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*J Immunol* 1999; 162:2341-2346; 
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Expression and Function of the Chemokine Receptors CXCR1 and CXCR2 in Sepsis¹

C. James Cummings, Thomas R. Martin, Charles W. Frevert, Joanne M. Quan, Venus A. Wong, Steven M. Mongovin, Tonja R. Hagen, Kenneth P. Steinberg, and Richard B. Goodman²

Neutrophils (polymorphonuclear neutrophils; PMN) and a redundant system of chemotactic cytokines (chemokines) have been implicated in the pathogenesis of the acute respiratory distress syndrome in patients with sepsis. PMN express two cell surface receptors for the CXC chemokines, CXCR1 and CXCR2. We investigated the expression and function of these receptors in patients with severe sepsis. Compared with normal donors, CXCR2 surface expression was down-regulated by 50% on PMN from septic patients (p < 0.005), while CXCR1 expression persisted. In vitro migratory responses to the CXCR1 ligand, IL-8, were similar in PMN from septic patients and normal donors. By contrast, the migratory response to the CXCR2 ligands, epithelial cell-derived neutrophil activator (ENA-78) and the growth-related oncogene proteins, was markedly suppressed in PMN from septic patients (p < 0.05). Ab specific for CXCR1 blocked in vitro migration of PMN from septic patients to IL-8 (p < 0.05), but not to FMLP. Thus, functionally significant down-regulation of CXCR2 occurs on PMN in septic patients. We conclude that in a complex milieu of multiple CXC chemokines, CXCR1 functions as the single dominant CXC chemokine receptor in patients with sepsis. These observations offer a potential strategy for attenuating adverse inflammation in sepsis while preserving host defenses mediated by bacteria-derived peptides such as FMLP. The Journal of Immunology, 1999, 162: 2341–2346.

¹ This work was supported in part by National Institutes of Health Grants HL51072, AI29103, HL30542, and GM37696; the American Heart Association; and the Medical Research Service of the Department of Veterans Affairs.

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³ Abbreviations used in this paper: PMN, polymorphonuclear neutrophils; ARDS, acute respiratory distress syndrome; ENA-78, epithelial cell-derived neutrophil activator; GRO, growth-related oncogene; ZAS, zymosan-activated human serum.
(24–26). Herein, we present data supporting these hypotheses. 

These data simplify our understanding of the molecular mechanisms for PMN recruitment in patients with sepsis and organ failure, and offer a rationale for targeting CXCR1 to block chemokine-mediated PMN migration while potentially preserving PMN recruitment to sites of bacterial infection.

Materials and Methods

Patient population

Patients in the intensive care units of Harborview Medical Center (Seattle, WA) were prospectively identified between December 1996 and April 1997 as having severe sepsis and organ dysfunction according to the American College of Chest Physicians/Society for Critical Care Medicine Consensus Conference definitions (28). Cardiovascular dysfunction was defined as systemic vascular resistance <800 dyne·s/cm², systolic blood pressure <90 mm Hg, or need for vasopressors. Hematologic dysfunction was defined as thrombocytopenia (platelets <100,000) or disseminated intravascular coagulation. Renal dysfunction was defined as urine output of <30 ml/h and an abrupt rise in creatinine. Hepatic dysfunction was defined as serum bilirubin >3.5 mg/dl. Metabolic dysfunction was defined as an unexplained anion gap >15 mEq/l or serum lactate >2 mmol/l. Central nervous system dysfunction was defined as a Glasgow coma scale value <12 unexplained by sedation. Patients were excluded if they were <18 yr of age, pregnant, neutropenic (<1000 white blood cells/μl), recently transfused (>3 U of blood within the preceding 24 h), known to have HIV infection, or entered into an interventional trial designed to ameliorate the systemic inflammatory response before sample acquisition. All subjects were enrolled within 96 h of the onset of severe sepsis and met the entry criteria at the time of enrollment. Eight normal nonsmoking volunteers served as controls. The study protocol was approved by the University of Washington human subjects committee, and informed consent was obtained from all subjects.

