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COMMENTARY

New Strategies for Chemokine Inhibition and Modulation

YOU TAKE THE HIGH ROAD AND I'LL TAKE THE LOW ROAD

Grant McFadden and David Kelvin**

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY, UNIVERSITY OF WESTERN ONTARIO, LONDON, ON N6A 5C1; AND THE JOHN P. ROBARTS RESEARCH INSTITUTE, LONDON, ON N6G 2V4, CANADA

ABSTRACT. Chemokines are low molecular weight cytokines that induce extravasation, chemotaxis, and activation of a wide variety of leukocytes. Members of the different chemokine families are defined by the orientation of specific critical cysteine residues, and are designated as C-X-C (e.g. interleukin-8), C-C (e.g. regulated upon activation normally T cell expressed and secreted, RANTES), or C (lymphotactin). All chemokines bind to members of a G-protein coupled serpentine receptor superfamily that span the leukocyte cell surface membrane seven times and mediate the biological activities of the individual ligands. Most chemokines possess two major binding surfaces: a high affinity site responsible for specific ligand/receptor interactions and a lower affinity site, also called the heparin-binding or glycosaminoglycan-binding domain, believed to be responsible for the establishment and presentation of chemokine gradients on the surface of endothelial cells and within the extracellular matrix. Although chemokines are clearly beneficial in wound healing, hemopoiesis, and the clearance of infectious organisms, the continued expression of chemokines is associated with chronic inflammation. Therefore, this class of cytokines are attractive targets for the creation of antagonists that abrogate one or more chemokine functions. It is envisioned that such antagonists could serve as a new class of anti-inflammatory drugs. In this commentary, we will discuss two different but related strategies for antagonizing chemokine-induced functions, namely, disruption of the low and high affinity binding sites. BIOCHEM PHARMACOL **54**;12:1271–1280, 1997. © 1997 Elsevier Science Inc.

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The recruitment of leukocytes from the circulation to sites of injury and infection is a key process in the physiological response to wound healing and the clearance of pathogenic organisms [1–5]. Recent advances in the understanding of the molecular mechanisms that regulate leukocyte recruitment have identified a complex interplay between leukocytes, cytokines, chemokines, adhesion molecules, and extracellular matrix components that is essential for directed leukocyte migration. Chemokines comprise an ever enlarging family of small molecular weight cytokines that have been recognized recently as key effector molecules that stimulate leukocytes to leave the circulation and migrate to the sites of inflammation and injury. This superfamily of cytokines has well over 30 distinct members, which bind to subsets of G-protein coupled serpentine receptors. In spite of obvious beneficial properties that chemokines have in the wound healing process and for the clearance of infectious organisms, they also can have pathophysiological consequences. It is thought that continued expression of chemokines stimulates the accumulation of leukocytes which, when appropriately activated, release injurious enzymes and oxidative radicals. Many inflammatory and immunological disorders, such as arthritis, asthma, reperfusion injury, and atherosclerosis, are characterized by increased levels of specific sets of chemokines [2–5]. Therefore, a likely target for suppression of inflammatory or immunological disorders is to inhibit chemokine expression or function, thereby limiting the degree of leukocyte infiltration [2–4, 6, 7]. In this commentary, we will focus on strategies and molecules that can function as chemokine antagonists by inhibiting their binding to either high affinity cell surface receptors or to low affinity binding sites within the extracellular matrix and on the surface of endothelial cells.

* Corresponding authors: Dr. Grant McFadden and Dr. David Kelvin, The John P. Robarts Research Institute, 1400 Western Road, London, Ontario N6G 2V4, Canada. For Dr. McFadden: Tel. (519) 663-3184; FAX (519) 663-3847; E-mail: mcfadden@rri.on.ca. For Dr. Kelvin: Tel. (519) 663-3812; FAX (519) 663-3847; E-mail: kelvin@sdri.uwo.ca.

