High Activity Suppression of Myeloid Progenitor Proliferation by Chimeric Mutants of Interleukin 8 and Platelet Factor 4*

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Thomas J. Daly‡§, Gregory J. LaRosa, Sylvia Dolicht, Theodore E. Maionet, Scott Cooper†, and Hal E. Broxmeyer¶

From the ‡Repligen Corporation, Cambridge Massachusetts 02139 and the ¶Departments of Medicine (Hematology/Oncology), Microbiology/Immunology, and the Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202-5121.

The proliferation of human myeloid progenitor cells is negatively regulated in the presence of certain members of the chemokine family of molecules. This includes interleukin 8 (IL-8) and platelet factor 4 (PF4), which in combination are able to synergize, resulting in cell suppression at very low concentrations of these molecules. A series of PF4 and IL-8 mutant proteins were analyzed in an in vitro colony formation assay for myeloid progenitor cells to assess domains of these proteins that are required for activity. Mutation of either of the two DLQ motifs within PF4 resulted in an inactive protein. Perturbations within the IL-8 dimer interface region also resulted in mutants that were incapable of suppressing colony formation. A class of chimeric mutants consisting of domains of either PF4 and IL-8, Groα and PF4, or Groβ and PF4 were observed to inhibit myeloid cell proliferation at concentrations which were between 500- and 5000-fold lower than either the IL-8 or PF4 wild-type proteins alone. These chimeric mutants possessed activities that were comparable to or better than the activity observed when IL-8 and PF4 were added together in vitro. One of these highly active chimeric proteins was observed to be 1000-fold more active than either IL-8 or PF4 alone in suppressing not only the proliferation but also the cell cycling of myeloid progenitor cells following intravenous injection of the mutant into mice. Examination of additional IL-8-based mutants in the colony formation assay, which centered on the perturbation of the amino-terminal "ELR" motif, resulted in the observation that the highly active IL-8 mutant required both aspartic acid at amino acid residue 4 and either glutamine or asparagine at residue 6. Single mutations at either of these positions resulted in mutants with myosuppressive activity equivalent to wild-type IL-8. Mutants such as IL-8M1 and IL-8M10 were observed to be significantly reduced in their ability to activate isolated human neutrophils, suggesting that separate mechanisms may exist by which myeloid progenitor cells and neutrophils are affected by chemokines.

Myelopoiesis is a complex, highly regulated process, which is dependent on the action of both positive and negative growth factors to control the proliferation of primitive morphologically indistinct cells from hematopoietic organs to supply functional end-stage blood cells. Factors that stimulate cell growth and differentiation have been well characterized and include the colony-stimulating factors (GM-CSF)\(^1\), granulocyte colony-stimulating factor, and macrophage colony-stimulating factor), erythropoietin, some of the interleukin family members (e.g. IL-1, IL-3, IL-4, IL-6, IL-9, IL-11) as well as other cytokines including Steel factor 1-3). A number of suppressor molecules have also been identified. These include E-type prostaglandins, H-ferritin, lactoferrin, interferons, tumor necrosis factors, and transforming growth factor-β (1-3). More recently, several members of the chemokine family of proteins including macrophage inflammatory protein 1α (MIP-1α), MIP-2α (Groβ), interleukin 8 (IL-8), platelet factor 4 (PF4), monocyte chemotactic and activating peptide (MCAF/MCP-1), and γ interferon-inducible protein, molecular weight 10,000 (γIP10), have been demonstrated to possess inhibitory activity toward the proliferation of immature stem/progenitor cells in vitro and in vivo (4-13).

Chemokines are a family of small inducible proteins possessing structural similarities and high amino acid identities (14-16). Although activity differences exist between the proteins, all are believed to possess chemoattractant properties for various cell types. The family is subdivided into two groups based on positioning of cysteine residues within the amino-terminal domain. The CXC group (2 cysteines with an intervening amino acid) includes IL-8, Groα, Groβ, NAP-2, PF4, ENA78, and γIP10. The three-dimensional structures of IL-8 and PF4 have been solved and show general structural identity (17, 18). Protein family members that possess the amino acid motif "ELR" within the amino terminus have all been observed to elicit potent neutrophil chemoattractant and stimulatory activities. This motif has also been shown to be required for specific interaction with either of the two IL-8 receptor proteins on the surface of neutrophils (19-21). The remaining members of the CXC subgroup display a more diverse activity profile, weak or no neutrophil chemoattracting activity, and less sequence homology to the ELR motif containing subgroup. Neither PF4 nor γIP10 have demonstrated significant neutrophil-related activities (22-25).

