Mac-1 (CD11b/CD18) and intercellular adhesion molecule-1 in ischemia-reperfusion injury of rat liver

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To inhibit their function have measurably reduced the extent of I/R injury to the liver (19). Such investigations suggest that recruitment of neutrophils into the liver is a critical step in the pathogenesis of I/R injury.

Neutrophil recruitment at sites of inflammation is mediated by a variety of adhesion molecules, which are cell-surface glycoproteins involved in cell-cell and cell-matrix interactions. Although many adhesion molecules have been identified, various in vitro studies have shown that during hepatic I/R the β2 integrin CD11b/CD18 (Mac-1) as well as their counterreceptor, intercellular adhesion molecule-1 (ICAM-1) (5), play a critical role in neutrophil transendothelial migration (9, 28) and adherence to hepatocytes (24). In vivo studies (7, 12, 18) have revealed that Mac-1 and ICAM-1 monoclonal antibodies given intravenously before and/or during reperfusion reduce liver injury in I/R and in endotoxic shock models (6, 17). However, the potential shortcomings of using antibodies administered in vivo to investigate the role of these adhesion molecules have not been fully discussed. For example, antibodies can have actions other than simply blocking the functional expression of their respective adhesion molecules: they may help attenuate liver injury by inhibiting complement activation (11). Likewise, antibodies may not block all the adhesive interactions of the targeted molecule, especially in the case of ICAM-1, which has multiple integrin binding sites (32). Therefore, there is a need to investigate not only the in vivo administration of antibodies against adhesion molecules, but also the expression profiles of these adhesion molecules in the development of liver injury.

Sinusoidal endothelial cells (SECs) in the liver are known to be particularly vulnerable to I/R injury (8, 13, 29). Changes in SECs have been reported (1, 15) to occur early in reperfusion, so these cells are thought to be the primary targets of this injury. Besides, recent investigations (4, 20, 27) have suggested that hepatocyte apoptosis may also be involved in liver I/R injury.
However, the relative importance of SEC injury and apoptosis in I/R injury remains unclear.

Our aim was to evaluate the role of Mac-1, ICAM-1, SEC damage, and hepatocyte apoptosis in the development of normothermic hepatic I/R injury in the rat. We investigated the chronological changes of Mac-1 and ICAM-1 expression simultaneously in two levels of hepatic I/R injury: transient, reversible injury and progressive, fatal injury, as determined by the duration of ischemia. We then studied the temporal relationship between the expression profiles of these molecules and the extent of liver cell damage, focusing particularly on SEC injury and the occurrence of hepatocyte apoptosis. Finally, the possibility that inhibition of apoptosis reduces neutrophil infiltration and subsequent liver injury after hepatic I/R was investigated in vivo using the caspase inhibitor Z-Val-Ala-Asp (OME)-CH$_2$F (ZVAD-fmk).

MATERIALS AND METHODS

Animals

For all the experiments, we used male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing 100–120 g. The animals were housed in Plexiglas cages in a temperature- and humidity-controlled room and allowed free access to food and water.

The experimental protocols followed the criteria of the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892].

Experimental Design

To minimize splanchnic congestion during total hepatic ischemia, we performed subcutaneous transposition of the capsule-scarified spleen (14, 25) to induce development of portosystemic collaterals between the spleen and the anterior abdominal wall. Four weeks later, the animals (200–250 g) were subjected to normothermic total hepatic ischemia. Rats were randomly assigned to either of two experimental groups according to the duration of ischemia: 30 min or 60 min of I/R. The animals were then anesthetized by halothane inhalation. Midline laparotomy was performed, and hepatic ischemia was induced by clamping of the hepatic artery, portal vein, and bile duct with an atraumatic bulldog clamp. During the ischemic period, 0.6 ml of saline solution was administered intravenously at 20-min intervals for volume replacement via the penile vein. Reperfusion was initiated by removal of the clamp. The wound was closed with 3-0 silk, and the rats were allowed to recover. Sham-operated control animals were treated in an identical fashion, apart from not being subjected to vascular occlusion. Body temperature was maintained with a heating pad during the surgery. The study consisted of three sets of experiments, as described below.

