

Chemokine Regulation of Neutrophil Function in Surgical Inflammation

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Background: Morbidity and even mortality correlate closely with major injury that causes a systemic inflammatory response. Cytokines and bioactive molecules present at the inflammatory site induce this response and regulate neutrophil proinflammatory responses. The CXC chemokines, important for neutrophil recruitment and activation, include interleukin 8 (IL-8), granulocyte chemotactic protein 2 (GCP-2), and epithelial cell-derived neutrophil attractant 78 (ENA-78). They induce neutrophil responses via 2 cell-surface receptors, CXCR-1 and CXCR-2. All 3 chemokines bind CXCR-2 with high affinity. Only IL-8 and GCP-2 bind CXCR-1 with high affinity.

Hypothesis: The CXC chemokines regulate neutrophil responses differently.

Methods: Pretreatment of neutrophils from healthy volunteers with IL-8, GCP-2, or ENA-78; measured IL-8–induced migration; and tumor necrosis factor α (TNF- α)–induced peroxide production.

Results: Flow cytometry and radioligand binding data

indicate that IL-8, GCP-2, and ENA-78 equivalently reduced CXCR-1 and CXCR-2 cell surface expression by 34% to 54%. All treatments decreased affinity of both receptors 1.5- to 2-fold. However, only IL-8 pretreatment inhibited chemotaxis to 10-nmol/L IL-8 (mean \pm SE inhibition, 62% \pm 6%). Although IL-8 and GCP-2, but not ENA-78, suppressed TNF- α –induced oxidant production (mean \pm SE inhibition, 42% \pm 8% and 40% \pm 23%, respectively), only GCP-2 inhibited the oxidative response to complement fragment C5a, and to the bacterial cell wall peptide *N*-formyl-methionyl-leucyl-phenylalanine.

Conclusions: The CXC chemokines regulate neutrophil proinflammatory functions differently. A thorough understanding of mechanisms for modulating neutrophil responses in inflammation will aid the development of interventions that reduce morbidity and mortality associated with severe trauma and sepsis.

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THE INFLAMMATORY environment is composed of multiple soluble mediators that regulate neutrophil function, and accordingly dictate the character of the inflammatory response. These cytokines possess the ability to stimulate neutrophils and to specifically regulate neutrophil responses to resident cytokines.¹⁻⁵ This regulation imposed by resident cytokines is likely time and concentration dependent as well as stimulant specific.

Chemoattractant cytokines that are particularly important for neutrophil or polymorphonuclear leukocyte (PMN) proinflammatory functioning have 2 cysteine residues separated by a single amino acid in their N-terminal region and are therefore classified as CXC chemokines.⁶ In contrast, the CC chemokines, which lack such separation, have no neutrophil-stimulatory activity. The CXC chemokines share 30% to 90% amino acid sequence homology, with molecular weights

ranging from 6 to 8 kd. Members of this family include interleukin 8 (IL-8); growth-related oncogene (GRO) α , GRO β , GRO γ ; neutrophil-activating peptide-2 (NAP-2), granulocyte chemotactic protein 2 (GCP-2); and epithelial cell-derived neutrophil attractant 78 (ENA-78).⁷⁻¹⁰ The chemokines induce neutrophil migration and secretion of cytotoxic granular contents and metabolites. Therefore, each may be important in neutrophil recruitment and activation in surgical inflammation. The differences in amino acid sequence and receptor-binding specificities, however, suggest that the CXC chemokines subserve important regulatory functions. Among the various chemokines, GCP-2 and IL-8 are functionally most similar, but share only 33% sequence similarity. In contrast, ENA-78 is 77% identical to GCP-2 but is functionally less similar than IL-8.¹¹

Two pertussis toxin–sensitive, G-protein–coupled CXC chemokine receptors have been identified on human neu-

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MATERIALS AND METHODS

PERIPHERAL BLOOD NEUTROPHIL ISOLATION AND PRETREATMENT WITH CHEMOKINES

Venous blood was drawn from healthy volunteers in syringes containing 0.25% EDTA (Sigma-Aldrich Corporation, St Louis, Mo). Blood cells were separated by dextran (Pharmacia, Columbus, Ohio) sedimentation and Ficoll histopaque (Ficoll-Hypaque; Sigma-Aldrich Corporation) density centrifugation as previously described.^{30,31} Neutrophil preparations were routinely greater than 95% pure with greater than 95% viability as assessed by trypan blue exclusion. For assays involving chemokine pretreatment, PMNs were pretreated at 1×10^7 cells/mL in indicated concentrations of chemokines for 15 minutes at 37°C before subjection to various assays. Hanks buffered saline solution was used as a vehicle control.

MODIFIED BOYDEN CHAMBER CHEMOTAXIS

Neutrophils were treated with IL-8, GCP-2, or ENA-78 for 15 minutes at 37°C in Hanks buffered saline solution with 0.1% bovine serum albumin (Sigma-Aldrich Corporation). After washing, PMN migration to various concentrations of IL-8 was measured using a modified Boyden chamber chemotaxis assay, as previously described.³⁰ Neutrophils were allowed to migrate into a 5- μ m micropore nitrocellulose filter (Neuroprobe) for 30 minutes at 37°C with 5% carbon dioxide before measuring the distance migrated by the leading front of cells.

FLOW CYTOMETRY ANALYSIS

Two million pretreated neutrophils were incubated with 1 μ g of mouse monoclonal antibodies directed against human CXCR-1 or CXCR-2 (PharMingen, San Diego, Calif) or isotype control antibodies (Accurate Chemical & Scientific Corporation, Westbury, NY) for 20 minutes at 4°C. Next, the cells were incubated in 1 μ g of fluorescent-labeled goat anti-mouse immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, Md) for 20 minutes at 4°C. After washing, neutrophils were resuspended in 1% paraformaldehyde and analyzed on a cytometer (Coulter Epics XL Flow Cytometer; Beckman Coulter, Inc, Fullerton, Calif).