The other half of the chemokine family is characterized by

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\(^{1}\) The abbreviations used are: GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; PF, platelet factor; MIP, macrophage inflammatory protein; MCP, monocyte chemotactic and activating peptide; γIP10, γ interferon-inducible protein (molecular weight 10,000); HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; CFU, colony-forming unit; BFU-E, burst-forming unit of erythroid progenitor cells; GM, granulocyte/macrophage; GEMM, multipotential cells.
the CC motif (two adjacent cysteine residues located within the amino terminus) and displays a much more diverse sequence homology profile. CC chemokines act predominantly on monocytes, although basophils, lymphocytes and eosinophils have also been reported to be target cells for various CC proteins including RANTES, MIP-1α, and MCP-1 (26–29). Compared to the CXC family, less is understood regarding domains within the proteins which are required for biological activity. However, recent structural information on MIP-1α should facilitate this understanding (30).

Activated platelets have been observed to release high concentrations of a high molecular weight proteoglycan complex consisting of chondroitin sulfate and PF4 (31). In addition to high affinity binding and neutralization of heparin, PF4 also has been observed to inhibit angiogenesis, inhibit bone resorption, and reverse the immunosuppressive effect of lymphoma cells (32–36). IL-8 has been observed to possess potent chemotactic and stimulating properties toward human neutrophils in vitro and has been shown to bind with high affinity to either of the two cloned human IL-8 receptors in vitro. In addition to these activities, IL-8 and PF4, as well as MIP-1α, MCP-1, Gro-β, and γIP10, were all observed to inhibit early myeloid progenitor cell proliferation at equivalent concentrations (>25 ng/ml) (10, 11). Several members of the chemokine family, including NAP-2, Gro-α, Gro-γ, RANTES, and MIP-1β did not possess any inhibitory activities in this assay. A third group of chemokines including Gro-α and Gro-γ (MIP-2α) blocked the inhibitory activity of IL-8 and PF4 (10). Similarly, MIP-1β was observed to inhibit the activity of MIP-1α (6, 10). Combinations of any of the two of the six active chemokines resulted in a synergistic decrease in the amount of each chemokine needed to inhibit proliferation (0.1 ng/ml of each chemokine), suggesting the possibility of a novel mechanism of action on the progenitors (10, 11). The low concentrations of PF4 and IL-8 required to elicit inhibition suggest the presence of protein-based receptors on the progenitor cells. To address this issue, a series of chimeric IL-8/PF4 mutants were expressed, purified, and tested for inhibitory activity toward immature subsets of myeloid progenitor cells.

MATERIALS AND METHODS

Isolation of Recombinant Proteins—The synthetic genes for human IL-8 (4, 14), and related mutants were expressed as non-fusion proteins in Escherichia coli (BL21) cells and grown in a 500-ml shaker flask containing 300 μg/ml kanamycin until an absorbance of 0.6 at 600 nm was reached. Cells were induced with isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37°C, followed by centrifugation at 14,000 g at 8°C. The precipitate from the centrifugation step was extracted with a 50-fold excess (v/v) of buffer containing 25 mM sodium acetate, pH 4.0, 8 M urea, and the column was washed with 25 mM sodium acetate, pH 4.0, to remove the urea. A second wash was performed using buffer containing 25 mM sodium acetate, pH 4.0, 0.5 mM NaCl. The protein was then eluted using buffer containing 50 mM Tris- HCl, pH 8.0, 0.1 mM NaCl. Fractions containing the appropriate chemokine protein were subjected to refolding overnight in the presence of 1 mM oxidized, 2 mM reduced glutathione at 25°C. Extent of refolding of the proteins was monitored through Poros analytical chromatography. The reduced protein was observed to elute from the Poros column at a different achondritine concentration relative to the refolded species. Refolded fractions were pooled and refolded by C4 semi-preparative reverse phase HPLC using a 0–100% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA). Peak fractions were pooled and lyophilized for concentration determination. Purity was assessed by Coomassie staining of SDS-polyacrylamide gel electrophoresis, analytical C4 reverse phase HPLC, and amino acid analysis. Small scale purifications typically yielded several milligrams of highly purified (>95% purity) material.

Isolation of Human Neutrophils—For isolation of human neutrophils, typically 22.5 ml of human blood was layered over 10 ml of Ficol 1119 and 10 ml of Ficol 1077 in a 50-ml polypropylene conical tube. The blood was centrifuged in a tabletop centrifuge for 20 min at 1800 rpm at 8°C. Following centrifugation, the neutrophil layer (located just above the pelleted red blood cell layer) was collected, washed in sterile phosphate-buffered saline (without Ca2+ and Mg2+; Life Technologies, Inc.), and further purified by centrifugation for 5 min at 3800 rpm at 4°C. The neutrophil fraction, which contains some contaminating red blood cells, was resuspended in 27 ml of sterile H2O, which served to lyse the remaining red blood cells. 3 ml of 10 X phosphate-buffered saline were added to the resuspended cells, which were pelleted by centrifugation at 8°C for 5 min at 1800 rpm. The pelleted neutrophils were resuspended in 10 ml of PBS and counted. Resuspended cells were kept on ice until needed for chemokine-dependent assays.

