Glycosaminoglycans Mediate Cell Surface Oligomerization of Chemokines

Arlene J. Hoogewerf,*,‡,§ Gabriele S. V. Kuschert,|| Amanda E. I. Proudfoot,‡ Frederic Borlat,‡ Ian Clark-Lewis,⊥ Christine A. Power,‡ and Timothy N. C. Wells‡

Department of Chemistry, University of York, Heslington, York YO1 5DD, England, University of British Columbia, Vancouver, British Columbia, Canada, and Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development SA, 14, chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland

Received May 14, 1997; Revised Manuscript Received August 7, 1997⊗

ABSTRACT: Chemokines are 8−10 kDa proteins involved in the control of leukocyte trafficking and activation. In free solution, chemokines are monomers at physiologic concentrations, although many multimerize at higher concentrations. Cell surface heparan sulfate may sequester chemokines, increasing their local concentrations and facilitating their binding to receptors expressed on leukocytes. In competitive binding assays using immobilized heparin, a 2−3-fold increase in the bound radiolabeled chemokine was seen with increasing concentrations of unlabeled chemokine in the nanomolar range. Unlabeled chemokine concentrations between 0.25 and 50 μM were needed to compete the bound radioactivity. This biphasic competition curve was not seen for *N*-methyl-L25 IL-8, a variant of IL-8 which is unable to dimerize. In addition, complexes of chemokine and heparin eluted from gel filtration columns with apparent molecular masses of 33−60 kDa, suggesting that chemokine multimerization had occurred. The physiological relevance of this multimerization process was seen from studies using human endothelial cells. The endothelial cell binding sites for IL-8, RANTES, and MCP-1 were deduced to be glycosaminoglycans since competition assays showed the biphasic curves and micromolar IC_{50} values seen in studies with immobilized heparin, and mRNA for known chemokine receptors was not detected. Furthermore, digestion of endothelial cell monolayers with glycosaminidases decreased chemokine binding by up to 80%. Glycosaminoglycans can act as modulators of the ligand binding affinity of chemokine receptor-bearing cells. Removal of glycosaminoglycans from CHO cells expressing chemokine receptors CXCR1, CCR1, or CCR2 resulted in 40−70% decreases in the binding of RANTES, MCP-1, IL-8, and MIP-1α. Our data show that cell surface glycosaminoglycans induce polymerization of chemokines, increasing their local concentration and therefore enhancing their effects on high-affinity receptors within the local microenvironment.

The movement of selected leukocyte populations from the blood, across the endothelial cell monolayer and basement membrane, and into the underlying tissue is an important component of an inflammatory response. This transendothelial migration is postulated to occur in a sequential three-step process (*1*). First, a loose adhesion between activated endothelial cells and leukocytes is mediated by selectins and their ligands. Second, the leukocytes are exposed to chemoattractant molecules on or near the endothelial cell surface, resulting in activation of several leukocyte processes, including enhanced integrin adhesiveness. Third, the enhanced adhesiveness results in the firm attachment of the leukocytes to the endothelium. Once firmly attached, the leukocytes are directed by a chemoattractant gradient to transmigrate between the endothelial cells, through the extracellular matrix, and into tissue affected by injury or infection. The control of the recruitment of specific leukocytes to inflammatory sites therefore involves the concerted action of various combinations of selectins, chemoattractants, and integrins (*2*). It is the multifactorial nature of the process which results in selectivity *in vivo*.

The chemokines, a family of small chemoattractant proteins, play a role in the activation and directional migration of specific leukocyte populations. The approximately 30 members of the family that have been identified can be divided into 2 main subclasses based on structure and chromosomal localization (reviewed in *3*). The CXC subclass mediates acute inflammatory reactions via mainly neutrophil attraction and activation, and includes interleukin-8 (IL-8),[1] neutrophil activating peptide-2 (NAP-2), and platelet factor-4 (PF-4). The CC subclass is implicated in chronic inflammation through the attraction of lymphocytes, eosinophils, basophils, and macrophages, and includes RANTES (regulated on activation, normal T-cell expressed and secreted) and macrophage inflammatory protein-1 (MIP-1) α and β, and monocyte chemoattractant protein-1 (MCP-1). Two novel chemokines, lymphotactin and fractalkine, are probably the prototypes of two minor subclasses of chemokines (*4, 5*). The chemokines are expressed by many cell types, including leukocytes and endothelial cells.

* Author to whom correspondence should be addressed.
‡ Glaxo Wellcome Research and Development SA.
§ Present address: Midland Certified Reagent Co., 3112A West Cuthbert Ave., Midland, TX 79701. Fax: (915) 694-2387. Email: arlene@oligos.com.
|| University of York.
⊥ University of British Columbia.
⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: RANTES, regulated on activation, normal T-cell expressed and secreted; MIP-1α, macrophage inflammatory protein-1α; MIP-1β, macrophage inflammatory protein-1β; MCP-1, monocyte chemoattractant protein-1; PF-4, platelet factor-4; IL-8, interleukin-8; IP-10, interferon-γ inducible protein-10; SDF, stromal cell derived factor; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; FCS, fetal calf serum; RT-PCR, reverse transcriptase−polymerase chain reaction; FGF, fibroblast growth factor; DARC, Duffy antigen receptor for chemokines.

S0006-2960(97)01125-2 CCC: $14.00 © 1997 American Chemical Society

RANTES, MIP-1β, and IL-8 have previously been localized to the vascular endothelium using immunohistochemical methods (*6*−*8*).

The chemokines interact with a family of seven-transmembrane receptors that are predominantly expressed on leukocytes. Nine human chemokine receptors have been identified as well as a number of viral homologues (reviewed in *3*). With the exception of CXCR1 and CXCR4, *in vitro* studies show that more than one chemokine is able to bind to a given receptor, and several receptors are able to bind a given chemokine. Thus, there appears to be a large amount of redundancy *in vitro*. However, leukocyte recruitment *in vivo* appears to be more specific. For example, in targeted gene deletion studies, mice which lacked the CC chemokine, MIP-1α, were not able to use other chemokines as a replacement to induce a response to a viral challenge (*9*). In addition, the chemokine IL-8 has been localized to the endothelium of postcapillary venules, the site where neutrophil recruitment often occurs (*10*), and MIP-1β has been localized to the high endothelial venules of lymph nodes, a site where lymphocytes are found (*6*). Therefore, both temporal and spatial distribution of chemokines may be responsible for the increased specificity observed *in vivo* compared with *in vitro*.

There are significant differences in the mechanisms of regulation of IL-8 and MCP-1 mRNA expression by endothelial cells (*11*), and the peaks of expression of these two chemokines occur at different times in the inflammatory process (*12*). In addition, chemokines that are produced by endothelial cells or platelets, which are often the inital cells responding to injury, may be localized on the vascular endothelium via interaction with glycosaminoglycans. Researchers have suggested that heparan sulfate glycosaminoglycans on endothelial cell surfaces localize and present chemokines to loosely-tethered leukocytes (*2, 13*). We have previously shown that chemokines bind to immobilized heparin and human umbilical vein endothelial cells (HUVECs) with different affinities (Kuschert et al., personal communication), and that the binding can be competed selectively by other glycosaminoglycan families. Altered expression of endothelial cell glycosaminoglycans has been observed in certain diseases such as atherosclerosis (*14, 15*), inflammatory bowel disease (*16*), and wound healing (*17*). This may promote the binding and presentation of chemokines that are selective for the particular glycosaminoglycans expressed, and thus contribute to a site-specific localization of chemokines.