Experiment 1. We determined the survival rate ($n = 11$ per group). Rats were considered to have survived if they remained alive on the seventh day after I/R.

Experiment 2. Rats from each group were reanesthetized by inhalation of halothane and killed by exsanguination at various times (3, 6, 12, or 24 h; $n = 5$ for each time point/group) after I/R. Heparin-anticoagulated whole blood was obtained via the vena cava at the time of killing. The portal vein was cannulated with PE-10 tubing and flushed with PBS. Liver tissues were sampled and fixed in 10% phosphate-buffered formalin for histological analysis. Other liver tissues were also obtained and embedded in OCT embedding medium. They were snap-frozen in liquid nitrogen and kept at −80°C until immunohistochemistry was performed. Additional specimens were taken for the analysis of apoptosis. They were fixed in 8% paraformaldehyde at 4°C for 18–24 h and then dehydrated in ethanol.

Experiment 3. Rats were divided into two groups. Group A animals (control group) received an intravenous injection into the dorsal penile vein of 0.3 ml PBS containing 1% DMSO, 2 min before induction of ischemia. In group B animals, 0.3 mg of ZVAD-fmk (Enzyme Systems Products, Livermore, CA) dissolved in 0.3 ml PBS containing 1% DMSO was administered under similar conditions. Both groups of rats were subjected to the same surgical procedure documented previously and given 60 min of I/R. The animals were killed at corresponding time points ($n = 3$ for each time point/group). Caspase 3-like activity, hepatocyte apoptosis, the number of neutrophils, and necrotic area were assessed in both groups.

Histology

Formalin-fixed portions of the liver were embedded in paraffin, and 5-µm-thick sections were cut. First, the neutrophils were stained using a naphthol AS-D chloroacetate esterase cytochemical staining kit from Sigma Diagnostics (St. Louis, MO) that identified specific leukocyte esterases. The staining procedure has been described in detail elsewhere (23). Neutrophils, identified by positive staining and nuclear morphology, were counted in 50 high-power fields (HPF; ×400) through an Olympus Labophot microscope. Next, the percent necrotic area was estimated by random evaluation of 10 HPF (×40) of parallel sections stained with hematoxylin and eosin. The necrotic areas and the nonnecrotic areas were mapped, and the percent necrotic area was estimated. Finally, the number of SECs was counted in 15 HPF (×400). SECs were identified by their location in the sinusoidal lumen and morphologically by their concavo-convex shape. Although this procedure may include in the count a number of dead SECs that retain their structure, the number of SECs per unit area is thought to roughly represent the degree of SEC damage.

ICAM-1 Immunohistochemistry

Frozen sections were cut at 5 µm in a cryostat, air dried for 1 h, fixed in cold acetone (4°C) for 10 min, and air dried for 10 min. The sections were covered with 1.5% blocking serum (Vector Laboratories, Burlingame, CA) and incubated for 20 min at room temperature. They were then incubated overnight at 4°C, first with the mouse monoclonal antibody to rat ICAM-1 (clone 1A29, Seikagaku, Tokyo, Japan) diluted 1:600 and next with the biotin-labeled horse anti-mouse antibody (Vector Laboratories) diluted 1:500. The slides were washed three times for 5 min each in PBS after each incubation. Endogenous peroxidase activity was blocked for 30 min in methanol containing 0.3% H$_2$O$_2$. After a 10-min wash in PBS, sections were covered with avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min at room temperature. Diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) was used as a chromogen. Substitution of the mouse IgG control antibody (Vector Laboratories) was used as a negative control. Sections were counterstained with hematoxylin and examined by light microscopy. The staining intensities of SECs and hepatocytes were scored by an examiner blinded to the groupings. The intensities were graded according to the amount of brown
stain precipitate as follows: 0, none; 1, trace; 2, mild; or 3, moderate.