RADIOLIGAND BINDING ASSAY

Carrier-free IL-8 (R&D, Minneapolis, Minn) or tyrosinated GRO α (RepliGen Corporation, Needham, Mass) was iodinated using the iodogen (Pierce Chemical Company,

Rockford, Ill) method. Specific activities of iodine 125-labeled IL-8 and ¹²⁵I-GRO α were 610.5×10^{10} Bq/mmol and 481×10^{10} Bq/mmol, respectively.

For radioligand competition studies, ¹²⁵I-IL-8 or ¹²⁵I-GRO α concentrations remained constant at 1 nmol/L, whereas the unlabeled IL-8 or GRO α concentrations varied from 0 to 50 nmol/L. Duplicate tubes containing 2×10^6 neutrophils were incubated 120 minutes at 4°C in 1-nmol/L radiolabeled chemokine with various concentrations of unlabeled homologous chemokine. Radioactivity of the cell pellets was counted in a gamma counter. Total receptors and dissociation constants (K_d) were determined by curve fitting to the Langmuire isotherm using commercially available software (Sigmaplot; Jandel Scientific, Inc, San Rafael, Calif).

ADHERENCE-DEPENDENT HYDROGEN PEROXIDE PRODUCTION

Ninety-six-well polystyrene tissue culture plates (Falcon-Primaria; Becton Dickinson Labware, Lincoln Park, NJ) were coated with 1 mg/well purified human plasma fibronectin (R&D) for 2 hours at 37°C and 5% carbon dioxide. Plates were washed with isotonic sodium chloride solution, then assay mix containing 0.5 mg of horseradish peroxidase, 1-mmol/L sodium azide, and 4 nmol of scopoletin in Krebs-Ringer solution with 0.1% glucose was added to the wells. The CXC chemokines were added to wells at a final concentration of 10 nmol/L. Polymorphonuclear leukocytes (1.5×10^4) then were added to the wells and allowed to settle for 15 minutes before stimulation with 10-ng/mL TNF- α , 10-nmol/L fMLP, or 10-nmol/L C5a. Hydrogen peroxide production was measured for 80 minutes as the decrease in fluorescence due to the oxidation of scopoletin. Tumor necrosis factor α normally has a lag time of 40 to 50 minutes before hydrogen peroxide production is appreciable. For this reason, only the data from 60 to 80 minutes are reported and are expressed as percentage of decrease from the control cells pretreated with Krebs-Ringer solution with glucose.

MATHEMATICAL CALCULATIONS AND STATISTICAL ANALYSIS

In flow cytometry experiments, the mean channel fluorescence of chemokine-pretreated cells was expressed as a percentage of buffer-pretreated cells. In chemotaxis experiments, the data reported represent the total distance migrated to IL-8. The percentage of inhibition was calculated from total migration in buffer- and chemokine-treated cells. All summarized data are represented as mean \pm SEM. Statistical analyses were performed using paired *t* tests and single-factor analysis of variance.

trophils, CXCR-1 and CXCR-2.^{12,13} These receptors are expressed at equal levels and share 32% sequence homology at the N-terminus and 77% over the rest of the protein.¹⁴⁻¹⁶ The N-terminal region of the receptor is a key determinant of ligand specificity, although other important sites have been identified.¹⁷⁻¹⁹ Of the neutrophil-activating chemokines, only IL-8 and GCP-2 induce neutrophil responses via CXCR-1.²⁰⁻²² On the

other hand, ENA-78, GRO α , NAP-2, IL-8, and GCP-2 induce neutrophil responses via CXCR-2 with similar efficacy (maximal chemotaxis or degranulation).²³

Originally isolated from osteosarcoma cells, GCP-2 is coproduced with IL-8, GRO α , and ENA-78 in these cells.^{11,22,23} Epithelial cell-derived neutrophil attractant 78 was initially isolated from the conditioned medium of a human alveolar type II epithelial cell line. Like IL-8,

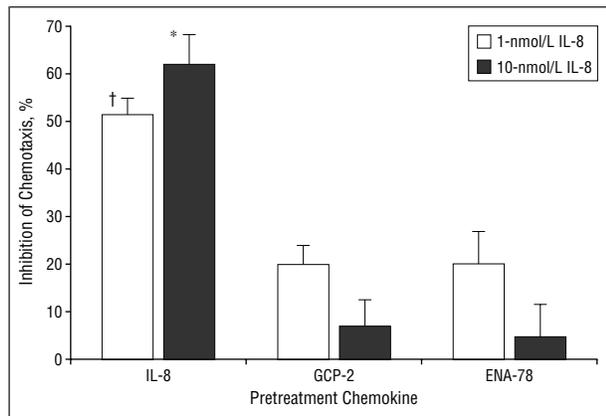


Figure 1. Polymorphonuclear leukocytes pretreated with Hanks buffered saline solution (control experiments), 10-nmol/L interleukin 8 (IL-8), or 100-nmol/L granulocyte chemotactic protein 2 (GCP-2) or epithelial cell-derived neutrophil attractant 78 (ENA-78) for 15 minutes were migrated to 1- or 10-nmol/L IL-8 for 30 minutes at 37°C with 5% carbon dioxide. Data are expressed as mean \pm SEM compared with controls. Asterisk indicates $P < .05$; dagger, $P < .001$; and CXC chemokines, chemokines with 2 cysteine residues separated by a single amino acid in their N-terminal region.

