Tumor Necrosis Factor–α Stimulates ICAM-1 Expression in Human Vascular Smooth Muscle Cells

Thierry Couffinhal, Cécile Duplaa, Laurence Labat, Jean-Marie Daniel Lamaziere, Catherine Moreau, Olga Printseva, and Jacques Bonnet

Human atherosclerotic plaques contain numerous smooth muscle cells (SMCs) that express intercellular adhesion molecule–1 (ICAM-1). Expression of ICAM-1 in different cells is known to be regulated by tumor necrosis factor–α (TNF-α), which has recently been found to be present in the intimal thickening of human arteries. Therefore, we studied the effect of TNF-α on ICAM-1 mRNA content and surface expression in cultured human aortic SMCs by using the methods of Northern blotting and immunofluorescence flow cytometry. Under basal conditions of cultivation, ICAM-1 mRNA was not revealed in SMCs. However, treatment of the cells with recombinant human TNF-α induced substantial levels of ICAM-1 mRNA. The content of ICAM-1 on the surface of SMCs also increased in a dose- and time-dependent manner after incubation with TNF-α. Twenty-four hours of treatment with 10 ng/mL TNF-α led to an approximately 10-fold increase in ICAM-1 surface expression in the SMCs. Under the same conditions, pretreatment of SMCs with TNF-α resulted in a twofold increase of their adhesiveness for monocytes. In the presence of anti–ICAM-1 monoclonal antibody 10F3, monocyte adhesion to TNF-α-pretreated SMCs was significantly inhibited, suggesting that the observed monocyte–SMC interaction involved the ICAM-1 expressed on SMC surfaces as a result of TNF-α stimulation. These results led us to propose that TNF-α may act as a regulator of functional ICAM-1 expression on the SMC surface and thus can increase the possibility of interactions between mononuclear cells and SMCs in atherosclerotic plaques. (Arteriosclerosis and Thrombosis 1993;13:407–414)

KEY WORDS • tumor necrosis factor–α • smooth muscle cells • intercellular adhesion molecule–1 • adhesion • monocytes • atherosclerosis

Intercellular adhesion molecule–1 (ICAM-1) is one of several recently described human cell adhesion molecules that play a critical role in the generation of inflammatory or immune responses.1-4 The interaction of ICAM-1 with its specific ligand, integrin leukocyte function-associated antigen–1 (LFA-1),5 has been shown to be critical for a number of adhesion events among leukocytes and between leukocytes and other cell types.6-12 ICAM-1 is constitutively expressed on the surface of some nonhematopoietic cells, including vascular endothelial cells (ECs), fibroblasts, and certain epithelial cells and on hematopoietic cells.1 In vitro proinflammatory cytokines, such as tumor necrosis factor–α (TNF-α), interferon gamma (IFN-γ), and interleukin 1 (IL-1) can increase baseline expression of ICAM-1 on different cell types13,14,15 or induce ICAM-1 expression on cells that normally do not express this molecule.6 Its de novo expression in the tissues involved in inflammatory processes or cellular immune responses and in T cell–independent injury has been demonstrated in several diseases.11-15 Recently it was demonstrated that smooth muscle cells (SMCs) from atherosclerotic lesions express ICAM-1.16-19 On the contrary, in the uninvolved arterial media, ICAM-1 was not found. A factor that might induce ICAM-1 expression in SMCs and the functional significance of the presence of this molecule on the surface of SMCs from atherosclerotic vessels are not known. In addition to numerous SMCs, human atherosclerotic plaques contain monocyte-derived macrophages and T lymphocytes,20-22 which can elaborate cytokines, including TNF, interleukins, and IFN-γ.23 The presence of TNF–α was demonstrated by immunocytochemical methods in human normal and atherosclerotic aortas.24,25 TNF–α is known to induce activation of SMCs, i.e., production of IL-1, release of prostaglandin, and secretion of biologically active TNF–α.26,27 However, it is not known whether TNF–α–mediated activation of vascular SMCs involves expression of the ICAM-1 gene. The aim of this work was to study the effect of TNF–α on ICAM-1 expression in cultured human aortic SMCs. We have found that TNF–α induces expression of functionally active (i.e., able to promote adhesion of monocytes) ICAM-1 on the SMC surface. These results led us to propose that TNF–α may act as a regulator of ICAM-1 expression on...
the SMCs surface and thus can increase the possibility of interactions between mononuclear cells and SMCs in atherosclerotic plaques.

