CCL11 (Eotaxin) Induces CCR3-Dependent Smooth Muscle Cell Migration

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Objective—CCL11 (Eotaxin) is a potent eosinophil chemoattractant that is abundant in atheromatous plaques. The major receptor for CCL11 is CCR3, which is found on leukocytes and on some nonleukocytic cells. We sought to determine whether vascular smooth muscle cells (SMCs) possessed functional CCR3.

Methods and Results—CCR3 mRNA (by RT-PCR) and protein (by Western blot analysis and flow cytometry) were present in mouse aortic SMCs. CCL11 induced concentration-dependent SMC chemotaxis in a modified Boyden chamber, with maximum effect seen at 100 ng/mL. SMC migration was markedly inhibited by antibody to CCR3, but not to CCR2. CCL11 also induced CCR3-dependent SMC migration in a scrape-wound assay. CCL11 had no effect on SMC proliferation. CCR3 and CCL11 staining were minimal in the normal arterial wall, but were abundant in medial SMC and intimal SMC 5 days and 28 days after mouse femoral arterial injury, respectively, times at which SMCs possess a more migratory phenotype.

Conclusion—These data demonstrate that SMCs possess CCR3 under conditions associated with migration and that CCL11 is a potent chemotactic factor for SMCs. Because CCL11 is expressed abundantly in SMC-rich areas of the atherosclerotic plaque and in injured arteries, it may play an important role in regulating SMC migration.

Key Words: chemokines ■ vascular smooth muscle ■ cell migration ■ arterial injury ■ Eotaxin (CCR11)
(MCP-1), may act as agonists for SMCs. CCL11 antigen has been found in SMCs of human atheroma and has been shown to be upregulated in the media of ischemic rat aorta. CCL11 has been reported to be elevated in the serum of patients with stable angina 24 hours after elective percutaneous transluminal angioplasty. CCR3 has also been identified in atherosclerotic plaques, predominantly in leucocytes. We now report that SMCs possess functional CCR3 and that CCL11 induces SMC migration. CCR3 and CCL11 are induced in SMCs of the arterial media and neointima after injury. CCL11 may play a role in mediating SMC migration in the injured arterial wall.

Methods

Cell Culture

Primary mouse (CB7Bl/6) SMCs were isolated by enzyme digestion as described and grown in DMEM supplemented with 10% FBS. SMCs were serially passaged before reaching confluence, and all experiments were performed on passages 4 to 9. THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in 25-mm-diameter DMEM supplemented with 10% FBS. The medium, with fresh serum containing growth factors, was changed every 2 days. Experiments were done in duplicate, with 3 to 5 plates used for each experiment. Medium, with fresh serum for 24 hours, was added to cultures at 24 hours after injury. CCL11 was measured using SPOT Advanced software (Diagnostic Instruments, Inc.). The wound area (immediately after wounding and at 24 hours after) was measured using SPOT Advanced software. Images (400x) were captured with a SPOT camera (Diagnostic Instruments, Inc.).

Migration

SMCsExpress CCR3 mRNA and Protein

SMCs were grown to 50% to 70% confluence in 0.3% serum in DMEM for 24 hours, and then treated with mouse CCL11 (100 ng/mL) for 1 hour. SMCs were harvested in PBS-based Cell Dissociation Buffer (Cat #13151 to 014, Invitrogen) and 5×10^5 cells were incubated at 4°C for 45 minutes in FACS buffer (PBS, 1% bovine serum albumin, 0.01% NaN₃) containing monoclonal anti-mouse CCR3 (FAB729F; R & D Systems). An irrelevant IgG2a antibody was used as an isotype control. Unbound antibody was removed by washing with 2 mL FACS buffer, and the cells were resuspended in the same buffer containing a 1:20 dilution of FITC-conjugated goat anti-mouse F(ab’)2 secondary antibody. After a 30-minute incubation, cells were washed with 2 mL FACS buffer and resuspended in 500 µL of the same. Surface expression was analyzed using a FACS Calibur flow cytometer, and data were processed using the CellQuest software program (Becton Dickinson).

Immunohistochemistry

Tissue staining was performed on 5-µm-thick parafomaldehyde-fixed, paraffin-embedded sections. After deparaffinization and hydration with PBS, sections were incubated in 3% H₂O₂ for 15 minutes, washed in PBS, and subsequently exposed to blocking serum for 5 minutes at room temperature. Antibodies to CCR3 (SC-7897; 1 µg/mL; Santa Cruz Biotechnology), smooth muscle α-actin (0.1 µg/mL; Sigma), and MOMA-2 (MCAS19G; 2mg/mL; Serotec Inc.), were applied for 2 hours at 37°C. Primary antibodies were detected with a goat anti-rabbit IgG antibody conjugated with biotin (Biogenex). Controls for each experiment included processing the specimens using the nonimmune IgG isotype as the primary antibody and omission of primary antibody. Sections from injured mouse femoral arteries were generated in a previous study as described.

