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Cutting Edge: Activity of Human Adult Microglia in Response to CC Chemokine Ligand 21

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The approximately 50 known chemokines are classified in distinct subfamilies: CXC, CC, CX3C, and C. Although the signaling of chemokines often is promiscuous, signaling events between members of these distinct chemokine classes are hardly observed. The only known exception so far is the murine CC chemokine ligand (CCL)21 (secondary lymphoid tissue chemokine, Exodus-2, 6Ckine), which binds and activates the murine CXC chemokine receptor CXCR3. However, this exception has not been found in humans. In this study, we provide evidence that human CCL21 is a functional ligand for endogenously expressed CXCR3 in human adult microglia. In absence of CCR7 expression, CCL21 induced chemotaxis of human microglia with efficiency similar to the CXCR3 ligands CXC chemokine ligand 9 (monokine induced by IFN-γ) and CXC chemokine ligand 10 (IFN-γ-inducible protein-10). Because human CCL21 did not show any effects in CXCR3-transfected HEK293 cells, it is indicated that CXCR3 signaling depends on the cellular background in which the CXCR3 is expressed. The Journal of Immunology, 2004, 172: 2744–2747.

In the peripheral immune system chemokines regulate migration of leukocytes expressing specific chemokine receptors (1). Based on conserved cysteine residues, chemokines and their receptors are subdivided into four families: CXC, CC, C, and CX3C chemokines (2). Chemokine signaling can be promiscuous and a variety of chemokines activate more than one chemokine receptor. However, this promiscuity is only observed within chemokine families, signaling between members of different chemokine families is no common phenomenon (3, 4). The only known exception so far concerns CC chemokine ligand (CCL)3 21 that binds and activates mouse CXCR3 (5–7). In the periphery, CCL21 and the related chemokine CCL19 (EBV-induced molecule-1 ligand chemokine) are known to be homeostatic chemokines that activate CCR7, whereas CXCR3 is activated by CXC chemokine ligand (CXCL)9, CXCL10, and CXCL11 chemokines that belong to the inflammatory chemokines (3, 4).

It is clear that chemokines and their receptors are also expressed in the CNS. Here chemokines, at least partially, control the infiltration of blood leukocytes into the brain and are involved in different brain pathologies (8–10).

Recently, we have described the induction of CCL21 expression in ischemic mouse neurons and the effects of CCL21 in microglia, suggesting the involvement of CCL21 in the communication between damaged neurons and microglia (11–13). Surprisingly, murine microglia responded to CCL21 stimulation in the absence of CCR7 via the alternative receptor CXCR3 (13–15), confirming results previously obtained in a recombinant expression system (5).

The validity of CCL21 as a neuronally derived activator of microglia in humans was challenged because activity of human CCL (hCCL)21 on human CXCR (hCXCR)3 has not been found (6, 7). However, none of these studies were conducted in (CCR7 lacking) primary human cells. Therefore, we studied possible effects of human CCL21 in primary adult microglia.

Materials and Methods

Cultured human primary microglia and HEK293

Human brain tissue was obtained by rapid autopsy. All patients or their next of kin had given written consent for autopsy during life, and for use of their brain tissue for research purposes. Microglial cell cultures were obtained as described previously (16). To induce proliferation of microglial cells, recombinant human GM-CSF (stock solution 300 µg/ml; Leukomax, Novartis, The Netherlands) was added (final concentration 25 ng/ml). Microglial cells immunostained (>90–98%) with anti-Mac1 (CD11b), LeuM5 (CD11c), LeuM3 (CD14), KP1 (CD68), and FcγRI (CD64), whereas they were negative for glial fibrillary acidic protein (GFAP). HEK293 cells were maintained in DMEM containing 10% FCS with 0.01% penicillin and 0.01% streptomycin in a humidified atmosphere (5% CO2) at 37°C.

Cloning of CXCR3 and transfection of HEK293 cells

Full-length murine CXCR3 (mCXCR3) was obtained from total RNA extracted from murine mixed glial cell cultures as described (17). Primers for the full-length sequence of mCXCR3 were designed (accession no. AF045146): forward primer: 5’-GCAAGTTCCCAACCCACAAGT-3’; backward primer:
yielded no signal (not shown). Scale bars with an isotype-matched Ab against GFAP or without primary Abs

Control experiments with an isotype-matched Ab against GFAP or without primary Abs yielded no signal (not shown). Scale bars = 50 µm.

