Shear Stress Increases ICAM-1 and Decreases VCAM-1 and E-selectin Expressions Induced by Tumor Necrosis Factor-α in Endothelial Cells

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Objective—Vascular endothelial cells (ECs) are subjected to shear stress and cytokine stimulation. We studied the interplay between shear stress and cytokine in modulating the expression of adhesion molecule genes in ECs.

Methods and Results—Shear stress (20 dynes/cm²) was applied to ECs prior to and/or following the addition of tumor necrosis factor (TNF)-α. Shear stress increased the TNF-α–induced expression of intercellular adhesion molecule-1 (ICAM-1) at both mRNA and surface protein levels, but decreased the TNF-α–induced expression of vascular adhesion molecule-1 (VCAM-1) and E-selectin. Transfection studies using promoter reporter gene constructs of ICAM-1, VCAM-1, and E-selectin demonstrated that these shear stress modulations of gene expression occur at the transcriptional levels. After 24-hour preshearing followed by 1 hour of static incubation, the effect of preshearing on TNF-α–induced ICAM-1 mRNA expression vanished. The recovery of the TNF-α–induced VCAM-1 and E-selectin mRNA expressions following preshearing, however, required a static incubation time of >6 hours (complete recovery at 24 hours). Pre- and postshearing caused a reduction in the nuclear factor-κB-DNA binding activity induced by TNF-α in the EC nucleus.

Conclusions—Our findings suggest that shear stress plays differential roles in modulating the TNF-α–induced expressions of ICAM-1 versus VCAM-1 and E-selectin genes in ECs. (Arterioscler Thromb Vasc Biol. 2004;24:1-8.)

Key Words: cytokine ■ E-selectin ■ endothelial cell ■ intercellular adhesion molecule-1 ■ shear stress ■ vascular adhesion molecule-1

Vascular endothelial cells (ECs) are constantly exposed to fluid shear stress, a tangential force generated by the velocity gradient in viscous fluid flow. The nature and magnitude of shear stress play a significant role in the homeostasis of the structure and function of the blood vessel. Recent evidence suggests that physiological levels of laminar shear stress modulate cellular signaling and EC function and are protective against atherogenesis. In human carotid and coronary arteries, atherosclerotic plaques are found in the vicinity of arterial bifurcations and bends, where the local flow is disturbed. In contrast, regions of artery that experience laminar non-oscillatory shear stress were protected from atherosclerosis. The cytokine tumor necrosis factor-α (TNF-α) is an important mediator of the inflammatory processes that occur during the progression of atherosclerosis. Produced by macrophages that infiltrate the lesion, cytokines such as TNF-α are known to induce the expression of many endothelial genes that contribute to the complex processes involved in atherogenesis. Well know examples include the transcriptional regulation of various adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. In contrast to laminar shear stress, cytokines are generally considered to be proatherogenic factors. Despite the intensive studies on the effects of fluid shear stress on ECs, the interplay of shear stress and cytokines in modulating EC gene expression and function has not fully been clarified. It has been shown that physiological levels of laminar shear stress inhibit the apoptosis of ECs induced by TNF-α and H₂O₂. This inhibitory effect of shear stress is mediated by signaling processes involving the glutathione redox system and NO. Kawai et al. found that the application of shear stress of 24 dynes/cm² for 24 hours augments the expression of tissue plasminogen activator (t-PA) induced by interleukin-1β (IL-1β) or TNF-α, but attenuates the secretion of plasminogen activator inhibitor-1 (PAI-1) induced by these cytokines. They further demonstrated that shear stress inhibits the TNF-α–induced endothelial expression of tissue factor gene at both mRNA and antigen levels, in a shear-intensity and exposure-time dependent manner. Tsao et al. reported...
that prior exposure to fluid flow for 4 hours decreases the EC adhesiveness induced by cytokines and lipoprotein and that this flow effect might be attributed in part to NO. A recent study by Surapisitchat et al demonstrated that pre-exposing ECs to shear stress of 12 dynes/cm² for 10 minutes inhibits cytokine-mediated activation of c-Jun NH₂-terminal kinase in ECs as a result of the activation of the extracellular signal-regulated kinase1/2 signaling pathway. Since atherosclerosis is a multifactorial disease involving a complex array of contributing factors, including shear stress and cytokines, it is of interest to investigate the effect of shear stress on endothelial function in the presence of cytokine stimulation.

