-fed group data on the same blots, and expressed as the percentage of pair-fed rats at time zero. Results derived from the three different blots were averaged to derive the mean (± SE) expression of each cytokine at each time point. Representative blots are shown in Figs. 2, 4, and 5.

Nuclear protein isolation, immunoblot, and gel mobility/supershift assays for NF-κB. Nuclear protein was isolated by NUN buffer (1.0 M urea, 0.33 M NaCl, 1.0% Nonidet P-40, 27.5 mM HEPES, pH 7.0, 1 mM DTT); protein concentrations were determined and treatment-related variations in p50 and p65 NF-κB, total Stat-3, and phosphorylated Stat-3 were analyzed by Western blot as described previously (45). In brief, proteins (40 µg/lane) were separated on 12% SDS-polyacrylamide gels by electrophoresis and transferred onto nylon membranes. After Coomassie blue staining of the gels and blot images, lane-lane equivalency in protein loading and transfer, the blots were probed with commercially available antibodies to the respective proteins and visualized by enhanced chemiluminescence. As detailed in the previous section, extracts from three different rats per feeding group per time point were evaluated and resultant blots were assessed by scanning laser densitometry. A representative Western blot that was probed for Stat-3 is shown in Fig. 7.

Gel mobility shift assays to evaluate treatment-related differences in NF-κB and Stat-3 DNA binding activities were performed as described (45). To assess NF-κB binding activity, an oligonucleotide probe containing the core NF-κB binding motif (5’-GGGACTTTCC-3’) from the mouse κ-light chain enhancer element (36) was used. An acute-phase response element probe containing sis-inducible factor binding sites (5’-GATCGGAATTCCTAGATC-3’) (46) was used to assess Stat-3 binding activity. Both probes were prepared by annealing the complementary oligonucleotides in annealing buffer [in mM: 67 Tris·HCl (pH 7.6), 13 MgCl2, 1.3 EDTA] at 100°C for 5 min. After incubation at room temperature overnight, the double-strand probes were end labeled with [γ-32P]ATP and T4 polynucleotide kinase. Labeled oligonucleotide probes in binding buffer [in mM: 10 HEPES (pH 7.5), 0.5 spermidine, 0.15 spermine, 5 EDTA, 10 dithiothreitol, and 0.35 sucrose] were incubated at room temperature for 15 min with 10 µg of nuclear protein (final reaction volume 20 µl) and loaded directly onto a 5% polyacrylamide gel in a buffer of 25 mM Tris borate (pH 8.0), 0.25 mM EDTA. In some experiments, antisera for p50 or p65 NF-κB or Stat-3 or preimmune sera (IgG) were added to reaction mixtures to determine the composition of protein-probe complexes. For these “supershift” assays, extracts were incubated with 1 µl of preimmune sera or an equal volume of anti-p50, anti-p65, or anti-Stat-3 antisera at 4°C for 30 min before addition of [γ-32P]-labeled probe. In all experiments, proteins were separated by electrophoresis at 200 V for 2 h at room temperature. Gels were dried and exposed to Kodak X-AR film with intensifying screens. Assays were repeated with extracts from three different rats per feeding group and evaluated by phosphorimage analysis to assure reproducibility of results. Representative autoradiographs for NF-κB and Stat-3 are shown in Figs. 3 and 7.

Serum cytokine measurements. Commercially available ELISA kits for rat TNF-α, IL-6, and IL-10 were used to determine treatment-related variations in serum cytokine levels. Standard curves for the respective rat recombinant cytokines were included in each assay; samples from three to four rats per feeding group per time point were evaluated, and samples from each rat were assayed in triplicate. Data were analyzed by ANOVA and are graphically displayed beneath the cytokine RNA data shown in Figs. 2, 4, and 5.

