Granulocyte-Colony Stimulating Factor and Lipopolysaccharide Regulate the Expression of Interleukin 8 Receptors on Polymorphonuclear Leukocytes*

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Interleukin 8 (IL-8) is a potent chemoattractant and activating factor for human polymorphonuclear leukocytes (PMN) and hence plays a critical role in the pathogenesis of acute inflammation. Two unique but homologous receptors for IL-8 have been cloned (IL-8RA and -RB), each of which binds the IL-8 ligand with high affinity. PMN stimulated by chemokines or lipopolysaccharide (LPS) exhibit changes in IL-8R mRNA and 125I-IL-8 binding. Granulocyte-colony stimulating factor (G-CSF) treatment of PMN enhances, and LPS inhibits, IL-8R mRNA expression. Similarly, 125I-IL-8 ligand binding to PMN is increased by G-CSF and decreased by LPS treatment. The stimulatory effect of G-CSF on IL-8R expression is transcriptional as it is inhibited by actinomycin D and is evident in nuclear run-on analyses. In contrast, LPS down-regulates IL-8R by both transcriptional and post-transcriptional mechanisms. The alterations in IL-8R expression are associated with similar changes in the IL-8-induced chemotactic responses of PMN. In conclusion, the two types of IL-8 receptor differ in their cellular distribution and are regulated in response to cytokines and LPS. Regulation of IL-8R expression by endogenous and exogenous immunomodulators may be important in the in vivo control of PMN effector functions in inflammation.

The recruitment and activation of polymorphonuclear leukocytes is the hallmark of acute inflammation. Chemoattractant factors produced at an inflammatory site regulate vascular adhesion, transendothelial migration, and the movement of leukocytes through the extracellular matrix. Interleukin 8 is the prototype of a family of chemoattractant cytokines known as chemokines (1), which regulate these migratory processes and determine the cellular composition of the inflammatory response by their target cell specificity (reviewed in Refs. 1 to 3). In particular, several members of the chemokine subfamily: IL-8, GRO/melanoma growth-stimulating activity, neutrophil-activating peptide 2 (NAP-2), and ENA-78 are neutrophil chemotactic (4–7). These chemokines are produced by a wide range of cell types, notably macrophages, in response to a diverse array of stimuli including proinflammatory cytokines, such as IL-1 and TNF, as well as LPS (1, 2).

Two receptors exhibiting high affinity binding (Kd ~2 nM) for IL-8 have been cloned, and hence have been designated as type A and B IL-8 receptors. The type A IL-8 receptor was cloned by direct expression from a PMN library (8) and binds IL-8 (9, 10). In contrast, the type B receptor was identified from HL60 myelomonocytic cells (11) and has subsequently been demonstrated to also bind GRO-a/melanoma growth-stimulating activity with high affinity (Kd ~2 nM), and NAP-2 and ENA-78 with lower affinity (9, 10). The IL-8R proteins are members of the rhodopsin superfamily of seven-transmembrane domain, G-protein-coupled receptors. They share 77% overall amino acid identity, including two matching regions of 105 and 64 amino acids, and their genes co-localize to chromosome 2q35 (12). The chemokine receptors, including the IL-8R and the recently cloned β chemokine receptors (13, 14), form a new subfamily of the rhodopsin receptor superfamily. IL-8RA is prominently expressed only in PMN, although the mRNA is detectable in differentiated HL60 cells (15). In contrast, IL-8RB is prominently expressed in PMN, and the mRNA is also widely distributed in myelomonocytic cell lines (U937, THP-1, and HL60), i.e., the urkat T cell line, as well as melanoma and fibroblast lines (15). In addition, we have recently obtained evidence that the IL-8R mRNAs are expressed in freshly prepared peripheral blood T cells, but are progressively diminished by incubation with or without anti-CD3 stimulation (16).

In order to better understand the physiological control of IL-8-mediated events in acute inflammation we have investigated the in vitro regulation of the IL-8 receptors A and B in human PMN.

