Monocyte chemoattractant protein-1 influences trauma-hemorrhage-induced distal organ damage via regulation of keratinocyte-derived chemokine production

Michael Frink,1 Ailing Lu,1 Bjoern M. Thobe,1 Ya-Ching Hsieh,1 Mashkoor A. Choudhry,1 Martin G. Schwacha,1 Steven L. Kunkel,2 and Irshad H. Chaudry1
1Center for Surgical Research, University of Alabama at Birmingham, Birmingham, Alabama; and 2Department of Pathology, University of Michigan Medical Center, Ann Arbor, Michigan
Submitted 14 September 2006; accepted in final form 30 October 2006

Frink M, Lu A, Thobe BM, Hsieh Y-C, Choudhry MA, Schwacha MG, Kunkel SL, Chaudry IH. Monocyte chemoattractant protein-1 influences trauma-hemorrhage-induced distal organ damage via regulation of keratinocyte-derived chemokine production. Am J Physiol Regul Integr Comp Physiol 292: R1110–R1116, 2007. First published November 9, 2006; doi:10.1152/ajpregu.00650.2006.—Leukocyte infiltration, mediated by chemokines, is a key step in the development of organ dysfunction. Lung and liver neutrophil infiltration following trauma-hemorrhage is associated with upregulation of monocyte chemoattractant protein-1 (MCP-1). Because MCP-1 is not a major attractant for neutrophils, we hypothesized that MCP-1 influences neutrophil infiltration via regulation of keratinocyte-derived chemokines (KC). To study this, male C3H/HeN mice were pretreated with MCP-1 antisum or control serum and subjected to trauma-hemorrhage or sham operation. Animals were killed 4 h after resuscitation. One group of trauma-hemorrhage mice receiving MCP-1 antisum was also treated with murine KC during resuscitation. Plasma levels and tissue content of MCP-1 and KC were determined by cytometric bead arrays. Immunohistochemistry was performed to determine neutrophil infiltration; organ damage was assessed by edema formation. Treatment with MCP-1 antisum significantly decreased systemic, lung, and liver levels of MCP-1 and KC following trauma-hemorrhage. This decrease in MCP-1 levels was associated with decreased neutrophil infiltration and edema formation in lung and liver following trauma-hemorrhage. Restitution of KC in mice treated with MCP-1 antisum restored tissue neutrophil infiltration and edema. These results lead us to conclude that increased levels of MCP-1 cause neutrophil accumulation and distant organ damage by regulating KC production during the postinjury inflammatory response.

neutrophils; inflammation; cell trafficking

THE MOST COMMON CAUSES OF death after multiple trauma are organ complications and severe infections leading to multiple organ dysfunction syndrome or multiple organ failure (1). Despite numerous advances in intensive care medicine, ischemia/reperfusion injury, sepsis, and organ dysfunction, leading to multiple organ failure, remain the major cause of death in trauma patients, as well as in patients following major surgery (11, 19, 21, 35).

Our previous studies have shown that trauma-hemorrhage leads to activation of resident immunocompetent cells in different organs (16, 45), resulting in an increased release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-6 (8). Increased levels of proinflammatory cytokines were associated with tissue damage caused by neutrophil infiltration (37, 48). Chemokines and their receptors play a pivotal role in mediating leukocyte transmigration from blood vessels to the inflamed tissue (30), which are released by both immunocompetent and intrinsic cells (13, 46). Neutrophils can release cytokines, enzymes, and oxygen radicals that result in tissue damage (3, 41). This inflammatory response causes tissue damage leading to organ dysfunction and failure (9). In our rodent models of trauma-hemorrhage, infiltration of neutrophils in lung and liver has been previously reported (48).

