Regulation of Vascular Cell Adhesion Molecule-1 and Intercellular Adhesion Molecule-1 in Human Vascular Smooth Muscle Cells

Thierry Couffinhal, Cécile Dupláa, Catherine Moreau, Jean-Marie Daniel Lamazière, Jacques Bonnet

Abstract Vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin are inducible proteins involved in cell-cell adhesion. Immunohistochimical studies have indicated that human atherosclerotic plaques contain smooth muscle cells (SMCs) that express ICAM-1 and VCAM-1. Recently, we demonstrated that SMCs in culture express a functionally active cytokine-inducible ICAM-1. SMCs and mononuclear cells participate in the local accumulation of cytokines and related growth factors in atherosclerotic lesions. Therefore, we determined the effects of different cytokines and growth factors on mRNA content and cell surface expression of VCAM-1, ICAM-1, and E-selectin in cultured human aortic SMCs by Northern blotting, quantitative polymerase chain reaction amplification, and immunofluorescence flow cytometry. Under basal conditions of cultivation, both VCAM-1 mRNA and membrane expression of VCAM-1 were low and were induced very little by interleukin-1β (100 U/mL). Platelet-derived growth factor or transforming growth factor-β decreased VCAM-1 mRNA basal expression. Treatment of SMCs with tumor necrosis factor-α (TNF-α) led to an increase in both VCAM-1 mRNA and cell surface expression for VCAM-1 in a dose- and time-dependent manner. Interferon-γ-induced a weak increase in VCAM-1 mRNA expression, with no synergistic effect on the stimulation by TNF-α. Various differences were noted between the expression of ICAM-1 and VCAM-1 genes, because interleukin-1β induced substantial amounts of ICAM-1 but not VCAM-1. The addition of interferon-γ delays the time at which peak expression of ICAM-1 in response to TNF-α stimulation occurs. Under our conditions, we did not detect any expression of E-selectin by SMCs. These results suggest that cytokines regulate VCAM-1 and ICAM-1 expression on arterial SMCs and could play an important role in the pathophysiology of inflammatory and immune processes in atherosclerosis. (Circ Res. 1994;74:225-234.)

Key Words • tumor necrosis factor-α • interleukin-1β • interferon-γ • platelet-derived growth factor • transforming growth factor-β • smooth muscle cells • vascular cell adhesion molecule-1 • intercellular adhesion molecule-1 • atherosclerosis

Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) are members of the immunoglobulin gene superfamily. VCAM-1 is a ligand for very late antigen-4, a β1 integrin expressed on monocytes and lymphocytes but not neutrophils; ICAM-1 is a ligand for lymphocyte function-associated antigen-1, a β2 integrin expressed on all leukocytes. Along with endothelial leukocyte adhesion molecule-1, now designed E-selectin, these molecules are inducible proteins involved in cell-cell adhesion (reviewed in References 1 and 2). VCAM-1 is present in activated endothelial cells (ECs), macrophages, lymphoid dendritic cells, and stromal fibroblasts in the bone marrow. Expression of this molecule is upregulated by tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-4, and perhaps other cytokines. ICAM-1 is constitutively expressed at low levels on the surface of hematopoietic cells and certain nonhematopoietic cells, including fibroblasts, vascular ECs, and epithelial cells. It is strongly upregulated by cytokines, endotoxin, or phorbol esters. E-selectin expression is restricted to cytokine-activated ECs.

Recent studies of cells found in human atherosclerotic lesions have identified certain smooth muscle cells (SMCs) bearing ICAM-1 antigens.5-5 SMCs of blood vessels in synovial tissues of patients with rheumatoid arthritis have also been shown to express ICAM-1 and VCAM-1.5 In addition to SMCs, human atherosclerotic plaques are characterized by two other major cell components, macrophages and T lymphocytes, derived from an infiltration of blood mononuclear cells through the endothelium into the arterial intima.7,8 Because the macrophage, along with T lymphocytes, is ubiquitous in atherosclerotic lesions, it has been suggested that inflammatory and immune reactions may be involved in atherosclerosis. Interactions between T lymphocytes and macrophages could participate in the activation of SMCs and macrophages, releasing cytokines (IL-1 and TNF-α) and growth-related factors (platelet-derived growth factor [PDGF] and transforming growth factor-β [TGF-β]). Some T lymphocytes may be activated and secrete interferon-γ (IFN-γ). These cytokines and growth-related factors have been detected immunohistochemically in human atheroma (reviewed in References 9 through 11) and could activate SMCs. We have recently demonstrated that a functionally active ICAM-1 is expressed on
cultured SMCs and is upregulated by TNF-α and IL-1 in a dose- and time-dependent manner.\textsuperscript{12,13}

