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Chemokine Receptor CXCR4 Expression in Endothelium

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The expression of chemokine receptor and viral coreceptor CXCR4 is reported in cultured endothelial cells and in arterial endothelium. A 1.9 kb transcript was cloned from cultured bovine aortic (BAEC) and human umbilical vein endothelial cells (HUVEC). CXCR4 mRNA was expressed at high levels in BAEC and HUVEC but was not expressed by cultured bovine arterial smooth muscle cells (BASM) or human umbilical vein smooth muscle cells (HUVSM). Western blotting with polyclonal antibodies demonstrated an approximate 46KD protein in endothelial cells only. *In situ* hybridization and immunocytochemistry (anti-CXCR4 monoclonal antibody 12G5) revealed both transcript and protein expression in cultured endothelial cells, and in the endothelium of normal aorta but not in aortic smooth muscle. The ligand for CXCR4, stromal cell derived factor 1 (SDF-1) stimulated mobilization of intracellular calcium at a moderate level (37% of the peak response to thrombin), confirming the expression of functional receptor at the endothelial surface. The involvement of CXCR4 in chemokine signaling, chemoattraction (through SDF-1), and its potential viral coreceptor activity suggest a multifunctional role in vascular homeostasis and pathophysiology. © 1998 Academic Press

Of the inflammatory molecules thought to play a role in atherogenesis, the most recent class is the chemotactic cytokine family known as chemokines, small proteins that stimulate chemotaxis (1). The family is divided into four classes based on the sequence around the first cysteine residue: CXC, CC, C or CX3C of which the α (CXC) and β (CC) classes are the largest (2,3). Chemokines are involved in the attraction and activation of mononuclear and polymorphonuclear leukocytes. Various members are synthesized and secreted from endothelial and smooth muscle cells when stimulated by appropriate cytokines (4,5). Their receptors are members of a superfamily of G protein-coupled receptors characterized structurally by seven transmembrane spanning domains and functionally by intracellular signaling via G proteins.

Various chemokines implicated in leukocyte recruitment during inflammation and atherogenesis have been shown to be constitutively expressed or inducible in vascular tissues (6), but in the vascular endothelium only the Duffy blood group antigen has been definitively identified as a chemokine receptor (7-9), there being indirect evidence for IL-8A receptor (10,11) and melanocyte growth stimulating activity (GRO/MGSA) receptor (8) and the leukocyte chemoattractant platelet activating factor (12).

An important additional function for some chemokine receptors is their utilization as coreceptors for HIV-1 infection of $CD4^+$ cells of the immune system (13-19). Different strains of HIV-1 use different chemokine receptors as entry cofactors. CXCR4 and CCR5 appear to be the most important coreceptors for macrophage-tropic and T-cell-tropic HIV-1 isolates, respectively (13,15,16). Dual tropic strains use CCR2b, CCR3, CCR5, and CXCR4, whereas a subset of macrophage-tropic isolates requires CCR3 (18).

The demonstration here of endothelial CXCR4 expression at the transcriptional, protein, and functional levels at the blood/tissue interface suggests a significant role in vascular pathobiology.

MATERIALS AND METHODS

Cell culture. HUVEC and HUVSM, isolated from umbilical cords, were used either in primary culture or within 5 passages. BAEC were used between passages 8 and 30; BASM were used within 10 passages. J774 and HL60 cells, and bovine pulmonary artery endothelial cells (BPAEC) were obtained from the American Type Culture Collection (ATCC) repository; BPAEC were used between passages

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12 and 18. BAEC, BASM, BPAEC, and J774 cells were cultured in Dulbecco's modified Eagle's medium (Gibco/BRL, Grand Island, NY) supplemented with 10% heat inactivated calf serum plus 2mM L-glutamine, 50U/ml penicillin, and 50mg/ml streptomycin (glu-pen-strep). HUVEC and HUVSM were cultured in Medium 199 (Gibco/BRL, Grand Island, NY) suplemented with 20% heat inactivated fetal calf serum, endothelial growth factor, heparin, and glu-pen-strep. HL60 cells were cultured in RPMI 1640 (Gibco/BRL) supplemented with 10% heat inactivated calf serum and glu-pen-strep.