CHEMOKINE FUNCTIONS

The most widely studied and recognized chemokine function is the stimulation of leukocyte chemotaxis. Generally speaking, this function is either assayed *in vitro* using a modified Boyden chamber in which directed cell migration can be enumerated, or with *in vivo* animal models where cellular influx can be monitored histologically or by intravital microscopy. The *in vitro* chemotaxis assay theoretically relies on the diffusion of chemokines through a soluble phase to establish a transient gradient that stimulates

directed cellular migration. In this assay system, C-X-C chemokines such as IL-8† induce neutrophil migration at concentrations in the low to middle nanomolar range, while C-C chemokines such as MCP-1 induce monocyte chemoattraction in a similar range of ligand concentrations. Other *in vitro* systems that measure directed leukocyte migration incorporate the use of extracellular matrix components, presumably to aid either in chemokine presentation or in adhesion events necessary for proper leukocyte locomotion. Alternatively, several *in vivo* assays for the induction of leukocyte movement and accumulation have been described with the more popular assays using injections of specific chemokines, or expression of cloned chemokines from viral vectors, followed by histological examination of the tissue to enumerate and classify the infiltrating leukocytes. Cellular functions stimulated by chemokines include leukocyte activation and granule release, oxidative burst generation, calcium influx, and upregulation of cellular adhesion, with the latter promoted by increased adhesion molecule expression or avidity [1–5].

A number of investigators have also shown chemokine modulation of *in vitro* and *in vivo* hemopoiesis [8]. MIP-1α and MIP-1β have received the most attention in this regard; however, the recent studies with SDF-1 in the mouse have shown that lymphopoiesis and myelopoiesis are both greatly hindered by disrupting the SDF-1 gene [9–12].

In some non-leukocyte populations (for example, endothelial cells, vascular smooth muscle cells, keratinocytes, and melanocytes), chemokines have been demonstrated to induce proliferation and migration. Furthermore, many C-X-C chemokines can either induce or inhibit angiogenesis [13–15]. At this time, the relationship between different functions ascribed to chemokines is not always clear, but some of these activities map to different chemokine epitopes, which correspond to high affinity and low affinity binding sites (see below).

CHEMOKINE STRUCTURES AND BINDING SITES

All chemokines, with the exception of lymphotactin, possess four conserved cysteine residues (see Fig. 1A). The configuration of the first two cysteine residues can be used to classify chemokines into two distinct subfamilies: the C-X-C (X refers to any intervening amino acid) and the C-C subfamilies. In the human, notable members of the C-X-C subfamily are PF4, IL-8, GROα, β, and γ, NAP-2, and IP-10. The GROs, IL-8, and NAP-2 can induce neutrophil migration, while IL-8 and IP-10 can also induce T cell migration. In addition, these C-X-C chemokines can either induce or inhibit angiogenesis. The C-C subfamily of chemokines appears to affect a wider array of leukocytes. For example, RANTES, MIP-1α, MIP-1β, MCP-1, MCP-2, MCP-3, and eotaxin stimulate migration or activation of one or more of the following leukocyte subsets: monocytes/macrophages, T cells, B cells, NK cells, stem cells, eosinophils, mast cells, and basophils.

X-ray crystallography and solution structural studies for IL-8, PF4, MIP-1β, and RANTES have demonstrated that monomeric forms of both the C-X-C and C-C chemokine families have a similar three-dimensional structure [7, 16–21]. Each of these contains a single α-helix at the C-terminus packaged against a three-stranded antiparallel β-sheet by hydrophobic interactions. The N-terminal amino acids preceding the first conserved cysteine have a large degree of movement, which renders structural studies difficult. The monomeric form is believed to be the major species found at physiological concentrations, and, based on numerous studies, monomers have been shown to be functional ligands. There is also substantial data to indicate that dimeric and multimeric forms of chemokines exist, but the functional relevance of these species is presently unknown. The N-terminal region is important for high affinity interactions with both the C-X-C and C-C chemokines (see Fig. 1B). The highly charged amino acids in the α-helix at the carboxyl terminal region appear to be important for low affinity interactions and have been called the heparin or GAG binding domain.

Like many cytokines and growth factors, chemokines utilize both high affinity and low affinity interactions to elicit full biological activity. Chemokine binding studies performed with labeled ligands have identified binding sites with affinities in the 500 pM to 10 nM range on the surface of neutrophils, monocytes, T cells, and eosinophils, as well as other non-leukocyte cell types [22–25]. Cloning these receptors has revealed that cell surface high affinity chemokine receptors all belong to the seven transmembrane G protein coupled receptor superfamily. This is consistent with biochemical data that indicate that chemokine-induced chemotaxis can, for most chemokines, be blocked by the G-α_i inhibitor pertussis toxin. Expression of cloned cDNAs for these receptors in COS cells or HEK293 cells, which normally do not express chemokine receptors, results in high affinity cell surface binding sites (500 pM to 10 nM K_d). Cloning of chemokine receptors has aided in the dissection of binding profiles for individual chemokines and chemokine receptors. In general, a single chemokine can bind to a limited number of closely related receptors, and a single receptor can bind a limited number of closely related chemokines. One notable exception to this latter rule is the Duffy antigen at the surface of red blood cells, which binds a much wider assay of chemokines but does not signal in response to any of them. Although a single leukocyte subset frequently expresses multiple chemokine receptors, during an *in vivo* inflammatory reaction receptor triggering is likely not restricted to a single chemokine/receptor interaction.

