Short Conceptual Overview

Takahiko Hara* and Kosuke Tanegashima

CXCL14 antagonizes the CXCL12-CXCR4 signaling axis

Abstract: CXCL12 and CXCL14 are evolutionarily conserved members of the CXC-type chemokine family. CXCL12 binds specifically to the G-protein-coupled receptor CXCR4 to induce the migration of primordial germ cells, hematopoietic stem cells, and inflammation-associated immune cells. In addition, CXCL12-CXCR4 signaling is often enhanced in malignant tumor cells and facilitates increased proliferation as well as metastasis. Although macrophage migration inhibitory factor and extracellular ubiquitin interact with CXCR4 as agonistic factors, CXCL12 was believed to be the sole chemokine ligand for CXCR4. However, a very recent report revealed that CXCL14 binds to CXCR4 with high affinity and efficiently inhibits CXCL12-mediated chemotaxis of hematopoietic progenitor and leukemia-derived cells. CXCL14 does not directly cross-compete with CXCL12 for the CXCR4 binding but instead inactivates CXCR4 *via* receptor internalization. Because both CXCL12 and CXCL14 are expressed during embryogenesis and brain development in mice, these two chemokines could function in an interactive fashion. We propose that the CXCL14 gene has been conserved from fish to man due to its role in fine-tuning the strength of CXCL12-mediated signal transduction. In addition to its biological implications, the above finding will be important for designing anti-cancer compounds targeting the CXCL12-CXCR4 signaling axis. In fact, a stabilized dimeric peptide containing the C-terminal 51–77 amino acid residues of CXCL14 has been shown to have stronger CXCL12 antagonistic activity than full-length CXCL14.

Keywords: chemokine; chemokine receptor; CXCL12; CXCL14; CXCR4.

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Abbreviations: GPCRs, G-protein-coupled receptors; iDCs, immature dendritic cells; NK, natural killer; MIF, macrophage migration inhibitory factor; HMGB1, high mobility group box1; CXCL14-KO, CXCL14-knockout.

Introduction

Chemokines are critical regulators of cell migration in development, homeostasis, and immune surveillance (1, 2). Two major chemokine classes, CC and CXC, are defined by the spacing of two N-terminal cysteine residues, which are either adjacent to each other or separated by one amino acid residue, respectively. In humans, there are at least 44 chemokine ligands that bind to 23 G-protein-coupled receptors (GPCRs) (3). Among the 17 known CXC-type chemokines, CXCL12 [also known as stromal cell-derived factor 1α (SDF-1α)] and CXCL14 (originally designated BRAK) are considered to be primordial chemokines based on their strong sequence conservation throughout evolution and their roles in homeostasis (4, 5). CXCL14 is composed of 77 amino acid residues with a molecular weight of 9.4 kDa, whereas CXCL12 is a 9-kDa protein of 75 amino acid residues. CXCL14 has been implicated in the mobilization of tissue macrophages/immature dendritic cells (iDCs)/natural killer (NK) cells, tumor suppression, and metabolic regulation (6). Although a large number of studies have investigated the structure and functions of the CXCL12 receptor (7–9), the nature of the CXCL14 receptor is not fully understood. Recently, we demonstrated that CXCL14 interacts with CXCR4 and blocks CXCL12-mediated cell migration (10). In this review, we describe the mechanism by which CXCL14 inhibits the CXCL12-CXCR4 signaling axis and discuss the physiological relevance of this antagonistic function of CXCL14.

CXCL12 receptors

CXCL12 is known to utilize CXCR4 and CXCR7 as functional receptors (5). CXCR4 plays a central role in

CXCL12-mediated cell migration, whereas CXCR7 mainly acts as a ligand-scavenging receptor, by which a sink of CXCL12 is locally generated (11–14). Because of this unique function, CXCR7 was recently designated ACKR3 (atypical chemokine receptor 3) (15). Interaction of CXCL12 with CXCR4 modulates the migration of primordial germ cells and neural progenitors in developing embryos, the bone marrow homing of hematopoietic stem cells, and the trafficking of a wide variety of immune cells, including T cells, pre-B cells, iDCs, and endothelial cells (7). Importantly, the CXCL12-CXCR4 signaling pathway has been shown to contribute to the malignant growth and metastasis of more than 20 different tumor cells derived from multiple tissue types, including lung, breast, prostate, and colon (2, 16, 17). Elevated levels of CXCL12 and its receptors are associated with poor prognosis in these cancers. These facts highlight the importance of gaining an accurate understanding how the CXCL12-CXCR4 axis is regulated.

