Novel CXCR2-dependent liver regenerative qualities of ELR-containing CXC chemokines

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ABSTRACT **Severe acute liver injury due to accidental or intentional acetaminophen overdose presents a major clinical dilemma often requiring liver transplantation. In the present study, liver regeneration after profound liver injury in mice challenged with acetaminophen was facilitated by the exogenous addition of ELR-containing CXC chemokines such as macrophage inflammatory protein-2 (MIP-2), epithelial neutrophil-activating protein-78 (ENA-78), or interleukin 8. Intravenous administration of ELR-CXC chemokines or N-acetyl-cysteine (NAC) immediately after acetaminophen challenge in mice significantly reduced histological and biochemical markers of hepatic injury. However, when the intervention was delayed until 10 h after acetaminophen challenge, only ELR-CXC chemokines significantly reduced liver injury and mouse mortality. The delayed addition of ELR-CXC chemokines to cultured hepatocytes maintained the proliferation of these cells in a CXCR2-dependent fashion after acetaminophen challenge whereas delayed NAC treatment did not. These observations demonstrate that ELR-CXC chemokines represent novel hepatic regenerative factors that exhibit prolonged therapeutic effects after acetaminophen-induced hepatotoxicity.—Hogaboam, C. M., Bone-Larson, C. L., Steinhauser, M. L., Lukacs, N. W., Colletti, L. M., Simpson, K. J., Strieter, R. M., Kunkel, S. L. Novel CXCR2-dependent liver regenerative qualities of ELR-containing CXC chemokines. *FASEB J.* 13, 1565–1574 (1999)**

Key Words: acetaminophen · acute liver injury · hepatic regeneration

THE DELETERIOUS EFFECTS of accidental or intentional (1) acetaminophen overdose often manifest many hours after major hepatic injury has occurred. When administered within 8 h (2), N-acetyl-cysteine (NAC),[2] a precursor of glutathione that is the standard medical treatment for acetaminophen overdose cases, effectively inhibits liver injury and prevents fulminant hepatic failure. However, because the onset of symptoms of acetaminophen overdose may be delayed or misinterpreted, the therapeutic window for NAC treatment of acetaminophen toxicity is frequently missed (3). Despite advances in supportive care, acute hepatic failure attributed to acetaminophen ingestion approaches 50% in the United Kingdom (4). A liver transplant will ensure patient survival in these cases (5), but the majority of patients affected by acetaminophen overdose do not fulfill all the necessary criteria for liver transplantation because they lack co-existent renal failure or severe acidosis (6). At present, considerable experimental attention is directed at elucidating factors that promote rapid and maximal liver regeneration after exposure of the liver to toxic or mechanical insults (7). Cytokines, particularly tumor necrosis factor α (TNF-α) (8) and interleukin 6 (IL-6) (9), are among newly described factors that possess unique liver regenerative qualities. Additional studies have shown that elevations in TNF-α and IL-6 in the damaged liver directly promote the *de novo* generation of ELR-containing CXC chemokines such as MIP-2 and ENA-78 (10, 11). These chemokines have been shown to contribute to the inflammatory process that follows acute hepatic injury (12, 13), but their role during the liver regenerative process remains largely unexplored. ELR-CXC chemokines are distinct from other CXC chemotactic cytokines because these mediators contain three amino acid residues, Glu-Leu-Arg (i.e., ELR), which are immediately next to the CXC motif, and the

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[2] Abbreviations: APAP, acetaminophen; AST, aspartate aminotransferase; CXCR2, CXC chemokine receptor 2; ELISA, enzyme-linked immunoassay; ELR-CXC, ELR-containing CXC chemokines; ENA-78, epithelial neutrophil-activating protein-78; HGF, hepatocyte growth factor; IL-8, interleukin 8; i.p., intraperitoneal; IP-10, interferon-inducible protein-10; i.v., intravenous(ly); MCP, monocyte chemoattractant protein; NAC, N-acetyl-L-cysteine; MIP-2, macrophage inflammatory protein-2; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

majority of ELR-CXC chemokines bind to CXC chemokine receptor 2 (CXCR2) (14).

Since ELR-CXC chemokines also exert mitogenic effects on a number of structural-type cells (reviewed in ref 15), we compared the therapeutic efficacy of NAC and hepatocyte growth factor (HGF) (7) with that of ELR-CXC chemokines in fasted mice challenged with acetaminophen. Superior therapeutic effects of MIP-2 [an interleukin 8-related chemokine present in rodents (16)], human IL-8, and ENA-78 were observed when any one of these ELR-CXC chemokines was intravenously (i.v.) infused into mice 10 h after acetaminophen challenge. Conversely, delayed NAC, IP-10 (a non-ELR-CXC chemokine), or HGF treatment did not attenuate liver injury in acetaminophen-challenged mice. It was also apparent from *in vitro* experiments that ELR-CXC chemokines had CXC receptor 2 (CXCR2) -dependent mitogenic effects on cultured hepatocytes. Taken together, these data suggest that ELR-CXC chemokines can reverse acetaminophen-induced liver injury and exert prominent therapeutic effects when NAC treatment is ineffective against acetaminophen-induced hepatotoxicity.

MATERIALS AND METHODS

Acetaminophen-induced hepatic injury

Female CD1 (6–8 wk of age) were purchased from Charles River Laboratories (Portage, Mich.) and maintained under specific pathogen-free conditions with free access to water and food prior to each experiment. Fresh suspensions of acetaminophen (Sigma Chemical Company, St. Louis, Mo.) were made daily by dissolving the compound in phosphate-buffered saline (PBS) warmed to 40°C. In all experiments, mice were allowed free access to water alone prior to an intraperitoneal (i.p.) injection of 400 mg/kg acetaminophen as described previously (17).

Experimental protocols

In the first set of experiments, groups of fasted CD1 mice were pretreated with preimmune rabbit serum or polyclonal rabbit anti-mouse antibody directed against the ELR-CXC chemokine receptor CXCR2; these mice were killed at 6, 48, and 144 h after acetaminophen challenge for histological examination of liver tissue. All polyclonal rabbit antibodies were generated and screened to ensure specificity prior to use as described previously in detail (18); the biological half-life of these immunoneutralizing antibodies was ~36 h (10). The anti-CXCR2 antibody inhibited KC-induced neutrophil influx into the peritoneum of mice and the MIP-2- and KC-induced angiogenesis in the rat cornea (R. M. Strieter, personal communication). Additional groups of mice (n=5–8 mice/group) were pretreated with 0.5 ml of preimmune rabbit serum or the same volume of polyclonal rabbit anti-mouse MIP-2 antibody 2 h prior to acetaminophen challenge. Mice were killed at 144 h after acetaminophen challenge and livers were prepared for histological examination.