MATERIALS AND METHODS

Receptor cDNAs—A 1050-base pair sequence of the IL-8RA cDNA (nucleotides 20–1030 of the published cDNA) was amplified in a polymerase chain reaction with two synthetic oligonucleotides, 5′-tgctgaaacctgaga-3′, 5′-gaatcagacagaga-3′, from human genomic DNA using a thermocycler oven (Bios Corp., New Haven, CT). The reaction conditions consisted of three cycles of 96°C × 1 min, 65°C × 1 min, 72°C × 1 min, followed by 30 cycles of 94°C × 30 s, 65°C × 45 s, 72°C × 30 s, and a final extension step of 72°C × 10 min. For Northern analyses, cDNAs encoding regions of divergence between IL-8RA and -RB were prepared. A 245-base pair AccI fragment (nucleotides 1–245) of leucylphenylalanine; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; CAT, chloramphenical acetyltransferase.
this IL-8RA cDNA and a 552-base pair HhaI-HindIII fragment of an IL-8RB clone previously isolated from a U937 myelomonocytic cell library (Invitrogen) (12) consisting of nucleotides 698-1250 of the published cDNA were chosen. These regions share only approximately 60% nucleotide homology with the analogous region of the other IL-8R.

Cells—Peripheral blood leukocytes enriched for mononuclear cells or for granulocytes were obtained from normal donors by leukapheresis. Granulocytes were purified by dextran sedimentation, followed by Ficoll gradient centrifugation and hypotonic lysis of red blood cells. PMN were collected, washed in phosphate-buffered saline, and resuspended at 2.5-5 x 10^6/ml in RPMI 1640 supplemented with 10% fetal calf serum. The purity of the PMN preparations was judged to be greater than 90-95% by morphological criteria; the remaining cells were typically lymphocytes.

Cell Stimulation—Purified PMN were incubated in tissue culture flasks (Costar) in the presence or absence of various cytokines or LPS. Prior to harvesting for RNA preparation, binding or chemotaxis assays cell viability was assessed by trypsin blue exclusion. The recombinant human cytokines used in these studies were obtained from the following sources: IL-8 was provided by Dr. M. Yamada of Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). TNF-α, GM-CSF, G-CSF, and GRO-α were provided by Dr. Craig Reynolds of the National Cancer Institute (Frederick, MD). The recombinant G-CSF was originally obtained from Amgen (Thousand Oaks, CA). Purified LPS (Escherichia coli 055:BS) was purchased from Difco Laboratories. Actinomycin D and fMLP were purchased from Sigma. Purified peripheral blood from volunteers was activated for 8 h on anti-CD3 (OKT3, Becton Dickinson) coated plates prior to harvesting for RNA.

Northern Blot Analyses—Total RNA was prepared from guanidinium thiocyanate-treated cell lysates using CsCl ultracentrifugation (17). The RNA was electrophoresed through 1% agarose/formaldehyde gels. The samples were transferred to nylon membranes (Nytran, Schleicher & Schuell), fixed by UV cross-linking, and hybridized overnight in a solution containing 1.39 M NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 0.2 M EDTA, 0.8 M each of dATP, dCTP, and dGTP. After two rounds of ethanol precipitation, the RNA pellets were resuspended in TES buffer (10 mM Tris-HCl, pH 7.4, 0.1% SDS, and 0.2% SSA) before estimation of the percentage of incorporation into the RNA (usually 70-80%). 10 µg of denatured plasmid cDNAs were slot-blotted onto nitrocellulose membranes, including full-length cDNAs for the IL-8RA and -B, β-actin (as above), and pGEM-3 as a negative control. Hybridization was performed for 48-72 h at 42°C in a freshly prepared solution (50% deionized formamide, 4 X SSC, 0.1% SSA, 100 µg of sonicated salmon sperm DNA, 5 X Denhardt's solution, 100 µg/ml of RNase, pH 7.0) containing 1-2 x 10^6 cpm/ml of labeled RNA. The membrane strips were washed three times for 20 min at room temperature in 2 X SSC, 0.2% SDS, followed by three washes for 30 min at 56°C in 0.1% SSA, 0.1% SSA. The membranes were exposed to Kodak XAR film for 1-2 weeks after which the autoradiographs were scanned by densitometer.

