Glycosaminoglycans Interact Selectively with Chemokines and Modulate Receptor Binding and Cellular Responses

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ABSTRACT: Chemokines selectively recruit and activate a variety of cells during inflammation. Interactions between cell surface glycosaminoglycans (GAGs) and chemokines drive the formation of haptotactic or immobilized gradients of chemokines at the site of inflammation, directing this recruitment. Chemokines bind to glycosaminoglycans on human umbilical vein endothelial cells (HUVECs) with affinities in the micromolar range: RANTES > MCP-1 > IL-8 > MIP-1α. This binding can be competed with by soluble glycosaminoglycans: heparin, heparin sulfate, chondroitin sulfate, and dermatan sulfate. RANTES binding showed the widest discrimination between glycosaminoglycans (700-fold), whereas MIP-1α was the least selective. Almost identical results were obtained in an assay using heparin sulfate beads as the source of immobilized glycosaminoglycan. The binding of chemokines to glycosaminoglycan fragments has a strong length dependence, and optimally requires both N- and O-sulfation. Isothermal titration calorimetry data confirm these results; IL-8 binds heparin fragments with a K_d of 0.39−2.63 μM, and requires five saccharide units to bind each monomer of chemokine. In membranes from cells expressing the G-protein-coupled chemokine receptors CXCR1, CXCR2, and CCR1, soluble GAGs inhibit the binding of chemokine ligands to their receptors. Consistent with this, heparin and heparin sulfate could inhibit IL-8-induced neutrophil calcium flux. Chemokines can therefore form complexes with both cell surface and soluble GAGs; these interactions have different functions. Soluble GAG chemokines complexes are unable to bind the receptor, resulting in a block of the biological activity. Previously, we have shown that cell surface GAGs present chemokines to the G-protein-coupled receptors, by increasing the local concentration of protein. A model is presented which brings together all of these data. The selectivity in the chemokine−GAG interaction suggests selective disruption of the haptotactic gradient may be an achievable therapeutic approach in inflammatory disease.

Chemokines are a family of small proteins mediating cellular recruitment in routine immunosurveillance and inflammation (*1*, *2*). In addition, chemokine receptors can act as fusion coreceptors for viruses such as HIV-1 (*3*). Almost 40 human chemokines have been identified, and these fall into two major subclasses, based on conserved cysteine residues at the amino terminus: the CXC subclass, of which IL-8 is the prototype; and the CC subclass, which includes monocyte chemoattractant protein-1 (MCP-1),[1] RANTES (regulated on secretion, normal T-cell expressed, and secreted), and macrophage inflammatory peptides 1α and β (MIP-1α and MIP-1β, respectively). More recently, two further subfamilies of chemokine have been identified: the C chemokine lymphotactin and the CX_3C chemokine known as neurotactin or fractalkine (*1*, *2*).

The interaction of chemokines with specific cell populations is mediated by G-protein-coupled seven-transmembrane receptors. Five human CXC chemokine, nine CC chemokines, and a CX_3C chemokine receptor have been identifed to date (*1*). In addition, the Duffy Antigen on erythrocytes has been shown to bind both basic CXC and CC chemokines (*4*).

Chemokines can also bind cell surface glycosaminoglycans at the vascular endothelium or in the extracellular matrix. This interaction has been suggested to play a role in the formation of immobilized, or haptotactic, gradients (*5*−*7*). Endothelial cells synthesize cell surface heparan sulfate chondroitin sulfate, and dermatan sulfate (*8*). Since the content and composition of cell surface proteoglycans depend on the location and type of endothelium (*9*, *10*), this may

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[1] Abbreviations: GAG, glycosaminoglycan; 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; NAP-2, neutrophil activating protein-2; IP-10, interferon-γ inducible protein-10; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle's Medium; CHO, Chinese hamster ovary; FCS, fetal calf serum; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SPA, scintillation proximity assay; WGA, wheat germ agglutin; FACS, fluorescence-activated cell sorter; HUVECs, human umbilical vein endothelial cells; GAG, glycosaminoglycan.

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also control the types of chemokines which can be immobilized at different inflammatory foci. GAGs are also found on leukocytes, and therefore may play a role in presenting the chemokines to the G-protein-coupled chemokine receptors on the same cell. Loss of cell surface glycosaminoglycan reduces the affinity of these cells for many chemokines (*11*). The low-affinity interaction with glycosaminoglycans can modulate the kinetics of the interaction of the chemokine with the G-protein-coupled receptor. Such a process may be central to the function of CCR5 as an HIV coreceptor, since the removal of heparan sulfate from human T cell lines means that the chemokines RANTES and MIP-1β cannot protect cells against HIV infection (*12*).

The heparin-binding properties of many chemokines have already been demonstrated. Data have been reported for the interaction of IL-8 (*13*−*16*), platelet factor-4 (*17*), interferon-γ inducible protein-10 (*18*), MIP-1β (*19*, *20*), MIP-1α (*21*), and RANTES (*20*) with heparin. To investigate the selectivity of the interaction, we have studied the interaction of four well-known chemokines, IL-8, MCP-1, RANTES, and MIP-1α, with four GAGs, heparin, heparin sulfate, chondroitin sulfate, and dermatan sulfate. We have characterized the binding of these chemokines to human umbilical vein endothelial cells (HUVECs), immobilized heparin, and determined the selectivity of this interaction in competition studies. In addition to the selectivity of immobilized GAGs, we show that soluble glycosaminoglycans inhibit chemokine receptor binding and cellular responses. Our results suggest that although immobilized GAGs may aid in the presentation of chemokines, soluble GAGs can form complexes with chemokines and play a specific inhibitory role.

EXPERIMENTAL PROCEDURES

Materials. Heparin−Sepharose, Sepharose CL-6B, and SMART system columns were from Pharmacia. HEPES buffer was from Gibco-BRL. Recombinant chemokines were expressed in *Escherichia coli* and purified by us as reported previously (*11*). MCP-3, MIP-1β, lymphotactin, and IP-10 were purchased from PeproTech (Princeton, NJ). [^{125}I]IL-8, [^{125}I]RANTES, [^{125}I]MIP-1α, and [^{125}I]MCP-1 were from Amersham (specific activity of 2000 Ci/mmol). HUVECs and endothelial growth medium were purchased from Clonetics. BSA was purchased from Sigma Chemical Co.

Glycosaminoglycan Sources and Description. Heparin (H-3125 or H-3393), heparan sulfate (H-5393), chondroitin sulfate (C-8529), dermatan sulfate (C-2413), and hyaluronic acid (H-0902) were purchased from Sigma. Size-fractionated heparin oligosaccharides were obtained from Alexis. Chemically modified heparins were purchased from Seikagaku. GAG sizes have been determined by the manufacturer, by gel filtration chromatography, or by low-angle laser light scattering as follows: heparin (9−12 kDa), heparan sulfate (7.5 kDa), chondroitin sulfate (45.5 kDa), dermatan sulfate (37 kDa), and chemically modified heparins (7.2 kDa). We verified these size determinations by gel filtration chromatography on Superdex 200 (data not shown). Unfractionated heparin has an average chain length of 40 saccharides (Sigma).

