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Anti-Fas Induces Hepatic Chemokines and Promotes Inflammation by an NF-κB-independent, Caspase-3-dependent Pathway*

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Agonistic antibodies against the Fas receptor, when administered to mice *in vivo*, cause significant apoptosis in the liver. In this study we show that anti-Fas antibody not only causes apoptosis of liver cells but also provokes hepatic inflammation. Two hours after injection of anti-Fas, when mice displayed evidence of caspase-3 activation and apoptosis, we found significant hepatic induction of the CXC chemokines macrophage inflammatory protein-2 and KC. Coincident with the chemokine induction was infiltration of the hepatic parenchyma by neutrophils. Neutralization experiments identified that chemokines were the cause of Fas-induced hepatic inflammation, with KC having the predominant effect. Chemokine induction in the livers of anti-Fas-treated mice was not associated with activation of NF-κB. Instead, it coincided with nuclear translocation of activator protein-1 (AP-1). AP-1 activation in liver was detected 1–2 h after anti-Fas treatment, suggesting a connection to the onset of apoptosis. When apoptosis was prevented by pretreating mice with a caspase-3 inhibitor, AP-1 activation and hepatic chemokine production were both significantly reduced. Hepatic inflammation was also reduced by 70%. Taken together, these findings indicate that Fas ligation can induce inflammation in the liver *in vivo*. Inflammation does not arise from Fas-mediated signaling through NF-κB; rather, it represents an indirect effect, requiring activation of caspase-3 and nuclear translocation of AP-1.

Fas (CD95/Apo1) is a 43-kDa cell surface glycoprotein belonging to the tumor necrosis factor receptor family (1). Ligation of the Fas receptor induces apoptosis by promoting proteolytic cleavage of intracellular caspases (2). *In vivo*, Fas mediates apoptosis in numerous tissues (3, 4); the liver seems especially sensitive, because systemic administration of agonistic anti-Fas antibody to mice causes massive death of hepatocytes. Animals treated with anti-Fas die within a few hours, with evidence of gross hemorrhage into the liver parenchyma (5).

Although engagement of Fas receptors typically results in apoptosis, Fas can also stimulate intracellular signaling pathways that lead to different responses. For example, when agonistic anti-Fas antibody is injected subcutaneously rather than systemically into mice, a robust local inflammatory response occurs (6). Similarly, when mouse peritoneum is inoculated with cells that express Fas ligand, local inflammation ensues (7). Some believe that Fas-induced inflammatory responses are triggered when neutrophils are the targets of apoptosis. In the peritoneum, for example, death of "sentinel" neutrophils can provoke inflammation caused simply by the release of the abundant cytokines within these cells (7). Another means by which Fas might induce inflammation, however, is to actively stimulate target cells to produce chemoattractants. This latter mechanism has been demonstrated in cell culture, in which many types of cells upon treatment with anti-Fas antibody begin to secrete the neutrophil chemoattractant interleukin-8 (8–11).

Interleukin-8 is a member of the chemokine superfamily of leukocyte chemoattractants. It belongs to the CXC subfamily of chemokines, named for the relative positions of two conserved cysteines near its N terminus (12). CXC chemokines are themselves separated into two groups, based on the presence or absence of a glutamic acid-leucine-arginine (ELR) motif upstream of CXC. The ELR sequence predicts potent chemotactic activity toward neutrophils (13). Among the ELR-containing CXC chemokines are interleukin-8, macrophage inflammatory protein-2 (MIP-2),[1] and growth-regulated oncogenes-α, -β, and -γ, as well as the rodent peptides cytokine-induced neutrophil chemoattractant and KC (14). CXC chemokines play a key role in neutrophilic inflammation in many organs *in vivo* (12). These compounds are induced by oxidants and cytokines by means of NF-κB and AP-1 consensus sequences within their promoter regions (15–17).

In many cells, ligation of the Fas receptor causes nuclear translocation of NF-κB (18, 19). Bronchiolar epithelial cells, which produce interleukin-8 in response to Fas ligation, also display Fas-mediated activation of NF-κB (11). This suggests that Fas may induce chemokine production by a mechanism involving NF-κB. Whether Fas signals chemokine production and inflammation in normal tissues is unknown. The objective of the current study was to determine whether Fas ligation

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[1] The abbreviations used are: MIP-2, macrophage inflammatory protein-2; AP-1, activator protein-1; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

induces neutrophilic inflammation of the liver and, if so, whether the inflammation is attributable to local induction of CXC chemokines. The results indicate that Fas causes hepatic inflammation in a process involving up-regulation of CXC chemokines. Fas-mediated chemokine induction in liver is not dependent on NF-κB but is controlled instead through the death pathway downstream of caspase-3.

