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Chemokine Cooperativity Is Caused by Competitive Glycosaminoglycan Binding

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Chemokines comprise a family of secreted proteins that activate G protein–coupled chemokine receptors and thereby control the migration of leukocytes during inflammation or immune surveillance. The positional information required for such migratory behavior is governed by the binding of chemokines to membrane-tethered glycosaminoglycans (GAGs), which establishes a chemokine concentration gradient. An often observed but incompletely understood behavior of chemokines is the ability of unrelated chemokines to enhance the potency with which another chemokine subtype can activate its cognate receptor. This phenomenon has been demonstrated to occur between many chemokine combinations and across several model systems and has been dubbed chemokine cooperativity. In this study, we have used GAG binding-deficient chemokine mutants and cell-based functional (migration) assays to demonstrate that chemokine cooperativity is caused by competitive binding of chemokines to GAGs. This mechanistic explanation of chemokine cooperativity provides insight into chemokine gradient formation in the context of inflammation, in which multiple chemokines are secreted simultaneously. The Journal of Immunology, 2014, 192: 3908–3914.

Chemokines are 8- to 12-kDa–sized secreted proteins that mediate the directed migration (chemotaxis) of leukocytes. The chemokine family encompasses nearly 50 members, which are classified based on the position of their conserved N-terminal cysteine residues (CC, CXC, CX3C, and C). Chemokines elicit intracellular responses via G protein–coupled receptors. Upon ligand binding, chemokine receptors activate G proteins of the Gαi family, leading to inhibition of adenylyl cyclases and mobilization of Ca2+ from intracellular stores. Furthermore, activated chemokine receptors bind to the scaffolding protein β-arrestin (1–3). Chemokine receptor activation mediates leukocyte chemotaxis toward lymphoid organs or sites of inflammation along a chemokine gradient that is established by binding of chemokines to membrane-tethered and extracellular matrix–associated glycosaminoglycans (GAGs) (4). GAGs represent a heterogeneous population of unbranched polysaccharides, with heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, and hyaluronic acid comprising the largest groups.

An interesting feature of chemokine biology is the ability of distantly related chemokines to enhance each other’s function (5–12). This behavior is referred to as chemokine cooperativity or chemokine synergy, but a clear mechanistic understanding of this phenomenon is lacking. We became interested in chemokine cooperativity while testing the ability of several chemokines to activate the chemokine receptor CCX-CKR. CCX-CKR is an atypical chemokine receptor in that it does not activate conventional G protein–mediated signaling pathways (3, 13, 14), but it does recruit the scaffolding protein β-arrestin2 upon activation by CCL19, CCL21, and CCL25 (3). We noted that several chemokines that did not activate or bind CCX-CKR directly enhanced the potency of CCL19 to activate CCX-CKR. We subsequently observed that multiple chemokine combinations can synergistically enhance the activation of typical (G protein–coupled) chemokine receptors, such as CCR7 and CXCR5. Through analysis of chemokine mutants that are deficient in GAG binding, we discovered that chemokine cooperativity is mediated by competitive binding of chemokines to GAGs.

Materials and Methods

The online version of this article contains supplemental material.

Abbreviations used in this article: BCS, bovine calf serum; CHO, Chinese hamster ovary; EFC, enzyme fragment complementation; GAG, glycosaminoglycan; [125I]-CCL19, [125I]-labeled CCL19; mtCXCL, mutant CXCL; wtCXCL12, wild-type CXCL12.

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Forskolin was from Sigma-Aldrich (Steinheim, Germany). Production of mutant CXCL (mutCXCL)12 (15), mutCXCL11 (16), CXCL12β (17), and CXCL12β (18) has been described previously.

**Cell culture**

The generation of the Chinese hamster ovary (CHO)-CCX-CKR cells has been described previously (3). These cells were maintained in DMEM F12 (PAA Laboratories, Colbe, Germany) supplemented with 10% v/v bovine calf serum (BCS; Hyclone, Logan, UT), 250 μg/ml hygromycin, 800 μg/ml genetin, 100 μM penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA). CHO cells stably expressing the β-arrestin2-EA fusion protein and G protein–coupled receptors C-terminally extended with geneticin, 100 U/ml penicillin, and 100 mg/ml streptomycin. L1.2 cells (American Type Culture Collection) were cultured in RPMI 1640 (Sigma-Aldrich) containing 10% v/v heat-inactivated BCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 2 mM l-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate (all from PAA Laboratories), and 50 μg/m-2 ME (Invitrogen).

