Negative transcriptional regulation of the VCAM-1 gene by fluid shear stress in murine endothelial cells

RISA KORENAGA,1 JOJI ANDO,1 KEISUKE KOSAKI,1 MASASHI ISSHIKI,1 YOSHIO TAKADA,2 AND AKIRA KAMIYA2

1Department of Cardiovascular Biomechanics and 2Institute of Medical Electronics, Faculty of Medicine, University of Tokyo, Tokyo 113; and 3Institute for Life Science Research, Asahi Chemical Industry Company, Fuji-City 416, Japan

Korenaga, Risa, Joji Ando, Keisuke Kosaki, Masashi Isshiki, Yoshio Takada, and Akira Kamiya. Negative transcriptional regulation of the VCAM-1 gene by fluid shear stress in murine endothelial cells. Am. J. Physiol. 273 (Cell Physiol. 42): C1506–C1515, 1997.—To explore the mechanism of shear stress-induced downregulation of vascular cell adhesion molecule 1 (VCAM-1) expression in murine endothelial cells (ECs), we examined the effect of shear stress on VCAM-1 gene transcription and assessed the cis-acting elements involved in this phenomenon. VCAM-1 mRNA expression was downregulated at the transcriptional level as defined by nuclear run-on assay and transient transfection of VCAM-1 promoter-luciferase gene constructs. The luciferase assay on the VCAM-1 deletion mutants revealed that the cis-acting element is contained between −694 and −329 bp upstream from the transcription initiation site. Gel shift assay using overlapping oligonucleotide probes of this region showed that oligonucleotides containing a double AP-1 consensus sequence (TGACTCA) formed distinct complexes with nuclear proteins extracted from shear-stressed cells. Mutation of either one or both of two AP-1 consensus sequences completely abolished the ability of the promoter to respond to shear stress. These results suggest that fluid shear stress downregulates the transcription of the VCAM-1 gene via an upstream cis-element, a double AP-1 consensus sequence, in murine lymph node venule ECs.

vascular endothelial cells; vascular cell adhesion molecule 1; nuclear factor activator protein-1; c-jun

VASCULAR ENDOTHELIAL CELLS (ECs) regulate the tonus of vessels by releasing various smooth muscle relaxing and contracting substances and display antithrombotic activity by producing anticoagulant and fibrinolytic mediators. They also actively interact with other cells via the growth factors, adhesion molecules, and extracellular matrix that they synthesize and secrete. In addition to their wide variety of functions, ECs are characterized by constant exposure to blood flow. This means that wall shear stress, a mechanical force generated by flowing blood, directly stimulates ECs. Studies in the last decade have shown that ECs change their morphology and functions in response to shear stress and that their responses are closely associated with blood flow-dependent phenomena such as angiogenesis, vascular remodeling, and atherosclerosis (1).

Recently, it has also become apparent that shear stress affects the gene expression of many bioactive molecules in ECs. For instance, shear stress upregulates gene expression of tissue plasminogen activator (tPA) (5), platelet-derived growth factor (PDGF)-A (6), nitric oxide synthase (NOS) (16), intercellular adhesion molecule 1 (ICAM-1) (15), transforming growth factor-β1 (TGF-β1) (17), monocyte chemotactic protein-1 (MCP-1) (23), C-type natriuretic peptide (CNP) (19), and both the Cu/Zn- and Mn-dependent isoforms of superoxide dismutase (SOD) (8, 26), whereas it downregulates gene expression of angiotensin converting enzyme (ACE) (21). Shear stress up- or downregulates the expression of genes encoding PDGF-B (11, 14, 20), endothelin (12, 22, 30), and thrombomodulin (TM) (13, 25), depending on the experimental conditions used. In terms of a molecular mechanism for the regulation of endothelial gene expression by shear stress, Resnick et al. (20) first identified a cis-acting shear stress-responsive element (SSRE) in the 5′-promoter region of the PDGF-B gene. A core-binding sequence within the SSRE (GAGACC) is also present in other genes that respond to shear stress, including the genes for tPA, NOS, ICAM-1, TGF-β1, MCP-1, TM, CNP, SOD, and ACE. This suggests a general mechanism for regulation of endothelial gene transcription by shear stress. It was later demonstrated, however, that cis-acting elements other than the GAGACC sequence were involved in the induction of TGF-β1 and MCP-1 genes by shear stress (17, 24). The PDGF-A gene, which does not contain GAGACC in its promoter, also responds to shear stress. Thus the cis-acting elements required for shear stress responsiveness appear to be diverse.