Reverse transcriptase-PCR (RT-PCR). Total RNA (5μg) was prepared from BAEC and first strand cDNAs were synthesized using a modified oligo dT primer and MMLV RT (United States Biochemical, Cleveland, OH; USB). The PCR product was synthesized using degenerate 5′ and 3′ primers for the 3rd and 6th transmembrane domains, respectively, of G-protein coupled receptors. Degenerate primers were derived, with modifications, from Libert *et al* (20).

Cloning and sequencing. Several distinct DNA fragments were generated by the second PCR reaction. A 389bp fragment was subcloned into plasmid Bluescript II KS (Stratagene, La Jolla, CA). Double stranded cDNA of the clone was sequenced by the Sanger dideoxy chain termination technique using Sequenase 2.0 (USB) and ^{35}S dATP (Amersham, Arlington Heights, IL). The sequence was identical to that of LCR −1 (21), now designated CXCR4. Total RNA was isolated from cultured cells by the guanidine thiocynate-acid-phenol method (22). RNA (10μg per lane) was size fractioned on a denaturing 1% agarose-formaldehyde gel and transferred to GeneScreen Plus (DuPont, NJ) for Northern blot hybridization analysis. The DNA probes were radioactively labeled with ^{32}P dCTP (Amersham) using Prime-It II (Stratagene). Blots were probed overnight at high stringency with a 389bp EcoRI-BamHI fragment of CXCR4 or a 1293bp fragment of rat GAPDH.

In situ hybridization. Rabbit thoracic aortas were obtained at the time of sacrifice, washed briefly in PBS, dissected, positioned, covered in OCT, and immediately immersed in isopentane and dry ice. 6-8μm cryostat sections were collected on gelatin/chromalum-coated slides. They were dipped in 4% paraformaldehyde (PFA)/PBS for 20 min at room temperature, followed by sequential dehydration in ethanol, and air-dried. Cells were grown to confluence on gelatin/chromalum-coated slides, washed in PBS and treated as described for the tissue slides. The protocol for *in situ* hybridization was based on that of Higgins and Wilson (23). The full coding sequence of CXCR4 was amplified from HUVEC RNA using primers specific for the 5′ and 3′ non-coding regions: forward primer 5′GAGGAATTC-CACGAGCGGCACAGGGTAGCAAAG3′; reverse primer 5′CTC-AAGCTTATT AGCTGGAGTGAAAACTTGAAGT3′.The product was cloned into Bluescript plasmid, sequenced, and shown to be identical to human CXCR4. A 569bp clone (bp 2-571) was used as a template to generate radiolabeled antisense and sense riboprobes with T7 and T3 RNA polymerase (Stratagene) respectively. Transcription reactions were performed according to the manufacturer's protocols using uridine ^{35}S-triphosphate labeled linearized plasmid DNA.

Immunocytochemistry. Confluent cultured endothelial cells and 6μm sections of rabbit thoracic aorta on gelatin-coated (0.1%) slides were stained with monoclonal anti-CXCR4 antibody (Mab 12G5; dilution 1:125), a gift of Dr. James Hoxie, Department of Pathology and Laboratory Medicine, University of Pennsylvania, using standard procedures.

Western blotting with polyclonal anti-CXCR4 antibodies. Synthetic peptides representing the carboxy terminus of CXCR4 were conjugated to KLH and used to generate rabbit polyclonal anti-CXCR4 antisera (HTI, Ramona, CA). Sera were affinity purified using synthetic CXCR4 peptides conjugated to Affi-Gel 10 (BioRad) and used for Western blotting of BAEC and BASM according to standard procedures. Detection was by enhanced chemiluminescence.

Intracellular calcium. Calcium mobilization was measured in Fura-2 loaded confluent monolayers of bovine aortic endothelial cells using a Xenon illumination source (OPTI-OUIP, Highland Mills, NY) coupled to a Nikon Diaphot Eclipse TE300 equipped with a VS4-1845 Intensifier and a CCD-200E camera (Video Scope International, Ltd., Sterling, VA). Post-acquisition analysis was performed using Axon Imaging Workbench software (Axon Instruments, Foster City, CA). Forty individual cells in a field of view were selected to image and the average intensity at wavelengths 340 nm and 380 nm was calculated for each cell referenced against calibration standards.

