Mechanisms and pathophysiological implications of sinusoidal endothelial cell gap formation following treatment with galactosamine/endotoxin in mice

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Ito, Yoshiya, Edward R. Abril, Nancy W. Bethea, Margaret K. McCuskey, Cathleen Cover, Hartmut Jaeschke, and Robert S. McCuskey. Mechanisms and pathophysiological implications of sinusoidal endothelial cell gap formation following treatment with galactosamine/endotoxin in mice. Am J Physiol Gastrointest Liver Physiol 291: G211–G218, 2006. First published March 30, 2006; doi:10.1152/ajpgi.00312.2005.—Neutrophil extravasation from sinusoids is a critical step for acute inflammatory tissue injury. However, the role of sinusoidal endothelial cells (SECs) in this process remains unclear. Matrix metalloproteinases (MMPs) have been shown to involve gap formation in SECs in several liver diseases. Therefore, the present study examined SEC modifications elicited by galactosamine (Gal)/endotoxin (ET). Treatment of male C3Heb/FeJ mice with Gal/ET or Gal/TNF caused the formation of numerous gaps in SECs at 4 h when no neutrophil extravasation occurred. Six hours after Gal/ET or Gal/TNF treatment, blood elements started to penetrate to the extrasinusoidal space through large gaps. Treatment with ET alone caused sinusoidal neutrophil accumulation but no gap formation, neutrophil extravasation, or hemorrhage. Gal/ET treatment increased hepatic MMP-2 and MMP-9 mRNA expression (6.7- and 11-fold, respectively). Pretreatment with 2-(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid, an MMP-2/MMP-9 inhibitor (5 mg/kg), minimized gap formation after Gal/ET and Gal/TNF treatment. The MMP inhibitor reduced injury only in the Gal/ET model mainly due to reduced TNF formation. The MMP inhibitor attenuated sinusoidal neutrophil accumulation at 6 h but failed to attenuate Gal/TNF-induced liver injury at 7 h due to excessive apoptosis. These results suggest that Gal/ET or Gal/TNF activates MMPs, which are responsible for SEC gap formation. Although the initial appearance of gap formation is independent of neutrophils, the gaps allow initial contact of neutrophils with damaged hepatocytes. In addition, MMP activation promotes neutrophil accumulation in sinusoids.

neutrophil; transmigration; matrix metalloproteinase; apoptosis; tumor necrosis factor

POLYMORPHONUCLEAR LEUKOCYTES (neutrophils) participate in the part of innate immune response against invading bacteria and are recruited into organs in response to tissue damage (16). Although the main function of neutrophils is to protect, under certain conditions, neutrophils can cause liver dysfunction and damage or aggravate an existing injury (27). Detrimental effects of hepatic neutrophil recruitment have been shown in models of ischemia-reperfusion (transplantation or Pringle maneuver) (23, 25), alcoholic hepatitis (3), sepsis and endotoxemia (24, 37), remote organ damage (20), and obstructive cholestasis (17, 18). Because of the dual role of neutrophils, the goal is to prevent neutrophil-induced tissue injury without compromising their host defense function. Therefore, it is important to better understand the mechanisms of how neutrophils are activated to cause tissue injury.

Several critical steps have been identified in the mechanism of neutrophil-induced liver injury. After systemic activation by cytokines, chemokines, or complement factors, neutrophils adhere to the endothelial lining of venules and sinusoids (14, 23, 35). If the neutrophils receive a chemotactic signal, they migrate out of the vasculature into the parenchyma and adhere to target cells (5, 38). This triggers the final activation of the neutrophil, resulting in degranulation and generation of reactive oxygen species including hypochlorite (19, 24, 25). Because only a fraction (35–60%) of all accumulated neutrophils actually extravasates, the number of accumulated neutrophils in the liver is of limited importance in the injury (5, 29). However, the extravasation and migration into the parenchyma are prerequisites for neutrophil-mediated injury (5). In contrast to many other organs in which neutrophils transmigrate from postcapillary venules, extravasation from sinusoids appears to be most important for hepatocellular injury (5, 17, 18). In addition, it was thought that the transmigration process depends on an intact sinusoidal endothelium. This was based on the observation that blocking ICAM-1, a critical adhesion molecule in the transmigration process, can prevent neutrophil-mediated injury during endotoxemia (10) and obstructive cholestasis (18). However, it is only modestly effective after ischemia-reperfusion (12, 39), where there is extensive sinusoidal endothelial cell (SEC) damage (13). More recently, it was recognized that SEC injury causes the formation of large gaps in the endothelial cell layer in a variety of experimental models including monocrotaline-induced venoocclusive disease (6) and acetaminophen hepatotoxicity (21, 36). The SEC injury precedes parenchymal cell injury and leads to hepatic microcirculatory dysfunction and hemorrhage (7, 21, 44) in these models. Treatment with inhibitors of matrix metalloproteinases (MMPs), including MMP-2 (gelatinase A) and MMP-9 (gelatinase B) (43), attenuated SEC damage and hemorrhage in the liver (8, 22). However, the development of SEC injury and gap formation elicited during endotoxemia and neutrophil-induced liver injury is unknown. Therefore, the objective of this investigation was to evaluate changes in SECs in a well-established mouse model of galactosamine (Gal)/endotoxin (ET)-induced liver injury and to assess the potential effects of an MMP inhibitor on SEC injury, hepatocellular apoptosis, and neutrophil-mediated liver damage.