Elastase Release Assay—Elastase release from human neutrophils was monitored using the fluorescent substrate MeO-Suc-Ala-Ala-Pro-Val-aminomethylcoumarin as described by Hebert et al. (37). Isolated human neutrophils were suspended in PBS buffer containing 0.02 mM Na2HPO4, pH 7.4, 0.15 mM NaCl, 0.2 mM Hepes, 1 mg/ml bovine serum albumin, 5 mM glucose at 37°C. Following incubation, cells were re-equilibrated at 37°C for 15 min. Chemokines at varying concentrations were added to the neutrophils while gently mixing. Following addition of the chemokines, the cells were pelleted and 0.75 ml of the resulting supernatant was added to 2.25 ml of PBS in the presence of 5 X 104 mg/ml of the elastase substrate (5 mg/ml stock in Me2SO; Sigma-Aldrich, St. Louis, MO) at 37°C, the upper chamber was removed and cells on the filter from the upper chamber were washed away. The filter was fixed with 100% ethanol, stained with a solution of 0.5% toluidine blue in 3.7% formaldehyde, and counted at 400× magnification.

Binding Studies—IL-8 was iodinated as described previously (39) or purchased from DuPont NEN. A stable transfectant CHO cell line, 4ABCH33 (21), expressing human neutrophil IL-8 receptor subtype B (hullRBR), was used in binding assays to test mutant chemokine binding. Details are described in Ref. 21.

Colony Formation Assays—As described previously (8, 9), 1 X 10⁴ low density (<1.077 g/cm³) normal human bone marrow cells were plated in 0.3% agar culture medium with 10% fetal bovine serum (HyClone, Logan, UT) with 100 units/ml recombinant human (rhu) GM-CSF plus 50 ng/ml rhu Steel factor (Immunex Corp., Seattle, WA) in the absence and presence of rhu chemokines for assessment of CFU-GM. For assessment of CFU-GEMM and BFU-E, cells were grown in 0.9% methylcellulose culture medium in the presence of rhu erythropoietin (1–2 units/ml) in combination with 50 ng/ml rhu Steel factor. Three plates were scored per concentration per experiment for CFU-GM, CFU-GEMM, and BFU-E colonies after incubation at 37°C in lowered (5%) CO2 and 5% O2 for 14 days. The combination of GM-CSF and Steel factor or erythropoietin and Steel factor allow detection of large colonies (usually >1000 cells/colony) which come from early, more immature subsets of myeloid lineage allowed for chemokine-dependent Myelosuppression. Levels of significance were determined using Student’s t distribution (two-tailed test).

In Vivo Testing of Chemokine Proteins—C3H/HeJ and BDF1 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were injected intravenously with 0.2 ml of saline/mouse or the stated amount of chemokine and sacrificed 24 h later. Femoral bone marrow was removed, treated with or without high specific activity tritiated thymidine, and plated in 0.3% agar cultured medium with 10% fetal bovine serum in the presence of 10% v/v pokeweed mitogen mouse spleen cell cultured medium as described previously (9). Colonies (>40 cells in a 7 days of incubation at 37°C) were scored and photographed by Student’s t distribution (two-tailed test). The number of progenitors in DNA synthesis (5 phase of the cell cycle) was estimated using the high specific activity (20 Ci/mmol) tritiated thymidine.
(50 μCi/ml) (DuPont NEN) kill technique and is based on the calculation in vitro of the reduction in the number of colonies formed after pulse exposure of cells for 20 min to "hot" tritiated thymidine as compared with control (McCoy's medium or a comparable amount of non-radioactive "cold" thymidine).

