Cutting Edge: Species Specificity of the CC Chemokine 6Ckine Signaling Through the CXC Chemokine Receptor CXCR3: Human 6Ckine Is Not A Ligand for the Human or Mouse CXCR3 Receptors

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*J Immunol* 1999; 162:3765-3769;
http://www.jimmunol.org/content/162/7/3765
The CC chemokine known as 6Ckine (SLC, Exodus-2, or TCA4) has been identified as a ligand for CCR7. Mouse 6Ckine has also been shown to signal through mouse CXCR3 and share some of the activities of IFN-\(\gamma\) inducible protein 10 and monokine induced by IFN-\(\gamma\). Nonetheless, human 6Ckine has not been shown to bind CXCR3 receptor or have angiostatic activity. In this study, we report that human 6Ckine does not induce a calcium flux in either human CXCR3 or mouse CXCR3 transfected cells, although it is an equally potent agonist as mouse 6Ckine and human macrophage inflammatory protein-3\(\beta\) in human CCR7 transfected cells. Mouse 6Ckine (but not human 6Ckine) is capable of competing with radio labeled IFN-\(\gamma\) inducible protein 10 for human CXCR3. In addition, radiolabeled human 6Ckine does not bind to either human CXCR3 or mouse CXCR3. Together these data suggest that human CC chemokine 6Ckine is not a ligand for the human or mouse CXC chemokine receptor CXCR3.


Chemokines constitute a group of small cytokines with common structural features that direct selective leukocyte migration and activation to inflammatory stimulus (1–4). Based on the number and arrangement of their conserved cysteines, chemokines can be divided into four classes. The CXC, CC, and CX3C groups have four conserved cysteines, whereas C chemokine (lymphotactin) has only two. CXC chemokines with the Glu-Leu-Arg (ELR)\(^2\) motif immediately before the CXC motif are potent chemoattractants for neutrophils, whereas those without the ELR motif are mostly directed to lymphocytes. Two of non-ELR CXC chemokines, IFN-\(\gamma\) inducible protein 10 (IP-10) and monokine induced by IFN-\(\gamma\) (MIG), were initially identified because of their dramatically enhanced expression in monocytes activated by IFN-\(\gamma\) or LPS (5). IP-10 and MIG have also been shown to be strongly angiostatic (6). Recently, CXCR3 was identified to be the receptor for IP-10 and MIG (7). By Northern blot analysis, the RNA for CXCR3 is highly restricted to activated T cells and NK cells. Therefore, IP-10 or MIG signaling appears to be an important mechanism for selective homing of activated/effector cells, which are known to accumulate preferentially at inflammatory sites (8), as well as in many tumors.

A novel CC chemokine, 6Ckine, with an unusual pattern of six conserved cysteines was identified using bioinformatics-based searches. Both mouse and human 6Ckine were cloned and are conserved between species (9). Recombinant mouse 6Ckine was expressed and shown to be chemotactic in vitro for thymocytes and activated T cells. Furthermore, mouse 6Ckine was shown to induce a calcium flux in mouse CXCR3-transfected cells, which was reported as an example of a CC chemokine binding to a CXC chemokine receptor (10). More importantly, mouse 6Ckine was also shown to have angiostatic activity, like IP-10 and MIG (known ligands for CXCR3 receptor). Nonetheless, human 6Ckine has not been shown to bind either human or mouse CXCR3 receptors.

With the interest in investigating the ligands that bind to and signal through human CXCR3, we have developed 293EBNA transfecants expressing high level of human CXCR3. Surprisingly, during testing of CXC and CC chemokines we have found and present here that human 6Ckine does not induce a calcium flux in human CXCR3 or mouse CXCR3 transfecants. It has little or no binding to human CXCR3 receptor from cross-competition.

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Received for publication November 10, 1998. Accepted for publication January 25, 1999.

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2 Abbreviations used in this paper: ELR, Glu-Leu-Arg; IP-10, IFN-\(\gamma\) inducible protein 10; MIG, monokine induced by IFN-\(\gamma\); MIP3\(\beta\), macrophage inflammatory protein-3\(\beta\).
with IP-10, although mouse 6Ckine has modest affinity. More convincingly, radiolabeled human 6Ckine does not bind to either human CXCR3 or mouse CXCR3 transfectants, although this radiolabeled chemokine binds its known receptor, human CCR7. Thus, 6Ckine may be an example of a chemokine whose receptor utilization exhibits species specificity, i.e., that mouse 6Ckine signals through CCR7 and CXCR3 but human 6Ckine binds to and signals solely through CCR7.

Materials and Methods

Materials

All chemokines except human interferon-inducible T cell α chemoattractant (I-TAC) (from Peprotech, Rocky Hill, NJ) were obtained from R & D Systems (Minneapolis, MN). Human 6Ckine was also obtained from two other suppliers: Research Diagnostics (Flanders, NJ) and Peprotech. Radiolabeled IP-10 was from NEN Life Science Products (Boston, MA).