To investigate the interplay between shear stress and cytokine stimulation in modulating EC gene expression and function, we analyzed the TNF-α-induction of three adhesion molecules (ie, ICAM-1, VCAM-1, and E-selectin) in ECs that had been previously exposed to laminar shear stress for various periods of time (preshearing). In addition, shear stress was applied to ECs after the TNF-α administration to examine the modulating effects of postshearing on TNF-α–induced gene expression. We found that both preshearing and postshearing of ECs and their combination augment the TNF-α–induced ICAM-1 expression at mRNA and protein levels, but attenuate the TNF-α–induced VCAM-1 and E-selectin expressions at mRNA and protein levels. Our present findings clearly demonstrate that the interplays between laminar shear stress and cytokine lead to differential modulations of the EC expressions of various adhesion molecules that serve similar function in vascular biology.

**Results**

**Both Preshearing and Postshearing of ECs Augment the ICAM-1 Gene Expression and Attenuate the VCAM-1 and E-selectin Gene Expression Induced by TNF-α**

Preshearing of ECs at 20 dynes/cm², a physiological level found in arteries, augmented the TNF-α–induced ICAM-1 mRNA expression in a shear time-dependent manner (Figure 1A). The normalized levels of the TNF-α–induced ICAM-1 mRNA in ECs that had been presheared for 4 and 24 hours were significantly increased as compared with the cells not presheared (P<0.05). In contrast to ICAM-1 expression, 4 or 24 hours of preshearing abolished the TNF-α–induced VCAM-1 mRNA expression to an extent similar to that in the control cells (P<0.01 versus ECs not presheared) (Figure 1B); however, preshearing had only a partial inhibitory effect on the TNF-α–induced E-selectin mRNA expression (P<0.05 versus ECs not presheared) (Figure 1C). These results indicate differential roles of laminar shear stress in modulating the TNF-α–induced ICAM-1 versus VCAM-1 and E-selectin gene expressions in ECs.

To further study the effects of shear stress on TNF-α–induced ICAM-1, VCAM-1, and E-selectin expressions, shear stress was applied to ECs either simultaneously with the addition of TNF-α or at 1, 2, or 3 hours after TNF-α addition. Shear stress application to ECs after the addition of TNF-α caused an augmentation of ICAM-1 mRNA expression (Figure 2A). The longer the duration of shearing, the greater was the increase in ICAM-1 mRNA expression. When the shear stress application was started simultaneously with the TNF-α addition (ie, postshearing for 4 hours), a maximal increase in ICAM-1 mRNA level was found. In contrast to the results on ICAM-1, the TNF-α–induced VCAM-1 (Figure 2B) and...
E-selectin (Figure 2C) mRNA expressions in ECs were attenuated by postshearing. The largest decreases of VCAM-1 and E-selectin mRNA expressions were seen when shear stress was applied for 3 hours (starting at 1 hour after TNF-α) or 4 hours (simultaneous exposure of ECs to shear stress and TNF-α). These results demonstrate that postshearing exerts the same differential effects as preshearing on the TNF-α-induced ICAM-1, VCAM-1, and E-selectin gene expressions in ECs.