RESULTS

Generation of IL-8- and PF4-derived Mutants—A series of chemokine mutants, based on either PF4 or IL-8 native sequences were constructed to examine the domains of these proteins, which are involved in suppression of myeloid progenitor cell proliferation. Fig. 1 shows the amino acid sequences of the mutant proteins, which were expressed, purified, and tested in the neutrophil-based assays as well as the myeloid progenitor colony formation assays. For IL-8-based mutants, emphasis was placed on regions surrounding the amino-terminal ELR domain. Mutations were also clustered in the region involved in IL-8 dimerization. For the PF4-based mutants, changes within the DLQ motif, located within the amino-terminal domain were examined. A second DLQ motif, located proximal to the putative heparin binding domain at a reverse β-turn near the carboxyl-terminal domain was also emphasized for mutation. Previously, several groups have reported that peptides containing this motif displayed activity toward suppression of progenitor cell proliferation (13, 40). Native sequence proteins as well as mutants were expressed as nonfusion proteins in E. coli (Novagen, pET system). Expression was induced using isopropyl-1-thio-b-D-galactopyranoside, and protein was initially observed as inclusion bodies following lysis of the cells. Proteins were purified from the inclusion bodies by extraction under reducing and denaturing conditions, ion exchange chromatography, a refolding procedure followed by reverse phase HPLC. Correct refolding of the chemokines was monitored by differential retention times using Poros chromatography. Identification of the purified protein was accomplished using amino acid analysis, mass spectrometry, and amino-terminal sequencing. Purity was determined by analytical reverse phase HPLC, mass spectrometry, and SDS-gel electrophoresis.

Assessment of Neutrophil Activities in Vitro—The ability of each of the chemokine proteins to activate neutrophils was tested using a degranulation assay, which followed chemokine-dependent release of elastase. Fig. 2A shows a summary of the activities of the purified chemokine proteins. IL-8, as well as IL-8M3, M4, M6, M7, and M64 all show significant elastase release activity, although compared to IL-8 wild-type, M4 was less active. The PF4-based mutant, PF4M2, displayed approximately 50% of the activity observed with the native sequence IL-8, demonstrating the requirement of the amino-terminal ELR domain for neutrophil-based activity. Some activity was observed with mutants PF4M1 and IL-8M1 only at concentrations of protein greater than 10^{-6} M (10 μg/ml). As expected, PF4, PF4-412, PF4-413, PF4-414, and PF4-421 showed no neutrophil elastase release activity at any of the concentrations tested.

Additional IL-8 mutants, designated IL-8M8 (ELQ), IL-8M9 (DLR), and IL-8M10 (DLN), which were developed to examine in greater detail the requirements surrounding the NH2-terminal ELR motif, were also tested in the elastase release assay in a concentration-dependent manner (Fig. 2B). As anticipated, all three displayed either significantly reduced activity or no ability to elicit degranulation of the isolated human neutrophils. Neither IL-8M8 or IL-8M10 elicited any release of elastase at concentrations as high as 4 \times 10^{-6} M (40 μg/ml) and 1.25 \times 10^{-5} M (100 μg/ml), respectively. IL-8M9 demonstrated the ability to release elastase, although at concentrations approximately 200-fold greater than for the native sequence IL-8. Perturbation of the ELR motif resulted in profound effects on the ability of these chemokine mutants to function on the neutrophil.

The ability of each of these mutant proteins to elicit chemotaxis of neutrophils was also examined. Each chemokine mutant was tested in a concentration-dependent manner for ability to elicit chemotaxis of isolated human neutrophils in a boyden chamber. Each concentration of each mutant was tested in triplicate in two separate experiments. Each data point was read in triplicate as well, for a total of 18 data points/concentration/chemokine. The results obtained demonstrate a direct correlation between the ability of the chemokine mutants to elicit chemotaxis of neutrophils and the ability to cause neutrophil degranulation as exhibited in the elastase release assay results (Fig. 3, A–D). With the exception of PF4M2, none of the PF4-derived mutants displayed any che-
motactic activity toward neutrophils. Similarly, with the exception of IL-8M1, which showed substantially reduced activity, all of the IL-8-derived mutants exhibited potent neutrophil chemotactic activity, although some reduction in activity was also observed for IL-8M4, IL-8M6, and IL-8M7. However, this decrease also correlated with the data obtained in the elastase release assay.

Binding of the IL-8-derived mutants to CHO cells containing the stably transfected IL-8 receptor subtype B was also performed. The B subtype receptor is able to bind with high affinity to IL-8 as well as other "ELR"-containing chemokines including Gro-α, Gro-β, and NAP-2. As shown in Table I, competition binding experiments utilizing \(^{125}\)I-labeled IL-8 and unlabeled mutant chemokine competitors demonstrated that each of the proteins that was able to activate the neutrophils was also able to bind to the neutrophil receptors. IL-8M1 and PF4M1, which displayed decreased ability to elicit elastase release from the neutrophils, showed a similarly decreased ability to compete with the labeled IL-8 for receptor binding.