Previous studies have shown that monocyte chemoattractant protein-1 (MCP-1) is upregulated under stress conditions such as trauma-hemorrhage (15). Since MCP-1 is a major attractant for macrophages and monocytes but not for neutrophils, the mechanism by which MCP-1 mediates neutrophil infiltration remains unclear. The CXC chemokines keratinocyte derived-chemokine (KC; aka, GRO-α and CXCL1) is a ligand for, and mediates neutrophil infiltration through, CXCR2 (13, 24, 36). Because other investigators have previously shown a connection between MCP-1 and KC (6, 12, 34), we hypothesized that MCP-1 influences neutrophil infiltration, resulting in tissue damage through regulation of KC production. To test this hypothesis, we evaluated the effect of MCP-1 antisum on KC levels, neutrophil infiltration, and tissue damage in lung and liver following trauma-hemorrhage.

MATERIALS AND METHODS

Animals and experimental groups. All animal studies were carried out in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Male C3H/HeN mice 8–12 wk old and weighing 19–23 g were obtained from Charles River Laboratories (Wilmington, MA). Mice were treated with either control serum or MCP-1 antisum. One group of mice was also treated with MCP-1 antisum and recombinant murine KC (R&D Systems, Minneapolis, MN) during resuscitation.

Treatment of mice with anti-MCP-1 antisum. Polyclonal anti-murine MCP-1 antisum was kindly provided by Dr. Steven L. Kunkel (University of Michigan, Ann Arbor, MI). To neutralize MCP-1 activity, 0.5 ml of rabbit anti-murine MCP-1 antisum or rabbit control serum was injected intraperitoneally 2 h before trauma-hemorrhage or sham operation. The time of administration and the dose of antisum were selected from previous studies and on the basis of the biological half-life of the antibody, that has been reported to be ~36 h (26). One group of mice received recombinant murine KC (1 μg/25 g body wt; R&D Systems) intravenously at the beginning of resuscitation.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Trauma-hemorrhage procedure. Mice in the trauma-hemorrhage groups were anesthetized with isoflurane (Minrad, Bethlehem, PA) and restrained in a supine position (22). A midline laparotomy was performed, which was closed in two layers with sutures (6-0, Ethicon, Somerville, NJ). Both femoral arteries and the right femoral vein were cannulated with polyethylene tubing (Becton Dickinson, Sparks, MD). Blood pressure was measured via one of the arterial lines using a blood pressure analyzer (Micro-Med, Louisville, KY). Within 10 min after awakening, animals were bled through the other arterial catheter to a mean arterial blood pressure of 35.0 ± 5.0 mmHg, which was maintained for 90 min. At the end of the procedure, the animals were resuscitated via the venous line with four times the shed blood volume in the form of Ringer's lactate. After removing the catheters, the incisions were flushed with lidocaine and were closed with sutures. Sham-operated animals underwent the same surgical procedures, but were neither hemorrhaged nor resuscitated.

Tissue harvesting. The animals were anesthetized with isoflurane at 4 h following sham operation or resuscitation in the trauma-hemorrhage groups, and blood was obtained via cardiac puncture using a syringe coated with EDTA (Sigma, St. Louis, MO). Blood was centrifuged (2,500g, 10 min, 4°C), and the plasma was stored at −80°C. Lung and liver were removed aseptically, frozen in liquid nitrogen, and stored at −80°C. Frozen tissue samples were thawed and suspended in 1% proteinase inhibitor cocktail (Sigma). The samples were sonicated on ice (Sonic Dismembrator, Fisher Scientific, Hampton, NH). The samples were then centrifuged at 12,000g for 10 min at 4°C. The supernatants were frozen and stored at −80°C until further assayed. Aliquots were used to determine protein concentration (Bio-Rad DC Protein Assay; Bio-Rad Laboratories, Hercules, CA).

Flow cytometric analysis of chemokine concentration. KC and MCP-1 concentrations in plasma and tissue protein were determined with cytokine bead array inflammatory kits using flow cytometry, according to the manufacturer’s instructions (BD Pharmingen, San Diego, CA), as described previously (15). Briefly, 50 μl of mixed capture beads were incubated with a 50-μl sample for 1 h at 25°C. Afterward, 50 μl of mixed PE detection reagent was added. After incubation for 1 h at 25°C in the dark, the complexes were washed twice and analyzed using the LSRII.
flow cytometer (BD Biosciences, Mountain View, CA). Data analysis was carried out using the accompanying FACSDiva and FCAP Array software (BD Biosciences). Tissue chemokine content was normalized to protein concentration.