EXPERIMENTAL PROCEDURES

Animals—Female Swiss-Webster mice (25–30 g of body weight) were obtained from Charles River Laboratories (Hollister, CA). All procedures were approved by the Committee on Animal Research at the University of California, San Francisco, and followed guidelines set by the American Veterinary Medical Association.

Induction of Apoptosis in Mice and Manipulation of Apoptotic/Inflammatory Responses in Vivo—The mice were injected intraperitoneally with purified hamster anti-mouse Fas monoclonal antibody (clone Jo2; Pharmingen, San Diego, CA). Five treatment groups received Jo2 in doses of 1, 2.5, 5, 10, and 50 μg. The control mice were treated with hamster nonimmune IgG (Pharmingen). Two hours after injection, the mice were killed for collection of liver tissue. A portion of each mouse liver was fixed in 10% buffered formalin and embedded in paraffin. The remainder was snap frozen in liquid nitrogen-cooled isopentane and stored at −80 °C until use.

For chemokine neutralization experiments, neutralizing monoclonal antibodies to murine KC or MIP-2 (0.2 mg in 0.1 ml) (R & D Systems, Minneapolis, MN), were injected intravenously into mice 15 min before intraperitoneal injection of Jo2. The control animals received nonimmune murine IgG (0.2 mg in 0.1 ml) (Pharmingen). The mice were killed 2 h after Jo2 injection.

For caspase-3 inhibition experiments, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (zDEVD-fmk, R&D Systems) was administered intraperitoneally to mice (0.5 mg in 0.1 ml of 1% Me_2SO) 15 min before Jo2. The control animals were injected with an equivalent dose of an inactive inhibitor (zFA-fmk, R&D Systems). The mice were killed 2 h after Jo2 injection.

Measurement of Caspase-3 Activity—The livers were homogenized in a solution of 25 mM HEPES, 5 mM EDTA, 5 mM $MgCl_2$, and 5 mM dithiothreitol supplemented with protease inhibitors. Caspase-3 activity was quantitated fluorometrically using a substrate conjugated with 7-amino-4-methyl coumarin (CaspACE assay system; Promega Corp., Madison, WI). To verify the specificity of the reaction, enzyme activity in each sample (90 μg of total protein) was measured in the presence and absence of the caspase-3 inhibitor Ac-DEVD-CHO. The results were expressed as pmol of substrate cleaved per mg of liver protein.

Immunohistochemistry—7-μm sections of frozen liver tissue were fixed for 10 min in acetone. Endogenous peroxidase activity was quenched with hydrogen peroxide. The sections were then incubated for 30 min with phosphate-buffered saline containing 1% BSA (PBS/BSA). For identification of neutrophils, the sections were treated with rat anti-mouse neutrophil antibody (Ly6G, Pharmingen) or an irrelevant control antibody diluted 1:100 in PBS/BSA. After 1 h, the slides were washed with PBS and treated with reagents to block nonspecific avidin-biotin binding (blocking kit; Vector Laboratories, Burlingame, CA). The sections were then incubated with biotinylated anti-rat IgG (Pharmingen; 1:100) followed by avidin-biotin complex (Vectastain ABC kit; Vector Laboratories). Bound antibody was detected with 3,3′-diaminobenzidine. The sections were counterstained with hematoxylin before coverslipping. The neutrophils were quantitated by direct counting of stained cells in 10 random high power (20×) fields. For identification of KC and MIP-2, the liver sections were fixed and pretreated as above and then incubated with anti-chemokine antibody (goat anti-mouse KC, R & D; goat anti-mouse MIP-2, R & D) or control antibody diluted 1:40 in PBS/BSA. After 1 h, the slides were washed and treated with horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnologies, Santa Cruz, CA). Bound antibody was detected with 3,3′-diaminobenzidine, and the sections were counterstained with hematoxylin as described above.

Assessment of Apoptosis by Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL)—Formalin-fixed sections of liver tissue (5-μm) were deparaffinized in xylene and hydrated in graded ethanols. The apoptotic cells were identified using the DeadEnd® *in situ* apoptosis peroxidase detection kit (Promega).

Quantitation of Chemokines in Liver Tissue—KC and MIP-2 were measured in whole liver homogenates using commercial sandwich enzyme-linked immunosorbent assays (Quantikine; R & D Systems). The raw data were normalized to total protein in the liver homogenates. The protein was quantitated by Bradford assay (Bio-Rad).