In the next set of experiments, mice were fasted for 12 h and received one dose of NAC, murine MIP-2, and the human proteins ENA-78, IL-8, HGF, or IP-10 either immediately or 10 h after acetaminophen challenge. Recombinant chemokines and HGF were obtained from R&D Systems (Minneapolis, Minn.) or Pepro Tech (Rockyhill, N.J.); NAC was obtained from Sigma. Mice were injected i.v. with 100 μg of NAC or 2 μg of recombinant protein dissolved in 0.5 ml of normal saline. All mice were subsequently killed 48 h after acetaminophen challenge, and serum and liver samples were removed.

In the final set of experiments, mice were fasted for 24 h and received MIP-2 (2 μg) or NAC (100 μg) via an i.v. injection either immediately or 10 h after acetaminophen challenge; liver and serum samples were removed from surviving mice 48 h after acetaminophen challenge. Liver and serum samples from the experimental groups and from mice challenged with an i.p. injection of PBS and treated i.v. with normal saline were processed as described below.

Chemokine ELISAs

Immunoreactive levels of CC and CXC chemokines were measured in liver homogenates using a modified double-ligand enzyme-linked immunoassay (ELISA) procedure as described in detail elsewhere (18). Before each ELISA, snap-frozen liver samples were thawed on ice, weighed, and homogenized in solution containing 2 mg of protease inhibitor (Complete; Boehringer Mannheim, Indianapolis, Ind.) per milliliter of normal saline. Previous studies in this laboratory have shown that Complete does not interfere with any of the chemokine ELISAs (18). Cell-free supernatants from the liver homogenates were loaded in duplicate onto 96-well microtiter plates coated with the appropriate capture antibody and blocked with 2% bovine serum albumin in PBS. Each ELISA consistently detected concentrations of chemokines below 10 pg/ml, and the specificity of the polyclonal detection and capture antibodies was confirmed prior to its use in an ELISA. Chemokines levels in liver homogenates were normalized to the weight of the liver sample assayed.

Serum aspartate aminotransferase

Acute hepatocellular injury results in elevated levels of aspartate aminotransferase (AST). Serum levels of AST were determined 48 h after mice were challenged with acetaminophen by Clinical Pathology at the University of Michigan Medical School (Ann Arbor, Mich.) using standardized techniques.

Histology

For histological assessment of hepatic injury, liver tissues were fixed in 4% paraformaldehyde for 24 h prior to routine histological processing. A pathologist assessed hematoxylin- and eosin-stained slides from acetaminophen-challenged mice in a blinded manner. Separate scoring systems were devised to indicate the area of liver necrosis and hemorrhage and the extent of hepatic inflammation. Detailed histological grading of hepatic injury, including area of liver necrosis and hemorrhage, was determined at 48 h after acetaminophen challenge. Hepatic inflammation was graded as absent, slight, moderate, or prominent.

Cultured hepatocyte proliferation

The effects of chemokines, NAC, and HGF on hepatocyte proliferation after a 24 h acetaminophen challenge were examined using normal murine liver cells obtained from American Type Culture Collection (ATCC CRL-1638;