In the present study, we sought to determine whether IL-1β, TNF-α, PDGF-BB, and TGF-β, which are themselves soluble products of monocytes, ECs, or SMCs, and IFN-γ, a product of T lymphocytes, regulate the expression of VCAM-1 and E-selectin on cultured human aortic SMCs. The results were compared with those for ICAM-1. As previously described for ICAM-1, we found that TNF-α induced VCAM-1 expression in SMCs. IL-1β had no effect on VCAM-1 expression, contrasting with the strong upregulation of ICAM-1 expression. IFN-γ associated to TNF-α modified the time course of the expression of ICAM-1 mRNA in response to TNF-α stimulation alone. A synergistic effect between TNF-α and IFN-γ was observed on ICAM-1 antigen expression. Both of the growth factors tested decreased these adhesion proteins, and we observed no expression of E-selectin on SMCs. These results suggest that cytokines act as regulators of VCAM-1 and ICAM-1 expression on arterial SMCs. The expression of these adhesion proteins on the surface of SMCs may thus facilitate interactions between mononuclear cells and SMCs in atherosclerotic plaques.

Materials and Methods

Cytokines and Monoclonal Antibodies

Recombinant human TNF-α (rhTNF-α), recombinant human IL-1β (rhIL-1β), and ultrapure natural human TGF-β were provided by Genzyme, Inc, Cambridge, Mass. Human IFN-γ was provided by Sigma Chemical Co, St Louis, Mo, and purified porcine PDGF-BB was provided by Boehringer Mannheim. Cytokine preparations were checked to contain <10 pg endotoxin per milligram of protein by the Limulus lysate assay (OQL-1000, Whittaker Bioproducts Inc, Walkersville, Md).

Murine monoclonal antibodies (mAbs) directed against human VCAM-1 (mAb 1G11) and against human ICAM-1 (mAb BBA3, IgG2 subtype) were provided by Immunotech, Oxford, UK. A murine mAb directed against rabbit SMCs (2P1A2, IgG2 subtype)\textsuperscript{44} was used for control purposes.

Cell Preparation and Culture

Human SMCs and ECs were isolated and cultivated as previously described.\textsuperscript{12,13,14,16} Briefly, human aortic SMCs, isolated by enzyme digestion of abdominal aortas, were grown in Ham's F10 medium (GIBCO BRL, Gaithersburg, Md) supplemented with 5% fetal calf serum, 5% heat-inactivated human serum, 5 mmol/L HEPES, 50 U/mL penicillin, and 50 mg/mL streptomycin, at 37°C in a 5% CO₂/95% air atmosphere. The morphology and growth pattern of the cells (“hills and valleys”) were typical of SMCs. All the cells were stained with anti–smooth muscle actin mAb (Sigma Immunochemicals, St Louis, Mo). Cultures were used at subconfluence (fifth day of growth) within the fifth passage. ECs, isolated from human umbilical vein, were used as a positive control for adhesion molecule expression (antigen and mRNA).

For the protein or mRNA analysis, SMCs and ECs were cultured in six-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ). Incubations with cytokines and related growth factors were carried out in 2 mL of growth medium supplemented with 1% and 0.5% fetal calf serum, respectively.

Immunofluorescence Flow Cytometry

Flow cytometry was performed with monodispersed suspensions of SMCs prepared by brief incubation in 5 mmol/L EDTA at 4°C after two washes with phosphate-buffered saline, pH 7.2, containing 1% of bovine serum albumin (BSA) with 0.2% of sodium azide (NaN₃), at 4°C. This buffer was used in all subsequent steps. Cells were resuspended in 100 μL of a final concentration, 10 μg/mL and incubated for 30 minutes at 4°C. After washing, cells were incubated in 100 μL of a buffer containing fluorescein isothiocyanate–conjugated goat anti–mouse IgG (Amersham) for 30 minutes at 4°C, washed three times, and resuspended in 500 μL of phosphate-buffered saline. The samples were analyzed immediately with a fluorescence-activated cell sorter (FACS analyzer ODAM-ATC 3000).\textsuperscript{12}

RNA Preparation Procedure

Total cellular RNA was prepared from confluent cell monolayers with a single-step acid guanidium thiocyanate/phenol/chloroform extraction method.\textsuperscript{17}