In Situ Hybridization—KC mRNA was detected in liver tissue using a homologous 200-bp cDNA sequence from the rat chemokine cytokine-induced neutrophil chemoattractant (20). The cytokine-induced neutrophil chemoattractant cDNA, subcloned into pGEM-4Z (Promega), was used as a template for transcription of single-stranded antisense and sense RNA probes containing [α-^{33}P]UTP (PerkinElmer Life Sciences). The labeled probes were applied to frozen sections of liver tissue as previously described (21).

RNase Protection Assay—Total RNA was isolated from liver tissue using TRI reagent (Molecular Research Center, Cincinnati, OH). KC, MIP-2, L_{32}, and glyceraldehyde-6-phosphate dehydrogenase mRNA were measured in 20 μg of total liver RNA using a Riboquant® multiprobe set (Pharmingen) labeled with [α-^{32}P]UTP (Amersham Biosciences). Protected mRNA fragments corresponding to the probes of interest were separated through 5% polyacrylamide-urea. The dried gels were applied to x-ray film (X-Omat AR-5; Eastman Kodak Co.). Autoradiographic signals were quantitated by scanning densitometry using NIH Image 1.61 software.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from liver tissue in Dignam C buffer (22). Electrophoretic mobility shift assay was performed as previously described (23). Briefly, NF-κB and AP-1 oligonucleotides were labeled with γ-^{32}P using T4 polynucleotide kinase and used as probes for nuclear protein binding. Protein-DNA binding reactions were carried out for 20 min on ice using 30 μg of nuclear extract and 20,000 cpm of probe.

RESULTS

Anti-Fas Antibody (Jo2) Induces Apoptosis and Hepatic Chemokine Production—When administered to Swiss-Webster mice *in vivo*, the agonistic anti-Fas antibody Jo2 caused dose-dependent apoptosis of liver cells. Mice receiving as little as 2.5 μg of Jo2 (approximately 0.1 mg/kg of body weight) exhibited apoptosis; this was demonstrated both by TUNEL staining and activation of caspase-3 (Fig. 1). When the dose of Jo2 was increased to 5 μg (0.2 mg/kg), the amount of apoptosis rose significantly. Apoptotic responses reached a maximum between 10 and 50 μg of Jo2 (0.4–2.0 mg/kg) (Fig. 1).

Coincident with apoptosis, Jo2 induced hepatic expression of mRNA encoding the CXC chemokines KC and MIP-2 (Fig. 2). Substantial increases in chemokine gene expression were evident even in response to low doses of the agonistic anti-Fas antibody (5 μg). *In situ* hybridization experiments showed that CXC chemokine mRNA was widely distributed in Jo2-treated liver, suggesting expression by hepatocytes (Fig. 3*b*). This was confirmed by immunohistochemistry (Fig. 3*d*). Accompanying the induction of KC and MIP-2 mRNA in Jo2-treated mouse liver was the production of KC and MIP-2 proteins (Fig. 4). KC and MIP-2 were detected at high levels in liver homogenates from mice receiving 5 μg of Jo2. At higher doses of Jo2, hepatic chemokine levels showed less induction; this was true despite significant up-regulation of their respective mRNAs (Fig. 2). The discrepancy between chemokine mRNA and protein levels at high doses of Jo2 is unexplained. It could be due to impaired protein translation (24) or accelerated protein degradation in apoptotic cells.

Jo2-treated Mice Exhibit Hepatic Neutrophil Infiltration—To explore the biological consequences of CXC chemokine induction in Jo2-treated mice, the livers were examined for evidence of neutrophil infiltration. Neutrophils were detected in Jo2-treated livers but not in livers from control mice (Fig. 5*a*). Neutrophils were most abundant in mice treated with 5 μg of Jo2, the dose of antibody that caused the greatest induction of KC and MIP-2 (Fig. 5*b*). 5 μg of Jo2 caused a 10-fold increase in hepatic neutrophils relative to controls. Overall, chemokine production and neutrophilic inflammation varied in parallel (Figs. 4 and 5). To determine which of the two chemokines is the dominant neutrophil chemoattractant in Jo2-treated liver, we performed experiments in which mice were injected with neutralizing antibodies against KC or MIP-2 15 min before Jo2.