† *Abbreviations:* IL-8, interleukin-8; RANTES, regulated upon activation normally T cell expressed and secreted; GAG, glycosaminoglycan; MCP-1, -2, and -3, monocyte chemoattractant-1, -2, and -3; MIP-1α and -1β, macrophage inflammatory protein-1α and -1β; SDF-1, stromal cell derived factor-1; PF4, platelet factor-4; GROα, β, and γ, growth related oncogene-α, β, and γ; NAP-2, neutrophil acting peptide-2; IP-10, γ-interferon-inducible protein-10; FGF, fibroblast growth factor; GM-CSF, granulocyte/macrophage colony stimulating factor; HIV-1, human immunodeficiency virus-1; and MIG, monokine induced by γ-interferon.

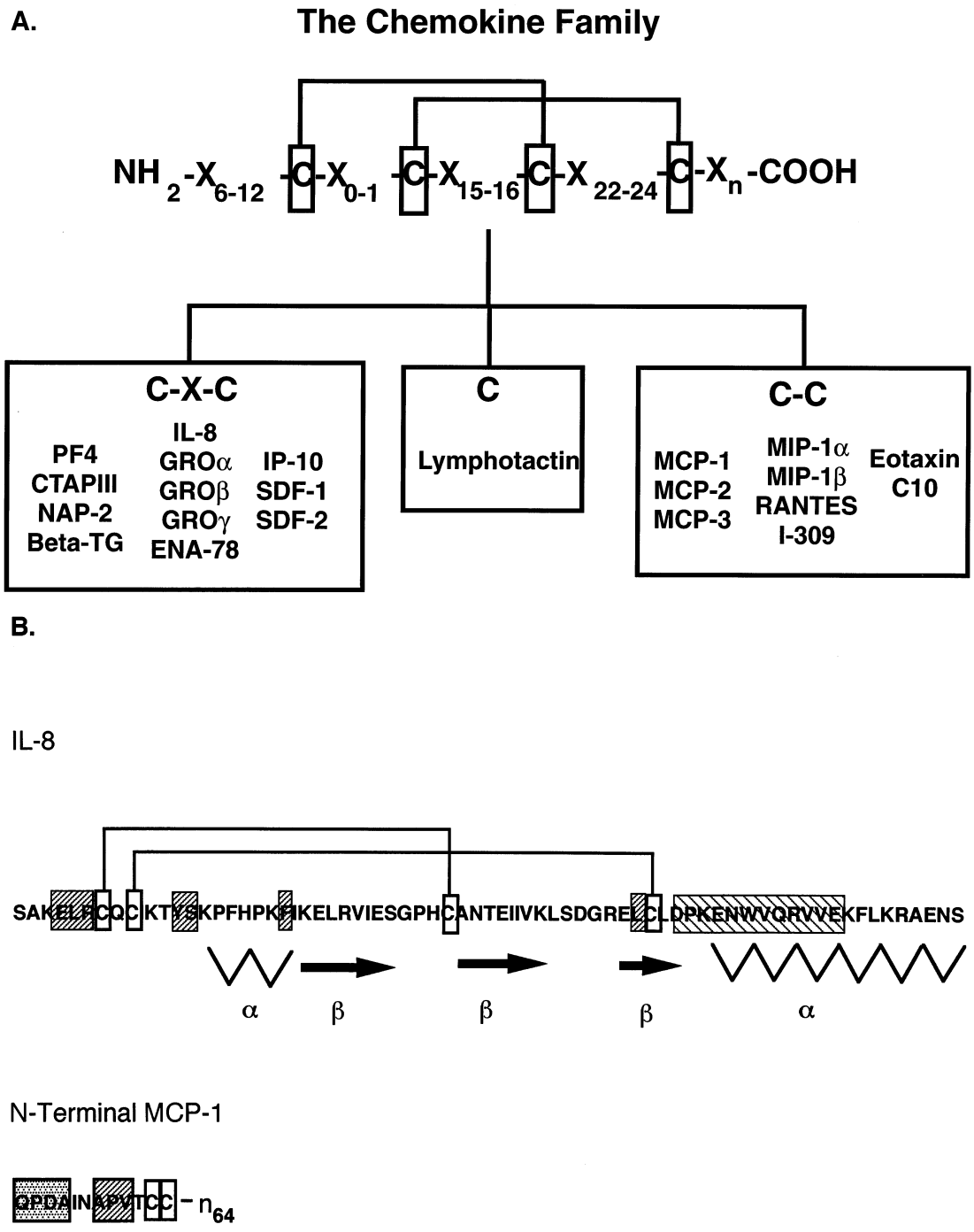

FIG. 1. (A) Schematic representation of the classification of the chemokine superfamily based on the arrangement of the first one or two conserved cysteine residues. Note: this is only a partial listing of the known chemokines. (B) Amino acid sequence of human IL-8 (top portion) and the N-terminal region of MCP-1 (bottom portion). The open boxes represent conserved cysteine amino acids. The left-hatched box and the overlapping α-helix outlines the region with low affinity binding characteristics. The right-hatched boxes represent areas of high affinity interactions in both IL-8 and MCP-1. The stippled box in MCP-1 represents the activation domain. The arrows indicate secondary β-sheets, while the waved lines represent α-helices.

Low affinity binding regions on chemokines have been recognized for many years and were exploited originally for biochemical purification [1–3]. As discussed in later sections, chemokines can bind to several molecules found in the extracellular matrix with affinities in the middle nanomolar to millimolar range. It is now thought that these low affinity interactions are biologically extremely important [26–28]. It is hypothesized that these low affinity associations probably play a role in presenting gradients of chemokines to cognate cell surface high affinity receptors, stabilizing the high affinity interactions, or stimulating cellular functions through low affinity signal transducing receptors.

TABLE 1. Potential strategies for modulation of chemokine ligands

Inhibitor affinity class	Mechanism	Example
(1) High affinity interactions		
(a) Neutralizing antibody	Prevent ligand/receptor engagement	See Table 2
(b) Chemokine mutant antagonist	Compete with ligand for receptor binding	See Table 2
(c) Small inhibitor molecule	Block receptor/ligand surface contacts	Under development