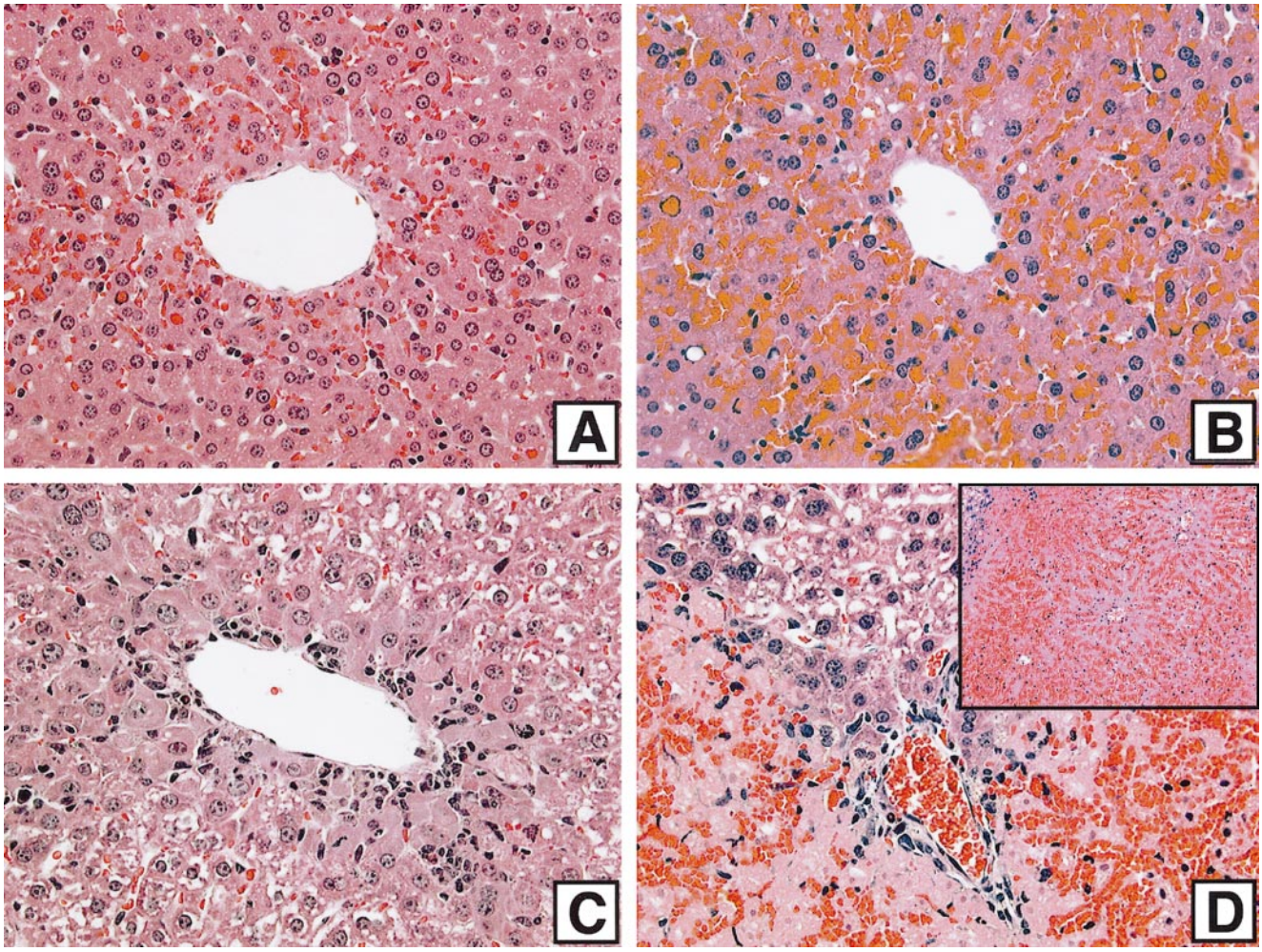

Figure 1. Histological appearance of liver in mice pretreated with either preimmune rabbit serum (*A, C*) or rabbit anti-mouse CXCR2 antibody (*B, D*) prior to acetaminophen challenge. Mice were fasted for 12 h and received preimmune serum or anti-CXCR2 antibody 2 h prior to an i.p. challenge with 400 mg/kg of acetaminophen. Liver tissue was removed at 6 h (*A, B*) and 48 h (*C, D*) after acetaminophen challenge. At both time points, profoundly greater hemorrhage and necrotic injury in the liver was observed in anti-CXCR2 antibody-treated mice compared to that in mice that received preimmune serum prior to acetaminophen challenge. Original magnification was 400× for each photomicrograph and 40× for the inset photomicrograph.

NmuLi). Hepatocytes were plated at a density of 1.0×10^5 cells/well in 6-well tissue culture plates and deprived of fetal bovine serum supplementation for 36 h prior to an experiment. Fasted hepatocytes were subsequently exposed to 2.5 mg/ml of acetaminophen and left untreated or were treated with NAC, MIP-2, ENA-78, IL-8, IP-10, or HGF. NAC at 1 mM or individual cytokines at 1 ng/ml were added either immediately or 10 h after the addition of acetaminophen. In additional *in vitro* experiments, normal serum or anti-CXCR2 antibody was also included in cultures of treated or untreated hepatocytes exposed to PBS or acetaminophen. [^{3} H]thymidine (10 μCi) was added to each well for the final 4 h of culture; 24 h after the addition of acetaminophen, suspensions of hepatocytes were prepared via cell lysis with 0.5% (v/v) Triton-X100. [^{3}H]thymidine incorporation was assessed by liquid scintillation counting on a Beckman counter (Beckman Instruments, Fullerton, Calif.).

Statistical analysis

Results are expressed as means ± standard error of the mean (SE) of 5–10 mice per group, and analysis of variance was used to detect significant differences between means. All statistical calculations were performed using GraphPad Prism 2.0 computer software (San Diego, Calif.); $P \leq 0.05$ was considered significant.

RESULTS

Endogenous CXCR2 and MIP-2 are required for hepatic regeneration after acetaminophen challenge

CXCR2 is the only CXC chemokine receptor that binds ELR-CXC chemokines in the mouse (14, 19). Thus, the role of endogenous CXCR2 in the hepatotoxic effects of acetaminophen was examined in fasted mice that received either neutralizing rabbit anti-mouse CXCR2 antibody or preimmune rabbit serum 2 h prior to acetaminophen challenge. As shown in **Fig. 1**, anti-CXCR2 antibody pretreatment markedly enhanced the degree of liver hemorrhage

TABLE 1. *Changes in hepatic levels of chemokines prior to and 48 h after acetaminophen challenge in mice*

Chemokine	Prior to acetaminophen[a] (ng/g tissue)	48 h post acetaminophen[b] (ng/g tissue)
MCP-1	1.0 ± 0.1	0.9 ± 0.3
MIP-1α	0.5 ± 0.1	0.1 ± 0.2
MIP-2	0.4 ± 0.1	0.8 ± 0.1‡
KC	0.4 ± 0.1	0.9 ± 0.2*

[a] Mice were fasted for 12 h prior to liver removal. Tissue samples were homogenized and supernatants were analyzed using ELISA. Data are mean ± SE of four representative experiments.

[b] Mice were fasted for 12 h prior to receiving an intraperitoneal injection of 400 mg/kg acetaminophen. Forty-eight hours later, liver was removed and prepared for ELISA. Data are mean ± SE of four representative experiments.

‡ $P = 0.047$ compared to levels in liver removed prior to acetaminophen.

* $P = 0.033$ compared to levels in liver removed prior to acetaminophen.

and necrosis at 6 h (Fig. 1*B*) and 48 h (Fig. 1*D*) after acetaminophen challenge compared to acetaminophen-induced injury at 6 h (Fig. 1*A*) and 48 h (Fig. 1*C*) in preimmune serum-treated mice. Although mice that received anti-CXCR2 antiserum alone did not show liver injury at any time point, marked liver necrosis was still apparent 6 days after acetaminophen challenge in mice that received anti-CXCR2 antibody (not shown). This was in marked contrast to the restored liver histology at this time in mice that received preimmune serum prior to acetaminophen challenge (not shown).

Endogenous hepatic levels of murine monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-2, and KC were determined by ELISA (**Table 1**). KC is a murine ELR-CXC chemokine with neutrophil chemotactic properties (12) that binds CXCR2 with ~10-fold less affinity than MIP-2 (19) The murine CC chemokines MCP-1 and MIP-1α were unchanged in liver homogenates from mice challenged 48 h earlier with 400 mg/kg of acetaminophen. In contrast, significant elevations in the murine ELR-CXC chemokines MIP-2 and KC were present in the same liver homogenates.

Elucidation of the role of increased MIP-2 levels in the liver was explored through the use of a polyclonal rabbit antibody directed against murine MIP-2 or preimmune rabbit serum to mice 2 h prior to acetaminophen challenge. In mice that did not receive acetaminophen, the presence of anti-MIP-2 antiserum or normal rabbit serum did not appear to exert any discernible injurious effect on the liver (not shown). Histological examination of liver samples on day 6 post-acetaminophen showed that endogenous MIP-2 was critical for normal liver regeneration in the mouse after acetaminophen challenge (**Fig. 2**). In contrast to mice that received preimmune serum (Fig. 2*A*), severe disruption of the hepatic architecture around central veins was readily apparent in mice that received anti-MIP-2 prior to acetaminophen challenge 6 days previously (Fig. 2*B*). Areas of necrosis encompassing > 50% of the total liver area were apparent in mice pretreated with anti-MIP-2 antibody (see inset to Fig. 2*B*).

