PAF and CD18 mediate neutrophil infiltration in upper gastrointestinal tract during intra-abdominal sepsis

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SEPSE S REMAINS ONE of the most commonly seen and most frequently fatal conditions in hospital intensive care units (ICU). One recent study of 154 ICU patients with sepsis noted an overall mortality rate of 34% (12). These authors and others have demonstrated that the mortality rate varies in proportion to the number of organ systems that fail subsequent to the onset of sepsis. The mortality rate in patients in whom only one organ fails ranges from 20 to 40%, whereas in those with failure of three or more organs it is 70 to 84% (12, 35). In light of such studies, the mechanisms by which sepsis leads to organ failure and subsequent mortality have become a major focus for researchers.

A growing body of evidence suggests that sepsis initiates the release of a variety of inflammatory mediators, which, among other actions, induce an increase in leukocyte-endothelial adhesion and extravasation into the tissues (10, 13, 32). After leukocyte uptake, increased release of leukotrienes, proteolytic enzymes, and reactive oxygen metabolites occurs. These substances produce microvascular injury, which when extensive may lead to organ failure (11, 16, 20, 34). In humans, neutrophils are a major class of white blood cells, accounting for 50 to 70% of all leukocytes, and may significantly contribute to microvascular dysfunction (9).

Platelet-activating factor (PAF), a glycerophospholipid produced by a variety of cells, including platelets, neutrophils, macrophages, basophils, and endothelial cells, has been suggested to be one of the critical inflammatory mediators responsible for the cardiovascular changes during sepsis (28). When administered exogenously, PAF produces a number of diverse responses including hypotension, activation of platelets and leukocytes, negative inotropic cardiac effects, and increased microvascular permeability (15, 16, 28, 30). In addition, PAF also stimulates leukocyte adhesion to endothelial cells in both in vitro and in vivo studies (3, 5, 15, 16, 34).

Specific adhesion molecules regulate these leukocyte-endothelial interactions. CD11-CD18 integrins, specifically CD11a-CD18 (LFA-1) and CD11b-CD18 (Mac-1), are believed to be very important in this process and have been studied in various systems (18, 25, 26, 31). Several investigators have demonstrated that when these adhesion molecules are blocked by specific monoclonal antibodies (MAb) (anti-CD18 MAb), leukocyte adhesion is attenuated (1, 8).

However, well-controlled studies of neutrophil infiltration under true septic conditions have not yet been reported. Therefore, the goal of this study was to examine the process of neutrophil infiltration using a cecal ligation and incision (CLI) model of intra-abdominal sepsis in rats. This approach is a modification of the standard cecal ligation and puncture (CLP) model (2, 6), which allows rapid induction of a septic state and therefore facilitates studies of specific inflammatory mediators and adhesion molecules that are endogenous to the septic condition. Specifically, we hypothesized that the release of endogenous PAF during CLI-induced sepsis is a major mediator of neutrophil infiltration into the gastrointestinal tract. We further hypothesized that CD18 was one of the major adhesion molecules involved in neutrophil infiltration during sepsis.

MATERIALS AND METHODS

All surgical and experimental procedures were conducted after approval from the Animal Care and Use Committee of the University of Kansas Medical Center. Guidelines set by
the National Institutes of Health and the Public Health Service policy on the humane use and care of laboratory animals were followed at all times. Male Sprague-Dawley rats (250–350 g) were fasted overnight before all surgical procedures.

Drugs and chemicals. Urethan, PBS, BSA, myeloperoxidase (MPO), hexadecyltrimethylammonium bromide (HTAB), hydrogen peroxide, α-dianisidine dihydrochloride, and PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, mixture of C-16 and C-18) were purchased from Sigma Chemical (St. Louis, MO). Anti-CD18 MAb (monoclonal mouse anti-rat ICAM-1, anti-CD54) were purchased from Genzyme (Cambridge, MA). WEB-2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo-[4,3-a][1,4]diazezin-2-yl]-1-(4-morpholinyl)-1-propanone) was a generous gift from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT).