Methods

Cytokines and Monoclonal Antibodies

Recombinant human TNF-α (rhTNF-α) was provided by Genzyme, Inc., Cambridge, Mass., and contained <10 pg endotoxin per milligram of protein by the Limulus lysate assay.

Human ICAM-1 was detected using monoclonal antibodies (mAbs) 10F317-19 and 84H10 (Immunotech). A mouse immunoglobulin G anti-rabbit SMC, 2P1A2,28 was used for control purposes.

Cell Preparation and Culture

Human aortic SMCs were isolated by enzyme digestion from the inner third of the tunica media of abdominal aortas obtained during aortofemoral bypass surgery.29 The cell cultures were grown in Ham’s F10 medium (GIBCO BRL) supplemented with 5% fetal calf serum (FCS), 5% heat-inactivated human serum, 5 mM N-hydroxymethylenepiperazine-N’-2-ethanesulfonic acid, 50 units/mL penicillin, and 50 mg/mL streptomycin at 37°C in a 5% CO₂/95% air atmosphere. The culture medium contained <0.40 pg/mL bacterial lipopolysaccharide as quantified by the Limulus amoebocyte lysate assay (QCL-1000, Whittaker Bioproducts Inc., Walkersville, Md.)30 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 12th passage.

ECs isolated by collagenase treatment of human umbilical veins31 were cultured in fibronectin-coated, six-well plates in medium 199 (GIBCO BRL) supplemented with 20% heat-inactivated FCS and used at the first passage at confluence. The cells were shown to be factor VIII–related antigen–positive as judged by immunofluorescent staining.

Human monocytes were isolated from residual leukocytes from a single platelet pheresis donor by counter-current centrifugal elutriation.32 The purity of the monocyte preparations was confirmed by May-Grünwald–Giemsa and nonspecific esterase staining.33 These preparations contained <3% lymphocytes. Cell viability tested by trypsin blue exclusion was higher than 95%.

For the protein or mRNA analysis, SMCs and ECs were cultured in six-well plates (Falcon, Becton Dickinson, Lincoln Park, N.J.). Incubation with rhTNF-α was performed in 2 mL growth medium supplemented with 5% FCS. For the adhesion assay, SMCs were cultured in 96-well plates (Falcon), washed three times in Ham’s F10 without serum and maintained in this medium, and then incubated with or without rhTNF-α before the adhesion assay.

Adhesion Assay

Monocytes were labeled with ¹¹¹In by incubation of cells seeded at 3–4×10⁶ cells/mL with 15–20 μCi/mL ¹¹¹In-oxine for 20 minutes at 37°C in Dulbecco’s modified Eagle’s medium without serum.34 The cells were washed four times and resuspended in Ham’s F10 medium without serum at a concentration of 2.5×10⁶ cells/mL.

After different preincubation tests (TNF-α and mAbs), SMCs in multwells were washed three times, and 100 μL of labeled monocytes (2.5×10⁵ cells) was added to each well of cultured SMCs. The plates were incubated at 37°C in a 5% CO₂/95% air atmosphere for 15 minutes, and then 100 μL of 2% glutaraldehyde in phosphate-buffered saline (PBS) was added to each well.35,36 At least 15 minutes elapsed before nonadherent monocytes were removed by two gentle washes with Immunowash (Immunowash Wash 12, Nune, Roskilde, Denmark). Adherent monocytes and SMCs were extracted by addition of 200 μL NaOH for 15 minutes at room temperature. The sodium hydroxide–eluted material was assayed for radioactivity in a gamma counter. Four replicates were measured for each sample. The results were expressed as percentage of total monocytes added, i.e.,

\[
\text{Percent adhesion} = \frac{\text{cpm harvested}}{\text{cpm added}} \times 100\%
\]

and expressed as the mean of three to five different experiments with four replicates per test.

