The Active Monomeric Form of Macrophage Inflammatory Protein-1α Interacts With High- and Low-Affinity Classes of Receptors on Human Hematopoietic Cells


Macrophage inflammatory protein-1α (MIP-1α) and its human homologue GOS19.1/LD78 are members of the C-C chemokine/intercrine family of secreted proteins. They have proinflammatory properties and also inhibit cell cycle progression of hematopoietic stem cells. Characterization of MIP-1α receptor(s) has been confused because of its reported aggregation to inactive forms. Using a defined monomeric form of MIP-1α that is biologically active for stem cell inhibition and induction of oxidative metabolism in polymorphonuclear cells, we report the detection of high- and low-affinity receptor classes on human leukemic CD34+ blast cells, promyelocytic cells, monocytes, peripheral blood neutrophils, and T cells. Both high- and low-affinity classes are expressed simultaneously in promyelocytes and neutrophils. The calculated kD for high-affinity receptors correlates with the concentrations of MIP-1α required to induce a biologic effect on stem cells and neutrophils. Cross-linking studies show that MIP-1α associates with two cell surface proteins with apparent molecular masses of 92 kD and 52 kD. Direct competition binding studies combined with studies on the inhibition of stem cells show that human and murine MIP-1α have different receptor-binding and biologic properties.

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ions of MIP-1α required to induce a respiratory burst. The HL-60 promyelocytic cell line also expresses two classes of receptors similar to those on neutrophils. We present evidence from cross-linking studies that MIP-1α binds to cellular proteins with calculated molecular masses of approximately 92 kD and 52 kD. The patterns of direct cross competition for MIP-1α binding by different members of the C-C chemokine family vary among the cell types examined, suggesting the expression of receptors with differing binding specificities.

MATERIALS AND METHODS

Reagents. Recombinant MIP-1α, MIP-1β, GOS19.1, ACT-2, RANTES, human IL-8, and goat anti-MIP-1α antibody were purchased from R&D Systems (Minneapolis, MN). MIP-1α was obtained either as pure recombinant protein in 30% acetonitrile buffer containing 0.1% trifluoroacetic acid or as a lyophilized powder containing bovine serum albumin (BSA) that was resuspended in phosphate-buffered saline (PBS). ACT-2 and RANTES were also purchased from PeproTech, Inc (Rocky Hill, NJ). BSA, disuccinimidyl suberate (DSS), ethylene glycol-bis (succinimidyl succinate) (EGS), phenylmethyisulfonyl fluoride (PMSF), pepstatin, aprotinin, EDTA, leupeptin, benzamidine, and 1,10-phenanthroline were obtained from Sigma (St Louis, MO). Disuccinimidyl tartarate (DST), dithio-bis (succinimidyl propionate) (DSP), dimethyl adipimidate (DMA), and dimethyl suberimidate (DMS) were obtained from Pierce (Rockford, IL). For oxidative metabolism studies, the dye 2’, 7’-dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR). The serum supplement TCH was purchased from Celox Corp (Hopkins, MN), Hanks’ Buffered Saline Solution (HBSS) from GIBCO BRL (Gaithersburg, MD), and phorbol 12-myristate 13-acetate (PMA) and N-formyl-Methionyl-Leucyl-Phenylalanine (fMLP) from Sigma. Polymorphprep and Nycoprep were purchased from Nycomed (Oslo, Norway; distributed by GIBCO BRL).

Chromatographic analysis of MIP-1α. For chromatographic analysis of the molecular forms of MIP-1α, a Protein PAK 125 column (Waters, Bedford, MA) was pre-equilibrated with 0.15 mol/L KCl, 0.05 mol/L Tris-HCl (pH 7.8). Twenty-microliter aliquots of protein samples were injected onto the column and eluted at a flow rate of 0.5 mL per minute. Protein elution was continuously monitored by absorption at 280 nm using a Beckman (San Ramon, CA) System Gold HPLC apparatus and 1-mL fractions were collected for counting on a Packard (Downers Grove, IL) gamma counter. The column was calibrated using a molecular mass marker kit from Sigma. For the analysis of small amounts of unlabeled MIP-1α, column fractions were spiked with BSA and proteins precipitated with 90% ammonium sulfate. After centrifugation, the pellets were redissolved in 50 mM/L Tris-HCl (pH 7.8) and then dialyzed against two changes (1 L each) of the same buffer. Samples were boiled in buffer containing 1% sodium dodecyl sulfate (SDS) with 5% 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 5% to 20% gradient polyacrylamide gels. Proteins were electrophoretically transferred onto nitrocellulose and analyzed by immunoblotting using goat anti-MIP-1α antibody.

Cell lines and cell culture. HL-60 (promyelocytic cells), Jurkatt (CD4+ T cells), THP-1 (monocytic cells), and U937 (monocytic cells) lines were obtained from American Type Culture Collection (ATCC; Rockville, MD). Cell lines were grown in RPMI 1640 (GIBCO BRL) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. FDCP-mix cells were the kind gift of M. Dexter (Manchester, UK) and were cultured in Fischer’s medium (GIBCO BRL) supplemented with 20% horse serum and either 50 U/mL murine IL-3 (GIBCO BRL) or 10% WEHI-conditioned medium as a source of IL-3.

Stem cell assays. The effects of chemokines in vitro on hematopoietic stem cells were determined using the colony-forming unit-A (CFU-A) assay34 that detects multipotent clonogenic cells of the colony-forming unit-spleen day-12 type. Briefly, cells from regenerating bone marrow of C57B16 male mice were incubated for 4 hours with culture medium, or culture medium containing varying concentrations of chemokines. The cultures were split, incubated for an additional hour in the presence or absence of cytosine arabinoside, and plated in the CFU-A assay to determine the number of viable clonogenic stem cells. The percentage of S-phase cells in each sample was calculated from the difference in colony numbers between untreated and cytosine arabinoside treated cultures. The details are given in Pragnell et al.24

Isolation of human bone marrow and peripheral blood cells. For the isolation of stem cells, peripheral blood cells as well as bone marrow cells were obtained (after obtaining appropriate informed consent) from a patient with an undifferentiated stem cell leukemia. At the time of harvest, these cells comprised greater than 90% of the peripheral blood and bone marrow cell populations. By flow cytometric analysis, the cells were phenotypically characterized as CD34+, CD33+, and HLA-DR+. For ligand binding studies, Ficol-Hypaque gradient centrifugation was used to further enrich for stem cells.