We recently demonstrated that fluid shear stress decreases cell surface expression of vascular cell adhesion molecule 1 (VCAM-1) in cultured murine lymph node venule ECs, leading to suppression of their adhesiveness to lymphocytes, and that VCAM-1 mRNA levels were also found to be downregulated by shear stress (2, 18). However, the GAGACC sequence (the PDGF-B SSRE) is not encoded within the VCAM-1 promoter, and the molecular mechanism of downregulation of VCAM-1 gene expression by shear stress, including the cis-elements and transcription factors involved, remains unclear. To investigate this negative regulatory mechanism, in this study, we cloned the genomic VCAM-1 gene from the cultured murine ECs and examined the direct effect of shear stress on VCAM-1 gene transcription by both nuclear run-on assay and luciferase assay using a reporter gene consisting of the VCAM-1 promoter coupled to luciferase. By deletion analysis and gel shift assay, we also localized a cis-acting element in the promoter that is critical for negative regulation by shear stress.
METHODS

Cell culture. ECs from lymph node venules of C57BL/6 mice (Japan SLC, Hamamatsu, Japan) were provided by M. Miyasaka (University of Osaka, Osaka, Japan). Methods of isolation and characterization of the cells have been described previously (27). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GibCO, Grand Island, NY) containing 20% fetal calf serum (GIBCO), 10 mM N2-hydroxylpiperazine-N’-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM 2-mercaptoethanol, 1% (vol/vol) 100X nonessential amino acids (Flow Laboratories, Irvine, Scotland), 100 U/ml penicillin, and 100 µg/ml streptomycin and passaged with 0.05% trypsin/2 mM EDTA in phosphate-buffered saline (PBS) when grown confluent. The cells had the configuration of a homogeneous monolayer resembling a cobblestone pavement and the ability to take up fluorescent acetylated low-density lipoprotein labeled with N-1-diotadecyl-1-3,3’,3’-tetramethylindocarbocyanine perchlorate (Biomedical Technologies, Stoughton, MA). Cells in their fourth to seventh passages were plated on a glass plate coated with collagen (0.1 mg/ml) (Cellmatrix I-A, Nitta Gelatin, Osaka, Japan) for use in the experiment.

Flow-loading apparatus. To apply controlled levels of shear stress to cultured cells, we used the same parallel plate type of flow chamber described previously (2). One side of the chamber was formed by a coverslip on which ECs were cultured. The other side was machined from a polyacrylate plate. These two flat surfaces were held ~200 µm apart by a silicone rubber gasket. The chamber had an entrance for the medium, and the entrance was connected to a reservoir by a silicone tube. The medium was perfused by a roller/tube pump (ATTO, Tokyo, Japan). The entire circuit was placed in an automatic CO2 incubator, and the flow-loading experiments were performed at 37°C in an atmosphere of 95% room air-5% CO2.

The intensity of wall shear stress (τ, dyn/cm2) on the EC layer was calculated by the formula τ = µ δQ/ab, where μ is the viscosity of the perfusate (0.0094 P at 37°C), Q is flow volume (ml/s), and a and b are cross-sectional dimensions of the flow path. Because the maximum Reynold’s number corresponding to the highest flow rate used in this study was ~40, we assumed that the flow was laminar.

RNA isolation and reverse transcriptase-polymerase chain reaction. Isolation of total RNA from murine ECs was accomplished by the acid guanidinium thiocyanate-phenol-chloroform extraction method. Reverse transcription of messages for VCAM-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were carried out using Moloney murine leukemia virus reverse transcriptase (GIBCO, Gaithersburg, MD). The obtained cDNA samples were then coamplified by using 12–24 cycles of polymerase chain reaction (PCR) with sense and antisense primers for VCAM-1 and GAPDH, AmpliTaq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT), and [α-32P]dCTP (Du Pont, Wilmington, DE). Each temperature cycle consisted of 95°C for 1 min, 60°C for 2 min, and 72°C for 3 min. After the PCR reactions, the amplification product was fractionated by electrophoresis on 8% polyacrylamide gel. The radioactivity of each band on the gel was measured with a GS363 molecular imager system (Bio-Rad Laboratories, Richmond, CA).

Changes in VCAM-1 mRNA levels were quantitatively estimated as follows. The radioactivity of each band was plotted against the number of PCR cycles on a semilogarithmic scale, which would form a sigmoid curve. From the curve, the cycle in which the operating range of the PCR was linear was selected, and the ratio of radioactivity between VCAM-1 and GAPDH in the cycle was calculated as a parameter of relative VCAM-1 mRNA levels.

Isolation of murine genomic VCAM-1 gene. Genomic DNA was extracted from cultured murine ECs with a TurboGen genomic DNA isolation kit (Invitrogen, San Diego, CA). Sau3AI-digested fragments (~20 kb) of the genomic DNA were ligated to the λEMBL3 arms (Stratagene, La Jolla, CA) and packaged with Gigapack II packaging extracts (Stratagene). The packaged λ-phage vectors were transfected into XL1-Blue MRA strain (Takara Biomedicals, Kyoto, Japan).