(d) Receptor homologs	Ligand diversion/inappropriate signaling	Herpesvirus chemokine receptors (e.g. cytomegalovirus US28)
(2) Low affinity interactions		
(a) Soluble chemokine binding proteins	Bind chemokine C-terminus and perturb chemokine gradient or extracellular localization	Poxvirus chemokine binding proteins? (e.g. myxoma T1/35 kDa)
(b) Heparin-like mimetics	Compete with sulphated proteoglycans for chemokine binding	Heparinylated BSA
(c) Small inhibitor molecule	Disrupt chemokine/matrix interactions	Non-sulphated aromatic polyanions
(d) Nonsignaling chemokine peptide	Compete with chemokine ligand for cell surface or matrix glycosaminoglycan binding sites	PF4 N-terminal deletions

ANTAGONISM OF HIGH AFFINITY INTERACTIONS

The easiest theoretical strategy for creating an antagonist of an individual chemokine is to develop or identify molecules that disrupt ligand binding to and/or activation of signaling receptors. The structural differences between high and low affinity binding sites require different strategies for the design of antagonists that can disrupt binding. Alternative strategies for disrupting chemokine functions, such as inhibition of chemokine expression or blockade of chemokine receptor stimulated signal transduction pathways, will not be explored here. Instead, we will focus on disruption of high affinity and low affinity interactions (see Table 1). In the case of the high affinity interactions, several strategies for modulation have been demonstrated, but in this commentary we consider examples of only two classes of reagents: neutralizing antibodies and ligand antagonists (see Table 2).

Antibodies as Antagonists of Chemokine Function

Neutralizing antibodies are one of the most useful inhibitors currently used in cytokine research. Several neutralizing polyclonal and monoclonal antibodies to chemokines and chemokine receptors have been produced and used successfully to limit or block pathophysiological conditions. For example, the WS-4 monoclonal to human IL-8 was shown to be effective in preventing neutrophil accumulation and tissue damage in a rabbit lung reperfusion-ischemia model [29]. WS-4 inhibits IL-8-induced neutrophil chemotaxis *in vitro* and prevents high affinity binding of IL-8 to neutrophils. Additional studies with this antibody showed that it was effective in treating acute glomerulonephritis, lipopolysaccharide-induced dermatitis, IL-1-induced arthritis, and delayed-type hypersensitivity [30–33]. Polyclonal antibodies directed to the rat C-C chemokine MCP-1 were able to inhibit the majority of leukocyte (macrophage) infiltration