Immunohistochemistry. Because myeloperoxidase is not exclusively found in neutrophils but also in macrophages (2, 40), the neutrophil content in lung and liver was determined by immunohistochemistry staining. Fresh tissue samples were fixed in 4% buffered formalin and embedded in paraffin. For quantitative analysis, 3-μm-thick slices were used. Every fifth of fifteen subsequent slices chosen by systematic uniformly random sampling was stained and analyzed. Sections were air-dried and incubated in 3% hydrogen peroxidase (Sigma). Endogenous biotin and avidin were blocked by applying a blocking kit (Vector Laboratories, Burlingame, CA). Sections were incubated with an anti-mGR-1 antibody (BD Pharmingen) overnight at 4°C in a humid chamber. The next day, sections were incubated with the biotin-coupled secondary anti-rat IgG antibody (Vector Laboratories) for 1 h followed by HRP-conjugated streptavidin (Vectastain kits, Vector Laboratories) for 1 h at 25°C in a humid chamber. Peroxidase was visualized by 3,3′-diaminobenzidine hydrochloride (DAB; Vector Laboratories). Cells were counterstained with hematoxylin. In negative controls, primary antibodies were omitted and replaced by a species-matched isotype (rat IgG2b; Serotec, Raleigh, NC). Slides were blinded and positive cells were counted in 15 high-power fields per animal at ×400 magnification by two investigators.

Table 1. KC restitution restores KC levels in plasma, lung, and liver

<table>
<thead>
<tr>
<th></th>
<th>KC</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T-CS</td>
<td>T-AS+KC</td>
</tr>
<tr>
<td>Plasma, pg/ml</td>
<td>607.8±95.4</td>
<td>631.6±139.0</td>
</tr>
<tr>
<td>Lung, pg/mg protein</td>
<td>292.6±42.9</td>
<td>341.9±49.1</td>
</tr>
<tr>
<td>Liver, pg/mg protein</td>
<td>63.2±11.1</td>
<td>50.7±5.4</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. Restitution of Keratinocyte-derived chemokine (KC) during resuscitation restored KC levels in plasma, lung, and liver in trauma-hemorrhage mice treated with monocyte chemotactant protein-1 (MCP-1) antiserum to that of trauma-hemorrhage mice treated with control serum (P > 0.05). Restitution of KC did not affect MCP-1 levels. Values of cytokine levels in sham animals treated with control or MCP-1 antiserum are shown in Fig. 1. T-CS, trauma-hemorrhage plus control serum; T-AS+KC, trauma-hemorrhage plus antiserum plus KC; T-AS, trauma-hemorrhage plus antiserum.
Determination of wet-dry ratios. Wet-to-dry weight ratios of lung and liver were used as a measure of tissue edema (15). Tissue samples were weighed immediately after removal (wet weight) and then subjected to desiccation in an oven at 80°C (Blue M, Asheville, NC) until a stable dry weight was achieved after 48 h. The ratio of the wet-to-dry weight was then calculated.

Statistics. Statistical analysis was performed using Sigma-Stat computer software (SPSS, Chicago, IL). The data were analyzed using one-way ANOVA and Student’s t-test, and differences were considered significant at *P* < 0.05. Results are expressed as means ± SE of 4–6 animals per group.

RESULTS

Plasma MCP-1 and KC concentrations. After trauma-hemorrhage, mice treated with rabbit control serum showed a significant increase in plasma MCP-1 and KC concentrations compared with control serum-treated sham animals (*P* < 0.05). Treatment with rabbit MCP-1 antiserum normalized MCP-1 and KC levels to that of shams (*P* < 0.05). In sham animals, MCP-1 antiserum resulted in decreased plasma MCP-1 levels as well (*P* < 0.05; Fig. 1, A and B). Plasma levels of IL-6 and TNF-α were increased following trauma-hemorrhage but were not affected by MCP-1 antiserum treatment (data not shown).