Previous studies have detected RANTES, MIP-1β, and IL-8 on endothelium using specific antibodies (*3, 7, 8*), suggesting that glycosaminoglycan presentation of chemokines is possible. However, from these studies it is not clear whether the chemokines are bound to glycosaminoglycans or to high-affinity seven-transmembrane receptors. The detection of binding of IL-8 to cultured endothelial cells has not been consistent (*18, 19*), although other CXC chemokines like interferon-γ inducible protein-10 (IP-10) and PF-4 have been identified as binding to proteoglycans on endothelial cells (*20, 21*). Thus, the purpose of the present study was to determine the nature of chemokine binding to endothelial cells. Our results show that the IL-8, MCP-1, MIP-1α, and RANTES bind to cultured endothelial cells principally by glycosaminoglycan-dependent mechanisms, and that glycosaminoglycans may serve to increase the local concentration of chemokines on cell surfaces by inducing chemokine oligomerization. We further show that glycosaminoglycans that are present on cells which express seven-transmembrane receptors influence the interaction of chemokines with these receptors.

EXPERIMENTAL PROCEDURES

Materials. Recombinant IL-8, MIP-1α, and RANTES were expressed in *E. coli* and prepared at the Geneva Biomedical Research Institute as described (*22, 23*), and MCP-1 was prepared by similar methods. [^{125}I]Chemokines (specific activity = 2000 Ci/mmol) were purchased from Amersham International. The monomeric *N*-methyl-L25 IL-8 was synthesized as described earlier (*24*). Heparin−Sepharose and Superdex 75 (PC 3.2−30 for SMART System) were from Pharmacia. Bovine serum albumin (BSA), heparinase I, heparinase III, chondroitinase ABC, heparin (H-3149), heparan sulfate (H-5393), low molecular weight heparin (H-3400), chondroitin sulfate (C-8529), and dermatan sulfate (C-2413) were purchased from Sigma. Human umbilical vein endothelial cells (passage 1, pooled), human microvascular endothelial cells (lung-derived, passage 4), and endothelial cell growth medium were from Clonetics. TRIZOL, DMEM/F12 media, and fetal calf serum were obtained from GIBCO BRL, and Reverse Transcriptase Kits were purchased from Promega. Amplitaq DNA polymerase and reaction buffer were purchased from Perkin-Elmer.

Immobilized Heparin Binding Assay. The immobilized heparin binding assay was performed as described in detail (Kuschert et al., personal communication), using binding buffer (50 mM HEPES, pH 7.4, containing 0.5% BSA, 5 mM $MgCl_2$, and 1 mM $CaCl_2$), 0.25−0.5 nM radiolabeled chemokines, and increasing concentrations of unlabeled chemokines or glycosaminoglycans. For RANTES, the binding buffer was supplemented with 0.15 M NaCl. Washes were performed with binding buffer containing 0.15 M NaCl for IL-8, MCP-1, or MIP-1α or containing 0.5 M NaCl for RANTES.

Human Endothelial Cell Culture and Binding Assays. Passage 2−6 (human umbilical vein endothelial cells) HUVECs or passage 6−8 human microvascular cells were seeded in 96-well plates at 5000 cells/well, using endothelial cell growth medium containing 2% fetal calf serum. The media were changed after 24 h, and cells were used for experiments 48 h after seeding. Prior to each experiment, the cells were rinsed with PBS, and potential heparin binding serum proteins were removed by incubating the cells for 10 min with PBS containing 50 μg/mL heparin. The heparin was removed by aspiration, and the cells were washed 3 times with PBS. In some experiments, the HUVEC monolayers were digested at 37 °C for 4 h with a mixture of 1.0 unit of heparinase I, 0.5 unit of heparinase III, and 0.5 unit of chondroitinase ABC in endothelial cell basal medium containing 0.5% BSA. Cell viability in the PBS- and glycosidase-treated cells was greater than 90%, as judged by trypan blue exclusion. For the binding experiments, cells were incubated in a total volume of 50 μL containing 0.25 nM [^{125}I]chemokine and increasing concentrations of glycosaminoglycan or chemokine competitors, using the same buffer conditions as described for the immobilized heparin binding assay. After incubation of the cells at 4 °C for 4 h, quick inversion of the 96-well plates was used to remove the unbound [^{125}I]chemokines and to wash the cells with

3 × 200 μL of buffer. The cells were lysed with 20 mM Tris, pH 8.0, buffer containing 0.2% Triton X-100. The lysed cells were transferred to 96-well plates, and the radioactivity was measured in a Wallac Microbeta counter after the addition of Optiphase scintillation fluid. For each experiment, a value for the background binding to the plates was determined by performing identical manipulations on plates which only received media at the time of cell seeding.

Chinese Hamster Ovary (CHO) Cell Culture, Chemokine Receptor Transfection, and Binding Assays. cDNAs encoding CXCR1, CCR1, and CCR2 were cloned (*25*, *26*) and stably transfected into CHO cells (Coulin et al., personal communication). Untransfected CHO cells were maintained in DMEM/F12 medium containing 10% heat-inactivated FCS, and G418 (300 μg/mL) was added to the media of the stably transfected cells. For the binding assays, adherent cells were detached with 1 mM EDTA/PBS, incubated with 50 μg/mL heparin in PBS for 10 min at 25 °C, and washed 3 times with binding buffer. The cells were resuspended in binding buffer at 4×10^6 cells/mL and were incubated with or without glycosidases (1.0 unit/mL heparinase I, 0.5 unit/mL heparinase III, and 0.5 unit/mL chondroitinase ABC) for 3 h at 37 °C. After the glycosidase digestion, the cell concentration was adjusted to 2×10^6 cells/mL with binding buffer. Cell viability was greater than 95%, as judged by trypan blue exclusion. Binding assays were performed in 96-well filtration plates in a total volume of 100 μL containing 50 μL of cells, 0.25 nM [^{125}I]chemokines, and increasing concentrations of unlabeled chemokines. After a 2-h incubation at 4 °C, the bound chemokine was separated from the free chemokine by vacuum filtration, and the cells were washed 3 times in binding buffer containing 0.5 M NaCl. Scintillation fluid was added, and the radioactivity in each well was measured using a Wallac Microbeta Counter. Data were analyzed using GraFit Software (*27*) and the equation described in the legend to Figure 6 (*28*), and a Student's *t*-test was used for statistical comparison of means.

Gel Filtration Chromatography. Gel filtration chromatography of chemokines was performed on a Superdex 75 PC3.2/30 column using the Pharmacia SMART system. Samples (50 μL) were chromatographed in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl at a flow rate of 0.1 mL/min. Protein elution was monitored by the absorbance at 280 nm. The column was calibrated with the following standards: BSA, 66 kDa; ovalbumin, 43 kDa; chymotrypsin, 25 kDa; cytochrome *c*, 12.4 kDa; ubiquitin, 8 kDa; and aprotinin, 6 kDa. The V_o was determined with Blue Dextran.

Heparin−Sepharose Chromatography. Chemokines were loaded onto a 1 mL heparin−Sepharose column (Pharmacia SMART System; heparin−Sepharose CL-6B) in a 50 mM Tris-HCl, pH 7.4, buffer containing 50 mM NaCl and 1 mM EDTA, and eluted with a 20 mL linear gradient (0−2 M NaCl in the same buffer) at a flow rate of 0.5 mL/min. Protein was monitored by the absorbance at 280 nm, and the concentration of NaCl was determined using an in-line conductivity meter calibrated with 50 mM Tris-HCl, pH 7.4, buffer containing 50 mM NaCl and 1 mM EDTA (0% conductivity) and 50 mM Tris-HCl, pH 7.4, buffer containing 2.05 M NaCl and 1 mM EDTA (100% conductivity).

