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Involvement of C-C Chemokine Ligand 2-CCR2 Interaction in Monocyte-Lineage Cell Recruitment of Normal Human Corneal Stroma

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Bone marrow-derived cells (BMCs) reside in the anterior stroma of the central and paracentral cornea, as well as all stromal layers of the peripheral cornea, in normal human eyes. We investigated the factors regulating the constitutive distribution of BMCs in normal human corneal stroma. Cultured human corneal kerocytes expressed several chemokines (growth-related oncogene/CXCL1–3, IL-8/CXCL8, and MCP-1/CCL2) in the Ab array study. CCR2 and CCR7 mRNAs were detected in BMCs by multiplex RT-PCR. Kerocytes/corneal epithelial cells and BMCs selected from normal human donor corneas by using magnetic beads expressed MCP-1/CCL2 and CCR2 protein, respectively. BMCs isolated from human corneal stroma showed a chemotactic response to MCP-1/CCL2 in the Boyden chamber assay. The chemotactic effect of kerocyte supernatant was inhibited by blockade of MCP-1/CCL2. This is the first work on constitutive expression of CCR2 by BMCs from the corneal stroma and MCP-1/CCL2 by kerocytes/epithelial cells. Our findings suggest that the interaction between MCP-1/CCL2 and CCR2 determines the distribution of constitutive BMCs in normal human corneal stroma. The Journal of Immunology, 2007, 178: 3288–3292.

Materials and Methods

Donor human corneas and isolation of CD45-positive and CD45-negative cells by magnetic beads

This study was conducted in accordance with the Declaration of Helsinki. Corneas were obtained from the Rocky Mountain Lions’ Eye Bank at 3–5 days after the death of the donors (aged 62–70 years) and were kept in Optisol GS storage medium (Bausch & Lomb) at 4°C until use. Corneas with focal or diffuse stromal opacity or with a history of corneal disease in the eye bank report were not used. For isolation of the corneal stroma, the endothelium and Descemet’s membrane were peeled away in a sheet from the periphery to center of the inner surface of the cornea with fine forceps, according to the procedure described previously (8). Then the epithelium was carefully removed from the stroma by scraping the outer surface of the cornea and used for total RNA extraction and flow cytometry after trypsin/EDTA treatment. Before isolation of corneal stromal cells, the peripheral cornea (including the limbal region) was dissected away from the stroma to avoid possible contamination by corneal limbal epithelial cells. Next, the stroma was cut into small pieces ~1 mm in diameter, which were incubated overnight at 37°C in serum-free basilar medium containing 0.02% collagenase (Sigma-Aldrich). After washing three times with PBS, a single-cell suspension was obtained by trituration with a fire-polished Pasteur pipette. CD45-positive cells were isolated by positive selection with a MACS (Miltenyi Biotec), according to the manufacturer’s instructions. For separation of CD45-positive and CD45-negative cells, the stroma from three to five corneas was processed simultaneously. The average number of CD45-positive cells per cornea was 10.5 ± 0.9 × 10⁴ (mean ± SD), as described elsewhere (1). Average BMC rate per isolated cells from corneal stroma was 91 ± 7% (mean ± SD; n = 5) in our preliminary study.
Antibodies
Mouse anti-human MCP-1/CCL2 (IgG2b; R&D Systems) mAb and mouse anti-human CCR2 polyclonal Ab (Abcam) was used for the immunochemical study.

Culture of keratocytes
After selection of CD45-positive cells from donor human corneas by using a MACS, keratocytes were isolated by negative selection and cultured to subconflunce in DMEM with 15% FCS. To detect chemokines and cytokines in the culture supernatant, keratocytes were washed twice in PBS and cultured in serum-free DMEM (basal medium). Then the supernatants harvested from cultures after incubation for 24 and 48 h were used for measurement of chemokine concentration and Boyden chamber assay, respectively.

Ab array technique
The keratocyte culture medium was analyzed with an Ab array, comprising a Human Angiogenesis Antibody 1 kit (20 angiogenic factors; Ray Biotech) and a Human Inflammatory Antibody Array III kit (40 inflammation-related mAbs, 20 cytokines), according to the manufacturer’s instructions. All of the plotted proteins are listed at www.raybiotech.com/images/map_all.htm. The membranes were washed twice with TBS and incubated in blocking buffer for 30 min. Keratocytes were cultured for 48 h and washed by basal medium three times. Then basal medium only was added in the well and incubated for 24 h. One milliliter of the keratocyte culture supernatant was added to each culture plate and was incubated overnight at 4°C. After decanting, the membranes were washed a total of five times with washing buffer. Then 1 ml of a 1/250 dilution of biotin-conjugated Ab was added to each plate, and incubated overnight at 4°C with shaking. The membranes were subsequently incubated with 2 ml/well of a 1/1000 dilution of HRP-conjugated streptavidin for 1 h incubation with the detection buffer for 5 min, and exposed to BioMax Light film (Eastman Kodak) for 1 min before processing. The signal intensities of individual spots were determined by densitometry (Gel-Pro analyzer software; Media Cybernetics), and the positive control signals on each membrane were used to normalize the intensity of the individual spots being compared on different membranes. For each spot, the net optical intensity was determined by using the Ray-Bio Antibody Array Analysis Tool (Ray Biotech). FCS-free medium was employed as a negative control.

