Human Vascular Smooth Muscle Cells Express Receptors for CC Chemokines


Abstract—Arteriosclerotic lesions are characterized by the accumulation of T lymphocytes and monocytes and the proliferation of intimal smooth muscle cells. Expression of the chemokine monocyte chemoattractant protein-1 (MCP-1) has been observed in arteriosclerotic plaques and has been proposed to mediate the transendothelial migration of mononuclear cells. More recently, MCP-1 has been proposed to affect the proliferation and migration of vascular smooth muscle cells (VSMCs). We have used reverse transcription–polymerase chain reaction (RT-PCR) to investigate chemokine mRNA expression in human arteriosclerotic lesions obtained from surgical biopsy of diseased vascular tissue and show, in addition to MCP-1, expression of the chemokine macrophage inflammatory protein-1α (MIP-1α) at higher levels than in “normal” aortic tissue. We have also used RT-PCR to characterize the expression of known chemokine receptors by primary human VSMCs. Messenger RNA for the MIP-1α/RANTES receptor, CCR-1, and the MCP-1/MCP-3 receptor, CCR-2, was expressed by unstimulated VSMCs grown under serum-free culture conditions for 24 hours. The receptors CCR-3, CCR-4, CCR-5, CXCR-1, and CXCR-2 were not expressed by VSMCs. The presence of functionally coupled receptors for MIP-1α on VSMCs was demonstrated by specific binding of biotinylated MIP-1α and increases in intracellular Ca2+ levels after exposure to this chemokine. Taken together, these results suggest that chemokines are likely to be involved in arteriosclerosis and may play a role in modulating the function of VSMCs in vivo. (Arterioscler Thromb Vasc Biol. 1998;18:397–403.)

Key Words: chemokines ■ receptors ■ humans ■ vascular smooth muscle cells ■ arteriosclerosis

Lymphocytes, macrophages, and associated cytokines are well accepted as orchestrators of many chronic inflammatory diseases, including asthma/allergy, arthritis, and multiple sclerosis. There is now accumulating evidence for the involvement of humoral and cellular immune reactions in the development of arteriosclerosis. Growth factors and cytokines derived from inflammatory cells can stimulate migration and proliferation of VSMCs and the formation of mature lesions.

Chemokines are now widely accepted as playing a pivotal role in the recruitment of leukocytes from the blood compartment into tissues, and their presence in human inflammatory disease has been widely documented. The chemokine superfamily can be sorted into four groups on the basis of the structural arrangement of four conserved cysteine residues. In the CXC family, the first two cysteines are separated by an intervening amino acid; these chemokines, which include IL-8, are generally involved in neutrophil and T-cell chemotaxis and activation. The CC chemokines, such as MCP-1, MIP-1α, and RANTES (regulated on activation, normal T cell expressed and secreted), have two adjacent cysteine residues and act more generally on monocyte/macrophages, lymphocytes, eosinophils, and basophils. The C and CX, C groups each have only one known member, both of which act on T cells. The biological responses to chemokines are mediated by a family of seven-transmembrane G-protein−coupled receptors. At least eight human CC chemokine receptors have been cloned (CCR-1 through CCR-8) and four receptors for the CXC chemokines (CXCR-1 through CXCR-4). The majority of these receptors have considerable overlapping ligand specificity and to date have generally been found on leukocytes.

The chemokine MCP-1 may play an important role in the establishment and propagation of arteriosclerosis, since mRNA and protein have been detected in arteriosclerotic plaques. In addition to being secreted by stimulated human macrophages, MCP-1 is also produced by cultured VSMCs and endothelial cells stimulated with cytokines and minimally modified low density lipoprotein. RANTES and MIP-1α expression has also been documented in a range of chronic inflammatory diseases. We and others have recently demonstrated that RANTES is expressed by stimulated human VSMCs and endothelial cells.