Neutrophils were isolated from heparinized blood by Ficol-Hypaque gradient centrifugation followed by sedimentation in 3% dextran sulfate, as previously described.27 Cells (95% to 98% neutrophils) were washed twice in PBS and kept at 23°C. For the analysis of oxidative metabolism, polymorphonuclear cells were isolated from heparinized blood with Polymorphprep. The neutrophil-rich layer was harvested and washed with HBSS. Red blood cells were then lysed using R&D Systems lysis buffer. The remaining cell suspension was washed and resuspended at 2 × 106 cells/mL in HBSS supplemented with 2% TCH.

For the isolation of T cells, peripheral blood was centrifuged over Nycoprep to obtain peripheral blood mononuclear cells. Monocytes were depleted by adherence to tissue culture flasks (Corning, Cornng, NY) for 1 hour at 37°C. Nonadherent cells were incubated with calcium-free and magnesium-free Dulbecco’s PBS containing 0.5% human IgG at room temperature for 15 minutes and layered on MicroCelleCtector CD4+ or CD8+ T-25 flasks (AIS, Santa Clara, CA) per the manufacturer’s instructions. Nonadherent cells were removed and approximately 10% adherent monocytes were added to each flask after gamma irradiation with 7,000 rad from a Cesium source. The cells were cultured in RPMI 1640 supplemented with 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (TCM). T cells were activated with 10 μg/mL PHA-P (Difco Laboratories, Detroit, MI) for 48 hours. At this time, the medium was replaced with fresh TCM containing 50 U/mL recombinant human IL-2. After an additional 48 hours in culture, the nonadherent T cells were harvested.

Iodination of MIP-1α. To radiolabel MIP-1α, aliquots of MIP-1α in acetonitrile buffer were lyophilized and resuspended in sodium tetraborate buffer (pH 8.5) at a concentration of 100 μg/mL. One microliter of [125I]Bolton-Hunter reagent (2,200 Ci/mmol, moniodinated; NEN, Boston, MA) was dried under nitrogen and 10 μL (1 μg) of the MIP-1α solution was added. The solution was incubated for 30 minutes on ice and the reaction was quenched with 490 μL ice-cold 0.2 mol/L glycine in borate buffer. The [125I]MIP-1α was purified by G-50 sephadex gel filtration. After radiolabeling, each preparation of [125I]MIP-1α was analyzed by SDS-PAGE and either silver staining or Western blotting followed by autoradiography. In all cases, a single protein with the same electrophoretic mobility as unlabeled MIP-1α was detected at approximately 8 kD.
[\[^{[\[^{125}\]]\]MIP-1\alpha\] binding and unlabeled ligand competition. Cells to be used for binding analyses were washed twice with PBS (pH 7.4) and resuspended at 2.0 to 5.0 x 10\(^6\) cells/mL in binding buffer consisting of Iscove’s modified Dulbecco’s medium (IMDM) containing 25 mmol/L HEPES and 4 mg/mL BSA (pH 7.4). The cells were incubated for 1 hour at 23°C with varying concentrations of [\[^{125}\]]MIP-1\alpha, ranging from 0.01 to 70 nmol/mL, in the presence or absence of a 100-fold excess of unlabeled MIP-1\alpha in a final volume of 400 \(\mu\)L. After incubation, 0.75 mL of an ice-cold mixture of 75% FBS in binding buffer was added to the cells, the tubes were vortexed, and the cells were pelleted by centrifugation. The supernatant fluids were aspirated and the radioactivity contained in the cell pellets was measured in a gamma counter. All binding experiments were performed in duplicate. Specific binding was defined as the amount of binding competed by a 100-fold excess of unlabeled MIP-1\alpha. Equilibrium binding data were analyzed according to the method of Scatchard and by weighted nonlinear least-squares curve fitting as described by Munson and Rodbard using the LIGAND Program. Objective statistical criteria (F test, extra-sum squares principle) were used to evaluate goodness of fit and for discriminating between receptor binding models. It is apparent that the maximal specifically bound values on the saturation curves are higher than the maximal values on the Scatchard plots (see Fig 3). This is the result of well-documented skewing in Scatchard curves with points at or close to saturation, which is automatically corrected for by the LIGAND Program.

For competition binding experiments using unlabeled ligands other than MIP-1\alpha, the cells were incubated for 1 hour at 23°C in binding buffer containing a constant amount of [\[^{125}\]]MIP-1\alpha and varying concentrations of unlabeled GOS19.1, ACT-2, MIP-1\beta, RANTES, or human IL-8 in the range of 0.01 to 70 nmol/mL. The cells were then further processed as described above.

Cross-linking of [\[^{125}\]]MIP-1\alpha to human hematopoietic cells and cultured cell lines. A total of 5 x 10\(^6\) cells in 1.0 mL binding buffer was incubated for 1 hour at 23°C with 0.4 to 1.25 nmol/mL [\[^{125}\]]MIP-1\alpha in the presence or absence of a 50- to 100-fold excess of unlabeled MIP-1\alpha. The cells were washed twice with ice-cold PBS (pH 7.4) and resuspended in 1.0 mL cold PBS (pH 8.3) to which DSS, DTM, DMS, DMP, or EGS was added to a final concentration of 1 mmol/L. The reaction was quenched after 30 minutes by the addition of 100 \(\mu\)L of ice-cold 1 mol/L Tris-HCl (pH 7.4), and the cells were pelleted by centrifugation and washed twice with cold PBS (pH 7.4). To reduce nonspecific binding and carryover of uncross-linked [\[^{125}\]]MIP-1\alpha, the pellets were resuspended in 1.0 mL of 70 mmol/L sodium acetate, 50 mmol/L NaCl (pH 4.0) and incubated for 10 minutes at 0°C. The samples were washed twice with ice-cold PBS (pH 7.4) and gently rocked for 20 minutes at 4°C in 1.0 mL lysis buffer (PBS, pH 7.4, containing 1% Triton X-100, 1 mmol/L PMSF, 10 \(\mu\)mol/L pepstatin, 1 mg/mL aprotinin, 2 mmol/L EDTA, 10 \(\mu\)mol/L leupeptin, 2 mmol/L benzamidine, and 2 mmol/L 1,10-phenanthroline). After centrifugation, the supernatant fluids were incubated overnight at 4°C with 5 \(\mu\)L of goat polyclonal antimurine MIP-1\alpha antibody and then further incubated with 250 \(\mu\)L of washed 10% Staphylococcus aureus cells bearing protein A (Pansorbin; Calbiochem) for 4 hours at 4°C. The samples were centrifuged, washed three times in lysis buffer, and resuspended in sample buffer containing 1% SDS and 5% 2-mercaptoethanol. Aliquots were electrophoresed through 5% to 20% gradient SDS-polyacrylamide gels and the dried gels were subjected to autoradiography.