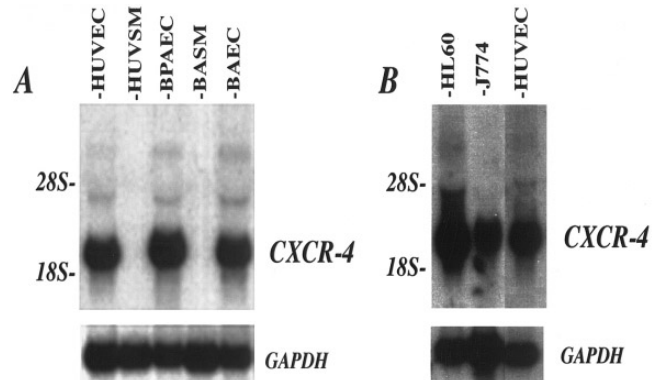

FIG. 1. CXCR4 expression in cells *in vitro.* Representative northern blots of total cellular RNA (10μg/lane) from vascular cells (1A) and mononuclear leukocytic cells (1B). Corresponding GAPDH expression in the same blots is shown for reference.

RESULTS

To identify novel serpentine receptors in bovine aortic endothelial cell (BAEC) RNA, degenerate primers for the highly conserved third and sixth transmembrane domains of known G protein-coupled receptors were used in the reverse transcriptase-polymerase chain reaction (RT-PCR). PCR products were then subcloned and sequenced. One clone, reported by us in preliminary form as endothelial serpentine orphan receptor-1 (ESOR-1) (24), was found to be identical to CXCR4.

CXCR4 mRNA was prominently expressed in HUVEC, BAEC, and BPAEC as a single 1.9 kb transcript (389bp fragment probe), but was undetectable in BASM and HUVSM (Figure 1A). The transformed cell lines HL60 promyelocytic cells and J774 macrophages expressed CXCR4 mRNA (Figure 1B). Expression of the appropriate transcript size in HL60 cells represented a positive control for the probe, HL60 being a cell line known to express CXCR4 (25,26).

Significant levels of CXCR4 mRNA were detected in northern blots of rabbit thoracic aorta and rat lung, but only traces were detectable in heart and spleen, and none in kidney (not shown). The basis of tissue heterogeneity, also noted by others (21,25, 27), is unclear and may reflect tissue regulation.

In situ hybridization of a variety of cells and tissues was performed using a 569bp CXCR4 probe. This larger probe was identical to the 389bp probe on northern