ELR-CXC chemokine vs. NAC therapy during acetaminophen challenge

The therapeutic effects of ELR-CXC chemokines were compared with those of NAC and HGF therapy during experimental acetaminophen hepatotoxicity.

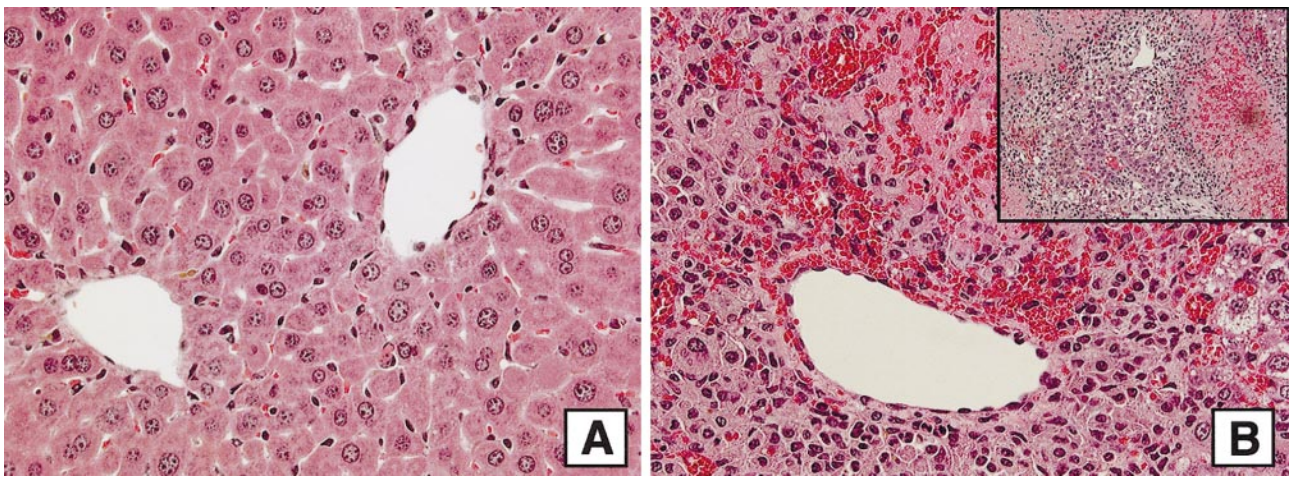

Figure 2. Histological appearance of livers from mice pretreated with preimmune serum (*A*) or polyclonal rabbit antibodies against murine MIP-2 (*B*). Mice were fasted for 12 h and received preimmune serum or neutralizing polyclonal antibody 2 h prior to i.p. injection with 400 mg/kg of acetaminophen. Mice were allowed free access to food after these treatments; 6 days later the liver was removed from each mouse for routine histological processing. Histological injury was absent in liver tissue removed from mice that received preimmune rabbit serum (*A*). Mice that received anti-MIP-2 antibody exhibited centrilobular hepatic injury and inflammation, and major areas of necrosis and hemorrhage were also apparent in the liver (see inset for B). Original magnification was 400× for the main photomicrographs and 40× for the inset photomicrograph.

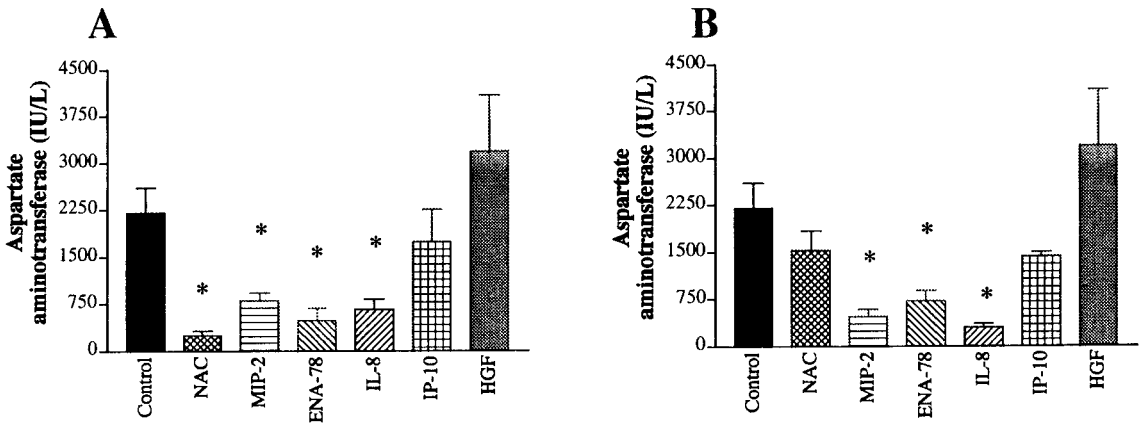

Figure 3. Serum aspartate aminotransferase (AST) activity in mice treated immediately after (*A*) or 10 h post (*B*) acetaminophen challenge. Mice were fasted for 12 h prior to i.p. injection with 400 mg/kg of acetaminophen and then allowed free access to food. 48 h later, blood was removed from each mouse for AST determination. When administered immediately after acetaminophen challenge, NAC was most effective at reducing AST levels compared to the other treatments. In addition, AST levels were significantly reduced in mice that received MIP-2, ENA-78, or IL-8, but not IP-10 or HGF (*A*). In contrast, when therapeutic intervention was delayed 10 h after acetaminophen challenge, only MIP-2, ENA-78, and IL-8 significantly reduced serum AST levels (*B*). Data are means ± SE of four separate experiments (n=5 mice/group); $*P \leq 0.05$ compared to control.