TABLE 2. Examples of known inhibitors of chemokine function

Molecule	Characteristics	Effective range of antagonism or inhibition	Blocked function or pathological condition
WS-4	Anti-IL-8 monoclonal antibody	1:1 Molar ratio of antibody to wild-type chemokine	Perfusion-ischemia, *in vivo* migration, glomerulonephritis
Anti-rat MCP-1	Polyclonal MCP-1 antibody		Glomerulonephritis, *in vivo* macrophage infiltration
IL-8 (6–72)	N-Terminal deletion	30:1 Molar ratio of mutant to wild-type chemokine	Neutrophil enzyme release Neutrophil-induced chemotaxis
MCP-1 (7ND)	N-Terminal deletion	4:1 Molar ratio of mutant to wild-type chemokine	MCP-1-induced chemotaxis
MCP-1 (9–76)	N-Terminal deletion	IC_{50} = 20 nM, K_d for binding = 8.3 nM	MCP-1-induced chemotaxis MCP-1 cell surface binding
Met-RANTES	Retention of Met in recombinant molecule	1.7:1 Molar ratio of mutant to wild-type RANTES	RANTES-induced chemotaxis Inhibition of RANTES and MIP-1α binding
RANTES (9–68)	N-Terminal deletion	30–300 nM range	Inhibition of *in vitro* HIV-1 infection

in a rat model of glomerulonephritis and improved excreted levels of protein and glomerulosclerosis [34]. Thus, neutralizing antibodies can act as effective antagonists of chemokine function in *in vivo* models of inflammation where specific chemokines are thought to be critical for the pathogenic phenotype. The major problem associated with using antibodies to antagonize chemokine function is that they must be customized or humanized before use in chronic human diseases. Furthermore, the ability of multiple chemokines to bind and activate a single receptor forces the development of a multiple antibody strategy or the use of cross-reactive antibodies in order to completely block or prevent pathological conditions.

N-Terminal Chemokine Mutants as Antagonists

Within the N-terminal region of C-X-C chemokines that induce neutrophil chemotaxis is a tripeptide ELR motif which, in the case of human IL-8, is positioned at residues 4–6 (see Fig. 1B) [1–3]. Mutagenesis studies of this motif show it to be essential for neutrophil migration and activation as well as high affinity binding [35–40]. Substitution of an ELR motif into PF4, which does not activate neutrophils and does not normally contain the ELR motif, changes PF4 to a neutrophil activating chemokine [37]. Additional studies have demonstrated that deletion of the N-terminal five amino acids of IL-8 produces an effective chemokine antagonist. A 30-fold molar excess of this variant is required for half-maximal inhibition of degranulation and partial inhibition of neutrophil chemotaxis [40].

N-Terminal C-C chemokine mutants appear to be more effective chemokine antagonists than their C-X-C counterparts, possibly due to the greater separation of the leukocyte activation domain and high affinity binding domain within the N-terminal region. For example, in the case of IL-8, the activation domain and binding domain more closely overlap [7, 35, 36, 39]. Truncation of the N-terminal region of MCP-1 yields an antagonist that inhibits chemotaxis at only a 4-fold molar excess [41, 42]. Truncation of amino acids 2–8 also creates an effective antagonist, with an IC_{50} of 20 nM and a K_d of 8.3 nM, which is only 3-fold higher than the affinity of wild-type MCP-1 to cell surface receptors [43]. Retention of the N-terminal initiating methionine in recombinant RANTES creates an effective antagonist for both RANTES and MIP-1α [44]. Met-RANTES antagonizes chemotaxis in a range of 1.7 to 1.0 molar excess and competes for binding of native RANTES and MIP-1α with IC_{50} values of 25 and 28 nM, respectively. Met-RANTES, however, could not inhibit IL-8-induced chemotaxis, showing this antagonist to have selectivity in antagonizing C-C but not C-X-C chemokines [44].

The recent discovery that chemokine receptors are used as co-receptors along with CD4 for HIV entry into monocytes and T cells has propelled new investigations into the use of chemokine antagonists as inhibitory agents against HIV infection [45–50]. In one of the first studies reported, the N-terminal mutant of RANTES can block infection of HIV in the 30–300 nM range, presumably by interfering with the viral gp120/CCR-2 interaction [51].