Reverse Transcriptase Polymerase Chain Reaction. HUVECs were maintained as indicated above, and RNA was prepared with the TRIZOL reagent according to the manufacturer's instructions. Reverse transcription of 1 μg of HUVEC RNA was performed with the AMV-reverse transcription kit, and PCR was performed on one-tenth of the RT reaction using 40 cycles of PCR (94 °C, 60 s; 55 °C, 90 s; 72 °C, 3 min) on a Crocodile III PCR machine (Appligene). The primers used to amplify the full-length coding sequence for the chemokine receptors CXCR1, CXCR2, CCR1, CCR2, CCR3, CCR4, CCR5, DARC, and GAPDH were the first 18−24 bases of the coding sequence (sense primer) and the last 18−24 bases of the coding sequence (antisense primer), based on the published sequences. All of these primer sets have successfully amplified the correct PCR product under the same cycling, buffer, and enzymatic conditions, using plasmids containing the appropriate chemokine receptor DNA as the template. PCR reaction products were electrophoresed on 1.0% agarose gels and visualized by ethidium bromide staining.

RESULTS

Competition of the Binding of Chemokines to Immobilized Heparin by Unlabeled Chemokines. Using binding assays with immobilized heparin and soluble glycosaminoglycan competitors, we had previously shown that chemokines selectively bind to heparin. To further understand the interaction of chemokines with glycosaminoglycans, we performed competitive binding assays with radiolabeled chemokines, immobilized heparin, and increasing concentrations of unlabeled chemokine competitors. Figure 1 shows the results of experiments performed with radiolabeled IL-8, MIP-1α, MCP-1, and RANTES. In contrast to what would be expected from a simple receptor−ligand interaction, we saw more complex competition curves. These curves had two phases; the first phase, which occurred at concentrations of 0.5−100 nM chemokine, involved an initial increase in the amount of radioactivity binding to the glycosaminoglycan. The second phase, which occurred between 100 nM and 50 μM, was a classical monophasic displacement curve. The initial increase in the quantity of radioactivity bound to the heparin occurred despite a decrease in the specific activity of the chemokine, caused by the dilution with unlabeled material. This implied that rather than simply increasing the proportion of receptors which were occupied, there was an increase in the number of ligands binding at each site. This situation can be modelled for a system where the ligand at each site was previously occupied by a monomer. For the simplest case, ligand dimerization on the receptor, the system can be described by

$$\mathrm{RL} + \mathrm{L} \overset{K_d}{\rightleftharpoons} \mathrm{RL}_2$$

The increase in radioactivity bound will parallel the increase in the proportion of sites which are occupied by dimers of ligands. The number of ligands bound per site originally occupied by a labeled monomer can be described by the equation:

$$F = ([\mathrm{RL}] + 2[\mathrm{RL}_2])/([\mathrm{RL}] + [\mathrm{RL}_2]) \qquad (1)$$

and therefore:

$$F = \{1 + 2([\mathrm{L}]/K_d)\}/(1 + [\mathrm{L}]/K_d) \qquad (2)$$

Since it was clear that chemokines were capable of forming higher order multimers than dimers, this model was extended

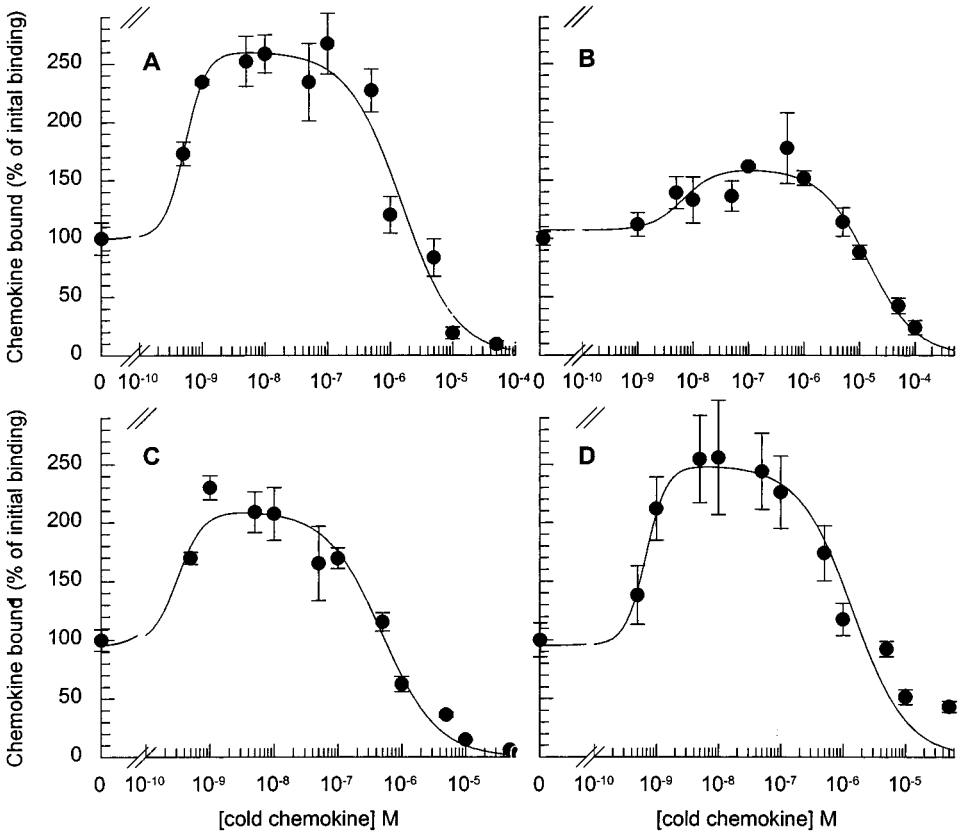

FIGURE 1: Chemokines multimerize on immobilized heparin. Radiolabeled chemokines were incubated with heparin−Sepharose beads and increasing amounts of the same unlabeled chemokine for 4 h as described under Experimental Procedures. Each data point represents the mean ± SEM of triplicate determinations in a single experiment, representative of at least two experiments. The data were analyzed with GraFit software using the equation $B_{obs} = B_i[(1 + n([L]/K_a)^{n-1})]/\{(1 + [L]/K_a)^{n-1}/[1 + ([L]/K_i)]\}$, where B_{obs} = cpm bound, B_i = initial cpm bound, n = Hill coefficient for oligomerization on heparin, [L] = concentration of competing chemokine, K_a = multimerization constant, and $K_i = IC_{50}$ for dimer competition of multimeric chemokine (eq 4, see text). (A) IL-8. (B) MIP-1α. (C) MCP-1. (D) RANTES.

to allow formation of a multimer containing n monomer subunits:

$$RL + (n-1)L \overset{K_d}{\rightleftharpoons} RL_n \quad (3)$$

which, by analogy with the Hill equation for cooperative interactions, can be described by the equation:

$$F = \{1 + n([L]/K_d)^{n-1}\}/\{(1 + ([L]/K_d)^{n-1}\} \quad (4)$$

At high concentrations of ligand, all the sites will be occupied by multimers, and normal competition kinetics will be observed, with $B = B_{max}/(1 + [L]K_i)$ where B = the observed bound radioactivity and B_{max} is the maximum activity seen. Provided that the binding site occupancy has reached its limit value before this concentration occurs (i.e., $K_i > 100K_d$), these two equations can be combined to give

$$F = \frac{1 + n([L]/K_d)^{n-1}}{\{(1 + ([L]/K_d)^{n-1}\}/(1 + [L]K_i)} \quad (5)$$