**Combinatory Effects of Preshearing and Postshearing on TNF-α-Induced ICAM-1, VCAM-1, and E-selectin Gene Expressions in ECs**

To investigate whether preshearing and postshearing exert additive effects on modulating TNF-α–induced gene expression in ECs, we also examined the mRNA expressions of ICAM-1, VCAM-1, and E-selectin under conditions where shear stress application was started 24 hours before TNF-α or 4 hours (simultaneous exposure of ECs to shear stress and TNF-α). These results demonstrate that postshearing exerts the same differential effects as preshearing on the TNF-α-induced ICAM-1 versus VCAM-1 and E-selectin gene expressions in ECs.

**Effects of Shear Stress on Stability of ICAM-1, VCAM-1, and E-selectin mRNA**

Since both preshearing and postshearing of ECs and their combination altered the TNF-α–induced surface expressions of these adhesive proteins on ECs, we investigated whether these shearing conditions also resulted in changes in the TNF-α–induced mRNA levels of ICAM-1, VCAM-1, and E-selectin. As shown in Figure 3, ECs treated with TNF-α for 4 hours under static condition significantly increased ICAM-1, VCAM-1, and E-selectin promoter activities by approximately 3.1-, 4.2-, and 3.2-folds compared with static control cells, respectively. Shear stress applied to ECs 4 hours before and 4 hours after TNF-α addition significantly enhanced the ICAM-1 promoter activities, but attenuated the VCAM-1 and E-selectin promoter activities induced by TNF-α (P<0.05 versus TNF-α-treated ECs without shearing). This indicates that transcription of the luciferase gene itself is not influenced by the TNF-α and shear stress treatments. These results clearly demonstrated that shear stress modulation of ICAM-1, VCAM-1, and E-selectin induction by TNF-α involves transcriptional regulation.

**Effects of Shear Stress on TNF-α–Induced Adhesion Molecule Expression on ECs**

Since both preshearing and postshearing of ECs and their combination altered the TNF-α–induced ICAM-1, VCAM-1, and E-selectin gene expressions, we investigated whether these shearing conditions also resulted in changes in the TNF-α–induced surface expressions of these adhesive proteins on ECs. Flow cytometric analysis was performed with the monoclonal antibodies against ICAM-1, VCAM-1, and E-selectin, and representative data are shown in Figure 4. Under static conditions, stimulation of ECs by TNF-α (100 U/mL) for 4 hours resulted in an increase in ICAM-1,
VCAM-1, and E-selectin surface expressions, with a mean fluorescence intensity of 230, 188, and 201, respectively, as compared with 97, 22, and 26 in untreated cells. Preshearing of ECs (20 dynes/cm²) for 24 hours augmented this TNF-α/H9251–induced ICAM-1 expression, with a mean fluorescence intensity of 307, but reduced the TNF-α–induced VCAM-1 and E-selectin expressions, with a mean fluorescence intensity of 19 and 93, respectively. When ECs treated with TNF-α were subjected to postshearing, augmented ICAM-1 and reduced VCAM-1 and E-selectin surface expressions were also found, with mean fluorescence intensities of 287, 68, and 112, respectively. As expected, the combination of preshearing and postshearing of ECs also enhanced ICAM-1 and attenuated VCAM-1 and E-selectin protein expressions induced by

Figure 3. Shear stress modulations of TNF-α–induced ICAM-1, VCAM-1, and E-selectin expressions are transcriptional event. Chimeras containing the promoter regions of ICAM-1 (−850 bp), VCAM-1 (−2296 bp), or E-selectin (−540) and the reporter gene luciferase were transfected into BAECs. The transfected cells were then treated with TNF-α (100 U/mL) for 4 hour under the static condition (TNF) or exposed to shear stress of 20 dynes/cm² for 4 hour before and 4 hour after TNF-α addition (S4+TNF+S4). As negative control experiments, BAECs transfected with empty control pGL2 were also subjected to TNF-α and shear stress treatments. Induction was measured as the luciferase activity in the experimental cells relative to those in static controls. Data are represented as mean ± SEM from 4 or 5 separate experiments. *P<0.05 versus static control ECs. #P<0.05 versus TNF-α-treated ECs without shearing.