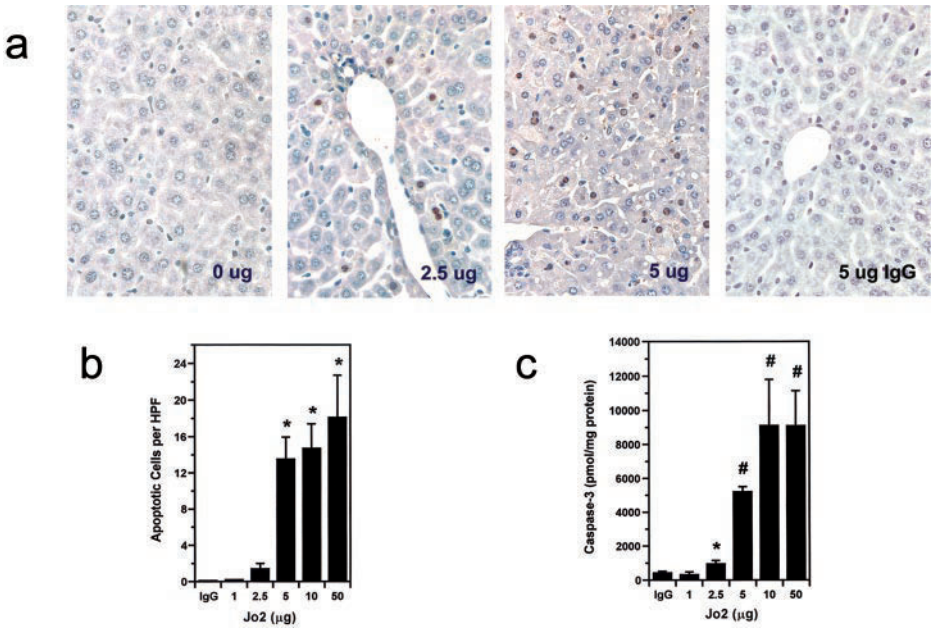

FIG. 1. **Dose-dependent apoptosis in mouse liver in response to anti-Fas.** The data depict apoptosis in mouse liver 2 h after Jo2 injection, as measured by TUNEL staining and quantitative assay of caspase-3 activity. *a*, photomicrographs of TUNEL stains from mice that received 0, 2.5, or 5 μg of Jo2 or 5 μg of control IgG. *b*, histogram showing the number of TUNEL-positive cells per high power field (*HPF*, 20×) as a function of Jo2 dose. Controls received 5 μg of IgG. *c*, histogram depicting caspase-3 activity in liver as a function of Jo2 dose. Controls received 5 μg of IgG. The values represent the means ± S.E. *, $p < 0.05$; #, $p < 0.001$ *versus* IgG control.

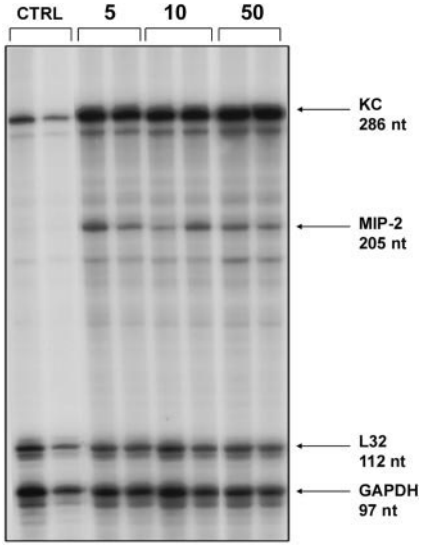

FIG. 2. **Induction of CXC chemokine mRNA in mouse liver after Jo2 injection.** The autoradiogram depicts the expression of KC and MIP-2 mRNA in mouse liver 2 h after injection of Jo2. Control mRNA signals encoding L_{32} and glyceraldehyde-6-phosphate dehydrogenase are at the *bottom* of the *gel*. Duplicate lanes represent samples from two different mice that received either Jo2 at doses of 5, 10, or 50 μg or 5 μg of control IgG (*CTRL*). As little as 5 μg of Jo2 (0.2 mg/kg) caused substantial induction of both KC and MIP-2 mRNA in the liver. Higher doses of Jo2 did not cause a progressive rise in KC or MIP-2 mRNA.

