

Functional Analysis of the Human Vascular Cell Adhesion Molecule 1 Promoter

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Summary

The vascular cell adhesion molecule 1 (VCAM-1) is a 110-kD member of the immunoglobulin gene superfamily expressed on the surface of interleukin 1β - or tumor necrosis factor α (TNF)-stimulated endothelial cells. The cell surface protein functions as an inducible adhesion receptor for circulating mononuclear leukocytes and some tumor cells. We have previously characterized the genomic organization of the *VCAM1* gene and described its chromosomal localization. In this report, the promoter of the *VCAM1* gene is characterized. New transcription of the *VCAM1* gene occurred when endothelial cells were treated with TNF. Fusion plasmids containing the 5' flanking sequence of the *VCAM1* gene and the chloramphenicol acetyltransferase reporter gene were used to identify *cis*-acting sequences that direct the cytokine-induced transcription. When transfected into bovine aortic endothelial cells, constructs containing 755 bp of the 5' flanking sequence were induced by TNF. Within the cytokine-responsive region of the core promoter were functional NF- κ B and GATA elements. Upstream of the core promoter, the *VCAM1* 5' flanking sequence contained a negative regulatory activity. NF- κ B-mediated activation of *VCAM1* gene expression may lead to endothelial expression of a mononuclear leukocyte adhesion molecule associated with initial events in the development of an atherosclerotic lesion.

Vascular cell adhesion molecule 1 (VCAM-1)¹ was first identified as an adhesion molecule induced on endothelial cells by inflammatory cytokines (IL-1 and TNF) or LPS (1, 2). The molecule binds cells expressing the integrin, very late antigen 4 (VLA-4), such as monocytes, T and B lymphocytes, basophils, and eosinophils, but not neutrophils (3). VCAM-1 is thought to participate in the recruitment of these cells from the bloodstream to sites of inflammatory responses. For example, VCAM-1 is upregulated on endothelium of post-capillary venules during rejection of cardiac allografts (4). Arterial expression of VCAM-1 is found in experimental models of atherosclerosis in the rabbit. The rabbit homologue of VCAM-1 is expressed locally on arterial endothelium overlying early foam cell lesions in dietary and heritable hypercholesterolemia (5). In addition to inflammatory and immune responses, VCAM-1 may participate in metastasis by facilitating adherence of VLA-4-expressing tumor cells to cytokine-activated endothelium (6). VCAM-1 may play a role in B cell development since it is constitutively expressed on follicular

dendritic cells in normal human lymph nodes (7) and on bone marrow stromal cells in the mouse (8). VCAM-1 can also activate human T cells in conjunction with antibodies defining the TCR (9–11). This suggests that the VCAM-1/VLA-4 pathway mediates signal transduction as well as cellular adhesion.

VCAM-1 was originally cloned from endothelial cells and was shown to contain six Ig domains (1). Subsequent analysis (12–14) revealed that a seven-Ig domain form is the predominant species made by endothelial cells. The new Ig domain, designated domain 4, is strikingly homologous to domain 1. mAb blocking studies (15), as well as experiments with chimeric constructs in which domains of VCAM-1 were replaced with analogous regions of intracellular adhesion molecule 1 (ICAM-1) (16), suggest that the seven-domain form contains two functional VLA-4 binding sites, whereas the six-domain form has one binding site. Structural analysis of the *VCAM1* gene proves that the two transcript forms of *VCAM1* containing six or seven domains are derived by alternative mRNA splicing (17). In an effort to further define the mechanism of cytokine inducibility, as well as the elements of the gene required for maintaining a cell type-restricted pattern of expression, a functional study of the promoter region of the *VCAM1* gene was undertaken.

¹ Abbreviations used in this paper: BAEC, bovine aortic endothelial cells; ELAM 1, endothelial-leukocyte adhesion molecule 1; ICAM-1, intracellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen 4.

Materials and Methods

Isolation and Culture of Bovine Aortic Endothelial Cells (BAEC). BAEC were isolated and maintained in culture using modifications of previously described procedures (18). Aortae were transported on ice in DME supplemented with calf serum (10%; vol/vol), penicillin (200 U/ml), and streptomycin (200 µg/ml). After rinsing once in fresh cold transport media, adventitia was removed and the ends were trimmed. The aorta was slit lengthwise and placed in an empty, sterile, 150-mm petri dish, lumen side up. The luminal surface was covered with 5 mg/ml dispase (*Bacillus polymyxa* grade II; Boehringer-Mannheim Biochemicals, Indianapolis, IN) in DME supplemented with 5% calf serum. The surface was kept moist by reapplication of the dispase solution. After 15 and 30 min, the surface was wiped with a sterile cotton swab. Each swab was immersed and shaken in 20 ml of DME supplemented with 10% calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). After centrifugation for 5 min at 650 g at 4°C, the pellet was resuspended in 10 ml of DME supplemented with 10% FCS, glutamine, and antibiotics, and plated onto a 100-mm tissue culture dish coated with gelatin. Dishes were coated by covering the surface with 2% gelatin (Sigma Chemical Co., St. Louis, MO), removing the excess, and allowing the plates to air dry. Cells were grown at 37°C in humidified 5% CO₂ and maintained in DME, supplemented with 10% FCS, glutamine, penicillin, and streptomycin, as described earlier (18). When cultures reached confluence (5–7 d), each 100-mm dish of cells was subcultured by trypsin dissociation and replated into a 150-cm² flask. At confluence, the flask of cells was subcultured 1:2. Confluent cultures in the two 150-cm² flasks of cells were subcultured into 24–30 100-mm tissue culture dishes and used for transfection. Further passage of BAEC in culture diminishes responsiveness of the cells to cytokine (H. J. Palmer and T. Collins, unpublished data). For experiments on cytokine induction, cells were exposed to recombinant human TNF-α (Genentech, San Francisco, CA) at a final concentration of 200 U/ml in complete media. COS and HeLa cells were originally obtained from the American Type Culture Collection (Rockville, MD), maintained in the same media, and were passaged every 3–5 d. Human umbilical vein endothelial cells were isolated and maintained in culture as described (19).

DNA Sequencing. Nucleotide sequence was determined by the dideoxynucleotide chain termination method with modified T-7 polymerase (United States Biochemical Corp., Cleveland, OH) and α-[³²S]dATP. Oligonucleotide primers were synthesized on a oligonucleotide synthesizer (381A; Applied Biosystems, Inc., Foster City, CA) and used without purification.