RESULTS

As reported by many other groups (reviewed in Refs. 27–29), rats fed ethanol in liquid diets develop a fatty liver without appreciable hepatic necrosis or inflammation. Also, as we (10–12) and others (16, 33, 40) have reported, ethanol feeding inhibits hepatocyte proliferation after PH. In the present study, this is evidenced by decreased hepatocyte incorporation of BrdU (Fig. 1A), mitoses (Fig. 1B), and restitution of liver mass (Fig. 1C) at 24–48 h after PH in ethanol-fed rats compared with controls.
Because TNF-α has been implicated in the pathogenesis of alcohol-induced liver injury but this particular ethanol feeding protocol does not overtly injure the liver, it was initially uncertain whether TNF-α expression would be increased in these ethanol-fed rats. To resolve this question, levels of TNF-α mRNA and protein were compared in the livers and sera of ethanol-fed rats and isoalcoholic controls before and after PH. As shown in Fig. 2, hepatic TNF-α mRNA levels increase within 15 min after PH in pair-fed rats, remain above baseline at 60 min post-PH, and decline to pre-PH levels by 3–6 h after PH. Compared with these pair-fed controls, ethanol-fed rats overexpress TNF-α mRNA before PH and for the first 15 min post-PH. Although hepatic expression of TNF-α mRNA is somewhat lower in ethanol-fed rats from 30 to 60 min after PH, it becomes greater again in ethanol-fed rats than controls at later time points (i.e., 3–6 h post-PH). Ethanol-associated increases in liver TNF-α mRNA expression are generally accompanied by increases in circulating levels of TNF-α protein. Hence, post-PH inductions of hepatic TNF-α mRNA and circulating TNF-α proteins are somewhat enhanced by chronic ethanol ingestion.

In normal rats, TNF-α mediates the activation of NF-κB that occurs within an hour after PH (44). Gel retardation assays (Fig. 3, A and B) and immunoblot analyses (data not shown) of liver nuclear extracts were done to determine whether ethanol influences regenerative induction of NF-κB. NF-κB DNA binding activity increases transiently at 30 min after PH in pair-fed rats (Fig. 3A). Supershift (Fig. 3B) and immunoblot analyses indicate that this increased NF-κB binding activity results, at least in part, from increases in nuclear accumulation of both p50 and p65. Surprisingly, increased NF-κB DNA binding activity is not observed after PH in ethanol-fed rats (Fig. 3A). Consistent with this finding, no increase in p50 or p65 expression is demonstrated by immunoblot analysis of post-PH liver nuclear extracts from ethanol-fed animals (data not shown).

The anti-inflammatory cytokine IL-10 is known to inhibit lipopolysaccharide (26) and TNF-α (42)-dependent activation of NF-κB in monocytic cells. To begin to evaluate the possibility that ethanol feeding may inhibit regenerative induction of NF-κB DNA binding activity by an IL-10-mediated mechanism, we compared IL-10 expression in ethanol- and pair-fed rats. As shown in Fig. 4, IL-10 mRNA increase transiently in the livers of pair-fed rats from 30 to 60 min after PH. Induction of hepatic mRNA for IL-10 is followed by increases in circulating IL-10 protein. We (34) reported similar post-PH induction of IL-10 in ad libitum-fed rats and suggested that such increases in IL-10 may play a role in limiting the TNF-α response after PH. Consistent with this theory, the post-PH increases in hepatic IL-10 expression temporally correlate with abrupt declines in NF-κB DNA binding activity in pair-fed animals. Regenerative induction of IL-10 mRNA and protein is increased in ethanol-fed rats compared with controls (Fig. 4). Superinduction of IL-10 correlates with decreased NF-κB DNA binding activity in the ethanol-fed group. Although this correlation does not definitively establish a cause-effect relationship between increased IL-10 expression and decreased NF-κB activity, the results are consistent with the possibility that overexpression of this cytokine may contribute to ethanol-associated inhibition of NF-κB activation.

Activation of NF-κB is essential for TNF-α dependent induction of IL-6 (48). Thus ethanol-related inhibition of NF-κB suggests that IL-6 expression may be decreased in ethanol-fed rats. If true, the latter could explain the antiregenerative actions of ethanol, because IL-6 is necessary for liver regeneration after PH (8). To evaluate the possibility that ethanol feeding may inhibit regenerative induction of IL-6, IL-6 expression was compared in ethanol-fed rats and pair-fed controls. As shown in Fig. 5, in pair-fed rats IL-6 mRNA begin to increase in the liver at ~30 min, peak at 60 min, and remain abundant for at least 6 h after PH. As we (1) and others (38) reported, these increases in hepatic IL-6 were greater in EtOH rats than controls from 1 to 12 h after PH (P < 0.01–0.001).
expression are followed by increases in circulating levels of IL-6 protein from 6 to 24 h after PH. In contrast, induction of IL-6 mRNA is delayed and attenuated in the livers of ethanol-fed rats. However, circulating levels of IL-6 protein are not comparably reduced. Indeed, quite the opposite is true: serum levels of IL-6 increase earlier after PH and are significantly greater in ethanol-fed rats than pair-fed controls at every time point evaluated during the initial 12 h after PH. The ethanol-related increases in serum IL-6 concentrations are apparent as early as 3 h after PH. At that time point, no IL-6 can be detected in control rat sera by ELISA but the mean serum IL-6 concentration in the ethanol-fed group is 57.5 ± 2.5 pg/ml (P < 0.027). Taken together, these findings suggest that extrahepatic tissues may be overproducing IL-6 in the ethanol-fed animals. To evaluate this possibility, cytokine gene expression was assessed in white adipose tissue (WAT) that was harvested from the epididymal fat pads of both groups. WAT was selected for scrutiny because other workers demonstrated that this tissue can express cytokines under certain circumstances (21).