Neutrophil isolation

Heparinized blood (40 ml) was obtained and transported immediately on ice to the laboratory. Plasma was obtained from an aliquot of heparinized blood within 20 min of acquisition and was stored at −20°C for subsequent analysis of chemokine concentrations by immunoassay. The remainder of the whole blood was layered onto a Ficoll-Hypaque density gradient (Mono-Poly Resolving Medium, ICN Biomedicals, Costa Mesa, CA), and neutrophils were isolated by centrifugation. RBC were eliminated by hypotonic lysis and dextran sedimentation. Each subject’s neutrophil sample was divided, and half was used for determination of CXCR1 and CXCR2 expression by flow cytometry. The other half was used for measurement of chemotaxis in vitro.

Flow cytometry

PMN were suspended at a concentration of 1 × 10⁶ cells/ml in ice-cold HBSS containing 0.1% NaN₃ and 0.1% BSA. To detect CXCR1, PMN were incubated with 1 μg/ml of mouse IgG2b mAb that is specific for CXCR1 (22). Anti-protein C mouse IgG2b Ab (Sigma, St. Louis, MO) served as a control for nonspecific binding. FITC-conjugated goat anti-mouse IgG F(ab)₂ (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a detecting Ab. To detect CXCR2, PMN were incubated with 10 μg/ml of affinity-purified polyclonal rabbit IgG F(ab')₂, that is specific for CXCR2 (22). Nonimmune rabbit IgG F(ab')₂ was used as a control, and FITC-conjugated goat anti-rabbit IgG F(ab')₂ was used as the detecting Ab (both from Jackson ImmunoResearch Laboratories). Flow cytometry was performed using a FACScan instrument (Becton Dickinson, Mountain View, CA) as previously described (22).

Neutrophil chemotaxis

PMN chemotaxis was measured as previously described (29). Briefly, PMN were labeled with 5 μM calcine-AM (Molecular Probes, Eugene, OR) and diluted to a concentration of 3 × 10⁶ cells/ml in RPMI 1640 medium (Sigma). PMN chemotactic activity for IL-8 is maximal between 10–100 nM in this assay. PMN migration was detected by measuring the fluorescence (Cytoflour II, PerSeptive Biosystems, Framingham, MA) of calcine-labeled PMN migrating through an 8.0-μm pore size polycyanoacrylne cofiiter during a 90-min incubation in a 96-well chemotaxis chamber (NeuroProbe, Cabin John, MD). Zymosan-activated human serum (ZAS; 10%; containing 1 × 10⁻⁸ M C5a by RIA) and FMLP (Calbiochem, San Diego, CA) was served as positive controls, and PBS served as a negative control. All samples were tested in triplicate.

Inhibition of chemotaxis was determined in the presence or the absence of an affinity-purified polyclonal rabbit IgG specific for CXCR1 that blocks binding of IL-8 to the CXCR1 receptor (22). PMN were incubated for 20 min at room temperature in the presence of 50 μg/ml of blocking Ab or nonimmune rabbit IgG (Jackson ImmunoResearch Laboratories). This concentration of anti-CXCR1 Ab is sufficient to block binding of 1 × 10⁻⁸ M IL-8 to cloned CXCR1 by competitive radioligand binding (22). The chemotactic index represents the percentage of the total PMN migrating. It was calculated from the mean fluorescence of PMN migrating toward chemotactant (IL-8, ZAS, or FMLP) minus the mean fluorescence of PMN migrating to PBS divided by the mean fluorescence of chambers containing 3 × 10⁻⁶ calcine-labeled PMN/ml (total cells available for migration) multiplied by 100. All conditions were tested in triplicate, and the values were averaged.

Plasma chemokine determinations

Plasma concentrations of IL-8, GRO-α, and ENA-78 were determined in 11 patients and 5 normal subjects by sandwich ELISA according to the manufacturer’s protocol (R & D Systems, Minneapolis, MN). Specimens with undetectable chemokine concentrations were assigned a value equal to the lower limit of detection to permit statistical analysis. Samples were stored at −20°C until assayed, and each sample was assayed in duplicate.

Statistical analysis

The expression of CXCR1 and CXCR2 on patient and normal PMN was compared using Student’s unpaired t test with unequal variance. Chemotaxis dose-response curves were compared by ANOVA with a Bonferroni/Dunn post-hoc analysis. Chemokine concentrations in plasma of patients and normal volunteers were compared using the Mann-Whitney U test for nonparametric data. Correlations between chemokine concentrations were determined using Spearman’s rank correlation. In all cases, p < 0.05 was accepted as significant.