The above studies indicate that N-terminal mutants of C-C chemokines may be effective antagonists presumably by competing for high affinity binding sites, although more complex interactions, including dominant negative effects and competition for low affinity sites, could also be involved. Furthermore, even peptides as short as 6 or 7 amino acids can display inhibitory properties [52], suggesting that further studies on smaller domain antagonists are warranted. Future *in vivo* studies are awaited to determine the effectiveness of this class of antagonists in inhibiting chronic inflammatory and immunological disorders (see also Refs. 53 and 54 for recent reviews on this subject).

ANTAGONISM OF LOW AFFINITY INTERACTIONS

Until quite recently it was not generally appreciated that the extracellular matrix itself plays a critical and dynamic role during leukocyte extravasation and trafficking within complex tissues [55–58]. In the case of chemokines, it was proposed only within the past 5 years that functional haptotaxis, namely the migration of responsive cells in response to a ligand gradient that is affixed upon the surface of endothelial cells or embedded within the extracellular matrix, required specific interactions between chemokines and specific GAG moieties [27, 59]. This chemokine/GAG interaction is believed to be critical, not only for the fixation of stable chemokine gradients, but also for the presentation and conformational activation of the ligands to their cognate serpentine receptors [26–28, 59, 60]. In fact, similar ligand/GAG interactions have been described for a variety of diverse cytokines and growth factors, including the various members of the FGF family, hepatocyte growth factor, pleiotrophin, transforming growth factor-β, interferon-γ, interleukins-3 and -7, GM-CSF, and vascular endothelial growth factor [61–65].

The situation with FGF is highly instructive. In the mid-1980s, FGFs were shown to bind heparin and heparan sulphate, and this interaction was proposed to protect the ligand from degradation and act as a reservoir for these ligands within the sub-endothelial cell matrix [66, 67]. In fact, the low affinity receptor for FGF is itself a cell surface heparan sulphate proteoglycan, and this binding is critical for dimerization of the high affinity receptors, possibly in a bridge-like arrangement [68, 69]. Thus, novel strategies to inhibit FGF activity during angiogenesis include the blockade of this lower affinity interaction with diverse agents such as metabolic inhibitors of heparan sulphate synthesis (or sulphation), by the use of heparan sulphate degrading enzymes (e.g. heparanases), or with synthetic mimetics of heparin/heparan sulphate [70]. Importantly, different classes of cell surface sulphated proteoglycans can have divergent effects on the activities of FGF, and there is evidence that at least some members of the FGF family possess both positive and negative regulatory elements

within the heparin-binding domain [71, 72]. Thus, it is now appreciated that FGF-mediated angiogenesis is just as dependent upon these lower affinity FGF/GAG interactions as on the higher affinity FGF/receptor interactions [69, 70]. For example, heparinoids such as suramin and pentosan polysulphate have been used to block angiogenic stimulation by basic FGF [70]. It is possible that similar types of reagents targeted to the heparin-binding domains of chemokines could be adapted to be comparably useful for perturbing chemokine functions as well. Below we consider four scenarios by which such low affinity interactions might be manipulated.

Soluble Chemokine Binding Proteins

Although secreted cellular proteins that interact with the heparin-binding domain of chemokines have not been specifically described, analogous soluble binding proteins that interact with the GAG-binding domains of acidic and basic FGF have been discovered [73–76]. Interestingly, a 17 kDa secreted FGF-binding protein (HBp17) isolated by heparin-affinity chromatography from A431-conditioned medium was shown to form inhibitory complexes with FGF and block receptor binding by both acidic and basic FGF, thereby preventing mitogenic stimulation of 3T3 cells [73]. This inhibitory effect of the HBp17 protein could be abrogated by heparin [73], suggesting that other ligands with structurally related heparin-binding domains, such as most chemokines, might also be inhibited by this protein; however, no experiments with chemokines and HBp17 have been reported.