Heparin−Sepharose Chromatography. Chemokines (10−50 μg) were loaded onto a 1 mL heparin−Sepharose column (Pharmacia SMART System) in 50 mM Tris-HCl buffer (pH 7.4) and eluted with a 25 mL linear gradient [0 to 2 M NaCl in 50 mM Tris-HCl buffer (pH 7.4)] at a flow rate of 0.5 mL/min. Protein was monitored by absorbance at 280 nm, and the concentration of NaCl was determined by using an in-line conductivity meter calibrated with 50 mM Tris-HCl buffer (pH 7.4) (0% conductivity) and 2 M NaCl in 50 mM Tris-HCl buffer (pH 7.4) (100% conductivity).

Human Umbilical Vein Endothelial Cell (HUVEC) Binding Assays. Passage 2−6 HUVECs were seeded in 96-well plates at a density of 5000 cells/well, using endothelial cell growth medium containing 2% fetal calf serum. The medium was 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 solution was removed by aspiration, and the cells were washed three times with PBS. The cells were incubated in a total volume of 50 μL containing 0.25 nM [^{125}I]chemokine and increasing concentrations of GAGs, using the same buffer conditions described above. After incubation of the cells at 4 °C for 4 h, a quick inversion of the 96-well plates removed the unbound [^{125}I]chemokines, and cells were washed with 3 × 200 μL of buffer. The cells were lysed with 20 mM Tris-HCl buffer (pH 8.0) containing 0.2% Triton X-100 and transferred to 96-well plates, and the radioactivity was measured as described above. The background level of binding to the plates was determined by performing identical manipulations on plates which only received medium at the time of cell seeding. The data were analyzed with GraFit Software (*22*), using the equation $B/B_{max}^{app} = 1/(1 + [L]/IC_{50})$, where B is the counts per minute bound, B_{max}^{app} is the counts per minute bound in the absence of competing ligand, [L] is the concentration of the competing ligand, and the IC_{50} is the amount of unlabeled competitor required to inhibit binding by 50%. Under the conditions that were used, it can be shown that $IC_{50} = [\text{radioligand}] + K_d$ (*23*).

Immobilized Heparin Competition Binding Assay. Competition experiments were performed in 96-well filter plates (Millipore MultiScreen MADVN6510, 0.22 μm pore size, low-protein binding) in a total volume of 100 μL/well. Each well contained 0.125−0.25 nM [^{125}I]chemokine, 5 nM unlabeled chemokine, heparin−Sepharose (Sepharose beads or binding buffer as a background control), and increasing amounts of GAGs (0−2 mg/mL). The mass of heparin on the beads was 1.5 μg/well for MIP-1α (corresponding to 187.5 μg of dry heparin−Sepharose), 0.015 μg/well for IL-8 and MCP-1, and 0.05 μg/well for RANTES, and equivalent amounts of Sepharose CL-6B beads or binding buffer were used for each chemokine in control experiments. The plates were incubated by shaking at 25 °C for 4 h in binding buffer [50 mM HEPES (pH 7.4) containing 0.5% BSA, 5 mM $MgCl_2$, and 1 mM $CaCl_2$]. The beads were washed three times with 200 μL of binding buffer containing 0.15 M NaCl under vacuum filtration. For RANTES, it was necessary to add 0.15 M NaCl to the binding buffer during the incubation and 0.5 M NaCl to the binding buffer for the washes to avoid high levels of nonspecific binding to the filter plate (*24*). The filters were air-dried; 30 μL of scintillation fluid was added to each well, and the radioactivity was measured in a Wallac Microbeta counter. Triplicate measurements were performed for each point.

Isothermal Titration Calorimetry of the IL-8 Heparin Complex. Isothermal titration calorimetry (*25*) was performed using the OMEGA instrument (Microcal, Northampton, MA). Titrations were performed by adding heparin or fragments to 1.8 mL of IL-8 solution in 50 mM HEPES buffer (pH 7.5) containing 5 mM $MgCl_2$ and 1 mM $CaCl_2$. The contents of the syringe were stirred at 400 rpm, and 10−21 injections of 5−12 μL of heparin were added. Data were processed using the software provided by the manufacturer [ORIGIN (*25*)]. This gave values for the binding stoichiometry, *n* (the number of IL-8 monomers bound to each heparin fragment), the dissociation constant, K_d, and the molar binding enthalpy, $\Delta H°$. The Gibbs free energy and the molar entropy of binding were calculated using the isotherm $\Delta G° = RT \ln K_d$.

Chemokine Receptor Binding Assay. cDNAs encoding CXCR1, CXCR2, and CCR1 were cloned and stably transfected into CHO cells, and binding assays were performed using the scintillation proximity assay format (*24*).

Human Neutrophil Purification. Human neutrophils were prepared from plasma by density gradient centrifugation on Ficoll-Paque, followed by hypotonic lysis of red blood cells. The cells were washed twice with PBS and resuspended at a density of 10^6 cells/mL in RPMI-1640 medium containing 10% heat-inactivated FCS and 20 mM HEPES (pH 7.4).

Calcium Mobilization Studies. Mobilization of intracellular calcium in neutrophils was performed essentially as described previously (*24*). Cells were loaded with FURA-2AM dye (2 μM) for 30 min at 37 °C in Krebs-Ringer buffer (pH 7.2) containing 0.1% BSA. Cells were washed after loading, resuspended at a density of 4×10^6 cells/mL, and stored in the dark until analysis was performed at 37 °C. Neutrophil fluorescence changes in response to 10^{-9} M IL-8 were detected in the presence of various GAGs using a Jasco FP-777 fluorimeter. Cells were not preincubated with heparin.

Elemental Analysis of Glycosaminoglycans. Elemental analyses of the glycosaminoglycan were performed on a Leco CHNS-932 instrument to determine the percentage mass of carbon, hydrogen, nitrogen, and sulfur. The values obtained were converted to molar ratios, and the approximate number of sulfate residues per disaccharide was determined using an average value of 13 carbon residues per disaccharide. The numbers of sulfate residues per disaccharide obtained were as follows: 2.4 for heparin, 2.4 for heparan sulfate (fast moving fraction), 0.8 for chondroitin sulfate, 1.0 for dermatan sulfate, 0.1 for completely desulfated, N-acetylated heparin, 0.9 for completely desulfated, re-N-sulfated heparin, and 1.2 for N-desulfated, N-acetylated heparin.

RESULTS

Chemokines Have Different Affinities for Heparin. The affinity of eight chemokines for heparin was investigated by elution of the chemokines from a heparin−Sepharose column with a linear NaCl gradient. There is a wide variation in heparin affinity among these eight chemokines. Of the chemokines that were tested, RANTES exhibited the highest affinity for heparin, requiring a concentration of 0.90 M NaCl to be eluted from the column. The elution of MIP-1α from the heparin−Sepharose column required the least NaCl (0.39 M), indicating that it has the weakest affinity for heparin. Other chemokines had intermediate values: 0.86 M for lymphotactin, 0.85 M for IP-10, 0.80 M for MCP-3, 0.60 M for IL-8, 0.60 M for MCP-1, and 0.56 M for NAP-2. The weak binding of MIP-1α reflects its overall negative charge, and contrasts with the other chemokines, which are highly basic.