Tissue MCP-1 and KC content. Trauma-hemorrhage mice treated with control serum showed increased MCP-1 and KC content in the lung compared with sham animals (*P* < 0.05). Pretreatment with MCP-1 antiserum decreased MCP-1 and KC to sham levels (*P* < 0.05; Fig. 2, A and B). Trauma-hemorrhage increased liver concentrations of MCP-1 and KC compared with sham operation in mice pretreated with control serum (*P* < 0.05). Similar to the lung, MCP-1 antiserum normalized MCP-1 content and decreased KC upregulation following trauma-hemorrhage (*P* < 0.05; Fig. 3, A and B). MCP-1 antiserum decreased lung and liver MCP-1 content in sham animals (*P* < 0.05; Fig. 2A and 3A). Content of IL-6 and TNF-α in lung and liver were increased following trauma-hemorrhage but were not affected by MCP-1 antiserum treatment (data not shown).

Effect of KC restitution on chemokine levels. To further establish the role of KC in mice pretreated with MCP-1 antiserum, animals were injected with recombinant murine KC.

Fig. 5. Quantification of liver neutrophil infiltration by immunohistochemistry. Immunohistochemistry procedure and data analysis were performed as described in Fig. 4. After trauma-hemorrhage, neutrophil (T-CS; B) infiltration was increased compared with sham animals (S-CS, A). Treatment with MCP-1 antiserum (T-AS; C) decreased the number of neutrophils (arrows) in the liver compared with mice injected with rabbit control serum (B). Restitution of KC (T-AS + KC, D) abrogated this effect.

Data are shown (E) as means ± SE; *n* = 4–6/group.

*P* < 0.05 vs. S-AS, S-CS, and T-AS.
Restitution with KC in MCP-1 antiserum-treated mice during resuscitation led to KC plasma levels comparable to control serum-treated animals (P > 0.05). Lung and liver KC content were restored after KC treatment compared with control serum-treated trauma-hemorrhage mice (P < 0.05; Table 1). Restitution of KC did not affect MCP-1, IL-6, or TNF-α levels (P > 0.05; Table 1; data not shown for IL-6 and TNF-α).

**Tissue neutrophil infiltration.** In sham animals, the treatment with antiserum had no effect on neutrophil infiltration in lung and liver. Following trauma-hemorrhage, more neutrophils were found in animals pretreated with control serum compared with sham animals (P < 0.05). Administration of MCP-1 antiserum resulted in attenuated neutrophil infiltration compared with control serum-treated trauma-hemorrhage mice (P < 0.05). The restitution of KC abrogated this effect (P < 0.05) (Figs. 4 and 5). Trauma-hemorrhage also increased neutrophil influx in the liver (P < 0.05). Treatment with MCP-1 antiserum decreased the number of GR-1 positive cells in trauma-hemorrhage mice (P < 0.05). Additionally, treatment with KC enhanced neutrophil influx in antiserum-treated trauma-hemorrhage mice (P < 0.05). No difference between control and antiserum treatment could be detected in sham animals (P > 0.05) (Figs. 4 and 5).

**Tissue wet-to-dry weight ratio.** In control serum-treated animals, trauma-hemorrhage resulted in a significantly higher wet-to-dry weight ratio compared with sham animals (P < 0.05). Antiserum treatment led to a significant reduction in lung edema formation compared with the control serum-treated trauma-hemorrhage group (P < 0.05). KC treatment abrogated the beneficial effects of MCP-1 antiserum on lung edema formation (Fig. 6A; P < 0.05). Administration of MCP-1 antiserum did not affect the wet-to-dry weight ratio in sham groups (P > 0.05). In animals treated with control serum, trauma-hemorrhage resulted in a significantly higher liver wet-to-dry weight ratio compared with sham animals. MCP-1 antiserum pretreatment following trauma-hemorrhage led to a significant reduction in edema formation compared with the control serum-treated trauma-hemorrhage group (P < 0.05). The restitution of KC levels in MCP-1 antiserum-treated animals resulted in edema induction similar to that of control serum-treated trauma-hemorrhage mice (Fig. 6B).