Flow Cytometric Studies

The extent of expression of Mac-1 on circulating neutrophils was determined by flow cytometry. Blood was lysed by 2 ml NH4Cl for 5 min at room temperature, centrifuged, and resuspended with Dulbecco’s PBS containing 1.0% bovine albumin and 0.1% NaN3. The cells were incubated for 15 min at 4°C (protected from light), first with biotinylated CD11b antibody (PharMingen, San Diego, CA) and next with streptavidin-FITC (PharMingen). The cells were washed with PBS after each incubation. Irrelevant isotype-matched primary antibodies (PharMingen) served as controls. The cells were analyzed in a Becton Dickinson FACScan flow cytometer (Mountain View, CA). Neutrophils were gated by the parameters of forward and side scatter.

In Situ Assay for DNA Fragmentation

Hepatocyte apoptosis was identified by detecting DNA fragmentation in situ in the serial sections adjacent to those stained with hematoxylin and eosin. DNA fragmentation was detected by performing a terminal deoxynucleotidyltransferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay, as described previously, with minor modifications (10, 25). The number of stained and unstained cells was counted in 15 HPF (%400) randomly chosen in periporal, midzonal, and pericentral fields. Approximately 1,500 hepatocytes were examined for each field, and the apoptotic index (AI) was defined as the number of stained cells per 100 hepatocytes (in %).

Caspase 3-Like Activity Assay

Liver tissue was homogenized in lysis buffer (MBL, Nagoya, Japan). The homogenates were centrifuged at 12,000 g for 10 min at 4°C and supernatants collected. Protein concentration was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The same amounts of protein from liver homogenates were dissolved in lysis buffer and stored at −70°C for subsequent analysis. Caspase 3-like activity was measured using a commercially available caspase 3 colorimetric protease assay kit (MBL), according to the manufacturer’s instructions. Caspase activity was determined by measuring the absorbance of paranitroaniline at 405 nm. Data are expressed as absolute fluorescence units per milligram of protein per hour, normalized against the data obtained from the sham-operated group.

Statistics

The results are expressed as means ± SE. ANOVA was performed to test the effects of group (30 or 60 min of ischemia) and time (3, 6, 12, or 24 h after reperfusion) and their interaction on the outcome variables. Least-square means were used to estimate the group-by-time-specific means and their linear contrasts. We used the χ² test to analyze the difference between survival rates in rats after 30 and 60 min of I/R. P < 0.05 was considered statistically significant.

RESULTS

Survival Rate

The survival rate on day 7 was 91% (10 of 11) in rats subjected to 30 min of I/R compared with 46% (5 of 11) in those subjected to 60 min of I/R (P < 0.05). The clinical status of rats that did not survive began to deteriorate within 6–12 h of reperfusion, and they all died by 48 h after reperfusion.

Histological Assessment

There was no significant necrosis in the sham-operated control livers. The rats subjected to 30 min of I/R demonstrated a mild hepatocellular necrosis in the midzonal region, whereas animals subjected to 60-min I/R showed marked liver necrosis in the midzonal and pericentral regions (Fig. 1). In rats subjected to 60 min of I/R, the necrotic area increased in a linear manner during the observation period. The necrotic area became evident after 12 h (P < 0.05 compared with controls), and at 24 h, it was six times bigger than the area observed in rats subjected to 30 min of I/R (P < 0.05; Fig. 2). The number of neutrophils observed in the liver did not change significantly after 30 min of I/R, although it appeared to reach a small peak at 6 h (29 ± 15 neutrophils/50 HPF; Fig. 3). After 60 min of
ischemia, the number of neutrophils increased linearly after reperfusion and reached a peak at 12 h (116 ± 42 neutrophils/50 HPF; Fig. 3). In the 30-min I/R group, liver necrosis and neutrophil infiltration were observed specifically in the midzonal region. In the 60 min I/R group, these features were distributed in both the midzonal and pericentral regions. The number of SECs decreased in both groups after I/R. In the 60-min I/R group, the number of SECs decreased linearly with time; the decrease was more marked at all time points than in the 30-min I/R group (Fig. 4).