Figure 4. Effects of shear stress on TNF-α–induced ICAM-1, VCAM-1, and E-selectin expressions on ECs. ECs either kept as control or treated with TNF-α (100 U/mL) for 4 hour (TNF) were analyzed by flow cytometer. In additional experiments, ECs were exposed to shear stress of 20 dynes/cm² for 24 hour before the TNF-α treatment (S24+TNF). Shear stress was applied to ECs simultaneously with the addition of TNF-α for 4 hour (TNF+S4). Shear stress was applied to ECs for 24 hour before TNF-α stimulation and continued for 4 hour in the presence of TNF-α (S24+TNF+S4). ECs incubated only with FITC-conjugated antibody were used as blank controls. Results are representative of duplicate experiments with similar results.
TNF-α on ECs. These results show that the differential effects of shearing (both pre- and postshearing and their combination) on TNF-α–induced ICAM-1 versus VCAM-1 and E-selectin gene expressions are accompanied by corresponding changes in their protein expressions on EC surface.

Reprogramming of ICAM-1, VCAM-1, and E-selectin Expressions in Presheared and TNF-α–Treated ECs After the Removal of Shear Stress

To analyze the reprogramming of the TNF-α–induced ICAM-1, VCAM-1, and E-selectin expressions in presheared ECs after the removal of shear stress, ECs sheared for 24 hours were either immediately treated with TNF-α for 4 hours under static conditions or subjected to static incubation for 1, 6, or 24 hours before the TNF-α treatment. As shown in Figure 5A, TNF-α treatment given immediately at the end of 24-hour preshearing caused an increase in ICAM-1 expression. When the 24-hour presheared ECs were subjected to 1 hour of static incubation, the TNF-α induction of ICAM-1 expression returned to the level seen in the cells not presheared (P < 0.05 versus static control ECs). These results indicate that the regulatory effects of shear stress on the TNF-α–induced gene expression are time-dependent, with the subsidence of the preshearing effects occurring more rapidly for ICAM-1 than VCAM-1 and E-selectin.

Shear Stress Attenuates NF-κB-DNA Binding Activity in the Nucleus Induced by TNF-α

Since the promoter regions of the ICAM-1, VCAM-1, and E-selectin genes contain the NF-κB binding domain that has been shown to be activated by TNF-α,13–14 we investigated whether the TNF-α–induced NF-κB-DNA binding activity was influenced by shear stress. Nuclear protein extracts from ECs that had been either (1) presheared for 24 hours prior to 1 or 4 hours of TNF-α treatment or (2) treated with TNF-α under flow conditions for 1 or 4 hours were prepared for electrophoretic mobility-shift assay (EMSA). The EMSA results obtained from incubating EC nuclear protein extracts with oligonucleotides corresponding to the NF-κB binding sequences showed that TNF-α treatment caused an increase in binding activity (Figure 6). Preshearing of ECs before TNF-α treatment or the simultaneous exposure of ECs to shear stress and TNF-α caused a decrease in NF-κB-DNA binding activities. This binding was specific for NF-κB, because it was abolished by coincubation of nuclear proteins with 20-fold unlabeled oligonucleotides. This specificity was further substantiated by the supershifting in gel mobility of the NF-κB-oligonucleotide complex after preincubation of nuclear proteins with an antibody to p65.