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MATERIALS AND METHODS

Animals and experimental protocol. Male C3HeB/FeJ mice (20–30 g body wt) were obtained from Jackson Laboratories (Bar Harbor, ME). The animals were allowed free access to food and water. The present study was performed in adherence to the National Institutes of Health guidelines for the use of experimental animals and followed the protocol approved by the Animal Care and Use Committee of the University of Arizona. Animals received an intraperitoneal injection of 100 μg/kg Salmonella abortus equi ET alone or in combination with 700 mg/kg Gal (Sigma, St. Louis, MO). Some animals were treated with the MMP-2/MMP-9 inhibitor 2-[(4-biphenylsulfonyl)-amino]-3-phenyl-propionic acid (Calbiochem, La Jolla, CA; 5 mg/kg ip) 0.5 h before Gal/ET treatment. The median inhibitory concentrations for MMP-2 and MMP-9 are 0.31 and 0.24 μM, respectively, in in vitro enzymatic assay using synthetic substrates (42). In another experiment, some animals were treated with an intravenous injection of TNF-α (10 μg/kg, Sigma) in combination with Gal (700 mg/kg ip). Blood samples were taken from the inferior vena cava. Sera were used for measurement of TNF-α and alanine transaminase (ALT) levels. Samples of the liver were homogenized immediately for caspase-3 activity measurement, snap-frozen in liquid nitrogen, or fixed in phosphate-buffered formalin. To assess liver cell injury, ALT activities were measured in sera using the test kit 68-B (Biotron Diagnostics, Hernet, CA). TNF-α activity in the serum was measured by quantifying cytolytic activity against the TNF-α-sensitive murine fibroblast WEHI 164 cells as previously described (21). Caspase-3 activities were determined using the synthetic fluorogenic acetyl-Asp-Glu-Val-4-methylcoumaryl-7-amide (Ac-DEVD-MCA) (Peptide Institute, Osaka, Japan) as described in detail (19, 29).

Histology. Formalin-fixed tissue samples were embedded in paraffin, and 5-μm sections were cut. Liver sections were stained with hematoxylin and eosin for evaluation of liver cell injury or with the naphthol AS-D chloroacetate esterase technique for evaluation of neutrophil infiltration in mouse livers as described previously (26). Neutrophil accumulation in the liver was quantified by counting the total number of neutrophils in 20 high-power fields.

In vivo microscopy. Animals were anesthetized with urethane (2 mg/g body wt sc). The hepatic microvascular responses were examined using established high-resolution in vivo microscopic methods (34). Briefly, a compound binocular microscope (Leitz, Wetzlar, Germany) adapted for in vivo microscopy was equipped to provide either transillumination or epi-illumination as well as video microscopy using a charge-coupled device camera (MTI, Michigan City, IN). With the ×80/1.0 numerical aperture water-immersion objective (Leitz ×80/1.00, Wetzlar, Germany) employed for these studies, microvascular events were observed and recorded for at least 30 s for subsequent off-line analysis using a Sony Betacam video tape recorder (Sony Medical Electronics, Park Ridge, NJ). The relative adequacy of blood perfusion through the sinusoids was evaluated by counting the number of perfused sinusoids in 10 periportal and 10 centrilobular regions in a standardized microscopic field (4,125 μm²) in each animal. To examine the interaction of leukocytes with the sinusoidal wall, quantification of leukocytes adhering to the endothelial lining of sinusoids was calculated in the same microscopic fields. A leukocyte was defined as adhering to the sinusoidal wall if it remained at this location for at least 30 s. Transmigrated leukocytes were also counted in the same microscopic field. The results were averaged, and the data were represented as the average number in each animal. To quantify the extent of hemorrhage, the area occupied by extrasinusoidal red blood cells (RBCs) was measured in the same microscopic fields using a computer-assisted digital-imaging processor (Scion Image; Scion, Frederick, MD). The results were expressed as extrasinusoidal area occupied by RBCs (μm²/10 regions).