In untreated CD1 mice, the mean serum AST level was increased ~20–fold above the baseline value of 99 ± 34 IU/l at 48 h after acetaminophen challenge (**Fig. 3*A, B***). Among the treatments used immediately after acetaminophen challenge, NAC treatment reduced AST levels by ~90% (Fig. 3*A*). In addition, all three ELR-CXC chemokines (one mouse and two human) significantly reduced serum AST levels by ~50%, but IP-10 (a non-ELR CXC chemokine) and HGF did not lower serum AST levels in acetaminophen-challenged mice. When treatment was delayed 10 h after acetaminophen challenge, MIP-2, ENA-78, and IL-8, but neither IP-10 nor NAC, significantly reduced serum AST levels (Fig. 3*B*).

The hepatic injury observed at 10 h (not shown) and 48 h (representative liver histology shown in **Fig. 4**) after acetaminophen challenge was characterized by intense necrosis and hemorrhage localized around central veins in the liver. In untreated mice, greater than 50% of the total liver mass was necrotic and exhibited hemorrhage (Fig. 4*A*). Marked attenuation of liver injury was observed in mice that received 2 μg of MIP-2 (Fig. 4*B*), ENA-78 (Fig. 4*C*), or IL-8 (Fig. 4*D*), but not IP-10 (Fig. 4*E*), HGF (Fig. 4*F*), or 100 μg of NAC (Fig. 4*G*), when these treatments were delayed until 10 h after acetaminophen challenge. The beneficial effects of delayed MIP-2, ENA-78, and IL-8 therapy were most marked around hepatic central veins, where healthy hepatocytes were readily apparent (Fig. 4*B–D*), and the histological appearance of liver sections in these treatment groups was similar to that observed in normal mice (Fig. 4*H*). In addition, necrotic injury in acetaminophen-challenged mice treated with ELR-CXC chemokines was either absent or comprised less than 10% of the liver area.

Delayed MIP-2 therapy protects mice from the lethal effects of acetaminophen

The lethal effects of a 400 mg/kg dose of acetaminophen were evident when mice were fasted for a longer period. In these experiments, only 25% of mice fasted for 24 h prior to acetaminophen challenge survived until day 2 (**Table 2**). NAC treatment of mice immediately after acetaminophen challenge increased mouse survival to 90%, whereas MIP-2 given at the same time spared ~50% of mice. When NAC treatment was delayed by 10 h after acetaminophen challenge, the survival rate in this group of mice was significantly reduced to 25%. However, 60% of mice that received a similarly delayed treatment of MIP-2 were protected from the lethal effects of acetaminophen (Table 2).

Mice that received NAC immediately after acetaminophen challenge had significantly lower levels of serum AST levels than mice that received MIP-2 at this time (**Fig. 5**). The converse was observed when the NAC and MIP-2 treatments were delayed for 10 h after acetaminophen challenge. Accordingly, mice in the delayed MIP-2 treatment group had significantly lower levels of serum AST compared to mice that received the delayed NAC treatment.

The histological appearance of liver tissue removed from these groups of mice corroborated the changes in serum AST. As shown in **Fig. 6**, the delivery of NAC to mice immediately after (Fig. 6*A*) but not 10 h post (Fig. 6*B*) acetaminophen

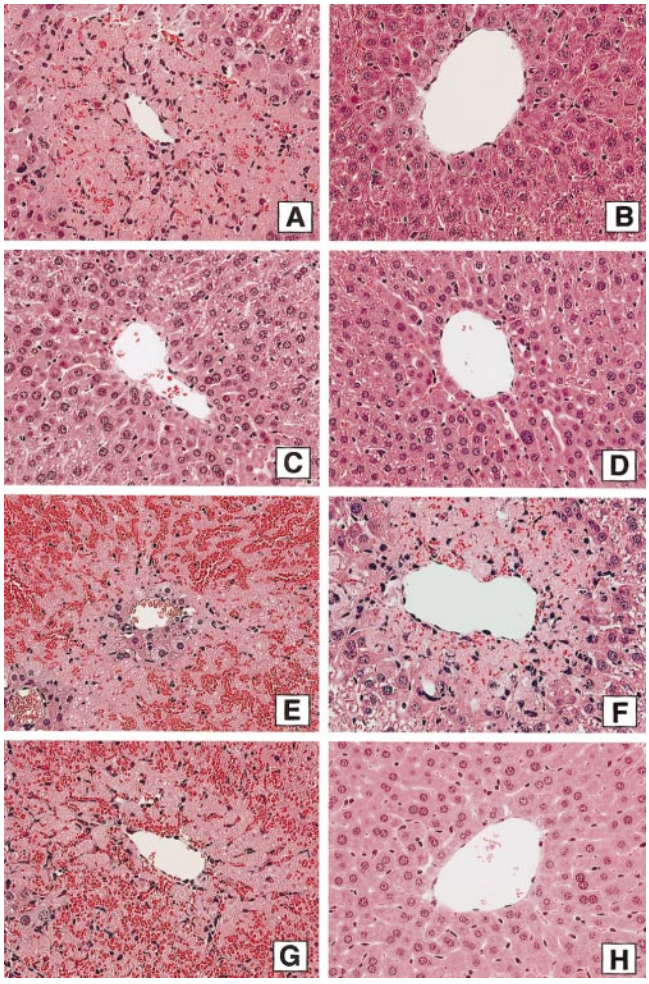

Figure 4. Histological appearance of livers from mice that were challenged with acetaminophen and received normal saline (*A*) or 2 μg of the following chemokines: murine MIP-2 (*B*), human ENA-78 (*C*), human IL-8 (*D*), human IP-10 (*E*), human HGF (*F*). Liver histology was taken from mice that received 100 μg of N-acetylcysteine (*G*). Mice were fasted for 12 h prior to i.p. injection with 400 mg/kg of acetaminophen, then allowed free access to food. In these experiments, however, saline, chemokines, or NAC were given by i.v. injection 10 h after acetaminophen challenge, and the liver was removed from each mouse for histological examination at the 48 h time point after acetaminophen. When treatment was delayed for 10 h, only mice that received MIP-2 (*B*), ENA-78 (*C*), or IL-8 (*D*) showed any major histological improvement, particularly in the centrilobular regions most sensitive to the hepatotoxic effects of acetaminophen. (*H*) Liver histology from a normal mouse. Original magnification was 400× for each photomicrograph.

markedly reduced liver injury. Although mice that received MIP-2 immediately after acetaminophen challenge exhibited marked liver injury at the 48 h time point (Fig. 6C), the liver appearance in mice that received the delayed MIP-2 therapy was markedly improved at this time point (Fig. 6*D*). Taken together, these findings suggested that when given immediately after a lethal dose of acetaminophen, NAC was clearly superior to MIP-2 treatment.