A 150-mer synthetic oligonucleotide encoding exon 1 of the murine VCAM-1 gene (4) was labeled with [α-32P]dCTP by random primer DNA labeling kit (Takara Biomedicals) and used as a probe. Approximately 106 recombinants from the λEMBL3 murine genomic DNA library described above were screened by plaque hybridization. Positive clones were identified, and one clone was analyzed further. Bacteriophage DNA was prepared, and BamHI-digested fragment (~7 kb) was subcloned in pUC19 plasmids (Takara Biomedicals), transfected into JM109 competent cells (Toyobo, Osaka, Japan), and amplified. The 2.4-kg DNA fragment obtained from the digestion of 7-kb fragment with Bgl II was subcloned in pUC19 plasmids, and their nucleotide sequences were determined by a cycle sequencer.

DNA sequencing. Template DNA was mixed with a terminator ready reaction mixture containing dye-labeled terminators and AmpliTaq DNA polymerase (PRISM ready reaction terminator cycle sequencing kit; Perkin Elmer) and synthetic oligonucleotide primers prepared with an Oligo 100 DNA synthesizer (Beckman Instruments, Fullerton, CA). PCR was carried out for 25 cycles in a thermal cycler (Perkin Elmer thermal cycler model 9600). The PCR products were then electrophoresed on 42.5% polyacrylamide gel, and base sequences were automatically determined by an Applied Biosystems 373 DNA sequencer (Perkin Elmer).

Nuclear run-on transcription assay. ECs were washed with ice-cold PBS, scraped, and pelleted by centrifugation at 1,500 revolutions/min (rpm) for 5 min. The cell pellet was resuspended in 1 ml NP-40 lysis buffer [10 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.5% (vol/vol) NP-40], incubated for 5 min on ice, and centrifuged at 3,000 rpm for 5 min. The nuclear pellet was washed once with 1 ml NP-40 lysis buffer and centrifuged again at 3,000 rpm for 5 min. Nuclei were resuspended in 100 µl of 50 mM Tris-HCl, pH 8.3, 5 mM MgCl2, 0.1 mM EDTA, and 40% (vol/vol) glycerol and frozen in liquid N2. The nuclei were thawed and reactivated in 100 µl reaction buffer consisting of 10 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 300 mM KCl, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, and 3.7 MBq [α-32P]JUTP (~111 TBq/mmol) for 30 min at 30°C. The 32p-labeled RNA was precipitated with trichloroacetic acid and purified with phenol-chloroform extraction. Plasmids containing murine VCAM-1 or human GAPDH fragment (Clontech) were linearized by restriction enzyme digestion and denatured at 95°C. The DNA was spotted onto nylon membranes and fixed with 0.4 N NaOH. Radiolabeled RNA was adjusted to 2.5 × 108 counts·min−1·ml−1 in a hybridization solution [5% SSPE, 50% formamide, 0.1% Denhardt’s solution, and 0.1% sodium dodecyl sulfate (SDS)] and hybridized to DNA immobilized on nylon membranes for 48 h at 42°C. Blots were washed twice in 2× SSPE and 0.1% SDS for 15 min at 42°C, once in 1× SSPE and 0.1% SDS for 30 min at 42°C, and twice in 0.1× SSPE and 0.1% SDS for 15 min at room temperature. Autoradiograms of the membranes were obtained using a GS363 molecular imager system (Bio-Rad).

Construction of reporter plasmids. To determine the transcriptional activity of the VCAM-1 gene, we used reporter plasmids.
plasmids containing the murine VCAM-1 promoter linked to the luciferase gene. A series of deletions was created through the 5' flanking sequences of the VCAM-1 gene by restriction enzyme digestion and subcloned into the luciferase reporter vector (pGL2-enhancer vector; Promega, Madison, WI). The following deletion constructs were generated.

pGLVCAM-1 (−3.7 luc) 3.7 kb of the VCAM-1 upstream region relative to the start of transcription was removed from the plasmid pUC19 containing 7-kb VCAM-1 fragment by digesting with Hind III and cloned into the Hind III digested pGL2-enhancer.

pGLVCAM-1 (−1.8 luc) 1,801 bp of the VCAM-1 upstream region was cut from 7-kb VCAM-1 fragment with Bgl II and Hind III and cloned into the Bgl II-Hind III digested pGL2-enhancer.

pGLVCAM-1 (−1.1 luc) 1,133 bp of the VCAM-1 upstream region was obtained by digesting 7-kb VCAM-1 fragment with Eco RI and Hind III and cloned into the Nhe I-Hind III digested pGL2-enhancer.

pGLVCAM-1 (−0.7 luc) 694 bp of the VCAM-1 upstream region was removed from the 7-kb VCAM-1 fragment with Mfe I and Hind III and cloned into the pGL2-enhancer treated with Bgl II and Hind III.

pGLVCAM-1 (−0.3 luc) 7-kb VCAM-1 fragment was digested with Sau3A I and Hind III. The 329 bp of the VCAM-1 upstream region was inserted into the Bgl II-Hind III digested pGL2-enhancer.