Electron microscopy. In a separate set of experimental animals (n = 3 in each experimental group), routine methods were used to prepare liver specimens for transmission (TEM) and scanning electron microscopy (SEM) (34). Livers were fixed by perfusion of portal veins with 0.1 M cacodylate buffer to wash out blood and, subsequently, to obtain fixation with 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. For TEM, small pieces of liver were washed in buffer, postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer at 4°C, dehydrated through a graded series of ethanol solutions, briefly rinsed in propylene oxide, and embedded in epoxy resin. Thin sections were cut on a Reichert Ultracut microtome (Leica, Deerfield, IL) and examined and photographed using a Philips CM-12S TEM (FEI, Mahwah, NJ). For SEM, pieces of perfused-fixed livers were dehydrated in a graded ethanol series, critical point-dried, fractured, sputter-coated with 10 nm gold, and examined using a FEI XL35 SEM. For each experimental group, three samples were studied by TEM and by SEM. In each of these samples, at least five fields selected at random were examined, and representative examples of the consistent morphological alterations in sinusoidal morphology were recorded.

MMPs activity assay. The liver was perfused with PBS and was removed from the animal. Then, the left lobe of the liver was sectioned into 0.5-cm blocks, snap-frozen in liquid nitrogen, and stored at −70°C until assays were performed. The levels of MMP-2 and MMP-9 were measured by a commercially available ELISA kit (Amersham Biosciences, Piscataway, NJ). Briefly, 25 mg of the liver tissue were homogenized in 50 mM Tris·HCl containing 1 mM monothioglycerol. The homogenized samples were centrifuged at 13,000 g for 10 min. According to the manufacturer’s instructions, 100 μl of either standard or experimental sample(s) were added to the designated wells and incubated overnight at 4°C. After incubation, the plate was washed four times and blotted dry. Aminophenylmercuric acetate was added to all wells, and the plate was incubated at 37°C. Then, the plate was read at a wavelength of 405 nm using an ELISA plate reader. Total MMP-2 and MMP-9 activity was calculated using the standard curve for this assay. The results were expressed as nanograms per gram of liver tissue.

Quantitative PCR analysis of MMP-2 and MMP-9. The liver was perfused with PBS and was removed from the animal. Part of the left lobe of the liver was cut into 0.1-cm blocks and placed into a cryovial containing 1.5 ml of RNA Later (Qiagen Valencia, CA), equilibrated at 4°C overnight, and then stored at −70°C until assays were performed. Total RNA was extracted from the tissue using a commercially available kit (Promega, Madison, WI). Total RNA was quantitated using a spectrophotometer 260/280 program and stored at −70°C. From the isolate RNA (50 ng/μl), a cDNA template was made using a reverse transcription reaction kit (Applied Biosciences, Foster City, CA) using the following PCR conditions: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. After amplification was complete, the cDNA was stored a 4°C until used. Quantitative PCR was performed using a Taqman gene expression assay for murine MMP-2, MMP-9 (Applied Biosystems), and β-actin (house keeping gene). Briefly, cDNA was added to Taqman master mix containing PCR water along with the experimental and control primers and probes (Applied Biosystems). The completed master mix (25 μl) was mixed and plated into a 0.50-ml microfuge tube. The tubes were placed into the holder of the Rotogene PCR thermocycler and run for 60 min using the following PCR conditions: 95°C for 2 min, 95°C for 20 s, and cycling for 40 cycles at 60°C for 1 min each cycle. On completion, the data were quantitated using the Rotogene software and are reported in comparative Ct concentration.

Statistical analysis. All data were expressed as means ± SE. Multiple comparisons were performed using one-way ANOVA with a post hoc Fisher test. Differences were considered to be significant for P values of <0.05.