Production of a 5' Deletion Series of the VCAM1 Promoter and Construction of Chloramphenicol Acetyltransferase Reporter Plasmids. A nested series of 5' deletions was generated. A 2,195-bp fragment, designated Ffl, which extends 28 bp downstream of the transcriptional start site, was created by restriction enzyme digestion of a 2.8-kb human genomic clone of VCAM1 (17) in pBluescript with SphI and BglI. Smaller constructs were generated by PCR using the same VCAM1 genomic clone as a template. A common 3' primer (R1) at +42 bp from the transcriptional start site and five different 5' primers were utilized to amplify the following regions: -755 (designated F0), -518 (F1), -258 (F2), -98 (F3), and -44 (F4) (Figs. 1 and 3 A). The ends of the SphI-BglI fragment and the PCR-generated fragments were filled using the Klenow fragment of DNA polymerase I and blunt end ligated (20) into the SmaI site of the reporter plasmid pCAT3 (21). The PCR fragments were phosphorylated by polynucleotide kinase before blunt end ligation into the SmaI site of pCAT3. Site-directed mutants were obtained

from PCR reactions using the 3' primer described above and the following 5' primers, which altered the underlined target sites by creating noncomplementary transversion mutants (i.e., G to T and C to A, and the reverse): F1M (AP-1) 5'-GAAGTTATGGTGTCCTTTTTTAAAAAAGTCAGGCTCAAAAAG-3'; F2M (GATA) 5'-ATGTCGACCCTTTTCGATTTCCAGTAAATCGAGCCTTTTGGAGTC-3'; F3M (NF-κB) 5'-TAAACTTTTTTCCCTGGC-TCTGCCCTGGGTTTCCCCTGAAATTTATTTAAATCC-3'. F0dNF was constructed from two separate amplification products; an 86-bp fragment generated from F4 and the reverse primer R1, and a 714-bp fragment containing a mutated NF-κB site and F3M-RP (GGATTTAAATAAATTCAGGGGAAACCCAGGCAGAGCCAGGGAAAAAAGTTTA). Both fragments were filled in with the Klenow fragment of DNA polymerase I and phosphorylated with polynucleotide kinase before blunt end ligation. The ligated product was then subjected to a second round of PCR with primers F0 and R1 to generate an 800-bp amplification product. The PCR amplification product was ligated into pCAT3 as described above. The DNA sequence of each construct was determined to confirm the primary structure.

Transient Expression of VCAM1 Promoter CAT Reporter Constructs. Subconfluent BAEC, HeLa, and COS cultures (10⁶ cells/100-mm culture dish) were transfected with each reporter construct (20 µg) by a modified calcium phosphate precipitation technique (22). Relative transfection efficiency was determined by cotransfection with pTKGH (5 µg), a human growth hormone construct. Samples of the medium were assayed for growth hormone by using a commercially available RIA kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). After transfection, cultures were washed twice with HBSS. Complete media with or without cytokine were replenished. Cells were harvested 48 h after transfection by trypsin dissociation, centrifuged (650 g for 5 min), washed once in PBS, and resuspended in 0.20 ml of 0.25 M Tris-HCl (pH 7.8) with 10 µg/ml aprotinin (Sigma Chemical Co.). Cells were lysed by sonication and one freeze-thaw cycle. Supernatants containing the cell extracts were obtained after centrifugation for 10 min at 14,000 g and stored at -20°C. Transfection of each VCAM1 promoter-CAT reporter construct was performed in duplicate for each experiment and the experiments were repeated at least three times using at least two independent plasmid preparations. Each experiment included a negative control, the promoterless plasmid, pCAT3, and a positive control, CMV-CAT.

Cotransfection of a GATA-2 Expression Plasmid. To assay for transactivation of the GATA site, 15 µg of a GATA-2 expression plasmid (pXM-h-GATA2, a generous gift of Drs. D. Wilson and S. Orkin, Children's Hospital, Harvard Medical School), 15 µg CAT reporter construct, and 5 µg pTKGH were cotransfected into subconfluent HeLa cells using the calcium phosphate precipitation technique described above.

Chloramphenicol Acetyltransferase Assay. Conversion of radiolabeled acetyl-coenzyme A to acetylated chloramphenicol was assayed by the two-phase fluor diffusion technique (20). For each sample, 15–30 µl of cell extract was incubated at 65°C for 15 min to inactivate endogenous transacetylases. The assay was performed in a reaction mixture of 1.25 mM chloramphenicol (Sigma Chemical Co.), 125 mM Tris HCl (pH 7.8), and 0.1 µCi of [³H]acetyl coenzyme A (DuPont Co., Wilmington, DE) in a reaction volume of 0.200 ml. The reaction mixture was overlaid with 5 ml of water-immiscible scintillation fluid (Econofluor; DuPont Co.) and incubated at 37°C for 2 h. The activity of the ³H-acetylated chloramphenicol was measured on a scintillation counter (Rackbeta; LKB Instruments, Gaithersburg, MD).