Transfection and luciferase assays. Murine ECs were seeded on a coverslip at a density of 1 × 10^6 cells/cm². The cells were transfected with constructs using Transfectam (Biosepra, Malborough, MA). The pSV-β-galactosidase vector (Promega) was cotransfected to monitor transfection efficiency. After incubation at 37°C for 24 h, the cells were then either exposed to shear stress or incubated under static conditions. The cells were washed twice with PBS, and 250 µl of lysis buffer (included in Promega's luciferase assay kit) were added. After 15 min at room temperature, the lysates were centrifuged at 12,000 rpm for 2 min. Luciferase activity was determined using 20 µl of the clarified lysate and 100 µl of luciferase (included in Promega's luciferase assay kit) were added. After 12,000 rpm for 2 min, the mixture was incubated for 1 h. The absorbance at 420 nm was measured by microplate reader (Bio-Rad model 3550). The luciferase activity of each sample was normalized to that of β-galactosidase before calculating the values reported in RESULTS.

Gel shift assay. To prepare nuclear extracts, control (static) or shear-stressed (3.5 dyn/cm² for 24 h) murine EC monolayers containing ~6 × 10⁶ cells were washed with PBS and suspended in hypotonic buffer [20 mM HEPES-KOH, pH 7.9, 5 mM KC1, 8 mM MgCl₂, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF); Sigma], and sediments were resolved in extraction buffer [20 mM HEPES-KOH, pH 7.9, 25% (vol/vol) glycerol, 0.5 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.5 µg/ml pepstatin A, and 1.3 µg/ml spermine]. After the centrifugation of 30 min at 21,000 rpm, the supernatants were then dialyzed against buffer [20 mM HEPES-KOH, pH 7.9, 10% (vol/vol) glycerol, 50 mM KC1, 0.5 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF], and the nuclear extracts obtained were kept frozen at −80°C until analysis.

Twenty-one oligonucleotides, which correspond to the sequences in the 5′ flanking region of the VCAM-1 gene (~700 and ~300 bp upstream from the transcription initiation site), were synthesized in an Oligo 100 DNA synthesizer. Each oligonucleotide was 30–34 bp long and contained a 10-bp overlapping stretch at their ends. These oligonucleotides were labeled using T₄ polynucleotide kinase (Toyobo) and [γ-32P]ATP. The binding reactions between radiolabeled oligonucleotides and nuclear extracts were allowed to proceed, and the reaction mixtures were electrophoresed through 7% polyacrylamide gels, using reagents supplied with the StrataGene GelShift assay kit. The protein-DNA complexes on the gel were analyzed with a GS363 molecular imager system.

In supershift assay, antibodies were added to the binding reaction, and the mixture was incubated for 30 min at room temperature before adding the labeled oligonucleotide. Antibodies against c-jun, c-fos, adenosine 3′,5′ cyclic monophosphate (cAMP)-responsive element binding protein-1 (CREB-1), cAMP-responsive element modulator-1 (CREM-1), activating transcription factor-2 (ATF-2), and retinoid acid receptor-α₁ (RAR-α₁) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Site-directed mutagenesis. Mutations were generated at AP-1 binding sites in the VCAM-1 gene promoter by the Kunkel method of site-directed mutagenesis. Single-stranded template DNA (ssDNA) was prepared by growing bacteriophage M13K07 (Takara) in a strain of Escherichia coli, CJ 236 (Takara) that had been transfected with the construct pGLVCAM-1 (−1.8 luc). Oligonucleotides corresponding to the wild-type sequence with specific mutations at sites indicated by asterisks in Fig. 6A were synthesized in an Oligo 100 DNA synthesizer and phosphorylated with T₄ polynucleotide kinase. The mutagenic oligonucleotides and ssDNA were annealed, and the polymerization/extension reaction was performed using E. coli DNA ligase (Takara) and T₄ DNA polymerase (Takara). The double-stranded DNAs obtained were transfected into J M109, and their sequences were confirmed. Luciferase assay was carried out in the murine ECs transfected with these mutagenized DNAs, which were cultured under static conditions or exposed to shear stress.

Statistical analysis. The results are expressed as means ± SD. The mean values obtained in the control and experimental groups were analyzed for significant differences by analysis of variance and the Bonferroni modification of the t-test. Differences were considered statistically significant at P < 0.05.