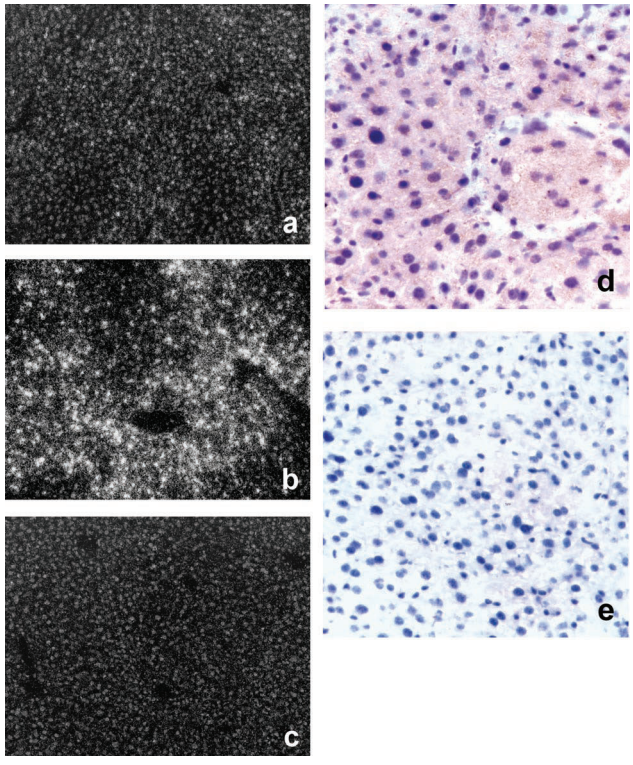

FIG. 3. ***In situ*** **hybridization and immunohistochemistry for KC in mouse liver sections.** Photomicrographs illustrate the localization of KC in mouse liver by *in situ* hybridization (*a–c*) and immunohistochemistry (*d* and *e*). Tissue was obtained 2 h after injection of either 5 μg of Jo2 or 5 μg of control IgG. A control liver incubated with a specific antisense KC cRNA probe (*a*) shows no hybridization signal. A Jo2-treated liver incubated with an antisense KC probe (*b*) shows diffuse KC transcripts throughout the parenchyma. A Jo2-treated liver incubated with a sense KC probe as a control (*c*) shows no specific hybridization signal. Anti-KC antibody identifies hepatocytes as the source of KC in the liver of a Jo2-treated mouse (*d*). Immunoreactive KC is not detectable in the liver of a control mouse (*e*). Similar data were obtained for MIP-2 (not shown). Original magnification was 10× for *a–c* and 20× for *d* and *e*.

Neither antibody influenced hepatic apoptosis (not shown). Anti-KC abolished hepatic neutrophil recruitment following Jo2 injection, but anti-MIP-2 had no effect on neutrophil accumulation in the liver (Table I).

Jo2 Does Not Induce NF-κB Activation in Liver but Promotes AP-1 DNA Binding Activity—Because Fas can activate NF-κB (18, 19) and NF-κB can in turn activate transcription of KC and MIP-2 (15, 16), we investigated whether NF-κB plays a role in Jo2-mediated induction of hepatic chemokines. We performed DNA binding experiments using nuclear extracts from mouse liver harvested 30 min, 60 min, and 2 h after Jo2 injection together with a consensus NF-κB oligonucleotide (Fig. 6*a*). No NF-κB binding was observed at any time after Jo2 injection. This was true despite strong NF-κB binding activity in positive control livers treated with lipopolysaccharide. As an alternative possibility, we considered that Jo2 might induce chemokine expression in liver by stimulating AP-1. AP-1 binding

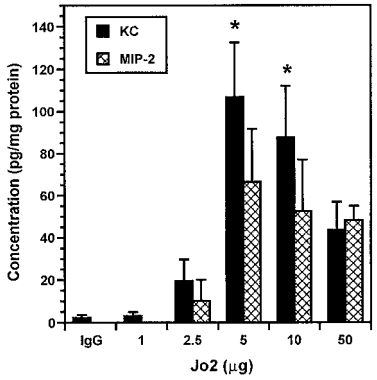

FIG. 4. **KC and MIP-2 in mouse liver after Jo2 injection.** Graph depicts KC and MIP-2 proteins measured by enzyme-linked immunosorbent assays in mouse liver homogenates 2 h after Jo2 injection. Control mice received 5 μg of IgG. KC and MIP-2 were maximally induced at a dose of 5 μg of Jo2. At higher doses, hepatic levels of both chemokines diminished. The values represent the means ± S.E. *, $p <$ 0.05, *versus* IgG control.