In Vitro Chemokine-dependent Suppression of Myeloid Progenitor Proliferation—Chemokines were assessed for the ability of each to suppress the proliferation of progenitor cells derived from the posterior iliac crest of normal healthy volunteers who had given informed consent. Chemokines were tested in a dose-dependent fashion, with concentrations ranging from 100 ng/ml (\(1 \times 10^{-8} \text{ M}\)) down to 0.001 ng/ml (\(1 \times 10^{-13} \text{ M}\)) in the assay. Cells were plated in the presence of rhu GM-CSF 100 units/ml \(\pm\) rhu Steel factor 50 ng/ml for granulocyte-macrophage progenitors (CFU-GM). Chemokines were added at the start of culture and colonies were permitted to develop for 14 days after which they were scored. Each chemokine mutant was tested in at least three separate experiments with three plates being scored per experimental point. The results obtained for the mutant chemokine proteins are summarized in
Table II and demonstrate three distinct categories of mutant activity. Several of the mutants showed little effect of the mutation on activity compared to either wild-type IL-8 or PF4. These include PF4-421 and IL-8M64. Both of these proteins as well as the wild-type PF4 and wild-type IL-8 inhibited progenitor cell proliferation at concentrations beginning between 10 and 25 ng/ml (approximately $1 \times 10^{-9}$ M). These results are comparable to previously published data (5, 6, 10, 11).

Several mutants were no longer able to inhibit the proliferation of the myeloid progenitors. Even at concentrations up to 100 ng/ml ($1 \times 10^{-8}$ M), no activity could be detected. These proteins include PF4M1, PF4-421 and IL-8M64. Both of these proteins as well as the wild-type PF4 and wild-type IL-8 inhibited progenitor cell proliferation at concentrations beginning between 10 and 25 ng/ml (approximately $1 \times 10^{-9}$ M). These results are comparable to previously published data (5, 6, 10, 11).

**Fig. 3.** Concentration dependence of chemokine mutants on neutrophil chemotaxis activity in vitro. Experiments were performed as described under “Materials and Methods.” A, samples include IL-8 WT, ●; IL-8M3, ○; PF4 WT, ■; IL-8M1, □. B, IL-8 WT, ●; IL-8M64, ○; PF4M1, ■; PF4M2, □. C, IL-8 WT, ●; IL-8M6, ○; IL-8M4, ■; PF4-426, □. D, IL-8 WT, ●; IL-8M7, ○; PF4-413, ■; PF4-421, □.
within the amino-terminal domain. Finally, PF4-412 and PF4-413, result from domain swaps with IL-8 and NAP-2, respectively, at the COOH-terminal DLQ region of PF4.

The third phenotype of chemokine mutant includes proteins possessing enhanced in vitro inhibitory activity toward proliferation of the CFU-GM population. The activities of these proteins, shown in Fig. 4 (A and B), are compared to the activities of either IL-8 or PF4 alone. The results are expressed as mean percent change from control ± 1 S.E. These proteins include PF4M2, IL-8M1, IL-8M10, PF4-414, and PF4-426.

### Table I

| Proteins | $K_i$ (nM) |
|----------|-----------|
| IL-8     | 3.0 ± 0.5 |
| IL-8M1   | 661.0 ± 189 |
| IL-8M3   | 22.1 ± 4.4 |
| IL-8M4   | 29.3 ± 7.2 |
| IL-8M6   | 35.3 ± 6.2 |
| PF4      | NC a |
| PF4M1    | 839.4 ± 202 |
| PF4M2    | 52.0 ± 11.8 |

a NC, no competition.

### Table II

Influence of PF4, IL-8, and their chimeric mutant proteins on colony formation by early subset of normal human bone marrow granulocyte-macrophage progenitor cells (CFU-GM)

5 × 10° low density (−1.077 g/cm³) normal human bone marrow cells were plated in 0.3% agar culture medium with 10% FBS (HyClone, Logan, UT) with 100 units/ml rhGM-CSF plus 50 ng/ml rh Steel factor (Immunex Corp., Seattle, WA) in the absence and presence of rhu chemokines. Three plates were scored per concentration per experiment for CFU-GM colonies after incubation at 37°C in lowered (5%) O₂ for 14 days. This combination of GM-CSF and Steel factor allows detection of large colonies (usually > 1000 cells/colony), which come from early more immature subsets of CFU-GM. The results are expressed as a mean % change from control ± 1 S.E. for the number of experiments shown in experiments in which the control number of colonies/10⁶ cells/ml ranged from 115 ± 5 (mean ± 1 S.E.) to 382 ± 10. The inhibition shown, while partial inhibition of total colony formation, is 90–100% inhibition of the Steel factor enhanced colony formation. Not shown is that, as previously reported for wild-type chemokines (5, 6, 10, 11), neither the wild-type nor the mutant chemokines in this table had an effect on colony formation stimulated only by GM-CSF.