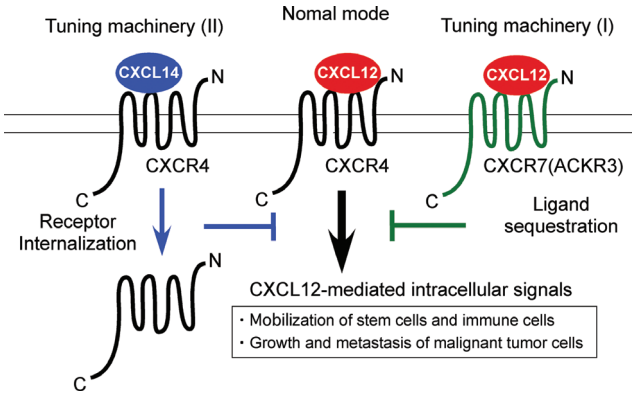

Figure 1 Proposed mechanism of CXCL14-mediated inhibition of the CXCL12-CXCR4 signaling axis.
The strength of CXCL12-CXCR4-mediated signaling can be modulated by sequestering the receptor CXCR7 or by adding the non-signaling ligand CXCL14.

CXCL12-interacting proteins and CXCR4 ligands

Since the discovery that CXCR4 is the main CXCL12 receptor, several other proteins have been found to interact with CXCR4 or CXCL12. First, CXCR7 binds CXCL12 with high affinity and acts as a decoy receptor to decrease the availability of CXCL12 (Figure 1), although the growth of certain tumor cells is stimulated by CXCL12 *via* CXCR7 (18, 19).

Previous studies revealed that macrophage migration inhibitory factor (MIF) and extracellular ubiquitin bind specifically to CXCR4 and induce cell migration (20–22). MIF is capable of chemoattracting primary T cells in a CXCR4-dependent manner; however, a 10-fold higher concentration of MIF was required to displace CXCL12 bound to CXCR4. Interestingly, MIF can also interact with CXCR2 (20). Therefore, MIF appears to act as a backup ligand for CXCR4 in the migration of immune cells to the site of inflammation.

In the case of ubiquitin, its binding affinity for CXCR4 is closer to that of CXCL12 (21). The regions at which ubiquitin and CXCL12 interact with CXCR4 appear to partially overlap and correspond to the extracellular loops 2/3 (22). In addition, ubiquitin-mediated chemotaxis was sensitive to treatment with AMD3100, a well-known CXCR antagonist (23). Nonetheless, the chemotactic activity of ubiquitin was weaker than that of CXCL12, and ubiquitin was unable to block CXCL12-mediated cell migration. Thus, the physiological role of ubiquitin-triggered CXCR4 activation remains to be determined.

Lastly, it was reported that high mobility group box1 (HMGB1) is associated with CXCL12, but not with CXCR4 (24). HMGB1 is a small nuclear protein released from necrotic or severely stressed cells. An HMGB1-CXCL12 heterocomplex is formed in the presence of subnanomolar concentrations of CXCL12 and strongly stimulates the migration of monocytic cells. Binding of the HMGB1-CXCL12 complex to CXCR4 triggers a conformational change distinct from that observed after the binding of CXCL12 alone. Therefore, HMGB1 plays an important role in the rapid recruitment of inflammatory cells to injured tissues.

CXCL14-CXCR4 interaction

In addition to the above molecules, we recently discovered that CXCL14 binds specifically to CXCR4 with high affinity (10). Although the chemotactic activity of CXCL14 is weak in human monocytic leukemia-derived THP-1 cells (25), CXCL14 strongly inhibited CXCL12-mediated chemotaxis of THP-1 cells and human bone marrow $CD34^+$ hematopoietic progenitor cells (10).

