Identification and Characterization of a Promiscuous Chemokine-binding Protein in a Human Erythroleukemic Cell Line*

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The erythrocyte chemokine receptor is a cell surface protein that binds a wide array of chemokines including interleukin-8 (IL-8), melanoma growth stimulating activity (MGSA), monocyte chemotactic protein-1 (MCP-1), and RANTES (Regulated on Activation, Normal T Expressed and Secreted). This protein has also been identified as the Duffy blood group antigen, a cell surface receptor for the malarial parasite Plasmodium vivax. In the present study, we have identified a chemokine receptor-like binding protein in a human erythrocyte cell line (HEL), which, based on its molecular properties, may be related to the erythrocyte chemokine receptor. Saturation binding studies with $^{125}$I-IL-8 revealed a single class of IL-8 binding sites in HEL cells with a $K_d$ of 7.4 ± 1.9 nM and a receptor density of 12,818 ± 965 binding sites/cell. In competition studies unlabeled IL-8, MGSA, MCP-1, and RANTES were fully able to inhibit the binding of $^{125}$I-IL-8 to HEL cells. Chemical cross-linking with radiolabeled IL-8 resulted in a cross-linked species of 60 kDa in membranes from HEL cells. The labeling was specific since it was inhibited by preincubation with 1 μM unlabeled IL-8 or MGSA. A monoclonal antibody (Fy6) to the human erythrocyte Duffy blood group antigen/chemokine receptor blocked the binding of IL-8 and other chemokines to the HEL cell chemokine receptor-like binding protein. Cell membranes from HEL cells and from erythrocyte ghosts were subjected to SDS-PAGE and analyzed by Western blotting with anti-Fy6. The antibody bound to a molecule with a molecular mass of 50 kDa in HEL cell membranes and 40 kDa in erythrocyte ghosts. Northern blot analysis of mRNA revealed that the HEL chemokine-binding protein hybridized to a cDNA probe to the Duffy antigen/chemokine receptor.

The Duffy blood group antigen is a cell-surface protein expressed in human erythrocytes that is required for the invasion of these cells by the human malarial parasite Plasmodium vivax (1). Besides this role, the Duffy antigen has also been shown to be a binding protein for a family of chemotactic polypeptides known as the chemokines (2). The chemokines, which include interleukin-8 (IL-8),* melanoma growth stimulating activity (MGSA), and monocyte chemotactic protein-1 (MCP-1), are a family of small secreted proteins involved in chemotraction of immune cells (3, 4). The intriguing double identity of the Duffy antigen was first revealed by the observation that erythrocytes from Duffy-negative individuals fail to bind IL-8 (2). In addition, a monoclonal antibody, Fy6, to the Duffy antigen (5) dose-dependently inhibits chemokine binding to human erythrocytes (2). The suspected relationship between these two proteins was recently confirmed with the cloning of the Duffy antigen (6). When the cDNA coding for the Duffy antigen is stably transfected into a human erythroleukemic cell line, K562, the expressed protein displays the chemokine binding profile of the erythrocyte chemokine receptor (7).

Given the importance of the Duffy antigen/chemokine receptor (DARC), both as a potential target for an antimalarial drug and as a binding protein that could play a role in the regulation of intravascular levels of chemokines, we surveyed a variety of cell lines to determine whether they expressed this protein. Here we report that a human erythroleukemic cell line, HEL (8), appears to express a protein that has the characteristic hallmarks of DARC, i.e. receptor binding of a wide array of chemokines, inhibition of this binding by the Fy6 antibody, cross-reactivity by Western blotting with Fy6 of a protein of a similar molecular mass, and hybridization of mRNA from HEL cells with a cDNA probe to DARC.

EXPERIMENTAL PROCEDURES

Materials—$^{125}$I-IL-8 (specific activity 2200 Ci/mmol) was from DuPont NEN. Unlabeled IL-8 and MGSA were purified as described previously (9, 10). Unlabeled RANTES and MCP-1 were from Peprotech. Reagents for electrophoresis were from Bio-Rad. Hepes and all other reagent-grade chemicals were from Sigma.

Cell Culture—HEL cells were obtained from the American Type Culture Collection and were maintained in RPMI 1640 medium containing 10% fetal calf serum. The cells were passaged weekly, and the medium was changed two additional times weekly. For binding assays the cells were collected, washed three times with RPMI 1640, and resuspended in binding buffer (RPMI 1640 containing 1% bovine serum albumin, 20 mM Hepes, pH 7.4). Cell viability was assessed by trypan blue exclusion, and cell number was determined by counting the cells in a hemacytometer.