Nuclear Run-Off Assay. Nuclei from $5-8 \times 10^7$ low-passage confluent human umbilical vein endothelial cells were utilized for nuclear run-off assays (23). Unstimulated cells and cells treated with 200 U/ml of TNF for 2 h were analyzed. Cells were washed twice with PBS, harvested by scraping, and centrifuged at 650 g for 5 min. The cell pellet was resuspended in 1 ml of 0.01 M Tris-HCl (pH 8.4), 1.5 mM MgCl₂, and 0.14 M NaCl, and lysed by the addition of 5% NP-40. Progress of lysis was monitored by trypan blue exclusion. Nuclei were obtained by centrifugation at 650 g for 1 min and were washed twice in 0.02 M Tris (pH 8.0), 20% glycerol, 0.14 M KCl, 0.01 M MgCl₂, 1 mM MnCl₂, and 14 mM 2-ME. Nascent transcripts were radiolabeled for 30 min at 30°C in 100 μ l of this buffer supplemented with 0.125 mCi α -[³²P]rUTP (New England Nuclear, Boston, MA) and the remaining three ribonucleotides (Pharmacia Fine Chemicals, Piscataway, NJ) at a final concentration of 1.0 mM. Nuclei were lysed in 1.75 ml of 4 M guanidine isothiocyanate, 0.25 M sodium citrate, 0.5% *n*-lauryl sarcosine, and 0.1 M β -ME. Chromosomal DNA was sheared by passage 10 times through a 22-gauge needle. Labeled RNA was recovered by centrifugation through 2.5 ml of 5.4 M CsCl and 0.025 M sodium acetate at 36,000 rpm for 16 h in a rotor (SW 41; Beckman Instruments, Inc., Palo Alto, CA). Labeled RNA pellets were washed with 70% ethanol and resuspended in 200 μ l of 100 mM Tris/10 mM EDTA buffer. Limited alkaline hydrolysis was achieved with the addition of 40 μ l 1 M NaOH and incubation on ice for 10 min, followed by neutralization with 48 μ l of 1 M Hepes (pH 7.9) and ethanol precipitation. Denatured target DNAs (5 μ g) consisted of: a human *VCAM1* partial cDNA (12), human endothelial-leukocyte adhesion molecule 1 (*ELAM1*) partial cDNA (24), rat brain tubulin cDNA (25), and a control plasmid pBS. DNAs were heat/alkali denatured and immobilized onto nitrocellulose in a slot blot apparatus (Schleicher & Schuell, Inc., Keene, NH). Filters were baked at 80°C for 2 h and prehybridized for 3 h at 65°C in 10 mM Tris (pH 8.0), 10 mM EDTA, 300 mM NaCl, 0.2 mg/ml Ficoll, 0.2 mg/ml polyvinylpyrrolidone, 10 U RNasin, and 0.1 mg/ml boiled salmon sperm DNA. Approximately 3×10^6 cpm labeled probe was added to the prehybridization mix and hybridized for 48 h at 65°C. Filters were washed in 2 \times SSC for 10 min at 37°C, followed by 50 min at 65°C. X-Omat film (Kodak) was exposed to the wet filters for 36 h at -70°C.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from $\sim 10^7$ control or 6-h TNF-treated BAEC prepared by the method of Schreiber et al. (26). Protein content was assayed utilizing the Bradford reagent (Bio-Rad Laboratories, Richmond, CA). Double-stranded oligonucleotides containing wild-type and mutant sequences of the GATA and NF- κ B recognition sequences were synthesized on an automated DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). Mutant sequences were identical to those used in the construction of mutant reporter constructs. Oligonucleotides used as probes were: GATA, 5'CTTTATCTTTCCAGTAAAGATAGCCT; NF- κ B, CCTTGAAGGGATTTCCTCC; and a mutated form, CCTTGAATTTATTTAAATCC. Wild-type sequences were end labeled with γ -[³²P]ATP and polynucleotide kinase. Each reaction contained 10 fmol of probe and 2-4 μ l (5 μ g protein) of nuclear extract. The buffer for GATA binding experiments contained 10 mM Hepes (pH 7.9), 5% glycerol, 50 mM KCl, 5 mM MgCl₂, 1.0 mM EDTA, 1 mM dithiothreitol, and 1 μ g poly (dI-dC) (Pharmacia Fine Chemicals). NF- κ B binding reactions were conducted in 50 mM Hepes (pH 7.9), 30% glycerol, 50 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 1 μ g poly(dI-dC) in a volume of 20 μ l. Binding reactions occurred at room temperature for 20 min. Complexes were resolved on 6.0% nondenaturing polyacrylamide gels in 50 mM Tris, 45 mM borate,

and 0.5 mM EDTA, pH 7.5 (0.5 \times TBE buffer). The samples were subjected to electrophoresis for 2-3 h at 10 V/cm. Gels were dried and autoradiographed at -70°C overnight.

Results

Structural Analysis of the *VCAM1* Promoter Region. Previous structural analysis of the *VCAM1* gene revealed a single transcriptional start site 25 bp downstream of a consensus TATAA box (17). Located near the transcriptional initiation site (Fig. 1) are several sequences that corresponded to previously defined elements. The sequence GGGATTTCCC, at -57 bp, is in complete agreement with the consensus sequence (GGGR [C/A/T]TYYCC) for a NF- κ B binding site (27). The structure of this site is identical to the functional NF- κ B site found in the acute phase response element of the angiotensinogen gene (28). A second NF- κ B-like site (GGGTTTCCC) is found 15 bp upstream from the consensus site (e.g., reference 29). The sequence TTTATCTTTCCAGTAAAGATAG, at position -255 to -235, contains one site conforming to the (T/A) TATC(T/A) consensus characteristic of recognition sites for members of the recently described GATA family of DNA binding factors (30); the second site contains the TATC core element in the opposite direction. The sequence TGACTCA at -490 bp from the transcriptional start site is identical to the AP-1 consensus sequence TGA(C/G)TCA (31). At position -216 is a purine-rich sequence (GAGGAAAAG) that contains the AGGAA core element of an Ets class (32) binding site. At position -724 is an octamer-like sequence ACTTGCAT.

The nucleotide sequence presented here extends the published sequence by 1,649 nucleotides in the 5' direction. Contained in this upstream sequence were putative binding sites for several classes of transcription factors. Two sites resembling octamer binding recognition elements were identified in the upstream sequence (Fig. 1). The sequence ATTTACAT, at both -1547 and -1175, resembles the binding site (ATTTGCAT) for Oct 1 and Oct 2, and is identical to the binding site for yeast MAT α 2 (33-35). Additionally, two Ets class binding sites were identified at positions -976 (AGGAAG) and -1026 (GAGGAA).

TNF Treatment of Endothelial Cells Results in Transcriptional Regulation of the *VCAM1* Gene. Nuclear run-off experiments were performed to determine whether the increase in VCAM-1 expression in response to TNF was mediated by changes in transcriptional initiation of the gene. Nuclei were isolated from untreated and TNF-treated human umbilical vein endothelial cells. Radiolabeled newly transcribed RNA from these nuclear preparations was hybridized to partial *VCAM1* or *ELAM1* cDNAs, a control tubulin plasmid, and the pBS plasmid backbone. As reported previously, transcription of the *ELAM1* gene was induced by TNF (36). *VCAM1* transcriptional activity was not detectable in uninduced human umbilical vein endothelial cells, but was strikingly increased after 2 h of TNF treatment (Fig. 2). No hybridization was seen between the endothelial nuclear RNA and the pBS plasmid alone. Similar results were obtained with BAEC (data