Cloning of human CXCR3

The cDNA encoding human CXCR3 was cloned by PCR using leukocyte cDNA (Clontech, Palo Alto, CA) as a template. The PCR primers were designed based on the published sequence (7) with incorporated restriction sites and a Kozak consensus sequence. The PCR product was digested with Kpn1 and Not1 and ligated into similarly digested and linearized mammalian expression vector pCEP4 (Invitrogen, Carlsbad, CA). The nucleotide sequences of human CXCR3 were confirmed by automated sequencing using an Applied Biosystems 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Expression of human CXCR3 in 293EBNA cells

293EBNA cells (Invitrogen) were transfected with 10 μg of DNA using a standard calcium phosphate procedure (Stratagene, La Jolla, CA) according manufacturer’s recommendation. The transfected cells were under selection with 300 μg/ml hygromycin (Boehringer Mannheim, Indianapolis, IN). After 7 days, the surviving colonies were pooled and continued with hygromycin selection. CXCR3 expression was verified by specific binding of radiolabeled IP-10. Fourteen days after the selection, the population was confirmed to be homogeneous by FAC5 using anti-human CXCR3 mAb (R & D Systems). For comparison, 293 cells expressing mouse CXCR3 were obtained from Soto et al. (10).

Radiolabeled IP-10 binding assay

293EBNA cells (200,000 cells/point) expressing human CXCR3 were resuspended in binding buffer (50 mM HEPES, 1 mM CaCl2, 5 mM MgCl2, 0.5% BSA) and incubated with 0.1 nM of radiolabeled human IP-10 in the presence of unlabeled chemokines in 96-well plates. After 2 h at room temperature, plates were spun down and cell pellets were resuspended in binding buffer supplemented with 0.5 M NaCl. Cells were spun through 10% glycerol in binding buffer with 0.5 M NaCl. Cell pellets were frozen in liquid nitrogen, clipped, and counted.

Radiolabeled human 6Ckine binding assay

Radiolabeled human 6Ckine binding assay was performed similarly as described above for radiolabeled IP-10 binding assay with the following modification. A total of 400,000 cells/point were resuspended in binding buffer supernatant of unlabeled chemokines in 96-well plates. After 2 h at room temperature, plates were spun down and cell pellets were resuspended in binding buffer supplemented with 0.5 M NaCl. Cells were spun through 10% glycerol in binding buffer with 0.5 M NaCl. Cell pellets were frozen in liquid nitrogen, clipped, and counted.

Measuring intracellular calcium using the FLIPR system

Parental 293EBNA cells and 293EBNA cells stably transfected with either human CXCR3 or human CCR7 were plated at a density of 80,000 cells per well in 96-well plates 24 h before assay. At the day of the assay, cells were loaded with the dye Fluo-3 in growth media and incubated for 1 h at 37°C. Cells were washed three to four times with HBSS supplemented with 20 mM HEPES and 1% BSA (FLIPR buffer; Molecular Devices, Palo Alto, CA) and left in a final volume of 100 μl of the same solution. A separate plate containing the chemokines at 3 x concentrations in FLIPR buffer was put in one chamber of the FLIPR. All FLIPR experiments were conducted with the following conditions: chamber temperature, 37°C; laser power, 300–500 mW; exposure of 0.4 s; sample addition after data point ten. Data
points were collected as follow: 60 samples at 1-s intervals followed by 20 samples at 6-s intervals.

**Results and Discussion**

To characterize the human CXCR3 receptor, we have engineered a 293EBNA transfectant expressing a high level of human CXCR3. Initially, stable expression of human CXCR3 in 293EBNA cells was verified by obtaining specific binding of radiolabeled IP-10; untransfected 293EBNA cells do not bind radiolabeled IP-10. Subsequently, the expression and homogeneity of the 293EBNA transfectant pool expressing human CXCR3 were confirmed by FACS. The human CXCR3 transfectant was estimated to express 100,000 receptors per cell with an affinity for radiolabeled IP-10 of about 1–2 nM from Scatchard analysis of a saturation binding experiment.

Because IP-10, MIG, and mouse 6Ckine were identified as ligands for CXCR3 by determining their abilities to induce calcium flux, we examined the ability of these CC and CXC chemokines to mobilize calcium in our human CXCR3 transfectant pool. Untransfected 293EBNA cells do not respond to any of chemokines tested in this experiment. Fig. 1 shows that both human IP-10 and MIG induced a calcium flux in a dose-dependent manner through human CXCR3, with significant flux detected as low as 100 pM for IP-10. Mouse 6Ckine weakly induced a calcium flux detectable at 10 nM chemokine. Recombinant human I-TAC, which has become available recently, was confirmed to be a functional ligand (data not shown) as reported (11). Unexpectedly, repeated experimentation has revealed that human 6Ckine does not induce flux through human CXCR3 up to 100 nM. The same results were observed when using human 6Ckine obtained from three commercial suppliers. In addition, human 6Ckine from one major supplier was confirmed to be authentic by N-terminal protein sequencing.