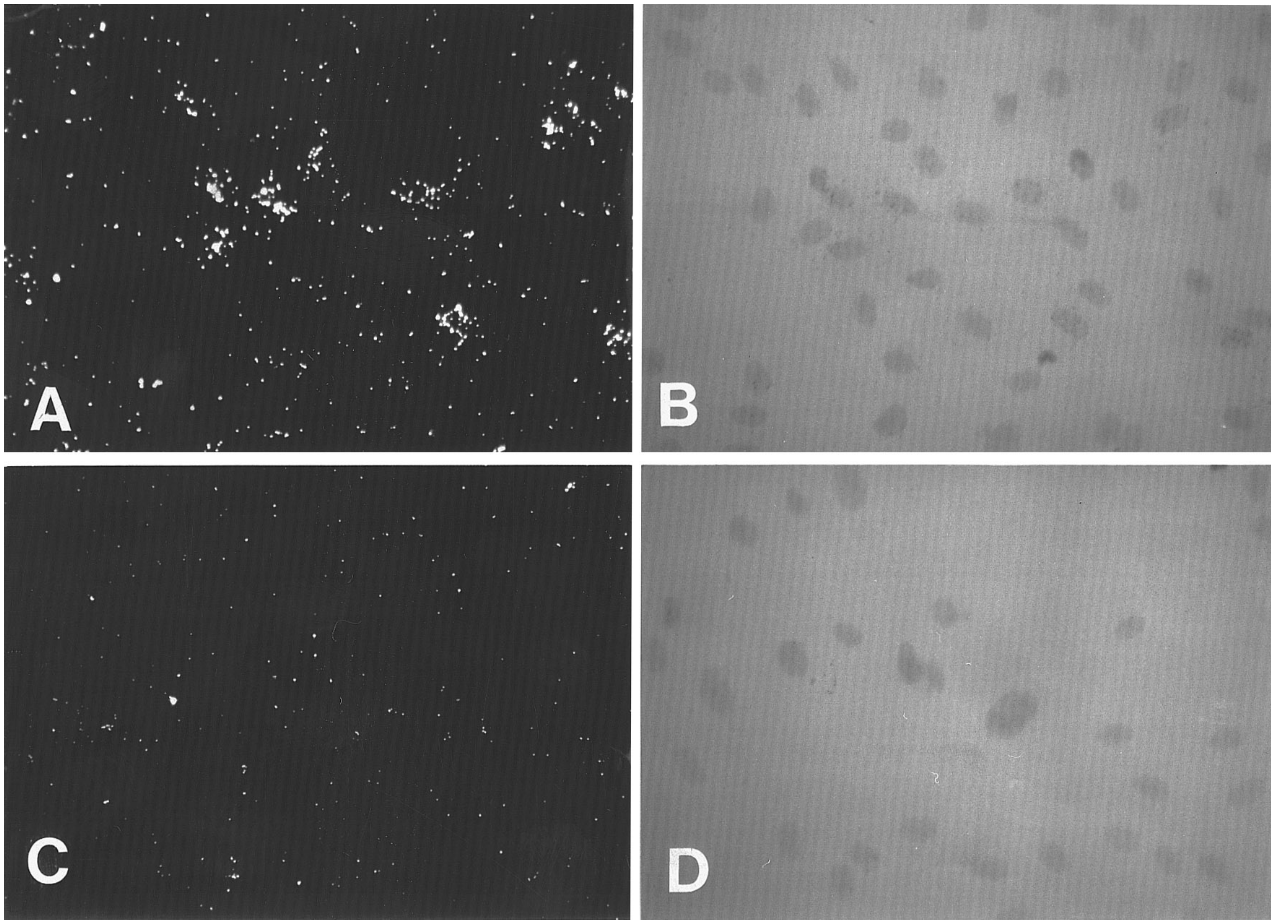

FIG. 2. Detection of CXCR4 by *in situ* hybridization of cultured human umbilical vein endothelial cells (HUVEC). Cells probed with antisense (A and B) and sense (C and D) CXCR4 cDNA are shown in dark-field and bright-field images, respectively. As is evident in panel A, some HUVEC expressed higher levels of CXCR4 than others within the same monolayer. Grain counts per cell were 38.2 ± 5.5 (SD) for cells expressing CXCR4, 4.1 ± 0.2 for non-expressors, and 3.1 ± 0.1 for cells probed with sense cDNA; n=10 for each group.

blots. Strong mRNA expression in cultured HUVEC and BAEC was confirmed by comparison of antisense and sense hybridizations (shown for HUVEC in Figure 2A where levels were approximately 10-fold higher in cells probed with antisense compared with sense control probe, Figure 2C). Heterogeneous expression of CXCR4 was prominent in HUVEC with some cells expressing much higher transcript levels than others in the same monolayer.

Some tissue endothelia expressed detectable levels of CXCR4. *In situ* hybridization of frozen sections of rabbit aorta revealed high grain counts over the endothelium compared with low levels throughout the remainder of the tissue (composed of smooth muscle cells and extracellular matrix) when hybridized with antisense probe (Figure 3). As was observed in cultured cells, some endothelial cells in the artery appeared to express higher levels than others (Figure 3B).

Western blotting of endothelial and smooth muscle cell proteins with polyclonal antiserum raised against the cytoplasmic C-terminal region of CXCR4 stained an endothelial protein of approximately 46KD, the predicted molecular weight of CXCR4 (Figure 4). This band was absent in BASM; however several other bands identified by affinity purified antiserum were common to both endothelium and smooth muscle cells, rendering it non-discriminatory for immunocytochemical microscopy. To circumvent this problem, a murine monoclonal antibody (12G5) against an extracellular epitope of CXCR4 was used as a probe for immunolocalization of CXCR4 protein.