-2167 |_{F_{FL}}---->
 GAATTC~~CAAAATGATATTT~~CAGTGGGGACAAGGCCAAACCATATATGTGCTTTATGCTACTAATAAAAAGGTTAAACCGAACAAAGTTCAAAGACAAAAC
 -2067 CAGTAGTACTTTATTTCAGACAGGTTAGTCTAAATCTGTAAACCTTATACTTGCAACTCTGATCATTCATTAATCTGC~~AAATTTTAATAAATGC~~TTATT
 -1967 TTAAGCTAAATGCTGAGATGAAAAATGAAACCATATGAGTTAGCAAAGTAGAAAAATATAGGCATATTAATCAGTAAATGCAGAATGAT~~TAAGCTCCAT~~
 -1867 CAATATGCACCTGTGTAGTGAGGCCACCGAGGAGGGTGAATCCTCTCAACCTGGGAGGAGCAGGTAGGACTTCAGATGTCACTCAACTCAAAGATATA
 -1767 GTGAGGGACTTGATCAAACATTTGCCAAGACCACTATGAGTTAAATGAATAGATTAGGCATTTCTCCAATGTTGCAAGCTTCGAATCATATCCAAACTCA
 -1667 GAACAACATAGCTTGGTCATAATGATCCCAAGGATCCTATTGGCCATTGTCTTTGAGCCTCAAAGGAACATATTA~~AAACTCCATAATACCCTTTT~~GATCT
 -1567 ATTCTGAAGTTAAGTAGTGA~~ATTACAT~~ATGATGATGACACAAACACTGTAAAGGACCTCTGGGTACTTGTTTATAAGCTAGTATTTCTGTAATCAATTTT
 -1467 TCTGATCCCTAGATATTTGGTAGGTGAAGTCATACCTATATATATCCCAACACCTAGAACAGCATCTCCAACCTATTTTTCCCTCCTGTCTTTTAGTGGG
 -1367 AGCCACATCAGTATCCAAGAGGAGATCCAGAAGCCTCTCCAACAGGATAGGACAGTTATAGATTCCAGACCTCAGCTATGGCCTTTGTACAGAGTACA
 -1267 AATGTTATATAGTACAAGTTTATTGTACACATCCCATGAGTCTCTGAGCTTTAGAATTTCTTGTAGAATTTAACAGTTTTTTCATGCCGT~~ATTACAT~~
 -1167 ATTATTGCTAGTATTTAGAATTTCTTCTCCAATGTATAACGTTTATTATTGCATTTTTGTATCCACTAAGTGGAAAAATCATGCATTAGATATTGTAG
 -1067 AAGTAGATACAACAATGAACAAGAAGCTGGTCTGACCATGAGAGGAAGTATGATCCAATGGGGGAGATAGACCTGCACGTGTTAATAAAAAGGAAGTGG
 -967 CTATTCCGGTTCTTTTTTGATGGGCAAGCATTTCGAAGGCCTTGGGCTATGTGTGTGCAAGGCTAAGCCAGTTAGTTAATGGGATTTTTTAAAAAAGG
 -867 CACTTCACTGGGGGGAAAAGGAACATAGAGTTGGTTATTGTC~~CCCTTGCC~~TATAATAAAAACTATATTTTTAATTTTTTAACTGGGTTTGCGGTTAAA
 -767 TCTCACAGCCCAAGAGATTGGCCACTTCAGATGGATTCCATACACTTGCATTTAAGTATGCAAAAAAATCCAATATCCAGCAATTTAACCAAATTATT
 -667 GGTAACCTTTCTAAAAA~~AAAAA~~ATGTTTCCCTGTTTTGGCAGCAATTTCAAGTTACAGTCCTTTACTTTTACTCAAGAAAATAGTTTCAA~~AAAGT~~
 -567 TGATGTTGTGTCTAAAGA~~AACTATTTTTATGAATAAATAAAA~~CTAAGAAGTTATGGTGTCCCTTTTTTAAAAA~~ATGACTCA~~TCAAAGA~~AAATAACTT~~
 -467 TTTCTTTCTCTGTGAAGAGAAAAAATTAATCTCTTTTAGAATTGCAAAACATATTTCTTGTATGGAGAAAATCAATTCACATGGCATAGTCGTTATTTA
 -367 TCCAGTTCAAAACCAGAGTAGAATTTACTACTCTGTCTCCATTTTTTCTCTCCCACCCCTTAACCCACATGGATTGAGAAAGCTTCATCTGCAAT
 -267 CAGCATTTGCTCTT~~ATCT~~TTCCAGTAA~~AGATAG~~CCTTTTGGAGTCGAAGATGAGGAAAAGCCTGTATTTTATAGTCTTGGAAAGTGTCTCTTTTGGCAGG
 -167 ACAGAGAGAGGAGCTTCAGCAGTGAGAGCACTGAAGGGGTTAATAGTGGAACTGGCTGGGTGTCTGT~~TAAACTTTTTTCCCTGGCTCTGCCCTGGGTT~~
 -67 ~~CCCCCTGAAGGGATTTCCT~~CCCGCCTCTGCAACAAGACCCCTT~~TATAA~~AGCACAGACTTCTATTTC ACTCCGGGTATCTGCATCGGGCCTCACTGGC
 33 TTCAGGAGCTGAATACCTCCAGGCACACAGGTGGGACACAAATAAGGGTTTTGGAACCACTATTTTCTCATCAGCAGCAACTTAA~~ATGCCTGG~~
 133 GAAGATGGTGTGATCCTTGGAGCCTCAAATATACTTTGGATAATGTTGCGACTT

Figure 1. 5' sequence of the human *VCAM1* gene. The transcriptional start site is designated -1 and the TATAA box is underlined. Locations of the reverse primer (R1) and the forward primers used in the construction of VCAM/CAT chimeras are indicated. Consensus AP1, GATA, NF- κ B, and the upstream octamer-like sites are boxed. The 5' end of the previously published sequence is indicated with an arrow.

not shown). From these results we conclude that TNF induces a rapid increase in *VCAM1* gene transcription.