**Time Course of ICAM-1 and Mac-1 Expression After Hepatic I/R**

ICAM-1 expression on SECs and hepatocytes was determined by immunohistochemistry using the monoclonal antibody 1A29. In control livers, the SECs stained weakly, but no staining was observed in both the hepatocytes (Fig. 5A). In the 30-min I/R group, ICAM-1 expression on SECs was upregulated significantly throughout the observation period (Figs. 5, B and C, and 6A). In the 60-min I/R group at 3 h after I/R, ICAM-1 expression had increased to a level similar to that seen in the 30-min group, but it decreased there- and remained significantly lower (Figs. 5, D and E, and 6A). ICAM-1 expression on hepatocytes after 60 min of I/R was upregulated throughout the observation period, although the difference between 30 and 60 min of I/R reached statistical significance only 6 and 24 h after I/R (Figs. 5, D and E, and 6B).

Flow cytometric analysis revealed that I/R induced a mild but linearly increasing upregulation of Mac-1 (CD11b/CD18) on the surface of circulating neutrophils in both groups of rats (Fig. 7). In animals subjected to 30 min of I/R, Mac-1 expression peaked at 6 h (141%) and remained almost stable until 24 h. In rats subjected to 60 min of ischemia, Mac-1 expression was 113% at 3 h. It then rose to 160% by 6 h and peaked at 183% 24 h after reperfusion. Mac-1 expression was significantly different between the two groups 24 h after reperfusion. Sham-operated rats showed only a slight increase of Mac-1 expression throughout the observation period.

**In Situ Assay for DNA Fragmentation**

There was a zonal difference in the chronological profiles of AI; AI was therefore expressed separately for the periportal, midzonal, and pericentral regions (Figs. 8 and 9). In the periportal region, there was a mild, albeit nonsignificant, peak in AI in both groups of rats at 3 h. In the 30-min I/R group, AI decreased to the control value thereafter; in the 60-min I/R group, it increased again from 12 h until the end of the observation period (Fig. 9A). In the midzonal region, the AI showed a similar chronological profile in the 30-min I/R group to that observed in the periportal region; in the 60-min I/R group, the AI increased linearly in this region throughout the observation period (Fig. 9B). In the pericentral region, the AI in the 30-min I/R group showed no significant increase; in the 60-min I/R group (Fig. 9C), it showed a profile similar to that observed in the midzonal region, i.e., a linear increase.
Attenuation of Neutrophil Infiltration and Hepatic Necrosis by ZVAD-fmk

In group A, caspase 3-like activity increased by 150% 3 h after hepatic I/R compared with the sham-operated group (Fig. 10). In contrast, caspase 3-like activity was markedly inhibited by ZVAD-fmk in group B, although the difference was not statistically significant due to the small number of animals. Likewise, administration of ZVAD-fmk significantly inhibited induction of hepatocyte apoptosis, as shown by the decreased number of TUNEL-positive cells in all zones 6 and 24 h after hepatic I/R ($P = 0.002$ and $P = 0.032$, respectively; Fig. 11). ZVAD-fmk treatment significantly reduced the number of neutrophils infiltrating into the liver 6 h after hepatic I/R ($P < 0.03$; Fig. 12A) and also attenuated the degree of hepatocellular necrosis, which was significantly lower in group B rats compared with group A animals 24 h after hepatic I/R ($P = 0.0001$; Fig. 12B).

DISCUSSION

The objective of this study was to clarify the involvement of Mac-1 and its receptor ligand ICAM-1 in liver cell impairment resulting from hepatic normothermic I/R injury. We investigated the chronological expression profiles of these adhesion molecules simultaneously in two different I/R models: 30 min of ischemia, which elicits a reversible impairment, and 60 min of ischemia, which results in fatal injury. We then studied the temporal relationship between the expression of adhesion molecules and various parameters of liver injury, including SEC damage and hepatocyte apoptosis. Finally, we verified whether an inhibition of apoptosis reduces the neutrophil infiltration and subse-
quent liver injury after hepatic I/R by using caspase inhibitor in vivo.