Synthesis of First-Strand cDNA

First-strand cDNA synthesis by reverse transcription was based on the procedure in Reference 18. An 11-μL reaction mixture containing 1 μg total RNA and 20 μg/mL oligo(deoxy)thymidine was heated at 70°C for 10 minutes and cooled. Sixty units of human placenta ribonuclease inhibitor (Amersham) and 200 U of Moloney’s murine leukemia virus RNAse H* reverse transcriptase (GIBCO BRL) were added in a final 20-μL reaction mixture containing (mmol/L) dNTP 1 each, dithiothreitol 10, Tris-HCl (pH 8.3) 25, KCl 75, and MgCl₂ 3, incubated for 1 hour at 42°C, heated for 5 minutes at 95°C, and diluted to 50-μL with double-distilled water.

Polymerase Chain Reaction

For amplification with a thermostable DNA polymerase,\textsuperscript{13,19} a 5-μL reaction mixture containing 5 μL of cDNA-RNA hybrids, 200 μmol/L of dATP, dCTP, and dGTP, 150 μmol/L of dTTP, 50 μmol/L of bio-11-dUTP (Sigma), 200 μmol/L Tris-HCl (pH 8.5), 2.5 mmol/L MgCl₂, 16 mmol/L (NH₄)SO₄, 150 μg/mL BSA, 1 μmol/L of each oligonucleotide primer, and 2.5 U of Thermus aquaticus polymerase (Bioprobe Systems) was overlaid with paraffin oil and subjected to defined temperature cycles. Routines of polymerase chain reaction (PCR) were carried out under the following conditions: 30 seconds of denaturation at 95°C, 5 seconds of annealing at 56°C, and 1 minute of extension at 72°C. The absence of contaminants was regularly checked by reverse-transcription PCR assays of control samples that lacked reverse transcriptase or RNA or only contained PCR reaction buffer without DNA.

Primers

The oligonucleotide primers for the PCR are shown in Table 1. Oligodeoxynucleotides were synthesized, according to published cDNA sequences for VCAM-1, E-selectin, ICAM-1, major histocompatibility complex HLA-DR α-chain, and human monocyte chemotactic protein-1 (MCP-1, murine JG gene).\textsuperscript{20,24} on an Applied Biosystems 381A DNA synthesizer and purified through a Sephadex G-25 column. Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers were provided by Clontech Laboratories, Inc, Palo Alto, Calif. The 5' and 3' VCAM-1 primers were chosen within exons 6 and 8, respectively, to avoid exon 5, which can be spliced.\textsuperscript{25} Human MCP-1 was amplified as a marker of PDGF-BB or TGF-β stimulation.\textsuperscript{26,27} Major histocompatibility complex class II, HLA-DR α-chain transcription assay, was used to confirm IFN-γ stimulation. As previously described,\textsuperscript{13,28} the linear phase of the exponential range of amplification was determined for each primer set to allow semiquantitative PCR analysis. The number of cycles was chosen in the linear phase of amplification: 18 cycles for G3PDH, MCP-1, and E-selectin; 20 for VCAM-1; and 22 for ICAM-1 and HLA-DR. The specificity of generated PCR products was attested by the correct size on the basis of the known cDNA sequences, the
TABLE 1. Oligonucleotides of 5′ Primers and 3′ Primers of Target Genes

<table>
<thead>
<tr>
<th>mRNA Species</th>
<th>Sense</th>
<th>Sequence (5′–3′)</th>
<th>Reference</th>
<th>Nucleotides</th>
<th>Size of PCR Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1</td>
<td>+</td>
<td>AGTGGTGCCCTGCTGTAATGG</td>
<td>20</td>
<td>1064–1083</td>
<td>700</td>
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<tr>
<td></td>
<td>−</td>
<td>CTGGTCTCTCGTCTCGGCT</td>
<td></td>
<td>1744–1763</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>+</td>
<td>GGGCAACAGAAGCCAGAAG</td>
<td>21</td>
<td>991–1010</td>
<td>863</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>CCGAACAGAGAAGAGAATG</td>
<td></td>
<td>1834–1853</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>+</td>
<td>GTCCCCCTCTAAAGTCATCC</td>
<td>22</td>
<td>105–124</td>
<td>943</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>AACCATTCCAGGCTCACCT</td>
<td></td>
<td>1028–1047</td>
<td></td>
</tr>
<tr>
<td>HLA-DRα</td>
<td>+</td>
<td>GGGCTCAGGAATCTAGGCC</td>
<td>23</td>
<td>70–89</td>
<td>859</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>ACTCGGGTGGGGTATAGG</td>
<td></td>
<td>909–928</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>+</td>
<td>CCGAGAGCCGCTGAGCTAACC</td>
<td>24</td>
<td>26–45</td>
<td>344</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>GCTGTCAGGGTGGCTCATG</td>
<td></td>
<td>350–369</td>
<td></td>
</tr>
<tr>
<td>G3PDH</td>
<td>+</td>
<td>TGAAGCTGCGATGTCACCAGATT</td>
<td>71–96</td>
<td>983</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>CATGGGCGATGACGATGCCACCC</td>
<td>1030–1053</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCR indicates polymerase chain reaction; + and −, sense or antisense oligonucleotide, respectively, relative to cDNA; VCAM-1, vascular cell adhesion molecule; ICAM-1, intercellular adhesion molecule; MCP-1, monocyte chemotactic factor; and G3PDH, human glyceraldehyde-3-phosphate dehydrogenase.

