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CXC Chemokines Suppress Proliferation of Myeloid Progenitor Cells by Activation of the CXC Chemokine Receptor 2

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IL-8 is one of the major mediators of the transendothelial migration of neutrophils from the circulation to the site of injury and infection. In this work we demonstrate that the CXC or α-chemokines, IL-8 and melanoma growth stimulatory activity (MGSA), induce myeloid suppression via direct action on progenitor cells, mediated by activation of the murine homologue of the CXC chemokine receptor-2 (CXCRII) or IL-8R B. We first show that proliferation of the IL-3-dependent murine myeloid progenitor cell line 32D is suppressed by human IL-8 and the functionally and structurally related peptide, MGSA. Second, we show for the first time the high endogenous expression of the murine CXCRII in 32D cells, as demonstrated by Northern blot analysis, binding to [125I]macrophage inflammatory protein-2, and macrophage inflammatory protein-2-induced calcium responses in 32D cells. Third, we demonstrate that IL-8 and MGSA induce a rise in intracellular calcium in 32D cells. The IL-8-induced Ca²⁺ response is desensitizing, since a second dose of IL-8 did not trigger a second calcium response. Other chemokines, including neutrophil-activating protein-2, platelet factor-4, RANTES, and macrophage chemotactic protein-1, neither suppressed the proliferation of 32D cells nor induced a rise in intracellular calcium. Finally, the IC₅₀ of IL-8- and MGSA-dependent suppression of proliferation of 32D cells is in good agreement with the EC₅₀ of IL-8- and MGSA-dependent activation of neutrophil Mac-1 up-regulation and chemotaxis. Our studies are consistent with the idea that IL-8 and MGSA suppress the proliferation of 32D cells by activation of murine CXCRII.

Cytokines modulate myelopoiesis by promoting or suppressing the proliferation and differentiation of myeloid progenitor cells. In contrast to the growth-promoting cytokines, the transmembrane signaling mechanisms of growth-suppressing cytokines are poorly understood. Exposure of bone marrow cells to chemokines (e.g., IL-8 and macrophage inflammatory protein-1 (MIP-1)α suppresses the proliferation of myeloid progenitor cells (1). Whether chemokines are acting on progenitor cells or on stromal cells of the bone marrow remains to be determined. Although previous studies have shown that several chemokines suppressed the proliferation of highly purified primary progenitor cells (2), the low frequency and heterogeneity of progenitor cells from bone marrow has precluded the determination of the transmembrane signaling mechanisms of chemokines that suppress the proliferation of progenitor cells.

Two major subfamilies of chemokines are distinguished on the basis of whether the first two cysteines are separated by a single residue (CXC or α-chemokines) or whether they are adjacent (CC or β-chemokines) (3). Chemokine receptors belong to the superfAMILY of G protein-coupled receptors encoded by cellular and viral genomes (4). The best characterized CXC chemokine is IL-8, a 72-amino acid peptide secreted in response to injury and infection. Multiple receptors have been identified to bind IL-8. Thus, IL-8 mediates the migration of neutrophils from the circulation to the site of injury by activation of IL-8R, subtypes A and B, or CXC chemokine receptors 1 and 2 (CXCRI and CXCRII) (5–7). The human CXCRII binds with high affinity to IL-8 and with low affinity to the structurally related CXC chemokines melanoma growth stimulatory activity (MGSA) and neutrophil-activating peptide-2 (NAP-2). The human CXCRII binds with high affinity to IL-8 and MGSA and with moderate affinity to NAP-2 (8). In contrast, murine neutrophils apparently express only the murine homologue of CXCRII. This receptor exhibits high affinity toward murine CXC chemokines MIP-2 and platelet-derived growth factor (PDGF) inducible gene, but low affinity to human IL-8 (9–12). Additionally, IL-8 is angiogenic and mitogenic for endothelial cells (13); however, the IL-8R subtype that mediates these effects is unknown. Both CXC and CC chemokines bind to the Duffy Ag of RBC (14) and to G protein-coupled receptors encoded by viral genomes. For example, IL-8 binds with high affinity to a G protein-coupled receptor encoded by Kaposi’s sarcoma B-associated herpes virus (15). Recent studies have suggested that the chemokine receptor that mediates the IL-8-dependent suppression of proliferation of bone marrow-derived progenitor cells appears to be IL-8R B or CXCRII (16). In this work, we show that IL-8 and MGSA suppress proliferation of the murine myeloid progenitor cell line 32D. The data are consistent with the idea that suppression of the proliferation of progenitor cells by IL-8 and MGSA is mediated by activation of the murine homologue of CXCRII.