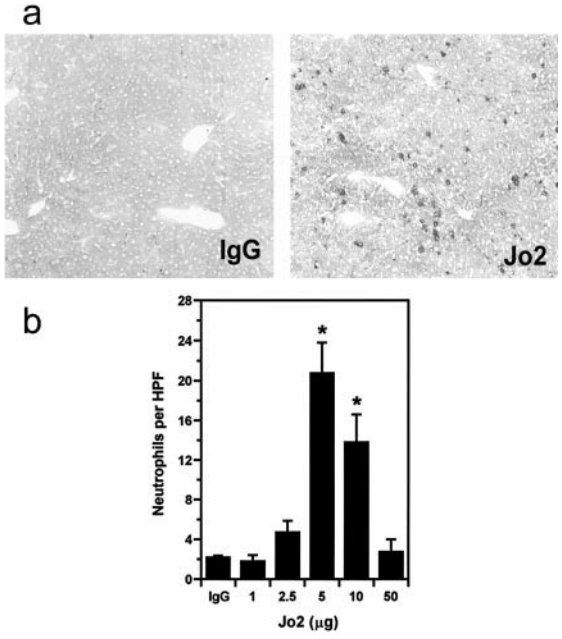

FIG. 5. **Neutrophilic inflammation of Jo2-treated livers.** *a*, photomicrographs depict immunoreactivity for neutrophils in mouse liver after injection with control IgG or Jo2. No neutrophils were detected in control mice, but abundant cells were identified in Jo2-treated liver with a diffuse distribution throughout the hepatic lobule. *b*, graph depicts the results of direct neutrophil counts in Jo2-treated livers. Neutrophils were most abundant in mice that received 5 μg of Jo2. The values represent the means ± S.E. *, $p < 0.05$, *versus* IgG control.

activity was first detected in nuclear extracts from mouse liver 1 h after Jo2 injection (Fig. 6*b*). AP-1 activation increased at 2 h. These results suggest that Jo2-mediated induction of hepatic chemokines is dependent on AP-1 rather than NF-κB.

Jo2 Mediates Hepatic Chemokine Expression and Neutrophil Recruitment Downstream of Caspase-3 Activation—The relatively late nuclear translocation of AP-1 in Jo2-treated livers suggests that activation of this transcription factor is not a direct consequence of Fas ligation. To determine whether caspase activation is required, we treated mice with the caspase-3 inhibitor zDEVD-fmk and monitored its effect on AP-1 in response to Jo2. zDEVD-fmk reduced caspase-3 activity in Jo2-treated liver by 90% (Fig. 7, *left panel*). Inhibition of caspase-3 was accompanied by elimination of Fas-mediated AP-1 DNA binding activity (Fig. 8). It was also accompanied by significant blunting of the Fas-mediated induction of KC and

TABLE I
Hepatic inflammation in Jo2-treated liver is reduced by anti-chemokine antibody

The values depict neutrophil counts/high power field in mouse liver 2 h after Jo2 injection. 15 min before Jo2 injection, mice were pretreated with 0.2 mg of neutralizing anti-chemokine antibody (anti-KC or anti-MIP-2) or an isotype-matched control IgG. Neutrophil infiltration was significantly reduced by anti-KC (−75%; $p < 0.01$). Anti-MIP-2 caused a modest but insignificant reduction in neutrophil recruitment to the liver. The values represent the means ± S.E.

Treatment	Neutrophils/HPF
Jo2 + anti-KC	3.56 ± 0.8[a]
Jo2 + control IgG	13.8 ± 3.4
Jo2 + anti-MIP-2	5.2 ± 2.4
Jo2 + control IgG	9.6 ± 4.1

[a] $p < 0.01$ *versus* control IgG.

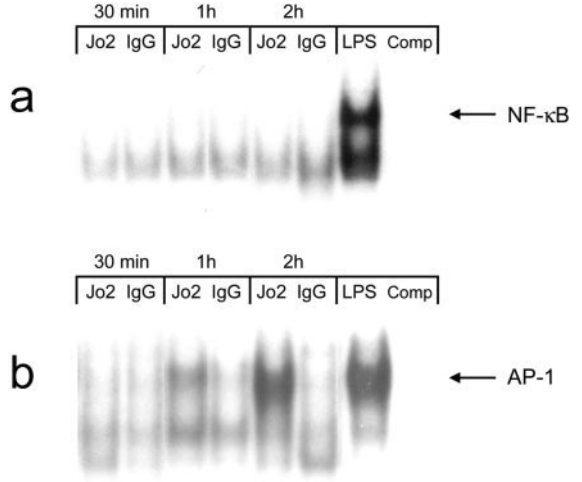

FIG. 6. **Electrophoretic mobility shift assay of liver nuclear extracts after treatment with Jo2.** Autoradiograms depict DNA binding of nuclear extracts from Jo2- or IgG-treated mouse livers to an NF-κB (*a*) or AP-1 (*b*) consensus oligonucleotide. *Panel a* shows no activation of NF-κB in Jo2-treated liver, at any time from 30 min to 2 h after injection. Lipopolysaccharide-treated mouse liver (*LPS*) serves as a positive control. The negative control represents lipopolysaccharide-treated liver incubated with labeled probe in the presence of a 200-fold excess of unlabeled competitor (*Comp*). *Panel b* shows that AP-1 DNA binding activity becomes detectable at 1 h after Jo2. AP-1 binding is increased at 2 h.