Results

Study population

We studied 14 patients and 8 normal subjects. The causes of sepsis and the number of dysfunctional organ systems are shown in Table I. The patients were critically ill, with APACHE II scores of 25.4 ± 10 (mean ± SD) and a 28-day mortality rate of 57%.

Chemokine receptor expression

CXCR1 and CXCR2 receptors were identified by flow cytometry. Fig. 1 compares receptor expression on PMN from a normal volunteer and a septic patient. The expression of CXCR1 was only slightly reduced, whereas the expression of CXCR2 was markedly

**Table I. Sources of sepsis and organ dysfunction in the study patients**

<table>
<thead>
<tr>
<th>Sepsis source(s)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumonia</td>
<td>9 (64)</td>
</tr>
<tr>
<td>Burn</td>
<td>3 (21)</td>
</tr>
<tr>
<td>Necrotizing Fasciitis</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>1 (7)</td>
</tr>
<tr>
<td>SBP</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Dysfunctional organ(s)</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>13 (93)</td>
</tr>
<tr>
<td>Respiratory</td>
<td>13 (93)</td>
</tr>
<tr>
<td>ALI</td>
<td>7 (50)</td>
</tr>
<tr>
<td>ARDS</td>
<td>6 (43)</td>
</tr>
<tr>
<td>CNS</td>
<td>8 (57)</td>
</tr>
<tr>
<td>Renal</td>
<td>5 (36)</td>
</tr>
<tr>
<td>Hematologic</td>
<td>3 (21)</td>
</tr>
<tr>
<td>Hepatic</td>
<td>3 (21)</td>
</tr>
<tr>
<td>Metabolic</td>
<td>2 (14)</td>
</tr>
</tbody>
</table>

a. SBP, spontaneous bacterial peritonitis, ALI, acute lung injury, ARDS, acute respiratory distress syndrome; CNS central nervous system.
reduced on the PMN from this septic patient. Similar changes were seen for each subject. The CXCR1 fluorescence intensity was normal (i.e., ±1 SD of the mean of CXCR1 fluorescence on PMN from normal donors) in nine of 14 septic patients, was reduced in four, and was increased in one. In contrast, CXCR2 fluorescence intensity was normal in only three of 14 septic patients and was significantly reduced (reduced by >1 SD from the mean of CXCR2 fluorescence on PMN from normal donors) in 11 of 14 septic patients.

The peak (mode) fluorescence intensity was averaged for the eight normal donors and the 14 septic patients (Fig. 2). CXCR1 expression on PMN from septic patients was not significantly different from that on normal PMN. By contrast, CXCR2 expression was significantly reduced (p < 0.005) on PMN from septic patients. Similar differences were seen when the data were expressed as the fold increase over control fluorescence. Thus, in contrast to the normal expression of CXCR1 receptor, CXCR2 receptor is significantly down-regulated on circulating PMN from septic patients.

Receptor function

The migration of PMN from 12 septic patients and seven normal donors toward five different CXC chemokines was measured (Fig. 3). The chemokine concentrations selected fell within a biologically relevant range (8). All the CXC chemokines were effective chemoattractants for PMN from normal subjects (Fig. 3, A–E, filled circles). The migratory response to IL-8 was robust in PMN from normal donors and septic patients (Fig. 3A). In contrast, PMN from septic patients demonstrated significantly reduced chemotactic activity to GRO-α, GRO-β, GRO-γ, and ENA-78 (Fig. 3, B–E, open circles), the chemokines that bind with high affinity to only CXCR2. These data indicate that the down-regulation of CXCR2 on PMN from septic patients, seen by flow cytometry (Fig. 2), is functionally significant.