Isolation of Erythrocytes and Production of Erythrocyte Ghosts—Human erythrocytes were isolated from whole blood as described previously (11). Ghosts were prepared as described earlier (12).

Receptor Binding Assays—HEL cells (2 x 10⁶ cells/ml) were incubated with $^{125}$I-IL-8 (0.2 nM) and varying concentrations of unlabeled ligands at 4 °C for 1 h. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicone/paraffin oil mixture as described previously (13). Nonspecific binding was determined in the presence of 1 μM unlabeled MGSA, melanoma growth stimulating activity; MCP-1, monocyte chemotactic peptide 1; MIP-1, macrophage inflammatory protein; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-cyclohexylamino/propane-1-sulfonic acid; MOPS, 4-morpholinopropanesulfonic acid.

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beled ligand. The binding data were curve-fit with the computer program LiGAND (14) to determine the affinity (KD), number of sites, and nonspecific binding.

Preparation of Cell Membranes—HEL cells were resuspended to a final concentration of 2 × 10^6 cells/ml in 59 mM Tris-HCl buffer, pH 7.4, containing 5 μg/ml of leupeptin and aprotonin, 0.1 mM phenylmethylsulfonyl fluoride, 0.05 mM Pefabloc, and 1 mM EDTA (lysis buffer). The cells were placed in a nitrogen cavitation chamber under 300 psi of pressure at 4 °C for 30 min. The lysed cells were then centrifuged at 5000 x g for 20 min. The cell pellet, which consisted of cell debris and nuclei, was discarded, and the supernatant was centrifuged at 48,000 x g for 30 min. After centrifugation the pellet was removed and resuspended to a final concentration of 1.5 mg/ml in lysis buffer and stored at -20 °C until further use.

Western Blot Analysis—Proteins were subjected to electrophoresis in 12% Novex pre-cast minigels and transferred electroeoretically to Problot (Applied Biosystems) in 10 mM CAPS, pH 11, containing 10% methanol for 1 h at a current of 250 mA. Following transfer, the blots were incubated for 90 min at room temperature in 25 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.1% Tween 20. The blots were then incubated overnight at 4 °C with a 1:1000 dilution of a monoclonal antibody to the Duffy antigen (Fy6). The blots were washed several times in Tris buffer and were incubated in a 1:20,000 dilution of horse-radish peroxidase-conjugated goat anti-mouse antibody (Tago Immunodiagnostics) for 1 h at room temperature. After extensive washing, the blots were developed by the ECL chemiluminescence procedure, according to the manufacturer's instructions (Amersham Corp.).

Cross-linking of IL-8 to Cell Membranes—150 μg of membranes were incubated in the presence of 5 nM 125I-IL-8, in the presence or absence of 1 μM unlabeled IL-8 or MGSA, for 1 h at 37 °C in PBS, pH 7.4. At the end of the incubation, the membranes were pelleted by centrifugation (100,000 x g, for 15 min), made up to the original volume in PBS, and chemically cross-linked with EDC at a final concentration of 1 M for 1 h at room temperature. The membranes were then pelleted as described above and solubilized in SDS sample buffer in the presence of 5% β-mercaptoethanol for 3 min at room temperature and then analyzed by 12% SDS gels.

Treatment of IL-8 Cross-linked Erythrocyte Membranes with N-Glycosidase F—HEL cell membranes (150 μg) were incubated with 125I-IL-8 in N-glycosidase F (20 unit/ml) in 50 mM sodium phosphate buffer, pH 7, for 4 h at 37 °C. The membranes were centrifuged, solubilized, and analyzed by SDS-PAGE as described above.

Northern Blot Analysis—Cell pellets of HEL cells were extracted with guanidinium isothiocyanate (15) and total cellular RNA was quantitated by measuring the absorbance at 280 nm. Aliquots of RNA were subjected to electrophoresis in a 1.2% agarose gel containing 20 mM sodium acetate, 5 mM sodium citrate, 1 mM EDTA, and 0.5% formaldehyde. The size-fractionated RNA was transferred to a nylon membrane. A complementary DNA/cDNA probe spanning the coding sequences of DARC (6) was labeled with [α-32P]CTP (3000 Ci/mol) using random hexanucleotide primers. The probe was allowed to hybridize to the membrane overnight at 42 °C in the presence of 50% formamide. Blots were washed under high stringency conditions (62 °C in 0.1 x SSC, 0.1% SDS) and exposed to XAR-5 film backed by an intensifying screen at -70 °C.