Recent data suggest the existence of several virus-encoded proteins that can interact with chemokines, either as cell surface serpentine receptor homologs or as secreted chemokine binding proteins [77]. Indeed, the recent discovery of chemokine-like ligands encoded by at least one poxvirus and two herpesviruses [78–80], as well as the observation that HIV-1 uses chemokine receptors as co-receptors [45], all affirm the notion that chemokines can affect the antiviral response for many classes of virus infections. The chemokine serpentine receptor homologs encoded by members of the herpesvirus and poxvirus families have been reviewed elsewhere [23, 77, 81, 82], and will not be considered further in this review. The discovery that certain secreted poxvirus proteins bind a wide spectrum of chemokines suggests that chemokine modulation by viruses might be a more extensively utilized strategy than previously suspected. To date, two families of virus-encoded secreted chemokine binding proteins have been discovered, both of which are expressed by members of the poxvirus family. The first example is a secreted 37 kDa glycoprotein encoded by the *M-T7* gene of myxoma virus, and originally defined as a homolog of the interferon-γ receptor family [83]. Although this secreted protein is indeed a potent species-specific inhibitor of interferon-γ [83, 84], knockout analysis of the *M-T7* gene indicated an unexpected linkage between expression of M-T7 and early leukocyte chemotaxis into virus-infected lesions [85]. *In vitro* studies with M-T7 indicate that the purified protein binds to many diverse members of the C-X-C, C-C, and C chemokine families via their conserved heparin-binding domains [86]. Given these dual activities of the M-T7 protein, however, it still remains to be formally demonstrated that the *in vitro* chemokine-binding property of M-T7 is the basis for the alterations in leukocyte influx into virus lesions *in vivo*.

A second distinct poxvirus chemokine-binding protein has been described recently, and is referred to as the T1/35 kDa family of secreted proteins [87]. Members of this larger family of secreted proteins are expressed by myxoma virus, Shope fibroma virus, rabbitpox, cowpox, and certain strains of vaccinia (Lister and Copenhagen); however, other vaccinia strains (e.g. WR) possess truncated homologs of T1/35 kDa [87–89]. As prototypic members, M-T1 of myxoma virus and the 35 kDa protein expressed by rabbitpox bind members of the C-X-C and C-C chemokine families (K_d = 73 nM for M-T1 binding to RANTES), and deletion analysis of the rabbitpox 35 kDa gene revealed that the knockout virus lesions are infiltrated by higher levels of extravasating leukocytes than the parental virus lesions [87]. Taken together, these results suggest that the T1 and T7 families of poxvirus proteins have the potential to interact with multiple chemokines located within the extracellular matrix, but definitive proof that these proteins can actually perturb chemokine functions during an inflammatory response *in vivo* remains to be demonstrated.

Heparin-Like Mimetics

For ligands such as the FGFs, or those chemokines that rely upon association with extracellular GAGs for correct biological presentation of the ligand to the receptors, the use of heparan-sulphate degrading enzymes such as heparanase (endo-β-D-glucuronidase) or metabolic inhibitors such as chlorate (to block sulphation of proteoglycans) provides a crude strategy to disrupt ligand distribution patterns in the extracellular environment [65]. Heparin or heparan sulphate directly competes for GAG interactions critical for T cell adhesion mediated by MIP-1β *in vitro* [59], and heparin itself can reduce FGF-mediated angiogenesis *in vivo* [65], but the extent to which any of the heparins or heparinoids can disrupt the establishment of chemokine gradients within the extracellular matrix milieu is unknown.

It is likely that the density of surface charge clusters on the C-terminal α-helix that forms the chemokine GAG-binding domain is critical for determining which classes of GAGs or their relevant mimetics might be the most appropriate inhibitor for these lower affinity interactions [26, 28]. The apparent K_d for heparin binding to different chemokines, for example, can range over several orders of magnitude (e.g. 5.5 μM for IL-8 vs 27 nM for PF4), and fractionated heparin subclasses can also vary widely in binding affinities to a single chemokine [26].

The potential use of heparins or heparin-mimetics to alter chemokine gradients within complex tissues faces

technical impediments associated with delivery strategies. Artificial proteoglycans, such as heparinylated-BSA, or natural proteoglycans such as CD44, have been used to facilitate MIP-1β-dependent binding of $CD8^+$-T cells *in vitro* [59], but it is unclear at this point whether such compounds could become useful *in vivo* chemokine inhibitors until strategies can be developed to deliver the active polypeptide outside the bloodstream into deeper tissues.

Small Molecular Weight Inhibitors

The use of small molecular weight drugs to bind cytokine ligands and disrupt interactions with extracellular GAGs has been best exploited with FGF-dependent angiogenesis [90]. For example, the heparinoids suramin and pentosan polysulphate both inhibit angiogenesis under conditions where heparin is either ineffective or even stimulatory [70]. Similarly, non-sulphated low molecular weight polyanions such as RG-13577 are able to block basic FGF binding to extracellular matrix, or heparin-Sepharose [65], and might very well provide a similar disruptive influence upon chemokine gradients. In the case of suramin, the anti-angiogenic capacity of the drug can also be shown to be targeted against vascular endothelial growth factor [91], which, like basic FGF, possesses heparin-binding domains similar to those of chemokines.