Preparation of RNA and RT-PCR
Total RNA was isolated from CD45-positive and CD45-negative cells in the corneal epithelium and stroma using Isogen reagent (Nippon Gene), and genomic DNA by using specific primers for different exons. Multiplex PCR kits (human CCR genes set-1, set-2, and CXCR gene set-1; Maxim Biotech) and a Human Inflammatory Antibody Array III kit (20 angiogenetic factors; Ray Biotech) were used for detection of chemokine receptors, according to the manufacturer’s instructions.

Immunochemical study
CD45-positive BMCs were isolated from donor corneal stromas by MACS, washed in PBS, and then attached to glass slides with a cytopsin machine. Then the cells on the slides were fixed in 100% cold acetone and processed for immunochemistry with FITC anti-human CCR2 mAb. Nonimmune FITC goat IgG was used as the negative control.

Boyden chamber assay
BMC migration was assayed by using a modified Boyden chamber (48 wells) technique. The supernatant of keratocytes cultured for 48 h with 1% FCS was placed in the lower wells (25 μl). Then 50 μl of a cell suspension (1 × 10⁴ BMCs/ml in RPMI 1640 medium with 1% FCS) was added to each of the upper wells of the chamber, which were separated from the lower wells by a polycarbonate polyvinylpyrrolidone-free microfilter (Nuclepore; Whatman). The pore size was 8 μm for the upper well filters and 5 μm for the lower well filters. After incubation of the chamber (60 min) at 37°C in a humidified atmosphere with 5% CO₂, the filters were removed and the upper sides were cleaned. Then the filters were fixed in methanol and stained with methyl green. Chemotaxis was quantified by counting all the cells that migrated completely through the pores of the filter, and the extent of migration was shown as the number of migrating cells per well. To assess the role of chemokines and chemokine receptors in the migration of BMCs, we added anti-human MCP-1/CCL2-blocking Abs to the upper wells.

Statistical analysis
One-way ANOVA and Scheffe’s multiple comparison test were used to compare mean values for the three groups, whereas unpaired Student’s t test was used to compare mean values for the two groups. The level of significance was set at p < 0.05. Analyses were performed using the Stat View statistical software package (version 5; Abacus Concepts).

Results
Chemokine production by cultured human keratocytes detected by the Ab array technique
To determine the chemokines produced by keratocytes, we analyzed 13 of chemokines in cultured human keratocytes isolated by negative selection with magnetic beads using the panleucokyte marker CD45. Based on the results of Ab array analysis with two array membranes, the appearance of representative membranes and the average net optical intensity of chemokines in the supernatant of cultured keratocytes are shown in Fig. 1. Mean optical intensity of positive spots from the culture supernatants was compared with those derived from the medium alone. Growth-related oncogene (GRO)/CXCL1–3 (the ligand of CXCR2), IL-8/CXCL8 (the ligand of CXCR1 and CXCR2), and MCP-1/CCL2 (the ligand of CCR2) proteins show high levels in the keratocyte culture medium (Fig. 1, A–E). The cultured keratocytes also produced cytokines and growth factor protein such as IL-6, tissue inhibitor of metalloproteinase (TIMP)-2, TGF-β1, TIMP-1, angiogenin, and basic fibroblast growth factor (Fig. 1F).

Chemokine receptor gene expression by BMCs in human corneal stroma detected by multiplex PCR
CD45-positive cells in the human corneal stroma are all BMCs, as reported elsewhere (1). To find the chemokine receptors expressed by BMCs in normal human corneal stroma, multiplex PCR was performed with primers for the CC, CXC, and CX3C chemokine receptors. As shown in Fig. 2, the genes for CCR2 (the receptor for MCP-1/CCL2 and MCP-3/CCL7) and CCR7 (the receptor for secondary lymphoid-tissue chemokine (SLC)/CCL21 and EBI 1-ligand chemokine (ELC)/CCL19) were detected in CD45-positive cells isolated from human corneal stroma. In contrast, the CXCR and CX3CR genes were not detected under any PCR conditions. The CCR11 gene (included in the primer set) was previously classified as a CC chemokine receptor, but has since been excluded from the chemokine family (9).
CCR2 protein expression in BMCs detected by immunocytochemistry

The immunocytochemical study showed that BMCs from human corneal stroma were strongly stained by FITC-labeled anti-human CCR2 Ab (Fig. 3A, arrows), but not by the control IgG (Fig. 3B). Immunocytochemistry was not performed for CCR7, because the genes for its ligands (SLC/CCL21 and ELC/CCL19) were not detected by RT-PCR in human keratocytes isolated from donor corneal stroma (data not shown).