Determination of oxidative metabolism in neutrophils. The detection of a respiratory burst in neutrophils was monitored by oxidation of the dye DCFH-DA. The nonpolar diacetate form of the dye readily diffuses into cells and is hydrolyzed to the nonfluorescent polar dichlorofluorescein (DCFH) by intracellular esterases. In the presence of hydrogen peroxide and peroxidase substrates, the dye is oxidized into the highly fluorescent compound DCF. Consequently, the mean level of fluorescence is proportional to the production of these reactive oxygen intermediates and can be monitored by flow cytometry.

Unseparated peripheral blood cells or isolated neutrophils were suspended at 2 x 10\(^6\) cells/mL in diluent buffer consisting of HBSS supplemented with 2% TCH. Cells (0.5-mL aliquots) were incubated with 5 \(\mu\)mol/L DCFH-DA for 15 minutes at 37°C followed by the addition of diluent buffer alone or diluent buffer containing varying concentrations of MIP-1\alpha, FMA, or IMLP. The cells were incubated at 37°C for various time intervals and aliquots analyzed for fluorescent DCF by flow cytometry. Data were collected using a Coulter Elite flow cytometer equipped with a 40 mW air cooled argon laser. Optical alignment of the instrument was performed daily using DNA Check Fluorescent Beads (Coulter, Hialeah, FL) with coefficients of variation less than 1.5 from the integrated signals of forward light scatter and green fluorescence. Cells were analyzed by forward and 90° light scatter properties and the population having the greatest 90° light scatter was electronically isolated. The fluorescence emitted at wavelengths between 505 and 550 nm was determined for each sample. A total of 10,000 to 20,000 events were collected for each sample and stored for further analysis. Quantitation of DCFH oxidation was represented as the mean cell fluorescence.

RESULTS

Physical characterization of MIP-1\alpha. MIP-1\alpha is known to form high molecular mass aggregates under certain conditions, some of which may be biologically inactive. Therefore, we determined the physical forms of both the [\[^{125}\]]MIP-1\alpha and unlabeled MIP-1\alpha used in our experiments. Figure 1A shows the size distribution of the [\[^{125}\]]MIP-1\alpha at concentrations of 1.4 nmol/L and 140 nmol/L as determined by HPLC chromatography. Ninety percent of the [\[^{125}\]]MIP-1\alpha was in a monomeric form, 7% was present as a dimer, and 3% as an aggregated complex (void volume), even at 140 nmol/L. Figure 1B shows a Western blot analysis of column fractions collected after fractionation of a 625 nmol/L aqueous solution of unlabeled MIP-1\alpha. The fractions represent column positions corresponding to the molecular masses of an aggregated complex (void volume, lanes 4 through 6), proteins in the 29 to 66 kD range (lanes 7 through 12), dimeric MIP-1\alpha (lanes 13 through 15), and monomeric MIP-1\alpha (lanes 16 through 18). The analysis shows that the MIP-1\alpha containing BSA (R&D Systems) when resuspended into an aqueous phase is almost entirely monomeric. Only small amounts of aggregated complex and the dimeric form could be detected. Thus, both the [\[^{125}\]]MIP-1\alpha and the unlabeled MIP-1\alpha used in our experiments were approximately 90% monomeric.

Binding of [\[^{125}\]]MIP-1\alpha to human hematopoietic cells and cultured cell lines. We were unable to consistently iodinate GOS19.1, the human homologue of MIP-1\alpha, without loss of specific binding capacity. Therefore, we used [\[^{125}\]]MIP-1\alpha for all binding studies. Although we used a murine ligand to analyze receptors on human cells, GOS19.1 consistently competed effectively for MIP-1\alpha binding to all human cell types tested. Binding of [\[^{125}\]]MIP-1\alpha to THP-1 cells was examined at 0°C, 10°C, and 23°C for periods of time ranging from 15 minutes to 90 minutes. [\[^{125}\]]MIP-1\alpha bound rapidly...
MULTIPLE RECEPTOR CLASSES FOR MIP-1α

Fig 1. HPLC analysis to determine the physical forms of MIP-1α present in 125I-MIP-1α and unlabeled MIP-1α preparations. (A) Size exclusion chromatography of 125I-MIP-1α at 1.4 nmol/L (●) and 140 nmol/L (□). Twenty microliters of 125I-MIP-1α either concentrated or diluted 1:100 in buffer was injected onto a Protein Pak 125 column (Waters) equilibrated with buffer containing 50 nmol/L Tris and 150 mmol/L KCl and eluted in the same buffer at 0.5 mL/min. Total column volume was 14.3 mL. One-milliliter fractions were collected and counted on a gamma counter. The position of the void volume was established using blue dextran. Peak 1 is the void volume that consistently appeared in fraction 7 in multiple experiments and at which the aggregate form eluted. Peak 2 is the dimeric form and Peak 3 is the monomeric form. The column was calibrated using BSA (66 kD), carbonic anhydrase (29.4 kD), cytochrome C (12 kD), and aprotinin (6.4 kD). (B) Western blot analysis of collected HPLC column fractions from a 625 nmol/L unlabeled MIP-1α aqueous preparation. Proteins were transferred onto nitrocellulose after reduced SDS-PAGE analysis and detected using a goat anti-MIP-1α primary antibody and rabbit anti-goat IgG (alkaline phosphatase conjugated) secondary antibody. Antibodies were diluted 1:500. Lanes 1 through 3, MIP-1α control standards at 5, 10, and 25 ng, respectively. Lanes 4 through 6, fraction 7 (protein mass > 88 kD); lanes 7 through 9, fraction 8 (protein mass of ~66 kD); lanes 10 through 12, fraction 9 (protein mass of ~29 kD); lanes 13 through 15, pooled fractions 12 and 13 (protein mass of ~16 kD); lanes 16 through 18, pooled fractions 18 through 22 (protein mass of ~6 to 8 kD). Lanes 6, 8, 11, 14, and 17 are 1:5 dilutions. Lanes 6, 9, 12, 15, and 18 are 1:20 dilutions. Monomeric MIP-1α is detected as an 8-kD molecular mass band.