This raised the question of how CXCL14 inhibits CXCL12-mediated signal transduction. Heterodimerization between CXCL12 and CXCL14 was disproved experimentally (26). It is also unlikely that they utilize common binding sites on CXCR4 based on the fact that the N-terminal region of CXCL12 is essential for the interaction with transmembrane helices of CXCR4 to trigger the conformational change (27), and CXCL14 is devoid of the corresponding N-terminal region (28). In addition, biologically

active concentrations (~100 nM) of CXCL14 did not cross-compete the high-affinity binding of ^{125}I-CXCL12 to CXCR4 in THP-1 cells (26), nor was high-affinity binding of ^{125}I-CXCL14 to CXCR4 inhibited by CXCL12 (10). Finally, AMD3100 did not interfere with CXCL14-triggered chemotaxis of THP-1 cells (K.T. and T.H., unpublished data).

CXCR4 and other chemokine receptors are known to be internalized after chemokine stimulation (29). We proposed that the internalization of CXCR4 is the molecular mechanism responsible for the inhibitory action of CXCL14 (26) (Figure 1). CXCL14-bound CXCR4 was internalized within 15 min, and ERK1/2 kinases were no longer activated by CXCL12 in these cells (26). The downstream processes activated by the CXCL14-CXCR4 interaction are apparently different from those of CXCL12-CXCR4.

CXCL14-CXCR4 interaction in development and homeostasis

Among all chemokine genes, only four (*CXCL8*, *CXCL12*, *CXCL13*, and *CXCL14*) are strongly conserved as far back as the elephant shark (5). Interestingly, *CXCR4* and *CXCR7* are also conserved from elephant shark to man. Because CXCL14 and CXCR7 play inhibitory roles in the CXCL12-CXCR4 signaling axis, they may also play important roles in fine-tuning its function (Figure 1). Although the *in vivo* roles of CXCR7 have been extensively studied (11, 13, 14, 30, 31), it remains unclear at which point CXCL14 modulates the CXCL12-CXCR4 axis in physiological settings.

Cxcl14 and *Cxcl12* mRNAs are co-expressed in early embryos and the fetal brain in chick and carp (4, 32). In the adult mouse brain, *Cxcl14* is specifically expressed in GABAergic interneurons in the dentate gyrus (33). CXCL14 was shown to inhibit the tonic and phasic effects of synaptically released GABA in neural progenitor cells (34). In sharp contrast, CXCL12 enhanced GABAergic transmission in these same cells. Because GABAergic interneurons play pivotal roles in postnatal neurogenesis, it will be important to elucidate the underlying molecular mechanism of these opposing effects of CXCL14 and CXCL12.

With regard to the neural function of CXCL14, CXCL14-knockout (CXCL14-KO) mice display behavioral abnormalities, such as a reduction of food intake without increased energy expenditure, which are established during the postnatal stage (35). Fasting-induced upregulation of the appetite-related genes *Npy* and *Agrp* was impaired in the hypothalami of CXCL14-KO mice. In addition, they took longer to start eating when placed in a novel environment. As *Cxcl14* is expressed not only in the hypothalamus but also in the cortex and hippocampus, CXCL14 could be required for the establishment of neural circuits that are closely linked with feeding behavior.

In adult mice, basal epidermal keratinocytes and squamous epithelial cells produce abundant amounts of *Cxcl14* mRNA (36, 37). Although many chemokines are involved in the initiation and amplification of atopic skin inflammation, *Cxcl14* expression in keratinocytes is suppressed in atopic dermatitis and psoriasis (38). Unexpectedly, the total numbers of macrophages and dendritic cells in the epidermis were not significantly different between CXCL14-KO mice and wild-type mice (39). Although steady-state immunity appeared to be normal in CXCL14-transgenic mice, they exhibited a higher incidence of collagen-induced arthritis due to an enhanced Th1 response and autoantibody production (40). Thus, we must be careful in defining the immune functions of CXCL14.