Receptor Binding Assays—125I-IL-8 binding was performed as described previously (19). In brief, 2 x 10^6 PMN were washed twice in chilled phosphate-buffered saline, resuspended in chilled RPMI 1640 with 10 mg/ml bovine serum albumin (Sigma), 25 µM HEPES, and then incubated in duplicate with 0.1 ng of 125I-IL-8 (specific activity 2200 Ci/mmole, DuPont NEN) in a total volume of 200 µl. The degree of nonspecific binding was determined by parallel incubation in the presence of a 500-fold excess of unlabeled IL-8. After incubation at 4°C for 2 h, the cells were pelleted by brief centrifugation and then washed twice with excess binding medium (as above). The tips of the tubes were cut, and radioactivity was counted in a γ counter. The nonspecifically bound radioactivity was subtracted from the total bound activity to establish specific binding.

Chemotaxis Assays—Cell migration was evaluated using a 48-well microchemotaxis chamber technique (20) with polycarbonate filters (5 µm pore size, Nucleopore Corp., Pleasanton, CA). PMN were resuspended in RPMI 1640 containing 1% bovine serum albumin (1 x 10^6 cells/ml, and 50 µl per well) was applied to the upper chamber. Chemoattractants were resuspended in the same medium and applied to the lower chamber. All responses were assayed in triplicate. Migration was allowed to continue at 37°C for 30 min in a 5% CO2, moist atmosphere. After the nonmigrating cells were washed from the upper surface of the filters, migrating cells were fixed in methanol and stained with Diff-Quik. The number of migrating cells in five high-powered fields were counted for each well.

RESULTS

The IL-8RA and -B mRNA Transcripts—In Northern analyses of total RNA prepared from PMN, the specific probes showed that the mRNA transcript for the IL-8RA was 2.4 kilobases and for IL-8RB was 3.1 kilobases. In addition, in some experiments, the IL-8RA probe hybridized at high stringency to an additional band at 2.2 kilobases, which may represent a transcript from the IL-8RA pseudogene (designated IL-8RAP; Ref. 21).

Regulation of IL-8 mRNA Expression by Cytokines and LPS—In initial experiments, purified human PMN were incubated for 6 h with a panel of different cytokines known to be present in acute inflammatory states, as well as the neutrophil chemoattractant fMLP, or with LPS. After each treatment, the viability of the cells was confirmed to be greater than 95% by Trypan Blue exclusion before harvesting for RNA. The Northern blots containing these RNAs were hybridized sequentially with the IL-8R probes, followed by the β-actin probe to assess the relative amounts of RNA loaded. This analysis demonstrated a substantial increase in specific mRNA for both IL-8R after treatment with G-CSF at 50 ng/ml and a dramatic reduction in the steady state IL-8R mRNA after PMN were treated with LPS at 10 ng/ml. A less marked down-regulation was found after stimulation with tumor necrosis factor α (Fig. 1).
The G-CSF and LPS effects were chosen for further study.

G-CSF and LPS Regulate IL-8R mRNA via Transcriptional Mechanisms—Changes in the steady-state level of RNA expression of a gene result from alterations in either the rate of transcription, the rate of degradation of the message, or a combination of both. After establishing the optimal doses of G-CSF and LPS (data not shown), the kinetics of the alterations in the steady state levels of IL-8R expression in PMN were investigated further. The mRNA expression of IL-8RA and -B was regulated in parallel in all experiments. Treatment of PMN with G-CSF for periods up to 18 h produced a dramatic increase (2–10-fold in four separate experiments) in steady-state IL-8R mRNA with a plateau reached at approximately 6–18 h. This contrasted with a slow but steady decline in the mRNA levels in untreated PMN (Fig. 2A). LPS treatment induced a significantly more rapid decline in the steady-state mRNA, evident after 1–2 h of treatment. Actinomycin D at a final concentration of 10 μM was added to PMN incubations in order to inhibit transcription. This treatment abrogated the up-regulation of IL-8R mRNA induced by G-CSF (Fig. 2B), whereas the addition of actinomycin D to untreated PMN induced further small decrease in steady-state mRNA levels. Nuclear run-on analyses confirmed the impression that G-CSF regulated IL-8R expression by enhancing transcription. The nuclear RNA signals for IL-8RA and -B in PMN were enhanced 4–6-fold in three separate experiments after 3–6 h of G-CSF treatment (Fig. 3). These data suggest that IL-8R mRNA expression in PMN is dependent on continuous transcription of the IL-8R genes, and, further, that the G-CSF effect is dependent upon enhanced transcriptional activity of the IL-8R genes.