In the TNF-α activation experiments, SMCs were washed three times in Ham’s F10 without serum and afterward were preincubated with TNF-α. In blocking experiments, SMCs were pretreated with TNF for 24 hours, and 1 hour before the end of this treatment, the anti–ICAM-1 mAb 10F3 or the unrelated mAb 2P1A2 (60 μg/mL) was added to the incubation medium. After activation, cells were washed three times and used for the adhesion assay.

Immunofluorescence Flow Cytometry

Flow cytometry was performed with monodispersed suspensions of SMCs prepared by brief incubation in 5 mM EDTA at 4°C, after two washes with PBS/bovine serum albumin (BSA) azide buffer (PBS, pH 7.2, containing 1% BSA with 0.2% NaN₃) at 4°C. This buffer was used in all subsequent steps. Cells were resuspended in 50 μL mAb (final concentration of 10 μg/mL) and incubated for 30 minutes at 4°C. After washing, cells were incubated in 50 μL of buffer containing fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulin G (Amersham) for 30 minutes at 4°C. Washed three times, and resuspended in 500 μL of buffer. The samples were analyzed immediately with a fluorescence-activated cell sorter (FACS analyzer ODAM-ATC 3000).

RNA Preparation Procedure

Total cellular RNA was prepared from confluent cell monolayers with a single-step acid guanidium isothiocyanate/phenol/chloroform extraction method.37

Polymerase Chain Reaction Conditions, Cloning, and Sequencing

The human ICAM-1 cDNA probe was prepared by polymerase chain reaction (PCR). Total cellular RNA was extracted from confluent human umbilical vein EC culture monolayers stimulated by rhTNF-α for 4 hours. RNA was primed by using oligodeoxynucleotide 12-18 cDNA synthesis on 1 μg of total RNA was accomplished by using avian myeloblastoma virus reverse transcriptase (Pharmacia) in a volume of 20 μL.38 cDNA’s were amplified by PCR39 using a primer set (5’-GTCCCGCCCT-
CAAAAGTCATCC, nucleotides 105–124 and 5'-AACCCCATTCAGCGTCACCT, nucleotides 1,047–1,066) for 28 cycles consisting of a 30-second denaturation at 95°C, 5-second annealing at 56°C, and 1-minute elongation at 72°C. The PCR products were subcloned into the HincII and Pst I sites of pBluescript (Stratagene OZYME). The DNA sequences of the inserts were determined by using the dideoxy chain-termination method with a modified T7 DNA polymerase (Sequenase, United States Biochemical). The β-actin probe was prepared in a similar way by PCR amplification with specific oligonucleotide primers (Clontech, Palo Alto, Calif.).

Northern Blot Analysis

Total RNA (30 μg) was electrophoresed through a 2% agarose–6% formaldehyde gel and transferred onto nylon membranes (Hybond N+, Amersham France SA). Blots were prehybridized in 50% formamide, 5× saline–sodium citrate (SSC), 5× Denhardt’s reagent, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 50 mM sodium phosphate buffer, pH 6.5, and 100 μg/mL sheared salmon sperm DNA for 2 hours at 42°C. cDNA probes for ICAM-1 and β-actin were purified by agarose gel electrophoresis and electrolution and were radiolabeled with ³²P by primer extension using random hexanucleotides to a specific activity of 3×10⁸ cpm/μg. Labeled probes, after denaturation at 95°C for 10 minutes, were added directly to the prehybridized solution. Blots were hybridized in this solution for 16 hours at 42°C and then washed at room temperature with 2× SSC/0.1% SDS and twice with 0.2× SSC/0.1% SDS at 55°C for 30 minutes. After hybridization, the blots were exposed to Kodak-X AR film at −70°C.