Binding of Chemokines to Human Umbilical Vein Endothelial Cells and Selective Competition for Binding by Soluble Glycosaminoglycans. RANTES, MIP-1β, and IL-8 have been shown by immunohistochemistry to bind to vascular endothelium (*19*, *20*, *26*). We investigated the binding of RANTES, MIP-1α, MCP-1, and IL-8 to glycosaminoglycans on endothelial cells and competition by the soluble GAGs: heparin, heparin sulfate, dermatan sulfate, and chondroitin sulfate (Figure 1). These four chemokines were able to reversibly bind to HUVECs under the same conditions used to detect binding to immobilized heparin. The binding of radiolabeled chemokines was competed for by increasing concentrations of heparin, heparan sulfate, chondroitin sulfate, or dermatan sulfate. The displacement curves were fitted to a single-site binding model as discussed previously, and the concentration required for 50% inhibition of binding (IC_{50}) was calculated. Of the four molecules, heparin was the most effective competitor. The IC_{50} values indicated that RANTES bound to the endothelial cells with the highest apparent affinity, requiring a heparin concentration of 0.9 μg/mL to reduce the level of binding by 50%. IL-8 and MCP-1 bound similarly but less tightly, with IC_{50} values of 19 and 17 μg/mL, respectively. MIP-1α had the lowest apparent affinity for HUVEC surfaces, requiring 40 μg/mL heparin to achieve a 50% reduction in the level of binding. The range in the IC_{50} values for the competition by different glycosaminoglycans was different for the four chemokines. RANTES exhibited the largest amount of selectivity, with a range of 750-fold between heparin and chondroitin sulfate. IL-8 and MCP-1 exhibited moderate selectivity, and MIP-1α binding to HUVECs exhibited only a 7-fold difference in the level of glycosaminoglycan competition.

To confirm these results, a solid phase competitive binding assay was used, incubating radiolabeled chemokines with heparin−Sepharose beads with increasing concentrations of soluble glycosaminoglycan competitor (*27*). Control experiments were performed by repeating the incubations in the presence of Sepharose CL-6B beads or without beads to confirm the specific binding of the chemokine to the heparin on the Sepharose beads (Figure 2). Although the final conditions of the assay were slightly different for each chemokine, the relative IC_{50} values obtained from the 96-well format assay corresponded well with the rank order of elution in heparin−Sepharose chromatography. In competion assays, the results were very similar to those seen in the HUVEC assay (Table 1). All four chemokines exhibited selectivity in their interaction with glycosaminoglycans, and the range of selectivity displayed by each chemokine varied. Again, RANTES was the most selective, with almost 3 orders of magnitude between the affinity for heparin and the affinity for chondroitin sulfate. MIP-1α exhibited the least selectivity with a 5-fold range. The IC_{50} values for IL-8 and MCP-1 were between 3.9 and 220 μg/mL, and 3.3 and 39 μg/mL, respectively, showing intermediate ranges. Further evidence of the selectivity of chemokines for glycosaminoglycans was seen in the variation in the rank order potency of the GAG competitors. For MIP-1α, the rank order of potency was as

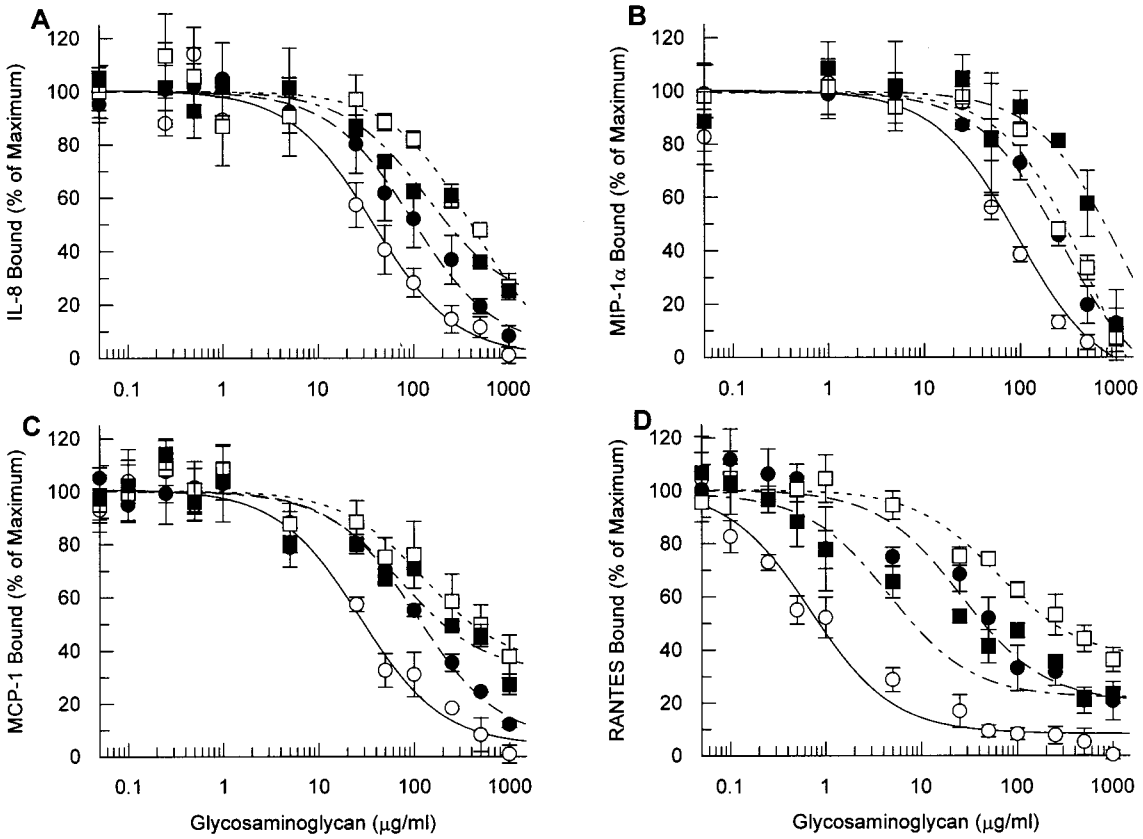

FIGURE 1: IL-8, MIP-1α, MCP-1, and RANTES bind to HUVECs, and glycosaminoglycans selectively compete for the binding. The binding of iodinated IL-8 (A), MIP-1α (B), MCP-1 (C), and RANTES (D) to HUVECs was performed as described in Experimental Procedures, and was competed by increasing concentrations of heparin (○), heparan sulfate (●), chondroitin sulfate (□), and dermatan sulfate (■). The data were analyzed with GraFit (*22*) using the equation $B/B_{max}^{obs} = 1(1 + [L]/IC_{50})$, where B is the counts per minute bound, B_{max}^{obs} is the counts per minute bound in the absence of competing ligand, [L] is the concentration of the competing ligand, and the IC_{50} = [radioligand] + K_d under the conditions of the experiment (*28*). The data are from one experiment representative of three.

follows: heparin > heparan sulfate > chondroitin sulfate > dermatan sulfate. The rank order of potency for MCP-1 and IL-8 was similar: heparin > heparan sulfate > chondroitin sulfate = dermatan sulfate. RANTES exhibited a very similar order, but interacts more strongly with dermatan sulfate than with heparan sulfate.