| Chemokine | Chemokine concentration (nM) | 10 | 5 | 2.5 | 1 | 0.1 | 0.01 | 0.001 | 0.0001 |
|-----------|-----------------------------|----|---|-----|---|-----|------|-------|--------|
| PF4WT     | −41 ± 3² | −40 ± 2² | −25 ± 7² | −10 ± 6 | −3 ± 2 |
| PF4M1     | −9 ± 10 | −5 ± 6 | −2 ± 2 | −6 ± 1 |
| PF4M2     | −43 ± 3² | −42 ± 2² | −46 ± 2² | −47 ± 2² | −42 ± 5² | −19 ± 8² | −3 ± 2 |
| PF4-412   | −6 ± 4 | −3 ± 3 | −1 ± 1 |
| PF4-413   | −19 ± 10³ | −14 ± 16 | −8 ± 8 | −2 ± 1 | +1 ± 1 |
| PF4-414   | −45 ± 1¹ | −44 ± 1¹ | −44 ± 2² | −43 ± 2² | −42 ± 5² | −43 ± 1² | −34 ± 8² | −3 ± 3 |
| PF4-421   | −42 ± 6² | −40 ± 4² | −29 ± 3³ | −25 ± 9³ | −8 ± 4 | +1 ± 1 |
| PF4-426   | −45 ± 3² | −44 ± 4² | −45 ± 1¹ | −46 ± 1¹ | −45 ± 3² | −44 ± 2² | −40 ± 3² | −20 ± 1³ |
| IL-8WT    | −42 ± 2² | −43 ± 2² | −21 ± 5² | −4 ± 2 | −3 ± 6 |
| IL-8M1    | −43 ± 4² | −43 ± 4² | −44 ± 1³ | −46 ± 2³ | −46 ± 2² | −46 ± 2² | −40 ± 1² | −25 ± 4³ |
| IL-8M3    | −8 ± 3 | −4 ± 3 | −1 ± 2 | −7 ± 2 |
| IL-8M4    | −1 ± 2 | −1 ± 2 | +1 ± 1 |
| IL-8M5    | −2 ± 2 | −1 ± 2 | +1 ± 1 |
| IL-8M7    | −43 ± 4² | −45 ± 2² | −25 ± 2² | −2 ± 3 | 0 ± 2 |
| IL-8M6    | −43 ± 1³ | −43 ± 2² | −27 ± 5³ | −10 ± 7 | −8 ± 5 | −5 ± 1 |

* Significant change from control, p < 0.01 (Student’s t test).
* Significant change from control, p < 0.05 (Student’s t test).
determine the role of specific amino acids in the synergistic myelosuppressive activity observed with IL-8M1 (IL-8 "DLQ"). These proteins include IL-8M8 (IL-8 "ELQ"), IL-8M9 (IL-8 "DLR"), and IL-8M10 (IL-8 "DLN"). These new mutants were tested along with IL-8 wild-type, PF4 wild-type, and IL-8M1 in assays designed to examine the concentration dependence of the inhibition of colony formation in the CFU-GM, CFU-GEMM, and BFU-E lineages. As shown in Table IV, there was no observable differences in activity between cell lineages for any of the chemokine proteins tested, demonstrating a broad

**Fig. 4. Summary of activities of chemokine mutants for in vitro inhibition of CFU-GM.**

A, comparison of highly active IL-8-derived mutants compared to IL-8 and PF4. Samples include IL-8 WT, ■; PF4 WT, □; IL-8M1, ○; IL-8M10, ●. B, comparison of highly active PF4-derived mutants compared to IL-8 and PF4. Samples include IL-8 WT, ■; PF4 WT, □; PF4M2, ○; PF4-426, ●; PF4-414, △.
The mutants that were inactive in the progenitor expression—either glutamine or asparagine are suitable for replacement of 10 ng/ml (IL-8M4, IL-8M6, and PF4-412 were incubated at varying concentrations) with either IL-8 or PF4. IL-8M6 was observed to suppress the proliferation of the progenitors at a concentration of 500 ng/ml, demonstrating that it is actually a weak agonist in this system. In the presence of 500, 250, or 50 ng/ml IL-8M3, no suppression of progenitor proliferation was observed. However, at the highest two concentrations, IL-8M3 was able to block the myelo-suppressive activity of IL-8. Under identical conditions, this mutant was unable to inhibit the ability of PF4 to suppress progenitor proliferation (Table IV). IL-8M3 is a mutant that contains several amino acids in the dimer interface region from MCP-1. That it inhibits IL-8 and not PF4 suggests that this mutant is able to interact with a cell-based receptor. In addition, the observation suggests that since it only affects IL-8 and not PF4, several different receptors with different specificity are likely to exist on the progenitor cell. Unfortunately, because of the low frequency of progenitor cells in bone marrow (1/1,000), it is not possible to get enough purified progenitors from bone marrow to perform adequate receptor binding studies.