and specifically at 23°C, with approximately 75% of maximal binding achieved by 15 minutes and equilibrium within 60 minutes (data not shown). Consequently, the incubation time for all steady state binding experiments was chosen to be 60 minutes. In multiple experiments binding was also performed in the presence and absence of 0.1% sodium azide, with no evidence for significant internalization of 125I-MIP-1α (data not shown).

To evaluate whether the affinity of MIP-1α for its receptor(s) was modified by the iodination process, self-displacement studies were performed using THP-1 cells. Binding curves were obtained using a constant amount of 125I-MIP-1α and increasing amounts of unlabeled MIP-1α, as well as increasing amounts of 125I-MIP-1α and a constant excess (100 to 200×) of unlabeled MIP-1α. Nonspecific binding did not exceed 10% of the free concentrations, showing that the monomeric form was the predominant binding species, because only 10% of the 125I-MIP-1α was present in the nonmonomeric form. Figure 2 shows that, when the results are plotted on a linear scale of bound over free MIP-1α versus a log scale of MIP-1α concentration, parallel curves are obtained. This is consistent with an equal affinity of the binding site for either 125I-MIP-1α or unlabeled MIP-1α. Thus, the iodination method used had negligible effects on the binding of MIP-1α to its receptor and the calculated kD values could be accepted. By constructing a self-displacement curve between the lines generated using 125I-MIP-1α and unlabeled MIP-1α, the specific activity for each 125I-MIP-1α preparation was determined. The specific activities ranged from 6.4 × 10⁷ to 2.5 × 10⁹ cpm/ng.

After determination of the specific activity, equilibrium binding studies with 125I-MIP-1α were performed on a variety of cell types. Representative results for THP-1 cells, human CD34+ cells enriched for stem and progenitor cells, and peripheral blood neutrophils are shown in Fig 3. Figure 3A shows binding of MIP-1α to THP-1 cells. Scatchard analysis of the equilibrium binding data in Fig 3B shows that the fitted line through the measured points best describes a one-site binding model. This was further confirmed by the sign test of the residual. In this representative experiment, a single class of high-affinity receptors with 1,300 sites per cell and a kD of 94 pmol/L was identified. Similar to THP-
49,600 sites per cell and a $K_d$ of 19 pmol/L was detected as well as a low-affinity class of receptors with 762 sites per cell and a $K_d$ of 305 pmol/L (Fig 3C and D). In contrast, two classes of receptors were detected on neutrophils. In the representative experiment shown in Fig 3E and F, a high-affinity class of receptors with 900 sites per cell and a $K_d$ of 1000 pmol/L was used as competitor. Cross-linking with $[^{125}]$MIP-la, GOS19.1 and MIP-1$\beta$ competed for binding of $[^{125}]$MIP-la. However, in contrast to THP-1 cells, ACT-2 was unable to compete at all for MIP-1$\alpha$ binding to neutrophils, whereas RANTES showed a limited ability to compete at high molar concentrations. A pattern of displacement similar to that for THP-1 cells was also observed with HL-60 cells (data not shown). The pattern of competition obtained with neutrophils is shown in Fig 4B. Unlabeled MIP-1$\alpha$, GOS19.1 and MIP-1$\beta$ competed for binding of $[^{125}]$MIP-la on THP-1 cells, whereas human IL-8 was unable to compete even at high molar concentrations. Similarly, GOS19.1 competed effectively for binding with $[^{125}]$MIP-la, whereas MIP-1$\beta$ and ACT-2 also competed, but at higher molar concentrations than GOS19.1. RANTES was unable to compete with $[^{125}]$MIP-la even at high molar concentrations. A pattern of displacement similar to that for THP-1 cells was also observed with HL-60 cells (data not shown). The pattern of competition obtained with neutrophils is shown in Fig 4B. Unlabeled MIP-1$\alpha$, GOS19.1 and MIP-1$\beta$ competed for binding of $[^{125}]$MIP-la. However, in contrast to THP-1 cells, ACT-2 was unable to compete at all for MIP-1$\alpha$ binding to neutrophils, whereas RANTES showed a limited ability to compete at high molar concentrations. These results show that the specificity of the receptors expressed on monocytic cells and HL-60 cells differs from those receptors expressed on neutrophils. Furthermore, although ACT-2 has been assigned as the human equivalent of MIP-1$\beta$, it clearly shows different receptor binding properties.

**Cross-linking studies.** $[^{125}]$MIP-1$\alpha$ was bound to various cell types at concentrations between 0.4 and 1.25 nmol/L and cross-linked to cell surface proteins to identify potential receptors by affinity labeling. A molecular mass of 8 kD was used to calculate the molecular mass of proteins found in complexes with $[^{125}]$MIP-1$\alpha$ after immune precipitation of lysed cells, SDS-PAGE, and autoradiography. Identical results were obtained by SDS-PAGE of cell lysates in the absence of immune precipitation, but protein bands were not as sharp, presumably because of overloading the gel lanes.