Functional Analysis of the *VCAM1* Core Promoter. A series of deletion mutant constructs was transfected into BAEC to determine whether DNA sequences immediately flanking the transcriptional start site of the *VCAM1* gene were sufficient for cytokine-induced expression in endothelial cells. Fusion plasmids containing portions of *VCAM1* 5' flanking sequence (Fig. 3 A), -2167 (Ff1), -755 (F0), -518 (F1), -258 (F2), -98 (F3), or -44 (F4), and the CAT reporter gene were constructed and introduced into low-passage BAEC. Each

of the Ff1, F0, F1, F2, and F3 VCAM-CAT constructs was induced by TNF (Fig. 3 B), confirming that the identified *VCAM1* promoter was cytokine inducible in BAEC. Primer extension analysis confirmed that transcriptional initiation was from the correct *VCAM1* transcription start site (data not shown). The -2167 chimera (Ff1) had the lowest level of reporter gene expression in unstimulated BAEC, equivalent to the promoterless pCAT3 control construct used in the construction of the reporter chimeras. Basal expression of the F0, F1, or F2 construct in unstimulated BAEC was higher than the Ff1 construct (Fig. 3 B). The Ff1 construct was induced by TNF, but the extent of induction was less than that obtained with constructs with further 5' deletions. This suggests the presence of a negative element(s) or a silencer(s) outside the -755 to +42 region of the *VCAM1* gene, which suppresses the expression of *VCAM1*. Treatment of cells with TNF overcomes the inhibitory effect of this negative element(s) and activates the promoter. Transfection of the 5' *VCAM1* CAT deletion constructs into other cells that could be induced by cytokine to express *VCAM1* revealed a similar inverse correlation between the amount of *VCAM1*

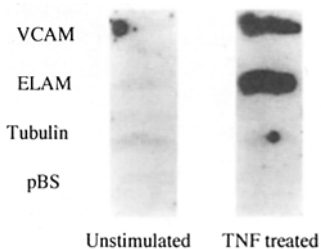


Figure 2. Nuclear run-off experiments in human umbilical vein endothelial cells, unstimulated or induced with 200 U/ml human TNF- α for 2 h. Target DNAs are partial *VCAM1* and *ELAM1* cDNAs, rat brain tubulin cDNA, and control pBS.

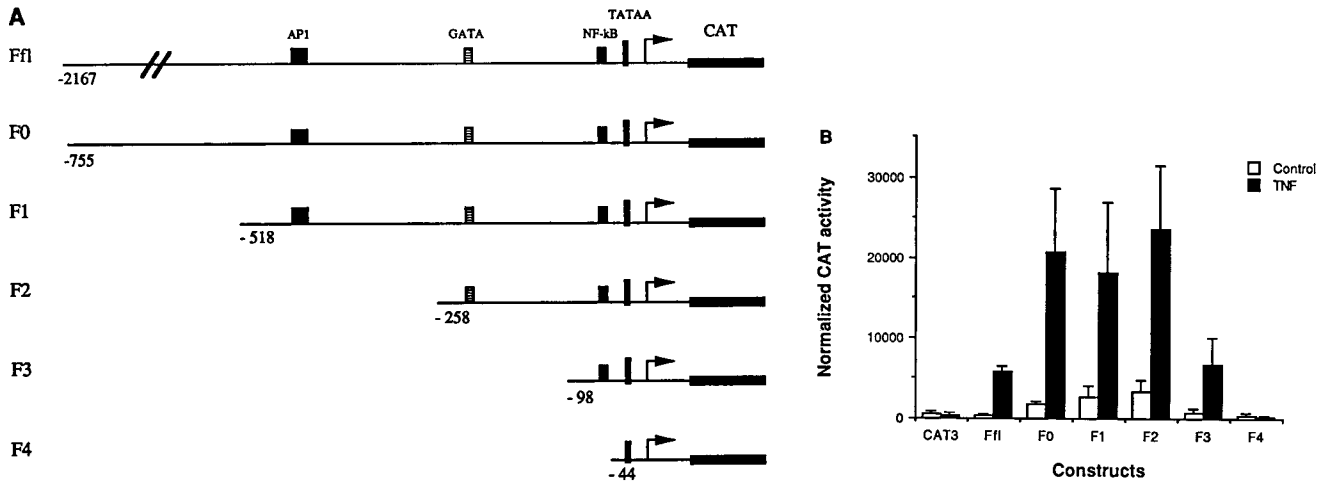


Figure 3. Deletion analysis of the *VCAM1* promoter. (A) Schematic diagram of the 5' deletion mutations in the *VCAM1* upstream regulatory regions. (B) Transfection of 5' deletion/CAT constructs into BAEC under unstimulated and TNF-induced (200 U/ml) conditions. 20 μ g of reporter plasmid was transfected by a modified calcium phosphate precipitation technique. 5 μ g of TKGH was cotransfected as a measure of relative transfection efficiency. Results are reported as the ratio of CAT activity to human growth hormone (cpm/ng/ml). Each experiment was repeated at least three times with each point assayed in duplicate.