RESULTS

Shear stress dependency of the flow-induced decrease in VCAM-1 mRNA levels. Total RNA was extracted from murine ECs exposed to shear stress (1.5 dyn/cm²) for 6 h, and changes in VCAM-1 mRNA levels were determined by reverse transcription-PCR. VCAM-1 mRNA levels decreased in response to flow loading, which agreed quite well with our previous work (2). To determine whether the flow-induced decrease in VCAM-1 mRNA levels is a specific response for shear stress, we carried out flow-loading experiments using two perfusates having different viscosities. The high-viscosity medium (DMEM with 5% dextran) or low-viscosity medium (DMEM alone) was perfused through the circuit, and changes in VCAM-1 mRNA levels were examined. VCAM-1 mRNA levels decreased as the flow velocity (shear rate) increased, but the decrease was greater at higher viscosity and higher shear stress (Fig. 1A). On the other hand, the curves for VCAM-1 mRNA levels plotted against shear stress formed a single line (Fig. 1B), indicating that the flow-induced decrease in VCAM-1 mRNA levels is shear stress dependent rather than shear rate dependent.
of 1.5–3 dyn/cm², which corresponds to physiological shear stress occurred at the relatively low shear stress pressure of 289 and 292 mosmol/kgH₂O, respectively. Note that specific gravity of 1.005 and 1.025, respectively, and an osmotic viscosity media had a viscosity of 0.945 and 3.780 cP, respectively, a DMEM plus 5% dextran (mol wt 162,000, Sigma). Low- and high-medium was DMEM alone, and high-viscosity medium consisted of cells (ECs) were exposed to laminar flow for 6 h. Low-viscosity lar cell adhesion molecule 1 (VCAM-1) mRNA levels. Endothelial Fig. 1. Shear stress dependency of flow-induced decreases in vascular cell adhesion molecule 1 (VCAM-1) mRNA levels. Endothelial cells (ECs) were exposed to laminar flow for 6 h. Low-viscosity medium was DMEM alone, and high-viscosity medium consisted of DMEM plus 5% dextran (mol wt 162,000, Sigma). Low- and high-viscosity media had a viscosity of 0.945 and 3.780 cP, respectively, a specific gravity of 1.005 and 1.025, respectively, and an osmotic pressure of 289 and 292 mosmol/kgH₂O, respectively. Note that VCAM-1 mRNA levels plotted against shear rate (A) at low and high viscosity are separate, whereas those plotted against shear stress (B) form an almost single line. Same experiments were repeated twice, and similar results were obtained. These results indicate that flow-induced decrease in VCAM-1 mRNA levels is shear stress dependent rather than shear rate dependent.

Marked downregulation of VCAM-1 mRNA levels by shear stress occurred at the relatively low shear stress of 1.5–3 dyn/cm², which corresponds to physiological levels of wall shear stress in small veins. It seemed likely, although not certain, that the ECs used are sensitive to venous levels of shear stress because they originated from venules of lymph nodes.

Sequence of the murine VCAM-1 promoter. We cloned the murine genomic VCAM-1 gene from the cultured murine ECs used in the present study. The promoter sequence −1,801 bp from the transcription initiation site is shown in Fig. 2. The transcription initiation site was determined by primer extension analysis. The sequences TGGGTTTCCC at −73 bp and AGGGATTTCCT at −68 bp are identical to the consensus sequence (GGG[CA/TT]YYCC) for the NFκB binding site. The sequence TGACTCA at both −481 and −461 bp perfectly matches the AP-1 consensus sequence (tumor promoting agent-response element). A sequence (CGTCA) with homology to cAMP response element (CRE) was identified at position −1420.

Direct effect of shear stress on VCAM-1 gene transcription. To determine whether shear stress directly influences VCAM-1 gene transcription, we performed a nuclear run-on transcription assay. Nuclei were prepared from static control or shear-stressed ECs, and transcription was allowed to continue in the presence of [³²P]UTP. Purified radiolabeled RNA was hybridized to cDNA immobilized on nylon membranes. Although high levels of transcription of the VCAM-1 gene could be detected in static control cells, after 24-h exposure to shear stress, transcription was decreased (Fig. 3A). Densitometry of individual spots of interest revealed that the density of VCAM-1 mRNA signal in shear-stressed cells decreased to 68 ± 1.4% (SD) of that in static controls (n = 3, P < 0.001). The transcription of GAPDH mRNA did not change significantly after exposure of the cells to shear stress. The density of shear-stressed cells was 91.4 ± 5.5% (SD) of that of static controls (n = 3, P = NS).

We also performed a luciferase assay to evaluate the effect of shear stress on VCAM-1 gene transcription. Murine ECs transfected with the reporter genes consisting of a 3.7-kb fragment of the murine VCAM-1 promoter coupled to luciferase were exposed to shear stress (3.5 dyn/cm²) for 24 h. The luciferase activity of shear-stressed cells was markedly lower than that of the static control cells (Fig. 3B). The reduction rate was ~48%, indicating that shear stress downregulates VCAM-1 mRNA transcriptionally. The reporter gene was also transfected to fetus bovine aortic ECs, and a similar luciferase assay was performed. The luciferase activity of the static bovine ECs was significantly lower (~24%) than that of murine ECs and further decreased to ~10% in response to shear stress. In contrast, similar luciferase activity was observed in murine ECs under both static and shear stress conditions after transfection of the pGL2-control or -enhancer vector that contained no VCAM-1 promoter, indicating that transcription of the luciferase gene itself is not influenced by the shear conditions used.

