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. **(*Arterioscler Thromb Vasc Biol*. 2004;24:1211-1216.)**

Key Words: chemokines ■ vascular smooth muscle ■ cell migration ■ arterial injury ■ Eotaxin (CCR11)

CCL11 (eotaxin) is a member of the MCP/eotaxin subfamily of CC chemokines[1] and is a potent chemoattractant for eosinophils,[2] basophils,[3] and Th2 lymphocytes.[4] CCL11 mRNA is expressed in a variety of tissues including heart, lungs, kidney, lymph nodes, thymus, and intestines.[5] CCL11 has also been identified in human lung epithelial cells,[6] pleural mesothelial cells,[7] bronchial airway epithelial cells,[8] and smooth muscle cells (SMCs).[9,10] CCL11 is upregulated in a variety of diseases associated with eosinophilic infiltrates (reviewed by Rankin et al[11]), including nasal polyposis, allergic rhinitis, inflammatory bowel disease, Hodgkin's disease,[12] and endometriosis.[13] CCL11 is expressed in airway SMCs, where it is regulated by a variety of cytokines, including tumor necrosis factor-α and interleukin-1β.[9,10]

CCL11 acts predominantly through CCR3, a member of the family of G-protein–coupled receptors with seven transmembrane spanning domains.[4,14–16] In addition to being the major receptor for CCL11, CCR3 also binds to CCL8 (MCP2), CCL7 (MCP3), CCL13 (MCP4), CCL5 (RANTES), CCL24 (eotaxin-2), CCL26 (eotaxin-3), and CCL15 (MIP5).[17–19] CCR3 has been identified on eosinophils,[16] basophils,[14] mast cells,[20] TH2 cells,[4] dendritic cells,[21] thymocytes,[22] brain microglial cells,[23] human airway epithelial cells,[24] and endothelial cells.[25–27] CCR3 is also a coreceptor for HIV-1,[28] and CCL11 inhibits CCR3-mediated HIV-1 infection.

SMCs are the predominant cellular element of the arterial media. In normal arteries, SMCs are in a quiescent, contractile state. In atherosclerosis, SMCs proliferate and migrate to form part of the intimal plaque.[29] Acute arterial injury, such as that produced experimentally in animals or accompanying human angioplasty, also results in proliferation and migration of SMCs, leading to the development of intimal hyperplasia.[30] Considerable effort has been expended on identifying molecules, such as platelet-derived growth factor (PDGF) and fibroblast growth factor that regulate vascular SMC proliferation and migration. However, the breadth and specific physiological roles of SMC activators remain to be fully elucidated.

A variety of CC chemokines have been identified in the atherosclerotic plaque and injured arteries.[31] In addition, several recent studies have suggested that CC chemokines, including CCL-1 (I-309),[32] CCL4 (MIP-1β), and CCL2

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(MCP-1), may act as agonists for SMCs. CCL11 antigen has been found in SMCs of human atheroma[33] and has been shown to be upregulated in the media of ischemic rat aorta.[34] CCL11 has been reported to be elevated in the serum of patients with stable angina 24 hours after elective percutaneous transluminal angioplasty.[35] CCR3 has also been identified in atherosclerotic plaques, predominantly in leukocytes.[33] 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[36] 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 mmol/L HEPES-buffered RPMI-1640 containing 10% FCS, 1 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. To measure proliferation, SMCs were suspended in 35-mm plates at 10^4 cells/plate and grown in DMEM +0.3% serum containing growth factors. The medium, with fresh growth factors, was changed every 48 hour. Duplicate wells were washed with PBS, trypsinized, and counted using a hemocytometer.

Migration

SMC migration was examined as described[37] using ChemoTX microchemotaxis chambers with 8-μm pores (Neuro Probe). Membranes were coated with 0.1 mg/mL of collagen (Vitrogen-100; Cohesion Technologies) in 0.1 mol/L HCl for 1 hour in 5% CO_2 at 37°C. SMCs (70% to 90% confluent) were trypsinized, washed 2× with PBS, resuspended in 0.3% FBS in DMEM, and added to the top wells (10^4 cells/50 μL). Recombinant mouse CCL11 (420-ME; R & D Systems, Minneapolis, MN) was added to the bottom chamber in 0.3% FBS and DMEM. After 6 hours at 37°C, nonmigrating cells were scraped from the upper surface of the filter. Cells on the bottom surface were fixed with methanol and stained with Diff-quick. (Baxter Scientific Products). The number of cells migrating to the lower surface was determined microscopically by counting 4 high power (400×) fields of 4 constant areas per well. In some experiments, SMCs were preincubated for 1 hour at 37°C with anti-CCR3 (MAB 1551; R&D Systems), anti-CCR2 (SC-7935; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or an irrelevant antibody of the same isotype before placing in the top wells.