ENA-78 also is produced by monocytes, endothelial cells, lung fibroblasts, and neutrophils.^{24,25} High steady state messenger RNA levels of GCP-2 and ENA-78 have also been detected in human heart and lung.¹¹ That these chemokines are derived from various cell types additionally supports the notion that chemokines are encountered by neutrophils in a temporally and/or spatially regulated manner as they migrate to sites of inflammation. However, it is also possible that neutrophils encounter chemokines concomitantly.

To date, considerable effort has been placed into identifying specialized functions of the individual CXC receptors. Results from such studies involving transfected cell lines reveal that each receptor is independently capable of mediating IL-8-induced chemotaxis.^{26,27} However, chemokine-specific regulation of PMN responses to various cytokines has not been identified. Several studies have shown that CXCR-2-specific ligands GRO α and NAP-2, but not IL-8 (which binds CXCR-1 and CXCR-2), potently suppress subsequent IL-8-induced degranulation, suggesting an important regulatory function of these ligands.^{28,29} Similar functions for other CXC chemokines remain to be characterized.

We reasoned that despite their similarities in structure, receptor binding, chemoattractant properties, and derivations, IL-8, GCP-2, and ENA-78 perform distinct regulatory functions. To test our hypothesis, we pretreated PMNs with IL-8, GCP-2, or the CXCR-2-specific chemokine ENA-78, and subsequently assessed chemotactic activity by measuring IL-8-induced migration. We also tested the ability of the CXC chemokines to modulate adherence-dependent oxidant production, an important event in neutrophil-mediated cytotoxicity and bactericidal activity. This response is mediated by receptors that bind tumor necrosis factor α (TNF- α), complement factor C5a, or the bacterial cell wall peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). It therefore accommodates the examination of chemokine effects on a range of neutrophil stimulants. Our findings demonstrate that IL-8, but not GCP-2, desensitized

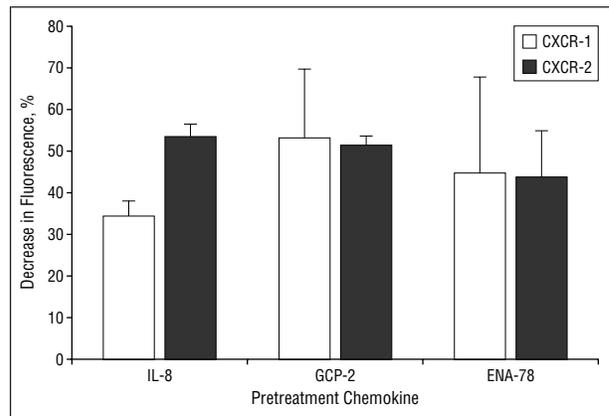


Figure 2. Polymorphonuclear leukocytes pretreated for 15 minutes in Hanks buffered saline solution (control experiments), 10-nmol/L IL-8, or 100-nmol/L GCP-2 or ENA-78 were incubated with monoclonal mouse anti-human chemokine receptor CXCR-1 or CXCR-2 antibodies, then fluorescein isothiocyanate-labeled secondary antibody. Data are expressed as mean \pm SEM compared with controls. Abbreviations are given in the legend to Figure 1.

PMN chemotactic responsiveness to IL-8. Although they suppressed TNF- α -induced oxidant production with similar efficacy, only GCP-2 inhibited the oxidative response to C5a and fMLP. Like IL-8 and GCP-2, ENA-78 substantially reduced cell surface expression of CXCR-1 and CXCR-2, but had no effect on IL-8-induced migration or stimulant-induced oxidant production.

RESULTS

CXC CHEMOKINES DIFFERENTIALLY SUPPRESS IL-8 RESPONSIVENESS

We initially sought to determine the effects of IL-8, GCP-2, and ENA-78 on neutrophil responses to subsequent IL-8 challenge. To this end, we pretreated PMNs with GCP-2 and ENA-78 for 15 minutes at 37°C and measured chemotaxis to IL-8. Migration to IL-8 was unaffected by pretreatment with 100-nmol/L GCP-2 or ENA-78, whereas pretreatment with a 10-fold lower concentration of IL-8 inhibited the response to subsequent 1- or 10-nmol/L IL-8 challenge by 54% \pm 3% and 62% \pm 6%, respectively (**Figure 1**). It has been speculated that as little as 20% of chemoattractant receptor occupancy is required to sustain chemotactic responsiveness.³² It therefore remained possible that although these chemokines have no effect on migration to IL-8, they could substantially decrease cell surface CXC receptor expression and thereby affect other cell functions. To explore this possibility, we performed flow cytometry studies using antibodies directed against CXCR-1 and CXCR-2.

CXC RECEPTOR DOWN-REGULATION AND AFFINITY MODULATION

Figure 2 shows a substantial reduction in CXCR-1 and CXCR-2 immunoreactivity following treatment with GCP-2, ENA-78, or IL-8. To quantify CXC receptor loss and to determine alterations in receptor affinity, we performed radioligand binding studies using ¹²⁵I-IL-8 or ¹²⁵I-GRO α . Pretreatment with IL-8, GCP-2, or ENA-78 caused a 1.5- to 2-fold decrease in receptor affinity (**Table**).

Summary of Pretreatments*

	Buffer	IL-8, 10 nmol/L	GCP-2, 100 nmol/L	ENA-78, 100 nmol/L
K_d nmol/L for ^{125}I -IL-8	1.0 ± 0.2	2.1 ± 0.3†	2.0 ± 0.04†	1.9 ± 0.1†
K_d nmol/L for ^{125}I -GRO α	1.0 ± 0.2	1.6 ± 0.2†	1.4 ± 0.03†	1.4 ± 0.3†

* K_d indicates dissociation constant; GRO α , growth-related oncogene α and ^{125}I , radioactively labeled with iodine 125. Other abbreviations are given in the legend to Figure 1. Data are given as mean ± SE.