RESULTS

Hepatic microvascular responses to Gal/ET. Treatment with Gal/ET caused significant increases in leukocyte adhesion to
the sinusoids in periportal and centrilobular regions (9.2- and 6.0-fold, respectively) after 6 h when compared with untreated controls (Fig. 1A). ET alone also increased leukocyte adhesion (7.9- and 7.4-fold, respectively). However, there was no significant difference in the number of adherent leukocytes between the two treatments. At that time, <10% of total number of hepatic neutrophils in Gal/ET-treated animals transmigrated, whereas no leukocyte transmigration was seen in ET-treated animals (Fig. 1B). In addition, no extravasated leukocytes were observed in Gal/ET-treated animals until up to 6 h after Gal/ET (data not shown). These results were consistent with our previous results obtained by histological methods (5). ET treatment caused a significant decrease in sinusoidal perfusion in periportal and centrilobular regions (13.4% and 10.6%, respectively) when compared with controls (Fig. 1C). The number of perfused sinusoids in Gal/ET-treated animals was further reduced when compared with ET-treated animals. At 7 h after Gal/ET, the sinusoidal perfusion in periportal and centrilobular regions was markedly decreased (54.8% and 55.9%, respectively) when compared with controls. The areas occupied by RBCs in the extrasinusoidal space were evident in Gal/ET-treated animals, whereas no infiltration of RBCs was shown in ET-treated animals (Fig. 1D). The infiltration of RBCs was not observed until up to 6 h after Gal/ET (data not shown). The hemorrhagic regions in Gal/ET-treated liver were further increased (~6.5-fold) at 7 h when compared with those at 6 h. Administration of Gal alone did not cause hepatic microcirculatory dysfunction, leukocyte infiltration, or hemorrhage (data not shown).

**SEM and TEM studies.** Because the infiltration of RBCs in the extrasinusoidal space indicated damage of SECs, the endothelium of the sinusoids was examined by means of electron microscopy. Minimal changes in SECs were shown 2 h after Gal/ET treatment, and the endothelium of the sinusoids exhibited fenestrae clustered in sieve plates in their cytoplasm (Fig. 2A). After treatment with Gal/ET or ET alone, neutrophils start to accumulate in the liver. The majority of neutrophils sequesters in the sinusoids between 2 and 3 h after the treatment (5). At 4 h after Gal/ET, however, SECs lost fenestrae and numerous gaps in SECs developed (Fig. 2B). The microvilli of the hepatocytes could be seen through these gaps. At this time point, most neutrophils had accumulated in the sinusoids but not in the extrasinusoidal space (5). At 7 h after Gal/ET, the gaps formed through SECs permitted the passage and accumulation of erythrocytes in the extrasinusoidal space (Fig. 2C). The Space of Disse was enlarged, and SECs were detached from the sinusoidal linings. ET treatment alone did not cause significant injury in SECs (Fig. 2D). Gal treatment failed to elicit gaps in SECs (data not shown). TEM also demonstrated intact SECs lining the sinusoids 2 h after Gal/ET treatment (Fig. 3A). Gaps were formed in the SEC lining at 4 h after Gal/ET, but the SECs exhibited no evidence of necrosis or apoptosis (Fig. 3B). At 5 h after Gal/ET, a larger loss of sinusoidal endothelium was seen, and SECs were detached.

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**Fig. 1.** Changes in liver microcirculation 6 h after treatment with endotoxin (ET) alone or in combination with galactosamine (Gal). The numbers of adherent leukocytes (A), transmigrated leukocytes (B), and perfused sinusoids (C) and the extrasinusoidal area occupied by red blood cells (RBCs; D) were measured in 10 periportal and 10 centrilobular regions in each animal. The extrasinusoidal area, which was occupied by RBCs, was quantified using Scion Image software. Data are the means ± SE from 5 mice per group. *P < 0.05. NS, not significant.
from the sinusoidal lining (Fig. 3C). There were few microvilli extending from the hepatocytes within the enlarged Space of Disse. The hepatocytes in the vicinity of the sinusoids exhibited vacuoles, which directly connected with the lumen of the sinusoids. The vacuoles appeared to contribute to the detachment of hepatocytes from the sinusoidal lining. At 7 h after Gal/ET, RBCs penetrated into the enlarged Space of Disse through the gaps in SECs (Fig. 3D). Erythrocytes accumulated in the Space of Disse as well as in the parenchymal tissue. Platelets also infiltrated in the sinusoids and in the extrasinusoidal space.