Length Dependence of the Chemokine−Glycosaminoglycan Interaction. For some heparin binding cytokines such as bFGF (*28*), hepatocyte growth factor (*29*), and vascular endothelial growth factor VEGF (*30*), it has been shown that a minimal heparin chain length is required for binding. We therefore used the solid phase binding assay to determine whether a minimal chain length is required for chemokines. Size-fractionated heparin oligosaccharides ranging from 6-mers to 20-mers were investigated for their ability to compete for the binding of radiolabeled IL-8 and MIP-1α to immobilized heparin. All of the oligosaccharides used in this study were able to compete for the binding of IL-8 and MIP-1α. The hexamer fraction exhibited IC_{50} values of 370 (200 μM) and 230 μg/mL (130 μM), respectively (Table 2). In the IL-8 experiment, increasing chain length reduced the IC_{50} values for all size-fractionated oligosaccharides, including the unfractionated crude heparin (average chain length of 40 saccharides). For MIP-1α, however, there was no decrease in the IC_{50} values with heparin chain length beyond the 18-mer.

Charge Dependence of Chemokine Interaction with Chemically Modified Heparin. The basic glycosaminoglycan structure is a repeating disaccharide unit consisting of a hexuronic acid (either glucuronic acid or its epimer iduronic acid) and D-glucosamine. The polysaccharide is substituted to a varying extent with N- and O-linked sulfate groups and N-linked acetyl groups (*31*). In vivo, the cell surface heparan sulfate is microheterogeneous due to various substitutions in the core disaccharide structure. To investigate the importance of N- or O-sulfation of heparin to its interaction with chemokines, heparins with both N- and O-sulfation, N-sulfation alone, O-sulfation alone, or no sulfation were tested for their ability to compete for the binding of the radiolabeled chemokines in the immobilized bead assay. Removal of the *N*-sulfate (and protecting the free amino with acetylation) increases the IC_{50} values 4−10-fold for IL-8, MCP-1, and MIP-1α (Table 3). For RANTES, the loss of N-sulfation had a more pronounced effect, with a 280-fold weaker IC_{50} value. The approximate number of sulfate groups per dissacharide, calculated from elemental analysis data, was 1.2 after the loss of N-sulfation compared to 2.4 for native heparin.

The effect of the loss of heparin O-sulfation was an increase in the IC_{50} values of 18−65-fold for IL-8, MCP-1, and MIP-1α. Again, the effect on RANTES binding was more pronounced, with an IC_{50} value 730-fold higher than that of native heparin. The loss of O-sulfation was therefore more detrimental to chemokine binding than the loss of N-sulfation, although the degrees of sulfation were similar for both molecules (0.9 per disaccharide). Finally, heparin, which lacks both *N*- and *O*-sulfates, competed very poorly,

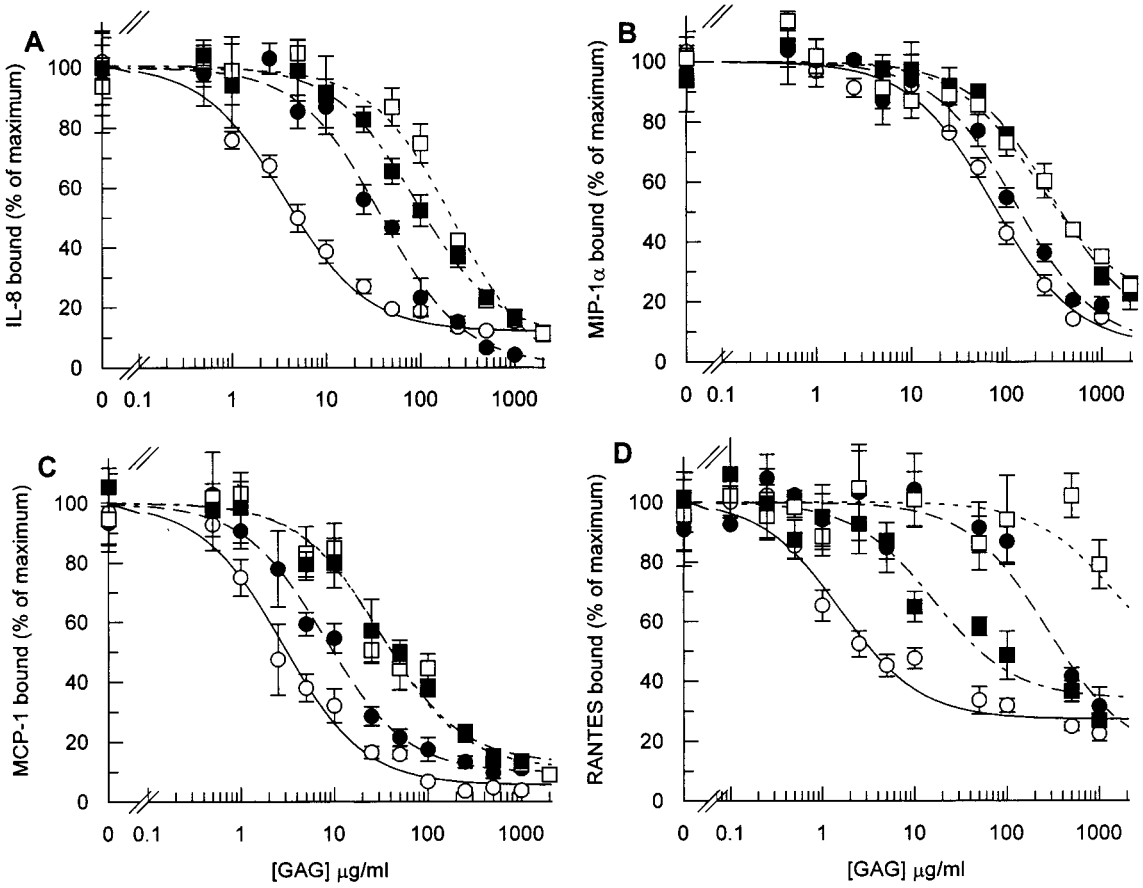

FIGURE 2: Chemokines can discriminate between glycosaminoglycan families. The binding of iodinated IL-8 (A), MIP-1α (B), MCP-1 (C), and RANTES (D) to immobilized heparin was competed for by increasing concentrations of heparin (○), heparan sulfate (●), chondroitin sulfate (□), or dermatan sulfate (■). Competition assays and data analysis were performed as described in Experimental Procedures. Each point represents the mean ± the standard error of the mean of triplicate values, and the data depicted in the graphs are representative of at least three experiments.