† $P < .05$ compared with buffer-treated groups, using the paired t test.

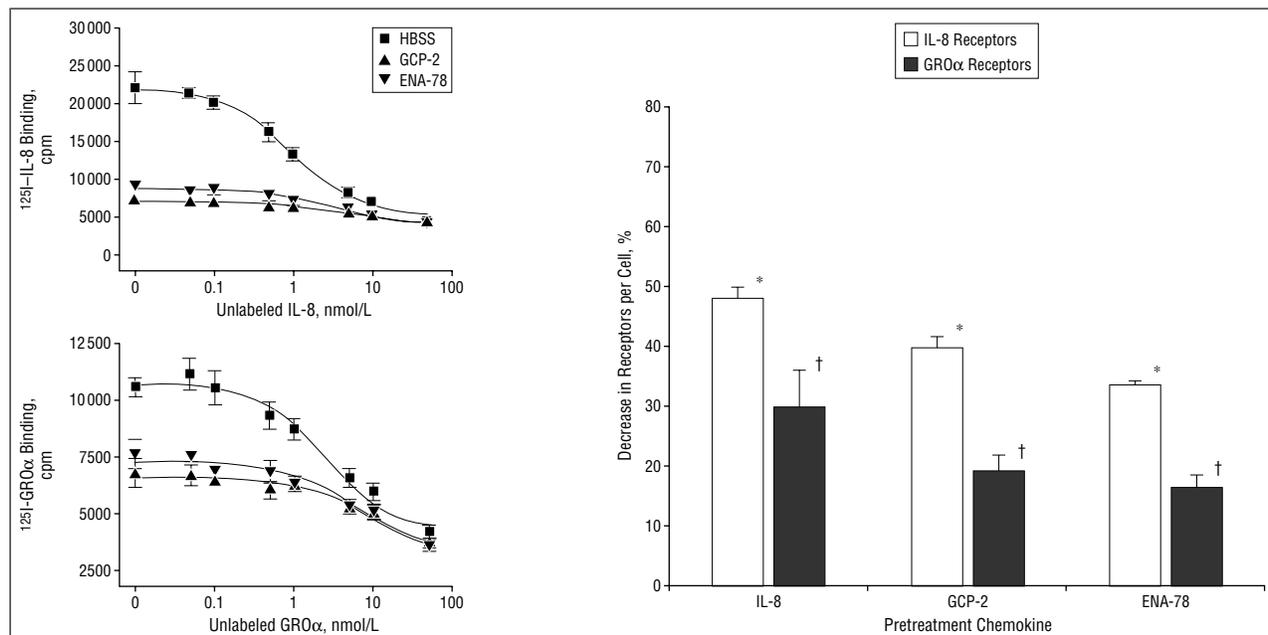


Figure 3. Polymorphonuclear leukocytes pretreated with 10-nmol/L IL-8 or 100-nmol/L GCP-2 or ENA-78 were subjected to homologous radioligand competition assays using iodine 125-labeled (^{125}I) IL-8 or ^{125}I -growth-related oncogene α (GRO α). Data are expressed as mean ± SEM from 3 different experiments. Asterisk indicates $P < .05$; dagger, $P < .02$; and HBSS, Hanks buffered saline solution (control experiments). Other abbreviations are given in the legend to Figure 1.

Figure 3 demonstrates that the loss of IL-8 receptors (eg, CXCR-1 and CXCR-2) as determined by results of radioligand binding assays parallels the findings of flow cytometry. Both assays indicate a loss of approximately 40% to 50% of total IL-8 receptors following pretreatment with either chemokine. However, there is a marked disparity between the loss of GRO α receptors (CXCR-2) when comparing the flow cytometry and radioligand binding results. Flow cytometry results show a greater decrease in CXCR-2 expression than is determined by radioligand binding results (approximately 50% and 20% losses, respectively). However, these differences were not statistically significant. The results from both assays demonstrate that loss of CXC receptors following GCP-2 or ENA-78 pretreatment is similar to that following IL-8 pretreatment, despite their inability to inhibit chemotaxis. This suggests that mechanisms other than receptor down-regulation are operative in IL-8 suppression of chemotaxis.

CXC CHEMOKINES DISPARATELY SUPPRESS OXIDANT PRODUCTION

Adherence-dependent oxidant production is a critical effector of PMN-mediated tissue injury. We therefore sought

to assess whether these chemokines could regulate this important neutrophil response. Polymorphonuclear leukocytes were plated on fibronectin-coated tissue culture plates that contained IL-8, GCP-2, or ENA-78. Following a 15-minute incubation at 37°C, PMNs were stimulated with 10 ng/mL TNF- α , 10-nmol/L fMLP, or 10-nmol/L C5a. **Figure 4** shows GCP-2, but not ENA-78, suppressed hydrogen peroxide production in response to TNF- α , fMLP, and C5a (40% ± 23%, 33% ± 11%, and 66% ± 14% inhibition, respectively). Interleukin 8 suppressed only the TNF- α -induced response by 42% ± 8%.