It has been reported by Soto et al. (10) that mouse 6Ckine signals through mouse CXCR3 receptor. However, the activity of human 6Ckine was not examined in this report. To obtain a clear picture of the species specificity of 6Ckine interaction with CXCR3, we compared our human CXCR3 transfectant directly with 293 cells expressing mouse CXCR3 (obtained from Soto et al.) by calcium flux stimulated with human and mouse IP-10, MIG, and 6Ckine. First, Fig. 2A shows that mouse MIG, mouse IP-10, and mouse 6Ckine signaled through mouse CXCR3 receptor, which is consistent with the previous finding by Soto et al. (10). Second, both human and mouse CXCR3 receptors did not respond to human 6Ckine, although they did respond to mouse 6Ckine, as shown in Fig. 2, A and B. Third, we have also observed that human IP-10 is a more potent ligand than human MIG, while mouse MIG is a more potent ligand than mouse IP-10 (CRG-2). This may lead to new information about differences in ligand usage for the CXCR3 receptor in mouse vs human cells.

One possibility to explain our unexpected results for human 6Ckine is that human 6Ckine proteins we used from all three different suppliers were not active. Because human macrophage inflammatory protein-3β (MIP3β) as well as 6Ckine were reported to be essentially equivalent in terms of cross-desensitization in calcium mobilization with human CCR7 and cross-competition in binding to CCR7 (12), we examined the ability of these ligands to mobilize calcium in our human CXCR3 transfectant and 293EBNA cells transfected with human CCR7 receptor. As illustrated in Fig. 3, both human MIP3β and 6Ckines (mouse and human) induced similar calcium fluxes through CCR7 in a dose-dependent manner, with detectable flux at 100 pM chemokine. Note that the concentration of mouse 6Ckine needed to induce detectable calcium flux in human CXCR3 transfectants was at least two orders of magnitude higher than in human CCR7 transfectants.

One other possibility for this intriguing result is that human 6Ckine might bind to human CXCR3 but not induce signaling (such as calcium flux). In fact, such results were previously reported for several chemokines, including eotaxin, which could serve as a CXCR3 antagonist (13). The human CXCR3 transfectant was assessed by competition of various chemokines against...
radiolabeled IP-10. Fig. 4 shows that human 6Ckine has no ability to compete with IP-10 for human CXCR3 up to 1000 nM, although mouse 6Ckine does compete with a lower affinity than IP-10 or MIG, which is consistent with our calcium flux data.

To prove human 6Ckine does not bind to human CXCR3 or mouse CXCR3 directly, we used radiolabeled human 6Ckine (from both NEN and Amersham) to perform receptor binding assays in both human CXCR3 and mouse CXCR3 transfected cells. Parental 293EBNA and human CCR7 transfected cells were used as controls. Fig. 5 shows that radiolabeled human 6Ckine does not bind to either human CXCR3 or mouse CXCR3 transfected cells, nor does it bind to parental 293EBNA cells, although the radiolabeled protein does bind to human CCR7 transfected cells as expected. Although calcium agonist activity on CXCR3 transfected cells coupled with binding competition studies using radiolabeled IP-10 provide compelling evidence that mouse 6Ckine can bind to and signal through both mouse and human CXCR3, absolute confirmation of binding will await direct binding determinations with radiolabeled mouse chemokine.

Taken together, the binding competition experiments, calcium flux data, and direct receptor binding results with radiolabeled human 6Ckine suggest that although the mouse 6Ckine has been
Nonetheless, we cannot assume that the function of chemokines these mouse and human chemokines remain to be discovered. There are no known human homologues (e.g., MCP-5, C10, protein (MCP)-2, and MCP-4); there are mouse chemokines for known mouse homologues (e.g., IL-8, monocyte chemoattractant protein (MCP)-2, and MCP-4). It is possible that more functional homologues of the mouse and human CXCR3 proteins are 86% identical overall and most of the differences are found at the aminoterminus. Further experimentation in swapping human and mouse CXCR3 domains is needed to prove that the differences in the sequences of the ligands may play a role in determining their binding specificities to CXCR3 receptor.

Moreover, there are human chemokines for which there are no known mouse homologues (e.g., IL-8, monocyte chemoattractant protein (MCP)-2, and MCP-4); there are mouse chemokines for which there are no known human homologues (e.g., MCP-5, C10, and MIP-1γ). It is possible that more functional homologues of these mouse and human chemokines remain to be discovered. Nonetheless, we cannot assume that the function of chemokines identified so far will cross species barriers. The potential lack of correlation between some mouse and human chemokines, and the first report here of apparently homologous chemokines utilizing different receptors in mouse and humans, raises a note of caution for predicting human biology from data obtained for chemokines and their receptors in the mouse.

Acknowledgments

We thank Drs. A. Zlotnik and L. Lanier (DNAX, Palo Alto, CA) as well as Dr. J. Hedrick (Schering-Plough Research Institute, Kenilworth, NJ) for helpful discussions. We also thank Drs. H. Soto and A. Zlotnik for providing 293 cells expressing mouse CXCR3 receptor.

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