The data obtained from the competition binding assays were fitted to this equation. The Hill coefficients obtained experimentally were 2.3 ± 0.2, 2.1 ± 0.1, and 2.6 ± 0.1 for IL-8, MCP-1, and RANTES respectively, and represent the mean of at least four determinations. Theoretically, the Hill coefficient is a function of the number of monomers in each complex and the cooperativity of the interaction, and is less than or equal to the number of monomers. Cooperative tetrameric proteins such as hemoglobin, pyruvate kinase, and glyceraldehyde-3-phosphate dehydrogenase all have Hill constants between 2.3 and 2.8 for tetramer interactions. The Hill coefficients for IL-8, MCP-1, and RANTES suggest that these three chemokines probably bind to immobilized heparin as tetramers at nanomolar chemokine concentrations. The Hill coefficient for MIP-1α was 1.4 ± 0.03, suggesting that MIP-1α was dimeric under the same conditions. The IC_{50} values for the displacement phase were 1.3 ± 0.3 μM, 4.7 ± 2.2 μM, 17 ± 4 μM, and 1.4 ± 0.1 μM for IL-8, MCP-1, MIP-1α, and RANTES, respectively.

An essential condition in this model of oligomerization on heparin is the ability of a chemokine to dimerize. If a competing chemokine was unable to dimerize, our model would predict a simple displacement of the radiolabeled chemokine from the immobilized heparin rather than an initial increase in radioactivity. To test this prediction, the binding of radiolabeled wild-type IL-8 to immobilized heparin was competed by increasing concentrations of unlabeled *N*-methyl-L25 IL-8, an obligate monomer of IL-8 (*24*). As can be seen in Figure 2, the binding curve obtained with the monomeric IL-8 did not display any oligomerization, in support of our model of oligomerization of chemokines on immobilized heparin.

Gel Filtration of Chemokine−Heparin Complexes. To assess the oligomerization of chemokines in the presence of heparin by another method, we examined the elution of various concentrations of chemokines from gel filtration columns in the absence and presence of low molecular weight heparin. When seven different concentrations of RANTES, MIP-1α, MCP-1, or IL-8 (0.5−25 μg in 50 μL) were

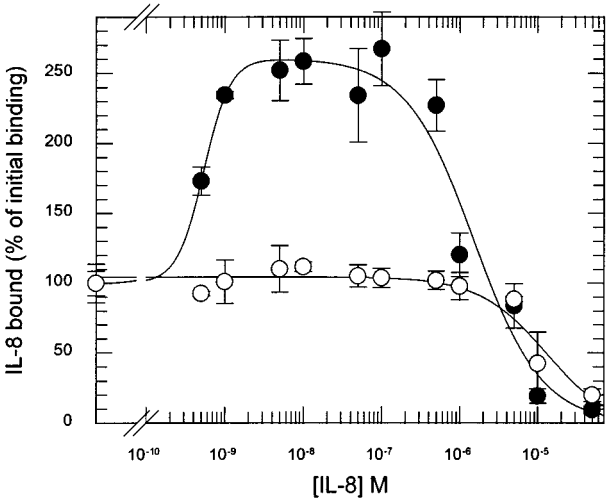

FIGURE 2: Dimerization is necessary for multimerization of IL-8 on immobilized heparin. Binding assays with radiolabeled wild-type IL-8 and increasing concentrations of unlabeled wild-type IL-8 (●) or *N*-methyl-L25 IL-8, an obligate monomer (○), were performed as described under Experimental Procedures. The data were analyzed using GraFit software and the multerimerization equation described in the legend to Figure 1. Each point represents the mean ± SEM of triplicate determinations, and the experiment shown is representative of three.

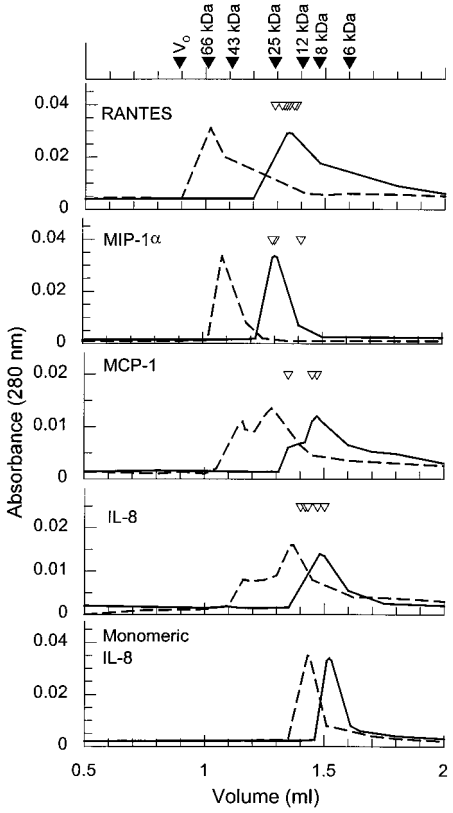

FIGURE 3: Chemokines elute with larger apparent masses in the presence of low molecular weight heparin. Chemokines were chromatographed on a Superdex 75 gel filtration column at concentrations between 0.01 and 0.5 mg/mL, as described under Experimental Procedures. The positions of the peaks are indicated by open triangles (▽). The elution profile of 6−8 μg of each chemokine in the absence (solid lines) or in the presence of 33 μg of low molecular weight heparin (dashed lines) is shown. Identical results were obtained in a second experiment.

subjected to gel filtration on Superdex 75 in the absence of heparin, a range of peaks representing monomers and dimers were obtained (Figure 3). For IL-8, the monomer and dimer eluted with apparent molecular masses of 8.9 and 12.6 kDa, respectively. The apparent molecular masses for monomeric and dimeric MCP-1 were 10 and 17.4 kDa. MIP-1α and RANTES eluted with larger apparent molecular masses: 12.6 and 13.5 kDa for monomers, and 19.8 and 19.9 kDa for dimers, respectively.

Similar experiments were performed with intermediate amounts of the chemokines (6−8 μg in 50 μL) in the presence and absence of low molecular weight heparin. The elution profiles in the absence of heparin indicated that for these concentrations, RANTES and IL-8 were monomeric and MIP-1α was dimeric, and that MCP-1 eluted both as monomers and as dimers (Figure 3, solid lines). When the same concentrations of chemokines were incubated overnight with a 4−5-fold mass excess of low molecular weight heparin, the chemokines eluted with much larger apparent molecular masses (Figure 3, dashed lines). The presence of low molecular weight heparin increased the apparent molecular masses of the chemokines from 19.8 and 19.9 kDa to 60 and 52 kDa, respectively, for RANTES and MIP-1α. When MCP-1 was incubated with low molecular weight heparin, the apparent mass was increased to two species of 24 and 40 kDa. IL-8 was eluted in two peaks of 15 and 33 kDa in the presence of low molecular weight heparin, compared to 9 kDa in the absence. These data suggest that chemokines form higher order multimers in the presence of heparin. It was also possible that the larger apparent masses obtained in the presence of heparin were merely the result of the addition of several polysaccharide chains bound to one IL-8 monomer. This could dramatically alter the aspect ratio of the complex, yielding results which deviate from the elution volume corresponding to spherical proteins. To confirm that the higher molecular masses observed in the presence of heparin were due to oligomerization, we repeated the experiment with the obligate monomer, *N*-methyl-L25 IL-8. The monomeric IL-8 alone eluted with an apparent molecular mass of ≈8 kDa. The addition of low molecular weight heparin shifted the elution of the monomeric IL-8 to 10.7 kDa (Figure 3, bottom panel), indicating that the low molecular weight heparin bound to monomeric IL-8 but failed to significantly increase its mass. To confirm the binding of monomeric IL-8 to heparin, monomeric and wild-type IL-8 were subjected to heparin−Sepharose affinity chromatography. Both monomeric IL-8 and wild-type IL-8 bound to heparin−Sepharose at pH 7.4, and were eluted with similar NaCl concentrations: 0.51 M NaCl for monomeric IL-8 vs 0.60 M NaCl for wild-type IL-8 (Figure 4). Therefore, the shift in elution from 8 to 10.7 kDa for monomeric IL-8 in the presence of low molecular weight heparin is consistent with an increased apparent mass due to bound glycosaminoglycan. The absence of higher molecular weight species is strong evidence that the higher molecular mass peaks (33−60 kDa) observed for the wild-type IL-8, MCP-1, MIP-1α, and RANTES were due to oligomerization of the chemokines in the presence of heparin.