TABLE 2. *Percent survival in CD1 mice fasted for 24 h and challenged with 400 mg/kg of acetaminophen*

Treatment	Immediately after acetaminophen[a]	10 h post acetaminophen[b]
Saline	25	25
NAC	90*	20
MIP-2	50*	60*

[a] Groups of $n = 12$ (saline), 13 (NAC), and 15 (MIP-2) mice were fasted for 24 h, challenged i.p. with 400 mg/kg acetaminophen, and immediately thereafter received 0.5 ml of saline, NAC or MIP-2 through i.v. injection. Survival was monitored over the subsequent 48 h.

[b] Groups of $n = 12$ (saline), 13 (NAC), and 10 (MIP-2) mice were fasted for 24 h, challenged i.p. with 400 mg/kg acetaminophen, and 10 h thereafter received 0.5 ml of saline, NAC, or MIP-2 through i.v. injection. Forty-eight hours later, liver was removed and prepared for ELISA.

* $P \leq 0.05$ compared with saline control.

However, delayed MIP-2 therapy was clearly more beneficial compared with a delayed NAC therapy as evidenced by reduced mouse mortality, serum AST, and liver injury in the delayed ELR-CXC chemokine therapy group.

ELR-CXC chemokines maintain hepatocyte proliferation via CXCR2

The liver-regenerative properties of ELR-CXC chemokines were also explored *in vitro* using a normal hepatocyte cell line. Untreated liver cells were susceptible to the toxic effects of acetaminophen as evidenced by significantly decreased cell proliferation (as monitored by [^{3}H]thymidine incorpo-

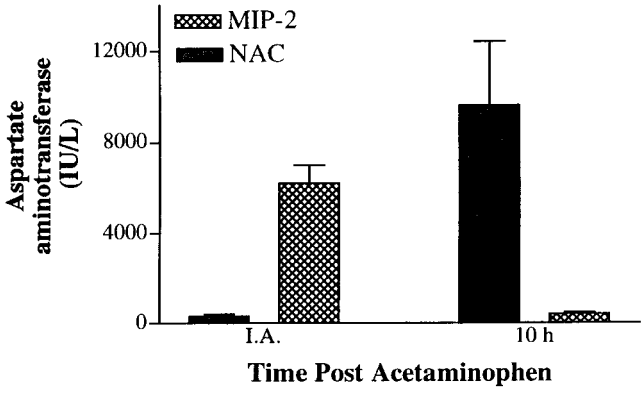

Figure 5. Serum aspartate aminotransferase (AST) activity in mice fasted for 24 h prior to MIP-2 or NAC treatment immediately after or 10 h after a 400 mg/kg acetaminophen challenge. Blood was removed from each mouse 48 h after acetaminophen challenge for AST determination. When administered immediately after acetaminophen challenge, NAC was more effective than MIP-2 at reducing AST levels. However, when the intervention was delayed 10 h, MIP-2 treatment was more effective than NAC treatment in the reduction of acetaminophen induced elevations in serum AST. Data are means ± SE of groups of 5 mice; *$P \leq 0.05$ compared to control.