Analysis of the cDNA-PCR–Amplified Products

Ten microliters of each PCR reaction mixture was electrophoresed in a 1.5% agarose gel and blotted onto nylon membrane Hybond N+ (Amersham) in 0.4N NaOH buffer. Gels were stained with ethidium bromide to check for successful transfer. Blots were preincubated in 100 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 3% BSA (wt/vol) for 30 minutes at 65°C, subsequently coupled to streptavidin–horseradish peroxidase complex (Amersham) in the same buffer for 30 minutes, and then washed extensively in buffer without BSA. Blots were stained in 100 mL of 50 mmol/L Tris-HCl (pH 7.5) containing 100 mg dianaminobenzidine (Sigma), 50 μL of 30% H2O2 (vol/vol), and 1 mL of 2% cobalt chloride (CoCl2) (wt/vol). Blue precipitates were formed onto filters, and reactions were stopped after 1 minute by washing in fresh 20 mmol/L Tris-HCl (pH 7.5). The blots were then air-dried.

Quantification of Detected Signals

The relative intensity (I) of the color and colored surface (S) of each signal of the PCR products were determined by digitized analysis, using a black-and-white CCD camera (Panasonic WV-52) and an image digitizer (PIP-matrox) installed in an IBM-compatible personal computer. The PCR product yield was calculated by fitting determined IxS to standard curves constructed for each target gene and G3PDH (with purified cDNA dilution series). Outside the linear range of video reading of the standard curve, dilutions of cDNA were performed. G3PDH, an invariant highly expressed gene, was used as an internal standard. VCAM-1, E-selectin, and ICAM-1 signals were normalized to G3PDH values. The data presented as relative values (target gene/G3PDH) were plotted against time. The results represent three independent amplifications for two separate studies.

Northern Blot Analysis

The human VCAM-1 cDNA probe was cloned using PCR. Briefly, total RNA from human umbilical vein ECs treated with 100 U/mL IL-1β for 6 hours was reverse-transcribed as described above. cDNA was amplified by PCR using a VCAM-1 primer set for 28 cycles. The 700-bp PCR product was purified, and both ends were converted to blunt ends with T4 DNA polymerase and subcloned into the HindII site of pBluescribe, according to standard procedures. The DNA sequence of the insert was determined by using the dyeoxy chain-termination method with modified T7 DNA polymerase (Sequenase, United States Biochemical).

SMCs stimulated with rhTNF-α in a dose- and time-dependent manner were harvested, and total RNA was isolated. Northern blot analysis was performed as previously described. Thirty micrograms of total RNA was electrophoresed on a 2% agarose/6% formaldehyde gel and then blotted onto nylon membranes. After prehybridization, membranes were hybridized with 32P-labeled VCAM-1 cDNA for 16 hours at 42°C. After washing, the blots were exposed to Kodak-X AR film at −70°C for 24 hours. All blots were rehybridized with β-actin to check RNA loading.

Results

VCAM-1 Expression on Cultured SMCs

Effect of rhTNF-α on VCAM-1 mRNA Content in Cultured SMCs

As determined by Northern blot analysis, VCAM-1 mRNA was undetectable in unstimulated, cultured human SMCs. Cells incubated for 24 hours with rhTNF-α (10 to 200 U/mL) showed a concentration-dependent increase in VCAM-1 mRNA level (Fig 1). Northern blot demonstrated that significant levels of VCAM-1 mRNA appeared in SMCs after a 24-hour induction with as little as 10 U/mL rhTNF-α. A plateau in VCAM-1 mRNA expression was reached at 40 U/mL. To determine the time-dependent increase in VCAM-1 mRNA, SMCs were treated with 200 U/mL of rhTNF-α. As shown in Fig 1, SMCs treated with rhTNF-α expressed VCAM-1 mRNA within 4 hours, continued to increase up to 24 hours, and then declined between 24 and 48 hours. Northern analysis showed that the VCAM-1 mRNA from SMCs was 3.2 kb long.