12G5 monoclonal antibody directed against an epitope of the second extracellular loop of the receptor revealed protein expression in cultured endothelial cells (Figure 5). CXCR4 protein expression varied in intensity and distribution from cell to cell, consistent with mRNA heterogeneity noted by *in situ* hybridization. In arterial sections (Figure 6), CXCR4 protein expression was restricted to the endothelium, consistent with the distribution of mRNA expression.

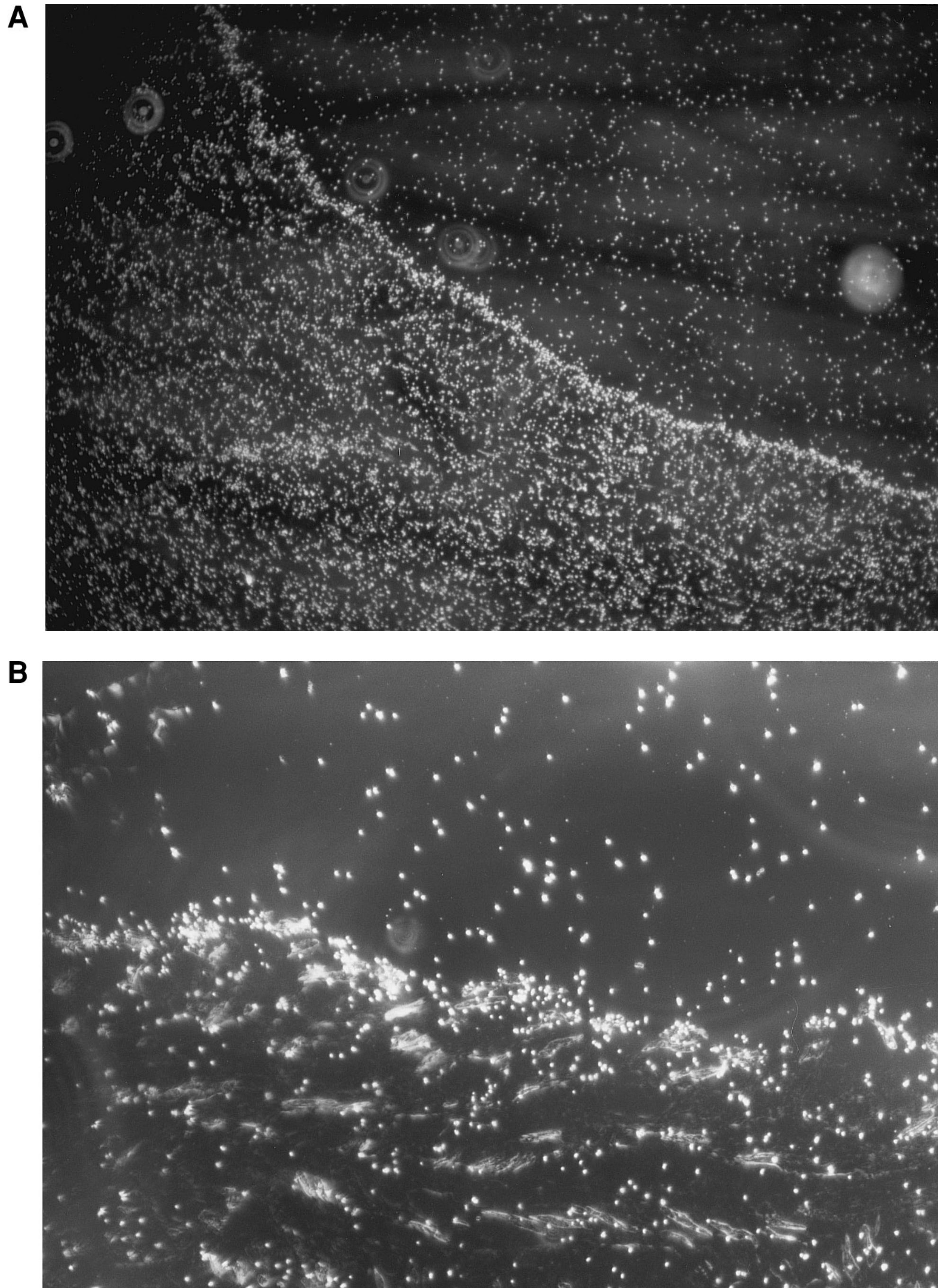

FIG. 3. Detection of CXCR4 gene expression in rabbit thoracic aortic tissue by *in situ* hybridization at (A) low, and (B) higher power. A band of CXCR4-expressing cells coincident with the endothelium is evident. The grain density over endothelium probed with sense cDNA was not significantly different to that over non-endothelial tissue (not shown).