Stock solutions of PAF (2 mg/ml in chloroform) were stored at −35°C until use. Immediately before each experiment an aliquot of the stock solution was evaporated to dryness under a gentle stream of air and resuspended in a PBS solution containing 0.2% serum albumin (pH 7.4) to minimize adherence of PAF to glass and plastic. The beaker containing the PAF solution was then placed in an ultrasonic cleaner (Fisher Scientific) for 1 min to solubilize PAF. Solutions of all other agents were freshly prepared on the day of the experiment.

Exogenous PAF experimental protocols. Rats were anesthetized with an intramuscular injection of 50% urethan (1.5 g/kg). Polyethylene cannulas (PE-50, 0.58 mm ID) were placed into the right jugular vein for drug and fluid administration, which was maintained at a rate of 2 ml/h by an infusion pump (Harvard Apparatus, South Natick, MA). An arterial cannula (PE-50) was placed in the right carotid artery and connected to a blood pressure analyzer (model 200, Digi-Med, Louisville, KY) for continuous measurement of arterial blood pressure throughout the experiment. Another cannula was placed in the left femoral artery to obtain blood samples. After surgery animals were monitored until mean arterial blood pressure (MABP) stabilized for at least 30 min before the initial infusion of PAF.

Rats were divided into five groups: 1) PAF-treated, 2) control, 3) PAF plus WEB-2086, 4) PAF plus anti-CD18, and 5) PAF plus anti-ICAM-1. PAF-treated rats received sequential infusions of graded doses of PAF (1.56, 6.25, 25, and 100 ng·kg⁻¹·min⁻¹) via the jugular vein cannula. Each dose was infused for 30 min, and each infusion (except the last) was followed by a 30-min recovery period. The experiment was terminated after the final PAF infusion.

The stomach, small intestines (duodenum, upper jejnum, lower jejnum, and ileum), and colon were harvested for MPO activity as will be described. Control animals were treated identical to PAF-treated rats except that only vehicle was injected during the four infusion periods. The PAF plus WEB-2086 group was similar to the PAF-treated group except WEB-2086 (250 µg·kg⁻¹·min⁻¹) was infused starting 10 min before the initial PAF infusion and continued throughout the experiment. PAF plus anti-CD18 rats were treated identical to PAF-treated rats except bolus injections of anti-CD18 MAb (5 µg) were given via the jugular vein 10 min before each PAF infusion. Similarly, in the PAF plus anti-ICAM-1 group, anti-ICAM-1 (5 µg) was injected intravenously before each PAF infusion.

CLP vs. CLP. Having established the pattern of neutrophil infiltration in the gastrointestinal tract after administration of exogenous PAF, we next wanted to examine neutrophil uptake during intra-abdominal sepsis. To accomplish this we developed the CLI model of intra-abdominal sepsis, which is a modification of the well-known CLP model (2, 6, 33). During CLI, fecal spillage is greatly increased by substituting cecal incision for cecal puncture. This modification is designed to speed the time course of intra-abdominal sepsis. Rats were divided into the following experimental groups: CLP, CLI, and control. All rats were anesthetized, and the jugular vein, femoral artery, and carotid artery were cannulated as previously described. Fluid was administered at 2 ml/h via the jugular vein, and blood pressure was recorded every hour. Blood samples were obtained for white blood cell (WBC) count and hematocrit every 2 h and just before the termination of the experiment, which occurred 13 h after the end of surgery. For the CLP group, following insertion of cannulas, a 2-cm midline abdominal incision was made and the cecum was gently exteriorized. The cecum was doubly ligated just inferior to the ileocecal valve with 6-0 silk suture. Positioning the ligature in this fashion avoids potential small bowel obstruction after the operation is complete and ensures hemostasis when the cecum is punctured (or incised). The cecum was punctured twice with an 18-gauge needle, and 2 ml of saline was injected into the cecum to increase the amount of fecal spillage. The cecum was restored to its normal position, and the abdomen was closed. The CLI group underwent similar surgical treatment as the CLP group except that the cecum was not punctured. Instead, the antimesenteric surface of the cecum was incised along its entire length and returned to the abdominal cavity. In control animals, the laparotomy was performed and the cecum was exteriorized. The cecum was then placed back into the abdominal cavity, and the abdomen was then closed.