Time Course of the Effects of Cytokines and Growth Factors on VCAM-1 Expression

To determine the different effects of cytokines and growth factors on VCAM-1 expression and the potential synergistic effect of IFN-γ, VCAM-1 membrane protein
and mRNA contents were analyzed by flow cytometry and semiquantitative PCR, respectively. Flow cytometry with the mAb directed against VCAM-1 on single-cell suspensions of confluent SMCs showed that SMCs constitutively expressed low levels of VCAM-1 antigens (corrected mean fluorescence, 4.4 arbitrary units), which was unaffected by incubation with PDGF-BB, TGF-β, or rhIL-1 in a dose-dependent (data not shown) and time-dependent (Table 2) manner. Exposure of cultured SMCs to 100 U/mL of rhTNF-α induced a time-dependent increase in VCAM-1 membrane expression. An increase in VCAM-1 expression was detectable at 4 hours (2.5-fold), reached a plateau at 24 hours (3.6-fold), and remained elevated between 24 and 48 hours (4.0-fold). IFN-γ (250 U/mL) induced a weak activation that was detectable at 24 hours and slightly increased up to 48 hours (1.8-fold). No additive or synergistic effects were observed in the presence of rhTNF-α and IFN-γ with respect to the stimulation by rhTNF-α alone on VCAM-1 antigen expression (Table 2 and Fig 2).

mRNA content analysis by semiquantitative PCR showed that unstimulated SMCs displayed a low constitutive expression of VCAM-1 mRNA. IL-1 (100 U/mL) induced a slight nonsignificant increase of VCAM-1 mRNA expression, maximum at 4 hours. After incubations with PDGF-BB or TGF-β, VCAM-1 mRNA decreased within 24 hours (PDGF-BB) or 4 hours (TGF-β) so that they were hardly detected (Figs 3 and 4a). However, rapid and strong mRNA induction of the MCP-1 gene after exposure to PDGF-BB or TGF-β demonstrated that these growth factors did stimulate SMCs (Fig 3). As shown by the Northern blot analysis, rhTNF-α (200 U/mL) induced a time-dependent increase in VCAM-1 mRNA. After exposure to 100 U/mL, a similar time-dependent increase in VCAM-1 mRNA was detected by PCR analysis (Figs 4b and 5).

The raised levels of mRNA were detectable after 1 hour, and levels continued to increase up to 24 hours, falling slightly thereafter. IFN-γ induced a very weak upregulation of mRNA expression from 1 to 48 hours (Fig 4b). The strong upregulation of HLA-DR α-chain expression after 24 hours showed that cells were activated by IFN-γ (Fig 5). Simultaneous administration of rhTNF-α (100 U/mL) and IFN-γ (250 U/mL) led to a slight overtranscription of VCAM-1 between 4 to 48 hours, relative to the effect of rhTNF-α alone, although the time course of induction was identical (Figs 4b and 5).

Statistical analysis showed that this upregulation was a simple additive effect of the two cytokines.

**ICAM-1 Expression on Cultured SMCs**

In previous studies, we observed a strong upregulation of ICAM-1 mRNA by TNF-α and IL-1β. To compare the effects of cytokines and growth factors on the induction of VCAM-1 and ICAM-1 protein and mRNA in SMCs, ICAM-1 expression was analyzed in the same experiments. To estimate the membrane expression of ICAM-1, single-cell suspensions of confluent SMCs were analyzed by flow cytometry. ICAM-1 basal expression was low (corrected mean fluorescence, 5.1 arbitrary units) and was slightly or not affected by either PDGF-BB (100 ng/mL) or TGF-β (20 ng/mL) (Table 2). Exposure of cultured SMCs to rhIL-1 induced an increase in ICAM-1 membrane expression in a dose-dependent (data not shown) and time-dependent (100 U/mL) manner. No increase was detectable at 1 hour, but expression peaked at 4 hours (3.6-fold), remaining elevated up to 24 hours. As previously reported, exposure of SMCs to 100 U/mL rhTNF-α for 24 hours induced a strong stimulation of ICAM-1 surface expression (5.6-fold) that was still elevated at 48 hours (4.5-fold). IFN-γ (250 U/mL) induced a small increase in ICAM-1 expression after 24 hours, which continued to rise up to 48 hours (2.6-fold). The combination of rhTNF-α (100 U/mL) and IFN-γ (250 U/mL) had more than additive effects on ICAM-1 expression (8.9-fold increase at 48 hours) (Table 2 and Fig 2).