Using the homobifunctional cross-linker DSS, two proteins with calculated molecular masses of 92 kD (100-kD complex) and 52 kD (60-kD complex) were detected after cross-linking $[^{125}]$MIP-la to THP-1 cells as shown in Fig 5A (lane 1). To determine whether the complexes detected were formed by specific binding of $[^{125}]$MIP-la, unlabeled MIP-1$\alpha$ was used as competitor. Cross-linking in the presence of a 50-fold excess of unlabeled MIP-1$\alpha$ greatly reduced the formation of radioiodinated complexes (lane 2). A similar pattern of affinity-labeled proteins was observed on CD34$^+$ cells and HL-60 cells using DSS (data not shown). We also examined the ligand-receptor complexes formed after cross-linking $[^{125}]$MIP-la to THP-1 cells using other homobifunctional agents with variable spacer arm lengths (Fig 5B). Cross-linking with EGS produced a similar profile of labeled bands, as observed with DSS, whereas only the 92-kD binding species was detected with DST. No complexes formed using DMA, DMS, or DSP. All cross-linked molecular mass complexes smaller in size than the 52-kD species were also detected in experiments using cell types (HeLa S3, Cos 7 and 8, and Colo 320 HSR) that showed no specific binding of MIP-1$\alpha$ (data not shown).

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**Table 1** summarizes the results of multiple binding experiments with THP-1 cells, U937 cells, HL-60 cells, neutrophils, CD34$^+$ cells enriched for stem and progenitor cells, and Jurkatt cells. The results are consistent with the presence of a single class of high-affinity receptors on stem and progenitor cells and monocytic cell lines and the expression of both high- and low-affinity receptors on neutrophils and HL-60 cells. A single class of low-affinity receptors was also detected on activated CD4$^+$ cells, whereas no specific binding to activated CD8$^+$ cells was observed (data not shown). Although Graham et al. have reported the presence of high-affinity receptors on the murine FDCP-mix progenitor cell line, we were consistently unable to detect specific binding of monomeric MIP-1$\alpha$ to this cell line when grown in logarithmic phase in the presence of IL-3 (data not shown).

**Competition binding assays.** To determine whether MIP-1$\alpha$ receptors also bind other C-C chemokines, we investigated the ability of different chemokines to compete for MIP-1$\alpha$ binding. Unlabeled GOS19.1, MIP-1$\beta$, ACT-2, or RANTES was used to directly compete with $[^{125}]$MIP-la for binding to THP-1 cells, human neutrophils, and HL-60 cells. Competition with unlabeled MIP-1$\alpha$ was used as a positive control, whereas competition with human IL-8 (a C-X-C chemokine) served as a negative control.

Figure 4A shows that, as expected, unlabeled MIP-1$\alpha$ competed effectively for binding of $[^{125}]$MIP-la on THP-1 cells, whereas human IL-8 was unable to compete even at high molar concentrations. Similarly, GOS19.1 competed effectively for binding with $[^{125}]$MIP-la, whereas MIP-1$\beta$ and ACT-2 also competed, but at higher molar concentrations than GOS19.1. RANTES was unable to compete with $[^{125}]$MIP-la even at high molar concentrations. A pattern of displacement similar to that for THP-1 cells was also observed with HL-60 cells (data not shown). The pattern of competition obtained with neutrophils is shown in Fig 4B. Unlabeled MIP-1$\alpha$, GOS19.1 and MIP-1$\beta$ competed for binding of $[^{125}]$MIP-la. However, in contrast to THP-1 cells, ACT-2 was unable to compete at all for MIP-1$\alpha$ binding to neutrophils, whereas RANTES showed a limited ability to compete at high molar concentrations. These results show that the specificity of the receptors expressed on monocytic cells and HL-60 cells differs from those receptors expressed on neutrophils. Furthermore, although ACT-2 has been assigned as the human equivalent of MIP-1$\beta$, it clearly shows different receptor binding properties.
Fig 3. Equilibrium binding curves and Scatchard plots for THP-1 cells, CD34+/CD33+/HLA-DR- stem cells and neutrophils. Cells (1 to 4 x 10^6) were incubated with varying concentrations of [125I]MIP-1α with or without a 100- to 200-fold excess of unlabeled MIP-1α as described in Materials and Methods. Scatchard transformations of the data were performed using the LIGAND Program. (A and B) The curves obtained with THP-1 cells; (C and D) with CD34+ cells enriched for stem and progenitor cells; and (E and F) with neutrophils. The binding curves (A, C, and E) are plotted as cpms of [125I]MIP-1α added. Total (e) and nonspecific (c) binding are shown. The Scatchard plots (B, D, and F) are plotted as the ratio of bound to free [125I]MIP-1α versus the concentration of bound [125I]MIP-1α. R, receptor number per cell.

Cross-linking in the absence of cells was also performed to determine whether the specific cross-linked bands identified represent aggregates of MIP-1α. As shown in Fig 5C, cross-linking [125I]MIP-1α alone using DST with increasing concentrations of unlabeled MIP-1α yielded several bands of higher molecular weight, none of which correlated with the 100-kD complex observed in the presence of cells. Cross-linking with DSS also yielded multiple higher molecular weight bands, none of which correlated with the 100-kD or 60-kD complex. Thus, although MIP-1α shows some tendency to self-associate in the presence of cross-linking agents, formation of the 100-kD and 60-kD cross-linked complexes was observed only in the presence of cells and formation of these complexes was greatly reduced by excess unlabeled MIP-1α, confirming the specificity of formation of these complexes.