The ability of vascular smooth muscle to respond to proinflammatory cytokines shows that it has characteristics of an immune-regulated tissue. There is also some recent evidence that VSMCs can respond to certain chemokines, including
IL-8, MCP-1, and TCA3.42–45 The purpose of this study was to determine whether other chemokines, in addition to MCP-1, are expressed in arteriosclerotic lesions and also whether isolated human VSMCs express receptors that enable them to respond to these chemokines. Here we report the expression of MIP-1α and RANTES, as well as MCP-1, mRNA in arteriosclerotic lesions. We also report that human VSMCs express MIP-1α/RANTES receptor CCR-1 and the MCP-1/MCP-3 receptor CCR-2. Binding and functional studies are described that demonstrate the presence of functionally coupled receptors for MIP-1α on VSMCs, CCR-1 being a candidate receptor.

**Methods**

**Reagents**

Cytokines and chemokines were obtained from Peprotec. All cell culture reagents and HI-FBS were purchased from GIBCO-BRL. Oligonucleotide primers were synthesized by Cruachem and GIBCO-BRL. Standard reagents were from Sigma or Fisons. Cytokines and chemokines were obtained from Peprotec. All cell culture reagents and HI-FBS were purchased from GIBCO-BRL. Oligonucleotide primers were synthesized by Cruachem and GIBCO-BRL. Standard reagents were from Sigma or Fisons.

**Isolation of Peripheral Blood Leukocytes**

Whole blood was obtained from normal donors, and standard methods were used to prepare PBMCs, CD18 neutrophils, and an eosinophil/basophil fraction. In brief, blood was centrifuged (10 minutes at 1500 g) to remove plasma. PBMCs were separated from red blood cells and neutrophils after density-gradient centrifugation on Lymphoprep (Nycomed). Red blood cells were removed from neutrophils by centrifugation through dextran T500. Eosinophils and basophils were separated from CD18 cells by negative selection, using magnetic beads coupled to anti–CD 16 antibodies (MACS, Miltenyi Biotec) as described by Hansel et al.38

**Isolation and Culture of SMCs**

SMCs were isolated from human saphenous vein by a standard explant method. Briefly, small moistened pieces of tissue (6 mm²) were placed in a tissue–culture flask and left to adhere for 2 hours, and then the base of the flask was carefully flooded with culture medium consisting of Dulbecco’s modified Eagle’s medium containing 15% HI-FBS, penicillin (10 U/mL), streptomycin (10 μg/mL), and fungizone (0.5 μg/mL) and left undisturbed for 2 to 3 weeks. Primary isolates were grown to confluence in culture medium and passaged using trypsin/EDTA. Cells were fixed for 10 minutes in ice-cold methanol and immunocytochemical analysis showed them to be positive for the smooth muscle cell–specific marker α-actin. Cells were used from five different patients between passages 2 and 9. To render cells quiescent, FBS was removed from the culture medium for 24 hours before all experiments. For RT-PCR studies, cells were treated for 3 to 4 hours with serum-free culture medium alone or unstimulated controls or with culture medium containing 15% HI-FBS or TNFα (30 ng/mL) before isolation of RNA. Treatments were applied to cells from the same donor.

**Biotinylated MCP-1 and MIP-1α Binding Studies**

Binding of MCP-1 and MIP-1α to VSMCs was measured using Fluorokine kits (R & D Systems) according to the manufacturer’s instructions. Briefly, VSMCs were grown to confluence in tissue–culture flasks coated with 1% gelatin and then depleted of serum for 24 hours. Cells were harvested with trypsin/EDTA, washed three times, and resuspended in PBS. We have previously found that trypsin treatment of MCP-1 receptor–transfected HEK 293 cells does not affect their ability to bind or respond to MCP-1.39 Biotinylated MCP-1α, MCP-1 (at final concentration of 150 nmol/L), or negative control protein (biotinylated soybean trypsin inhibitor) was incubated at 4°C with 1×10⁶ cells, and binding was detected by using avidin–FITC. Cells were washed and analyzed by flow cytometry on a FACS Vantage cell sorter (Becton Dickinson), using excitation at 488 nm and fluorescence emission of 520 nm. The specificity of MIP-1α binding was tested either by preincubating the biotinylated MIP-1α with anti-human MIP-1α antibody for 15 minutes at room temperature or by the addition of 100-fold excess of nonbiotinylated MIP-1α.