**DISCUSSION**

Neutrophil infiltration is a critical step in the posttraumatic immune response. Chemokines play a key role in mediating extravasation of neutrophils to the site of inflammation (14). Moreover, neutrophil influx is believed to be responsible for induction of tissue damage following trauma-hemorrhage (41). Because MCP-1 is upregulated following trauma-hemorrhage, we examined the mechanism of how MCP-1 might mediate neutrophil infiltration following trauma-hemorrhage. Blocking MCP-1 resulted in decreased plasma KC concentrations, as well as lower KC contents in the lung and liver. This was associated with less neutrophil infiltration demonstrated by immunohistochemistry. MCP-1 antiserum treatment also prevented organ damage, as measured by edema induction. Restitution of KC in MCP-1 antiserum-treated mice during resuscitation not only resulted in increased neutrophil infiltration, but also led to tissue edema similar to trauma-hemorrhage mice treated with control serum. An effect of MCP-1 antiserum on other cytokines, which are upregulated following trauma-hemorrhage, such as IL-6 and TNF-α, was excluded. These findings collectively suggest that upregulation of MCP-1 in lung and liver following trauma-hemorrhage increased KC production, which, in turn, led to enhanced neutrophil infiltration. The release of reactive metabolites by neutrophils is known to cause tissue damage and edema formation (9), which may be the mechanism in our model.

Another possibility of inducing tissue damage measured by tissue edema may be initiated by increased levels of KC itself. Injection of recombinant KC resulted in necrosis and increased synthesis of type I collagen without infiltration of neutrophils, indicating a direct hepatotoxic effect of KC (39).

The chemokine MCP-1 is upregulated after multiple conditions in humans, such as sepsis (7) and after LPS injection (31), as well as in various animals models. MCP-1 primarily attracts macrophages and monocytes via its receptor CCR2 (12, 17). Despite the fact that the chemotactic effect of MCP-1 on neutrophils in vitro is debatable (18, 47), blocking of MCP-1 resulted in decreased neutrophil infiltration following trauma-hemorrhage. These findings are consistent with results from other investigators indicating an effect of MCP-1 on neutrophil infiltration (12, 23, 33, 38). After ischemia-reperfusion injury (I/R), blocking of CCR2, the main receptor for MCP-1, resulted in decreased rolling, adherence, and transmigration of neutrophils (33). Furthermore, administration of a peptide-
based MCP-1 receptor antagonist delayed neutrophil clearance in a rat nephritis model (23). In CCR2-deficient mice (CCR2/-), a decreased expression of MCP-1 was associated with reduced macrophage infiltration and less tissue damage following renal I/R (12). Following cecal ligation and puncture, neutrophil infiltration was shown to be mediated by MCP-1; in contrast, neutrophil trafficking was independent after airway instillation of bacterial LPS (38). However, the exact mechanism by which MCP-1 influences neutrophil infiltration is not yet clear.

Although differential expression and regulation of MCP-1 and KC was demonstrated (42), some investigators described a connection between MCP-1 and KC (6, 12). During renal I/R in CCR2/- mice, both, MCP-1 and KC were downregulated compared with wild-type mice (12). After administration of a KC-inhibiting antibody in wild-type mice, reduced neutrophil accumulation in the ischemic kidneys was observed in the same model (28). Biswas and Sodhi (6) demonstrated an upregulation in KC mRNA expression in murine peritoneal macrophages after stimulation with MCP-1. These data are consistent with our findings demonstrating that blocking of MCP-1 decreased KC production following trauma-hemorrhage. Regarding the results of studies showing a down-regulation of MCP-1 and KC in CCR2/- mice during renal I/R, this effect is most likely mediated via MCP-1 activation of CCR2 (12). However, it remains unknown whether MCP-1 regulates KC and neutrophil infiltration via CCR2. Experiments involving blockade of CCR2 or using knockout of CCR2 may be useful to answer these questions.