As shown in Fig. 6, the expression of several cytokine mRNA differs dramatically in the WAT of ethanol- and pair-fed rats. TNF-α, IL-6, and IL-10 are not expressed basally in WAT of pair-fed animals, but WAT expression of each of these cytokines is significantly increased by 3 h after PH. In contrast, TNF-α mRNA are relatively abundant in WAT from ethanol-fed rats even before PH. In addition, in the ethanol-fed group, increases in TNF-α, IL-6, and IL-10 mRNA begin to occur as early as 30 min after PH and are amplified and prolonged relative to those noted in the pair-fed controls. These results suggest that extrahepatic tissues such as WAT may be important sources of circulating cytokines after PH, particularly in ethanol-fed rats.

Although serum concentrations of IL-6 protein are relatively increased in the ethanol-fed rats compared with the controls after PH, hepatic expression of IL-6 appears to be attenuated by ethanol feeding. Thus it remained uncertain whether IL-6-dependent events would be induced normally in the livers of ethanol-fed animals. To explore the implications of ethanol-associated alterations in IL-6 expression, we compared Stat-3 phosphorylation and DNA binding activity in liver nuclear extracts from ethanol- and pair-fed rats. Stat-3 was selected for scrutiny because others showed that IL-6 is responsible for the inductions of Stat-3 phosphorylation and DNA binding activity that follow PH (7, 8, 38). Consistent with this fact and with the previously cited evidence that ethanol ingestion does not reduce serum concentrations of IL-6 proteins, neither the nuclear accumulation of phosphorylated Stat-3 (Fig. 7A) nor the induction of Stat-3 DNA binding activity (Fig. 7B) was inhibited in the ethanol-fed group.

Fig. 6. Effect of ethanol consumption on cytokine expression by white adipose tissue. Total RNA was isolated from epididymal fat of PF and ETOH rats at various time points after PH. Variations in levels of TNF-α, IL-6, IL-10, and GAPDH mRNA were evaluated in separate semiquantitative RT-PCR reactions using oligonucleotide primers that were specific for each cytokine. After agarose gel electrophoresis and transfer to membranes, PCR products were visualized by hybridization with oligonucleotide probes specific for respective cytokines. Results shown are representative of 4 RT-PCR experiments, each of which used input RNA from different pairs of ETOH and PF rats at these time points. Serum concentrations of IL-6 were greater in ETOH than controls from 1 to 24 h after PH (P < 0.05–0.001).
Stat-3 has been implicated as a prerequisite for hepatocyte progression through the cell cycle after PH (8, 44). Consistent with this theory, induction of cyclin D1, a gene that is important for G1-S transition in hepatocytes (2, 24, 32), is inhibited in IL-6 null mice, in which regenerative induction of Stat-3 is also aborted (8). Given this background information that links Stat-3 induction with cell cycle progression after PH, we expected that cyclin D1 mRNA would accumulate normally in the livers of ethanol-fed rats after PH. Surprisingly, this was not the case. Steady-state mRNA levels of cyclin D1 and several other genes (e.g., cdk-1, cyclin D3, and p53) increase in the livers of pair-fed rats by 24 h after PH, as was reported for ad libitum-fed rodents (2). However, little, if any, induction of these genes can be detected by Northern blot analysis of hepatic RNA isolated from ethanol-fed rats at various time points after PH (Fig. 8). Cyclin mRNA and proteins may be discordantly regulated after PH (2). However, our preliminary observations suggest that induction of cyclin D1 protein is also inhibited by ethanol ingestion (data not shown). Thus it appears likely that ethanol-associated decreases in cyclin D1 expression contribute to the ability of ethanol to arrest cell cycle progression at the G1/S boundary. These results complement the data shown in Fig. 1 and indicate that regenerative activation of Stat-3 is not sufficient to ensure that hepatocytes will progress from G1 into S phase after PH.