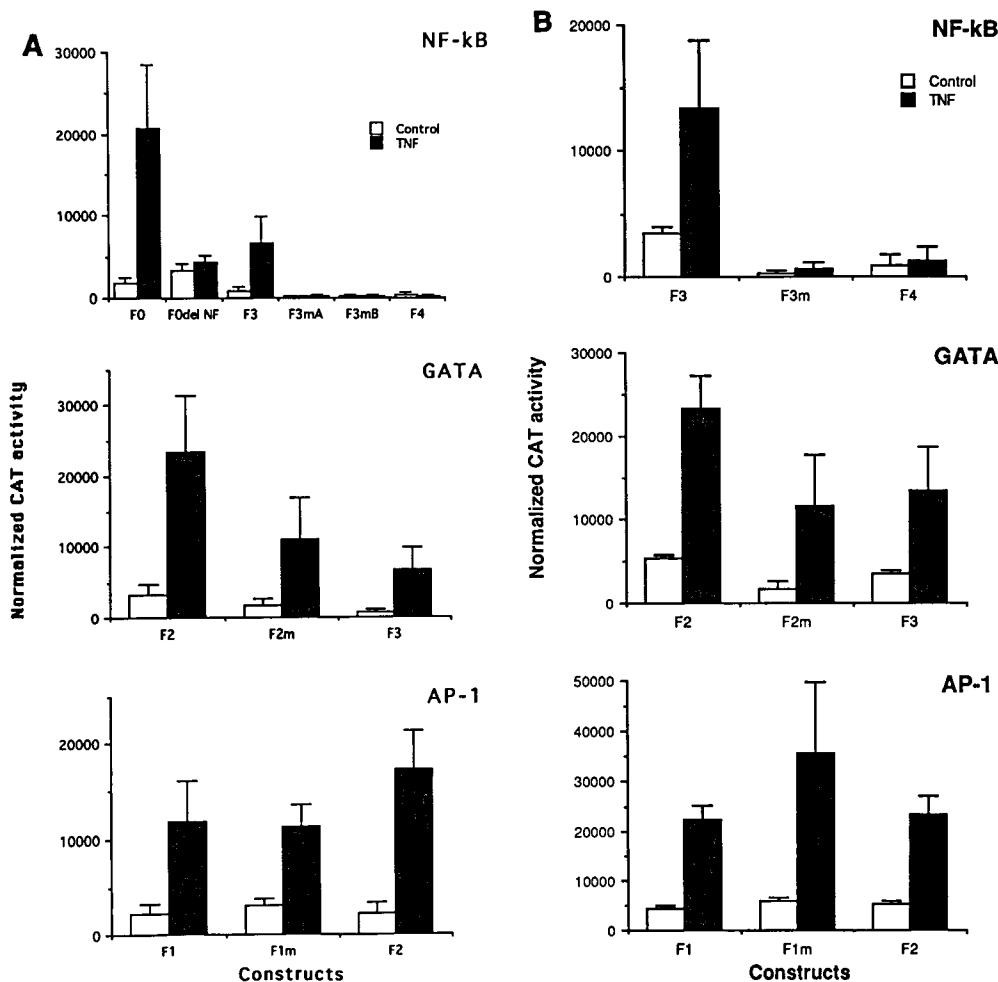


Figure 4. Analysis of transcriptional control elements in the *VCAM1* promoter. Reporter plasmids containing mutated recognition sites (see Materials and Methods) were transfected and assayed in BAEC (A) and COS cells (B). Results are reported as the ratio of CAT activity to human growth hormone (CPM/ng/ml).

5' flanking sequence and either basal or induced marker gene activity (data not shown). This suggests that at least some of the negative effect is not cell type specific. The F0, F1, and F2 deletion constructs showed approximately equivalent levels of induced activity after TNF treatment of cells (Fig. 3 B). The F3 deletion construct shows ~50% of the TNF-induced activity of the F0, F1, and F2 constructs. The F4 construct was not basally active and was not induced by cytokine, as expected for a chimera containing only the TATAA box and no other upstream sequences.

Role of the NF- κ B Binding Site in the TNF Induction of VCAM-1 Gene Transcription. The data presented above suggest that TNF-induced expression of the *VCAM1* gene is required in part by 5' flanking sequences present between the -258 to -98 and the -98 to -44 regions of the core promoter. Within this *VCAM1* minimal promoter region were sequences that conform to consensus NF- κ B and GATA elements. The NF- κ B transcription factor family is known to play a role in cytokine induction of many genes (reviewed in references 27, 29, and 37). To further investigate the role of these elements in TNF-induced *VCAM1* expression, constructs were generated that specifically altered these sites.

Deletion analysis was used to determine if the *VCAM1* NF- κ B sequences were a functional promoter element. Block mutations of the homologous *VCAM1* sequence were generated in the F3 chimera (F3mA and F3mB). The sites were changed by noncomplementary transversion mutation. The activity of mutant (F3mB) was compared with that of the wild-type F3 fusion construct in BAEC and COS cells (Fig. 4, A and B). Changes in the structure of the consensus NF- κ B element at -57 totally abolished both inducible and basal transcription in either cell type. Similarly, specific changes in the upstream NF- κ B site (F3mA) abolished basal and inducible transcription. An additional site-directed mutant, F0dNF, contained all of the sequence in the F0 (-755) construct except the bases of the 3' NF- κ B element. TNF-induced reporter gene expression from this deletion construct was absent, suggesting that an NF- κ B-like factor binding to this site is necessary for TNF induction of *VCAM1* gene transcription. The pattern of expression of the *VCAM1* NF- κ B deletions in COS (Fig. 4 B, and data not shown) and HeLa cells (data not shown) in uninduced and in TNF-induced cells was similar to that seen with BAEC. These results indicate that at least part of the mechanism(s) involved in cytokine induction of *VCAM1* in endothelial cells is functional in these nonendothelial cell types.

Gel shift assays were performed to identify nuclear proteins specifically recognizing the 3' *VCAM1* NF- κ B element. A labeled DNA fragment spanning this region was incubated with BAEC extract prepared from control and TNF-treated BAEC in the presence of specific, mutated specific, or nonspecific competitor DNAs (Fig. 5). Two specific DNA-protein complexes were observed with extracts from TNF-treated endothelial cells. Addition of excess unlabeled DNA spanning the NF- κ B element of the promoter blocked both of the TNF-inducible DNA-protein complexes. Formation of these complexes was not blocked by heterologous compe-

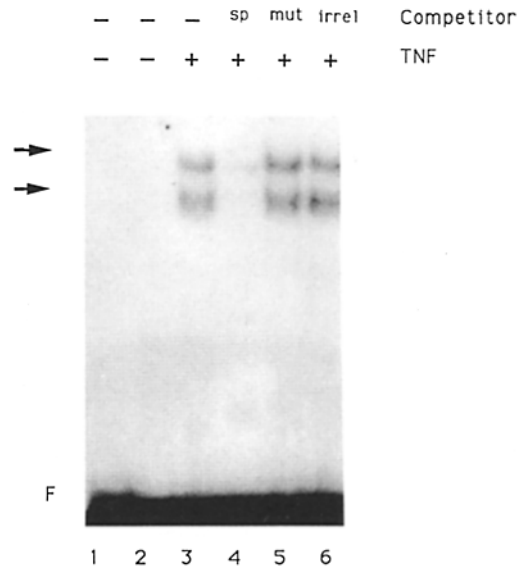


Figure 5. Binding of an endothelial NF- κ B-like factor to the *VCAM1* NF- κ B element. Nuclear extracts from uninduced (lane 2) and TNF-stimulated BAEC (lanes 3-6) were incubated with 10 fmol of labeled double-stranded oligonucleotide probe containing the *VCAM1* NF- κ B recognition site. Competitor oligonucleotides were present at 50-fold molar excess and include specific competitor (lane 4), competitor with a mutated NF- κ B binding site (lane 5), and an irrelevant double-strand oligonucleotide (lane 6). Specific DNA-protein complexes (arrows); unbound free probe (F).

titors, or by a DNA fragment containing a mutated *VCAM1* NF- κ B site. These results confirm the importance of this element of the *VCAM1* gene in regulation of cytokine induction of *VCAM1* and demonstrate that endothelial cells make protein(s) capable of binding to this promoter element. The nature of these putative NF- κ B family members is under investigation.

Role of the GATA Sites in TNF Induction of VCAM1 Gene Transcription. The data presented above demonstrate that an important element for TNF-induced *VCAM1* expression is present within the -258 to -98 region of the promoter. Within this region (-255 to -235) is the sequence TTATCTTTCCAGTAAAGATAG, which contains two putative sites: GATA and TATC, characteristic of recognition sites for members of the recently described GATA family of DNA binding factors (30). To determine if either of these sites plays a role in *VCAM1* gene expression, block mutations of the homologous *VCAM1* sequence were generated in the F2 chimera. Each base between nucleotides 253-251 and 239-237 was changed by noncomplementary transversion mutation. The activity of this mutant (F2m) was compared with the wild-type F2 fusion construct in both BAEC and COS cells (Fig. 4, A and B). Changes in the sequence of the GATA elements decreased both basal and TNF-induced transcription in both cell types by ~50%.