**Enzyme fragment complementation assays**

EFC assays were performed, as described previously (3). Briefly, cells were dispensed in a 384-well white CulturPlate (Perkin Elmer) with 1 × 10⁴ cells in 15 μl OPTImem (Invitrogen) containing 1% BCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Following overnight culturing in a humidified incubator (37°C, 5% CO₂, 95% humidity), cooperative and receptor-engaging chemokines were added in 5 μl OPTImem each, followed by 90-min incubation at 37°C. Subsequently, cells were disrupted by addition of 12.5 μl PathHunter Detection Reagent (Discoverx) in the formulation specified by the manufacturer. Following 1-h incubation at room temperature in the dark, luminescence was measured using a Victor3 multilabel plate reader (Perkin Elmer).

**Radioligand binding**

Radioligand-binding experiments were performed, as described previously (3). For saturation-binding experiments, 25 × 10⁴ CHO cells in 100 μl culture medium were dispensed in a poly-L-lysine-coated 96-well culture plate (Greiner) and incubated overnight in a humidified incubator (37°C). Medium was replaced by 50 μl nonradiolabeled chemokine in 20 mM HEPES, 5 mM MgCl₂, 1 mM CaCl₂, and 100 mM NaCl (pH 7.4) at 4°C (assay medium), followed by addition of 50 μl [¹²⁵I]-CCL19 in assay medium. Plates were incubated for 3 h at 4°C and then washed with ice-cold assay medium supplemented with 500 mM NaCl. Cells were disrupted by addition of 150 μl radioluminescence assay buffer (Sigma-Aldrich), and radioactivity in lysates was measured with a Compugamma gamma counter (Perkin Elmer).

**Bare-filter migration (chemotaxis) assays**

A total of 30 μl chemokine in RPMI 1640 (Sigma-Aldrich) containing 0.1% BSA (assay buffer) was added to the lower compartment of a 96-well ChemoTx plate (Greiner) and incubated overnight in a humidified incubator (37°C). Twenty-five microliter assay buffer from the lower well was then transferred to a white 96-well CulturePlate Plate (Perkin Elmer), followed by addition of 25 μl calcein AM (1 μg/ml final concentration) in assay buffer. Fluorescence intensities were measured on a Victor3 plate reader.

**Data analysis**

Sigmoidal dose-response curves were plotted using GraphPad Prism 6.0 software. All data are presented as averages ± SEM. Statistical significance of observed differences was determined using Student’s t test or two-way ANOVA, as indicated in the figure legend and indicated in the figures with asterisks (*). A p value < 0.05 was regarded as statistically significant.

**Results**

**CXCL13 enhances the ability of CCL19, CCL21, and CCL25 to activate CCX-CKR**

Previous studies have suggested that CXCL13 is a ligand for CCX-CKR (13, 19). We therefore tested the ability of this chemokine to activate or inhibit CCX-CKR in CHO cells genetically engineered to express complementary β-galactosidase mutants coupled to CCX-CKR and the scaffolding protein β-arrestin2 (CHO-CCX-CKR cells). The recruitment of β-arrestin2 to activated CCX-CKR enables enzyme fragment complementation (EFC) between the two β-galactosidase mutants, thus allowing measurement of receptor activation by assessing β-galactosidase activity (3, 20). In accordance, when CHO-CCX-CKR cells were treated with CCL19, CCL21, and CCL25, they responded with a concentration-dependent increase in β-galactosidase activity (3) (Fig. 1A). In contrast, CXCL13 did not activate CCX-CKR in these cells (Fig. 1A, filled circles). CXCL13 was also unable to displace radiolabeled CCL19 ([¹²⁵I]-CCL19) from CHO cells expressing CCX-CKR (Supplemental Fig. 1A, filled circles). Thus, CXCL13 is unlikely to be a ligand for CCX-CKR. Nonetheless, we found that CXCL13...