Effect of MMP inhibition on SEC after Gal/ET treatment. Because MMPs have been shown to be involved in gap formation of SECs elicited by hepatotoxicants including monocrotaline (8) and acetaminophen (22), we measured MMP-2 and MMP-9 in the liver after Gal/ET treatment. Total activity of MMP-2 in the liver was increased (2.3-fold) 7 h after Gal/ET treatment, but MMP-9 did not change significantly.

Fig. 2. Scanning electron micrographs of the sinusoids of the liver treated with Gal/ET. A: 2 h after Gal/ET, sinusoidal endothelial cells (SECs) exhibit fenestration organized in sieve plates. B: 4 h after Gal/ET, SECs lose fenestrae and develop gaps in their cytoplasm. Note the hepatocyte microvilli visible through gap in SECs. C: 7 h after Gal/ET, numerous RBCs accumulate in the extrasinusoidal space. D: 5 h after ET alone, SECs maintain fenestration in sieve plates. Bars indicate 2 μm.

Fig. 3. Transmission electron micrographs of the sinusoids (S) of the liver treated with Gal/ET. A: 2 h after Gal/ET, no injury to hepatic sinusoidal endothelium and maintained integrity of the sinusoidal lining. B: 4 h after Gal/ET, partial destruction of the endothelium (arrowheads). C: 5 h after Gal/ET, loss of endothelium and dehiscence of SEC from the sinusoidal lining. Note the loss of microvilli on parenchymal cells. Vacuoles can be seen to communicate with sinusoidal lumen (arrows). D: 7 h after Gal/ET, RBCs penetrate into the space of Disse through gaps (arrow) in SECs. Platelets also accumulate in the Space of Disse (arrowheads). Original magnifications: ×17,500 (A), ×13,000 (B), ×10,500 (C), ×13,000 (D).
We tried to determine the active forms of MMPs; however, those levels were below the detection levels at any time point (data not shown). The levels of MMP-2 mRNA expression in the liver were increased 3 (3-fold), 6 (6-fold), and 7 h (6.7-fold) after Gal/ET when compared with those in untreated liver (time = 0; Table 1). Hepatic MMP-9 expression also increased at 3 (3-fold), 6 (3-fold), and 7 h (11-fold). To determine whether inhibition of MMPs attenuates SEC injury in response to Gal/ET, animals were treated with the MMP-2/-9 inhibitor 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid. Pretreatment with 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid suppressed the formation of gaps in the cytoplasm of SECs 4 and 6 h after Gal/ET treatment (Figs. 4, A and B). Concomitantly, this compound reduced ALT levels by 55.7% 7 h after Gal/ET (Table 2). Because endotoxemia is a TNF-dependent model of liver injury, we measured peak plasma TNF levels 1.5 h after Gal/ET treatment. 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid inhibited TNF production by 33.3% (Table 2). This suggests that its inhibitory effect on liver injury elicited by Gal/ET could be, at least in part, attributed to the inhibition of TNF production. To test this hypothesis, 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid was administered to animals treated with Gal/TNF.

### Table 1. Changes in MMP-2 and MMP-9 after Gal/ET treatment

<table>
<thead>
<tr>
<th>Time (h) After Gal/ET Treatment</th>
<th>Total MMP-2 activity, ng/g tissue</th>
<th>Total MMP-9 activity, ng/g tissue</th>
<th>MMP-2 mRNA (comparative Ct concentration)</th>
<th>MMP-9 mRNA (comparative Ct concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>96 ± 16</td>
<td>21 ± 3</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>111 ± 18</td>
<td>20 ± 5</td>
<td>0.9 ± 0.1*</td>
<td>0.6 ± 0.1*</td>
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<tr>
<td>6</td>
<td>153 ± 18</td>
<td>25 ± 4</td>
<td>1.8 ± 0.2*</td>
<td>0.6 ± 0.2*</td>
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<tr>
<td>7</td>
<td>220 ± 30*</td>
<td>28 ± 6</td>
<td>2.0 ± 0.0*</td>
<td>2.2 ± 0.1*</td>
</tr>
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</table>

Values are means ± SE from 3–4 mice per time point. Expression of mRNAs for matrix metalloproteinases (MMPs) and MMP activities was measured by quantitative PCR and ELISA. *P < 0.05 vs. controls (time 0). Ct, threshold cycle; Gal, galactosidase; ET, endothelin.