Table 1: Glycosaminoglycan Competition of the Binding of Chemokines to HUVECs and Immobilized Heparin[a]

GAG	IL-8	*n*	MCP-1	*n*	MIP-1α	*n*	RANTES	*n*
heparin								
HUVECs	19 ± 6	4	17 ± 6	3	40 ± 16	3	0.9 ± 0.2	3
immobilized heparin	3.9 ± 0.2	8	3.3 ± 0.4	10	77 ± 5	14	1.8 ± 0.5	6
heparan sulfate								
HUVECs	44 ± 21	3	85 ± 24	3	150 ± 40	3	30 ± 7	3
immobilized heparin	37 ± 8	3	8.9 ± 1.4	5	130 ± 20	4	160 ± 60	3
chondroitin sulfate								
HUVECs	630 ± 190	3	400 ± 10	3	200 ± 30	3	680 ± 440	4
immobilized heparin	220 ± 30	4	37 ± 7	5	260 ± 60	5	1200 ± 200	4
dermatan sulfate								
HUVECs	380 ± 70	3	200 ± 80	3	280 ± 80	3	12 ± 3	3
immobilized heparin	200 ± 10	4	39 ± 6	4	380 ± 100	5	22 ± 4	4

[a] Binding of radiolabeled chemokines to immobilized heparin or HUVECS was competed for by increasing concentrations of GAG, either heparin, heparin sulfate, chondroitin sulfate, or dermatan sulfate, as described in the text. Data were fitted to a single-site competition model as described in the text and presented as IC_{50} values (micrograms per milliliter) ± the standard error of the mean.

with IC_{50} values 50−140-fold higher than tat of native heparin for IL-8, MCP-1, and MIP-1α, and at least 2000-fold higher for RANTES. Elemental analysis showed that the approximate number of sulfate groups per disaccharide was 0.1.

Isothermal Titration Calorimetry of the IL-8−Heparin Complex. The results of the binding assays were further confirmed by studying the interaction of IL-8 and heparin fragments using isothermal titration calorimetry. Initial experiments showed that optimal concentration for the calorimetry was 30 μM for IL-8 at pH 7.5, with 9000 MW heparin (Alexis). Under these conditions (Figure 4), the dissociation constant K_d equals 0.98 ± 0.07 μM and the stoichiometry equals 6.5 ± 0.4 IL-8 monomers per heparin fragment. Reducing the protein concentration to 5 μM reduced the signal-to-noise ratio, making the data much harder to interpret, but had little effect on the values that were measured (K_d of 0.6 ± 0.3 μM and a stoichiometry of 5.65). Increasing the IL-8 concentration to 90 μM gave results similar to those of the 30 μM study (K_d of 0.97 ± 0.11 μM and a stoichiometry of 6.8). We therefore used 30 μM IL-8 as our reference condition. Under these conditions,

Table 2: IC_{50} Values for the Competition of IL-8 and MIP-1α from Immobilized Heparin by Sized-Fractionated Heparin Oligosaccharides[a]

chemokine	heparin competitor	IC_{50} (μg/mL)	IC_{50} (μM)	*n*
IL-8	6-mer	370 ± 50	200	3
	8-mer	160 ± 30	65	3
	10-mer	120 ± 30	38	3
	12-mer	100 ± 20	29	3
	14-mer	70 ± 3	17	3
	16-mer	41 ± 11	8.5	3
	18-mer	45 ± 15	8.3	3
	20-mer	17 ± 6	2.8	3
	heparin (≈40-mer)	3.9 ± 0.3	0.3	7
MIP-1α	6-mer	230 (220−240)	130	2
	8-mer	240 (210−270)	100	2
	10-mer	100 (90−110)	33	2
	12-mer	120 (90−150)	33	2
	14-mer	90 (60−120)	22	2
	16-mer	78 (59−98)	16	2
	18-mer	40 (29−50)	7.4	2
	20-mer	50 (30−71)	8.3	2
	heparin (≈40-mer)	77 ± 5	6.5	14

[a] The values represent the mean IC_{50} value and the standard error of the mean or range (in parentheses). *n* is the number of experiments that were carried out.

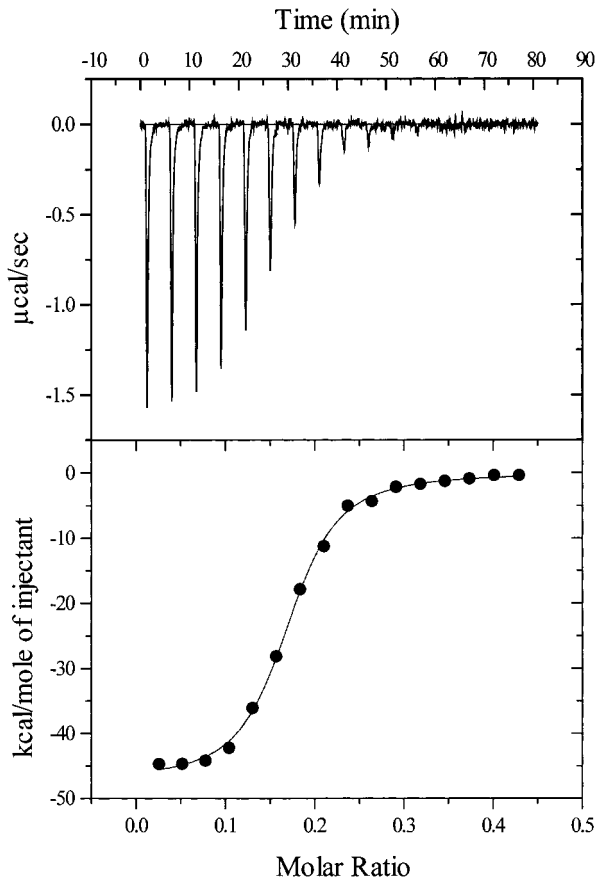

FIGURE 3: Binding isotherm for the titration of IL-8 with Alexis MW 9000 heparin. A 150 μM heparin solution was titrated into a 15 μM IL-8 solution (1.4 mL) using sixteen 7 μL injections spaced at 4 min intervals at 25 °C. The area under each injection heat was integrated and plotted in the bottom panel. The solid line represents a nonlinear least-squares fit of the reaction heat for each injection using a single-binding site model.

the reaction is exothermic, and has a significant entropic gain ($\Delta S = 10.5 \pm 5.4$ J mol^{-1} K^{-1}) presumably due to the release of bound solvent. The isothermal calorimetry experiments were repeated for a number of heparin fragments with different lengths (Table 5). The number of saccharide units required for the binding of each IL-8 monomer can be seen to vary between 4 and 5. The dissociation constant decreases with increasing heparin chain length, from 2.63 to 0.39 μM. This decrease is reflected primarily in the enthalpy of binding. Under the conditions of the experiments, a slow precipitation process (over 4−20 min) was observed, which indicates the formation of higher-order complexes. The precipitation was not sufficiently rapid to have any influence on the rapid heats obtained following injection of the heparin fragments into the IL-8 solution, and therefore, its energetics are not measured during the calorimetry experiment. Stoichiometry data obtained using the *N*-methylleucine 25 derivative of IL-8 [which does not dimerize in free solution (*32*)] were almost identical to those of the wild-type protein: $n = 4.4 \pm 0.8$ and 10.0 ± 0.7 for MW 5000 and 18000 heparin, respectively. However, no precipitation was observed under these circumstances. The K_d values for *N*-methylleucine 25 IL-8 were smaller (35 ± 9 and 5.9 ± 0.4 μM for MW 5000 and 18000 heparin, respectively), consistent with previous results (*11*).