![Figure 1](http://atvb.ahajournals.org/) ICAM-1 mRNA in tumor necrosis factor (TNF)-α-stimulated cultured human aortic smooth muscle cells (SMCs) and endothelial cells (ECs) from umbilical vein. Panel A: Northern blot. Lane 1, SMCs, no treatment; lanes 2–4, SMCs incubated with TNF-α (0.5–10 ng/mL) for 24 hours (lane 2, 0.5 ng/mL; lane 3, 2 ng/mL; and lane 4, 10 ng/mL); lanes 5–8, SMCs incubated (30 minutes to 48 hours) with 10 ng/mL TNF-α (lane 5, 30 minutes; lane 6, 4 hours; lane 7, 24 hours; and lane 8, 48 hours); lane 9, ECs, no treatment; lane 10, ECs incubated with TNF-α (10 ng/mL) for 4 hours. Each lane was loaded with 30 μg total RNA. All the blots were probed for ICAM-1 and reprobed for β-actin. Under basal conditions of cultivation, ICAM-1 mRNA was not detected in SMCs (lane 1), whereas incubation with recombinant human TNF-α induced expression of ICAM-1 in a dose- and time-dependent manner (lanes 2–8). In ECs, a small amount of ICAM-1 mRNA was present without any stimulation, and it increased dramatically after incubation with TNF-α. Data from one of three independent experiments are presented. Panel B: Relative values of the signal intensity determined from the Northern blots. Line plot a, dose-dependence curve. SMCs were preincubated for 24 hours with 0.5–10 ng/mL TNF-α. Line plot b, time-course curve. SMCs were preincubated with 10 ng/mL TNF-α for 0.5–48 hours. The relative values of the signals were determined by densitometry as described in “Methods.” ICAM-1, intercellular adhesion molecule-1.
Quantification of mRNA

The relative intensity of the signals from each lane of the Northern blots was determined by analysis of digitized data. Radiographic images were observed through a black-and-white charge-coupled device camera (Panasonic WV-52) and converted to a set of numerical values through an image digitizer (PIP-matrox) installed in an IBM-compatible personal computer. DNA fingerprints were quantified by densitometry and surface analysis. All blots were first hybridized with the ICAM-1 probe and then rehybridized with β-actin to control RNA loading. The data are presented as relative values (ICAM-1/β-actin) and plotted against time or dose. The results demonstrated are representative of three separate studies. The results were reproducible with repeated measurements and linear within the range of the RNA loadings used.

Statistical Analysis

Statistical analysis was performed by unpaired Student's t test or analysis of variance. Results are expressed as mean±SD.

Results

Effect of rhTNF-α on ICAM-1 mRNA Content in Cultured Smooth Muscle Cells

Unstimulated, cultured human SMCs contained no detectable mRNA for ICAM-1 as determined by Northern blot analysis. Cells incubated for 24 hours with rhTNF-α (0.5–10 ng/mL) showed a concentration-dependent increase in ICAM-1 mRNA level (Figure 1). Densitometry of the Northern blot demonstrated that significant levels of ICAM-1 mRNA were induced with as little as 0.5 ng/mL rhTNF-α. A plateau in ICAM-1 mRNA expression was reached at 2 ng/mL TNF-a. From the Northern blot analysis, ICAM-1 mRNA was identified as a 3.3-kb species. Figure 1 shows the time course of increase of the ICAM mRNA level after incubation of the SMCs with 10 ng/mL TNF-α. ICAM-1 mRNA appeared in SMCs as soon as 4 hours, continued to increase up to 24 hours, and then declined between 24 and 48 hours (Figure 1). In parallel studies, the ICAM-1 mRNA of rhTNF-α-stimulated ECs was of similar size and strongly upregulated after 4 hours of incubation.