Materials and Methods

Cell cultures

The murine IL-3 dependent 32D cell line was provided by Dr. J. Greenberger, University of Pittsburgh Medical School (Pittsburgh, PA). 32D cells were maintained in RPMI 1640 plus 15% heat-inactivated FBS and...
15% conditioned medium from the murine myelomonocytic cell line WEHI-3B as a source of crude IL-3 (17). Purified IL-3 was a gift from Amgen, Inc. (Thousand Oaks, CA). Cells were cultured at 37°C in a 5% CO₂ atmosphere and were maintained at a cell density of 0.5 \times 10^6 \text{ cells/ml}.

Murine stem cell lines CCE and D3 were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM supplemented with 10% FCS and 1000 U/ml of leukemia inhibitory factor (Life Technologies, Grand Island, NY).

**Agar colony assays**

Cultures of 32D cells were conducted as described by Metcalf (18). In brief, 300 cells were seeded in 35-mm petri dishes containing 1 ml of Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated FBS, 0.3% agar and IL-3, or 10% conditioned medium from the cell line WEHI-3B. Chemokines resuspended in PBS or an equal volume of PBS were added to the empty culture dish before addition of the cell suspension in agar medium. Colonies were scored on days 7 and 14 of culture.

**Intracellular calcium measurements**

Exponentially growing 32D cells were harvested by centrifugation and resuspended in a solution containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM NaHPO₄, 5 mM glucose, 20 mM HEPES (pH 7.4), 1 mg/ml BSA, and 1 mM probenecid. Cells \( (10^7/\text{ml}) \) were loaded with 5 \( \mu \text{M} \) of the calcium-sensitive dye indo-1/AM for that room temperature as previously described (19). Intracellular calcium levels were monitored at 37°C with a spectrofluorometer (Perkin-Elmer 650–10S, Norwalk, CT) using an excitation wavelength of 330 nm and an emission wavelength of 405 nm.

**Northern blot analysis**

Total RNA extracted from \( 10^7 \) cells using the Ultraspec RNA isolation system (Biotecx Laboratories, Houston, TX) was fractionated on 1% agarose-formaldehyde gels, blotted to nylon membranes, and cross-linked by brief exposure to UV irradiation. Membranes were hybridized to the murine CXCR2 cDNA labeled by the random priming procedure as described by the manufacturer (Pharmacia, Piscataway, NJ).

**Expression of murine CXCR2 in COS-7 cells**

The cDNA encoding the murine CXCR2 was synthesized by PCR as described previously (9) and subcloned into the expression vector pRC/CMV (Invitrogen, San Diego, CA). COS-7 cells were transiently transfected by the DEAE-dextran procedure using 10 \( \mu \text{g} \) of recombinant plasmid (5). Recombinant Tyr-MIP-2 was expressed in Escherichia coli and purified using a heparin column as described previously (20). Labeled \([^{125}\text{I}]\)MIP-2 was prepared by the chloramine-T procedure (5). Binding of \([^{125}\text{I}]\)Tyr-MIP-2 to transfected COS-7 cells was performed as previously described (8).

**Results and Discussion**

IL-8- and MGSA-induced suppression of proliferation of 32D cells

To elucidate the receptor signaling mechanisms of chemokine-induced suppression of myeloid progenitor cells, it is necessary to characterize tissue culture models of precursor cells that respond to growth factors. For this study, we have chosen the 32D cell line derived from bone marrow myeloid precursor cells. This cell line exhibits many features of normal hemopoietic progenitor cells. In
particular, the survival, proliferation, and regulation of the cell cycle of 32D cells requires the presence of CSFs. Additionally, this cell line exhibit clonogenic cell self-renewal (18).

The proliferation of 32D cells is readily monitored by agar colony assays. In Figure 1 we show that colony formation was dependent upon the concentration of IL-3 in the culture medium. Compact spherical colonies of various sizes were formed after 7 days in culture. No colony formation was observed in the absence of IL-3. Similar colony formations were observed after addition of conditioned medium from cultures of the cell line WEHI-3B, a source of crude IL-3. This result is in good agreement with those of previous studies on the requirement of IL-3 for the proliferation and survival of 32D cells (18). We then tested the effects of recombinant human chemokines on the colony formation of 32D cells. We found that chemokines, including IL-8, MGSA, NAP-2, platelet factor-4 (PF4), RANTES, and MCP-1. These results are distinct from those studies with myeloid progenitor cells from bone marrow of mice and humans (1, 16). Thus, CXC chemokines (e.g., IL-8, MIP-2, PF4, and IFN-γ-inducible protein and CC chemokines (e.g., MIP-1α and MCP-1) produced marked suppression of proliferation of progenitor cells derived from murine (BFU-E) granulocyte-macrophage CFU, erythrocyte burst-forming unit, and multipotent CFU granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) (16). The distinct pharmacologic profiles of the 32D cell line vs the heterogeneous population of progenitor cells from bone marrow are most likely due to the expression of chemokine receptors in a cell type-specific fashion. The 32D cell line may have derived from a subset of murine progenitor bone marrow cells that only express receptors for IL-8 and MGSA. Alternatively, the immortalized 32D cells may have lost the receptors for the other suppressive chemokines.