In common with the low membrane expression of ICAM-1, PCR analysis showed a rather low constitutive expression of ICAM-1 mRNA on unstimulated SMCs. PDGF-BB decreased ICAM-1 mRNA expression at 4 hours, and TGF-β decreased ICAM-1 mRNA expression at 4 and 24 hours (Figs 3 and 6a). After incubation of the SMCs with 100 U/mL rhIL-1β, ICAM-1 mRNA appeared within 1 hour, continued to increase up to 4 hours, and then returned almost to basal levels between 4 and 24 hours (Fig 6a). rhTNF-α (100 U/mL) produced a time-dependent increase in mRNA, which appeared more delayed but less transient than the mRNA stimulation observed after rhIL-1 treatment (Fig 6b). IFN-γ produced a slight rise in ICAM-1 mRNA that was detected between 1 and 4 hours and increased slightly between 24 and 48 hours. Simultaneous exposure of SMCs to rhTNF-α and IFN-γ induced a complex response in comparison to rhTNF-α alone. rhTNF-α associated with IFN-γ induced a slight increase of ICAM-1 mRNA within 1 hour, although there was no change in mRNA levels between 1 and 4 hours. A strong but transitory rise in mRNA content was observed between 4 and 24 hours, subsequently falling between 24 and 48 hours (Figs 5 and 6b). Statistical analysis showed that the
slight upregulation of ICAM-1 mRNA, observed after 1 hour of costimulation, corresponded to a simple additive effect of rhTNF-α and IFN-γ. At 4 and 48 hours, incubation of SMCs with the two cytokines led to a significant decrease in ICAM-1 expression compared with the sum of the effects of rhTNF-α and IFN-γ separately. In contrast, the stimulation of ICAM-1 mRNA at 24 hours of coexposure was significantly greater than the sum of the effects obtained after rhTNF-α and IFN-γ alone (Fig 6b).

**E-selectin Production by SMCs**

SMCs failed to elaborate E-selectin at any point examined (flow cytometry analysis and PCR amplification). As previously described, ECs showed a strong upregulation of E-selectin expression (protein and mRNA) after exposure to rhIL-1 (data not shown).

**Discussion**

ICAM-1 and VCAM-1 are expressed by cellular components of the arterial wall, including ECs, macrophages, and fibroblasts. ICAM-1 and VCAM-1 are induced or upregulated by proinflammatory cytokines such as IFN-γ, TNF-α, and IL-1β. E-selectin is transiently expressed on ECs in response to the same cytokines. In the present study, we produced evidence for the regulation of VCAM-1 at both mRNA and protein levels.
protein levels in vascular SMCs by various cytokines. Recent studies in human arthritis have identified certain SMCs bearing the VCAM-1 antigen.\(^6,^36\) Under the conditions of our experiments, the constitutive expression of VCAM-1 in human cultured SMCs was rather low, albeit detectable at both mRNA and protein levels by PCR and flow cytometry but not by Northern blot analysis. In a previous study, we found that ICAM-1 expression in SMCs was strongly upregulated by TNF-\(\alpha\) in a dose- and time-dependent manner.\(^12\) We show here that TNF-\(\alpha\) induced a strong upregulation of VCAM-1 expression at both the mRNA and protein levels. However, TNF-\(\alpha\) led to a much more rapid rise in the expression of ICAM-1 than VCAM-1. The ICAM-1 mRNA expression peaked after 4 hours, slowly declining over the next 48 hours. In contrast, VCAM-1 mRNA expression rose between 1 and 24 hours and then slowly declined over the subsequent 24 hours. Protein expression, however, remained elevated for at least 48 hours. IFN-\(\gamma\) slightly increased the expression of VCAM-1 and ICAM-1 protein. The IFN-\(\gamma\)-induced changes were delayed and quantitatively weaker than those observed with TNF-\(\alpha\). It is conceivable that these somewhat slow and modest inductions of VCAM-1 and ICAM-1 depend on an autocrine/paracrine pathway in SMCs. The overinduction of VCAM-1 mRNA induced by TNF-\(\alpha\) and IFN-\(\gamma\) from 4 to 48 hours was a result of the additive effect of the two cytokines. This modest increase and the same time course of expression could explain the absence of synergistic or additive effect on VCAM-1 membrane expression. In contrast with the expression of VCAM-1 membrane protein, the combination of TNF-\(\alpha\) and IFN-\(\gamma\) had a synergistic action on ICAM-1 protein expression at 48 hours. This synergistic effect could be the consequence of the delayed peak expression of ICAM-1 mRNA at 24 hours. However, with no increase in the steady-state level of mRNA, the upregulation is both delayed and transient. This has been observed for IL-8 mRNA after stimulation of keratinocytes by both TNF-\(\alpha\) and IFN-\(\gamma\).\(^33\) Surprisingly, IL-1\(\beta\) did not induce significant expression of VCAM-1 at any time or even in a dose-dependent manner (1, 5, 20, and 100 U/mL), although PCR detected a slight but transient VCAM-1 expression at \(\approx\)4 hours (Fig 4a). IL-1\(\beta\) was biologically active, since it upregulated ICAM-1 even at concentrations as low as 5 U/mL. E-selectin appeared to be noninducible in SMCs under the conditions studied.