**FIGURE 1.** CXCL13 increases the ability of CCL19 to activate CCX-CKR. (A) CHO cells stably expressing CCX-CKR fused to a peptide fragment of β-galactosidase and β-arrestin2 coupled to a complementary β-galactosidase mutant (CHO-CCX-CKR cells) were treated with increasing concentrations of CCL19 (○) or CXCL13 (●) before measurement of β-galactosidase activity. In addition, CHO-CCX-CKR cells were treated with increasing concentrations of CXCL13 in the presence of 2 nM CCL19 (▲). Asterisks (*) indicate statistically significant differences (two-way ANOVA, p < 0.05) between cells treated with 2 nM CCL19 in the presence and absence of CXCL13. (B) CHO-CCX-CKR cells were treated with increasing concentrations of CCL19 in the presence of 1000 nM CXCL13, 100 nM CXCL13, or corresponding vehicle, followed by measurement of β-galactosidase activity. pEC₅₀ values were as follows; vehicle: 8.6 ± 0.2 (4), 100 nM CXCL13: 9.1 ± 0.2 (4), and 1000 nM CXCL13: 9.4 ± 0.1 (4) (average ± SD [n]). Differences in pEC₅₀ values between vehicle-treated cells and cells treated with either 100 or 1000 nM CXCL13 reached statistical significance (Student’s t test, p < 0.05).
concentration dependently enhanced the potency with which CCL19 activated CCX-CKR (Fig. 1). This effect was not limited to activation of CCX-CKR by CCL19, as similar results were observed in experiments using CCL21 and CCL25 (Supplemental Fig. 1B, 1C).

Secondly, we measured β-arrestin2 recruitment to CCX-CKR using a bioluminescence resonance energy transfer approach (3). For this, we transiently transfected HEK293T cells with vectors encoding CCX-CKR genetically extended with Renilla luciferase and β-arrestin2 fused to yellow fluorescent protein. Similar to results for the β-galactosidase complementation assay, CXCL13 increased the potency with which CCL19 activated CCX-CKR (Supplemental Fig. 1D). Thus, CXCL13 cooperates with CCL19, CCL21, and CCL25 in activating CCX-CKR.

Chemokine cooperativity is specific for certain chemokine combinations

We then tested whether the cooperative effect of CXCL13 was specific for certain chemokine pairs or chemokine receptors. To this end, we used CHO cells that complement β-galactosidase following recruitment of β-arrestin2 to the Goi-coupled chemokine receptors CCR7 (CHO-CCR7), CCR5 (CHO-CCR5), and CXCR5 (CHO-CXCR5). CXCL13 enhanced the potency with which CCL19 induced β-arrestin recruitment and Goi activation by CCR7 in CHO-CCR7 cells (Fig. 2A, 2B). Reciprocally, CCL19 enhanced the potency of CXCL13 toward activation of CXCR5 (Fig. 2C). However, both CCL19 and CXCL13 were incapable of potentiating CCL3-induced activation of CCR5 (Fig. 2D).

Next, a panel of chemokines was tested for their ability to cooperate with CCL19 in activation of CCX-CKR. Several chemokines other than CXCL13, including CXCL12 and CXCL11, were found to cooperate with CCL19, whereas several others (CCL3, CCL4, and CCL23) did not (Fig. 2E). Essentially the same pattern was observed when CCX-CKR was activated with CCL21, as follows: CXCL13, CXCL12, and CXCL11 cooperated with CCL21, whereas CCL3 and CCL4 did not (Supplemental Fig. 2).

CXCL13 enhances CCL19-mediated chemotaxis

Next, we used a transwell migration assay to test whether the enhanced receptor activity translated to a more potent chemotactic

FIGURE 2. Distinct chemokine combinations display cooperativity. (A) CHO-CCR7 cells were treated with increasing concentrations of CCL19 in the presence of 1000 nM CXCL13, 100 nM CXCL13, or corresponding vehicle, followed by measurement of β-galactosidase activity. (B) CHO-CCR7 cells were treated with 0.5 μM forskolin and increasing concentrations of CCL19 in the presence of 1000 or 100 nM CXCL13, followed by measurement of cAMP levels. (C) CHO-CXCR5 cells were treated with increasing concentrations of CXCL13 in the presence of 1000 nM CCL19, 100 nM CCL19, or corresponding vehicle, followed by measurement of β-galactosidase activity. (D) CHO-CXCR5 cells were treated with ascending concentrations of CCL3 and 1000 nM CCL19, 1000 nM CXCL13, or corresponding vehicle, followed by measurement of β-galactosidase activity. (E) CHO-CCX-CKR cells were treated with 2 nM CCL19 in the absence or presence of 1000 nM of the indicated chemokines (x-axis), followed by measurement of β-galactosidase activity. Asterisks (*) indicate statistically significant differences (Student t test, p < 0.05).
response. We used murine L1.2 cells, a pre-B lymphoma cell line, which migrated toward a gradient of CCL19 (Fig. 3A), implying that these cells endogenously express CCR7. However, the L1.2 cells did not migrate toward CXCL13 (Fig. 3A). In accordance with our functional assays, 1 μM CXCL13 significantly potentiated CCL19-mediated chemotaxis in these cells (Fig. 3B).