Competition of Chemokine Binding to HUVECs by Excess Unlabeled Chemokine. RANTES, MCP-1, MIP-1α, and IL-8 bound to HUVECs, and this binding could be selectively competed by soluble glycosaminoglycan competitors (Kuschert et al., unpublished data). To identify the binding site, we performed competitive binding assays on HUVECs with radiolabeled chemokines, and competed the binding with unlabeled chemokines under similar conditions as were used for the immobilized heparin binding assays. The results, shown in Figure 5 (closed circles), show that each of these

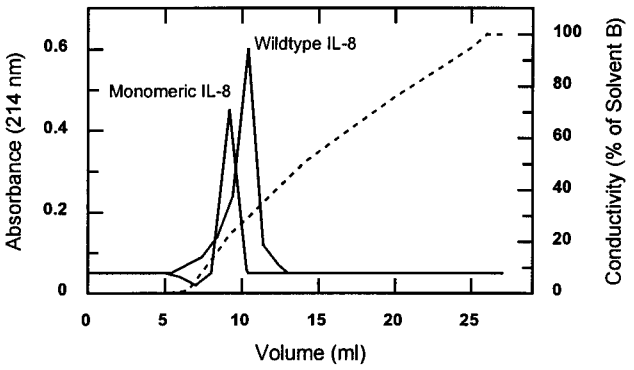

FIGURE 4: Wild-type and monomeric IL-8 have similar affinities for heparin−Sepharose. Wild-type (30 μg) and monomeric IL-8 (16 μg) were loaded onto a heparin−Sepharose column and eluted with a linear NaCl gradient as described under Experimental Procedures. The elution of protein was monitored by the absorbance at 214 nm (solid lines), and the [NaCl] required for elution was determined by an in-line conductivity meter (dotted line).

chemokines bound to the HUVEC monolayers and was competed by the unlabeled ligand. The biphasic curves at low nanomolar concentrations of IL-8, MCP-1, and RANTES for chemokine binding to HUVEC monolayers were similar to the curves observed with immobilized heparin. The Hill coefficients were 2.5 ± 0.6, 2.1 ± 0.5, and 3.0 ± 0.4 for IL-8, MCP-1, and RANTES, respectively (mean of four to seven determinations). The IC_{50} values for the displacement from HUVECs or immobilized heparin were similar for IL-8 and RANTES, but differed by 10-fold for MCP-1 (Table 1). The results for MIP-1α were clearly different. MIP-1α did not oligomerize on the HUVEC monolayers, and the IC_{50} values for the displacement of MIP-1α from immobilized heparin and HUVECs were different by more than 2 orders of magnitude (Table 1).

When the cells were treated with a mixture of heparinase I, heparinase III, and chondroitinase ABC, glycosidases which remove heparan sulfate, chondroitin sulfate, and dermatan sulfate, the binding of all four chemokines to the HUVECs was reduced (Figure 5, open circles). The initial binding of IL-8 to HUVECs and the maximum observed binding were both reduced by 50%. For MIP-1α, the initial binding and maximum binding were reduced to 40% of control levels. For MCP-1, the initial binding was reduced to 32% of control levels, and the maximum binding due to polymerization was reduced by 80%. The initial binding of RANTES to the glycosidase-treated HUVECs was 40% of control levels, and the maximum binding due to polymerization was reduced by 80%. Thus, the loss of heparan sulfate, chondroitin sulfate, and dermatan sulfate dramatically reduced the binding of the chemokines to the cells.

It was important to determine if HUVECs cultured under these conditions could express any of the known chemokine receptors which might be responsible for the observed binding. HUVECs were cultured on uncoated tissue culture flasks for 48 h, the same conditions used for the binding assays. RNA was prepared from these cells, and RT-PCR