Role of PAF, CD18, and ICAM-1 in CLI. Rats were anesthetized and cannulas were placed in the jugular vein, femoral artery, and the carotid artery as previously described. Fluid (2 ml/h) was administered via the jugular vein. Rats were randomly assigned to the following experimental groups: 1) CLI, 2) control, 3) CLI plus WEB-2086, 4) CLI plus anti-CD18, and 5) CLI plus anti-ICAM-1. Five hours after the cecum was replaced into the abdomen, blood samples were obtained for WBC count and hematocrit and tissues (stomach, duodenum, upper and lower jejunum, ileum, and intestines) were harvested for MPO activity. The CLI and control animals were prepared as previously described. The CLI plus WEB-2086 group was treated similar to the CLP group except WEB-2086 (250 µg·kg⁻¹·min⁻¹) was infused just before the cecum was opened and continued throughout the experiment. The CLI plus anti-CD18 group was also treated identical to CLI rats except bolus injections of anti-CD18 MAb (5 µg) were administered via the jugular vein just before cecal incision and then every hour. Similarly, in the CLI plus anti-ICAM-1 group, anti-ICAM-1 (5 µg) was injected before cecal incision and then every hour.

WBC count and hematocrit. WBC counts were performed using a Coulter Counter model ZM system (Coulter Electronics, Hialeah, FL), whereas hematocrit was determined using an IEC MB microcapillary tube reader (Fisher Scientific).

Measurement of MPO activity. Tissue MPO levels have been shown previously to correlate with neutrophil levels in tissues and are therefore a good marker of neutrophil infiltration in different organs (4, 14). At the end of each experiment, rats were killed to harvest stomach, small intestines (duodenum, upper jejunum, lower jejunum, and ileum), and colon.
results

Exogenous PAF experiments. Our initial experiments examined the effect of exogenous PAF administration on systemic blood pressure and gastrointestinal neutrophil infiltration. MABP remained stable over time in the control group (Table 1). PAF significantly decreased MABP and increased neutrophil infiltration in the stomach, duodenal, and upper jejunum as reflected by increases in tissue MPO activity (Table 1). In contrast, MPO activity in the distal small bowel and colon was not significantly increased. The continuous infusion of the PAF antagonist WEB-2086 significantly attenuated the PAF-induced decreases in MABP and increases in MPO activity as shown in Table 1. MABP remained stable throughout the experiment and tissue MPO activity showed no significant increase above controls in any organ. Although pretreatment with MAb to CD18 did not prevent PAF-induced hypotension, it significantly inhibited the PAF-induced increase in MPO levels in the stomach and duodenum. Similarly, an antibody to ICAM-1 did not prevent PAF-induced hypotension but significantly inhibited the PAF-induced increase in MPO in these two tissues.

CLI model of sepsis. Our first studies concerning intra-abdominal sepsis compared hemodynamic parameters in rats after CLI and CLP. Whereas MABP remained stable throughout the experiment in the control group, this parameter decreased significantly in rats undergoing either CLI or CLP (Fig. 1). Blood pressure decreased within 6 and 10 h among rats undergoing CLI and CLP, respectively. Similarly, hematocrit did not change in control rats but significantly increased 4 and 10 h after CLI and CLP, respectively (Fig. 2). Additionally, WBC counts of control animals remained stable throughout the experiment but were significantly altered in animals undergoing either CLP or CLI (Fig. 3). WBC decreases became statistically significant after 8 h in the CLP group, whereas it only took 10 h for the WBC count to decrease in the CLI group.

CLI produced significant increases in MPO activity in stomach and duodenum (Table 2). However, no significant increases in the jejunum, ileum, or colon were observed.