Table III

Influence of IL-8, IL-8M1, and PF4 on absolute numbers and cycling status of granulocyte-macrophage progenitor cells (CFU-GM) in vivo in femurs of C3H/HeJ and BDF1 mice.

Results shown are for two experiments with 3 mice/group (each mouse assessed individually) and expressed as mean ± 1 S.D. Mice were injected intravenously with 0.2 ml/mouse of saline or the stated amount of chemokine and sacrificed 24 h later. Femoral bone marrow was removed, treated with high specific activity tritiated thymidine and plated in 0.3% agar cultured medium with 10% FBS in the presence of 10% (v/v) pokeweed mitogen mouse spleen cell cultured medium. Colonies (>40 cells/aggregate) were scored after 7 days of incubation.

Table IV

Influence of IL-8 mutant proteins IL-8M3, IL-8M4, IL-8M6, and PF4 mutant protein PF4-412 on colony formation by bone marrow CFU-GM and the activity of wild-type IL-8 and PF4.

Results are expressed as mean ± 1 S.E. with percent change from control shown in parentheses. Cells were plated in 0.3% agar culture medium with 10% (v/v) pokeweed mitogen and the activity of wild-type IL-8 and PF4.

* Significant percent change from control, p < 0.005.

Number in parentheses indicates percent change from control.

Significant percent change from control, p < 0.025.
**DISCUSSION**

A series of chemokine mutants have been cloned, expressed, purified, and evaluated for in vitro myelosuppressive activity. Of the proteins examined, one group of proteins have been identified that are able to inhibit myeloid progenitor cell proliferation at very low concentrations. The activities of these individual mutants appeared comparable to or greater than the activity observed previously when low concentrations of IL-8 and PF4 were added together (10). These proteins include PF4M2, PF4-414, PF4-426, IL-8M1, and IL-8M10. These synergistic mutants were found to be active at concentrations as low as 0.001 ng/ml (−1 × 10−13 M monomer concentration). PF4M2 contains the NH2-terminal ELR motif from IL-8 with the remaining COOH-terminal domains from PF4. It has been shown to possess both neutrophil-related activities as well as an ability to bind heparin and inhibit the proliferation of cultured endothelial cells. Conversely, IL-8M1 contains the amino-terminal DLQ motif from PF4 with the remaining COOH-terminal domains from IL-8. This potent mutant displayed significantly reduced neutrophil binding, chemotaxis and activation activities. Comparable to native IL-8, IL-8M1 binds to heparin with an affinity that is significantly reduced relative to PF4. Because this protein is inactive on neutrophils, but highly active on progenitor cells, it is likely that progenitor-related activity occurs via a different mechanism than that which occurs on neutrophils.

IL-8M10 was another highly active mutant in the myeloid progenitor proliferation assay. This mutant contains the sequence DLQ as a replacement for ELR in wild-type IL-8. The activity of this mutant is similar to IL-8M1 and demonstrates that either glutamine or asparagine in this position is well tolerated on the progenitor cell. IL-8M9, which contains DLR, displayed significant activity. A closer analysis, however, reveals that substitution of the third arginine residue with glutamine has been demonstrated previously to block PF4- and IL-8-dependent inhibition of proliferation (10), suggesting that Gro-α is able to bind to the progenitor cell. It is possible that the combination of the DLQ motif from PF4 with the sequence ACNPASPIK is sufficient to generate a molecule that possesses an activity analogous to the combination of two of the active chemokine proteins (10). It is suggested that correct combinations of domains from various chemokine proteins elicit a synergistic activity on myeloid progenitor cells.

This hypothesis is supported by mutant PF4-426. This mutant contains three point mutations, each of which replaces an arginine residue with glutamine. The result is a highly active protein, which on first glance is a simple PF4 mutant. A closer analysis, however, reveals that substitution of the third arginine residue at position 49 with glutamine results in the generation of the second dlq-ELR sequence IATLKNGQK, which is identical to a sequence within Gro-β. Gro-β has been demonstrated previously to be able to synergize with PF4 in the progenitor proliferation assay (10). The results demonstrate that correctly placed domains that result in chimeric chemokines are able to elicit an enhanced suppressive activity on myeloid progenitor cells.