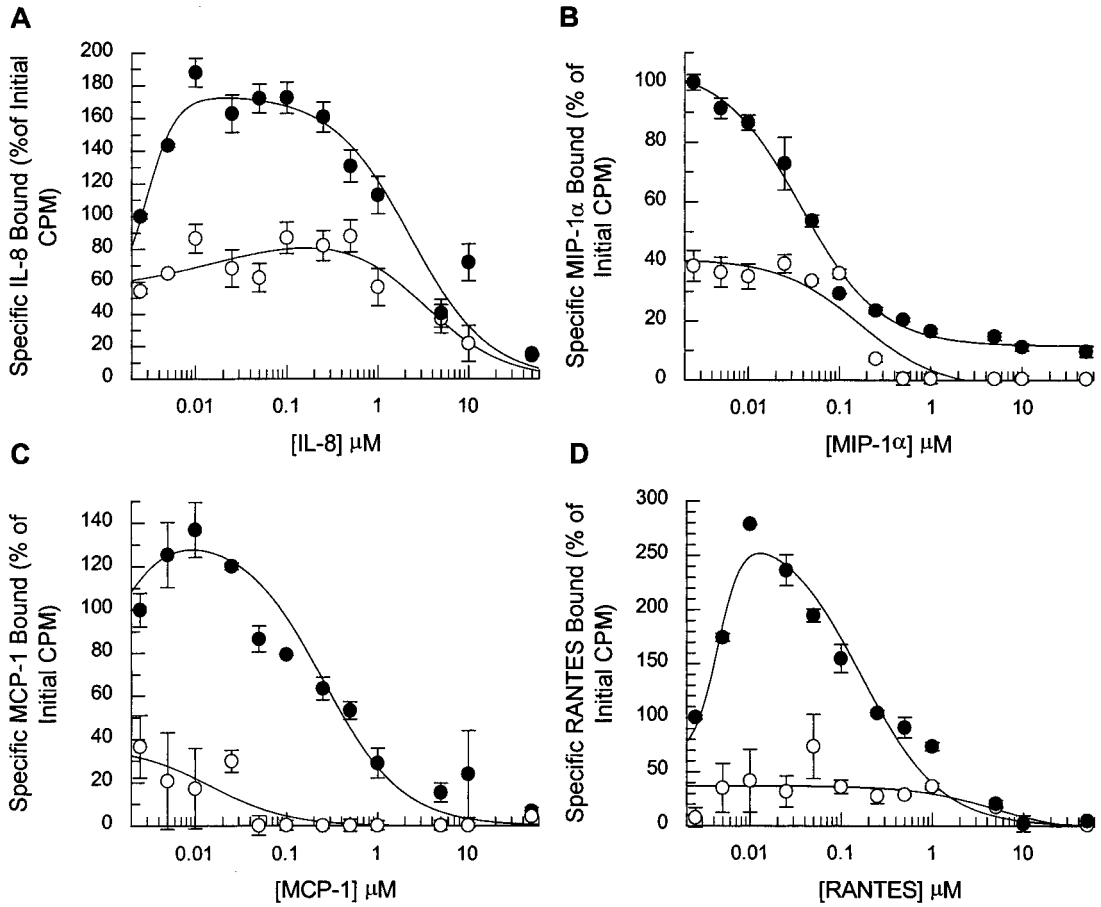

FIGURE 5: Chemokine binding to and multimerization on HUVEC monolayers is largely dependent on the presence of intact glycosaminoglycans. HUVECs were seeded in 96-well plates and prepared for binding assays as described under Experimental Procedures. Binding of radiolabeled IL-8 (panel A), MIP-1α (panel B), MCP-1 (panel C), and RANTES (panel D) was determined on HUVEC monolayers that were treated with PBS (●), or with monolayers that were treated with a mixture of heparinases I and III and chondroitinase ABC (○). Data were analyzed with GraFit software (*27*) using the equation described in the legend to Figure 1. Each point represents the mean ± SEM of triplicate values after the subtraction of background binding. The experiment shown is representative of at least four.

Table 1: Multimerization of Chemokines on Immobilized Heparin and HUVECs[a]

	IL-8	MCP-1	MIP-1α	RANTES
IC_{50} values				
immobilized heparin	1.3 ± 0.3 μM	4.7 ± 2.2 μM	17 ± 4 μM	1.4 ± 0.1 μM
HUVECs	2.0 ± 0.8 μM	0.7 ± 0.3 μM	47 ± 11 nM	2.4 ± 0.8 μM
Hill coefficients				
immobilized heparin	2.3 ± 0.2	2.1 ± 0.1	1.4 ± 0.03	2.6 ± 0.1
HUVECs	2.7 ± 0.4	2.3 ± 0.4	none	3.1 ± 0.4
multimerization constants				
immobilized heparin	18 ± 16 nM	3.4 ± 1.7 nM	25 ± 17 nM	1.0 ± 0.1 nM
HUVECs	33 ± 14 nM	29 ± 15 nM	none	49 ± 12 nM

[a] The binding of radiolabeled chemokines to immobilized heparin or HUVECs was competed by increasing concentrations of the same unlabeled chemokine. The data were analyzed using the multimerization equation described in the text. The data are the mean ± SEM of 4−7 separate experiments for the HUVECs, and at least 3 experiments for the immobilized heparin.

was performed using primers for the known human chemokine receptors. No PCR products for CXCR1, CXCR2, CCR1, CCR2, CCR3, CCR4, CCR5, and Duffy antigen receptor for chemokines (DARC) were obtained, although 1.0 kb PCR products for GAPDH could be clearly seen (data not shown). This indicates that the known human chemokine receptors either were not expressed or were expressed at levels below the limits of detection.

Finally, in order to determine if similar binding occurs on other types of endothelial cells, similar binding experiments were performed with RANTES on human microvascular endothelial cells. The binding curves obtained showed the same biphasic binding patterns with multimerization constants near 50 nM, similar IC_{50} values (4.4 μM for microvascular cells vs 2.4 μM for HUVECs; $n = 4$ experiments), and a similar reduction in RANTES binding to glycosidase-treated cells. Thus, the binding patterns observed were not unique to HUVECs.

Effect of Cell Surface Glycosaminoglycans on the Binding of Chemokines to Cells Transfected with Seven-Transmembrane Chemokine Receptors. The multimerization of chemokines on cell surface glycosaminoglycans could act to raise the local concentration of the chemokine in the area where contact with high-affinity receptors is likely to occur. Under these circumstances, it would be predicted that if glycosaminoglycans were absent, higher concentrations of unlabeled ligand would be required to displace a radiolabeled ligand, since any benefit of the glycosaminoglycan to increase local concentrations would not be realized. To test this prediction, CHO cells expressing recombinant receptors were incubated with glycosidases to remove heparan sulfate, dermatan sulfate, and chondroitin sulfate, and competitive binding assays were performed, using concentrations of unlabeled ligand up to 500 nM. The binding curves are shown in Figure 6. Typical ligand displacement curves are observed, except that for RANTES, MCP-1, and IL-8, there was a slight, but consistent, increase in the bound radioactivity between 25 and 100 nM. This concentration range is similar to the range where chemokine oligomerization occurred in the endothelial cells. In general, the maximum level of binding was significantly reduced in the cells which lacked glycosaminoglycans. The IC_{50} values were also higher for cells which lacked glycosaminoglycans, although this trend was only statistically significant for RANTES (3.6 ± 1.9 nM vs 53 ± 6 nM for control vs glycosidase-treated cells; $n = 4$ experiments; $p \leq 0.01$) and MIP-1α (4.4 ± 2.3 nM vs 12 ± 4 nM for control vs glycosidase-treated cells; $n = 4$ experiments; $p \leq 0.05$). These data show that glycosaminoglycans increase the apparent affinity of the receptors for chemokines by a factor of 2−15-fold. These data are consistent with a role of cell surface glycosaminoglycans to bind chemokines, thereby increasing the local chemokine concentration and affecting the interaction with high-affinity receptors.

DISCUSSION

These experiments show that the chemokines IL-8, MCP-1, and RANTES bind to cell surface glycosaminoglycans on cultured HUVECs, and suggest that vascular endothelial cell surface glycosaminoglycans *in vivo* are capable of binding chemokines. Our results lend support to the model which suggests that vascular glycosaminoglycans sequester and present chemokines to loosely bound leukocytes (*2*). Others have shown that three chemokines, RANTES, IL-8, and MIP-1β, were localized to endothelium by immunochemistry (*6*−*8*), but the identity of the binding site was not known. The binding of IP-10 and PF-4 to cell surface glycosaminoglycans, and the binding of RANTES to subendothelial extracellular matrix, has previously been demonstrated, but this is the first documentation of IL-8, MCP-1, and RANTES binding to endothelial cell surface glycosaminoglycans.

Identifying that the binding site on HUVECs for RANTES, MCP-1, and IL-8 involved glycosaminoglycans was based on three observations. First, the competition by unlabeled chemokine of the binding of radiolabeled chemokine to HUVECs followed the same biphasic binding curve as observed for the immobilized heparin. The binding of the same chemokines to cells stably transfected with seven-transmembrane receptors did not follow the same biphasic pattern. Second, the IC_{50} values for the competition from the endothelial cells were in the low micromolar range, rather than the low nanomolar IC_{50} values which are typical for binding to seven-transmembrane receptors. The IC_{50} values seen with HUVECs were similar to those observed for the interaction between chemokines and immobilized heparin. Third, digestion of cell surface glycosaminoglycans with three enzymes which remove heparan sulfate, chondroitin sulfate, and dermatan sulfate reduced the binding of these chemokines to HUVECs by 50−80%.