5′-CGAGCTAGCCCAACTACCGGAAGGAGGA-3′. The PCR product was cloned into pCRIL.1 (Invitrogen, Breda, The Netherlands), sequenced, and subcloned into pCDNA3.1 for transfection experiments. Plasmids containing hCXCR3 were a kind gift of C. Tensen (Vrije Universiteit Medical Center, Amsterdam, The Netherlands) and B. Moser (Theodor-Kocher Institute, Bern, Switzerland) (18). One microgram of the plasmids was transfected with 6 µl of Fugene (Roche Molecular Biochemicals, Almere, The Netherlands) in HEK293 cells. Stable transfected cells were selected with G418 500 µg/ml. Mock transfections were performed with pCDNA 3.1 containing the reversed CXCR3 sequence.

Immunocytochemistry

Cells were seeded on poly-L-lysine-coated glass cover slides and fixed in 4% paraformaldehyde for 30 min. After rinsing in PBS and preincubation in PBS containing 0.3% Triton X-100 (PBS’ ) and 5% normal goat serum, slides were incubated in PBS’ with 2% normal goat serum and human CD11b (Mac-1; Chemicon International, Hofheim, Germany) or mouse anti hCXCR3 (R&D Systems, Abingdon, U.K.) for 2 h and visualized with a Cy3-labeled goat anti-mouse secondary Ab. Control experiments for all immunocytochemical stainings were done by incubating cells with an isotype-matched primary Ab against GFAP (Chemicon International) or in the absence of primary Abs.

RT-PCR

RNA preparation and reverse transcriptase were performed as described previously (17). Two microliters of cDNA were amplified in the PCR using the following two primer pairs for CCR7: forward, 5′-ACCAATGAAAAGCGT

GCTG-3′; backward, 5′-GGCGGATGAGAGGATGACAA-3′; 977 bp), and forward, 5′-TGAGGTCAGGAGGATTACA-3′; backward, 5′-CATGCACTGGAAGAGCTCA-3′ (355 bp) with an annealing temperature of 56°C and 35 cycles. As a control for cDNA quality, experiments with primers for the housekeeping gene GAPDH: forward, 5′-CATCTGGCACACCACGTG

CTTAG-3′; backward, 5′-GCCTGTTACCCAGCGCTTCTTGATG-3′ were done in parallel with an annealing temperature of 60°C and 28 cycles.

In situ hybridization

The cellular distribution of CXCR3 mRNA was investigated by in situ hybridization experiments as described earlier (17) using CXCR3 digoxigenin-conjugated sense and antisense probes.

Chemotaxis assays

Migration of microglia and CXCR3-transfected HEK293 cells in response to CXCL10, CXCL16, CCL19, CCL21, and CX3C chemokine ligand (CX3CL1) (R&D Systems) was determined as described earlier (17). For experiments in the presence of CXCL10, microglia were first preincubated with the upper chamber of the microchamber. Microglial cells (1.5 × 10⁴) or 3 × 10⁵ transfected HEK293 cells were applied per well (50 µl) in the upper chamber. Migrated cells were counted and data are presented as percentages of control migration.

Statistical analysis

Statistical analysis and comparison of the different groups was done with the Tukey ANOVA test for multiple comparison (SPSS, Chicago, IL) with a significance level of 0.05.

Results and Discussion

Expression of CXCR3 but not CCR7 in human microglia and CXCR3-transfected HEK293 cells

The presence of CXCR3 in stably transfected HEK293 cells and microglia was confirmed by immunocytochemistry (Fig. 1) and in situ hybridization (Fig. 2). Stably transfected HEK293 cells showed intense immunostaining for hCXCR3 (Fig. 1A) and mCXCR3 (Fig. 1B). Nontransfected HEK293 cells gave negative staining results (not shown). In cultured microglia, >92% of the cells were found positive for CD11b confirming the identity of these cells (Fig. 1C). Parallel stainings for CXCR3 resulted in >95% CXCR3-positive cells (Fig. 1D).

Control experiments with an isotype-matched (IgG1) Ab against GFAP (Chemicon International), or without primary Abs did not show staining (data not shown). As already shown for CXCR3 and other chemokine receptors in glial cells, immunostaining for CXCR3 was predominantly located in the cytoplasm with minor staining the membrane (17, 19, 20).