BMCs were localized to the anterior layer of the central and paracentral corneal stroma, as previously described (1). Keratocytes and corneal epithelial cells were isolated after removal of CD45-positive cells from donor corneas and were shown to express the MCP-1/CCL2 gene by RT-PCR (Fig. 4A). Intracellular expression of MCP-1/CCL2 in keratocytes and corneal epithelial cells was evaluated by flow cytometry, because immunocytochemistry and immunohistochemistry did not show any staining for MCP-1/CCL2. Flow cytometric analysis of cells permeabilized with saponin showed a positive reaction to anti-human MCP-1/CCL2 mAb in both keratocytes and corneal epithelial cells isolated from donor corneas, demonstrating that there is at least intracellular expression of MCP-1/CCL2 by these cells (Fig. 4B). These findings suggested that MCP-1/CCL2 and CCR2 may possibly be the chemokine and chemokine receptor involved in regulating the distribution of BMCs in human corneal stroma.

Boyden chamber assay

To test whether CCR2 ligands, MCP-1/CCL2, MCP-2/CCL8, and MCP-3/CCL7 are candidate chemokines for chemotaxis assay, we measured the concentrations of these chemokines in the culture supernatant of human keratocytes. Cultured keratocytes produced 1240 ± 490 pg/ml (mean ± SD; n = 4) MCP-1/CCL2 protein measured by using ELISA kits (R&D Systems), whereas concentrations of MCP-2/CCL8 and MCP-3/CCL7 were undetectable level. Therefore, the Boyden chamber assay was performed to test
whether CCR2 and its ligand MCP-1/CCL2 had a role in the migration of BMCs. When human rMCP-1/CCL2 was added to RPMI 1640 medium in the lower wells of the chamber, it showed chemotactic activity for a human monocytic cell line (THP-1) and BMCs, whereas the medium alone did not (Fig. 5A). Supernatant from keratocytes cultured with 1% FCS showed significantly higher chemotactic activity for BMCs than control medium (RPMI 1640 with 1% FCS) (Fig. 5B). When blocking Ab to MCP-1/CCL2 was added to the supernatant of cultured keratocytes in the lower wells, MCP-1/CCL2 mAb, but not control IgG, significantly inhibited the migration of BMCs. These findings demonstrated that keratocytes produce a chemotactic factor for BMCs in the corneal stroma and that the interaction between MCP-1/CCL2 and CCR2 on BMCs has a role in human corneal stroma.

**Discussion**

Based on our hypothesis that a certain chemokine and its receptor may determine the constitutive distribution of BMCs in the normal human corneal stroma, we searched for chemokines and chemokine receptors by the Ab array and multiplex RT-PCR methods. The Ab array is a method based on enzyme-linked immunosassay that enables us to simultaneously analyze various proteins at minute levels. We detected six chemokines in the culture medium of corneal keratocyte by using this system, among which GRO/CXCR1–3, IL-8/CXCR8, and MCP-1/CCL2 showed high levels. Multiplex RT-PCR demonstrated that CCR2, the receptor for MCP-1/CCL2 and CCR7, and the common receptors for SLC/CCL21 and ELC/CCL19 were candidate chemokine receptors in stromal BMCs. Both the MCP-1/CCL2 gene and protein were detected in keratocytes and BMCs, whereas the CCR2 gene and protein were detected in BMCs derived from normal donor corneas. BMCs bearing CCR2 in the human corneal stroma showed a chemotactic response to rMCP-1/CCL2. The chemotactic effect of the supernatant of cultured keratocytes was inhibited by MCP-1/CCL2-blocking Abs. The exact mechanism underlying the distribution of BMC in the central and paracentral anterior stroma of the human cornea is still unknown (1). The fact that not only cultured human corneal epithelium (10, 11), but also donor corneal epithelium (as shown in this study) constitutively expresses the MCP-1/CCL2 gene and protein may contribute to explaining the localization of BMC in the anterior corneal stroma. All of these findings may help to explain the constitutive localization of BMCs to the anterior stroma of the central and paracentral regions vs all stromal layers at the periphery of avascular normal human corneas.