Chemokine cooperativity is dependent on GAG binding

Our analysis revealed that CCL3 and CCL4 did not induce chemokine cooperativity (Fig. 2E). These chemokines bind to several GAGs with relatively low affinity compared with other chemokines (e.g., CXCL8, CCL2, and CCL5) (21, 22). To investigate the importance of GAG binding for chemokine cooperativity, we tested the ability of GAG-binding–deficient chemokine mutants to cooperate in CCX-CKR activation. Such mutants have been described for CXCL12 and CXCL11 (15, 16, 23). In sharp contrast to wildtype CXCL12 (wtCXCL12), a GAG binding-deficient CXCL12 mutant (CXCL12K24S,H25S,K27S, hereafter referred to as mtCXCL12) did not cooperate with CCL21 in the EFC assay (Fig. 4A). Likewise, we found that mtCXCL12 did not cooperate with CCL21 in activating CCX-CKR in the bioluminescence resonance energy transfer assay (Supplemental Fig. 3). Similarly, a GAG-binding–deficient mtCXCL11 (CXCL11 50s or CXCL11K57A,K59A,R62A) was impaired in its ability to cooperate with CCL21 in activating CCX-CKR (Fig. 4B). Both mtCXCL12 and mtCXCL11 were capable of activating their cognate receptors with potencies and efficacies comparable to wild-type chemokines (Supplemental Fig. 4).

Chemokine cooperation is mediated by both chemokine monomers and dimers

Chemokine heterodimerization has previously been suggested to underlie chemokine cooperativity (10). GAG binding and dimerization of chemokines are intimately linked processes (18, 22). Indeed, the CXCL12-GAG binding interface significantly overlaps with the CXCL12 (homo)dimerization surface, and mtCXCL12 is not only impaired in its ability to bind to GAGs but also in its ability to dimerize (18). To differentiate between dimerization and GAG binding as potential mechanisms for chemokine cooperativity, we used a CXCL12 mutant that forms obligate monomers (termed CXCL121) and a constitutively dimeric mutant of CXCL12 (CXCL122) (17, 18, 24, 25). CXCL121 and CXCL122 are strictly monomeric and dimeric at concentrations up to 10 μM (17, 24). Both mutants bound to heparin, although CXCL121 did so with a ∼10-fold lower potency than wtCXCL12 (17–18). When tested in the EFC assay on CHO-CCX-CKR cells, both CXCL121 and CXCL122 cooperated with CCL21 in the activation of CCX-CKR (Fig. 4C). In conclusion, both monomeric and dimeric CXCL12 proteins can mediate chemokine cooperativity.

Chemokine cooperation is caused by competitive GAG binding

The simplest model that explains the necessity for GAG binding in chemokine cooperativity is one in which the cooperative chemokines displace the receptor-engaging chemokine from binding to GAGs. This scenario would result in elevated concentrations of soluble receptor-engaging chemokines that are available to bind and activate receptors. To test whether CXCL12 and CXCL13 compete with CCL19 for binding to GAGs on the cell surface, we monitored the binding of [125I]-CCL19 to CHO cells. The CHO cells did not express CCX-CKR or CCR7, as determined by RT-PCR (data not shown), whereas they abundantly express GAGs (23, 26). Incubation of CHO cells with [125I]-CCL19 led to a concentration-dependent increase in cell-associated radioactivity, a large portion of which could be competed for by adding excess (1 μM) unlabeled CCL19 (Fig. 5A). wtCXCL12 was also capable of displacing [125I]-CCL19 from CHO cells, whereas mtCXCL12 was not (Fig. 5A). Similarly, CXCL13 displaced 0.5 nM [125I]-CCL19 from both CHO and HEK293T cells, whereas the noncooperative chemokine CCL3 did not affect [125I]-CCL19 binding to these cells (Fig. 5B and data not shown). These data indicate that cooperative chemokines compete with CCL19 for GAG binding on cells, whereas noncooperative chemokines do not (Fig. 6).

Discussion

Chemokine cooperativity has previously been suggested to be caused by convergence of intracellular signaling pathways downstream of receptor activation (5–7, 11, 12). Our data indicate that chemokine cooperativity is not strictly dependent on the receptor of the cooperative chemokine. For example, mtCXCL12 and mtCXCL11 fully activate their respective G protein–coupled receptors (Supplemental Fig. 4) but do not cooperate with CCL21 (Fig. 4). Furthermore, RT-PCR analyses and cAMP measurements indicated that CHO-CCX-CKR cells do not express CXCR5 or CXCR4, the cognate Gαi-coupled receptors for CXCL13 and CXCL12, respectively (data not shown). Our data concur with previous evidence suggesting that activation of the receptor for the cooperative chemokine is not necessary for cooperativity (8, 9).