Effect of Soluble Glycosaminoglycans on the Binding of Chemokines to G-Protein-Coupled Receptors. One possible mechanism of action of cell surface glycosaminoglycans is presenting the chemokine to its receptor. In an extreme version, this presentation requires formation of a chemokine, glycosaminoglycan, and receptor complex. For this to happen, the binding sites for the receptor and glycosaminoglycan need to be spatially distinct, and at least uncompetitive in terms of binding. We therefore investigated the effect of soluble glycosaminoglycans on chemokine binding to their G-protein-coupled receptors. A scintillation proximity assay for [^{125}I]IL-8 binding to membranes from CHO cells which had been transfected with the cDNAs for CXCR1 and CXCR2 and for [^{125}I]MIP-1α binding to membranes from CHO cells which had been transfected with CCR1 cDNA was used (Figure 4). Unlabeled IL-8 competed with the binding of [^{125} I]IL-8 to CXCR1 and CXCR2 with IC_{50} values of 1.9 ± 0.4 and 0.5 ± 0.1 nM, respectively, and unlabeled MIP-1α competed for the binding of [^{125}I]MIP-1α to CCR1 with an IC_{50} of 0.3 ± 0.05 nM, consistent with the published data for these receptors. All of the tested glycosaminoglycans competed for the binding of these two chemokines to their high-affinity receptors with a rank order of potency similar to the one observed in the immobilized heparin competition assay. Heparin exhibited the most potent competition for the binding of radiolabeled IL-8 and MIP-1α to the appropriate membranes, with IC_{50} values for CXCR1, CXCR2, and CCR1 of 23 ± 10, 5 ± 2, and 7 ± 2 μg/mL, respectively, and the IC_{50} values for heparan sulfate were 2−3-fold higher (Table 5). The IC_{50} values for chondroitin sulfate and dermatan sulfate were approximately 10-fold lower, demonstrating a selectivity in the competition for receptor binding by these GAGs. Radiolabeled heparin did not bind to untransfected cells or to cells transfected with CCR1 or CXCR1 with any quantitative differences.

Effect of Soluble Glycosaminoglycans on Intracellular Calcium Mobilization in Neutrophils. Since, in recombinant cell systems, soluble glycosaminoglycans compete with chemokine binding to its receptor, we examined their effects on a response in primary cells. Previous studies have

Table 3: Competition of the Binding of Radiolabeled Chemokines to Immobilized Heparin by Chemically Modified Heparins[a]

	native heparin N- and O-sulfate	N-desulfated, N-acetylated heparin O-sulfate	completely desulfated, re-N-sulfated heparin N-sulfate	completely desulfated, N-acetylated heparin none
SO_3/disaccharide	2.4	1.2	0.9	0.1
IC_{50} (μg/mL)				
IL-8	3.9 ± 0.2	34 (33−35)	260 (240−280)	370 (260−480)
MCP-1	3.3 ± 0.4	18 (13−23)	180 (100−260)	470 (400−540)
MIP-1α	77 ± 5	280 (270−290)	1400 (1200−1600)	4100 (1800−6400)
RANTES	1.8 ± 0.5	540 (420−650)	1300 (1000−1600)	>5000

[a] The immobilized heparin competitive binding assay was performed as described in Experimental Procedures. Each value represents the mean IC_{50} value and the range or standard error of the mean of at least two experiments performed in triplicate.

Table 4: Isothermal Titration Calorimetry Results for IL-8 Binding to Five Commercially Available Size-Fractionated Heparins[a]

heparin	chain length (saccharides)	n	K_d (μM)	$\Delta H°$ (kJ/mol)	$\Delta G°$ (kJ/mol)	$\Delta S°$ (J mol^{-1} K^{-1})
Sigma LMW (MW 3000)	10	2.08 ± 0.10	2.63 ± 0.27	−28.1 ± 1.4	−31.8 ± 0.3	12.4 ± 5.3
Alexis LMW (MW 5000)	16	3.83 ± 0.26	2.36 ± 0.41	−29.2 ± 1.2	−32.1 ± 0.5	9.5 ± 3.3
Sigma (MW 6000)	20	4.69 ± 0.18	1.55 ± 0.21	−30.6 ± 0.7	−33.2 ± 0.4	8.7 ± 3.4
Alexis Fr I (MW 9000)	30	6.5 ± 0.4	0.98 ± 0.07	−31.2 ± 1.8	−34.3 ± 0.2	10.4 ± 5.4
Alexis Fr III (MW 18000)	60	12.0 ± 0.6	0.39 ± 0.03	−34.6 ± 0.9	−36.5 ± 0.2	6.4 ± 2.5

[a] Experiments were performed at 25 °C in 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, and 1 mM $CaCl_2$. The fitting parameters n, K_d, and ΔH were obtained by a nonlinear least-squares fit of the reaction heats after each injection using a single-binding site model. The values are the means of at least three experiments.

disagreed in this regard (*13*, *16*, *33*, *34*) with one group showing a small enhancement of activity, and three groups showing inhibition. Neutrophils were stimulated with 1 nM IL-8 in the presence or absence of various GAGs, and the induced calcium flux was monitored. We observed a dose-dependent decrease in the extent of calcium mobilization when heparin was added (Figure 5). The signal was 45% of control levels with the addition of 40 μg/mL heparin, and 6% of control levels with 400 μg/mL heparin. In a separate set of experiments, the abilities of different GAGs to inhibit neutrophil chemotaxis were compared. Control neutrophils exhibited a calcium flux of 750 ± 50 (arbitrary limits); inhibition with 50 μg/mL heparin reduced this to 76 ± 13 units. Heparin sulfate was less effective as an inhibitor (184 ± 81 units), and chondroitin sulfate was the least effective (455 ± 185 units). Thus, the rank order of potency of GAG inhibition of neutrophil calcium mobilization agreed with the ability of these molecules to compete for binding of IL-8 to its receptor. In the absence of IL-8, the glycosaminoglycans do not induce calcium mobilization in neutrophils (data not shown). Thus, soluble GAGs selectively inhibit IL-8-mediated neutrophil calcium mobilization, in proportion to their affinity for IL-8.

DISCUSSION

The interaction of chemokines with both endothelial and leukocytic cell surface glycosaminoglycans is important for at least two reasons. First, it allows the formation of immobilized gradients of chemokines (haptotactic gradients), and second, it facilitates the receptor binding process. We have demonstrated that four representative members of the chemokine family interact diversely with the different types of GAGs that are present on endothelial and other cells, and this may contribute to the selectivity of leukocyte recruitment.

Members of the chemokine family exhibit a wide variation in their affinity for heparin, measured either by heparin−Sepharose chromatography or using the solid phase binding assay on heparin beads. Each chemokine can distinguish between the various glycosaminoglycans expressed on the endothelium, such as heparan sulfate, chondroitin sulfate, and dermatan sulfate. We have shown that endothelial cells bind these four chemokines and the binding is competed for by various glycosaminoglycans. The data show that there is specificity in the chemokine−GAG interaction beyond that predicted purely by electrostatics. Furthermore, dermatan sulfate and chondroitin sulfate have similar levels of sulfation, yet interact with RANTES with very different IC_{50} values (22 vs 1200 μg/mL, respectively). Finally, studies with chemically modified heparins show that RANTES in particular is exquisitely sensitive to the loss of one sulfate from each disaccharide. If the IC_{50} values are an accurate reflection of the K_d of the complex, then this represents more than 3 kcal/mol, more than would be expected from a simple electrostatic interaction, suggesting a role for H-bond formation (*35*). A minimum level of heparin sulfation does appear to be necessary, however, since the completely desulfated, N-acetylated heparin interacted very poorly with chemokines. In addition to an overall minimum degree of sulfation, the location of the sulfation is important. For all four chemokines, O-sulfation was more important than N-sulfation.