Using RT-PCR experiments, we were not able to detect expression of CCR7 in both microglia and CXCR3-transfected HEK293 cells (Fig. 2). As a positive control to the two different sets of primers, lymph node tissue was used where pronounced
experiments with human microglia: infected HEK293. The maximum migratory response was observed in response to the CCR7 ligand CCL19. The maximum migratory response observed with CCL21 was comparable to those for other CXCR3 ligands CXCL9 and CXCL10. The migratory response of 1 nM CCL21 was significantly inhibited by the presence of 10 nM CXCL10. No effect of 10 nM CXCL10 was found on the migratory response of 1 nM CX3CL1. HEK293 cells transfected with mCXCR3 migrated toward both CXCL9, and CXCL10 (data not shown), and CCL21 in a dose-dependent manner. hCXCR3-transfected HEK293 cells showed chemotaxis in response to CXCL9 and CXCL10 (data not shown) but not to CCL21. The graphs provide the results from a typical experiment; similar results were obtained in at least three independent experiments. Data are given in mean ± SEM, for experiments with human microglia: n = 4 and with transfected HEK293: n = 6. *Significant different from control values (p ≤ 0.05).

Effects of hCCL21 and other ligands for CXCR3 on chemotaxis of human microglia

The functional activity of CXCR3 in murine microglia in response to CXCL9 (10) and CCL21 was demonstrated recently (13, 15, 17). Similar as murine microglia, human microglia migrated in response to CCL21 in a concentration-dependent manner (Fig. 3A). The maximum migratory response was found, between 0.1 and 1 nM, which was comparable with migratory responses for CXCL9 or CXCL10 (Fig. 3A) (17). Moreover, CCL21-induced chemotaxis was sensitive to pertussis toxin, indicating the involvement of G protein-coupled chemokine receptors (Fig. 3A). Again, similar to our findings in murine microglia, hCXCR3 had no effect in human microglia (Fig. 3A).

Using CXCR3-deficient mice, it was demonstrated that mCXCR3 is the responsible chemokine receptor for murine CCL21 in microglia (15). To further address this also in human microglia, we first tried to block CXCR3 signaling by Ab treatment. However, our hCXCR3 Ab did not influence the migration of hCXCR3-transfected HEK203 cells in response to CXCL9 and 10 (data not shown) and was therefore not suitable. Next, a desensitization approach was performed because we have shown previously, that CCL21 and CXCL10 cross-desensitize their signaling in mice, indicating that both ligands activate the same receptor (13). It is shown here that the effect of CCL21 was absent in microglia in the presence of 10 nM CXCL10 (Fig. 3B), whereas the chemotactic activity of another chemokine, CX3CL1, was not affected (Fig. 3B). These results strongly suggest that CXCR3 is desensitized by the presence of 10 nM CXCL10 and the lack of a CCL21 response indicates that CXCR3 is the responsible chemokine receptor for CCL21 in human microglia.

bCCL21 did not activate hCXCR3 in HEK293 cells

HEK293 cells were transfected with murine or human CXCR3 and investigated for possible responses to CCL21 and other CXCR3 ligands. In agreement with previously published findings, it was found that HEK293 cells transfected with mCXCR3 responded to CCL21 and CXCL9 in a concentration-dependent manner (Fig. 3C). HEK293 cells transfected with hCXCR3 migrated when exposed to CXCL9 or CXCL10, but no migratory response was observed to CCL21 (Fig. 3D) (5, 6). Migration of mock-transfected HEK293 was never observed (data not shown).

Signaling of hCXCR3 is different in microglia

Pharmacological differences of chemokine receptors expressed in different expression systems have already been published (see CCR5 signaling in various cells; Refs. 21-23). Our results suggest that the pharmacology of hCXCR3 depends on its cellular background. Because we did not detect a microglia-specific splice variant for CXCR3 in mice and humans (data not shown), other mechanisms must account for the here-presented difference. Post-translational modifications (i.e., palmitoylation), which may influence signaling cascades are known for chemokine receptors (24, 25). Moreover, interactions with regulator of G protein signaling protein may influence chemokine receptor functioning (26) or chemokine receptor heterodimerization might change the pharmacology of hCXCR3 in microglia as it was shown for CCR2 (27).

bCCL21 might be important in human brain inflammation

Hence, the mechanism accounting for the effects of hCCL21 in human microglia requires further investigation. However, the presented results show that hCCL21 induces functional responses in human microglia similar to those in murine cells. This shows for the first time functional activity of hCXCR3 in...
response to hCCL21. Because hCCL21 expression was recently detected in cerebrospinal fluid of patients with neurodegenerative diseases (28), it is suggested that hCCL21 is involved in neuroimmune signaling also in humans.

References