**Functional effect of CXCR1-blocking Ab**

PMN from 14 septic patients and eight normal donors were incubated in the presence of nonimmune rabbit IgG (50 μg/ml) or affinity-purified rabbit anti-human CXCR1 polyclonal IgG (50 μg/ml). This concentration of Ab is sufficient to block binding of radiolabeled IL-8 (up to 1 × 10⁻⁸ M) to recombinant CXCR1
The median concentration of GRO-α levels were undetectable in all five normal subjects (plasma from septic patients was 0.157 ng/ml (19 pM), whereas plasma concentrations of ENA-78 were normal in patients with sepsis. As predicted, there have been detected in the plasma of septic patients (35) and can be down-regulated (anti-CXCR2; hatched bars) or control Ab (nonimmune IgG; solid bars), and migration was measured toward 1 × 10^{-8} M concentrations of IL-8 and FMLP and a 10% concentration of ZAS. Anti-CXCR1 significantly inhibited chemotaxis of both normal and septic PMN to IL-8 (\( p < 0.005 \)), but the effect was more pronounced with septic PMN. As expected, anti-CXCR1 had minimal effect on PMN migration to FMLP (\( p = 0.05 \) with normal PMN, nonsignificant with septic PMN) and ZAS (\( p = \text{NS} \)).

Plasma chemokine levels

Plasma concentrations of IL-8, GRO-α, and ENA-78 were determined by immunoassay in plasma from 11 septic patients and five normal donors (Fig. 5). The median concentration of IL-8 in plasma from septic patients was 0.157 ng/ml (19 pM), whereas levels were undetectable in all five normal subjects (\( p < 0.005 \)). The median concentration of GRO-α was 0.170 ng/ml (20 pM) in plasma from septic patients compared with 0.030 ng/ml (4 pM) in that from normal subjects (\( p = 0.07 \)). By contrast, the median concentration of ENA-78 was 0.140 ng/ml in plasma from septic patients compared with 0.020 ng/ml (2.6 pM) in that from normal subjects (\( p = 0.005 \)). By contrast, the median concentration of GRO-α was 0.170 ng/ml (20 pM) in plasma from septic patients compared with 0.030 ng/ml (4 pM) in that from normal subjects (\( p = 0.07 \)). By contrast, the median concentration of ENA-78 was 0.140 ng/ml in plasma from septic patients compared with 0.020 ng/ml (2.6 pM) in that from normal subjects (\( p = 0.005 \)). By contrast, the median concentration of GRO-α was 0.170 ng/ml (20 pM) in plasma from septic patients compared with 0.030 ng/ml (4 pM) in that from normal subjects (\( p = 0.07 \)). By contrast, the median concentration of ENA-78 was 0.140 ng/ml in plasma from septic patients compared with 0.020 ng/ml (2.6 pM) in that from normal subjects (\( p = 0.005 \)).

Discussion

The major goal of this study was to investigate the effect of severe sepsis on the expression and function of the two CXC chemokine receptors on circulating PMN. We found that CXCR2 expression was reduced by 50% in septic patients, whereas CXCR1 expression was preserved. Similarly, we found that the chemotactic responses to the CXCR2 chemokines which bind with high affinity to only CXCR2 (GRO-α, β, and ENA-78) were markedly suppressed in PMN from septic patients, whereas the chemotactic response to IL-8, which binds with high affinity to either CXCR, was preserved. Finally, specific blockade of CXCR1 had a more pronounced suppressive effect on the chemotactic function of PMN from septic patients than on that of PMN from normal donors. Taken together, these observations indicate that CXCR2 is functionally down-regulated in severe sepsis, leaving CXCR1 as the dominant receptor for mediating the effects of the CXC chemokines in PMN from these patients.

Previous reports (13, 21, 30) demonstrate that in normal PMN, CXC receptors are transiently internalized following in vitro stimulation by IL-8. Subsequently, CXCR1 is rapidly re-expressed on the cell surface, whereas CXCR2 is re-expressed at a considerably slower rate (21). The primary rationale for this study was to investigate the relevance of these in vitro observations for patients whose circulating PMN were stimulated in vivo by an active inflammatory process. We prospectively defined a population of patients with severe sepsis and organ dysfunction that we predicted would have elevated circulating chemokine concentrations. We found chemokine values that were elevated to an extent similar to those reported by others (31–34). Moreover, we found that CXCR2 was significantly down-regulated in these patients. In addition, there were trends suggesting that higher plasma chemokine concentrations were correlated with lower CXCR2 surface expression. The plasma chemokine concentrations were considerably less than those required for receptor down-regulation in vitro (21), suggesting that CXCR2 may be modulated by very low chemokine concentrations in vivo in patients with severe sepsis.