Three dimensional imaging of receptor distribution in cultured BAEC by deconvolution microsopy following permeabilization and 12G5 immunocytochemistry demonstrated an extensive intracellular distribution of CXCR4 protein (not shown). Aggregates of receptors were distributed throughout the cytoplasm as well as at the cell surface suggesting the presence of significant amounts of CXCR4 unavailable for ligand binding. However, functional CXCR4 expression at the cell surface was demonstrated in cultured endothelial cells by stimulation of intracellular calcium mobilization with the CXCR4 ligand SDF-1β (5μg/ml; Figure 7). The peak $[Ca^{++}]i$ responses averaged 37% of those evoked in the same cells by 4U/ml of thrombin.

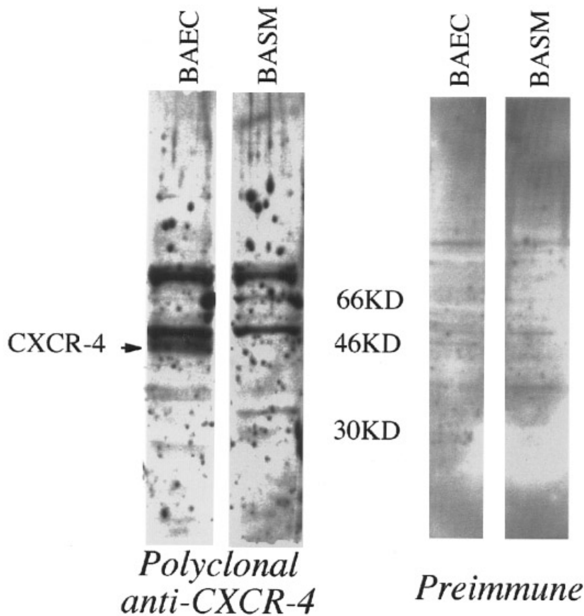

FIG. 4. Western blot of BAEC and BASM demonstrating expression of an endothelial-specific 46KD protein corresponding to CXCR4. Affinity purified rabbit polyclonal antiserum raised against a cytoplasmic peptide sequence of CXCR4.

DISCUSSION

The demonstration of chemokine receptor CXCR4 expression in blood vessel endothelial cells adds a new endothelial chemokine receptor to considerations of vascular physiology and pathology. Its localization in endothelium, an important cellular interface between blood and other tissues, and its absence in smooth muscle of the arterial media is of potential importance in the inflammatory aspects of atherogenesis and possibly in viral infection.

The Duffy blood group antigen, a multispecific chemokine receptor expressed on renal postcapillary endothelium (7) appears to be the only other chemokine receptor unambiguously associated with the endothelium to date. In addition, interleukin 8 and GRO/MGSA have also been reported to bind to specific sites on endothelium (10-12). CXCR4 was first cloned from bovine brain, designated as locus ceruleus receptor-1 (LCR-1), and incorrectly proposed to bind neuropeptide Y (27). Subsequently it was independently cloned from lymphocytes (21), spleen (25) and leukocytes (designated as Lestr) (26) and reclassified as an orphan receptor (28,29). We initially cloned it from endothelial cells as a member of the G protein coupled receptor superfamily and reported it in abstract form as an orphan receptor (24).

The ligand for CXCR4 has recently been identified to be SDF-1 (30), a chemoattractant for lymphocytes and monocytes (31,32). SDF-1α and β, derived from the SDF gene by alternative splicing, is expressed in all murine tissue cells except blood cells and unlike most other chemokines its expression is not augmented by pro-inflammatory stimulants such as LPS and IL-1 (32). In