CLI during PAF blockade with WEB-2086. Administration of WEB-2086 during CLI significantly affected hemodynamic parameters.
Fig. 2. Hematocrit in experimental groups. CLP and CLI both induce a significant increase in hematocrit over time compared with controls. CLI produces a significant increase in hematocrit more rapidly than does CLP. *P < 0.05 vs. control.

Fig. 3. White blood cell (WBC) count in experimental groups. CLP and CLI both induce a significant decrease in WBC over time compared with controls. Decrease in WBC first becomes significant at 8 h in CLI group and at 10 h in CLP group. *P < 0.05 vs. control.

DISCUSSION

Several investigators have suggested a central role for leukocytes as a mediator of organ failure in general and gastrointestinal mucosal injury, in particular during sepsis (15, 17, 24, 30). Adhesion of activated leukocytes to the microcirculation appears to be a precursor to mucosal injury under these conditions. Preliminary investigations into the mechanism of leukocyte adhesion and activation have focused on specific inflammatory mediators [PAF (15, 24), tumor necrosis factor (30)] that may provoke leukocyte adhesion. The role of adhesion molecules (ICAM-1, CD11/CD18) involved in this process has also been studied (19, 24, 27). These studies generally involve intravenous administration of an exogenous substance to stimulate leukocyte adhesion. Our findings extend these studies by using an animal model of sepsis which is clinically relevant. We examined the effect of PAF during both exogenous administration and endogenous release precipitated by the creation of intra-abdominal sepsis in this animal model.

Infusion of exogenous PAF alone produced hemodynamic changes and neutrophil infiltration into organs of the gastrointestinal tract. Systemic blood pressure decreased in a dose-related fashion (data not shown), and neutrophil infiltration in the upper gastrointestinal tract was significantly increased. Neutrophil uptake in the stomach, duodenum, and upper jejunum was attenuated by a PAF antagonist (WEB-2086), a MAb against CD18, and a MAb against ICAM-1. These results suggest that neutrophil infiltration in the upper gastrointestinal tract stimulated by the inflammatory mediator PAF involves the leukocyte integrin CD18 interacting with ICAM-1.

To determine the role of endogenous PAF in inducing neutrophil infiltration in the gastrointestinal tract during sepsis, we first considered using the CLP model of sepsis introduced by Chaudry et al. in 1979 (6). This model examines sepsis secondary to perforation of an intra-abdominal viscus. Perforation of an intra-abdominal viscus and resultant systemic sepsis may occur in several common disorders, including appendicitis, diverticulitis, and colon cancer. Therefore, this model has great clinical relevance. The model is also easy to prepare because it is unnecessary to grow and quantify bacteria or prepare an inoculum for infusion. Instead, the septic condition is induced by a relatively simple surgical procedure. In addition to these advantages, the CLP model has been validated over many years and has become a gold standard among sepsis models (2, 6, 33).

Despite these advantages, the CLP model does have limitations. Because many of the physiological changes during CLP require many hours, animals are conscious during much of this time, which makes serial or continuous data collection cumbersome. Generally, early sepsis does not develop until 10 h after CLP, and late sepsis, as defined by decreased MABP, may not appear until 16 h or more after operation (2, 6, 33). Our
modifications of the model to perform cecal incision were designed to speed the time course of the septic condition. By increasing the amount of fecal spillage, cecal incision allows a more rapid induction of the septic state. CLI alters the timing but not the overall changes in blood pressure, WBC count, and hematocrit seen during CLP. These results suggest that although similar processes are occurring after CLI and CLP, the process is accelerated after CLI.

In both CLI and CLP groups, hematocrit levels rose throughout the experiment. Evaporative fluid losses from surgical preparation of this model could not account for such changes because no increase in hematocrit was seen in the control group. The increased hematocrit seen in the CLI and CLP groups likely represents hemoconcentration occurring secondary to increases in microvascular permeability with subsequent leaking of intravascular fluid into the interstitial space. This phenomenon is known to occur clinically during sepsis (23).

During CLP and CLI, WBC counts decreased by 10 and 8 h, respectively. This marked leukopenia is consistent with the significant leukocyte infiltration into tissues that occurred during sepsis in both models.