Effect of rhTNF-α on ICAM-1 Surface Expression in Smooth Muscle Cells

To estimate the expression of ICAM-1 on the cell surface, single-cell suspensions of confluent SMCs were analyzed by flow cytometry with the anti-ICAM-1 mAb 10F3 (Table 1). ICAM-1 basal expression on SMCs was rather low and varied slightly, depending on the growth phase. Corrected mean fluorescence levels of 8.0 and 3.1 were obtained for cells cultured for 3 days (log phase of growth) and 6 days (confluence), respectively. Stimulation of confluent SMCs with rhTNF-α (0.25–10 ng/mL) for 24 hours induced a concentration-dependent increase of ICAM-1 expression. At 0.25 ng/mL, we observed a 3.3-fold and at 10 ng/mL a 10-fold increase in ICAM-1 mean fluorescence level (Figure 2 and Table 1). The dependence of ICAM-1 surface expression in SMCs on the time of preincubation with TNF-α is shown in Figure 3. An increase in ICAM-1 expression was not detectable at 30 minutes but became apparent at 4 hours. Expression continued to increase up to 24 hours (11-fold) and thereafter remained at a constant level up to 48 hours. Similar results were observed with the anti-ICAM-1 mAb 84H10 (data not shown). Stimulation of ECs by 10 ng/mL TNF-α for 6 hours also resulted in a significant increase of ICAM-1 surface expression (Table 1).

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Analysis of rhTNF-α-Induced Adhesion of Smooth Muscle Cells for Monocytes and Effect of Anti-ICAM-1 Monoclonal Antibody on Monocyte Adhesion to Smooth Muscle Cells

Adhesion of monocytes to untreated SMCs was rather low (10.8±1.4%) and was affected slightly by a mAb specific for ICAM-1 (9.4±1%). After stimulation for 24 hours with rhTNF-α, SMC layers showed a dose-related enhancement of adhesiveness for monocytes (Figure 4). A plateau level was reached at 1.5 ng/mL TNF-α (20.6±1.1%). Figure 5 shows the time course of increase of SMC adhesiveness in response to incubation of confluent SMCs with 10 ng/mL rhTNF-α. Little increase of monocyte adhesion was observed after 30 minutes (14.8±8.8%), whereas a plateau level was reached after 4 hours of TNF stimulation (19.6±0.4%). In three matched experiments, pretreatment of SMCs with the anti-ICAM-1 mAb 10F3 significantly inhibited the adhesion of monocytes to SMCs stimulated with rhTNF-α (10 ng/mL) for 24 hours (16.6±0.7% versus 21.5±2%, n=12, p<0.005; Figure 6). In control experiments, the unrelated mAb 2P1A2 did not affect TNF-stimulated adhesion of monocytes (data not shown).
FIGURE 2. Effect of recombinant human tumor necrosis factor-α (rhTNF-α) concentration on ICAM-1 surface expression in human cultured smooth muscle cells. Cells were treated with various doses of rhTNF-α (0, 0.25, 0.5, 1, or 10 ng/mL) for 24 hours, dispersed by EDTA at 4°C, labeled with monoclonal antibody 10F3, and assessed for ICAM-1 expression by flow cytometry. The data are displayed as histograms with relative fluorescence as a linear scale on the x axis and cell number as a linear scale on the y axis. Data from one of three similar experiments are presented. ICAM-1, intercellular adhesion molecule-1.

Discussion

In previous work, low levels of constitutive ICAM-1 expression by SMCs have been demonstrated. Under the conditions of our experiments, the constitutive expression of ICAM-1 in human arterial SMCs was rather low, although detectable. We demonstrate here that TNF-α induces a significant increase of both ICAM-1 mRNA and the protein content in cultured SMCs in a dose- and time-dependent manner. The mRNA appeared in SMCs after treatment with low doses of TNF-α (0.5 ng/mL) and was maintained at a high level for approximately 24 hours, while the protein expression was maintained for at least 48 hours. mRNA content was significantly decreased after 24 hours of TNF stimulation; therefore, the ICAM-1 expression induced in SMCs appeared to be transient. This transient expression of ICAM-1 appears to be a general property of this molecule and has been described in several cell types, including ECs and renal tubular cells. In vitro, ICAM-1 can be induced in many cell types by various proinflammatory cytokines, including TNF-α, IFN-γ, and IL-1 or by phorbol esters. However, the immunomodulatory effects on ICAM-1 expression are highly dependent on cell lineage. ICAM-1 is strongly upregulated by TNF-α in ECs, fibroblasts, renal tubular cells, and SMCs (this study). On the contrary, in epidermal keratinocytes or bronchial epithelial cells, ICAM-1 expression is strongly upregulated by IFN-γ whereas TNF-α has little or no effect. As detected by Northern blot analysis, the size of ICAM-1 mRNA in different cells varies from 1.6 to 3.3 kb. We have found that in SMCs as in ECs, ICAM-1 mRNA was 3.3 kb.