Alternatively, chemokine cooperativity has been suggested to originate from chemokine heterodimerization (9, 10). Our obser-
In this study, we demonstrate a determining role for GAG binding in chemokine cooperativity. Our data suggest that cooperative chemokines compete for binding to GAGs, thereby raising the effective free concentration of chemokine that can engage its receptor (Fig. 6). Other researchers performed experiments on heparinase-treated cells to test whether GAGs influence chemokine cooperativity and observed no effect of heparinase treatment on chemokine cooperativity (8, 10). Similarly, pretreatment of CHO-CCX-CKR cells with a mixture of heparinases did not influence the ability of CXCL13 to cooperate with CCL19 (data not shown). A flaw in this approach is that GAGs form a large heterogeneous population of molecules, and it is experimentally challenging to ascertain that all of the GAGs relevant to chemokine cooperativity are removed by treatment with heparinase and chondroitinase mixtures. Our analysis with GAG-binding–deficient mutants represents a method of analysis that lacks this pitfall. A remaining challenge will be to deduce which GAG subtypes are actually involved in chemokine cooperativity. Such
FIGURE 6. A model for chemokine cooperativity. (A) In the absence of cooperative chemokines, CCL19/CCL21 binds to its seven-transmembrane receptors (CCX-CKR or CCR7) or to membrane-tethered GAGs. (B) In the presence of cooperative chemokines, CCL19/CCL21 is competed from the GAGs, raising the free concentration of CCL19, which results in enhanced association of CCL19/CCL21 with its receptors and consequent receptor activation.

GAGs may be identified by studying the ability of chemokines to compete for binding on arrays or columns containing immobilized synthetic or purified GAGs (21, 27).

Another outstanding question is whether chemokine cooperativity occurs in vivo. Although systemic, homeostatic levels of CCL19, CCL21, CXCL13, and CXCL12 are generally much lower than the chemokine concentrations required to induce chemokine cooperativity in our functional assays, local concentrations of chemokine may well exceed the concentrations necessary for cooperativity to occur. Of note, CCL19, CCL21, CXCL12, and CXCL13 have exceptionally broad and overlapping expression patterns, which would provide many potential opportunities for synergistic interactions. For instance, CCL19, CCL21, CXCL12, and CXCL13 are expressed on the luminal side of high endothelial venules (28), and CCL21, CXCL12, and CXCL13 are expressed in lymph nodes (29–31). Indeed, CXCL12 has been shown to enhance the CCR7-mediated recruitment of naive T cells from high endothelial venules into the underlying lymphoid tissue (5). Furthermore, CCL19, CCL21, CXCL12, and CXCL13 are expressed locally during chronic inflammation (32–34), and individual chemokine concentrations within these inflamed sites may very well exceed the threshold for cooperativity to occur. Additionally, previous work has suggested that chemokine mixtures can concertedly induce cooperativity at low individual chemokine concentrations (10). Because multiple chemokines are expressed concomitantly during inflammation and homeostatic trafficking (35–37), the concentration of several cooperative chemokines combined may be sufficient for chemokine cooperativity to occur.

How would chemokine cooperativity influence chemokine function, and how will chemokine cooperativity be altered by (patho)physiological processes? Because chemokine cooperativity would allow chemokines to activate their cognate receptors at lower chemokine concentrations, it is likely to extend the range from which chemokines can induce recruitment of leukocytes. Another implication from our data is that alterations in GAG composition or abundance, as have been observed to occur in several pathologies (38–41), might alter chemokine cooperativity. Lastly, our data present a new perspective on therapeutic targeting of the chemokine system. Through modulation of cooperativity, therapeutic strategies aimed at inhibiting chemokine–GAG interactions might establish a broader effect on leukocyte trafficking than targeting chemokine receptors. For instance, chemokine cooperativity is likely to be modulated by administration of heparin or GAG derivatives, which have previously been shown to affect chemokine gradient formation (42, 43). Furthermore, small molecules or Abs targeting chemokines, such as those described for CXCL12 (44–46), may have applicability as cooperativity-disrupting drugs. Future endeavors will have to elucidate how chemokine cooperativity is modulated in such instances.

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