Although these experiments demonstrate that the chemokines bind to cell surface glycosaminoglycans, they do not define which types of glycosaminoglycans are responsible. We previously demonstrated that IL-8, MCP-1, MIP-1α, and RANTES have differential selectivities for glycosaminoglycan families (Kuschert et al., personal communication). It is likely that based on the selectivity of a particular chemokine for the different glycosaminoglycans, different chemokines will bind with differing affinities, or may not bind at all, to

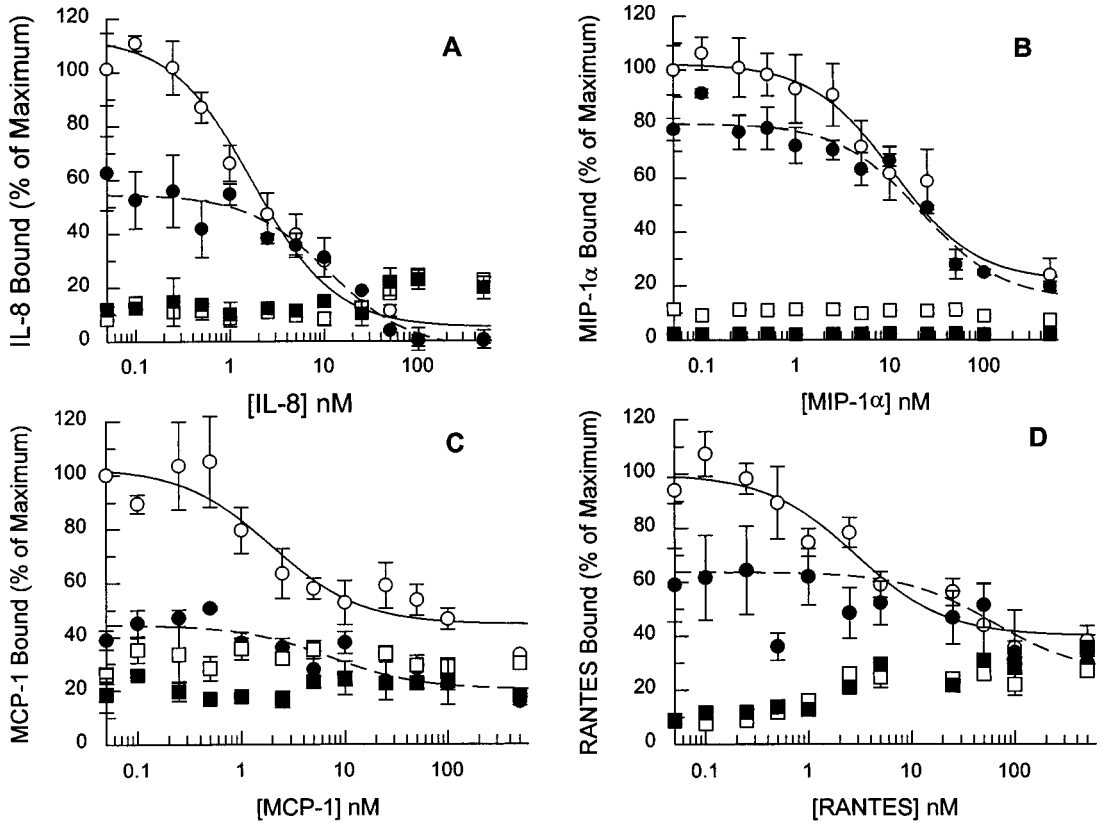

FIGURE 6: Binding of chemokines to cells expressing seven-transmembrane chemokine receptors is reduced in the absence of intact glycosaminoglycans. Control cells (■, □) or cells expressing recombinant seven-transmembrane chemokine receptors (●, ○) were digested either with a mixture of heparinase I, heparinase III, and chondroitinase ABC (●, ■) or with PBS (○, □). The binding of [^{125}I]IL-8 to CXCR1 (A), [^{125}I]MIP-1α to CCR1 (B), [^{125}I]MCP-1 to CCR2 (C), and [^{125}I]RANTES to CCR1 (D) was examined. The data were analyzed using GraFit software using the equation $B/B_{max}^{app} = 1/(1 + [L]/IC_{50})$, where B = cpm bound, B_{max}^{app} = cpm bound in the absence of competing ligand, [L] = competing ligand, and IC_{50} is the amount of unlabeled competitor required to inhibit binding by 50%. Each point represents the mean ± SEM of triplicate determinations. The data shown from a single experiment are representative of 3−4 experiments.

the glycosaminoglycans present on the vascular endothelium in a particular inflammatory condition. Studies with HUVECs which were digested with only heparinases or only chondroitinases reduced the binding of RANTES by only 10−40% (data not shown). This suggests that for certain chemokines, more than one type of glycosaminoglycan is responsible for the binding. Thus, if the HUVECs were digested with heparinases alone, it is likely that RANTES could bind to the remaining dermatan sulfate, since RANTES has a higher affinity for dermatan sulfate than for heparan sulfate (Kuschert et al., personal communication).

In contrast to the binding of IL-8, MCP-1, and RANTES to HUVECs, the IC_{50} values for competition assays with MIP-1α were nanomolar, and the curves were not biphasic, suggesting that the primary binding site for MIP-1α on HUVECs was most likely not a glycosaminoglycan. Glycosidase treatment did result in a lower maximum binding for MIP-1α on the HUVECs. This was very similar to the lower maximum binding observed for the binding of MIP-1α to the glycosidase-treated CHO cells expressing CCR1, suggesting that the MIP-1α binding to HUVECs was to a high-affinity receptor rather than to cell surface glycosaminoglycans. Since RT-PCRs for CXCR1, CXCR2, CCR1−5, and DARC were negative on endothelial cells grown under normal conditions, either the binding of MIP-1α to these cells could be to a known receptor whose expression was too low to be detected by RT-PCR, or the binding could be to a novel site.

Another important observation from this study is that chemokines oligomerize on heparin and cell surface glycosaminoglycans. This observation is based on several lines of evidence. First, a biphasic binding curve is observed for all four chemokines using immobilized heparin. The data obtained from several experiments fit a mathematical model of positive cooperativity in ligand binding to a receptor. The absence of this cooperativity when an obligatory monomeric form of IL-8 was used implies that IL-8 must be able to dimerize in order to display the increase in radioactivity, consistent with our interpretation of oligomerization. Second, gel filtration of the chemokines in the presence and absence of low molecular weight heparin showed that the chemokines eluted with much larger molecular weights in the presence of low molecular weight heparin. The shifts in elution times observed in the presence of heparin were not due to the binding of several heparin chains to a monomer, as was confirmed by experiments with the obligatory monomeric IL-8.

The role of heparin-induced oligomerization of proteins in the control of biological activity has been observed for the fibroblast growth factors (*29*, *30*) and hepatocyte growth factor (*31*), which also oligomerize in the presence of heparin. There are at least two possible physiological roles for heparin-induced oligomerization of chemokines. First, glycosaminoglycans induce chemokine oligomerization, and this acts to raise the local concentration of a chemokine. This may have implications wherever glycosaminoglycans are found, including vascular endothelial surfaces, basement membranes, extracellular matrices, and on the surface of leukocytes which express the seven-transmembrane receptors. Glycosaminoglycans in the basement membrane could bind

chemokines produced by activated endothelial cells or cells adjacent to the foci of inflammation, raising the local concentration, and contributing to a gradient for the transmigration of leukocytes.

Our data showing a decrease in the maximum binding and a lowered affinity of chemokines to 7-transmembrane receptors after glycosidase treatment are consistent with the role of glycosaminoglycans to increase the local concentration. Since glycosaminoglycans are expressed on all cells, this result is not surprising. There is a precedent for heparin to increase the affinity of the fibroblast growth factors (FGF) for their high-affinity receptors. Although FGF can bind to its receptor in the absence of heparin, the affinity is increased in the presence of heparin (*29*). More importantly, however, is the observation that heparin induces dimerization of FGF receptors, allowing phosphorylation of the receptor tyrosine kinase and subsequent mitogenic stimulus for the affected cells (*30*).