CXCL14-CXCR4 interaction in cancer

The involvement of CXCL14 in tumorigenesis was first suspected when *CXCL14* mRNA expression was shown to be repressed in various malignant cancer tissue samples and several tumor cell lines (36, 41, 42). Recent studies also demonstrated that *CXCL14* is epigenetically silenced in lung adenocarcinoma and prostate cancer-derived cells (43, 44). Moreover, CXCL14 protein is rapidly degraded by the ubiquitin-proteasome system in several cancer cell lines (28). The unique amino acid sequence of CXCL14, Val^{41}-Ser-Arg-Tyr-Arg^{45}, is a target site for this degradation. Taken together, these findings show that the expression of CXCL14 is negatively regulated at transcriptional and post-translational levels in cancer cells.

When a CXCL14 expression vector was stably introduced into head-and-neck carcinoma-derived HSC-3 and lung carcinoma cell-derived H23 cell lines of human origin, the tumor-forming activities of these cells in immunodeficient mice were dramatically suppressed (43, 45). The growth of mouse melanoma and lung carcinoma-derived cells was also impaired in CXCL14-transgenic mice (46). These experimental data suggest a tumor-suppressive function for CXCL14. There are three potential mechanisms by which CXCL14 may act as a tumor suppressor. First, CXCL14 may strengthen anti-tumor immune surveillance by recruiting activated NK cells (47). Second, CXCL14 is a potent inhibitor of angiogenesis mediated by vascular endothelial growth factor (48), which is required for neovascularization within solid tumors. Third, CXCL14 can weaken the CXCL12-mediated growth of malignant tumor

cells by inducing CXCR4 internalization (26). As *CXCL14* mRNA is strongly expressed in inflammatory and stromal cells adjacent to tongue carcinomas and prostate tumors (36, 49), all of these possibilities are theoretically valid. It is of note that *CXCL14* mRNA expression is induced by gefitinib, a powerful anti-cancer drug that targets the EGF receptor (50).

In contrast to the tumor-suppressive role of CXCL14, expression levels of *CXCL14* in prostate and pancreatic cancers were higher than in respective normal tissues (51, 52). In fact, breast cancer cells acquired invasive characteristics associated with ROS-mediated upregulation of CXCL14 (53), and *CXCL14* was shown to promote the growth and invasiveness of breast and pancreatic cancer cells (52–54). Moreover, NIH3T3 cells expressing *CXCL14* effectively enhanced the growth and migration of the prostate cancer cell line LNCap by secreting secondary cytokines and chemoattractants (49). It remains to be determined whether CXCR4 is involved in these pro-tumor functions of CXCL14.

Chemical modification of CXCL14

CXCL14 represents a potential strategy for the pharmacological inhibition of the CXCL12-CXCR4 axis, especially for anti-cancer therapy. AMD3100 is one of the most specific and efficient inhibitors available for disrupting the interaction between CXCL12 and CXCR4 (55). However, because CXCL12 is essential for the homing of hematopoietic stem cells to the bone marrow niche, treatment with AMD3100 results in bone marrow failure. CXCL14 or its derivatives might have some advantages over preexisting CXCL12 inhibitors because they are tethered in the extracellular matrix, and thereby not systemically effective.

Using a deletion mutant of CXCL14 and a CXCL14-CXCL12 chimeric peptide, we found that the C-terminal 51–77 amino acid residues of CXCL14 are required for the inhibition of CXCL12-mediated chemotaxis and CXCR4 binding (26) (Figure 2). Although the CXCL14-CXCL12 chimeric peptide did not compete with ^{125}I-CXCL14 for CXCR4 binding, it inhibited CXCL12-mediated cell migration and displayed chemotactic activity (K.T. and T.H., unpublished data). Presumably, the CXCL12-derived C-terminal region in this artificial peptide interacts with CXCR4 to induce these effects.