Localization of the shear stress-responsive regions of the VCAM-1 promoter. The fact that shear stress suppresses VCAM-1 gene transcription suggests the presence of cis-acting regions within the VCAM-1 promoter that are responsive to shear stress. To localize these regions, a series of nested 5’-deletion mutations of the VCAM-1 promoter coupled to luciferase was generated (Fig. 4A) and transfected into murine ECs. The transfected cells were then exposed to shear stress (3.5 dyn/cm²) for 24 h, and their luciferase activity was measured. As shown in Fig. 4B, the cells transfected with constructs −3.7, −1.8, −1.1, or −0.7 luc showed a marked decrease in luciferase transcription in response
to shear stress, whereas transfection of construct −0.3 luc containing 329 bases of the promoter abolished responsiveness to shear stress, although not completely. These findings indicate that the shear stress-responsive regions are located between −694 and −329 bp of the 5' flanking region of the murine VCAM-1 gene.

The actual levels of reporter gene transcription varied somewhat according to the nested deletions. Although the exact reason for that was not investigated, it seems that many cis-elements, such as enhancers and silencers, which up- or downregulate transcription, may exist at various locations in the 5'-upstream region of the murine VCAM-1 gene.

Oligonucleotides bearing the AP-1 binding sites interact with nuclear proteins from ECs exposed to shear stress. To examine the presence of transcriptional factors that bind to the shear stress-responsive regions, we performed gel shift assays in which nuclear extracts from static or shear-stressed cells were incubated with each of 21 radiolabeled oligonucleotides synthesized based on the sequences of −694 and −329 bp upstream from the transcription initiation site of the VCAM-1 gene. Three oligonucleotides bearing the transcription factor AP-1 binding sites (−472 to −443 bp, −492 to −463 bp, and −481 to −452 bp) were able to form distinct complexes with nuclear protein derived from either static or shear-stressed murine ECs (Fig. 5A). Gel shift assays, which were performed with oligonucleotides bearing the mutated AP-1 consensus sequences as a labeled probe and nuclear extracts from shear-stressed cells, showed the complete disappearance of the protein-DNA complexes formed by the oligonucleotide containing native AP-1 binding site sequences (Fig. 6B). These findings indicate that a double AP-1 consensus element is responsible for the shear-induced downregulation of murine VCAM-1 gene transcription.

AP-1 consensus elements are essential for shear-mediated downregulation of reporter gene expression. To test whether the AP-1 binding sites are critical for the VCAM-1 gene response to shear stress, we constructed several chimeric genes with site-specific mutagenesis at the AP-1 binding sites and assayed luciferase in the murine ECs transfected with these mutants. Mutant-1 and mutant-2, which had a mutated distal and proximal AP-1 binding site, respectively, and mutant-3, in which both sites were mutated, abolished the downregulation of VCAM-1 gene transcription in response to shear stress seen in the wild-type gene (Fig. 6A). Gel shift assays, which were performed with oligonucleotides bearing the mutated AP-1 consensus sequences as a labeled probe and nuclear extracts from shear-stressed cells, showed the complete disappearance of the protein-DNA complexes formed by the oligonucleotide containing native AP-1 binding site sequences (Fig. 6B). These findings indicate that a double AP-1 consensus element is responsible for
VCAM-1 gene response to shear stress in murine lymph node venules.

Mutant-1 and mutant-3 enhanced transcriptional activities in response to shear stress more than twofold. A gel shift assay demonstrated that shear stress induces nuclear proteins that bind to the CRE consensus sequence (CGTCA), which exists at position 21420 in the 5′-upstream region of murine VCAM-1 gene (Fig. 6C). Mutant-4, in which both CRE and AP-1 binding sites were mutated, did not exhibit the shear stress-induced enhancement of transcriptional activity seen in mutant-3, indicating that CRE is involved in the mechanism by which shear stress enhances the transcription in the AP-1 binding site mutant, i.e., the positive effect of CRE on transcription may become dominant in shear-stressed cells when the negative effect of the AP-1 binding site is blocked by site-specific mutagenesis.

DISCUSSION

Our previous study demonstrated that laminar flow suppresses the adhesiveness of murine lymph node venule ECs to lymphocytes by downregulating the expression of VCAM-1 at both the mRNA and cell surface protein levels (2, 18). The present study confirmed that downregulation of VCAM-1 mRNA is a specific response to shear stress and not to flow velocity or shear rate. When cells are exposed to fluid flow, two distinct effects can occur: 1) mechanical stimulation of the cells by the direct force of flow, shear stress; and 2) an indirect effect of flow that changes the concentration of agonists at the endothelial surface. In regard to this latter effect, if certain agonists, e.g., adenine nucleotides, bradykinin, and angiotensin, are removed rapidly by degradation at the cell surface, a diffusion boundary layer will exist near the cell surface, and this layer will become thinner as flow velocity increases, i.e., flow will increase the amount of such agonists reaching the cell surface, and consequently modulate cell functions, flow velocity or shear rate dependently.