MCP-1/CCL2 (also known as monocyte chemotactic and activating factor) was originally identified as a key molecule for the chemotaxis and activation of macrophages (12, 13). MCP-1/CCL2 was found to be potently angiogenic when implanted into rabbit corneas, and it is the strongest ligand for CCR2 (14). CCR2 is a cognate receptor of MCP-1 that is expressed on monocytes, activated and memory T lymphocytes, and macrophages (15). Human corneal keratocytes exposed to appropriate stimulants express a high level of MCP-1/CCL2 (16, 17). Moreover, a study of human corneal endothelial cells as well as cultured human corneal endothelial cells also concluded that MCP-1/CCL2 was produced when cells were stimulated (18, 19). These findings suggested that CCR2-expressing cells can migrate into all layers of the cornea under inflammatory conditions, whereas MCP-1/CCL2 regulates the constitutive distribution of BMCs, as well as accumulation of leukocytes under inflammatory conditions. Clinically, functional interaction of MCP-1/CCL2 in corneal cells and CCR2-expressing BMCs and memory T cells in cornea may have a central role in a variety of corneal inflammatory diseases, such as herpetic stromal keratitis, interstitial keratitis, diffuse lamellar keratitis after laser in situ keratomileusis, and subepithelial infiltrates after epidemic keratoconjunctivitis. Moreover, MCP-1/CCL2-CCR2 interaction may also be associated with immune reaction involving allore cognition and allorejection in corneal transplantation.

The distribution of BMCs in normal tissues should not be random due to specific factors that allow the recruitment of marrow cells to each tissue. This process may involve various adhesion mechanisms.
molecules, including selections, integrins, and their corresponding vascular ligands, as well as the large family of chemokines and their receptors (20). In addition to MCP-1/CCL2-CCR2 as a critical determinant of the BMC distribution in the corneal stroma, we identified liver and activation-regulated chemokine/MIP-3/α/CCL20-CCR6 as a possible key factor explaining the constitutive existence of myeloid DC in normal human corneal epithelium (S. Yamagami, unpublished observation). These findings suggest that specific chemokines accurately control the distribution of constitutive BMCs with different phenotypes in each part of cornea. However, the reason that MCP-1/CCL2 expressed in the corneal epithelium does not attract CCR2-positive cells into the epithelial layer is unclear. Immunological processes, adhesion molecules, or vascular ligands may restrict leukocyte influx into the anterior segment of the eye before local leukocyte trafficking via chemokine-chemokine receptor interactions. Alternatively, cells of the monocyte lineage bearing CCR2 may change their phenotypes in the ocular microenvironment before migrating into the corneal epithelium, because monocytes can differentiate into immature DCs after appropriate cytokine stimulation (21).

Correlations between chemokine production and corneal pathology have been reported in various experimental models. Liver and activation-regulated chemokine/CCL20 and its receptor CCR6 is a possible functional chemokine receptor pair in murine herpetic stromal keratitis (22). RANTES/MIP-1β and their receptor CCR5 is a chemokine receptor combination involved in DC recruitment in inflamed mouse corneal epithelium (23). In a mouse corneal transplantation model, CC chemokines (such as RANTES/CCL5, MIP-1α, and MCP-1/CCL2) may be associated with the effector phase of corneal allograft rejection (24) and with the recruitment of innate immune cells in the early period after high-risk corneal transplantation (25). Moreover, blockade of proinflammatory cytokines suppresses chemokine expression and promotes corneal allograft survival (26). These findings suggest that chemokine expression may be associated with a variety of ocular inflammatory conditions. Apart from the data obtained in murine inflammation models, our findings provide the first clues to explain the regulation of the constitutive distribution of cells by chemokine receptor interactions in the normal human cornea.

In summary, MCP-1/CCL2 in keratocytes and CCR2 in BMCs were detected as a candidate chemokine-chemokine receptor combination by the protein array assay, multiplex RT-PCR, and RT-PCR. Keratocytes and corneal epithelial cells from normal donor corneas express MCP-1/CCL2. Supernatant of cultured keratocytes has a chemotaxin effect on CCR2-expressing BMCs isolated from normal human corneal stroma. This chemotaxis could be suppressed by blockade of MCP-1/CCL2 in a Boyden chamber assay. Our findings are the first evidence of the constitutive expression of CCR2 by BMCs in the corneal stroma and the expression of MCP-1/CCL2 by normal keratocytes/epithelial cells, suggesting that the interaction between MCP-1/CCL2 and CCR2 may determine the constitutive distribution of BMCs in normal human corneal stroma.

Disclosures
The authors have no financial conflict of interest.

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