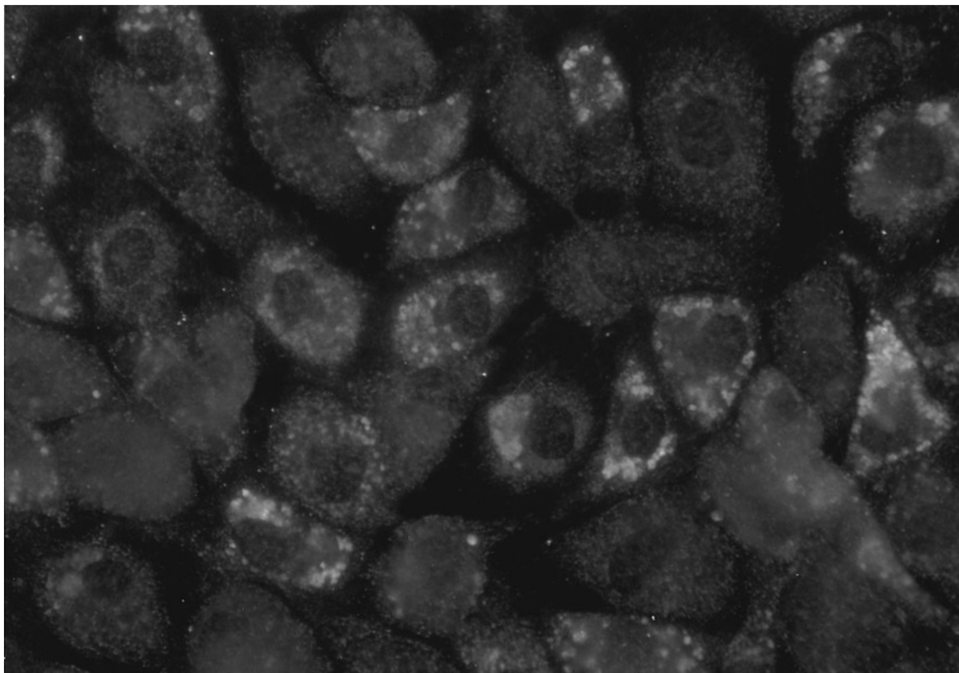

FIG. 5. Expression of CXCR4 protein by immunocytochemical localization in cultured HUVEC in confluent monolayer. Monoclonal 12G5 antibody.

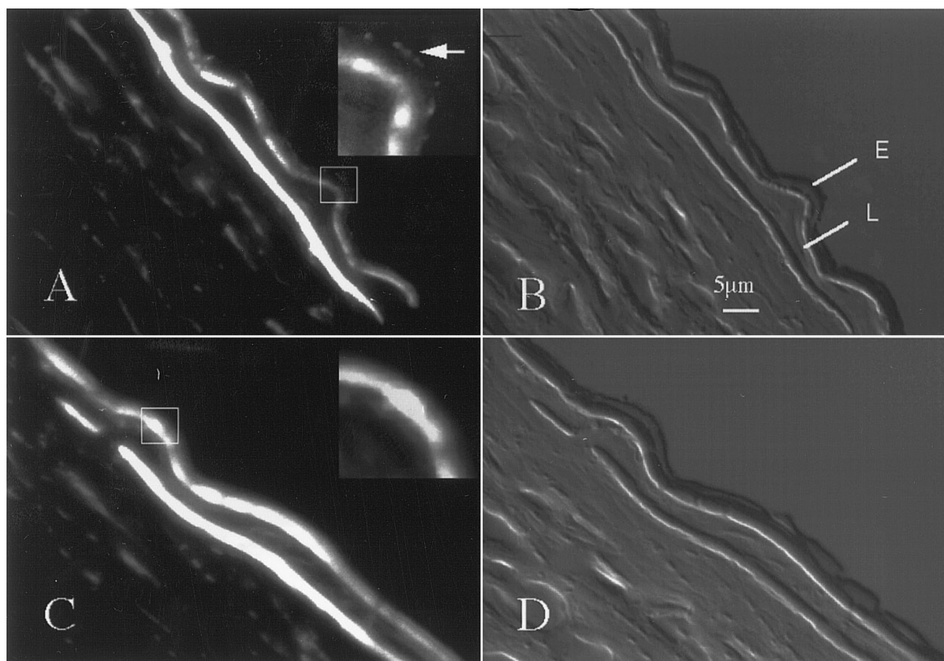

FIG. 6. Immunocytochemistry of normal rabbit aorta with anti-CXCR4 monoclonal antibody (A). Control sections (C) omitted the primary antibody. Differential interference contrast images of the same sections are shown in panels B and D demonstrating the presence of intact endothelium (E). Intense staining of the first two elastic lamellae (L) in A and C was non-specific.