RESULTS AND DISCUSSION

To determine whether any human erythroleukemic cell lines expressed the erythrocyte DARC, we screened a variety of cell lines, including K562, and HEL cells, for 125I-IL-8 binding. Of the cell lines tested, only the HEL cells showed specific 125I-IL-8 binding (Fig. 1). These cells, which were originally derived from a patient with Hodgkin's disease, carry the phenotypic markers of erythroid cells that include the ability to synthesize globin (8). Fig. 1 shows the specific binding of 125I-IL-8 to HEL cells as a function of ligand concentration, and the inset shows the Scatchard analysis of the binding data. 125I-IL-8 bound to the HEL cells increased as the amount of ligand increased toward the level of saturation. Scatchard analysis of the binding data gave a linear plot consistent with a single class of binding sites with a KD of 7.4 ± 1.9 nM and a receptor density of 12,818 ± 965 binding sites/cell. This KD for IL-8 binding compares favorably with that previously obtained for IL-8 binding to the human erythrocyte DARC, which ranges from 5 to 10 nM with a receptor density of 5000 sites/cell (11, 16, 17).

The specificity of 125I-IL-8 binding to HEL cells was tested by incubation in the presence of increasing concentrations of unlabeled chemokines (Fig. 2). As shown in Fig. 2 increasing concentrations of unlabeled IL-8, MGSA, RANTES, and MCP-1 dose-responsively displaced 125I-IL-8 binding to HEL cells. The KD values for this displacement were around 5–10 nM for the unlabeled chemokines. In contrast, excess unlabeled MIP-1α or MIP-1β displaced the specifically bound 125I-IL-8 very poorly. The 125I-IL-8 binding profile to HEL cells is very similar to that obtained for the erythrocyte DARC (16, 17), except that the nonspecific binding in HEL cells was around 35–40% compared to 5–15% in erythrocytes. Thus, based on the ligand binding profiles, the HEL chemokine-binding protein is similar to the erythrocyte DARC.

To further explore the molecular properties of the HEL chemokine-binding protein, we chemically cross-linked membranes from HEL cells with 125I-IL-8 in the presence and absence of 1 μM unlabeled IL-8 and MGSA (Fig. 3). Analysis by SDS-PAGE revealed the covalent labeling of a protein with approximate molecular mass of 60 kDa (Fig. 3, lane 1). The inclusion of unlabeled IL-8 or MGSA decreased its intensity of labeling (Fig. 3, lanes 2 and 3). If one molecule of IL-8 binds to one receptor, the molecular mass of the HEL cell chemokine-binding protein is 52 kDa compared to a molecular mass of 39 kDa for the erythrocyte DARC (16, 17). It is likely that these differences in molecular mass of these two proteins reflect differences in glycosylation. Indeed, treatment of HEL membranes with N-glycanase reduces the molecular mass of the HEL chemokine-binding protein from 52 to 38 kDa (Fig. 4), while the molecular mass of the erythrocyte DARC is only reduced by 7 kDa under the same conditions (16).

The human erythrocyte DARC has been identified as a 39-kDa protein by Western blotting with a monoclonal antibody to the Duffy antigen, Fy6 (16). To determine whether Fy6 would similarly cross-react with the HEL chemokine-binding protein, we carried out immunoblotting experiments with HEL cell membranes and compared their immunoreactivity with Fy6 to that of the erythrocyte ghosts (Fig. 5). Immunoblotting of the erythrocyte ghosts confirmed that the Fy6 antibodies bound to a protein with a molecular mass of 40 kDa (Fig. 5). In contrast the Fy6 antibodies bound to a protein with a molecular mass of 50 kDa in the HEL membranes. Thus, the molecular mass of the HEL cell chemokine-binding protein obtained by immunoreactivity with the Fy6 antibody is very similar to that obtained by chemical cross-linking with radiolabeled IL-8 (Fig. 3).

We have shown previously that the antibody Fy6 dose-reponsively inhibited the binding of IL-8 and other chemokines...
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Fig. 2. Inhibition of $^{125}$I-IL-8 binding to HEL cells. HEL cells (4 x $10^6$ cells/ml) were incubated for 1 h at 4 °C with $^{125}$I-IL-8 (0.2 nM) in the absence or presence of increasing concentrations of unlabeled IL-8, MGSA, RANTES, MCP-1, and MIP-1α. The binding reactions were stopped as described under "Experimental Procedures." The data in this figure are calculated by subtracting nonspecific binding (binding in the presence of 1 μM unlabeled ligand) from total binding (binding in the absence of unlabeled ligand). This is taken to represent 100% binding, and the data for other experimental points are all expressed as percentages of this initial binding.