To differentiate the direct and indirect effects of flow, we performed flow-loading experiments with two perfus-

Fig. 3. Effect of shear stress on VCAM-1 gene transcription. A: nuclear run-on transcription assay. Nuclei (1 × 10⁷ cells) were harvested from murine ECs either incubated under static conditions (static) or exposed to a shear stress of 3.5 dyn/cm² (shear) for 24 h. Labeled RNA was hybridized to cDNA for murine VCAM-1, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (positive control) and plasmid pUC19 (negative control). After exposure to shear stress, transcription of VCAM-1 gene decreased, but GAPDH gene transcription did not change. There was no hybridization to pUC19 control. Same experiments were repeated three times, and similar results were obtained. B: luciferase assay. Murine or bovine ECs transfected with murine VCAM-1-luciferase construct were exposed to laminar shear stress (3.5 dyn/cm² for 24 h; shear) or incubated under static conditions for same period of time (static). As negative control experiments, murine ECs transfected with pGL2-control or pGL2-enhancer vector were also exposed to shear stress. pGL2-control contains simian virus 40 (SV40) enhancer, promoter, and luciferase gene, and pGL2-enhancer contains SV40 enhancer and luciferase gene. Luciferase activity is expressed as percentage of static control of murine ECs. Data are means of 3 separate coverslips.

VCAM-1 gene response to shear stress in murine lymph node venules.

Mutant-1 and mutant-3 enhanced transcriptional activities in response to shear stress more than twofold. A gel shift assay demonstrated that shear stress induces nuclear proteins that bind to the CRE consensus sequence (CGTCA), which exists at position –1420 in the 5′-upstream region of murine VCAM-1 gene (Fig. 6C). Mutant-4, in which both CRE and AP-1 binding sites were mutated, did not exhibit the shear stress-induced enhancement of transcriptional activity seen in mutant-3, indicating that CRE is involved in the mechanism by which shear stress enhances the transcription in the AP-1 binding site mutant, i.e., the positive effect of CRE on transcription may become dominant in shear-stressed cells when the negative effect of the AP-1 binding site is blocked by site-specific mutagenesis.

Fig. 4. Deletion analysis of murine VCAM-1 promoter. A: schematic diagram of 5′-deletion mutations in VCAM-1 upstream regulatory regions. B: luciferase assay. A series of 5′-deletion/luciferase constructs was transfected into cultured murine ECs, which were then exposed to a shear stress (3.5 dyn/cm²) for 24 h or maintained under static conditions for same period of time. Cells transfected with –3.7 luc, –1.8 luc, –1.1 luc, or –0.7 luc constructs showed significantly decreased relative luciferase activity in response to shear stress (*P < 0.001, n = 6), whereas cells transfected with –0.3 luc construct showed no significant change.
ates of different viscosity. The results demonstrated that the decrease in VCAM-1 mRNA levels occurred to a greater extent at higher viscosity or higher shear stress at a given flow velocity, indicating that the VCAM-1 mRNA response is shear stress dependent rather than shear rate dependent.

Our nuclear run-on assay clearly demonstrated that the downregulation of murine VCAM-1 gene expression by shear stress is transcriptionally mediated. Luciferase assay using the reporter gene containing the promoter of a cloned murine VCAM-1 gene also revealed that transcriptional activity was markedly suppressed by shear stress. Although it is unclear whether shear stress alters the stability of VCAM-1 mRNA, it seems certain that suppression of transcription is involved. When bovine fetus aortic ECs were transfected with the same reporter gene, the basal transcription level was markedly lower than that of murine ECs, but suppression of murine VCAM-1 gene transcription occurred in response to shear stress. This indicates that there is a mechanism that specifically enhances basal transcription of the VCAM-1 gene in murine lymph node venule...
ECs, but that murine and bovine ECs may possess common transcriptional factors that can be activated by shear stress. In vivo, lymph node venule ECs, a common site of leukocyte emigration, constitutively express abundant VCAM-1 on their cell surface, and display high adhesiveness for lymphocytes. Thus the inhibitory effect of shear stress on VCAM-1 expression might be noticeable in such ECs expressing VCAM-1 at high basal levels. There are certain other situations in which shear stress exerts an inhibitory effect on VCAM-1 expression. Varner et al. (28) showed that preconditioning human umbilical vein ECs with shear stress inhibits cytokine (interleukin-1β)-induced VCAM-1 gene expression. Walpola et al. (29) observed that when blood flow was decreased by surgical manipulations in rabbit carotid arteries in vivo, endothelial VCAM-1 expression greatly increased, indicating that at physiological levels of shear stress blood flow might have been suppressing VCAM-1 expression in the presurgical arteries.

By analyzing the 5′-promoter of the murine VCAM-1 gene, we found that the cis-acting element responding to shear stress is present between −694 and −329 bp upstream from the transcription initiation site that contains two AP-1 consensus sequences, TGACTCA. Gel shift assay showed that oligonucleotides bearing a single AP-1 binding site is a cis-acting element responding to shear stress.