There was a marked histological difference in liver damage, as assessed by the area of necrosis, between rats subjected to 30 and 60 min of I/R (Fig. 2). This difference closely paralleled the survival results. The number of neutrophils infiltrated into the liver showed a chronological profile and intergroup, i.e., 30 vs. 60 min I/R, difference similar to those observed in the liver injury, except that the peak of neutrophil infiltration preceded the development of liver injury by 6 to 12 h in both groups of I/R (Fig. 3). These findings support the notion that neutrophils contribute to hepatic I/R injury (19).

Immunohistology revealed that ICAM-1 expression on SECs was enhanced from the baseline value in both I/R groups (Figs. 5 and 6A). Paradoxically, however, the magnitude of its upregulation was inversely correlated with the degree of neutrophil infiltration and liver injury when the two groups were compared. We believe that this apparent discrepancy can be attributed to the SEC damage that occurs during I/R. As reflected in the reduction in SEC numbers in our study (Fig. 4), these cells are considered to be selectively injured in I/R (8, 13, 29). This SEC damage is most likely independent of the upregulation of adhesion molecules, because changes to SECs were observed as early as 5 min after reperfusion, when the levels of expression of the adhesion molecules had not yet risen (1, 15). We believe that a large number of SECs died or lost the ability to express ICAM-1 after 60 min of I/R. In contrast, the marked difference in ICAM-1 expression on hepatocytes between the two I/R groups appeared to correlate well with the degree of neutrophil infiltration and liver injury (Figs. 5 and 6B). In various models of neutrophil-induced liver parenchymal cell injury, ICAM-1 has been reported to play a pivotal role in the transmigration of neutrophils through the endothelial layer and in adhering them firmly to the hepatocytes. Taken together, these results suggest that, in a model of liver I/R injury in which the endothelial lining is impaired in its early phase, the interaction of ICAM-1 and β2 integrins (lymphocyte function-associated antigen-1 and Mac-1) is not an essential step for neutrophils to pass through the endothelial layer. Rather, the role of ICAM-1 in liver I/R injury is thought to lie in the adherence of neutrophils to hepatocytes. In this context, the attenuation of hepatic I/R injury by the administration of anti-ICAM-1 antibody reported previously (7, 12, 18) was most likely caused by the
blockage of binding of neutrophils to hepatocytes, thus reducing the adherence-dependent cytotoxicity of the neutrophils.

Both 30 and 60 min of I/R led to increasing upregulation of Mac-1 on circulating neutrophils during the reperfusion period (Fig. 7). However, the magnitude of this upregulation and the intergroup differences were mild compared with the upregulation of ICAM-1 expression on hepatocytes and the degree of liver injury. This finding is in line with that of Jaeschke et al. (18), who reported moderate upregulation of Mac-1 on circulating neutrophils up to 8 h after partial ischemia of the rat liver for 45 min. Interestingly, this moderate upregulation of Mac-1 presents a clear contrast with the rapid and sustained 300% increase after endotoxin injection also reported by Jaeschke and co-workers (31). In different experimental models of inflammatory liver injury, Mac-1 has been reported (17, 18) to be responsible for transendothelial migration through interactions with its ligands and for adherence-dependent cytotoxicity by the activation of neutrophils to generate inflammatory mediators such as reactive oxygen and elastase. Our results indicate that the role played by Mac-1 in liver I/R injury is mainly the latter. In a situation where the endothelial layer is severely impaired, neutrophils may not need a marked upregulation of Mac-1 to get direct access to hepatocytes. In other words, moderate upregulation of Mac-1, as shown in our study and previous ones, may be enough for the neutrophils to generate cytotoxic mediators once they are in close apposition or adherent to hepatocytes. The protective effect of anti-Mac-1 antibodies reported in earlier studies (12, 18) may lie in their functional inactivation of neutrophils in the liver.