Another class of mutants appeared neither inactive or significantly reduced in inhibitory activity in the assay. All of the
IL-8 mutants which contain mutations within the dimerization domain of IL-8 were either inactive or significantly reduced in activity, suggesting that this region and perhaps more specifically the sequence ELRV plays a role in suppression of proliferation of progenitor cells. These three IL-8 mutants all elicited elastase release and were able to chemoattract neutrophils, demonstrating that they are likely to be correctly refolded in a manner analogous to the native sequence IL-8. Although IL-8M3, IL-8M4, and IL-8M6 contain mutations within the dimer interface of IL-8, only IL-8M4 appears to be monomeric in solution at 0.1 mg/ml (1 × 10⁻⁵ μm) concentration (data not shown). Another monomeric IL-8 mutant, IL-8M64, possessed equivalent activity as wild-type IL-8 on both neutrophils and progenitor cells, suggesting that oligomeric state may not be a critical factor for activity on myeloid progenitor cells. Furthermore, at concentrations in the range of 10⁻¹¹ μm, wild-type IL-8 is likely to exist predominantly as a monomeric species in solution (42). The data from this class of mutant suggest that activity may not be oligomeric state-dependent but rather a result of a specific amino acid sequence within the protein or correct protein folding, which is required for myeloid suppression.

Another mutant that lacked the ability to inhibit progenitor proliferation was PF4M1. This protein contains a single point mutation (DLQ to DLR) within the amino-terminal domain. It is unclear whether this amino acid change results in a direct effect on the interaction with the progenitor cells. However, crystal structure data demonstrate that spatially, the DLQ motifs of two PF4 monomers (the A and D subunits) lie adjacent to each other (17). The glutamine residues in particular are situated side by side in the intact tetramer. Replacement of these glutamine residues with the positively charged arginine groups may result in charge repulsion and an altered oligomeric conformation of the protein. The DLQ motif of PF4 appears to be highly important with regard to activity on the progenitor cells. The region surrounding the COOH-terminal DLQ motif of PF4 also appears important for myeloid cell growth suppression. Two mutants, PF4-412 and PF4-413 were generated, which replace this domain with the analogous regions of either IL-8 or NAP-2, respectively. The resulting loss of activity suggests that this region also is involved either in direct interaction with progenitor cells or is required for proper folding of the protein. Both of these mutants were observed, however, to inhibit endothelial cell proliferation in vitro and to bind heparin at concentrations comparable to wild-type PF4.

Fig. 5 is a summary of the domains that have been identified to be involved in this activity. In IL-8, the amino-terminal ELR motif appears to be required for activity, especially if inserted into PF4. The dimer interface region of IL-8 also appears critical for suppressive activity. In PF4 both of the DLQ motifs appear necessary for this protein to inhibit myeloid progenitor proliferation. Loss of either of these domains results in loss of activity of PF4. Combinations of IL-8 and PF4, PF4 and Groα, as well as PF4 and Groβ result in synergistic effects. It is anticipated that other combinations of chemokines may also generate additional synergistic mutants.

A number of chemokine receptors have been identified to date, including the two human neutrophil IL-8 receptors and the Duffy antigen on erythrocytes (43–45). Recent work by Cacalano et al. (46) has shown that deletion of a murine gene with high homology to the two human IL-8 receptors results in a mouse with a phenotype of elevated levels of B cells, macrophages, and mature neutrophils, suggesting that the receptor plays a role in the negative control of development of blood cell components. However, neither of the two identified human IL-8 receptors displays the activity we observed with either the mutant chemokines or native sequence chemokine family members that has been observed with the progenitor cells (46). This would suggest that the neutrophil receptors may not be involved in the regulation of progenitor cell proliferation. Furthermore, no receptor has as yet been identified as being specific for platelet factor 4. Since the activity pattern of the IL-8 and PF4 mutants does not correlate with the activity profile observed on neutrophils, it is possible that a new family of receptors may exist on the progenitors. Furthermore, the observation that IL-8M3 was able to inhibit the suppressive activity of IL-8 but not PF4 suggests that it is not a single receptor, but possibly a family of receptors that are responsible for interaction with each chemokine. One potential model for the mechanism of action of the chemokines on progenitor cells that is supported by our data, in conjunction with work by Broxmeyer et al. (10), suggests that two or more different occupied receptors, each with a high affinity for a specific chemokine (such as IL-8 or PF4) and a weaker affinity for each of the other active chemokines, interact with each other, leading to signal transduction and suppression of progenitor cell cycling. Since a minimum of two bound receptors with different specificities would be required for this synergistic suppression, it is likely that chimeric chemokines such as IL-8M1 are simultaneously interacting with two distinct receptors with different specificities with high affinity, leading to the synergistic phenotype observed. The apparent weaker activity observed with a single chemokine such as IL-8, may result from specific binding to the high affinity IL-8 receptor and nonspecific binding to a much lower affinity PF4 (or other chemokine) receptor. Further work in this area will provide insight into the mechanism of chemokine-dependent myeloid progenitor cell regulation.

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