Biologic activities of C-C chemokines. To investigate the biologic effects mediated by MIP-1α receptors, the effects of varying concentrations of MIP-1α and related chemokines on proliferating murine stem cells and human neutrophils were analyzed. Murine stem cells were used as a model, because there is no convenient assay for proliferating human stem cells. Figure 6 shows that a 4-hour exposure of proliferating murine stem cells to 1.2 nmol/L MIP-1α significantly reduces the S-phase component, as previously reported.21 In repeated experiments, we observed that higher doses of MIP-1α (2.5 to 6.25 nmol/L) consistently showed decreased activity (data not shown). At concentrations between 300 and 600 pmol/L, both GOS19.1 and ACT-2 also greatly reduced the proportion of stem cells in S-phase. Again, at higher concentrations (2.5 to 4.0 nmol/L), biologic activity was lost. The reasons for the decrease in activity observed at higher
control. Figure 7 shows a representative time course for the blood neutrophils, exposure of the cells to PMA (1 nmol/L and 100 nmol/L), over 90 minutes, and was comparable to that obtained with MIP-1α, although much lower, was approximately linear.

brane and directly stimulates protein kinase C. The rate for PMA and fMLP, both potent inducers of oxidative metabolism, in neutrophils with the release of free radicals, we were interested in determining whether MIP-1α also induces oxidative metabolism in neutrophils. As positive controls, we used the response observed with PMA and fMLP, both potent inducers of oxidative metabolism. The response to diluent alone was used as a negative control. Figure 7 shows a representative time course for the increase in release of free radicals in unseparated peripheral blood neutrophils, as detected by fluorescence of DCFH after exposure of the cells to PMA (1 nmol/L and 100 nmol/L), fMLP (1 nmol/L), and MIP-1α (1.25 nmol/L). The mean cellular fluorescence obtained with diluent alone increased only slowly over the time course studied. As expected, the increase in free radical product obtained with PMA was rapid and massive, because PMA bypasses the cellular membrane and directly stimulates protein kinase C. The rate for MIP-1α, although much lower, was approximately linear over 90 minutes, and was comparable to that obtained with fMLP. Table 2 shows the results of several experiments in which the rates of production of free radicals induced by MIP-1α and fMLP in unseparated peripheral blood neutrophils and isolated neutrophils were compared by measuring the levels of mean cellular fluorescence above the background value (diluent alone) at 90 minutes. Although the rate induced by MIP-1α was low (2 to 6 times the background rate), it was consistent and varied from between 25% to 70% of the rate obtained with 1 nmol/L fMLP. A consistent increase in free radical production was obtained with 125 pmol/L MIP-1α, suggesting that the high-affinity receptors expressed on neutrophils may mediate the induction of oxidative metabolism.

**DISCUSSION**

MIP-1α belongs to the C-C family of chemokines with proinflammatory and chemotactic properties. It has a broad spectrum of biologic activities on a variety of cell types, including stem cells, monocytes, basophils, T cells, and mast cells. An understanding of the mechanisms by which MIP-1α induces its diverse biologic effects requires characterization of specific receptors for MIP-1α on responsive cells. One major drawback to such an analysis is the tendency for MIP-1α to form high molecular mass aggregates with unknown biologic activity. In this report, we present evidence for different classes of MIP-1α receptors expressed on different human hematopoietic cell types using a defined monomeric biologically active form of MIP-1α.

Using HPLC, we show that the [125I]MIP-1α preparations concentrations of these chemokines are not known. In contrast, relatively high concentrations (2.5 nmol/L) of MIP-1β were required to inhibit stem cells, with little effect observed at picomolar concentrations, again underlying a difference in the biologic activities of ACT-2 and MIP-1β.

Because many cytokines have been reported to induce a respiratory burst in neutrophils with the release of free oxygen radicals, we were interested in determining whether MIP-1α also induces oxidative metabolism in neutrophils. The response to diluent alone was used as a negative control.

**Table 1. [125I]MIP-1α Binding Affinities and Receptor Numbers on Hematopoietic Cells**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>( kd )</th>
<th>Receptors per Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1</td>
<td>80 ± 25 pmol/L</td>
<td>1,370 ± 370</td>
</tr>
<tr>
<td>U937</td>
<td>170 ± 60 pmol/L</td>
<td>1,420 ± 650</td>
</tr>
<tr>
<td>HL-60*</td>
<td>29 ± 26 pmol/L</td>
<td>720 ± 560</td>
</tr>
<tr>
<td></td>
<td>10 ± 4 nmol/L</td>
<td>49,800 ± 27,700</td>
</tr>
<tr>
<td>Neutrophil1</td>
<td>15 ± 3 pmol/L</td>
<td>380 ± 200</td>
</tr>
<tr>
<td></td>
<td>5 ± 2 nmol/L</td>
<td>22,100 ± 13,900</td>
</tr>
<tr>
<td>CD34+, CD33+, HLA-DR+</td>
<td>305 pmol/L</td>
<td>900</td>
</tr>
<tr>
<td>Jurkat</td>
<td>3.4 nmol/L</td>
<td>4,072</td>
</tr>
</tbody>
</table>

Summary of results of MIP-1α equilibrium binding experiments. Where indicated, the value is reported as the average value of separate experiments (N = 3 to 8) ± standard error of the mean. An F test was used to calculate the P values, which were then used to distinguish between a one-site model or a two-site model.

* P = .003.

† P < .0001.

Fig 4. Competition by other ligands for binding of [125I]MIP-1α to THP-1 cells and neutrophils. Two hundred microliters of 0.5 nmol/L [125I]MIP-1α diluted in binding buffer was added to 1 to 2 × 10⁶ cells in 200 µL to which 5 µL of unlabeled ligand was added and the cells incubated as described in Materials and Methods. (○) MIP-1α, (▼) GOS19.1.1, (■) MIP-1β, (□) ACT2, (♦) RANTES, and (△) HL-8. The data were plotted as counts per minute (CPM) bound versus concentration of the unlabeled ligand. (A) is for THP-1 cells and (B) is for neutrophils.
MULTIPLE RECEPTOR CLASSES FOR MIP-1α

Fig. 5. Cross-linking of [125I]MIP-1α to THP-1 cells. (A) [125I]MIP-1α was covalently cross-linked to THP-1 cells in the absence (lane 1) or presence (lane 2) of excess unlabeled MIP-1α using 1 mmol/L DSS. The cells were lysed, the lysates were immunoprecipitated with a goat anti-MIP-1α antibody, and the immunoprecipitates were visualized by SDS-PAGE and autoradiography. (B) Cross-linking of [125I]MIP-1α to THP-1 cells using cross-linking agents with varying linker arm lengths. Lane 1, 1 mmol/L DST (6.4Å); lane 2, 1 mmol/L DMA (8.6Å); lane 3, 1 mmol/L DMS (11.0Å); lane 4, 1 mmol/L DSS (11.4Å); lane 5, 1 mmol/L DSP (12.0Å); lane 6, 1 mmol/L EGS (16.0Å). DSP is a 2-mercaptoethanol cleavable cross-linker. (C) Cross-linking of [125I]MIP-1α in the absence of cells. [125I]MIP-1α was incubated with increasing concentrations of unlabeled MIP-1α in the presence of 1 mmol/L DST. Lane 1, 50 ng/mL unlabeled MIP-1α; lane 2, 100 ng/mL unlabeled MIP-1α; lane 3, 200 ng/mL unlabeled MIP-1α. In (A and B), arrows indicate specific cross-linked complexes with apparent molecular masses of 92 kD and 52 kD. M, 13C-labeled protein molecular mass markers (in kilodaltons).