COMMENT

It is generally accepted that neutrophils exist in the following 4 prominent populations: the circulating pool; the marginating pool, loosely tethered to microvasculature; the pool firmly adhered to endovasculature; and transmigrated neutrophils in the parenchyma. It is believed that the circulating and particularly the marginated pools provide an abundant source of neutrophils capable of being recruited rapidly to sites of injury or infection. After injury, neutrophils from these pools firmly attach to the endovasculature and migrate into the parachymal spaces,

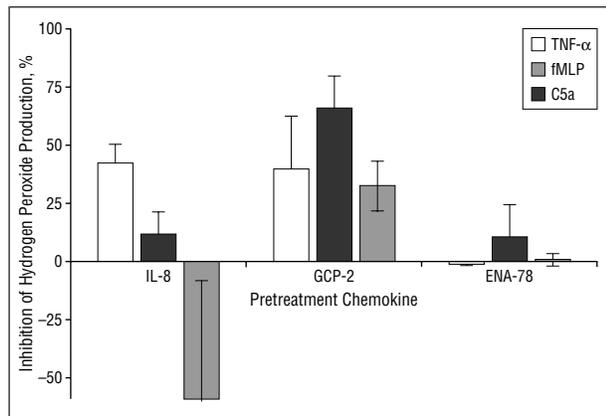


Figure 4. Polymorphonuclear leukocytes were plated on fibronectin-coated wells containing 100-nmol/L IL-8, GCP-2, or ENA-78 at 37°C for 15 minutes before stimulation with 10 ng/mL tumor necrosis factor α (TNF- α), 10-nmol/L complement factor C5a, or 10-nmol/L N-formyl-methionyl-leucyl-phenylalanine (fMLP). Data are expressed as mean \pm SEM from 3 experiments. Other abbreviations are given in the legend to Figure 1.

thus progressing to the latter 2 populations.^{33,34} Mechanisms regulating the initiation and termination of neutrophil progression through these populations are largely unknown.

Following traumatic injury or endotoxemia, plasma levels of several proinflammatory cytokines, including IL-8, are significantly elevated.³⁵⁻³⁷ It is possible that elevated plasma IL-8 levels serve as a biological timer of neutrophil recruitment to inflammatory loci. The effects of an IL-8 concentration spike may be particularly important for the circulating and marginated neutrophil pools in that they potentially terminate neutrophil recruitment from these pools to sites of inflammation. We sought to examine this possibility by exposing suspension-phase neutrophils to IL-8 and measuring subsequent IL-8-induced migration.

At sites of inflammation, transmigrated neutrophils, presumably in an adherent phase, encounter a milieu of soluble mediators. Activated macrophages and epithelial cells present at the site produce several CXC chemokines in addition to IL-8.^{24,25} We therefore measured adherence-dependent oxidant production to assess the modulatory effects of various chemokines on this neutrophil population's responses to inflammatory stimuli.

We showed that pretreatment with IL-8, GCP-2, or ENA-78 causes substantial loss of cell surface CXCR-1 and CXCR-2 expression with concomitant decrease in receptor affinity. Pretreatment with 10-nmol/L IL-8, but not 100-nmol/L GCP-2 or ENA-78, resulted in ablation of migration to subsequent maximal stimulatory doses of IL-8 (10 nmol/L). The mechanisms involved in differential inhibition of IL-8-induced chemotaxis by these chemokines are currently unknown. However, homologous desensitization of the CXC receptors has been the subject of several recent investigations. The CXC receptors are desensitized by protein kinase C-dependent phosphorylation of the C-terminal region of the receptor.³⁸⁻⁴⁰ Various stimulants, including fMLP, C5a, TNF- α , and GCP-2, have been shown to desensitize IL-8-induced calcium transients.^{1,2,11,41} The finding that GCP-2 had no significant effect on IL-8-induced chemotaxis suggests that

Statement of Clinical Relevance

Acute respiratory distress syndrome has emerged as a leading cause of morbidity and mortality for trauma and burn patients with otherwise survivable injuries. Neutrophil recruitment and disordered oxidant production in the alveolar interstitium are believed to be central to the extracellular matrix destruction and cytotoxicity associated with this condition. We present evidence that, in addition to inducing neutrophil migration, select CXC chemokines induce important anti-inflammatory responses in neutrophils. In this connection, the CXC chemokines participate in the modulation of the inflammatory response. Interleukin 8 and GCP-2 inhibit adherence-dependent oxidant production. However, of the chemokines tested, only IL-8 desensitized neutrophil migration to subsequent IL-8 challenge. We believe that both of these inhibitory responses are mediated via only 1 of the 2 CXC receptors, CXCR-2. We previously showed that loss of CXCR-2 is commensurate with severity of traumatic injury.⁴⁴ Loss of CXCR-2-mediated regulation may be an important prognostic indicator in these patients. Moreover, our present findings suggest that anti-inflammatory drugs can be modeled after the chemokines to selectively inhibit chemotaxis and/or oxidant production by differentially activating CXCR-2.

signaling for intracellular calcium elevation and chemotaxis are independently inhibitable.

We also present novel evidence that CXC chemokines suppress stimulant-induced oxidant production. Interleukin 8, ENA-78, and GRO α previously have been shown to enhance or to prime the oxidative response to stimulants such as fMLP.¹⁴ Moreover, in suspension-phase neutrophils, some CXC chemokines are capable of inducing an oxidative response.^{42,43} Herein we show that IL-8 and GCP-2 inhibit TNF- α -induced oxidant production, an adherence-dependent response. Granulocyte chemotactic protein 2, but not IL-8 or ENA-78, also inhibits C5a- and fMLP-induced oxidant production in adherent neutrophils. These findings further support the notion of receptor discrimination among different high-affinity ligands. Granulocyte chemotactic protein 2 and IL-8 in this instance have distinctly different regulatory effects on neutrophil responses to IL-8, C5a, and fMLP.