MIP-2 in mouse liver (48 and 78%, respectively, $p < 0.05$; Fig. 7, *middle panel*). Mice treated with zDEVD-fmk exhibited a substantial reduction in the neutrophilic inflammatory response to Jo2 (70%, $p < 0.01$; Fig. 7, *right panel*). These results indicate that caspase-3 is a critical mediator of hepatic inflammation during apoptosis. They suggest that inflammation arises from caspase-3-mediated activation of AP-1, which induces chemokines that stimulate neutrophil recruitment to the liver.

DISCUSSION

The current study shows that in Fas-mediated liver injury *in vivo*, engagement of Fas receptors not only causes cell death but also stimulates local production of proinflammatory chemokines. Chemokine production and cell death are activated in parallel in response to anti-Fas, suggesting that the two processes are closely related. The chemokines induced in response to Fas are biologically active; KC in particular causes active recruitment of neutrophils to the hepatic parenchyma. Together these findings indicate that *in vivo*, tissue inflammation can result from what was once considered a pure apoptotic stimulus.

Apoptosis has classically been viewed as a silent mode of cell

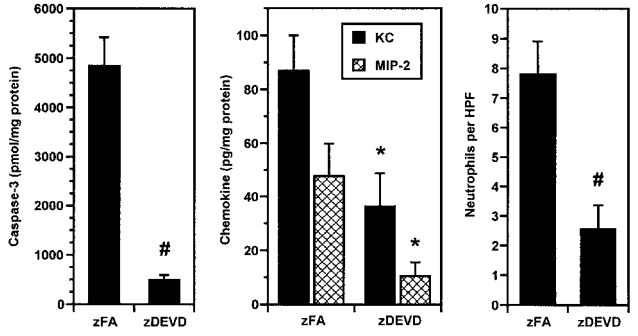

FIG. 7. **Caspase-3 inhibition reduces chemokine induction and neutrophilic infiltration in Jo2-treated liver.** Graphs depict caspase-3 activity (*left panel*), KC and MIP-2 (*middle panel*), and neutrophil counts (*right panel*) in mouse liver 2 h after Jo2, following pretreatment with either the caspase-3 inhibitor zDEVD or the control compound zFA. zDEVD markedly reduced caspase-3 activation in response to Jo2. ZDEVD also significantly impaired the hepatic induction of KC (−58%) and MIP-2 (−78%). These events were accompanied by reduced neutrophil recruitment to the hepatic parenchyma (−70%). The values represent the means ± S.E. *, $p < 0.05$; #, $p < 0.01$, *versus* zFA.

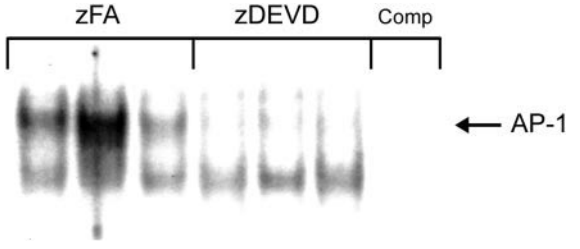

FIG. 8. **Inhibition of caspase-3 eliminates AP-1 DNA binding activity in the livers of Jo2-treated mice.** Autoradiogram illustrates AP-1 activation in the livers of mice pretreated with either the caspase-3 inhibitor zDEVD or the control inhibitor zFA prior to Jo2 injection. Mice pretreated with zFA exhibit AP-1 DNA binding activity at 2 h after Jo2. By contrast, mice pretreated with zDEVD exhibit no AP-1 DNA binding at 2 h. No DNA binding is observed in nuclear extracts incubated with a 200-fold excess of cold competitor (*Comp*). Each lane represents the liver nuclear extract from a separate mouse.

death, in which affected cells are cleared by their neighbors or professional phagocytes without eliciting an inflammatory response (25–27). Apoptotic cells are recognized by cell surface display of compounds such as long chain carbohydrates, thrombospondin, or anionic phospholipids, which interact with receptors on phagocytic cells in a manner that avoids induction of proinflammatory cytokines (28, 29). However, despite the mechanisms that have evolved for inconspicuous removal of apoptotic cells, recent studies indicate that phagocytes can produce inflammatory cytokines upon ingestion of apoptotic bodies (30). In addition, apoptotic cells themselves are capable of producing cytokines. Cells from colon (8), synovium (9), brain (10), and lung (11) have each been reported to produce CXC chemokines upon exposure to Fas agonists or tumor necrosis factor in culture. Their ability to provoke inflammation *in vivo*, however, has not been demonstrated. Our study shows that in the intact liver, anti-Fas stimulates hepatocytes to produce chemokines, which in turn results in hepatic inflammation.