Migration was also examined using a scrape-wound assay as described.[38] SMCs (passages 6 to 10) were grown to confluence on 60 mm dishes. Cells were made relatively quiescent by incubating in DMEM +0.1% BSA for 24 hours. Seven linear wounds were made in each plate using a 200-μL pipette tip. Cells were then washed 1× with PBS and then incubated with CCL11 in DMEM +0.1% BSA. In some experiments, SMCs were preincubated for 1 hour at 37°C with anti-CCR3 or an irrelevant antibody before wound formation. Images (40×) were captured with a SPOT camera (Diagnostic Instruments, Inc.). The wound area (immediately after wounding and at 24 hours after) was measured using SPOT Advanced software. Experiments were done in duplicate, with 3 to 5 plates used for each condition. Statistical analyses were done using a one-way ANOVA with post hoc Dunnett's multiple comparisons.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from SMCs using an RNA mini-prep kit (QIAGEN Inc.) and treated with DNA-free (cat # 1906, Ambion). CCR3 primers (sense-5′-ATGGCATTCAACACAGATG-3′, antisense-5′-AATCCAGAATGGGACAGTG3′) were synthesized by Gene Link. The conditions for amplifications were: 15 minutes at 95°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 1 minute at 72°C; followed by a final extension for 10 minutes at 72°C. RT-PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining. RT-PCR products were ligated into the TA3 vector (Invitrogen) and sequenced.

Western Blot Analysis

SMC were washed 2× with cold PBS and lysed in 50 mmol/L TRIS·HCl, 150 mmol/L NaCl, and 1% Triton X-100. Cell lysates were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences). Blots were blocked with 1% milk fat overnight at 4°C, incubated for 1 hour with rabbit polyclonal anti-CCR3 (1 μg/mL), washed with PBS/0.1% Tween 20, and incubated with secondary antibody (goat anti-rabbit IgG, SC-2004; Santa Cruz Biotechnology). Immunoreactive bands were visualized using an enhanced chemiluminescence Western blotting detection kit (Amersham Biosciences).

Flow Cytometry

SMCs grown to 50% to 70% confluence were incubated 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% NaN_3) 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 FACSCalibur flow cytometer, and data were processed using the CellQuest software program (Becton Dickinson).

Immunohistochemistry

Tissue staining was performed on 5-μ-thick paraformaldehyde-fixed, paraffin-embedded sections. After deparaffinization and hydration with PBS, sections were incubated in 3% H_2O_2 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 (MCA519G; 2mcg/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.[39]

Results

SMCs Express CCR3mRNA 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

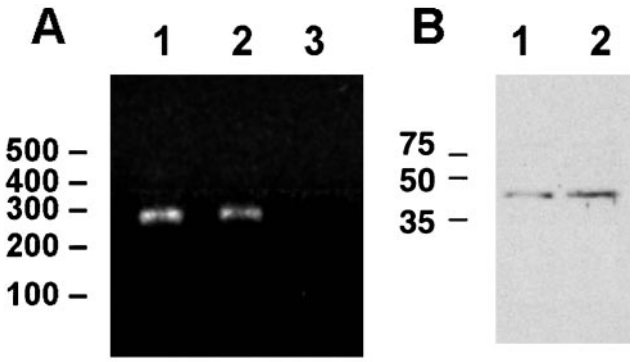

Figure 1. Analysis of CCR3 mRNA and protein in mouse SMCs. A, RT-PCR. Total RNA from aortic SMCs of passage 7 and passage 16 (lanes 1 and 2, respectively) were amplified using a primer pair specific for a 287-bp fragment derived from the mouse CCR3 cDNA. In lane 3, the reverse transcriptase was omitted. B, Western blot analysis was performed using a polyclonal antibody to CCR3. Lane 1, protein extracts from mouse aortic SMC; lane 2, extracts from THP-1 cells.