**Effect of MMP inhibition on liver injury after Gal/TNF.** Treatment with Gal/TNF caused the formation of numerous gaps in the cytoplasm of SECs at 4 h (Fig. 4C), which is consistent with the results elicited by Gal/ET. Pretreatment with 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid prevented Gal/TNF-induced gap formation (Fig. 4D). Table 3 summarizes the effect of MMP inhibitor on ALT, neutrophils, and caspase-3 activity after Gal/TNF treatment. At this time point (4 h after), no significant differences in ALT levels, the number of neutrophils, and hepatic caspase-3 activity were observed between the Gal/TNF-treated groups with or without 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid pretreatment (Table 3). At 6 h after Gal/TNF treatment, ALT levels were moderately increased when compared with controls. Pretreatment with 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid did not change ALT levels (Table 3). Because TNF-induced parenchymal cell apoptosis is a critical component of the injury in this model, hepatic caspase-3 activities were determined. Gal/TNF caused a significant increase (20.3-fold) in caspase-3 activity, and the MMP inhibitor did not affect the levels of caspase-3 (Table 3). Concomitantly, Gal/TNF significantly increased to 48.3-fold the total number of neutrophils in the liver, and neutrophils were sequestered in the sinusoids (Table 3). Pretreatment with 2-[(4-biphenylsul-
fornyl) amino]-3-phenyl-propionic acid reduced by 60% the number of neutrophils accumulated in sinusoids after Gal/TNF. After Gal/TNF treatment for 7 h, ALT levels were extensively elevated, and the total number of neutrophils increased to 31.5-fold. The MMP inhibitor failed to significantly reduce ALT levels or the number of accumulating neutrophils.

**DISCUSSION**

The objectives of the present study were to examine the changes in SECs elicited during endotoxemia and to evaluate the involvement of MMPs in this effect. Using in vivo microscopic and electron microscopic studies, we showed that SECs exhibited numerous gaps in their cytoplasm before the occurrence of neutrophil transmigration and of parenchymal cell damage. These results suggest that SECs are an early target for Gal/ET-induced injury. Inhibition of MMP-2 and MMP-9 reduced the gap formation in SECs during endotoxemia, suggesting that MMPs play a role in this process.

**SEC gap formation and neutrophil cytotoxicity.** The sequence of events, which leads to severe liver injury in the Gal/ET model, starts with the early formation of TNF-α between 0.5 and 3 h. TNF-α is directly responsible for neutrophil activation and recruitment into the liver (10, 40) and the transcriptional activation of CXC chemokines (9) and adhesion molecules (28). After treatment with Gal/ET, neutrophils start to accumulate in the liver. The majority of neutrophils seques ters in the sinusoids between 2 and 3 h after (5). Around 5–6 h after Gal/ET treatment, many parenchymal cells are undergoing apoptosis (33), which is the signal for neutrophils to extravasate into the tissue and aggravate the injury (29, 31). Neutrophils, which accumulate in sinusoids, are generally primed for reactive oxygen formation but not fully activated (2, 25). Only extravasated neutrophils generate hypochlorous acid and cause cell injury (17, 18). Because massive hemorrhage correlated with neutrophil transmigration and cytotoxicity, we hypothesized that damage to the endothelial cell lining of the sinusoid is secondary to the neutrophil transmigration process or represents collateral damage of the attack on dying hepatocytes (29, 30). However, the current data suggest a completely different interpretation. First, large gaps in the sinusoidal lining develop well before the onset of neutrophil cytotoxicity. Second, the SECs do not show morphological evidence of cell injury, i.e., there is no evidence of apoptosis or necrosis, which is consistent with a previous report after Gal/TNF (41). These findings also agree with earlier data, where no evidence of caspase activity or DNA fragmentation was found in nonparen chymal cells at 6 h after Gal/ET treatment (29). Thus SEC gap formation appears to be due to an alteration in the cytoskeleton, which affects fenestration (4) rather than cell injury. When massive hemorrhage through the gaps has taken place at 7 h after Gal/ET treatment, the architecture of the sinusoids is destroyed. Neutrophils are present in sinusoids at the time of gap formation, i.e., most neutrophil accumulation precedes gap formation. However, the fact that this does not happen after treatment with ET alone, where similar numbers of neutrophils are present in sinusoids (5), suggests that primed neutrophils are not responsible for this effect on SEC. In addition, there is no evidence for a neutrophil-induced oxidant stress at the time of gap formation (19). Thus SEC gap formation after Gal/ET administration is unlikely caused by these inflammatory cells. Moreover, the absence of SEC gaps after ET alone suggests that gap formation does not affect neutrophil recruitment into the liver.