Results

SMCs Express CCR3 mRNA and Protein

To determine whether SMCs possess CCR3 mRNA, RT-PCR was performed with CCR3 specific primers. A band of the expected size (287 bp) was seen in lanes containing SMC RNA (Figure 1A). The RT-PCR product was subcloned and sequenced and found to be 100% homologous to CCR3 cDNA (GenBank accession #NM_009914.1) Western blot analysis of mouse SMC extracts with an antibody that recognizes mouse and human CCR3 demonstrated a single band of ≈40kDa, similar to that found in extracts of human eosinophils (Figure 1B). The presence of CCR3 on the
surface of SMC was confirmed by fluorescent cytometry using a monoclonal antibody against CCR3 (Figure 2).

**CCL11 Induces SMC Migration**

To help establish a biologic role for CCR3 on SMCs, migration assays were performed using a modified Boyden chamber. CCL11 induced concentration-dependent SMC migration that was similar in extent to that seen with PDGF-BB or 10% FBS (Figure 3A). Enhanced SMC migration was not seen when CCL11 was placed on both sides of the membrane or when CCL11 was present only in the top wells (Figure 3B). This indicates that the effect of CCL11 on SMC is predominantly chemotactic rather than chemokinetic. CCL11-mediated SMC chemotaxis was substantially blocked by pretreatment with a monoclonal antibody to CCR3 (Figure 3C). In contrast, an antibody to CCR2 and an irrelevant IgG failed to inhibit SMC chemotaxis.

To examine further the effect of CCL11 on migration, scrape-wound assays were performed on SMC monolayers. As shown in Figure 4, CCL11 induced a 73±19% increase in SMC migration at 24 hours. Antibody to CCR3 almost completely blocked this increase.

To determine whether CCL11 mediates proliferation, 10⁴ SMCs were plated in 35-mm dishes and cell counts were obtained at 24 hour intervals in the presence of CCL11 or other growth factors. CCL11 had no effect on SMC proliferation (Figure 5). In contrast, PDGF or 10% FBS produced substantial increases in cell number. CCL11 had no incremental or decremental effect on PDGF-mediated SMC proliferation.
Figure 3. CCL11 induces mouse SMC chemotaxis. A, Mouse CCL11 in 0.3% FCS at the concentrations indicated (ng/mL) were placed in the bottom wells of a modified Boyden Chamber. 10⁴ SMCs were placed in the top wells. The chamber was incubated for 6 hours at 37°C and then analyzed for migration as described in Materials and Methods. For comparison, recombinant PDGF-BB (1 ng/mL) or 10% FBS were also used as chemoattractants. 0.3% serum was used as negative control. *P < 0.005 compared with 0 CCL11. B, Checkerboard analysis in which CCL11 (C; 100 ng/mL) was placed in the bottom, top, or both wells as indicated. *P < 0.005 compared with 0.3% FCS (–). C, SMC were incubated with antibodies against CCR3 (anti-CCR3), CCR2 (anti-CCR2) (1 μg/mL), or irrelevant IgG for 30 minutes at 37°C before being placed in the Boyden chamber. Results are expressed as SMC/hpf ± SEM and represent triplicate experiments *P < 0.005 compared with 0.3% FCS. **P < 0.005 compared with 0.3% FCS.

Figure 4. CCL11 induces mouse SMC migration. SMCs were grown to confluence and then incubated in DMEM + 0.1% BSA for 24 hours. Linear wounds were made in each plate and SMCs were then incubated with DMEM + 0.1% BSA alone (control) or in the presence of CCL11 (concentration in ng/mL indicated on the X axis). In some experiments, SMCs were preincubated for 1 hour with anti-CCR3 (CCR3 Ab) or an irrelevant antibody (IgG) before wounding and treatment with 10 ng/mL CCL11. The wound area was measured immediately after wounding and at 24 hours. Migration is expressed as the % decrease in wound area in 24 hours compared with control. Experiments were done in duplicate, with 3 to 5 plates used for each condition. *P < 0.001, 10 ng/mL CCL11 compared with control; **P < 0.001, 10 ng/mL eotaxin + CCR3 Ab compared with 10 ng/mL eotaxin alone; #NS, 10 ng/mL eotaxin + IgG compared with 10 ng/mL eotaxin alone.
CCR3 Is Induced in SMC in Response to Arterial Injury

The above data suggested that CCL11 mediates SMC migration. We therefore sought to determine whether CCR3 and CCL11 were present in SMCs in vivo under conditions associated with migration, such as after arterial injury. As shown in Figure 6, CCR3 and CCL11 antigens were not detected in the normal arterial wall. In contrast, CCR3 and CCL11 antigens were abundant in the media of mouse femoral arteries 5 days and 7 days after wire injury. CCR3 and CCL11 staining were associated with α-actin positive cells (data not shown). No macrophages were seen in the media at 5 or 7 days as determined by staining with MOMA-2 (data not shown). CCR3 and CCL11 staining were also abundant in the neointimal SMCs 28 days after injury.

Discussion

This report describes the presence of CCR3 in cultured mouse vascular SMCs and demonstrates that CCL11, a CCR3 ligand, induces SMC chemotaxis. This is the first report to our knowledge that CCL11 acts as an agonist for vascular SMCs. We also report that CCR3 antigen is present in the SMCs of the shoulder region of atherosclerotic plaques and is induced in medial SMCs after arterial injury, both states in which SMCs assume a migratory phenotype.