**Measurement of Intracellular Ca²⁺ Elevation**

VSMCs were seeded onto sterile glass coverslips coated with 0.1% type I collagen. The cells were allowed to adhere at 37°C overnight in serum-free medium. Washed cells were loaded with fura 2-AM (Molecular Probes) mixed with an equal volume of 25% pluronic F-127 (Molecular Probes) diluted to 5 μmol/L in HBSS containing BSA (1 mg/mL) and Ca²⁺/Mg²⁺ (1 mmol/L) at 37°C for 30 minutes. Cells were washed and left to equilibrate in HBSS for 30 minutes before use. The calcium flux was measured using a PTI dual wavelength spectrofluorimeter (Photon Technology International) with excitation at 340 and 380 nm and emission at 510 nm using a fiber-optic light guide to illuminate a microscope stage. Elevation in Ca²⁺ was assessed as an increase in the ratio of the signals at the two excitation wavelengths. Coverslips were placed in a heating chamber at 37°C and bathed in 500 μL HBSS. Groups of approximately five cells were selected, and the Ca²⁺ flux in response to 500 nmol/L chemokine was measured. Thrombin (4 U/mL) was used as a positive control to confirm cell viability.

**RNA Isolation**

Total cellular RNA was extracted from VSMCs, PBMCs, neutrophils, eosinophils/basophils, and tissues, using a commercial solution containing guanidinium thiocyanate (RNAsol, Tel-Test) as described by the manufacturer. Human surgical biopsy specimens (~50 to 200 mg) from abdominal aortic aneurysm and carotid endarterectomy (Gloucestershire Royal Hospital) and control tissue from organ transplant donors (Harefield Hospital) were snap-frozen and stored in liquid N₂ immediately after surgical isolation. These were added to a solution of RNAsol while still frozen and were homogenized using an Ultra-Turax (IKA-Labortechnik). The homogenate was cleared by centrifugation (10,000 g, 10 minutes) and product size for each gene-specific primer pair used are shown in the Table. The conditions for amplification were 5 minutes

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**Table: Reagents**

- HI-FBS = heat-inactivated fetal bovine serum
- IL = interleukin
- MCP = monocyte chemoattractant protein
- MIP = macrophage inflammatory protein
- PBMC = peripheral blood mononuclear cells
- RT-PCR = reverse transcription–polymerase chain reaction
- TNF = tumor necrosis factor
- VSMC = vascular smooth muscle cell
at 94°C, 35 cycles of 1 minute at 94°C, 1 minute at 55°C to 58°C, 2 minutes at 72°C, followed by an extension for 10 minutes at 72°C. PCR products were resolved by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. Images were captured by video camera and GRABBIT software (UV Products).

Gene-specific oligonucleotide primers were designed using Laser-gene software (DNASTAR). To control for genomic DNA contamination in PCR reactions, sense and antisense primers were designed to span an intron-exon splice site. Chemokine receptor genes, however, do not contain intron sequences. As a consequence, an identical parallel PCR reaction was performed, in which reverse transcriptase was omitted from the reverse transcription reaction.

**Results**

**Chemokines MIP-1α, RANTES, and MCP-1 Are Expressed in Arteriosclerosis**

To determine whether other chemokines, in addition to MCP-1, are expressed in arteriosclerosis, we have examined biopsy specimens of aortic, iliac, and carotid arteries, which were isolated during surgical procedures for vascular disease (abdominal aortic aneurysm and carotid endarterectomy). Each specimen was assessed histopathologically and shown to exhibit extensive arteriosclerotic lesions. Messenger RNA expression was measured by RT-PCR for MIP-1α, MCP-1, MCP-3, and RANTES, relative to the loading control gene GAPDH. As shown in Fig 1, mRNA for MIP-1α, MCP-1, and RANTES was readily detected in all specimens of diseased tissue, as was GAPDH mRNA; MCP-3 mRNA expression could not be detected. The normal tissue used as a control (thoracic aorta obtained from organ transplant donors) did not express MCP-3 or MIP-1α. Some of the specimens showed low expression of MCP-1, and RANTES was detected in all normal tissues examined. No obvious arteriosclerotic lesions were detected in control specimens.