On the other hand, gap formation in SEC may facilitate the recognition of dying cells by neutrophils. Neutrophils located in sinusoids have access to the cell surface of hepatocytes through these gaps. Similar to phagocytosis of apoptotic bodies by macrophages, which is triggered by the recognition of the modified cell surface (phosphatidyl serine exposure) (11), neutrophils may recognize apoptotic hepatocytes and then extravasate. This hypothesis is also supported by our recent observation that despite the massive formation of CXC chemokines in this model, these chemotactic factors are not involved in neutrophil extravasation (9). In contrast, the direct contact of the neutrophils with apoptotic hepatocytes may be the stimulus for its extravasation. Because in healthy liver tissue there is very little room in the Space of Disse for the neutrophil to migrate, SEC gap formation and vacuolation in hepatocytes may provide the initial space needed for extrava-

| Table 2. Effects of MMPs inhibition on plasma levels of ALT and TNF after Gal/ET treatment |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | Gal/ET          | Gal/ET + MMPI   |                                |                                |                                |
| ALT, IU/l                       | 2.794±357       | 1.238±105*     |                                |                                |                                |
| TNF, ng/ml                      | 39±4            | 26±1*          |                                |                                |                                |

Values are means ± SE from 4–6 mice per group. Plasma levels of alanine aminotransferase (ALT) and TNF were measured at 7 and 1.5 h after Gal/ET treatment. *P < 0.05 vs. Gal/ET-treated animals. MMP1, MMP-2 and -9 inhibitor, 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid.

| Table 3. Effects of MMP-2/MMP-9 inhibitor on ALT, hepatic neutrophil accumulation, and caspase-3 activity after Gal/TNF treatment |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | Time (h) After Gal/TNF Treatment |
|                                | 0               | 4               | 6               | 7               |
|                                | MMP(−)          | MMP(+)          | MMP(−)          | MMP(+)          |
|                                |                 |                 |                 |                 |
| ALT (U/l)                      | 29±7            | 94±16           | 57±17           | 351±104         | 107±53          |
|                                |                 |                 |                 | 5,563±1944*     | 6,659±1248*     |
| Neutrophils, n/20 (HPF)        | 3±1             | 112±17*         | 71±10*          | 145±6*          | 58±11           |
|                                |                 |                 |                 | 207±24*         | 171±14*         |
| Liver caspase-3, ΔF=min⁻¹·mg⁻¹| 31±12           | 93±26           | 79±24           | 630±83*         | 595±55*         |
|                                |                 |                 |                 | ND              | ND              |

Data are the means ± SE from 4–9 animals per group. Mice were pretreated with 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid, an MMP-2/MMP-9 inhibitor (MMPI) 30 min before Gal/TNF treatment. *P < 0.05 vs. time 0, †P < 0.05, NS; not significant, ND, not determined.
sating neutrophils. Thus these early changes in SEC and hepatocytes appear to initiate and facilitate the extravasation of neutrophils. Interestingly, in other models where extravasated neutrophils are present, e.g., hepatic ischemia-reperfusion injury (23) or acetaminophen hepatotoxicity (32), there is either severe SEC damage (13) or SEC gap formation (21, 36). Further studies are needed to clarify the role of SEC gap formation in the process of neutrophil extravasation in acute inflammation.