Expression of murine CXCR2 in 32D cells

Chemokines bind to a subfamily of G protein-coupled receptors that are expressed in a leukocyte-specific fashion. For example, receptors for CXC chemokines, such as IL-8 and MGSA, are highly expressed in neutrophils, whereas receptors for CC chemokines, including RANTES, MIP-1α, MIP-1β, and MCP-1, are expressed in monocytes, eosinophils, lymphocytes, and basophils (3). In addition, both CXC and CC chemokines have been shown to bind to the Duffy Ag of RBCs and to receptors encoded by viral genomes (14, 15, 21). It has been argued that separate receptor signal transduction mechanisms mediate the IL-8-induced suppression of proliferation of myeloid progenitor cells and the activation of neutrophils (1, 22). In this work, we examined the expression of the murine homologue of CXCR2 in 32D cells. First, we conducted Northern blot analysis of RNA extracted from the murine stem cell lines CCE and D3, murine neutrophils, and 32D cells, and probed with cDNA encoding the murine CXCR2. As shown in Figure 3, the stem cell lines did not express CXCR2 mRNA, whereas neutrophils and 32D cells expressed high levels. The level of expression of CXCR2 mRNA in 32D cells was similar to that in neutrophils. Most importantly, we demonstrate that 32D cells express active CXCR2, since the ligand for the murine

**FIGURE 4.** MIP-2-induced increase of intracellular calcium in 32D cells. Increasing amounts of recombinant Tyr-MIP-2 were added to 32D cells loaded with indo-1 as described in Materials and Methods. The final concentrations of Tyr-MIP-2 were: a, 2 nM; b, 5 nM; c, 10 nM; d, 20 nM; e, 40 nM; and f, 60 nM.

**FIGURE 5.** Binding of [125I]Tyr-MIP-2 to COS-7 cells transfected with murine CXCR2 cDNA. Transfected cells were incubated with [125I]Tyr-MIP-2 (1 nM) at 4°C for 2 h in the presence of increasing concentrations of unlabeled Tyr-MIP-2 and IL-8.
homologue of CXCR2, MIP-2, induced a rise in intracellular Ca\(^{2+}\) in a dose-dependent fashion (Fig. 4). In addition, the receptor cDNA amplified from RNA from 32D cells appeared to be identical with the murine homologue of CXCR2 that we previously described (9).

COS-7 cells transfected with the murine IL-8 receptor exhibited high affinity binding to [\( ^{125}\)I]MIP-2, with an apparent \( K_i \) of 2 nM (Fig. 5). This represents the first report demonstrating a high level of expression of CXCR2 in a nonneutrophil cell line. However, trace quantities of CXCR1 and CXCR2 mRNAs have been detected by reverse transcription-PCR in several cell types (23).

The high expression of murine CXCR2 mRNA in 32D cells suggests that IL-8 and MGSA mediate myelosuppression via the same
receptor that is expressed in neutrophils. In Figure 6, we show that IL-8 and MGSAP trigger a rise in intracellular calcium in 32D cells. The estimated EC_{50} for the IL-8-induced rise of intracellular calcium was 25 nM (Fig. 7); this agrees with the ID_{50} for the IL-8-dependent suppression of proliferation, suggesting that mobilization of intracellular calcium is one of the signaling mechanisms that regulates the proliferation of 32D cells.

32D cells pretreated with IL-8 failed to respond to a second dose of IL-8 and addition of MGSAP, indicating that IL-8 induced desensitization of the IL-8 receptor. In contrast, cells pretreated with MGSAP failed to respond to a second dose of MGSAP, but responded well to addition of IL-8. These observations are consistent with our and other recent studies, suggesting that MGSAP induces a weaker desensitization signal than IL-8. For example, IL-8 triggers a faster rate of internalization of IL-8R B than MGSAP, indicating that IL-8 induced de-sensitization of the IL-8 receptor. Based on these results, MGSAP promotes the suppression of myeloid progenitor cell by a non-invasive calcium transduction mechanism similar to that induced for neutrophil activation. This cell model should be suitable to examine the downstream molecular mechanisms of the IL-8-dependent suppression of proliferation of progenitor cells.

References


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