LPS De-stabilizes IL-8R mRNA—The half-life \( t_{1/2} \) of the IL-8R mRNA was determined by treatment of PMN in the presence or absence LPS after prior incubation with actinomycin D. Least squares regression analysis of the normalized autoradiographic data was used to calculate the numerical value of the \( t_{1/2} \). In untreated PMN, the IL-8R mRNA \( t_{1/2} \) was 3.6 h (S.E. 0.6 h; n = 3), whereas in the presence of LPS the half-life was reduced to approximately 2.2 h (S.E. 0.4 h; n = 3; Fig. 4). The estimated half-lives for the IL-8RA and -B mRNAs in these experiments were not significantly different. The nuclear run-on analyses also suggested that LPS inhibited IL-8R transcription within 3 h of treatment (2–3-fold in three separate experiments; Fig. 3). Furthermore, the addition of LPS, in combination with G-CSF treatment of PMN, abrogated the up-regulation associated with G-CSF, resulting in a similar down-regulation of transcriptional activity to that seen with LPS treatment alone (Fig. 3). Taken together, these data suggest that LPS inhibits IL-8R expression by a combination of transcriptional inhibition and decreasing mRNA stability.

G-CSF and LPS Also Regulate IL-8 Receptor Binding on PMN—The expression of IL-8R on the surface of PMN was estimated by measurement of \(^{125}\text{I}-\text{IL-8} \) binding. As both IL-8R demonstrate similar high affinity for the IL-8 ligand, these data are likely to reflect the combined binding capacity of both IL-8R types on the PMN surface. Fig. 5A shows that G-CSF treatment produced a sustained increase in IL-8 binding which was approximately 2-fold greater after 20 h in treated PMN in comparison with untreated cells. In contrast, LPS treatment rapidly reduced IL-8 binding on PMN.
G-CSF and LPS Regulate IL-8 Chemotactic Responses in PMN—The ability of PMN to migrate in response to IL-8 in a standard chemotaxis assay was studied in order to examine the possible functional consequences of the alterations in IL-8R expression. G-CSF treatment produced a significant increase in the number of PMN migrating in response to an IL-8 chemotactic gradient (Fig. 5B). LPS treatment was associated with a rapid reduction in the IL-8 chemotactic response. Similar results were obtained when GRO-α was used as the chemotactic stimulus (data not shown), confirming that the expression and functional response of IL-8RB (which binds GRO) is regulated in this system.

DISCUSSION

The IL-8Rα and -B are abundantly expressed on human PMN. The IL-8R mRNAs are reciprocally regulated in response to stimuli such as G-CSF and LPS, which are likely to be present in sites of acute inflammation.