Small molecular weight polyanions could, in theory, disrupt chemokine gradients imbedded upon GAG matrices within tissues, and this is an area ripe for further exploration. A major advantage exploited by this strategy is the fact that the majority of C-C and C-X-C chemokines are believed to possess similar C-terminal protein folding domains that define the GAG-binding site, and hence such compounds have the potential to circumvent the projected redundancies of multiple chemokines known to be co-ordinately induced by pro-inflammatory stimuli.

Nonsignaling Chemokine Peptides

Several members of the chemokine family, such as IP-10, PF4, and MIG were characterized by their ability to inhibit pro-angiogenic chemokines, and lack the critical N-terminal ELR motif believed to be responsible for proper receptor triggering [14, 15, 92]. For example, IP-10 binds *in vitro* to cell surface heparan sulphate (K_d = 25 nM), as does PF4, and this binding correlates with the angiostatic properties of these ligands as measured *in vivo* [92]. In fact, an even more potent inhibitor of endothelial cell proliferation can be created from these ligands by N-terminal deletions which completely ablate the signaling properties of these ligands. In the case of PF4, such deletion variants no longer bind to high affinity receptors and can only be biologically characterized by the inhibitory properties of the truncated protein [93]. The possibility exists that naturally N-terminal cleaved variants of the ELR-minus C-X-C chemokines might also be powerful natural inhibitors of the positively signaling chemokines by competing for available GAG binding sites in the extracellular matrix [13]. Indeed, the availability of ligand variants that bind GAGs but not high affinity receptors reaffirms the notion that the lower affinity binding domains shared by most chemokines are a worthy target for further drug development.

CONCLUSIONS

There is general consensus that chemokines play pivotal roles in orchestrating leukocyte migration into diseased or damaged tissues. It is useful to note that viruses have also apparently discovered chemokines and their receptors [77]. There are now three examples of chemokine-like genes encoded by DNA viruses (molluscum contagiosium, murine cytomegalovirus, and Kaposi's sarcoma-associated herpesvirus), but whether these viral proteins are actually ligand agonists or antagonists remains to be deduced [78–80]. Several members of the poxvirus and herpesvirus families also express seven transmembrane-spanning serpentine receptors that, at least for the cytomegalovirus and herpesvirus saimiri examples, are *bona fide* high affinity receptors for chemokine signaling [23, 81, 82]. In the case of the secreted chemokine-binding proteins expressed by poxviruses, the binding domains for the viral proteins with chemokines remain to be mapped, but the lack of specificity exhibited by the viral proteins for binding to the various C-X-C and C-C chemokines suggests involvement of the low affinity GAG-binding domain rather than the high affinity site. We suggest that the high and low affinity binding domains each could be effective targets for immunosubversion strategies by viruses and possibly other pathogens as well.

The most widely studied chemokine inhibitors to date have been neutralizing antibodies and ligand antagonists ([53, 54] and Table 2). The specificity of these reagents, however, suggests that inflammatory disorders characterized by multiple, or redundant, chemokine expression profiles will be relatively more refractory to treatment. The technical advantage for drugs targeted to the low affinity GAG-binding domain is that most chemokines tested bind to heparan sulphates, implying that such strategies would be predicted to exhibit less specificity for chemokine family distinctions than the high affinity site modulators. Thus, drugs of this class might not block chemokine/receptor engagement *per se*, but any disruption of chemokine localization within the extracellular matrix could nevertheless provide an effective block for directed leukocyte taxis within tissues.

To conclude, proper chemokine function during *in vivo* inflammatory responses depends both upon high affinity interactions with specific chemokine receptors and lower affinity interactions with critical proteoglycans such as heparan sulphate and chondroitin sulphate. This point was reaffirmed recently by the observation that mutagenesis of the MIP-1α GAG binding site generates a ligand variant still capable of stem cell inhibition, but which cannot bind to the CCR-1 receptor or stimulate chemotaxis of monocytes [94]. This apparent interdependence between the two

chemokine binding domains further emphasizes the rationale for developing newer generations of drugs targeted against each domain, rather than to just the high affinity binding sites alone.

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