Because we were primarily interested in neutrophil adhesion and infiltration during sepsis, we examined patterns of neutrophil infiltration, as measured by MPO activity, after CLI. MPO activity rose in the stomach and duodenum but not in the distal small intestine. The ability of WEB-2086 to prevent neutrophil infiltration by the gastrointestinal tract strongly suggests that endogenous PAF plays a major role in neutrophil uptake in these organs. In the absence of WEB-2086, CLI significantly increased neutrophil infiltration in the stomach and duodenum. When the actions of PAF are inhibited by WEB-2086, MPO levels remained stable in both of these organs. These results suggest that PAF is a major mediator of leukocyte infiltration into these tissues.

The effects of WEB-2086 and anti-CD18 MAb on MABP, hematocrit, and WBC count during CLI-induced sepsis may provide some indirect evidence as to the pathophysiology of the hemodynamic changes associated with sepsis. PAF is known to decrease MABP, decrease WBC count, and increase hematocrit (7). Thus we hypothesized that PAF may be a mediator of these pathophysiological events in sepsis. The PAF antagonist WEB-2086 significantly attenuated changes in MABP, hematocrit, and WBC count after CLI. The CD18 integrins, on the other hand, would not be expected to have a great effect on systemic hemodynamics. However, anti-CD18 MAb significantly attenuates changes in MABP and hematocrit compared with CLI alone. These results are consistent with the belief that leukocyte infiltration, which is partially dependent on CD18, enhances production of inflammatory mediators from leukocytes and endothelial cells, which have a significant effect on systemic hemodynamics (21, 22, 29). Thus by preventing leukocyte infiltration and activation, anti-CD18 MAb may block the release of mediators from leukocytes and thereby attenuate some of the systemic changes seen during CLI-induced sepsis. It is interesting that neither anti-CD18 nor anti-ICAM-1 antibodies blocked leukopenia even though they prevented the uptake of neutrophils in the upper gastrointestinal tract. It is possible that CLI increases leukocyte infiltration into other organs via a mechanism that is not CD18 dependent. Another possible explanation is that leukopenia is due to decreases in circulating lymphocytes and is not primarily due to changes in neutrophil levels.

In summary, our results suggest that neutrophil infiltration is significantly increased in the stomach and duodenum during CLI-induced intra-abdominal sepsis. The inflammatory mediator PAF and the adhesion molecules CD18 and ICAM-1 are important mediators of this neutrophil infiltration in these organs. PAF appears to be a systemic mediator of hemodynamic changes associated with sepsis, including decreased MABP, increased hematocrit, and decreased WBC count. CD18 may contribute to the systemic hemodynamic changes of sepsis by facilitating leukocyte uptake and subsequent release of inflammatory mediators from activated leukocytes.

Table 3. MABP, hematocrit, and WBC count after CLI

<table>
<thead>
<tr>
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<th>MABP, mmHg</th>
<th>Hematocrit, %</th>
<th>WBC Count, ×10⁶/ml</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>106 ± 2</td>
<td>47.2 ± 0.8</td>
<td>10.7 ± 0.8</td>
</tr>
<tr>
<td>CLI</td>
<td>47 ± 1*</td>
<td>61.7 ± 1.6*</td>
<td>5.7 ± 0.7*</td>
</tr>
<tr>
<td>CLI + WEB-2086</td>
<td>103 ± 7†</td>
<td>49.5 ± 1.1†</td>
<td>10.2 ± 1.0†</td>
</tr>
<tr>
<td>CLI + anti-CD18</td>
<td>76 ± 4†</td>
<td>55.5 ± 1.2†</td>
<td>7.7 ± 1.8</td>
</tr>
<tr>
<td>CLI + anti-ICAM</td>
<td>54 ± 8*</td>
<td>58.3 ± 2.4*</td>
<td>5.2 ± 1.2*</td>
</tr>
</tbody>
</table>

Data are means ± SE (U/g tissue); n = 6 rats. WBC, white blood cell. *P < 0.05 compared with controls. †P < 0.05 compared with CLI alone.
REFERENCES