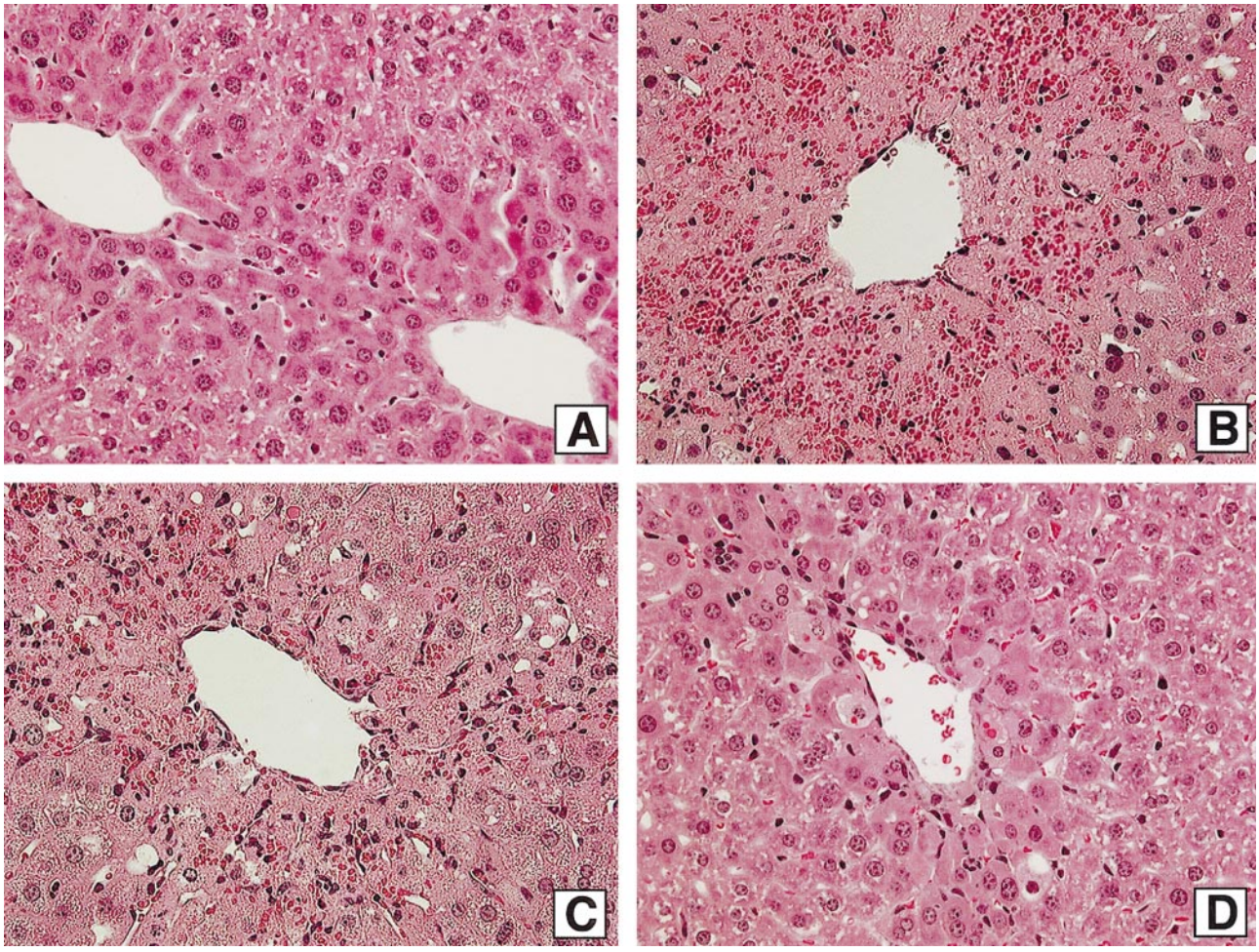

Figure 6. Histological appearance of livers from mice that were challenged with acetaminophen and received 100 μg of NAC (*A, B*) or 2 μg of murine MIP-2 (*C, D*) by i.v. injection either immediately after or 10 h later. Mice were fasted for 24 h prior to i.p. injection with 400 mg/kg of acetaminophen and then allowed free access to food. 48 h later, the liver was removed from each mouse for histological examination. When given immediately after acetaminophen, NAC treatment (*A*) provided superior hepatic protection to MIP-2 treatment (*C*). The delayed administration of NAC did not attenuate hepatic injury in acetaminophen-challenged mice, but mice that received MIP-2 at this time showed major restoration in the centrilobular regions most sensitive to acetaminophen. Original magnification was 400× for each photomicrograph.

ration) after exposure to acetaminophen for 24 h (**Fig. 7*A, B***). Although NAC did not alter the proliferation of liver cells exposed to PBS, the addition of NAC to liver cell cultures immediately after acetaminophen maintained the proliferation of these cells at levels measured in cell cultures challenged with PBS (Fig. 7*A*). Likewise, MIP-2, ENA-78, IL-8 and HGF did not change the proliferation of liver cells exposed to PBS, but MIP-2 and ENA-78 prevented the drop in liver cell proliferation after exposure to acetaminophen for 24 h. The non-ELR CXC chemokine IP-10, which binds CXCR3, lowered the proliferation rate of liver cells exposed to PBS by 40% compared to control cultures with PBS, but the proliferative response of liver cells was not further reduced by the exposure of these cells to acetaminophen. A 10 h delay in the treatment of the liver cells after acetaminophen challenge significantly reduced liver cell proliferation in cultures treated with NAC compared to the respective PBS control cultures (Fig. 7*B*). In contrast, all other cultures of liver cells exposed to acetaminophen that received delayed cytokine or chemokine treatment showed [^{3}H]thymidine incorporation comparable to cytokine- or chemokine-treated cultures exposed to PBS.

The mitogenic properties of ELR-CXC chemokines were also dependent on their ability to bind to CXCR2, since the presence of a rabbit anti-mouse CXCR2 antibody significantly reduced the mitogenic effects of MIP-2, ENA-78, and IL-8 on acetaminophen-challenged liver cells (**Fig. 8**). The presence of anti-CXCR2 antibody also significantly reduced [^{3}H]thymidine incorporation by NAC-treated liver cells exposed to acetaminophen for 24 h. However, the mitogenic effects of IP-10 and HGF on acetaminophen-challenged liver cells were independent of

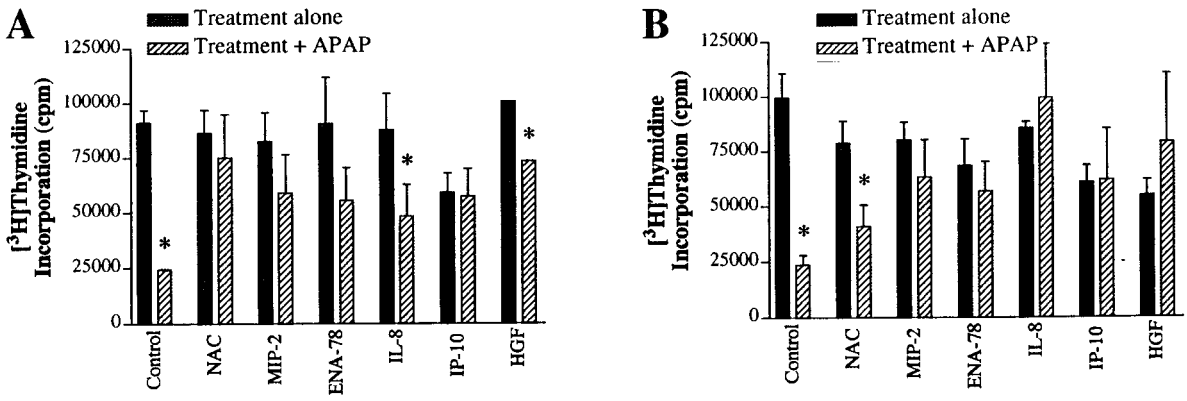

Figure 7. Proliferation of cultured liver cells exposed to saline (control), NAC, MIP-2, ENA-78, IL-8, IP-10, or HGF in the presence or absence of acetaminophen. Liver cells were treated with NAC (1 mM), cytokines, or chemokines (all at 1 ng/ml) either immediately after (*A*) or 10 h after (*B*) the addition of PBS or acetaminophen (2.5 mg/ml). 4 h before the end of a 24 h exposure of the liver cells to PBS or acetaminophen, cultures were pulsed with 10 μCi of [^{3}H]thymidine/well and [^{3}H]thymidine incorporation was determined by liquid scintillation counting. Data are mean ± SE of three separate experiments. *$P \leq 0.05$ compared to respective cultures of hepatocytes that received NAC or cytokine treatment alone. APAP: acetaminophen.

CXCR2 function. Overall, these *in vitro* data suggested that CXC chemokines possess hepatoprotective effects after acetaminophen challenge due partly to their CXCR2-dependent mitogenic effects on liver resident cells.