other studies, we have noted that exposure of endothelial cells to bacterial polysaccharide (LPS) or IL-1 resulted in transient downregulation of CXCR4 mRNA expression. These observations are similar to LPS-induced transient downregulation of neutrophil IL-8 receptors (33) with which CXCR4 shares 34% homology. Although Il-8 binds to non-heparin elements on the endothelial surface (13) and is implicated in endothelial mitogenesis and chemoattraction (34), IL-8 receptor mRNA has not been detected in endothelial cells.

Whether CXCR4 functions principally in inflammatory circumstances is unclear; it is expressed at modest levels in normal endothelium. It is also unclear why the receptor is limited to the endothelium and is absent in the rest of the normal vessel wall. SDF-1/CXCR4 may play a role in lymphocyte and monocyte adhesion to the endothelium by presentation of immobilized chemokine to circulating leukocytes.

CXCR4 mRNA levels correlate with HIV-1 permissiveness in diverse human cell types that co-express the primary HIV receptor CD4 (13-19). However, evidence that endothelial cells either express CD4 or become infected by the virus is limited (35). Endothelial cells isolated from human liver sinusoids were permissive for HIV-1 (36), and also were reported CD4 positive. However, a CD4-independent route of entry has been suggested by Mankowski et al (37) who, using a simian immunodeficiency virus macaque model, detected viral RNA in brain capillary endothelial cells where a neurovirulent strain of the virus replicated productively. Infection was not blocked by soluble CD4. Furthermore, Moses and Nelson (38) have reported that cultured human brain capillary endothelial cells, unlike aortic or umbilical endothelial cells, can be permissively infected with a T-cell tropic HIV1 strain through a mechanism independent of CD4 or galactosylcerebroside, suggesting the presence of an alternative HIV receptor on endothelium. In other studies,

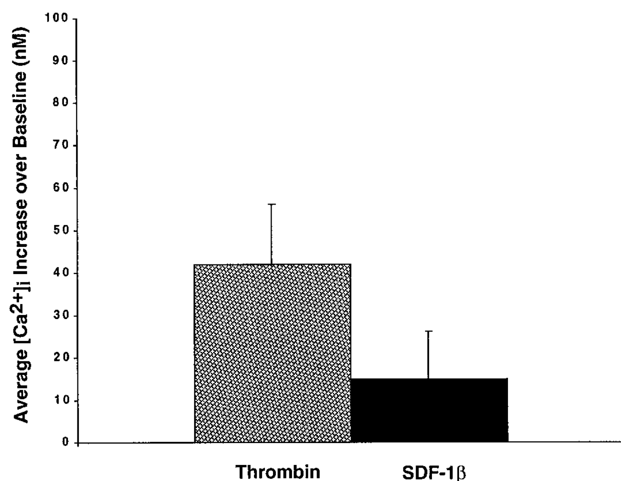

FIG. 7. CXCR4-mediated calcium signaling in BAEC compared to thrombin activation. (n=80).

HIV p24 antigen was detected in endothelium of placental tissue from HIV positive mothers (39), HIV infection was noted in brain capillary endothelium of AIDS patients with CNS dysfunction (40), and vascular endothelium in HIV-1 positive lymph nodes expressed epitopes of the HIV regulatory protein *tat* (41) It is suggested that viral infection of endothelium may be facilitated by the presence of CXCR4 coreceptor. In such circumstances, CXCR4 may enable widespread tissue infection by a mechanism that is not limited to the transmigration of infected leukocytes and, as the gatekeeper cell at the blood-tissue interface, the endothelium would become a useful target for chemokine-related intervention in the extravascular dissemination of HIV-1.

It is unlikely that endothelial CXCR4 has evolved solely to facilitate pathogen infection. Although the roles of CXCR4/SDF-1 in inflammation and atherogenesis are unknown, SDF-1 signaling via CXCR4 will likely involve the activation of multiple second messenger pathways similar to other members of the G protein-coupled receptor superfamily.

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