Responses to chemoattractant cytokines, including IL-8, may be desensitized by continued stimulation (22), confirming that the chemokine receptors are regulated via agonist-dependent mechanisms as has been shown for other rhodopsin family members (reviewed in Ref. 23). In the present work, we have demonstrated that the two IL-8R also exhibit regulation in response to exogenous stimuli. Treatment of PMN with G-CSF resulted in increased IL-8R mRNA expression, as well as IL-8 binding and chemotactic responsiveness. G-CSF has been shown to enhance PMN survival in vitro by inhibition of apoptosis (24). However, the up-regulation of IL-8R expression we have demonstrated is not explicable simply on the basis of improved PMN survival, as the IL-8R mRNA and chemotactic response to IL-8 are significantly increased above baseline levels in G-CSF-treated PMN (Figs. 2 and 5). G-CSF enhances IL-8R expression by a transcriptional mechanism, as actinomycin D treatment blocked its effect, and nuclear run-on studies showed increased IL-8R signals after G-CSF treatment of PMN. This regulatory pathway, if direct, may indicate a novel G-CSF-responsive transcriptional regulatory element in the IL-8R genes. Indeed, we have recently isolated, sequenced, and characterized the genomic structure of the IL-8RB gene (25). IL-8RB promoter region-CAT constructs demonstrated enhanced expression in HL60 cells after treatment with G-CSF, thus providing evidence for a direct transcriptional effect of G-CSF on the IL-8RB gene. Comparison of the promoter region of this gene with that of another PMN chemoattractant recep-
tor, the fMLP receptor, has demonstrated the presence of several novel, but highly conserved, sequence motifs, which may represent tissue-specific transcriptional regulatory elements (25). Interestingly, these motifs were not detected in the promoter region of the IL-8RA gene (26).

Down-regulation of IL-8R expression and IL-8 responsiveness was demonstrated when PMN were incubated with LPS. This treatment induced a significant decrease in the half-life of the IL-8R mRNA, consistent with an effect on the stability of the message. In addition, IL-8R transcriptional activity appeared to be inhibited in nuclear run-on studies. LPS also has been shown to enhance the survival of PMN in vitro (24), and to induce the expression of several genes including IL-1β (27) and TNF-α (28). Hence, the inhibitory effect of LPS on IL-8R expression contrasts with these findings and is not related to reduced PMN survival. Hormone-induced reduction in mRNA stability has been documented for several G-protein-coupled receptors including the β2-adrenergic receptor and thrytropin-releasing hormone receptor (23). Although the mechanisms of this altered RNA stability are not yet clear, the 3'-untranslated region of the mRNA for the β2-adrenergic receptor, as well as several other rhodopsin family members, contain AU-rich elements correlated with highly regulated, short-lived mRNAs (23, 29). We have identified multiple regions of AU-rich sequence in the 3'-untranslated region of the IL-8RB gene (25).

The inhibitory effect of LPS on the chemotactic response of PMN to IL-8 was detected earlier than expected from the alteration in the mRNA. This may have been attributable in part to clumping and adhesion of PMN to the upper side of the polycarbonate filter. We have observed similar clumping effects with fMLP, yet LPS treatment enhances fMLP receptor mRNA expression in PMN and augments the PMN chemotactic response to fMLP. Similarly, the stimulatory effect of G-CSF on chemotactic responses to IL-8 was more rapid than would be expected from the transcriptional regulation. These apparent discrepancies may relate to differences in assay sensitivity or to independent effects of these stimuli on receptor turnover. Nevertheless, our data suggest that regulation of the IL-8R after some hours of PMN exposure to G-CSF or LPS is correlated with the transcriptional regulatory mechanisms defined here.

Our findings suggest that stimuli occurring in vivo in acute inflammation may alter the expression of IL-8R on PMN. We have provided preliminary evidence for the functional significance of this regulation in PMN chemotaxis. The capacity of G-CSF to stimulate PMN functions, including phagocytosis and superoxide generation, may be mediated in part by enhanced responsiveness to IL-8. Interestingly, exposure of PMN to LPS may result not only in the synthesis and release of a secondary cascade of proinflammatory cytokines including IL-1β, TNF-α, and IL-8 (30), but also in anti-inflammatory effects leading to decreased PMN responsiveness such as IL-1RA production (30) and down-regulation of IL-8R as we have documented here. Perhaps these immunomodulatory effects of LPS may preferentially direct PMN toward bacterially derived chemotraactants, such as fMLP. The kinetics and magnitude of these respective PMN responses may be important determinants of the outcome of acute neutrophilic inflammation.

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