To create a better CXCL12 inhibitor, it is ideal to eliminate the chemokine function of CXCL14. We engineered various peptides of the C-terminal 51–77 region of CXCL14 (CXCL14-C) and used an activating anti-CXCL14 monoclonal antibody (MAB730)-assisted chemotaxis assay of

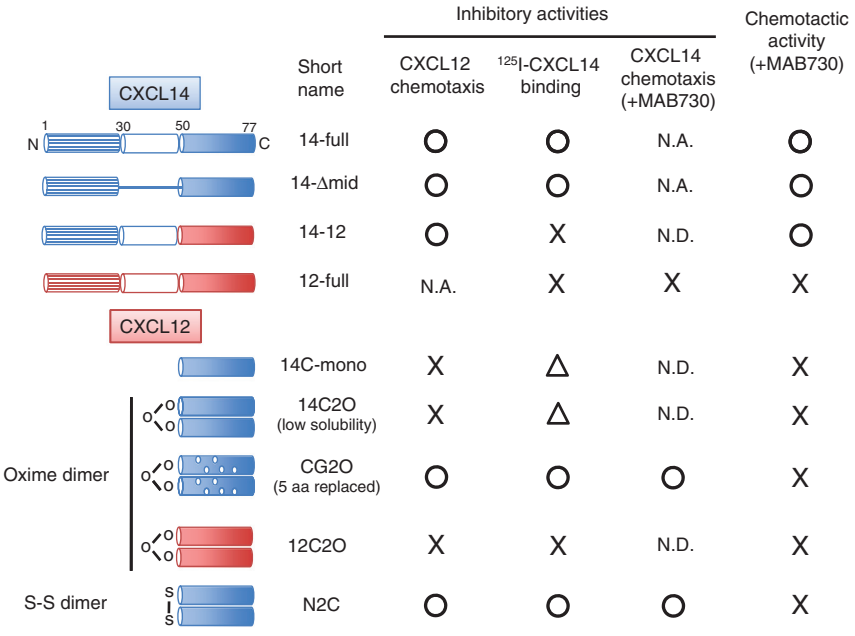

Figure 2 Structure-function relationship of the CXCL14 derivatives.
We analyzed the inhibitory activities of the indicated CXCL14-derived synthetic peptides on CXCL12-mediated chemotaxis, the binding of iodinated CXCL14, and MAB730-assisted CXCL14-mediated chemotaxis. In addition, the chemotactic activity of each peptide was examined in the presence of MAB730. All experiments were carried out using CXCR4-transfected THP-1 cells. 14C2O and 12C2O are C-terminal oxime dimer peptides of CXCL14 and CXCL12, respectively. CG2O and N2C correspond to oxdmCXCL14-Cc/g and ssdmCXCL14-Cc/g, respectively, as described in the previous report (26). N.A., not applicable; N.D., not determined.

THP-1 cells to evaluate their chemokine activity (25). As summarized in Figure 2, CXCL12-inhibitory activity was present in N2C (ssdmCXCL14-C), an S-S dimer peptide of CXCL14-C. In addition, CG2O (oxdmCXCL14-Cc/g), an oxime-linked dimer peptide of the CXCL14-C, was shown to be even more potent than full-length CXCL14 based on IC_{50} values (4.9 vs. 11.5 nM) determined in an ^{125}I-CXCL14 binding inhibition assay (26). Importantly, neither N2C nor CG2O exhibit chemotactic activity by themselves. Although *in vivo* evaluation of their effectiveness will be necessary, this CXCL14-based molecular approach is a feasible method for developing new inhibitors of the CXCL12-CXCR4 axis. Besides, CXCL14-derived peptides could exhibit synergistic effects when combined with preexisting CXCL12 inhibitors because CXCL14 has a distinct binding site on CXCR4 from CXCL12.

Remaining questions

CXCL14 binds to CXCR4 with high affinity and inhibits the CXCL12-triggered activation of CXCR4. However, CXCR4 alone is not sufficient to drive CXCL14-mediated signaling because normal T cells and T cell leukemia-derived Jurkat cells, both of which express CXCR4 on cell surface, did not respond to CXCL14 in the MAB730-assisted chemotaxis assay (K.T. and T.H., unpublished data). Thus, the question remains as to how CXCL14 exerts biological activities, such as chemoattraction of iDCs/NK cells and metabolic regulation. Further studies will be necessary to identify additional receptor(s) or signal transducer(s) that work together with CXCR4 to uncover hidden regulators of the evolutionally conserved chemokine system.

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