We propose that the CXC chemokines function within what we term a multiple ligand-single receptor regulatory system. The CXC chemokines, although competitively binding the same receptor(s), subserve independent and important regulatory functions. The data suggest that chemokine receptors discriminate between ligands and accordingly mediate ligand-specific responses. Despite reports that IL-8 and GCP-2 induce chemotaxis with similar efficacy, our data suggest that they disparately inhibit IL-8-induced chemotaxis. Although they equipotently decreased cell surface CXC receptor expression and TNF- α -induced oxidant production, only GCP-2 inhibited C5a- or fMLP-induced oxidant production.

Broadly, our data suggest that the existence of multiple molecules that bind to the same promiscuous receptor permits ligand-specific regulation of cell re-

sponses. The operation of such a multiple ligand–single receptor regulatory system in neutrophils allows the character of the inflammatory response to be coordinated temporally and spatially by the composition of the inflammatory milieu. We suspect that the CC chemokines, which similarly bind promiscuous receptors, differentially modulate monocyte and lymphocyte immune responses. Exploitation of the anti-inflammatory and immunosuppressive actions of these chemoattractant receptors may prove beneficial in the development of novel therapeutic interventions.

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REFERENCES

- Asagoe K, Yamamoto K, Takahashi A, et al. Down-regulation of CXCR2 expression on human polymorphonuclear leukocytes by TNF-alpha. *J Immunol*. 1998; 160:4518-4525.
- Tomhave ED, Richardson RM, Didsbury JR, Menard L, Snyderman R, Ali H. Cross-desensitization of receptors for peptide chemoattractants: characterization of a new form of leukocyte regulation. *J Immunol*. 1994;153:3267-3275.
- Dunlevy JR, Couchman JR. Interleukin-8 induces motile behavior and loss of focal adhesions in primary fibroblasts. *J Cell Sci*. 1995;108:311-321.
- Ben-Baruch A, Grimm M, Bengali K, et al. The differential ability of IL-8 and neutrophil-activating peptide-2 to induce attenuation of chemotaxis is mediated by their divergent capabilities to phosphorylate CXCR2 (IL-8 receptor B). *J Immunol*. 1997;158:5927-5933.
- Campbell JJ, Foxman EF, Butcher EC. Chemoattractant receptor cross talk as a regulatory mechanism in leukocyte adhesion and migration [process citation]. *Eur J Immunol*. 1997;27:2571-2578.
- Baggiolini M, Dewald B, Moser B. Interleukin-8 and related chemotactic cytokines: CXC and CC chemokines [review]. *Adv Immunol*. 1994;55:97-179.
- Koch AE, Kunkel SL, Harlow LA, et al. Epithelial neutrophil activating peptide-78: a novel chemotactic cytokine for neutrophils in arthritis. *J Clin Invest*. 1994; 94:1012-1018.
- Ahuja SK, Murphy PM. The CXC chemokines growth-regulated oncogene (GRO) α , GRO β , GRO γ , neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. *J Biol Chem*. 1996;271:20545-20550.
- Imaizumi T, Albertine KH, Jicha DL, McIntyre TM, Prescott SM, Zimmerman GA. Human endothelial cells synthesize ENA-78: relationship to IL-8 and to signaling of PMN adhesion. *Am J Respir Cell Mol Biol*. 1997;17:181-192.
- Jones SA, Moser B, Thelen M. A comparison of post-receptor signal transduction events in Jurkat cells transfected with either IL-8R1 or IL-8R2: chemokine mediated activation of p42/p44 MAP-kinase (ERK-2). *FEBS Lett*. 1995;364:211-214.
- Van Damme J, Wuyts A, Froyen G, et al. Granulocyte chemotactic protein-2 and related CXC chemokines: from gene regulation to receptor usage. *J Leukoc Biol*. 1997;62:563-569.
- Lee J, Horuk R, Rice GC, Bennett GL, Camerato T, Wood WI. Characterization of two high affinity human interleukin-8 receptors. *J Biol Chem*. 1992;267:16283-16287.
- Vita N, Lefort S, Brouilaud MJ, Magazin M, Guillemot JC, Ferrara P. Functional linkage of the GRO β and IL-8 receptors on the surface of human neutrophils. *Eur Cytokine Netw*. 1993;4:197-204.
- Green SP, Chuntharapai A, Curnutte JT. Interleukin-8 (IL-8), melanoma growth-stimulatory activity, and neutrophil-activating peptide selectively mediate priming of the neutrophil NADPH oxidase through the type A or type B IL-8 receptor. *J Biol Chem*. 1996;271:25400-25405.
- Ahuja SK, Shetty A, Tiffany HL, Murphy PM. Comparison of the genomic organization and promoter function for human interleukin-8 receptors A and B. *J Biol Chem*. 1994;269:26381-26389.
- Murphy PM. Neutrophil receptors for interleukin-8 and related CXC chemokines. *Semin Hematol*. 1997;34:311-318.
- Suzuki H, Prado GN, Wilkinson N, Navarro J. The N terminus of interleukin-8 (IL-8) receptor confers high affinity binding to human IL-8. *J Biol Chem*. 1994; 269:18263-18266.
- Ahuja SK, Lee JC, Murphy PM. CXC chemokines bind to unique sets of selectivity determinants that can function independently and are broadly distributed on multiple domains of human interleukin-8 receptor B: determinants of high affinity binding and receptor activation are distinct. *J Biol Chem*. 1996;271:225-232.
- Wu L, Ruffing N, Shi X, et al. Discrete steps in binding and signaling of interleukin-8 with its receptor. *J Biol Chem*. 1996;271:31202-31209.