used in our binding studies are almost entirely monomeric at concentrations up to 140 nmol/L. [125I]MIP-1α was prepared by lyophilizing recombinant protein dissolved in acetonitrile buffer and resuspending it in sodium tetraborate buffer. Unlabeled MIP-1α (supplied by R&D Systems), when lyophilized and reconstituted into an aqueous phase, is also almost entirely monomeric at concentrations tested up to 625 nmol/L. Ninety percent of our [125I]MIP-1α is monomeric and nonspecific binding accounts for 10% of the total binding of MIP-1α to monocytes. Our results strongly suggest that the active form of MIP-1α is a monomer, unlike the related chemokines IL-8 and PF4, which appear to act as dimers and tetramers, respectively.40,41 Our results contrast with a recent report that showed that MIP-1α from R&D Systems forms inactive aggregates when directly diluted into phosphate buffer from acetonitrile buffer at concentrations greater than 1.2 nmol/L.32 At this time, little is understood about aggregate formation, but it is conceivable that aggregates with distinct properties may form under different conditions. In addition, there clearly appears to be variability in the physical state of MIP-1α in different commercial preparations. We have tested four separate preparations of MIP-1α and found only two to be monomeric, further emphasizing the need to characterize each preparation before study. Thus, aggregate formation has confused the interpretation of previous attempts to characterize the biologic activity and receptor interactions of MIP-1α. We show here that monomeric MIP-1α is biologically active and is able to bind to different affinity classes of receptors present on different blood cell types.

Using monomeric MIP-1α, we show for the first time the presence of a single class of high-affinity receptors on primary (leukemic) human stem and progenitor cells with a CD34+, CD33−, HLA-DR− immunophenotype (kd = 305 pmol/L; 900 receptors per cell). Unfortunately, these cells were completely quiescent and could not be tested for a proliferative response to MIP-1α. We are currently establishing culture conditions that will promote their propagation. Although we were unable to directly examine the response of proliferating human stem cells to MIP-1α, the calculated kd for MIP-1α on human stem and progenitor cells correlates with the picomolar concentrations of MIP-1α required to...
inhibit proliferating murine stem cells. At picomolar concentrations, GOS19.1 and ACT-2 also inhibit murine stem cells, but inhibition tends to be reduced at higher concentrations of these chemokines. Wright et al have shown that human bone marrow-conditioned medium contains an activity similar to murine SCI, which is able to inhibit murine stem cells. We suggest that both GOS19.1 and ACT-2 contribute to this activity. Interestingly, MIP-1\(\beta\) is unable to effectively inhibit murine stem cells. Thus, MIP-1\(\beta\) has distinct properties compared to ACT-2. To date, because of difficulties in obtaining sufficient numbers, it has not been possible to directly analyze MIP-1\(\alpha\) receptors present on primary murine stem cells. However, using the IL-3-dependent murine FDCP-mix progenitor cell line, Graham et al reported the presence of 20,000 to 30,000 MIP-1\(\alpha\) receptors per cell, with a \(k_d\) of 250 pmol/L. These investigators used a value of 100 \(k_d\) for the molecular mass of MIP-1\(\alpha\) (aggregate form) and it is possible these estimates are inaccurate. We have consistently been unable to detect any specific binding of monomeric MIP-1\(\alpha\) to FDCP-mix cells growing in log phase, although we used the same clone and apparent growth conditions as Graham et al. We are unable to explain the difference in results at this time, except to note that progenitor cell populations are difficult to propagate without losing their progenitor-like properties. It is interesting to note that, when we deprive our FDCP-mix cells of IL-3, we are able to detect two affinity classes of MIP-1\(\alpha\) receptors with properties similar to those expressed on neutrophils and HL-60 cells. We speculate that the cells may terminally differentiate under these conditions.

In multiple experiments, we detect a single class of high-affinity receptors for MIP-1\(\alpha\) on human monocytic cell lines. THP-1 cells exhibit 1,370 \(\pm\) 370 receptors per cell, with a \(k_d\) of 80 \(\pm\) 25 pmol/L, whereas U937 cells exhibit 1,420 \(\pm\) 550 receptors per cell, with a \(k_d\) of 170 \(\pm\) 60 pmol/L. This is in agreement with another report of high-affinity receptors for MIP-1\(\alpha\) on THP-1 cells. In contrast, we show that neutrophils express two classes of receptors. A high-affinity class is present with an average of 380 \(\pm\) 200 receptors per cell and a \(k_d\) of 15 \(\pm\) 3 pmol/L, as well as a low-affinity class with 22,100 \(\pm\) 13,900 receptors per cell and a \(k_d\) of 5 \(\pm\) 2 nmol/L. HL-60 cells show a similar pattern of receptor expression as neutrophils. We also observe the presence of a single class of low-affinity receptors on the CD4\(^+\) Jurkatt cell line (\(k_d\) = 3.4 nmol/L; 4,072 receptors per cell) and on activated peripheral blood CD4\(^+\) T cells (\(k_d\) = 1 nmol/L; 1,311 receptors per cell, data not shown).

The receptors present on isolated neutrophils are biologically active and mediate a respiratory burst. In some experiments, we have been able to detect a respiratory burst with as little as 12.5 pmol/L MIP-1\(\alpha\) (data not shown), strongly suggesting that the high-affinity receptor mediates this response. Recently, McColl et al have reported that 100 nmol/L MIP-1\(\alpha\) induced calcium mobilization in isolated neutrophils. However, these investigators were unable to detect an increase in oxidative metabolism. We cannot fully explain this difference in results, except to note that we were unable to obtain a consistent response with neutrophils until using Polymorphprep isolation rather than the more commonly used method of centrifugation through Ficoll-Hypaque followed by dextran sulfate sedimentation.