**Chemokine Receptor mRNA for CCR-1 and CCR-2 Are Expressed by Human VSMCs**

With the use of RT-PCR and by designing gene-specific primers, we were able to distinguish between closely related members of the chemokine receptor family, which share 20% to 80% sequence identity. Gene-specific primer pairs were validated by examining their expression in PBMC, neutrophil, and eosinophil/basophil cell fractions of human peripheral blood (Fig 2). CCR-1, -2, -4, and -5 mRNA was significantly expressed by PBMCs. CCR-3 was predominantly expressed by the eosinophil/basophil fraction, while both CXCR-1 and CXCR-2 were expressed by neutrophils. The authenticity of each RT-PCR product was confirmed by DNA sequencing.

To determine whether chemokine receptors are expressed by VSMCs, human VSMCs were isolated from saphenous vein and cultured by explant. The results of representative experiments from three individual patients (a, b, and c) are shown in Fig 2. Both CCR-1 and CCR-2 mRNA was significantly expressed by unstimulated VSMCs (cultured for 24 hours in

<table>
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<th>Product Length, bp</th>
<th>Sequence</th>
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the absence of serum: lane 1, patients a, b, and c), at levels comparable to those found in peripheral blood cells under identical culture conditions. None of the other chemokine receptor mRNAs tested were detected.

We have examined whether chemokine receptor mRNA was differentially regulated by mitogenic stimuli or proinflammatory cytokines. VSMCs were treated for 3 to 4 hours with media containing either 15% HI-FBS or 30 ng/mL TNFα. This concentration of TNFα induces chemokine expression in VSMCs.29 The results were inconclusive; in one culture stimulation with FBS appeared to downregulate the expression of both CCR-1 and CCR-2 mRNA (Fig 2, patient a; compare lanes 1 and 2); however, this result was not found in the other two cultures examined (patients b and c). Stimulation with TNFα did not appear to regulate receptor mRNA expression. None of the other chemokine receptor mRNAs tested were detected after stimulation with either FBS or TNFα.

MIP-1α Binds to Human VSMCs

The ligands known to bind to CCR-1 are MIP-1α, RANTES, MCP-3, and to a lesser extent MCP-1.14,20,49 The known ligands for CCR-2 are MCP-1 and MCP-3.15,19 Human VSMCs were incubated with biotinylated MIP-1α or MCP-1 to determine whether receptors for these ligands were expressed on the cell surface. Bound ligand was measured by flow cytometry. MIP-1α was found to bind to VSMCs, shown as a rightward shift in the fluorescence peak compared with the negative control (Fig 3). Binding was completely blocked by preincubation of the biotinylated MIP-1α with anti–MIP-1α antibody or coincubation with nonbiotinylated MIP-1α (1.5 μmol/L). The analysis included 20 mg/mL prooxidant iodide to enable the exclusion of nonviable cells. Results are representative of five separate experiments in which the mean fluorescence intensity for MIP-1α binding was 26±3.3 and 15±2.3 for antibody displacement.

Identification of a Functional MIP-1α Response in Human VSMCs

Chemokine-stimulated elevation of cytosolic Ca2+ concentration is a characteristic response of most leukocyte chemokine receptors. Transient Ca2+ elevation in adherent VSMCs could be reproducibly induced by 500 nmol/L MIP-1α. The amplitude of the response was variable, but a typical example is shown in Fig 4a. A lower concentration of 100 nmol/L MIP-1α did not induce a response. Previous experience with inflammatory cells would suggest that the relatively prolonged Ca2+ elevation observed after exposure to MIP-1α is indicative of a sustained Ca2+ influx response. This finding contrasts with the transient response to thrombin, which appears to be mainly composed of Ca2+ mobilization followed by a brief Ca2+ influx. A smaller Ca2+ elevation occurred in response to 500 nmol/L MCP-1 (Fig 4b) but was not seen consistently in all
mented, but we and others have also shown in in vitro studies the identification of the cell types producing chemokines. Chemokine expression of mononuclear cells into atherosclerotic lesions. The presence of mRNA for MIP-1 has previously been described in carotid plaque and coronary arteries obtained from transplanted hearts. We have shown in this study that in addition to MCP-1, the chemokines MIP-1 and RANTES are also expressed in spontaneously occurring atherosclerosis from a number of sites, namely the abdominal aorta and the carotid and iliac arteries. RANTES mRNA was also found in arteriosclerotic lesions from a number of sites, namely the abdominal aorta and carotid and iliac arteries. RANTES is also expressed in spontaneously occurring arteriosclerosis.