CCR3 is a member of the CC chemokine family of G-protein–coupled receptors. Like other chemokine receptors, CCR3 has been found predominantly on leukocytes, including eosinophils, basophils, mast cells, TH2 cells, dendritic cells, and thymocytes. However, several reports have demonstrated that CCR3 is also present on nonleukocyte-derived cells, such as brain microglial cells, airway epithelial cells, and human brain and microvascular endothelial cells. The current study provides further evidence that CCR3 has a broad spectrum of expression and plays a role in the biology of nonleukocytic systems.

CCL11 antigen is abundant in SMCs of atherosclerotic plaques, and CCL11 mRNA is upregulated in SMCs of rat aortic allografts exposed to prolonged ischemic storage. In the atherosclerotic plaque, the expression of CCL11 was not associated with an eosinophilic infiltrate, leading the authors to speculate that it might be playing a role distinct from eosinophil chemotaxis. Data supporting a role for CCL11 distinct from leukocyte chemotaxis has recently been provided by Salcedo and coworkers, who demonstrated that CCL11 induced chemotaxis of human microvascular endothelial cells. Antibodies to CCR3 inhibited CCL11-mediated endothelial cell chemotaxis by ≈70%. CCL11 induced blood vessel formation in chick chorioallantoic membranes and in matrigel plugs in association with an eosinophilic infiltrate. However, CCL11 also induced endothelial sprouting from rat aortic rings in the absence of eosinophilia, suggesting that the chemokine might have a direct effect on endothelial cell migration in vivo. The current study provides further evidence that CCL11 has nonleukocytic targets. Most intriguingly, it suggests that in addition to stimulating endothelial cell migration, CCL11 also induces SMCs to migrate. This is in contrast to growth factors, such as PDGF or vascular endothelial growth factor, which induce SMCs and endothelial cell migration, respectively, but not both. CCL11 could thus play a particularly important role in processes, such as neovascularization in ischemic tissues, that are characterized by endothelial and SMC migration and proliferation.

Recent studies have demonstrated that vascular SMCs possess chemokine receptors, including CCR5, CCR8, and CXCR4. Activation of CCR5 by CCL4 (MIP-1β) or the HIV envelope protein, gp120, results in mobilization of intracellular calcium and induces tissue factor, the initiator of...
coagulation. 

Activation of CXCR4 by CXCL12 (SDF)-1 or gp120 also induces tissue factor. The induction of tissue factor appears to be dependent on activation of mitogen activated protein kinases (MAPKs) and protein kinase C (PKC). Activation of human CCR8 by CCL1 (I-309) promotes SMC migration. CCL2 (MCP-1) also acts as an agonist for SMCs, inducing tissue factor, cytokine production, and in some studies proliferation. There is controversy as to whether these responses are mediated by the known CCL2 (MCP-1) receptor, CCR2. The current study demonstrates that SMCs also possess CCR3 and, most importantly, that this receptor mediates SMC migration, further suggesting that chemokines as a class may play an important role in SMC biology.

The migration of SMCs from the arterial media to the intima is a crucial event in the initiation and progression of the atherosclerotic plaque and is also thought to play a key role in the development of intimal hyperplasia after arterial injury (reviewed by Schwartz). A variety of molecules have been shown to induce SMC migration, including angiotensin II, fibroblast growth factor, and transforming growth factor-β. PDGF, in particular, has been shown to play a key role in migration of SMCs from the media to the intima after arterial injury. In the current study, CCL11 and PDGF induced similar levels of migration of cultured SMCs. The current study thus demonstrates a new role for CCR11 as an SMC chemoattractant. Although it is difficult to directly examine SMC migration in vivo, the immunohistochemical studies in Figure 5 demonstrate that CCR3 and CCL11 are minimally expressed in uninjured SMCs, but are abundant in the medial SMCs 5 days after femoral arterial injury. This corresponds to the peak of medial SMC proliferation and the initiation of SMC migration from the media to the intima. This migration and the subsequent proliferation of intimal SMCs results in intimal hyperplasia and luminal narrowing (reviewed by Clowes et al). CCR3 and CCL11 are also abundant in the neointimal SMCs, which are also undergoing migration and proliferation. This study thus raises the possibility that the upregulation of CCR3 and CCL11 by injury may have important consequences in mediating SMC migration. Additional studies will be needed to fully elucidate the importance of CCR3 and CCL11 in mediating in vivo SMC activation.

Figure 6. Expression of CCR3 antigen in injured mouse femoral arteries. Samples were obtained from a previous study in which C57Bl/6 mice had undergone wire-induced femoral arterial injury. Sections from uninjured arteries (A, B), arteries harvested 5 days (C, D), 7 days (E, F), and 28 days after injury (G through J) were stained with antibodies to CCR3 (left) or CCL11 (right). Magnification=40× for A, B, E, F, G, and H and 200× for C, D, I, and J. Sections are representative of studies done on 3 different animals.
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