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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 (CXCR2) 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 CXCR2 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 CXCR2. The Journal of Immunology, 1998, 160: 906–910.

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4 Abbreviations used in this paper: MIP-1, macrophage inflammatory protein-1; CXCR, CXC chemokine receptor; MGSA, melanoma growth stimulatory activity; NAP-2, neutrophil-activating peptide-2; PF4, platelet factor-4; MCP-1, macrophage chemotactic protein-1.
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 × 10⁶ 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 (10⁷/ml) were loaded with 5 μM of the calcium-sensitive dye indo-1/AM for 1 hour at 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 10⁷ 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 μg of recombinant plasmid (5). Recombinant Tyr-MIP-2 was expressed in *Escherichia coli* and purified using a heparin column as described previously (20). Labeled [¹²⁵I]MIP-2 was prepared by the chloramine-T procedure (5). Binding of [¹²⁵I]Tyr-MIP-2 to transfected COS-7 cells was performed as previously described (6).

**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 multipotential 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 MIP-2, the murine homologue of MGSA, suppressed the proliferation of 32D cells, although at lower concentrations than IL-8 or MGSA (data not shown). No effects were observed with other chemokines, including 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 multipotential 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.
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).

**FIGURE 6.** IL-8- and MGSA-induced increase of intracellular calcium in 32D cells. IL-8 and MGSA were added at a final concentration of 250 nM to cells preloaded with indo-1.

**FIGURE 7.** Dose response of IL-8-induced increase of intracellular calcium. Increasing amounts of rIL-8 were added to 32D cells loaded with indo-1 as described in Materials and Methods. The final concentrations of IL-8 were: a, 10 nM; b, 25 nM; c, 50 nM; d, 125 nM; and e, 250 nM.

**FIGURE 8.** 32D cells pretreated with pertussis toxin failed to respond to IL-8. IL-8 (250 nM) was added to untreated and pertussis toxin-treated 32D cells loaded with indo-1. The ATP concentration added to the cells was 0.5 mM.

**FIGURE 9.** MIP-2 desensitized the IL-8 B-dependent calcium response. 32D cells were pretreated with MIP-2 (5 nM), and then IL-8 was added at a final concentration of 250 nM.
receptor that is expressed in neutrophils. In Figure 6, we show that IL-8 and MGSA 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-affinities of IL-8 and MGSA to the murine CXCR2, we examined COS-7 cells transfected with the murine CXCR2 cDNA. IL-8 with pertussis toxin (Fig. 8), indicating that the murine homologue fails to induce a rise in intracellular calcium in 32D cells pretreated IL-8-dependent calcium response in 32D cells pretreated with pertussis toxin (Fig. 8). IL-8, suggesting that the murine CXCR2 undergoes homologous desensitization (Fig. 8). NAP-2, PF4, RANTES, and MCP-1 failed to elicit a calcium response in 32D cells, consistent with their lack of effect on the suppression of proliferation of 32D cells. In neutrophils, CXCR1 and CXCR2 are coupled to pertussis toxin-sensitive G proteins. We have analyzed the sensitivity of the IL-8-dependent calcium response in 32D cells pretreated with pertussis toxin. Similar to the results obtained in neutrophils, IL-8 fails to induce a rise in intracellular calcium in 32D cells pretreated with pertussis toxin (Fig. 8). IL-8, indicating that the murine homologue of CXCR2 is coupled to G_{i} proteins. To determine the relative affinities of IL-8 and MGSA to the murine CXCR2, we examined the effect of IL-8 on the binding of recombinant IL-8 and MGSA. This result is in good agreement with those of previous studies indicating that the murine homologue of CXCR2 exhibits low affinity for human IL-8 (8–12).

The relationship between the apparent affinity of IL-8 to the murine CXCR2 and the EC_{50} of the IL-8-dependent suppression of proliferation is consistent with previous studies in murine neutrophils. For example, the apparent K_{d} of IL-8 binding to murine neutrophils is several orders of magnitude higher than the EC_{50} of IL-8 for Mac-1 up-regulation and chemotaxis (10). Of importance, IL-8 failed to induce a calcium response in cells pretreated with MIP-2 (Fig. 9), indicating that MIP-2 and IL-8 activate the same receptor. This observation plus the good correlation of the pharmacologic profile of chemokine-induced suppression of proliferation of 32D cells with the chemokine-induced calcium responses in 32D cells strongly suggest that IL-8- and MGSA-mediated myeloid suppression is via activation of CXCR2 in 32D cells. Further support for this idea is provided by a recent observation with a cell line derived from 32D named 32D-GR (26) that does not express CXCR2. IL-8, MGSA, and MIP-2 neither induced an increase in intracellular calcium nor suppressed the proliferation of 32D-GR cells (data not shown). These results are in good agreement with a recent report indicating that IL-8 failed to induce myelosuppression of bone marrow cells from mice deficient in CXCR2 (16).

In conclusion, this work demonstrates that the IL-8-dependent suppression of progenitor cell proliferation is mediated by direct action on the progenitor cells. The data suggest that IL-8 and MGSA promote the suppression of myeloid progenitor cells by triggering a 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.

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