Since growth-related factors are the other main activators of SMCs, we studied the effects of two important growth factors. PDGF-BB is a product of endothelial cells and macrophages, and TGF-\(\beta\) is derived from macrophages and SMCs. They are known to interact with SMCs via specific receptors.\(^37,^39\) Under the conditions studied, PDGF-BB and TGF-\(\beta\) appeared to inhibit VCAM-1 and ICAM-1 gene expression. In preliminary experiments, we tested increasing concentrations of PDGF-BB (1, 10, 20, and 100 ng/mL) and TGF-\(\beta\) (1, 10, and 20 ng/mL) on ICAM-1 antigen expression. No effects were observed at any concentration tested. Therefore, we used 100 ng/mL for PDGF-BB and 20 ng/mL for TGF-\(\beta\), biologically active concentrations in SMCs, to examine the potential time dependence of VCAM-1 and ICAM-1 stimulation. The biologic activity...
Regulation of VCAM-1 and ICAM-1 in SMCs

Graphs showing time course of vascular cell adhesion molecule-1 (VCAM-1) mRNA expression in cultured human aortic smooth muscle cells stimulated by cytokines or growth-related factors. Cultured human smooth muscle cells were incubated with 100 U/mL recombinant human interleukin-1 (rhIL-1), 100 ng/mL platelet-derived growth factor (PDGF), or 20 ng/mL transforming growth factor-β (TGF-β) (a) or with 100 U/mL recombinant human tumor necrosis factor-α (rhTNF-α), 250 U/mL interferon-γ (IFN-γ), or a combination of rhTNF-α and IFN-γ (b). The results are expressed as the ratio of VCAM-1 to human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) for each incubation time. Results are representative of three independent polymerase chain reaction amplifications on two separate studies.

Studies on epidermal keratinocytes, dermal fibroblasts, mesangial cells, and human umbilical vein ECs or microvascular ECs have shown that the effects of immunomodulatory agents on VCAM-1 and ICAM-1 expression are highly dependent on cell lineage. VCAM-1 expression of the two growth factors was checked by the stimulation of MCP-1 gene expression. This gene is expressed at low levels in vascular SMCs (24 hours in 0.5% serum). It was strongly induced by PDGF-BB and TGF-β with the time course described by other authors.27,28

Fig 5. Southern transfer of biotinylated polymerase chain reaction products. Cultured human smooth muscle cells were incubated with 100 U/mL recombinant human tumor necrosis factor-α (rhTNF-α), 250 U/mL interferon-γ (IFN-γ), or a combination of rhTNF-α and IFN-γ. cDNA was subjected to separate amplification for intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), HLA-DR α-chain, and human glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Ten microliters of reaction mixture was electrophoresed, transferred, and stained. The target gene amplified and the stimulation are indicated. Upregulation of major histocompatibility complex HLA-DR α-chain expression demonstrates the biologic activity of IFN-γ.
is minimally expressed on resting human umbilical vein ECs, and its expression is strongly upregulated by IL-1α and TNF-α but not by IFN-γ. In microvascular ECs, VCAM-1 does not increase after IL-1α treatment, contrasting with the strong upregulation of ICAM-1. Dermal fibroblasts upregulate ICAM-1 but not VCAM-1 after TNF-α stimulation. ICAM-1 is strongly upregulated by TNF-α and IL-1 and only weakly upregulated by IFN-γ in human umbilical vein ECs and SMCs (in the present study). In dermal fibroblasts and renal tubular cells, ICAM-1 is strongly upregulated not only by TNF-α and IL-1 but also by IFN-γ. In contrast, in epidermal keratinocytes or bronchial epithelial cells, ICAM-1 expression is strongly upregulated by IFN-γ, whereas TNF-α has little or no effect. We demonstrate here that IL-1β stimulation of SMCs did not induce VCAM-1, contrasting with the strong upregulation of ICAM-1 induced by this cytokine and the strong upregulation of both genes by TNF-α. These results suggest that, in SMCs, ICAM-1 and VCAM-1 are differentially regulated in a tissue-specific manner. However, our results do not show whether this is due to a fundamental difference in gene regulation or to differences in regulatory extrinsic factors such as cytokines synthesized by SMCs acting via an autocrine/paracrine pathway.