<table>
<thead>
<tr>
<th>Mutant constructs</th>
<th>Reduction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.48</td>
</tr>
<tr>
<td>M-1</td>
<td>2.03</td>
</tr>
<tr>
<td>M-2</td>
<td>1.17</td>
</tr>
<tr>
<td>M-3</td>
<td>2.40</td>
</tr>
<tr>
<td>M-4</td>
<td>1.15</td>
</tr>
</tbody>
</table>

A double AP-1 binding site is a cis-acting element responding to shear stress. A: wild-type (WT) or various mutants (M-1, M-2, M-3, and M4) with site-specific mutations indicated by asterisks were transfected into murine ECs for luciferase assay. Reduction rate is ratio of luciferase activities (normalized for transfection efficiency) for shear-stressed cells to those for static controls. Similar results were obtained in 2 separate experiments. B: gel shift assay of complexes formed by oligonucleotide probes bearing native double AP-1 consensus sequences (lanes 1 and 2) or mutated ones like M-3 (lanes 3 and 4), M-2 (lanes 5 and 6), and M-1 (lanes 7 and 8) with nuclear extracts from shear-stressed cells. Lanes 1, 3, 5, and 7, no nuclear extracts; lanes 2, 4, 6, and 8, nuclear extracts from shear-stressed cells (3.5 dyn/cm² for 24 h). Arrow indicates shifted band, protein-DNA complexes. Nuclear proteins bound to oligonucleotide containing double native AP-1 consensus sequences (TGACTCA) but not to those with mutated consensus sequences (GGA-CTTG). Essentially similar results were obtained in 2 separate experiments. C: gel shift assay of complexes formed by oligonucleotide containing double native AP-1 consensus sequences (TGACTCA) but not to those with mutated consensus sequences (GGA-CTTG). Essentially similar results were observed in the 5′-upstream region of murine VCAM-1 gene. Similar results were observed in 2 separate experiments.
shear-stressed cells, suggesting that shear stress increases nuclear protein binding to the AP-1 consensus sequences. Furthermore, site-specific mutations indicate that both the proximal and distal AP-1 consensus element are critical for negative regulation of VCAM-1 transcription by shear stress. The molecular mechanism of shear-mediated gene regulation involving AP-1 is also seen in the MCP-1 gene, whose promoter contains two AP-1 binding sites, but there are some differences between the two (24). Shear stress upregulates transcription of the MCP-1 gene, and only one of the two AP-1 binding sites is shear responsive. AP-1 consensus elements have been known to be capable of acting as either transcriptional enhancers or silencers (3).

The products of the nuclear protooncogenes c-fos and c-jun form protein dimers that exhibit powerful binding activity to AP-1 consensus elements on gene promoters. Several reports indicate that shear stress increases c-fos and c-jun at both protein and mRNA levels in ECs (7, 10). The present gel shift assay revealed a marked increase of proteins that specifically bind to oligonucleotides with AP-1 consensus sequence in the nuclei of shear-stressed cells. We carried out supershift assay to examine whether c-fos and c-jun are involved in the protein-DNA complexes. The results demonstrated that antibody to c-jun peptide, which recognizes both phosphorylated and unphosphorylated forms of c-jun peptide, partially inhibited the formation of the protein-DNA complexes, whereas antibody to c-fos had no effect on the binding reaction. This suggests that c-jun dimers may be involved, at least in part, but there are other proteins that can form these complexes. The increase in the amount of protein-DNA complexes detected by gel shift assay does not necessarily mean that the same phenomenon occurs in vivo, i.e., the AP-1 binding sites in VCAM-1 promoter are occupied by proteins. From evidence now at hand, it seems difficult to tell which is more important for the regulation of murine VCAM-1 gene transcription by shear stress, the increase in the amount of transcriptional factors, or modifications of the factors, e.g., phosphorylation. Recently, transcription factor NFκB has been found to be activated by shear stress and to interact with the PDGF-B SSRE, leading to upregulation of PDGF gene expression in bovine ECs (9). There are two NFκB binding sites in the proximal region of murine VCAM-1 promoter, but interestingly, these sites are not implicated in the VCAM-1 gene response to shear stress. Therefore, further studies identifying cis-acting elements and transcription factors involved in a variety of EC responses to shear stress are needed to clarify the molecular mechanism for shear-mediated regulation of endothelial gene expression. Such studies will also provide us with new insights into blood flow-dependent phenomena such as angiogenesis, vascular remodeling, and atherosclerosis.

We thank Dr. Michael A. Gimbrone, J. R., Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, for advice and criticism.

This work was partly supported by Grants-in-Aid for Scientific Research and for Scientific Research on Priority Areas from the Japanese Ministry of Education, Science, and Culture, a research grant for cardiovascular diseases from the Japanese Ministry of Health and Welfare, and research funds from Tsumura.

Address for reprint requests: J. Ando, Dept. of Cardiovascular Biomechanics, Faculty of Medicine, Univ. of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

Received 4 November 1996; accepted in final form 30 June 1997.

REFERENCES