Figure 4. Elevation in intracellular Ca\(^{2+}\) in adherent VSMCs caused by MIP-1/α and MCP-1. Adherent smooth muscle cells were loaded with fura 2-AM, washed, and Ca\(^{2+}\) flux was measured by changes in ratio of the fluorescence signals at the excitation wavelengths of 340 and 380 nm using a spectrofluorimeter linked to a microscope. Cells were left to equilibrate at 37°C in HBSS (500 μL). They were then stimulated with 20 μL MIP-1/α (a) or MCP-1 (b) to give a final concentration of 500 nmol/L. When Ca\(^{2+}\) had returned to basal levels, the cells were restimulated with 4 U/mL thrombin, which was used as a positive control. The example shown is of a representative experiment.

 Cultures examined. Cells were viable, as shown by their ability to respond to thrombin. The chemokines IL-8 and RANTES, tested at both 100 and 500 nmol/L, did not cause Ca\(^{2+}\) elevation (data not shown).

Discussion

The monocyte chemoattractant protein MCP-1 was the first chemokine to be described in arteriosclerotic plaques. It has been proposed that MCP-1 plays a key role in the recruitment of mononuclear cells into atherosclerotic lesions. The presence of mRNA for MCP-1/α and RANTES has previously been described in carotid plaque and coronary arteries obtained from transplanted hearts. We have shown in this study that in addition to MCP-1, the chemokines MIP-1/α and RANTES are also expressed in spontaneously occurring arteriosclerosis from a number of sites, namely the abdominal aorta and the carotid and iliac arteries. RANTES mRNA was also expressed in all control samples (aorta); however, while apparently healthy, it is possible that these tissue specimens were affected by early arteriosclerotic events. These chemokines, in addition to being chemoattractive for monocytes, are also potent chemoattractants and activators of T lymphocytes, which are present in arteriosclerotic lesions. The role of the T cell in arteriosclerosis is still unclear, but cytokines produced by these lymphocytes undoubtedly affect vascular-immune interactions.

Our studies with atherosclerotic tissue do not enable identification of the cell types producing chemokines. Chemokine production from inflammatory cells has been well documented, but we and others have also shown in in vitro studies that smooth muscle cells can be potent sources of chemokines, including IL-8, MCP-1, RANTES, and MIP-1α, when stimulated with proinflammatory cytokines. These chemokines can also be produced by stimulated endothelial cells.

We have shown that cultured human VSMCs constitutively express mRNA for the chemokine receptors CCR-1 and CCR-2. The recognized ligands for CCR-1 are MIP-1α, RANTES, MCP-3, and to a lesser extent MCP-1, while CCR-2 binds MCP-1 and MCP-3. Binding studies clearly show that our VSMCs express an MIP-1α binding protein on the cell surface. In addition to CCR-1, known MIP-1α binding proteins include CCR-4 and CCR-5. However, the absence of mRNA for these receptors implies that CCR-4 and CCR-5 is not the identity of the MIP-1α binding protein.

Our data are therefore consistent with the possibility that CCR-1 mRNA is correctly processed and expressed on the VSMC surface; however, absolute identity must await detection at the protein level.