The ability of heparin fragments to compete for chemokine binding is dependent on the length, where longer heparins are better competitors than short competitors. The increase in affinity with chain length appears to be incremental with chain length. Our data confirm the observation that oligosaccharides of six or more sugars can inhibit IL-8−heparin binding in a nitrocellulose binding assay (*15*). However, it is clearly dependent on the precise nature of the disaccharide units as we have shown previously for IL-8 (*14*). Chondroitin sulfate and dermatan sulfate are 3 times longer than heparin, and compete 10−600-fold less efficiently with IL-8, MCP-1, and RANTES. Although the size of glycosaminoglycans is important, it is clearly not the only determinant of selectivity.

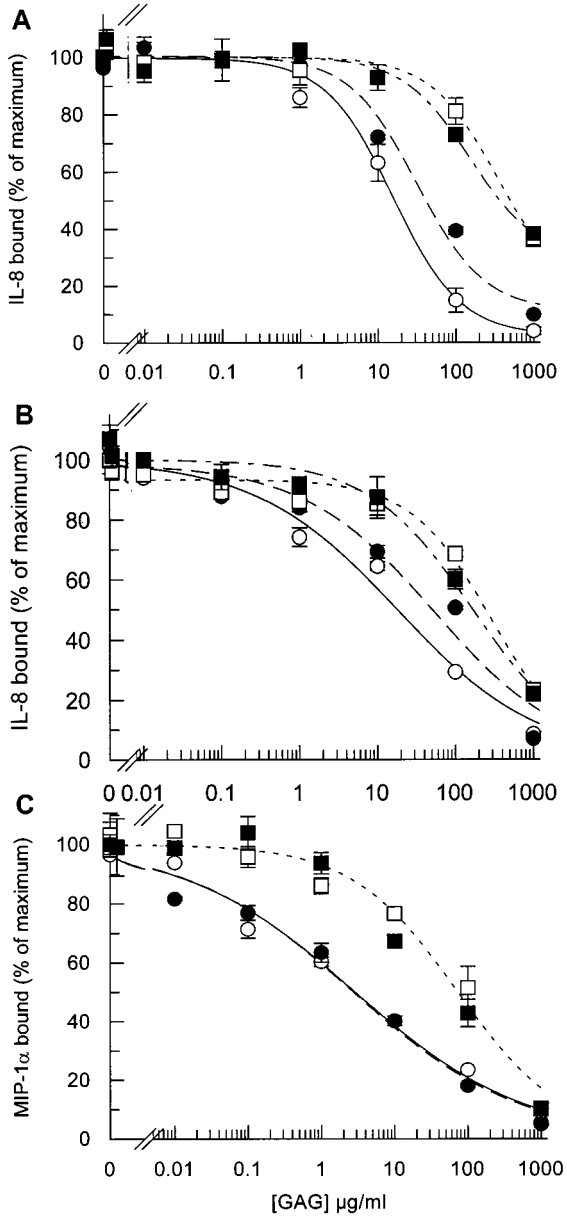

FIGURE 4: Soluble GAGs selectively inhibit the binding of IL-8 and MIP-1α to membranes from cells transfected with chemokine receptor cDNA. Membranes prepared from stably transfected CHO cells expressing CXCR1, CXCR2, or CCR1 were used in a scintillation proximity assay performed as described in Experimental Procedures. The binding of IL-8 to CXCR1 (A) and CXCR2 (B) and binding of MIP-1α to CCR1 (C) were competed for with increasing concentrations of heparin (○), heparan sulfate (●), chondroitin sulfate (□), and dermatan sulfate (■). Each point represents the mean ± the standard error of the mean of duplicate values from two to four independent experiments.

Studies of the formation of the IL-8−heparin complex using isothermal titration calorimetry confirm the significance of the interaction. The data show that four or five saccharide units are required for the binding of each IL-8 monomer. The values obtained in calorimetry suggest a direct interaction between GAG and chemokine with a K_d in the low micromolar range. The data do not provide direct meaurements of the aggregation state of the heparin in this complex, but a number of suggestions emerge. First, the affinity of the IL-8 for heparin increases as the stoichiometry increases, indicating that there is some contact between the IL-8 monomers (resulting in an increase of more than 6-fold in the binding affinity, with more than 1 kcal/mol of additional binding energy being released). Second, calculations for platelet factor 4 (*36*) predicted a minimum size of eight saccharides per protein monomer. Our results of four or five saccharides per monomer suggest a far more intimate connection between the protein and saccharides. Third, the observation that the stoichiometry of the *N*-methylleucine 25 IL-8 binding to heparin is identical to that of the wild-type protein is surprising. It would suggest that the dimer interface observed in the NMR and crystallographic studies is not used in the formation of the initial complex in the isothermal titration calorimetry experiments. Alternatively, the heparin fragment is able to help stabilize the formation of dimers of *N*-methylleucine 25 IL-8. The observation that precipitation of the heparin−IL-8 complex occurs after several minutes indicates that higher-order complexes can be formed, involving multiple heparin fragments. Since the precipitation is not seen in the *N*-methylleucine 25 IL-8 studies, we suggest that it is these higher-order complexes which are the cause of the enhanced binding effects seen in previous studies where we chemokine binding from heparin−Sepharose or HUVECs competes against that with unlabeled chemokine (*11*).

Table 5: IC_{50} Values for the Competition of Chemokines from Their High-Affinity Receptors by Soluble Glycosaminoglycans[a]

receptor	radiolabeled chemokine	competitor	IC_{50} (μg/mL)	*n*
CXCR1	IL-8	heparin	23 ± 10	3
		heparan sulfate	64 ± 24	3
		chondroitin sulfate	220 ± 90	3
		dermatan sulfate	220 ± 110	3
CXCR2	IL-8	heparin	5 ± 2	3
		heparan sulfate	11 ± 7	3
		chondroitin sulfate	130 ± 30	3
		dermatan sulfate	100 ± 40	3
CCR 1	MIP-1α	heparin	7 ± 2	4
		heparan sulfate	24 ± 10	3
		chondroitin sulfate	90 ± 10	3
		dermatan sulfate	51 ± 21	3

[a] The competitive binding assay was performed as described in Experimental Procedures. Each value represents the mean IC_{50} and the standard error of the mean of at least two experiments performed in triplicate.

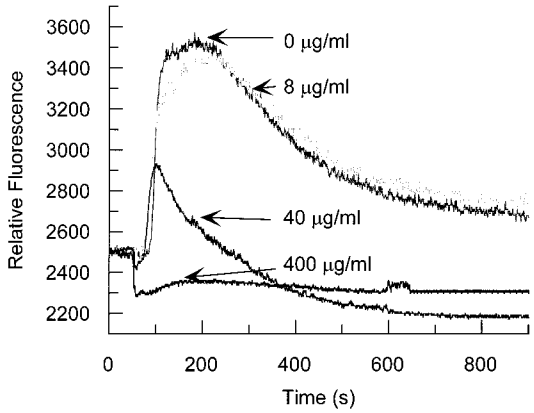

FIGURE 5: Intracellular Ca^{2+} mobilization in neutrophils stimulated with 10^{-9} M IL-8 is inhibited by heparin. The level of calcium mobilization in Fura-2-AM-loaded neutrophils was measured with a fluorimeter following the addition of 10^{-9} M IL-8 and increasing concentrations of heparin. Data are means of triplicate values and are representative of at least three experiments.