**DISCUSSION**

There is now compelling evidence that the injury-related cytokines, TNF-α and IL-6, promote liver regeneration and, therefore, are required for recovery from...
liver injury (1, 8, 13, 14, 44). Habitual consumption of ethanol is one of the most common causes of liver disease in the world (15), and it is conceivable that this occurs because ethanol can both injure the liver and impair regeneration. The present study extends existing knowledge about the effects of ethanol on liver regeneration by demonstrating that ingestion of ethanol in subhepatotoxic amounts (27–29) permits normal-to-enhanced induction of proregenerative cytokines, e.g., TNF-α and IL-6, after a regenerative stimulus (e.g., PH). The results also identify the importance of extrahepatic sites such as WAT as sources of these cytokines after liver injury, particularly in individuals who have been chronically exposed to ethanol.

Although chronic ethanol ingestion does not inhibit injury-related increases in TNF-α or IL-6 expression, it does selectively impede cytokine-initiated signal transduction during the regenerative response. Induction of hepatic NF-κB, an event that requires activation of type 1 TNF-α receptors after PH (44), is blocked in ethanol-fed rats. It is tempting to speculate that ethanol-associated overexpression of IL-10 may contribute to the decreased induction of hepatic NF-κB, because IL-10 is known to block TNF-α-dependent activation of NF-κB in other settings (26, 42). The role of NF-κB as a regulator of hepatocyte proliferation after PH is unclear because another group showed that supplemental IL-6 can restore hepatocyte DNA synthesis after PH in TNF receptor type 1 null mice, although these animals exhibit decreased NF-κB DNA binding activity (44). This finding prompted those investigators to conclude that NF-κB is not necessary for hepatocyte DNA synthesis after PH. On the other hand, there is recent evidence that treatment of a cultured hepatocyte cell line with purified specific inhibitor κB promotes apoptosis (3). Furthermore, another group has observed that pretreatment with inhibitors of NF-κB results in massive apoptosis of hepatocytes in the G2/M stage of the cell cycle after PH (D. Brenner, personal communication). Our work documents relatively decreased liver mass in the ethanol-fed group after PH, but these tissues were not evaluated for apoptosis. However, the possibility that ethanol-related inhibition of NF-κB may promote hepatocyte apoptosis is provocative and merits careful evaluation in future studies.

It is also important to emphasize that ethanol-associated TNF-α “resistance” appears to exhibit tissue selectivity, because induction of IL-6, a TNF-α- and NF-κB-regulated event, is somewhat inhibited in the liver but markedly enhanced in the WAT of ethanol-fed rats. The present study did not evaluate NF-κB activity in WAT. Thus it is not clear whether decreased induction of this factor occurs in the fat of ethanol-fed rats as it does in the livers of these animals. If induction of adipose NF-κB DNA binding activity was preserved in fat, however, this could explain the observed discrepancy between hepatic and adipose tissue IL-6 expression in the ethanol-fed group. Alternatively, other signals (e.g., mediated by cAMP or prostaglandins) that regulate IL-6 by both transcriptional and posttranscriptional mechanisms (47) may help to increase IL-6 expression when NF-κB activity is inhibited. In any case, upregulation of adipose IL-6 expression is associated with increased circulating levels of IL-6 protein in ethanol-fed animals. The latter may function hormonally to compensate for the delayed induction of hepatic IL-6. This is suggested by at least two lines of evidence (i.e., Western blot and gel mobility shift analyses) that indicate that post-PH activation of Stat-3 in the liver, an IL-6-dependent process (8), proceeds normally in the ethanol-fed group.

Others (8, 44) suggested that IL-6-mediated activation of Stat-3 is necessary for the induction of later events, including cyclin D1 expression, that permit hepatocytes to exit G1 and enter replicative phases of the cell cycle. However, the present results demonstrate that increases in serum IL-6 and the resultant activation of hepatic Stat-3 are not sufficient to ensure induction of many different late G1 events after PH. These findings complement evidence that ethanol ingestion inhibits hepatocyte incorporation of BrdU and mitoses. Taken together, these data suggest that additional factors are necessary for hepatocytes to escape G1. Ethanol feeding appears to inhibit one or more of these other factors. Thus injury-related cytokines such as TNF-α and IL-6 act predominantly as “initiation” factors, working to move quiescent (G0) hepatocytes into the prereplicative (G1) phase of the cell cycle. Although initiation is critically important for eventual hepatocyte replication, factors other than TNF-α and IL-6 appear to be required for hepatocytes to “progress” out of G1 and into S phase, where DNA is actually replicated.

This work was supported in part by National Institutes of Health Grants R01-AA-09347, R01-AA-10154, and K-020173 to A. M. Diehl. Address for reprint requests: A. M. Diehl, 912 Ross Bldg., Johns Hopkins Univ. School of Medicine, 720 Rutland St., Baltimore, MD 21205.

Received 17 February 1998; accepted in final form 11 May 1998.

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