TNF-α, IL-1β, IFN-γ, PDGF, and TGF-β are pleiotropic mediators known to regulate many genes. Although the mechanisms underlying their gene regulatory actions are not well understood, transcription initiation of eukaryotic genes in general is regulated by the interaction of multiple trans-acting transcription factors with cis-acting elements located primarily in the 5′-flanking region of genes. TNF-α and IL-1 are known to activate the ubiquitously expressed transcription factor NF-κB. Several potential NF-κB–binding sequences have been identified in the 5′-regulatory region of VCAM-1 and ICAM-1 genes. The absence of increase in VCAM-1 gene transcription after IL-1β treatment suggests that transcription of the VCAM-1 gene in SMCs may require more than NF-κB activation. PDGF-BB and TGF-β, which appear to influence other aspects of the behavior of SMCs such as proliferation, migration, and matrix production, did not induce...
either of these two genes. PDGF and TGF-β have been shown to stimulate the expression of c-jun and c-fos genes, which encode components of the AP-1 complex. Despite the presence of AP-1 sites in VCAM-1 and ICAM-1 promoters, no response to PDGF-BB and TGF-β was observed. Thus, it appears that the expression of VCAM-1 and ICAM-1 in SMCs depends mainly on cytokines derived from monocytes and macrophages.

Vascular SMCs provide structure and contractility to the blood vessel. In their differentiated “contractile” state, they lie embedded in the medial layer of the arterial wall, where they constitute the only cell type. During atherogenesis or vascular regeneration after balloon angioplasty, a subpopulation of medial SMCs develops the ability to migrate and regains the capacity to proliferate (reviewed in References 15, 52, and 53). Vascular SMCs can generate locally acting cytokines and matrix proteins, whereas inflammatory mediators released by surrounding cells, macrophages, T lymphocytes, and ECs contribute to the milieu in which growth and differentiated properties are regulated and synchronized. The detection of T cells in the atherosclerotic plaque next to SMCs bearing class II antigens is indicative of activation of T lymphocytes with the consequent secretion of IFN-γ. TNF-α and IL-1 have been found to be secreted by monocytes, ECs, and SMCs (reviewed in Reference 10). Thus, in vivo the expression of ICAM-1 and VCAM-1 in SMCs of the lesions may be induced by these cytokines. However, the phenotypic reversion of SMCs to a synthetic phenotype could depend on a switch in the differentiation program and/or an inability of the intimal cells to maintain the differentiated state. In vivo, ICAM-1 is expressed in medial SMCs of the human fetal aorta. ICAM-1 and VCAM-1 are absent from adult medial SMCs and appear again in the cells of atherosclerotic lesions. ICAM-1 and VCAM-1 expressions in intimal cells may thus have a bearing on the general switch in the differentiation program. Whatever the effects, the functional significance of VCAM-1 and ICAM-1 on the surface of vascular SMCs is intriguing.

In the arterial wall, SMCs are covered by a highly organized and continuous extracellular matrix, the basement membrane. Apart from indirect interactions due to synthesis and release of cytokines and growth-related factors, direct interactions between SMCs and surrounding cells through the fenestrated basement membrane have been suspected. VCAM-1 and ICAM-1 are respective ligands for lymphocyte function–associated antigen-1 and very late antigen-4, which are expressed on the surface of monocytes/macrophages and T lymphocytes. These interactions have been implicated in some lymphocyte functions, including cytotoxic T-cell killing and the adherence of lymphocytes and monocytes to ECs, fibroblasts, or SMCs. Modulation of the expression of ICAM-1 and VCAM-1 on SMCs by cytokines could thus play an important role in the pathophysiology of inflammatory and immune processes in atherosclerosis.

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