Connections between apoptosis and inflammation *in vivo* are being recognized with increasing frequency. For example, classical inflammatory stimuli such as ischemia reperfusion (31, 32), galactosamine-endotoxin (33), and bleomycin (34) typically cause apoptosis before the onset of leukocyte infiltration. When apoptosis is inhibited in these experimental models, the leukocyte response disappears; this suggests that programmed cell death plays an essential role in the completion of the inflammatory response. On the other hand, the concept that apoptotic agents can induce tissue inflammation by themselves, in the absence of independent inflammatory signals, has not been extensively explored (35, 36). One group recently reported that smooth muscle cells in the carotid artery, triggered to undergo apoptosis by activation of the Fas-associated death domain, produced CXC chemokines, and elicited an inflammatory response in the vessel wall (36). We provide evidence that similar events occur in the liver in response to an agonistic anti-Fas antibody and define the pathway that leads from Fas through chemokines to inflammation.

Fas ligation caused simultaneous induction of MIP-2 and KC in mouse liver. This is in keeping with the common modes of gene regulation within the CXC chemokine subfamily (15–17). Recruitment of neutrophils to the liver, however, was attributable primarily to KC alone. Why KC would be preeminent in eliciting a neutrophil response is uncertain, given that both KC and MIP-2 interact with the same receptor (CXCR2) on murine neutrophils (37). There is some evidence that KC and MIP-2 differ as neutrophil chemoattractants *in vivo* (38, 39). For example, although KC and MIP-2 are both induced in the lung in response to *Nocardia asteroides* infection (40) and bleomycin treatment (41), neutralizing antibodies against MIP-2 have little effect on tissue inflammation. This parallels exactly what we found with MIP-2 neutralization in liver. In our study, neutralizing antibodies against KC abrogated the inflammatory response to Fas ligation. Thus, KC represents the primary neutrophil chemoattractant mediating Fas-induced hepatic inflammation.

In our experiments, anti-Fas-induced hepatic chemokine production is independent of NF-κB. Although in some cells Fas is believed to stimulate chemokine secretion by activating NF-κB (11), this appears not to be the principal pathway to Fas-induced chemokine production by hepatocytes. In cultured hepatocytes, anti-Fas antibody is capable of activating NF-κB (42); in the liver *in vivo*, however, anti-Fas induces only modest nuclear translocation of NF-κB, even at lethal doses (43). Our experiments employed a relatively low dose of anti-Fas antibody. Thus, the likelihood of activating NF-κB in hepatocytes was low. More importantly, at doses of anti-Fas that caused maximal hepatic chemokine induction, NF-κB DNA binding was minimal or absent.

Fas-induced chemokine production in the liver did coincide with nuclear translocation of AP-1. AP-1 was activated relatively late after Jo2 treatment, suggesting that it was not a direct consequence of Fas ligation as reported in some cells (44, 45). The time course suggested instead that AP-1 was activated in dying cells as part of a stress response (46). In the intact liver, Fas induced CXC chemokines and neutrophilic inflammation only after activation of caspase-3. Although this is at odds with cell culture data that Fas induces chemokines independently of apoptosis (9, 36), it is consistent with several reports *in vivo* that caspase inhibition prevents inflammation as well as cell death (31, 32, 34, 47).

Interestingly, despite numerous studies investigating Fas-mediated apoptosis in liver, hepatic inflammation has not previously been recognized (5, 33). The explanation for this may lie in the fact that chemokine induction in response to Fas appears dose-dependent (Fig. 4). At very low doses of anti-Fas, there is probably too little apoptosis to induce hepatic chemokines; at high doses, apoptosis is massive but there is no inflammation, perhaps because of a caspase-3-dependent block in chemokine translation (24). Only at moderate doses of anti-Fas does apoptosis stimulate an inflammatory response (Fig. 5). This intermediate situation may be most relevant to organ injury *in vivo*, in which moderate levels of apoptosis can continue over prolonged intervals. In this scenario, ongoing apoptosis could be an

essential component of a chronic inflammatory response.

In summary, the current experiments demonstrate that Fas ligation induces hepatic chemokine expression, which in turn causes neutrophilic inflammation of the liver. Chemokines and inflammation are not induced directly by Fas but instead are consequences of caspase-3 activation. The results suggest that CXC chemokines are induced as part of a stress response to cell death, which involves nuclear translocation of AP-1. An important consequence is neutrophil recruitment, which can potentiate organ injury by release of oxidants and proteinases (48). To the extent that inflammation represents a maladaptive response to apoptosis, inhibition of apoptosis holds promise as a strategy to limit inflammatory responses. Indeed, caspase inhibitors may have broad clinical utility as modulators of both acute and chronic inflammatory diseases.

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