We confirm that GAGs can alter the biological function of chemokines, selectively inhibiting IL-8-mediated calcium mobilization in neutrophils. Our results are in agreement with previous data obtained from three different laboratories that demonstrate heparin inhibition of leukocyte responses (*16*, *33*, *34*). More recently, heparin was found to inhibit neutrophil calcium mobilization induced by several individual chemokines, including NAP-2, IL-8, and a hybrid AELR/PF4 chemokine (*33*). However, one study reported that soluble heparan sulfate, but not heparin, enhanced neutrophil responses to IL-8 (*13*). It is not yet clear if variations in experimental protocol may account for these discrepancies, such as the source or variation in soluble glycosaminoglycans added to the neutrophil assays or perhaps the activation state of the neutrophils used in the assay. The role of the cell surface GAG can be summarized as enhancement of the local concentration of chemokine in the locality of the receptor, and this enhances chemokine binding to the receptor as we and others have shown previously (*11*, *12*, *18*). The data in this study show that the chemokine−cell surface GAG interaction has selectivity in itself, and therefore may act as a mechanism for further enhancing the selectivity of chemokine networks in recruiting specific cell populations in a specific inflammatory event.

In vivo, there is a selective loss of heparan sulfate in inflammatory situations such as transplant rejection (*41*). In contrast, the vessel wall content of glycosaminoglycans after balloon angioplasty is increased 4−10-fold (*42*, *43*). There is also an increase in the levels of chondroitin sulfate and dermatan sulfate and a decrease in the level of heparan sulfate in atherosclerotic veins and aortas compared to normal vessels (*44*). Since RANTES has a higher affinity for dermatan sulfate than for heparan sulfate, it would be expected that RANTES would bind to atherosclerotic tissues, whereas IL-8 or MIP-1α would exhibit weaker binding. Likewise, MCP-1 also has relatively low IC_{50} values for both chondroitin sulfate and dermatan sulfate, and would be expected to be preferentially retained in atherosclerotic vessels, and indeed, MCP-1 has been identified in atherosclerotic tissue (*45*). Further studies will be needed to determine if the differential expression of GAGs in vivo correlates with a differential retention of chemokines.

Soluble glycosaminoglycans have already been shown to reduce inflammation levels in experimental animal models. Low doses of heparin are able to inhibit delayed-type hypersensitivity reactions, adjuvant arthritis, allergen-induced lung eosinophil infiltration, and thioglycollate-elicited neutrophil accumulation (*46*−*48*). This reduction in the level of inflammation could be due to the blockade of chemokine-mediated leukocyte activation, which we have demonstrated in vitro. However, heparin can bind to several molecules implicated in inflammation in addition to the chemokines, including L- and P-Selectin (*49*) and heparanase (*50*), and can also inhibit mast cell degranulation (*51*). Hence, the mechanism by which heparin inhibits these inflammatory events is likely to be complex. With the more recent advances in the understanding of heparin structure, heparin lacking its anticoagulant properties is now available. Further studies with chemokines and glycosaminoglycans may help to identify discrete binding sites within heparin, thus allowing more specific antagonism of the specific heparin-binding molecules involved in inflammation.

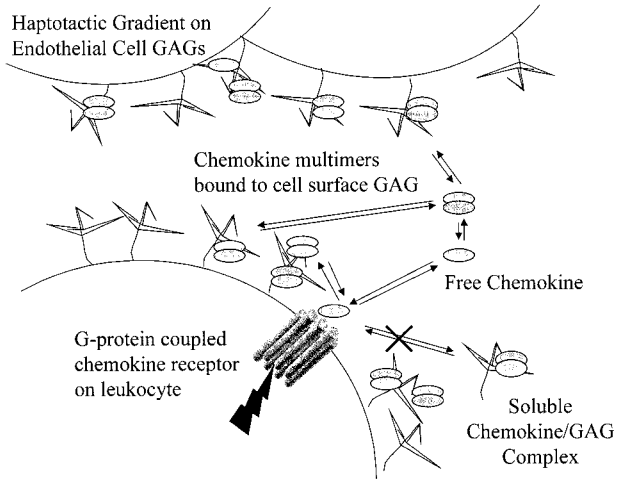

FIGURE 6: Model for the role of glycosaminoglycans in modulating chemokine activity. Cell surface glycosaminoglycans on the leukocyte can form complexes with chemokines that enhance the local concentration of chemokine in the vicinity of the G-protein-coupled receptor. These chemokines come from the haptotactic gradient on the endothelium. However, the complex of the chemokine with a soluble glycosaminoglycan is unable to bind to the G-protein-coupled receptor, and as such, the soluble GAG competes with the receptor for free chemokine.

Our data therefore suggest that cell surface GAGs play a role different from that of the GAGs free in solution. Our model is shown in Figure 6. Free chemokine can interact reversibly with its seven-transmembrane receptor and drive a cellular response. The presence of cell surface GAGs on the leukocyte provides an array of low-affinity interactions for the chemokine, which help to raise the local concentration of the chemokine in the vicinity of the seven-transmembrane receptor. These chemokines could have been passed from the haptotactic gradient on the endothelial cells, or simply could be present in the bulk medium. Thus, although there is no formation of a ternary glycosaminoglycan−chemokine−G-protein-coupled receptor complex, the cell surface glycosaminoglycan can still act to enhance the local chemokine concentration. A biochemical analogy for this is the way that the light-harvesting complex captures photons for photosynthesis. The GAGs are thus acting as an antenna to help concentrate the chemokine close to the receptor. Since there is selectivity in the chemokine−GAG interaction, it is reasonable to suggest that cell surface GAGs may contribute to selectivity, by only concentrating a selection of chemokines.

However, if the chemokine is complexed with soluble GAGs, this complex will be unable to bind to the receptor. This is largely an electrostatic effect since both the N-terminus of the receptor and the glycosaminoglycan are very acidic. The amino acid side chains responsible for receptor and heparin binding are spatially distinct for many chemokines (*14*, *37*−*40*). It may be possible to find specific fragments of GAGs that bind the chemokine and do not prevent the complex binding to the G-protein-coupled receptor. So far, we have not found such a molecule, but this may explain the results reported by Webb et al. (*13*).

In summary, we have shown that the glycosaminoglycans that are expressed on endothelial cell surfaces interact selectively with chemokines, and that structural features of the glycosaminoglycans are important in these interactions.

These interactions are significant enough for soluble glycosaminoglycans to compete for the binding of chemokines to their high-affinity receptors, and to inhibit downstream receptor-mediated cell responses. Therefore, the selectivity in the interactions between glycosaminoglycans and chemokines may be exploited in identifying molecules which are capable of reducing the level of inflammation in vivo.

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