Chemokine-stimulated cytosolic Ca\(^{2+}\) elevation appears to be a consequence of signaling via phospholipase C–coupled G-protein receptors. MIP-1α consistently caused an elevation of intracellular Ca\(^{2+}\) in VSMCs. We were unable to detect a similar increase in response to another CCR-1 ligand, RANTES. The MIP-1α response was not detected at concentrations below 500 nmol/L, and since RANTES has a lower potency than MIP-1α for CCR-1, still higher concentrations of RANTES may have been needed to elicit a response. However, such higher concentrations were not tested because it was considered that the physiological relevance would be questionable. It is also possible that in VSMCs the RANTES response is not coupled to an elevation in intracellular Ca\(^{2+}\). We have previously observed that this is certainly the case for primary T lymphocytes, which exhibit chemotactic responses but fail to show Ca\(^{2+}\) elevation in response to RANTES.

It must also be considered that the MIP-1α receptor expressed was not CCR-1 but another as yet unidentified chemokine receptor. MCP-1 caused a much smaller Ca\(^{2+}\) flux, which was not seen consistently in all cells examined. Since both CCR-1 and CCR-2 can bind MCP-1, this functional response could be occurring via either receptor, although CCR-1 has a lower affinity for MCP-1. The presence of a functional MCP-1 receptor seems to contradict the inability to detect MCP-1 binding to VSMCs when a fluorokine kit was used, which we have previously shown detects MCP-1 binding in receptor-transfected HEK cells. The binding assay may be insufficiently sensitive to pick up binding if receptor density per cell is low. Others have reported problems detecting chemokine binding to cells that produce functional responses to the same chemokine.

An increase in cytosolic Ca\(^{2+}\) and inositol lipid hydrolysis are signals often associated with cell proliferation and migration. These functional responses have been previously shown in VSMCs stimulated with IL-8, MCP-1, or TCA3. Yue et al showed that IL-8 was able to stimulate the growth of rat and human VSMCs and the migration of rat VSMCs, although it is unclear how these signals may be transmitted; Schonbeck et al were unable to measure IL-8 binding to human VSMCs. This observation is in agreement with our inability to detect
CXCR–1 or -2 receptor mRNA. MCP-1 also has functional effects on VSMCs, but these seem to vary depending on the species used. In rat VSMCs, MCP-1 appears to inhibit growth and mitogen-activated protein kinase activity.61 Since we completed these studies, Xu et al59 reported that porcine VSMCs have mRNA for the MCP-1 receptor CCR-2, and MCP-1 was reported to induce VSMC migration and proliferation. In addition, Luo et al62 have demonstrated that the CC chemokine TCA3 but not MCP-1 or MIP-1α can induce proliferation of rat VSMCs, although all caused some migration.

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In our study on human VSMCs, we have demonstrated that MIP-1α and to a lesser extent MCP-1, but not IL-8 or RANTES, induce an elevation in cytosolic Ca2+, together with a clear demonstration of MIP-1α binding. The functional significance of this response is currently being investigated.

Our results suggest that CCR-1 and possibly CCR-2 are expressed constitutively on quiescent cultured human VSMCs and are not upregulated by proinflammatory cytokines. Preliminary results suggest that FBS may be able to downregulate chemokine receptor mRNA expression in VSMCs, although the functional significance remains to be investigated. It may be speculated that chemokines and their receptors play a role in homeostasis in the normal vessel and/or in response to injury. MCP-1 mRNA is rapidly upregulated 1 to 4 hours after injury to rabbit aorta, preceding the migration and proliferation of VSMCs.63 Low levels of particular chemokines may prevent the normal VSMC layer from proliferating. MIP-1α is an unusual chemokine in this respect, since it has been shown to inhibit the proliferation of several cell types, including hematopoietic stem cells, dermal keratinocytes, and spermatogonia.64–66 It remains to be seen how MIP-1α affects VSMC function.

Although chemokines were initially identified for their ability to induce selective recruitment of leukocyte populations, they have now been shown to have a broad range of functions, including the modulation of HIV cellular uptake,4,65 keratinocyte proliferation,66 effects on the vasculature such as angiogenesis, and aspects of smooth muscle cell function such as chemotaxis and proliferation.67 Further studies are clearly needed to determine whether chemokine receptors on VSMCs play a role in affecting the course of arteriosclerosis in vivo. Nevertheless, the VSMC must now be regarded as a truly immune-responsive cell able to both produce and respond to chemokines.

Acknowledgments
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References


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