Human MIP-1\(\beta\) has been reported to bind to the same receptor as MIP-1\(\alpha\) on human monocytes and T cells using direct binding competition. However, other previous attempts to analyze whether different members of the C-C family of chemokines share the same receptor have used desensitization studies in which the biologic response of cells to one chemokine is suppressed by prior exposure to another. The interpretation of such studies is complicated by the possibility that MIP-1\(\alpha\) receptors belong to the seven membrane-spanning domain G-protein linked receptor class. If related, receptors share the same heterotrimeric G-protein complexes. If this were so, activation and downregulation of a heterotrimeric complex by ligand binding to one receptor...
species could render the complexes unavailable to a second receptor species. Direct competition for binding of MIP-1α to receptors by other chemokines overcomes this ambiguity. We report for the first time different direct competition binding patterns by MIP-1β; ACT-2, the presumed human homologue of MIP-1β; and RANTES for MIP-1α receptors on human monocytes and neutrophils. The pattern of competition to monocytic cell lines is different than that observed to neutrophils. Using THP-1 cells that express a single class of high-affinity receptors, binding is effectively competed by MIP-1α (self-competition), GOS19.1, MIP-1β, or ACT-2. There is no detectable competition by RANTES (a C-C chemokine) or human IL-8 (a C-X-C chemokine). However, using neutrophils, MIP-1α, MIP-1β, and GOS19.1 still compete effectively, but there is no detectable competition by ACT-2. As expected, human IL-8 does not compete, but RANTES shows partial competition. Thus, MIP-1β appears to share the same receptors for MIP-1α and GOS19.1 on both monocytes and neutrophils (albeit with a lower affinity), whereas ACT-2 shares the same receptors only on monocytes. The results also show that ACT-2 behaves quite differently than MIP-1β. Although ACT-2 has been assigned as the human homologue of MIP-1β, these results, taken together with the results on stem cell inhibition, raise questions as to their equivalence. It remains probable that not all members of the C-C chemokine family have been identified.

Our studies have not addressed whether MIP-1α receptors internalize after ligand binding. It has previously been shown that receptors for IL-8 and MCAF undergo internalization that is not prevented by azide treatment. Our attempts to prevent MIP-1α receptor internalization by binding at 0°C gave binding curves with poor fits when analyzed using the LIGAND Program. Thus, it is possible we may have underestimated the number of MIP-1α receptors present, but this should not affect the interpretation of our results.

Receptors for the C-X-C and C-C chemokine families have been investigated by several groups and a number of receptors have been cloned. Two distinct high-affinity receptors for IL-8 have been cloned and shown to be members of the G-protein coupled class of receptors with seven membrane spanning domains. Recently, two groups cloned low-affinity receptors for MIP-1α with identical nucleotide sequences from HL-60 cDNA libraries by hybridization of either redundant oligonucleotides representing conserved coding regions of the transmembrane domains from G-protein coupled receptors, or probes from analogous regions of the IL-8 receptor. The cloned receptor had a predicted molecular mass of approximately 41 kD. Neote et al. expressed this receptor in human embryonic kidney cells and concluded that it bound MIP-1α with an affinity of approximately 4 to 10 nmol/L and mediated a calcium flux in the transfected cells. Competition studies demonstrated that MIP-1β competed with high affinity and RANTES with lower affinity for the binding of MIP-1α, whereas competition with ACT-2 was inefficient. These properties are identical to the low-affinity receptors we find on isolated neutrophils, and we suggest that this is the molecular species cloned by this group. To date, there are no reports of cloned high-affinity receptors for MIP-1α. It is possible that this is a consequence of the cloning strategies used or that the intracellular environment affects the affinity and specificity of the receptors being expressed. Further biologic and molecular

Table 2. Neutrophil Oxidative Metabolism Measured by DCF Fluorescence

<table>
<thead>
<tr>
<th></th>
<th>PMA</th>
<th>1 nmol/L</th>
<th>10 nmol/L</th>
<th>0.125 nmol/L</th>
<th>1.25 nmol/L</th>
<th>10 nmol/L</th>
<th>100 nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unseparated</td>
<td>7.0</td>
<td>10.1</td>
<td>10</td>
<td>10</td>
<td>2.0</td>
<td>3.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated (N = 2)</td>
<td>10</td>
<td>6.2</td>
<td>5.7</td>
<td>2.0</td>
<td>1.8</td>
<td>3.4</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Data represent experiments using unseparated peripheral blood cells or isolated neutrophils. Data are expressed as the fold increase in mean fluorescence of DCF in the presence of fMLP or MIP-1α as compared with the mean fluorescence of DCF alone (negative control) at 90 minutes ± the standard deviation.

Abbreviation: ND, not done.

* Value obtained from a single subject; data shown represent a single subject at the respective concentrations.

† Both unseparated peripheral blood cells and isolated neutrophils were examined in these two subjects.
characterization of receptors that bind MIP-1α and other members of the C-C chemokine family are necessary to resolve these questions.

The affinity-labeled cell surface proteins detected by cross-linking [125I]MIP-1α to THP-1 cells at concentrations between 400 pM/mL and 1.25 nM/mL implicate two potential MIP-1α receptor binding proteins with calculated masses of 52 kD and 92 kD. The 92-kD species is detected by all cross-linking agents that yield specific complexes, whereas the 52-kD species is detected only by EGS or DSS. The reasons for the difference in patterns remains unknown. The cross-linked complexes detected in all cell types examined are the same size regardless of whether the cells express a single affinity class of receptors or two affinity classes. Further experiments using an extended range of MIP-1α concentrations may resolve the question of which molecular mass species represents the high-affinity receptor protein.

ACKNOWLEDGMENT

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The active monomeric form of macrophage inflammatory protein-1 alpha interacts with high- and low-affinity classes of receptors on human hematopoietic cells

BR Avalos, KJ Bartynski, PJ Elder, MS Kotur, WG Burton and NM Wilkie