Fig. 3. Covalent cross-linking of $^{125}$I-IL-8 to HEL cell membranes. Membranes prepared from HEL cells were incubated with 5 nM $^{125}$I-IL-8 in the absence (lane 1) and in the presence (lane 2) of 1 μM unlabeled IL-8 and 1 μM unlabeled MGSA (lane 2). After 1 h at 4 °C, the membranes were washed free of unbound label. The bound $^{125}$I-IL-8 was cross-linked by incubation with EDC for 1 h at 4 °C and then washed with PBS before analysis by SDS-PAGE. 150 pg of protein were applied to the gel. After electrophoresis the gels were dried down and subjected to autoradiography.

Fig. 4. Effect of N-glycanase on the mobility of the $^{125}$I-IL-8 cross-linked HEL cell chemokine-binding protein on SDS gels. Membranes prepared from HEL cells were cross-linked to $^{125}$I-IL-8 as described above and treated with (lane 2) and without (lane 1) N-glycanase as described under "Experimental Procedures." The reaction products were solubilized with SDS sample buffer and run on a 10% gel.

Fig. 5. Western blot analysis of HEL cell membranes. 150 μg of HEL cell membranes and erythrocyte ghosts were subjected to SDS-PAGE, transferred electrophoretically to Problot, and analyzed as indicated under "Experimental Procedures."

Since immunologic and ligand binding analyses demonstrated that HEL cells expressed a DARC-like molecule, we carried out a Northern blot analysis with a cDNA probe corresponding to the coding sequence of the DARC. RNA extracted from HEL cells contained an RNA species of approximately 1.2 kilobase pairs that annealed to the DARC cDNA probe under high stringency conditions (Fig. 7). The RNA transcript present in HEL cell RNA comigrated with that isolated from human kidney from a Duffy-positive individual. These data further suggest that HEL cells express a chemokine-binding protein that is identical to the DARC.

The identification of a human chemokine receptor-like binding protein in HEL cells is especially interesting since the DARC mRNA has been identified in brain, kidney, and spleen.
The internalization of chemokines by the DARC presumably serves to clear them from the circulation, and after internalization they are either degraded or recycled to the cell surface by retroendocytosis (18). Thus, the DARC-like binding protein in HEL cells may be more reflective of the DARC expressed in non-erythroid tissues, both in form and function, and could prove to be an interesting cell type to study the intracellular fate of the internalized chemokines.

It will also be interesting to determine whether the HEL chemokine-binding protein can bind the malaria parasite and, if so, whether the parasite can invade these cells. The malarial parasite P. vivax has an absolute requirement for globin, and since HEL cells can be induced to express this protein (8), they might presumably support the growth of the parasite. Should this prove to be the case, then HEL cells, since they are a cultured cell line, could be a useful cell line in which to study malarial invasion. In addition, since HEL cells are nucleated, it should prove possible to study the gene regulation and expression of the chemokine-binding protein in these cells, something that is not possible in the non-nucleated erythrocytes. These studies could be useful in helping to clarify the mechanisms of malarial invasion of the erythrocyte. Finally, it should also be possible to examine whether the HEL chemokine-binding protein can signal in response to IL-8. With respect to signaling, preliminary experiments indicate that IL-8 and MGSA have no measurable effects on the ability of HEL cells or of erythrocytes to flux Ca²⁺ (data not shown). It is possible that the major role of the DARC in erythrocytes is as a clearance receptor (to remove chemotactic and inflammatory peptides from the blood) (11) rather than as a signaling receptor. The significance of the erythrocyte chemokine receptor’s role as a sink for chemokines, however, must be interpreted in the light of individuals who lack expression of this protein on erythrocytes (1), yet appear to be able to mount a normal immune response. However, although the erythrocytes of Duffy-negative individuals do not express the DARC, we do not know if this protein is expressed on their non-erythroid tissues (kidney, spleen, etc.). Thus, it is possible that the Duffy-negative phenotype is restricted to erythrocytes, as an adaptive response to resist malaria, and that the non-erythroid tissues of Duffy-negative individuals express the protein where it plays a full role in the normal inflammatory response.

REFERENCES


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