Severe inflammatory reaction with subsequent necrosis, as shown histologically and by elevated serum aminotransferases, is a well known phenomenon associated with liver I/R injury, and recent studies (4, 20, 27) have focused on the role of apoptosis in its development. In our study, in situ staining for apoptotic cells by the TUNEL method revealed a number of characteristics of hepatocyte apoptosis after I/R: a clear contrast between the two I/R groups (60 > 30 min); a zonal distribution (mostly in the midzonal region in the 30-min I/R group and in the midzonal and pericentral

Fig. 10. Caspase-3-like activity was investigated in sham-operated rats and group A (control rats) and group B (ZVAD-fmk-treated rats), 3 h after 60 min of I/R. Data are expressed as means ± SE; n = 3 for each group. In group A, caspase-3-like activity increased by 150% compared with that in sham-operated rats, and ZVAD-fmk administration (group B) suppressed caspase 3-like activity almost to control values, although the difference between groups A and B was not statistically significant due to the small number of rats.

Fig. 11. Suppression of hepatocyte apoptosis by caspase inhibitor (ZVAD-fmk) administration. Hepatocellular apoptosis was counted in 45 HPF (×400), 3, 6, and 24 h after 60 min of ischemia in control and ZVAD-fmk-treated groups. The degree of apoptosis was expressed as the AI, calculated as the number of TUNEL-positive cells/100 hepatocytes (in %). Data are expressed as means ± SE; n = 3 at each time point/group. #P < 0.05 for control vs. ZVAD-fmk group.

Fig. 12. Number of neutrophils infiltrating into the liver (A) and % liver necrosis (B) evaluated 3, 6, and 24 h after 60 min of ischemia in group A (control rats) and group B (ZVAD-fmk-treated rats). Data are expressed as means ± SE; n = 3 at each time point/group. #P < 0.05 for control vs. ZVAD-fmk group.
regions in the 60-min I/R group); and a chronological profile (no significant increase in the 30-min I/R group but a linearly increasing pattern in the 60-min I/R group; Figs. 8 and 9). These characteristics showed a temporal and zonal coincidence with those observed in neutrophil infiltration and hepatocellular necrosis (Figs. 2 and 3). These results suggest that there is a link between excessive parenchymal cell apoptosis and the neutrophil-induced inflammatory and necrotic reaction. To verify this hypothesis, the effect of inhibition of apoptosis on attenuation of the later phase of liver I/R injury, characterized by massive necrosis, was investigated using the caspase inhibitor ZVAD-fmk. Rats given ZVAD-fmk showed lower caspase 3-like activity, as well as hepatocyte apoptosis. Furthermore, the extent of neutrophil infiltration and hepatocellular necrosis in these animals was less than that in the control animals. These findings strongly suggest that excessive parenchymal cell apoptosis is a signal for the neutrophil-induced inflammatory and necrotic reaction in rat liver I/R injury. A similar link between parenchymal cell apoptosis and transendothelial migration of neutrophils has been reported (21) in murine models of endotoxin and Fas antibody-induced liver injury.

Interestingly, a small peak of hepatocyte apoptosis was observed in the early reperfusion phase, specifically in the pericentral and midzonal regions, after 30 min of I/R. These cells were thought to be shed in a later phase without eliciting secondary necrosis, because the scale of apoptosis was within the range of the inherent clearance mechanisms of the liver. This transient emergence of apoptosis is most likely caused by a mechanism different from that which induced an upsurge of apoptosis, leading to massive liver necrosis, in the 60-min I/R group. Kupffer cells may be the culprits for this phase of apoptosis, because these cells, which are most abundant in the perportal region, are known to release proapoptotic mediators such as tumor necrosis factor-α and are thought to be involved in I/R injury in its early phase, unlike neutrophils (2, 3, 22). Further study is needed to clarify these concepts.

In conclusion, our data indicate that, in liver I/R injury in which SECs are specifically impaired, interaction between ICAM-1 on SECs and Mac-1 on neutrophils is not an essential step for neutrophil transmigration through the endothelial layer. Rather, ICAM-1 and Mac-1 play a role in adhering neutrophils firmly to hepatocytes and activating them. Excessive parenchymal apoptosis may be a signal for this neutrophil-induced inflammatory and necrotic reaction.

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