Other mechanisms may also contribute to the down-regulation of CXCR2 in patients with severe sepsis. Cytokines such as TNF-α have been detected in the plasma of septic patients (35) and can down-regulate CXCRs on PMN in vitro (36). TNF-α may also induce proteolytic degradation of CXCR2 (37). Hypoxic conditions in vitro can affect PMN cell surface expression of CXC receptors (38). Granulocyte CSF up-regulates the transcription and expression of both receptors, and LPS down-regulates each receptor by
decreasing transcription and reducing the half-lives of their mRNAs (36). In the complex cytokine milieu of sepsis, all these mechanisms may contribute to CXCR expression on PMN. Our studies were designed to examine the net effect of these multiple mechanisms of CXCR regulation on circulating PMN in vivo under clinically relevant conditions. We found that CXCR2 expression and function are down-regulated on circulating PMN from patients with sepsis.

Our observations are relevant to the mechanisms of PMN emigration from the bloodstream in patients with severe sepsis and organ dysfunction. Although Sjoerjima and colleagues (39) showed in chronic stable lung disease that CXCR down-regulation can occur as a result of the process of migration, we have shown that changes in receptor expression occur on circulating PMN even before they migrate into the tissues in critically ill patients with systemic inflammation.

Multiple CXC chemokines are produced in the organ tissues of septic patients. For example, patients with sepsis-related ARDS have significantly increased concentrations of IL-8, ENA-78, and GRO-α in their bronchoalveolar lavage fluids (8, 40). Interestingly, in patients with ARDS, the average concentrations of ENA-78 and GROα are higher than that of IL-8 (8, 40). A similarly broad spectrum of CXC chemokines is produced by macrophages simply by stimulation with endotoxin in vitro (41). Another important CXC chemokine, granulocyte chemotactic peptide-2, binds with high affinity to CXCR1 and is a potent chemoattractant for PMN, although its characterization in clinical fluids is limited (42–44). The host-derived signals for PMN recruitment to tissues appear to be highly redundant.

Despite the multiplicity of CXC chemokines, however, the data presented here suggest that the GRO proteins and ENA-78 may contribute little to PMN recruitment in septic patients. GRO-α, β, and -γ and ENA-78 bind to and signal via CXCR2 (17). In keeping with this, the chemotaxis of normal PMN to GRO-α (10 nM) is inhibited only by Abs to CXCR2, not by Abs to CXCR1 (23). Our observations extend these findings and demonstrate their relevance to human disease. It is likely that down-regulation of CXCR2 expression in vivo contributes significantly to the suppressed chemotaxis responses to the GRO proteins and ENA-78 that we measured. Regardless of the mechanism, PMN from septic patients respond poorly to the cognate ligands of CXCR2 when ligands are present at concentrations ≤10 nM. This may help explain the success of therapeutic interventions targeting only IL-8 in some animal models of inflammation (9–11).

Ab inhibition of CXCR1 was functionally more effective in PMN from patients with sepsis than in normal PMN. IL-8 can bind with high affinity and stimulate chemotaxis via either CXCR (17), although it stimulates chemotaxis of normal PMN largely via CXCR1 (22, 23). The present studies confirm this prior finding in normal PMN. Additionally, we demonstrated that chemotaxis of PMN from patients with sepsis is more profoundly inhibited than that of normal PMN in the presence of CXCR1 Ab. These experiments further support the functional significance of CXCR2 down-regulation in septic PMN. Chemotaxis to FMLP was well preserved in septic PMN and, as expected, was minimally affected by CXCR1 blockade.

Thus, we have shown that CXCR2 is functionally down-regulated in patients with severe sepsis, perhaps in part by ligand-induced receptor internalization occurring in the circulation, yet CXCR1 remains functional with normal cell surface expression. These data simplify an otherwise complex and redundant system of CXC chemokines and receptors and focus attention on the importance of CXCR1 in sepsis. These studies suggest that a CXCR1 receptor-targeted strategy to limit inflammation in patients with sepsis will reduce PMN migration to CXC chemokines, yet preserve PMN responsiveness to bacterial products.

Acknowledgments

We thank Michelle Goodman, Doreen Anardi, Donna Davis, and Patsy Treece for assistance with patient identification, and Ellen Caldwell for assistance with statistical analysis. We thank Drs. Donald C. Foster and Gary Rosenberg for their guidance in cloning the IL-8R and in isolating the CXCR1 mAb.

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