In contrast to our results, other investigators describe MCP-1-induced neutrophil transendothelial migration independent of the presence of KC. In a murine model of acute septic peritonitis, blocking of MCP-1 had no effect on peritoneal levels of KC but decreased neutrophil infiltration via regulation of leukotriene B4 produced by residential macrophages (26). MCP-1 was also shown to increase ICAM-1 expression on endothelial cells after hepatic I/R, which may be another potential pathway modulating neutrophil infiltration (20, 44). Following hemorrhagic shock, lung neutrophil accumulation was not associated with an upregulation of ICAM-1 expression; however, hemorrhagic shock enhanced lung neutrophil infiltration in ICAM-1-deficient mice (37). In our study, the salutary effect of the MCP-1 antiserum was abrogated by restitution of KC, which indicates an indirect effect of MCP-1 on neutrophil influx and tissue damage via regulation of KC.

The question as to what extent KC contributes to neutrophil infiltration was raised in various studies (4, 24, 29). In a two-hit model, blockade of CXCR2 resulted in less neutrophil infiltration and attenuated lung tissue content of KC (24). A pivotal role of MIP-2, another CXCR2 ligand but not KC, was shown in the same model (25). However, it was convincingly shown in most recent publications that CXCR2 is activated only in the presence of both ligands (32). Nevertheless, experiments in which KC is blocked appear to be necessary to prove its contribution in neutrophil infiltration in this model. Neff et al. described the involvement of the Fas/FasL system during acute lung inflammatory response in increased KC production ultimately leading to enhanced neutrophil influx and tissue damage (29). Injection of recombinant KC in healthy mice did not activate neutrophils or recruiting them to the liver (4), indicating that neutrophils were primed through other factors following trauma-hemorrhage. This is supported by results from Engelhardt et al. (10), who showed, using a human wound healing model, elevated levels of growth-related oncogene-α (GRO-α, human homologue to KC) at the site of injury 7 days after clearance of neutrophil infiltration.

Although we have shown that Kupffer cells are the main source of MCP-1 following trauma-hemorrhage (15), the precise cellular source of KC production remains unclear. Because MCP-1 primarily attracts macrophages and monocytes, it could be argued that decreased KC levels may be due to diminished number of tissue macrophages. However, neutrophils releasing oxygen radicals were shown as the major cause of tissue damage following trauma-hemorrhage (41). In a model of local infection, it was shown that KC not only acts as a neutrophil chemoattractant but that neutrophils were identified as a major source of KC (13). Other investigators described the modulation of CINC-1 (rat homologue of KC) release from hepatocytes by Kupffer cells (27). Peritoneal mesothelial cells mediated neutrophil infiltration via GRO-α after stimulation with IL-17 (43). However, peritoneal macrophages from normal animals showed an upregulation of KC expression after stimulation with MCP-1 (6). Thus, one can assume that residential macrophages are activated by increased MCP-1 levels. In our model, activation of alveolar macrophages and Kupffer cells may lead to enhanced release of KC, which mediates neutrophil infiltration in the affected organs.

Our data demonstrate that MCP-1 causes organ damage via upregulation of KC production, which could be initiated directly by the chemokine itself and via neutrophil infiltration. Because studies have shown that neutrophil depletion reduced mortality in a rat model of hemorrhagic shock (5), inhibition of KC may have beneficial effect via both routes. Modulation of the MCP-1/KC axis may constitute a novel therapeutic approach in treating posttraumatic immune response.

ACKNOWLEDGMENTS

The authors wish to thank Bobbi Smith for valuable help in editing the manuscript.

GRANTS

This work was supported by National Institutes of Health Grant-R01-GM-37127. Dr. Schwacha is supported in part by National Institutes of Health Grant K02-AI-49960.

REFERENCES

R1116 MCP-1 INFLUENCES ORGAN DAMAGE VIA KC