To confirm a role for GATA factors in the basal and induced expression of the *VCAM1* gene, cotransfection experiments with a GATA-2 expression construct (38) were per-

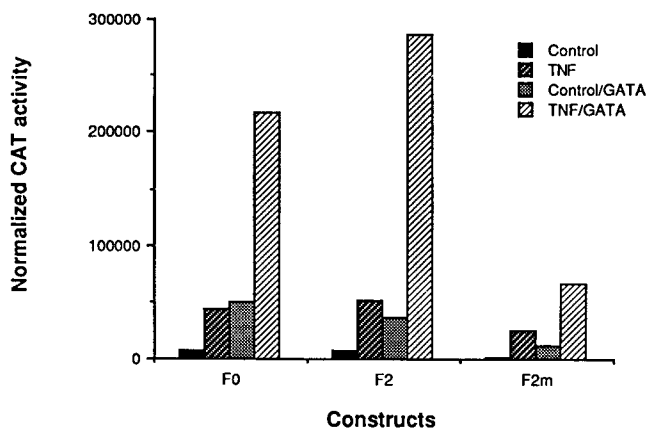


Figure 6. Transactivation of *VCAM1* reporter constructs by a human GATA-2 expression vector. HeLa cells were cotransfected with 15 μ g of reporter plasmid, 15 μ g of the GATA-2 expression plasmid pXM-hGATA, and 5 μ g of a normalization plasmid.

formed. In the presence of TNF, transcriptional activity increased fivefold in cells cotransfected with the F0 or F2 *VCAM1* reporter construct and the GATA-2 expression plasmid (Fig. 6). Cotransfection of the expression vector with a construct bearing a mutant GATA site, or a 5' deletion of the GATA region, decreased the effect. Cotransfection of the expression vector backbone plasmid pMT2 had no effect on reporter gene transcription. The GATA expression vector did not *trans*-activate the promoterless pCAT3 construct; additionally, there was no *trans*-activation of a human growth hormone gene placed under the transcriptional control of a thymidine kinase promoter.

Gel shift assays were performed to identify nuclear proteins specifically recognizing the GATA sequences from positions -256 to -230 of the promoter. A labeled DNA fragment spanning this region was incubated with BAEC extract in the presence of specific and nonspecific competitor DNA. A DNA-protein complex was observed in the absence of specific competitors (Fig. 7). Addition of excess unlabeled DNA spanning this region of the gene blocked formation of the labeled DNA-protein complex. Formation of the complex was not blocked by heterologous competitor (data not shown). When a similarly sized oligonucleotide probe containing the endothelin 1 promoter GATA site (38, 39) was incubated with the same nuclear extract, a DNA-protein complex was formed that comigrated with that seen with the *VCAM1* probe (Fig. 7). From these experiments, we conclude that a GATA factor acts as a positive regulator of TNF-inducible transcription of the *VCAM1* gene.

Together these results suggest that there are at least three separate clusters of elements within the *VCAM1* 5' flanking sequence involved in cytokine-induced expression of the gene: a negative regulatory element(s), the GATA sites, and the NF- κ B binding sites. Thus, cytokine induction of *VCAM1* gene transcription appears to involve interaction between a combination of *cis*-acting elements and the *trans*-acting factors that interact with these elements.

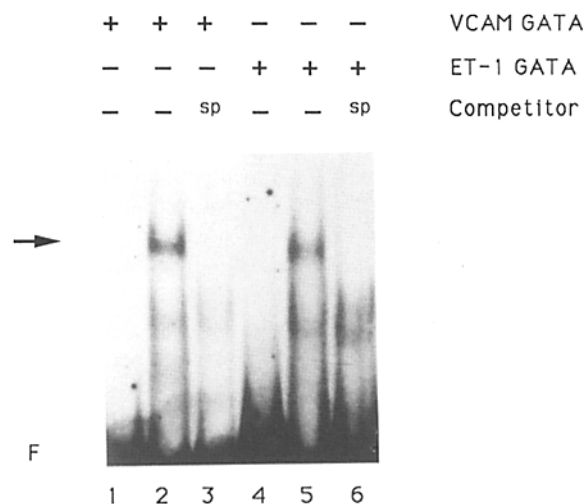


Figure 7. Binding of an endothelial GATA-like factor to the *VCAM1* GATA element. Labeled double-strand oligonucleotide probe containing the *VCAM1* GATA (lanes 1-3) and endothelin 1 GATA (lanes 4-6) recognition sites were incubated with nuclear extracts from uninduced BAEC (lanes 2 and 3, and 5 and 6). Specific competitor oligonucleotides were present at 50-fold molar excess (lanes 3 and 6). Specific DNA-protein complexes (arrows); unbound free probe (F).

The AP1 Site in the VCAM1 Promoter Is Not Required for TNF Induction. AP1 sites have been shown to mediate transcriptional responses to cytokines such as TNF (40). Additionally, *c-fos*-*c-jun* complexes are induced after TNF-mediated activation of cultured endothelial cells (41). At -490 in the *VCAM1* promoter is the sequence TGACTCA, which is identical to the AP1 consensus sequence TGA(C/G)TCA (31). Inspection of the sequence flanking the AP1 core element A(TGACTCA)T reveals a T 3' to the consensus. The function of each base pair in the consensus element and the flanking sequence on the binding of *c-fos* and *c-jun* has been investigated (42). The presence of the 3' T diminishes *in vitro* binding of *c-fos* and *c-jun* by >75%. To test whether the *VCAM1* AP1 site was necessary for the high level of gene expression in BAEC, a block mutation was introduced into the AP1 site of the F1 construct. The activity of this mutant (F1m) was compared with that of the wild-type F1 construct in both BAEC and COS cells (Fig. 4, A and B). Structural alteration of the AP1 site had little effect on either basal or TNF-induced reporter gene expression in BAEC. A small increase of both basal and inducible expression of CAT was observed in COS cells with construct F1m. The F2 deletion construct, which deleted the AP1 site (along with 260 bases of surrounding sequence) also had a similar small increase in CAT activity. These observations indicate that the AP1 site in the *VCAM1* promoter is not necessary for TNF-induced transcription. The AP1 site is not required for efficient *VCAM1* gene expression in endothelial cells. In the β globin HSII region, erythroid specificity appears to be due to binding of a tissue-specific factor (NFE2) to an AP1-like site (43). Since alteration of the *VCAM1* AP1 site had no effect on the cell type specificity of expression (Fig. 4, A and B), it is unlikely

that endothelial cells make a lineage-specific protein that acts through the AP1 site in the *VCAM1* gene.