In vivo, ICAM-1 is expressed in medial SMCs of the human fetal aorta; however, it is absent from adult...
FIGURE 4. Line plot showing tumor necrosis factor–α (TNF-α)–induced stimulation of monocyte adhesion to smooth muscle cells (SMCs) and its dependence on TNF-α concentration. Human cultured SMCs were grown to confluence in 96-well plates and treated with the indicated concentration of recombinant human TNF-α for 24 hours. The values presented are the means of three experiments with four assays in each experiment. Data are expressed as mean±SD (n=12, *p<0.01 vs. control).

FIGURE 5. Line plot showing tumor necrosis factor–α (TNF-α)–induced stimulation of monocyte adhesion to smooth muscle cells (SMCs) and the effect of preincubation time. SMCs in 96-well microtiter plates were treated with 10 ng/mL recombinant human TNF-α for various indicated times. Adhesion tests were performed as described in the legend for Figure 4. The values presented are the means of three experiments with four assays in each experiment. Data are expressed as mean±SD (n=12, *p<0.01 vs. control).

FIGURE 6. Bar graph showing effect of monoclonal antibody (mAb) 10F3 on recombinant human tumor necrosis factor–α (rhTNF-α)–stimulated smooth muscle cell (SMC) adhesiveness to monocytes. Human cultured SMCs were treated with medium or 2 ng/mL rhTNF-α for 24 hours. During the last hour of this incubation, anti–ICAM-1 mAb 10F3 (60 μg/mL) was present in the incubation medium. Then SMCs were washed three times and used for adhesion assays as described in the legend for Figure 4. Results are expressed as a percentage of the variation of adhesion observed in the presence of mAb 10F3 and/or TNF-α over the basal level of monocyte adhesion on unstimulated SMCs (Δ adhesion %). Presented results are the mean of three independent experiments. ICAM-1, intercellular adhesion molecule–1.

monocytes, ECs, and SMCs, and it was found to be attached to cell membranes or accumulated on intense and extended extracellular deposits in both the necrotic and fibrotic areas of the plaque. Thus, in vivo expression of ICAM-1 in SMCs of the lesion may be induced by this cytokine. SMCs from intimal thickenings are known to undergo phenotypic changes and to express a phenotype somewhat similar to that of cells from fetal arteries (reviewed in References 50–52). Expression of ICAM-1 in SMCs from atherosclerotic lesions may be relevant to this general switch in the differentiation program and the inability of intimal cells to maintain a differentiated state.

ICAM-1 is a ligand for LFA-1 expressed on the surface of monocytes/macrophages. Under the conditions of our experiments, approximately 10% of monocytes adhered to unstimulated SMCs. This adhesion was slightly affected (12.9% of inhibition) by the anti–ICAM-1 mAb 10F3. This effect is probably due to an inhibition of constitutive ICAM-1 in cultured SMCs. Importantly, ICAM-1 expressed in SMCs after stimulation with TNF-α was able to increase adhesiveness of the SMC layer for monocytes twofold by a pathway that is at least partially ICAM-1 dependent. SMCs and monocyte-derived macrophages are the two major cell types found in the atherosclerotic plaque, and therefore, in the subendothelial intima, direct contact (adherence) between SMCs and mononuclear cells infiltrating the plaque is possible. Expression of ICAM-1 on the SMC surface would be critical for such an event. Conceivably, possibilities for such direct adherence are limited because of the highly organized and continuous extracellular matrix substance, the basement membrane, covering the SMCs. However, the presence of ICAM-1 on the SMC surface would allow direct adherence between SMCs and the infiltrating mononuclear
cells, monocytes, and lymphocytes via ICAM-1–LFA-1 interactions and thus could play an important role in the pathophysiology of inflammatory or immune processes in atherosclerosis.

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