Mechanisms of SEC gap formation. The central role of neutrophil extravasation for a potential additional injury by these leukocytes (5), together with the emerging concept that SEC gaps may be a prerequisite for neutrophil extravasation, suggest that the mechanism of gap formation could be an important target to control neutrophil-induced tissue injury. Previous data indicate that SEC dehiscence from the Space of Disse in a model of sinusoidal obstruction syndrome correlates with activation of MMPs and is prevented by an MMP inhibitor (8). In addition, the same MMP-2/-9 inhibitor attenuated microvascular injury and hemorrhage during acetaminophen hepatotoxicity (22). Therefore, we tested this inhibitor and observed an almost complete prevention of SEC gap formation. In the Gal/ET model, we also observed a reduction in endotoxin-induced TNF formation. This finding is consistent with earlier reports showing that the TNF-converting enzyme, which cleaves membrane-bound TNF, is a metalloproteinase (15). However, because the MMP inhibitor also prevented SEC gap formation by Gal/TNF, the inhibition of SEC gap formation was not due to the inhibition of TNF liberation. On the other hand, the significant hepatoprotective effect of the MMP inhibitor in the Gal/ET but not in the Gal/TNF model suggests that an attenuated TNF release may have been a significant factor in this protection. This is consistent with previous observations that TNF is a central mediator for apoptosis (33) and hepatic neutrophil recruitment (10) in this model. Because parenchymal cell apoptosis then triggers neutrophil cytotoxicity (29), any modulation of TNF formation can have substantial effects on the overall liver injury. In addition, the present study showed that the magnitude of liver injury was somewhat greater in animals treated with Gal/TNF when compared with Gal/ET. Because the peak TNF levels in Gal/TNF-treated animals appear to be higher than those in Gal/ET-treated animals and because its time point seems to be earlier, it is likely that the liver injury was more extensive in Gal/TNF-treated animals. Furthermore, the extensive release of ALT is observed at later time points if there is an ongoing process of hepatocellular apoptosis or secondary necrosis (19).

The reduced SEC gap formation by an MMP inhibitor indicates that MMPs are activated and released after Gal/ET treatment. This conclusion is confirmed by the increased mRNA levels of MMP-2 and MMP-9 in the liver 3–7 h after Gal/ET treatment and enzyme activity of MMP-2 in the liver 7 h after. The reasons for discrepancy between mRNA levels and enzyme activity for MMPs after Gal/ET remain unclear; however, others reported (45) even earlier activation of MMPs (∼2 h after Gal/TNF treatment) when measured in plasma. In addition, we observed mainly activation of MMP-2, but Wielockx et al. (45) reported activation of MMP-2 and MMP-9. The differences could be due to the high doses of TNF used and the measurement of MMPs in plasma as a more sensitive indicator of MMP activity compared with liver tissue activities. Nevertheless, our tissue measurements confirmed that MMPs are activated in the liver after Gal/ET treatment.

The MMP inhibitor had no effect on hepatocellular apoptosis after Gal/TNF treatment as indicated by the high caspase-3 activities in the liver at 6 h. Caspase activation is critical for TNF-mediated apoptosis in this model and correlates with the number of dUTP nick-end labeling-positive cells, DNA fragmentation, and the number of cells with apoptotic morphology (19, 29). These findings are in contrast to a previous report where a broad-spectrum MMP inhibitor (BB-94) substantially reduced apoptosis after Gal/TNF (45). In addition, gene knockout mice of different MMPs showed reduced apoptosis after Gal/TNF (45). Because similar experimental models were used in both studies, it appears that the MMP-2/-9 inhibitor used in our studies may not inhibit MMPs critical for apoptosis, despite the fact that it is fully effective in reducing SEC gap formation. Interestingly, our MMP-2/-9 inhibitor attenuated hepatic neutrophil accumulation similar to the previously used BB-94 (45). This would suggest that MMP activation supports hepatic neutrophil accumulation. Because neutrophil accumulation in sinusoids can be directly triggered by TNF-α (1, 10) and is independent of adhesion molecules (26), it was postulated that the initial neutrophil accumulation occurs due to mechanical trapping (28). The fact that MMP inhibitors attenuate TNF-mediated neutrophil accumulation in sinusoids at 6 h suggests that MMP activation may modify either the sinusoidal lining and/or the neutrophil in such a way that it promotes neutrophil trapping in these capillaries. More investigations are necessary to investigate this mechanism in more detail. However, the current data demonstrate that the MMP effect on hepatic neutrophil accumulation is independent of SEC gap formation.

In summary, our data showed that SEC gap formation is a phenomenon that occurs after Gal/ET or Gal/TNF treatment but not after ET alone. Hepatic neutrophil accumulation preceded the appearance of gap formation, suggesting that primed neutrophils are not responsible for the initial appearance of gap formation, although the large gaps in SEC allow initial contact of neutrophils with damaged hepatocytes. SEC gap formation is mediated by the activation of MMPs. In addition, MMP activation promotes neutrophil accumulation in sinusoids independent of SEC gap formation. Thus activated MMPs may be an important therapeutic target to control neutrophil-mediated liver injury during acute inflammation.

GRANTS

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REFERENCES

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