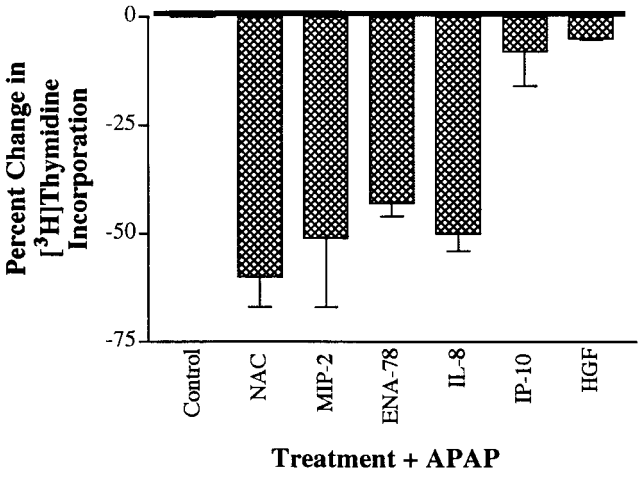

Figure 8. Role of CXCR2 in the mitogenic effects of saline (control), NAC, MIP-2, ENA-78, IL-8, IP-10, or HGF in cultures of acetaminophen-challenged liver cells. Liver cells were deprived of serum for 36 h prior to exposure to acetaminophen (2.5 mg/ml); NAC (1 mM), cytokine, or chemokine (all at 1 ng/ml) were added 10 h later. Rabbit preimmune serum or rabbit anti-mouse CXCR2 antibody was added concomitantly with NAC, cytokine, or chemokine treatment. 4 h before the conclusion of a 24 h exposure of the liver cells to PBS or acetaminophen, cultures were pulsed with 10 μCi of [^{3}H]thymidine/well; [^{3}H]thymidine incorporation was determined by liquid scintillation counting. Acetaminophen is abbreviated to APAP. Data are mean ± SE of three separate experiments.

DISCUSSION

Acetaminophen is a widely used analgesic and antipyretic medication that is generally perceived to be nontoxic. However, large or repeated doses of acetaminophen cause profound liver injury (1), potentially leading to liver failure (20). Because the symptoms of acetaminophen overdose commonly mimic common illnesses, the real diagnosis and the initiation of NAC therapy may be delayed beyond the time in which NAC effectively reduces liver injury (21). After the therapeutic window of NAC is passed, liver transplantation is often the only clinical intervention that will ensure the survival of these patients. Unfortunately, liver transplant recipients require intensive management to avoid potential complications due to rejection of the hepatic transplant (22). A novel therapeutic strategy that reduces the need for liver transplantation after acetaminophen overdose may be found in the inherent regenerative processes of the liver (23). Despite evidence that the liver possesses a tremendous capacity to regenerate after hepatic injury, few biological substances have been identified that, when delivered several hours after liver injury has occurred, promote a rapid hepatocyte proliferative response to counteract the profound hepatic necrosis that follows acetaminophen-induced toxicity.

Centrilobular hepatocytes have the important role of metabolizing many of the drugs introduced into the liver (24). When exposed to acetaminophen, the hepatocyte uses glutathione to neutralize the toxic effects of the primary metabolite of acetaminophen, N-acetyl-*p*-benzoquinoneimine. The toxic effects of this metabolite can be reversed with the addition of NAC, but the efficacy of this compound declines precipitously as hepatocytes

succumb to the toxic effects of N-acetyl-*p*-benzoquinoneimine. Delayed NAC treatment for acetaminophen-induced hepatotoxicity fails, in part, because this drug fails to trigger the restoration of the critical mass of hepatocytes needed for liver function. This was confirmed in the present study by the failure of delayed NAC treatment to prevent acetaminophen-induced liver damage observed *in vivo* and the decreased liver cell proliferation observed *in vitro* after acetaminophen challenge. Consequently, at the latter stages of drug-induced hepatotoxicity, the proliferation of the normally quiescent hepatocyte is paramount to prevent fulminant hepatic failure (23). However, many of the factors that promote the proliferation of cultured hepatocytes do not necessarily exhibit liver regenerative effects *in vivo* (7). The *in vitro* data presented herein showed that ELR-CXC chemokines, HGF, and IP-10 effectively maintained hepatocyte proliferation in the presence of toxic levels of acetaminophen. More important, the mitogenic effect of these factors was still evident when these mediators were added to hepatocytes with prior exposure to acetaminophen for 10 h. However, unlike the ELR-CXC chemokines, neither the immediate or delayed injection of HGF or IP-10 into acetaminophen-challenged mice promoted rapid liver restoration after acetaminophen-induced injury. Thus, these data demonstrate that ELR-CXC chemokines possess novel hepatic regenerative properties that were consistently observed during *in vivo* and *in vitro* acetaminophen challenge of hepatic tissue.

CXC chemokines are widely recognized as inflammatory cell recruitment factors (15). Nevertheless, the list of functions attributable to CXC chemokines has recently expanded and newer biological effects of these mediators include angiogenesis (25) and mitogenesis (16). In particular, the newly described properties of ELR-CXC chemokines are attributed mainly to the ELR motif (25, 26). ELR-CXC chemokines are specific for CXCR1 and CXCR2, whereas the non-ELR-CXC chemokines (that lack angiogenic and mitogenic effects) typically bind to CXCR3 and CXCR4. Several lines of evidence suggest that ELR-CXC chemokines may by critical participants in generalized wound healing responses. First, elevated levels of ELR-CXC chemokines and CXCR2 are invariably found in damaged tissues, including the liver (27) and skin (28). Second, ELR-CXC chemokines have previously been shown to have potent mitogenic effects on several tissue resident cells, including keratinocytes (29), epithelial cells (16), endothelial cells (30), and hepatocytes (11). Third, many of the previously mentioned cells exhibit *de novo* synthesis of ELR-CXC chemokines after inflammatory stimuli. For example, hepatocytes (31) and hepatic stellate cells (13) have been shown to generate ELR-CXC chemokines after exposure to acetaminophen or other inflammatory stimuli. Liver injury precipitated by hepatectomy is also associated with dramatic increases in MIP-2 and ENA-78 (10), the absence of which after antibody-mediated immunoneutralization significantly impairs normal liver regeneration (11). The precise cellular mechanism by which ELR-CXC chemokines facilitate hepatocyte proliferation is being investigated. Thus, the novel hepatocyte regenerative properties of ELR-CXC chemokines may represent another viable therapeutic intervention in the acetaminophen-damaged liver that may obviate or postpone the need for liver transplantation.

CONCLUSION

We have identified ELR-CXC chemokines that possess therapeutic properties in the acutely damaged liver after acetaminophen challenge. The data shown herein suggest that ELR-containing CXC chemokines promote rapid liver regeneration after drug-induced acute injury. Overall, ELR-CXC chemokines such as human IL-8 and ENA-78 may have tremendous clinical potential in reducing the need for liver transplantation and the mortality associated with acetaminophen-induced fulminant liver failure. FJ

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