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

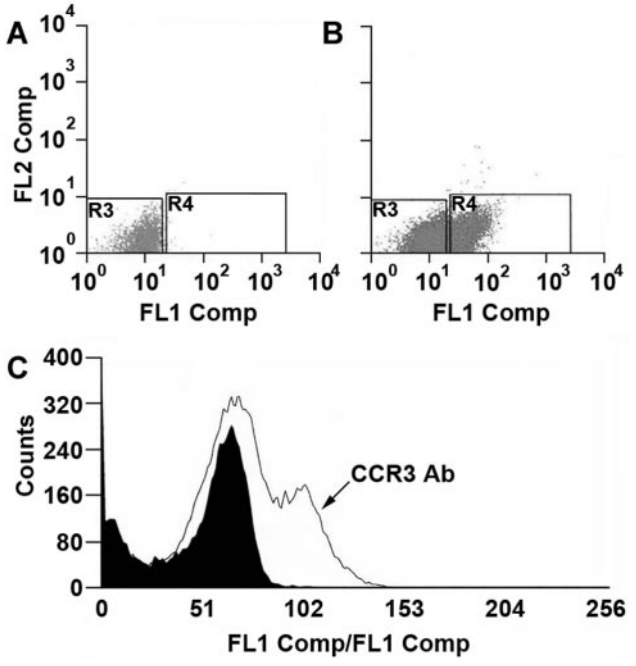

Figure 2. Fluorescent cytometry of mouse vascular SMCs. SMCs were examined by flow cytometry for the expression of CCR3 using FITC-labeled CCR3 and control antibodies. A, IgG control; B, CCR3 antibody; C, Composite showing labeling with control (area in black) and CCR3 (arrow) antibodies. Electronic gates (*R*, R3 & R4) represent forward scatter and delineate shift in fluorescence.

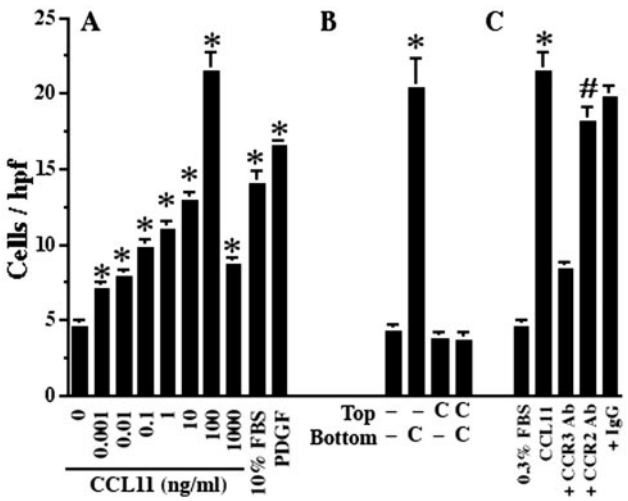

Figure 3. CCL11 induces mouse SMC chemotaxis. A, Mouse CCL11 in 0.3% FCS at the concentrations indicated (ng/mL) were placed in the bottom wells of a modified Boyden Chamber. 10^4 SMCs were placed in the top wells. The chamber was incubated for 6 hours at 37°C and then analyzed for migration as described 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. #$P<0.05$ compared with 0.3% FCS.

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 irrelevent 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, 10^4 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.

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 CCRL11 antigens were not

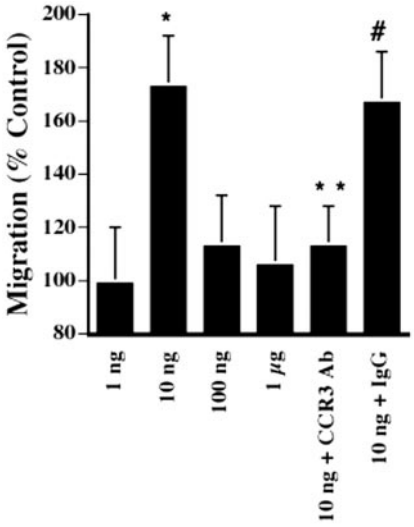

Figure 4. CCL11 induces mouse SMC migration. SMCs were grown to confluence and then incubated in DMEM +0.1% BSA for 24 hours. Linear wounds were made in each plate and SMCs were then incubated with DMEM +0.1% BSA alone (control) or in the presence of CCL11 (concentration in ng/mL indicated on the X axis). 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 CCRL11. 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.