- Wuyts A, Proost P, Lenaerts JP, Ben-Baruch A, Van Damme J, Wang JM. Differential usage of the CXC chemokine receptors 1 and 2 by interleukin-8, granulocyte chemotactic protein-2 and epithelial-cell-derived neutrophil attractant-78. *Eur J Biochem*. 1998;255:67-73.
- Wuyts A, Van Osselaer N, Haelens A, et al. Characterization of synthetic human granulocyte chemotactic protein 2: usage of chemokine receptors CXCR1 and CXCR2 and in vivo inflammatory properties. *Biochemistry*. 1997;36:2716-2723.
- Froyen G, Proost P, Ronsse I, et al. Cloning, bacterial expression and biological characterization of recombinant human granulocyte chemotactic protein-2 and differential expression of granulocyte chemotactic protein-2 and epithelial cell-derived neutrophil activating peptide-78 mRNAs. *Eur J Biochem*. 1997;243:762-769.
- Proost P, De Wolf Peeters C, Conings R, et al. Identification of a novel granulocyte chemotactic protein (GCP-2) from human tumor cells: in vitro and in vivo comparison with natural forms of GRO, IP-10, and IL-8. *J Immunol*. 1993;150:1000-1010.
- Walz A, Strieter RM, Schnyder S. Neutrophil-activating peptide ENA-78. *Adv Exp Med Biol*. 1993;351:129-137.
- Walz A, Schmutz P, Mueller C, Schnyder-Candrian S. Regulation and function of the CXC chemokine ENA-78 in monocytes and its role in disease. *J Leukoc Biol*. 1997;62:604-611.
- Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B. Both interleukin-8 receptors independently mediate chemotaxis: Jurkat cells transfected with IL-8R1 or IL-8R2 migrate in response to IL-8, GRO α and NAP-2. *FEBS Lett*. 1994; 341:187-192.
- Chuntharapai A, Kim KJ. Regulation of the expression of IL-8 receptor A/B by IL-8: possible functions of each receptor. *J Immunol*. 1995;155:2587-2594.
- Petersen F, Flad HD, Brandt E. Neutrophil-activating peptides NAP-2 and IL-8 bind to the same sites on neutrophils but interact in different ways: discrepancies in binding affinities, receptor densities, and biologic effects. *J Immunol*. 1994; 152:2467-2478.
- Harter L, Petersen F, Flad HD, Brandt E. Connective tissue-activating peptide III desensitizes chemokine receptors on neutrophils: requirement for proteolytic formation of the neutrophil-activating peptide 2. *J Immunol*. 1994;153:5698-5708.
- Solomkin JS, Nelson RD, Chenoweth DE, Solem LD, Simmons RL. Regulation of neutrophil migratory function in burn injury by complement activation products. *Ann Surg*. 1984;200:742-746.
- Solomkin JS, Tindal CJ, Cave CM, Zemlan F. Regulation of TNF-alpha receptor expression on human neutrophils by various cell activating factors. *J Surg Res*. 1994;56:261-266.
- De Togni P, Bellavite P, Della BV, Grzeskowiak M, Rossi F. Intensity and kinetics of the respiratory burst of human neutrophils in relation to receptor occupancy and rate of occupation by formylmethionylleucylphenylalanine. *Biochim Biophys Acta*. 1985;838:12-22.
- Huber D, Balda MS, Matter K. Transendothelial migration of neutrophils. *Invasion Metastasis*. 1999;18:70-80.
- Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood*. 1996; 88:146-157.
- Solomkin JS, Bass RC, Bjornson HS, Tindal CJ, Babcock GF. Alterations of neutrophil responses to tumor necrosis factor α and interleukin-8 following human endotoxemia. *Infect Immunol*. 1994;62:943-947.
- Goodman ER, Kleinstein E, Fusco AM, et al. Role of interleukin 8 in the genesis of acute respiratory distress syndrome through an effect on neutrophil apoptosis. *Arch Surg*. 1998;133:1234-1239.
- Jiang JX, Tian KL, Chen HS, Zhu PF, Wang ZG. Plasma cytokines and endotoxin levels in patients with severe injury and their relationship with organ damage. *Injury*. 1997;28:509-513.
- Richardson RM, DuBose RA, Ali H, Tomhave ED, Haribabu B, Snyderman R. Regulation of human interleukin-8 receptor A: identification of a phosphorylation site involved in modulating receptor functions. *Biochemistry*. 1995;34:14193-14201.
- Mueller SG, Schraw WP, Richmond A. Activation of protein kinase C enhances the phosphorylation of the type B interleukin-8 receptor and stimulates its degradation in non-hematopoietic cells. *J Biol Chem*. 1995;270:10439-10448.
- Mueller SG, White JR, Schraw WP, Lam V, Richmond A. Ligand-induced desensitization of the human CXC chemokine receptor-2 is modulated by multiple serine residues in the carboxyl-terminal domain of the receptor. *J Biol Chem*. 1997; 272:8207-8214.
- Mueller SG, Schraw WP, Richmond A. Melanoma growth stimulatory activity enhances the phosphorylation of the class II interleukin-8 receptor in non-hematopoietic cells. *J Biol Chem*. 1994;269:1973-1980.
- Jones SA, Wolf M, Qin S, Mackay CR, Baggiolini M. Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes: NADPH oxidase

and phospholipase D are activated through IL-8R1 but not IL-8R2. *Proc Natl Acad Sci U S A*. 1996;93:6682-6686.

43. Walz A, Meloni F, Clark Lewis I, von Tscherner V, Baggiolini M. $[Ca^{2+}]_i$ changes and respiratory burst in human neutrophils and monocytes induced by NAP-1/interleukin-8, NAP-2, and GRO/MGSA. *J Leukoc Biol*. 1991;50:279-286.
44. Quaid GA, Cave C, Robinson C, Williams MA, Solomkin JS. Preferential loss of CXCR-2 receptor expression and function in patients who have undergone trauma. *Arch Surg*. 1999;134:1367-1372.