A second possible physiologic relevance of heparin-induced chemokine oligomerization is to affect the interaction with receptors. Many chemokines interact with their receptors as monomers (*32*, *33*), since the monomer−dimer equilibrium constant in solution is micromolar and chemokines are active at nanomolar concentrations. However, in studies with chemokine variants, it was determined that MCP-1 functions as a dimer (*34*, *35*). Our data suggest that the ability to form dimers on cell surface glycosaminoglycans occurs at physiologic nanomolar concentrations. Thus, although chemokines are able to interact with receptors as monomers (*24*), it is possible that due to glycosaminoglycan-induced oligomerization, chemokines can achieve higher order oligomers *in vivo*, and they may interact with receptors differently as dimers or tetramers, or heparin−chemokine complexes.

In summary, we have shown that glycosaminoglycans are capable of binding MIP-1α, MCP-1, IL-8, and RANTES, and that the binding of glycosaminoglycans to chemokines results in the oligomerization of chemokines. Glycosaminoglycan-induced oligomerization of chemokines is consistent with its proposed role to increase local concentrations of chemokines. This may be important wherever glycosaminoglycans are located, including the vascular cell surface, the basement membrane, and on the cells which express chemokine receptors. Notably, we show here that cell surface glycosaminoglycans influence the interaction of chemokines with their seven-transmembrane receptors.

ACKNOWLEDGMENT

We thank Erik Whitehorn, Emily Tate, Ron Barrett, and Tanya Chernov-Rogan of Affymax for preparation of stably-transfected cell lines expressing chemokine receptors.

REFERENCES

1. Springer, T. A. (1994) *Cell 76,* 301−314.
2. Butcher, E. C. (1991) *Cell 67,* 1033−1036.
3. Power, C. A., and Wells, T. N. C. (1996) *Trends Pharmacol. Sci. 17,* 209−213.
4. Kelner, G. S., Kennedy, J., Bacon, K. B., Kleyensteuber, S., Largaespad, D. A., Jenkins, N. A., Copeland, N., Bazan, J. F., Moore, K. W., Schall, T. J., and Zlotnik, A. (1994) *Science 266,* 1395−1399.
5. Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D. R., Zlotnik, A., and Schall, T. J. (1997) *Nature 385,* 640−644.
6. Tanaka, Y., Adams, D. H., Hubscher, S., Hirano, H., Siebenlist, U., and Shaw, S. (1993) *Nature* 254, 79−82.
7. Huber, A. R., Kunkel, S. L., Todd, R. F., III, and Weiss, S. J. (1991) *Science 254,* 99−102.
8. Wiedermann, C. J., Kowald, E., Reinisch, N., Kaehler, C. M., von Luettichau, I., Pattison, J. M., Huie, P., Sibley, R. K., Nelson, P. J., and Krensky, A. M. (1993) *Curr. Biol. 3,* 735−739.
9. Cook, D. N., Beck, M. A., Coffman, T. M., Kirby, S. L., Sheridan, J. F., Pragnell, I. B., and Smithies, O. (1995) *Science 269,* 1583−1585.
10. Rot, A. (1992) *Immunol. Today 13,* 291−294.
11. Brown, Z., Gerritsen, M. E., Carley, W. W., Strieter, R. M., Kunkel, S. L., and Westwick, J. (1994) *Am. J. Pathol. 145,* 913−921.
12. Kilgore, K. S., Flory, C. M., Miller, B. F., Evans, V. M., and Warren, J. S. (1996) *Am. J. Pathol. 149,* 953−961.
13. Witt, D. P., and Lander, A. D. (1994) *Curr. Biol. 4,* 394−400.
14. Marquezini, M. V., Strunz, C. M., Dallan, L. A., and Toledo, O. M. (1995) *Cardiology. 86,* 143−146.
15. Wasty, F., Alavi, M. Z., and Moore, S. (1993) *Diabetologia 36,* 316−322.
16. Murch, S. H., MacDonald, T. T., Walker-Smith, J. A., Levin, M., Lionetti, P., and Klein, N. J. (1993) *Lancet 341,* 711−714.
17. Yeo, T.-K., Brown, L., and Dvorak, H. F. (1991) *Am. J. Pathol. 138,* 1437−1450.
18. Petzelbauer, P., Watson, C. A., Pfau, S. E., and Pober, J. S. (1995) *Cytokine 7,* 267−272.
19. Schonbeck, U., Brandt, E., Petersen, F., Flad, H.-D., and Loppnow, H. (1995) *J. Immunol 154,* 2375−2383.
20. Luster, A. D., Greenberg, S. M., and Leder, P. M. (1995) *J. Exp. Med. 182,* 219−231.
21. Rybak, M. E., Gimbrone, M. A., Jr., Davies, P. F., and Handin, R. I. (1989) *Blood 73,* 1534−1539.
22. Alouani, S., Gaertner, H. F., Mermod, J.-J., Power, C. A., Bacon, K. B., Wells, T. N. C., and Proudfoot, A. E. I. (1995) *Eur. J. Biochem. 227,* 228−234.
23. Proudfoot, A. E. I., Power, C. A., Hoogewerf, A. J., Montjovent, M-O., Borlat, F., and Wells, T. N. C. (1996) *FEBS Lett. 376,* 19−23.
24. Rajarathnam, K., Sykes, B. D., Kay, C. M., Deward, B., Geiser, T., Baggiolini, M., and Clark-Lewis, I. (1994) *Science 264,* 90−92.
25. Power, C. A., Meyer, A. M., Nemeth, K., Bacon, K. B., Hoogewerf, A. J., Proudfoot, A. E. I., and Wells, T. N. C. (1995) *J. Biol. Chem 270,* 19495−19500.
26. Lusti-Narasimhan, M., Power, C. A., Allet, B., Aluoani, S., Bacon, K. B., Mermod, J.-J., Proudfoot, A. E. I., and Wells, T. N. C. (1995) *J. Biol. Chem. 270,* 2716−2721.
27. Leatherbarrow, R. J. (1992) GraFit Version 3.01, Erithicus Software Ltd., Staines, U.K.
28. Cheng, Y., and Prusoff, W. H. (1973) *Biochem. Pharmacol. 22,* 3099−3108.
29. Pantoliano, M. W., Horlick, R. A., Springer, B. A., Van Dyk, D. E., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D., and Sisk, W. P. (1994) *Biochemistry 33,* 10229−10248.
30. Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladbury, J. E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., and Lax, I. (1994) *Cell 79,* 1015−1024.
31. Zioncheck, T. F., Richardson, L., Liu, J., Chang, L., King, K. L., Bennett, G. L., Fügedi, P., Chamow, S. M., Schwall, R. H., and Stack, R. J. (1995) *J. Biol. Chem. 270,* 16871−16878.
32. Burrows, S. D., Doyle, M. L., Murphy, K. P., Franklin, S. G., White, J. R., Brooks, I., McNulty, D. E., Scott, M. O., Knutson, J. R., Porter, D., Young, P. R., and Hensley, P. (1994) *Biochemistry 33,* 12741−12745.
33. Paolini, J. F., Willard, D., Consler, T., Luther, M., and Krangel, M. S. (1994) *J. Immunol. 153,* 2704−2717.
34. Zhang, Y., and Rollins, B. J. (1995) *Mol. Cell. Biol. 15,* 4851−4855.
35. Schnitzel, W., Monschein, U., and Besemer, J. (1994) *J. Leukocyte Biol. 55,* 763−770.

BI971125S