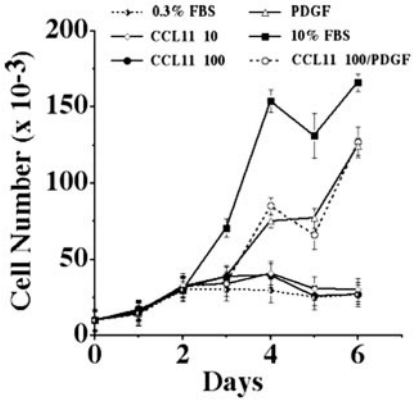

Figure 5. CCL11 does not modulate SMC proliferation. SMCs were plated in 35-mm wells and treated with CCL11 (10 ng/mL or 100 ng.ml) PDGF (1 ng/mL), CCL11 (100 ng/mL) + PDGF (1 ng/mL), 10% FCS, or 0.3% FCS. Duplicate plates were counted on the days indicated. The medium, with fresh growth factors, was replaced every 48 hours. Results are expressed as cell number ± SEM and represent the average of duplicate experiments.

detected in the normal arterial wall. In contrast, CCR3 and CCRL11 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 ∝-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 induced in medial and neointimal SMCs after arterial injury, a state 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,[33] and CCL11 mRNA is upregulated in SMCs of rat aortic allografts exposed to prolonged ischemic storage.[34] 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.[33] Data supporting a role for CCL11 distinct from leukocyte chemotaxis has recently been provided by Salcedo and coworkers,[27] 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.[27] 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,[40,41] CCR8,[32] and CXCR4.[41] 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.[40,41] Activation of CXCR4 by CXCL12 (SDF)-1 or gp120 also induces tissue factor.[41] 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.[32] CCL2 (MCP-1) also acts as an agonist for SMCs,

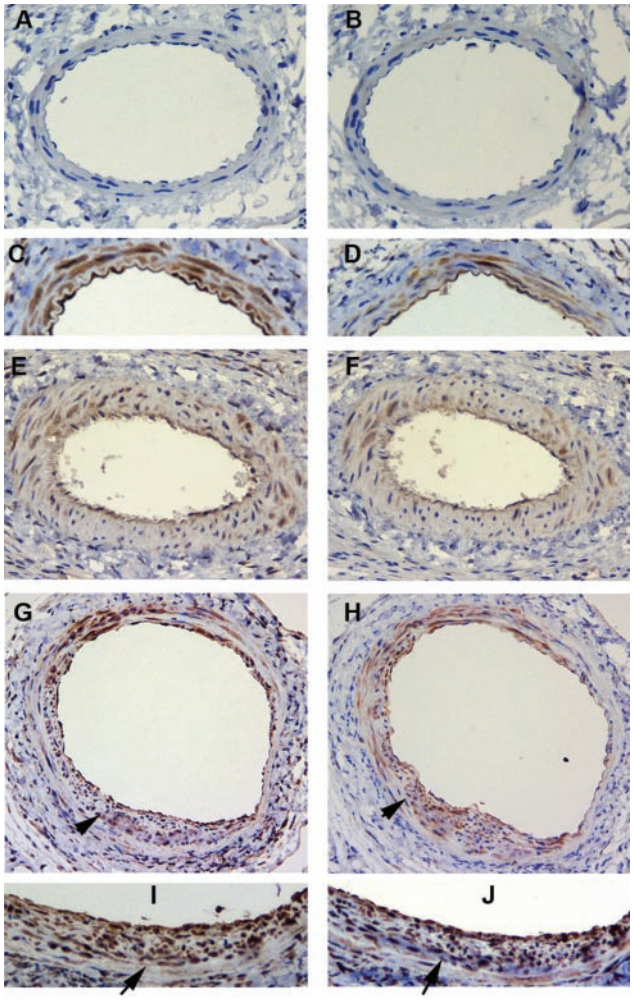

Figure 6. Expression of CCR3 antigen in injured mouse femoral arteries. Samples were obtained from a previous study in which C57Bl/6 mice had undergone wire-induced femoral arterial injury.[39] Sections from uninjured arteries (A, B), arteries harvested 5 days (C, D), 7 days (E, F), and 28 days after injury (G through J) were stained with antibodies to CCR 3 (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.

inducing tissue factor,[42] cytokine production,[43,44] and in some studies proliferation.[43,45] There is controversy as to whether these responses are mediated by the known CCL2 (MCP-1) receptor, CCR2.[42,43] 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[46]). 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.[47] 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[30]). 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.

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