Discussion

VCAM-1 is a member of the Ig gene superfamily that functions as an adhesion receptor and a signal transducer to leukocytes expressing the ligand VLA-4. The previously described induction of VCAM-1 protein expression on endothelial cells after cytokine treatment (1) was shown here to correlate with a similar regulation of transcription initiation of the *VCAM1* gene. The deletion and block mutation experiments define two functionally important elements: a pair of GATA sites and two NF- κ B sites. These sites in the core promoter of the *VCAM1* gene are important for the cytokine-induced expression of the gene.

In the *VCAM1* promoter, two GATA elements are found ~ 10 bp apart in opposite transcriptional orientation. Analysis of other promoters reveals a similar arrangement of GATA sites. The γ -globin promoter contains two functional GATA elements (CAGATATTTGCATTGAGATAGT) that are 10 bp apart (44). The GATA-1 promoter contains two functional GATA recognition elements (CTGATAAGACTTATCTG) that, as in the *VCAM1* gene, are placed in opposite transcriptional orientation (45). The GATA family of transcription factors is known to interact with other *trans*-acting factors. Within erythroid-specific promoters, GATA elements appear to cooperate with CACCC boxes to direct gene expression (30). The analysis of the *VCAM1* promoter presented here suggests that factors binding to the GATA sites and transcription factors recognizing the NF- κ B sites may interact to generate full *VCAM1* cytokine responsiveness.

While GATA elements in *VCAM1* promoter are important positive regulators of expression, they cannot be primarily responsible for the restricted cell type pattern of VCAM-1 expression. Two arguments support this idea. First, a diversity of cells express members of the GATA family of transcription factors. The GATA family contains at least three members (reviewed in reference 30). Endothelial cells, as well as a variety of other cells, express GATA-2 (38). Second, structural alterations in the GATA site did not preferentially alter the level of *VCAM1* expression in endothelial cells (Fig. 4, A and B). Additionally, identification of an upstream region of the *VCAM1* promoter that contains a negative element suggests that additional regulatory components may be present elsewhere in the gene. These regulatory elements may interact with the GATA binding factor to confer lineage restriction. Such a model has recently been suggested for platelet factor 4 (PF4). In this gene, an upstream tissue-specific silencer/enhancer element interacts with a GATA site present in the core promoter to restrict PF4 gene expression to megakaryocytes (46).

Another functional element in the *VCAM1* promoter are the NF- κ B binding sites. NF- κ B, a ubiquitously expressed transcription factor, is involved in the increased expression of many cytokine-induced genes (reviewed in references 27, 29, and 37). Like VCAM-1, expression of ELAM-1 and ICAM-

1 are upregulated in endothelial cells by inflammatory cytokines (reviewed in reference 47). Structural analysis of the *ELAM1* and *ICAM1* genes revealed putative NF- κ B binding sites in the 5' flanking sequences (48–50). Deletion analysis of the *ELAM1* promoter revealed that cytokine induction of *ELAM1* gene expression required the NF- κ B site (36). An NF- κ B-like factor was induced in cytokine-treated endothelial cells that bound to the NF- κ B element in the *ELAM1* promoter (36, 49). However, the nature of the forms of NF- κ B binding to elements in the promoters of the genes for leukocyte adhesion molecules remains to be determined. Specificity determined by the association of NF- κ B components has recently been shown to provide a mechanism to selectively regulate genes containing NF- κ B sites (51). Definition of the forms of NF- κ B interacting with the promoters of *VCAM1* and *ELAM1* genes may help explain the different temporal and anatomic patterns of expression of these leukocyte adhesion molecules (reviewed in reference 47).

It is tempting to speculate that NF- κ B-mediated activation of VCAM-1 expression may play a role in the development of atherosclerosis. Formation of the atherosclerotic lesion involves the adherence and transmigration of circulating monocytes into the arterial intima (reviewed in references 52 and 53). VCAM-1 and ICAM-1 are known to bind monocytes in monolayer adhesion assays in vitro and bind mononuclear cells in vivo (reviewed in reference 47). Expressions of VCAM-1 and ICAM-1 are upregulated in endothelium adjacent to early foam cell lesions in the aorta of experimental models of atherosclerosis. ICAM-1 expression has been identified in human atherosclerotic plaques (54). Thus, expression of these leukocyte adhesion molecules may be an important event in early atherosclerosis (5). Since NF- κ B controls the cytokine-induced expression of these leukocyte adhesion molecules, NF- κ B may be important in the initiation of the atherosclerotic lesion. Consistent with the proposal that multiple factors can initiate atherosclerosis (reviewed in references 52 and 53), a variety of signals can activate NF- κ B (reviewed in references 27, 29, and 37). For example, these NF- κ B-activating signals can emanate from cell surface receptors for cytokines (IL-1 β , TNF- α , and β) or LPS, from second messenger pathways, or protein kinase C activation. More specifically, reactive oxygen intermediates are associated with the formation of atherosclerosis and have been shown to activate NF- κ B (55). Additionally, oxidized lipids are involved in the formation of atherosclerotic lesions (reviewed in reference 56), and activate NF- κ B (Dr. Alan Fogelman, UCLA School of Medicine, personal communication). Similarly, viruses such as Herpes virus and cytomegalovirus have been implicated in atherosclerosis (57, 58) and these agents can activate NF- κ B (reviewed in reference 37). Thus, this transcriptional mediator is an attractive candidate for linking the otherwise seemingly diverse etiologic agents associated with atherosclerosis to the expression of a mononuclear-selective leukocyte adhesion molecule that has been implicated in the initiation of lesion formation. Additionally, endothelial cells at sites of developing atherosclerotic lesions are believed to have increased expression of multiple cytokines (59) and

growth factors (52). Since NF- κ B sites are found in the promoters of a variety of relevant soluble (e.g., IL-6, IL-8, TNF- α , G-CSF, GM-CSF, and gro), as well as cell surface (e.g., MHC class I and II) mediators, activation of a single

pleiotropic transcriptional factor like NF- κ B in endothelial cells could increase expression of multiple pathophysiologically relevant agents.

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