DISCUSSION

H. Hank Simms, MD, Providence, RI: I congratulate Mr Williams on his fine presentation of complex material. This is another in a series of insightful papers from Dr Solomkin's laboratory investigating the regulation of the human inflammatory response, in this case, regulation of neutrophil proinflammatory functioning by CXC chemokines. I have a few questions for you.

Do you have an explanation for the different profiles of receptor down-regulation? Why is it that only IL-8 suppresses CXCR-1?

Second, what is the explanation for the apparent discrepancy regarding the magnitude of the loss of the CXCR-2 in the radiolabeled GRO α experiments between the flow cytometry and radiolabeled ligand assays, 50% vs 20%, respectively? That seems like a rather large difference to me.

Third, are matrix proteins, in this case, fibronectin, required for the biological response to the chemokines regarding H₂O₂ [hydrogen peroxide] production?

You have concluded in your discussion that pretreatment with IL-8 "ablates" migration of subsequent IL-8 exposure; however, I think a conclusion of this magnitude is not completely supported by the data, because the degree of homologous desensitization that you have shown is on the order of 50% to 65%. Parenthetically, our laboratory has failed to show homologous or heterologous desensitization using IL-8 or GRO α when apoptosis was the biological end point. So do you think your chemokine data is biological end point dependent?

You have implied in your discussion that you believe that chemokine receptors are being internalized via receptor phosphorylation. To rule out shedding or to corroborate this hypothesis, have you performed experiments in the presence of cytoskeleton stabilizers such as phalloidin, cytochalasin D, or taxol?

Lastly, the most dramatic differences with IL-8 vs GCP-2 signaling for H₂O₂ production is with C5a stimulation. I would ask you to speculate whether it is plausible that the differences in oxidant production are not due to a multiple ligand-single receptor regulatory system in vivo but potentially a multiple ligand-multiple regulatory protein system, at least regarding complement, such as factors H, I, or the C3b convertase, which would determine the bioavailability of the C5a substrate.

Mr Williams: Thank you, Dr Simms, for your very provocative questions and comments.

With regard to your first question as to why there is a differential down-regulation of CXCR-1 where only IL-8 pretreatment will induce that down-regulation, this is not an uncommon finding. In fact, several studies have shown that CXCR-2 is preferentially down-regulated in response to a variety of stimuli, including the chemokines C5a, fMet-Phe [formylmethionyl phenylalanine], and, as we recently reported, TNF- α . So it does appear that CXCR-2, its expression is a little more labile, or at least it is more easily internalized.

In fact, Richardson³⁸ had published some results that suggested that one of the reasons why CXCR-2 is down-regulated more easily than CXCR-1 may be phosphorylation-dependent, and that may be one of the reasons, or at least the explanations, for why certain chemokines, such as IL-8, will

down-regulate CXCR-1 as opposed to the other CXC chemokines and that the CXCR-1 is more resistant to internalization. However, with longer exposure, we may actually observe that CXCR-1 would be internalized. We have examined time points up to 120 minutes using GRO α and NAP-2, and neither one of them still was able to down-regulate CXCR-1.

I would suspect that if we were to pretreat with GCP and ENA-78, which, if you recall from the slides, down-regulated the receptor CXCR-1, but it had a rather wide error bar, my suspicion is that if we pretreated for a longer period of time with those chemokines that we would indeed see a substantial down-regulation of CXCR-1. Those experiments remain to be performed still.

Regarding the discrepancy between the flow cytometry and the radioligand binding studies, we believe that one of the discrepancies—it was a 50% down-regulation by flow cytometry vs approximately a 20% down-regulation by radioligand binding studies—one explanation for this is that we believe that with the radioligand binding studies, we may have binding of GRO α to the receptor as a dimer. Although those differences were not significant, there was a substantial difference, 50% vs 20%.

You asked another question about the dependence on matrix proteins, and, indeed, we have only tested this in neutrophils in terms of suppression of oxidant production. We have only tested it in neutrophils that were adherent to fibronectin at this time. And one of the reasons why we chose to use a matrix-dependent assay is that there are discrepant findings, or at least reports in the literature that suggest that in suspension phase cells, these chemokines can actually stimulate oxidant production, or even, at least at the very minimum, prime the response to stimulus such as fMet-Phe.

We believe that this may be an artifact and that the treatments for the suspension phase cells actually use cytochalasin B and disrupts the actin cytoskeleton, probably to the similar degree that adherence will do the same thing: cause cytoskeletal reorganizations. So it may actually be matrix protein dependent. We have not done it in suspension phase cells for that reason.

The comment in the article about CXC chemokines or, particularly, the IL-8, ablating the chemotactic response, basically may be a typographical error. What happened originally, as we examined our data, we initially would subtract out the buffer response, the spontaneous migration, and when you do that, it would appear as if the chemotactic response was ablated. However, we thought that that might be a little misleading, so what we would do is instead of subtracting out the spontaneous migration, we would just measure the maximum distance migrated by the neutrophils following exposure to these chemokines.

The observation that you had in your laboratory where you found that IL-8 does not desensitize the ability of subsequent exposure to IL-8 to suppress apoptosis is an interesting finding and that it is biological end point dependent—I think that's the phrasing you used for that—and I agree with you wholeheartedly. I believe that it is biological end point dependent, as we tried to point out in our presentation, and that is one of the reasons why we used 2 different functional assays, chemotaxis and oxidant production. As you see, we had different responses for the functions that were measured. So we do believe that it is biological end point dependent.

And then, finally, your question regarding the regulatory proteins, it is possible that one of the mechanisms by which these chemokines exert their differential effects may not necessarily be receptor dependent. That is a possibility and it is even plausible, however, we have not examined that possibility to this date.