Discussion

Our present study provides several lines of evidence that the physiological level of laminar shear stress plays differential
roles in mediating the TNF-α-induced ICAM-1 and VCAM-1 and E-selectin expressions in ECs. First, both preshearing and postshearing of ECs and their combination augmented the TNF-α-induced ICAM-1 mRNA expression, but decreased the TNF-α-induced VCAM-1 and E-selectin expressions. Second, shear stress increased the ICAM-1 and decreased the VCAM-1 and E-selectin promoter activities induced by TNF-α, whereas the mRNA stability of these adhesion molecules was not affected by shear stress. Third, these differential effects of shear stress on the TNF-α-induced ICAM-1 versus VCAM-1 and E-selectin protein expressions were shown by flow cytometric analysis. Fourth, when ECs that had been presheared for 24 hours were subjected to 1 hour of static incubation, their TNF-α-induced ICAM-1 mRNA expression returned to the same level as in the cells not presheared. The recovery of the TNF-α-induced VCAM-1 and E-selectin mRNA expressions, however, required a longer static incubation time (complete recovery at 24 hours). These results suggest that the regulatory effects of shear stress on the TNF-α-induced gene expressions are transient and that the effect lasts for a shorter period for the augmentation of ICAM-1 than the suppression of VCAM-1 and E-selectin. Finally, the TNF-α-induced NF-κB-DNA binding activity was reduced in the nucleus of ECs subjected to either preshearing or postshearing. This finding suggests that shear stress plays a role in modulating the cytokine-induced signal transduction, especially at the transcriptional level.

The effect of preshearing on the TNF-α-induced ICAM-1 expression is a function of the duration of preshearing. This time dependence might explain the difference between our results on the enhanced TNF-α-induced ICAM-1 expression by 24 hours of preshearing (Figure 4) and the results from the study by Taso et al.9 that preshearing of ECs for 4 hours did not increase the TNF-α-induced ICAM-1 protein expression on ECs. Our previous studies11,16 demonstrated that ECs exposed to shear stress for 24 hours induced their ICAM-1 surface protein expression. Thus, 24-hour preshearing exerts an additive effect to enhance the TNF-α-induced ICAM-1 expression (Figure 4). The inhibitory effect of preshearing on the TNF-α-induced VCAM-1 expression seems to be less dependent on the duration of preshearing. Thus, the TNF-α-induced VCAM-1 expression is inhibited by preshearing for either 4 hours9 or 24 hours (Figure 4).

The augmentation of the TNF-α-induced ICAM-1 expression by shear stress was not persistent, as indicated by the rapid return of the TNF-α-induced ICAM-1 expression to that of the static level within 1 hour after stoppage of the preshearing. Thus, a continuous application of shear stress to ECs is needed to sustain its regulatory effect on the TNF-α-induced gene expression in ECs. The recovery of the shear stress inhibition of the TNF-α-induced VCAM-1 and E-selectin expressions was slower than that of ICAM-1 after stoppage of the preshearing.

If such shear-induced modulation of EC gene expression in vitro is also operative in the arterial tree in vivo, the VCAM-1 and E-selectin genes may be rendered quiescent in the arterial ECs exposed to a constant shear flow, thus the shear stress has anti-inflammatory and antiatherogenic functions.

Our current study shows that postshearing also augmented the TNF-α-induced ICAM-1 and attenuated the TNF-α-induced VCAM-1 and E-selectin expressions in ECs. This suggests that shear stress exerts regulatory effects on the TNF-α-induced gene expressions regardless of whether it is applied before or after the addition of TNF-α. Whether the preshearing and postshearing share identical signaling pathways in ECs to modulate the TNF-α-induced gene expressions remains unclear. Our present study demonstrated that the combination of preshearing and postshearing did not exert more regulatory effect than that of preshearing alone on the TNF-α-induced ICAM-1, VCAM-1, and E-selectin mRNA expressions (Online Figure I versus Figure 1), suggesting that...
the effects of preshearing and postshearing on the TNF-α–induced gene expressions are not additive.

The mechanisms by which shear stress exerts differential effects on the TNF-α–induced ICAM-1 versus VCAM-1 and E-selectin expressions in ECs remain unclear. Cytokines and mechanical forces are well known mediators of gene expression in cells via activation of transcriptional factors, including NF-κB. Molecular cloning of ICAM-1, VCAM-1, and E-selectin has provided evidence for the presence of NF-κB–binding domains in the promoter regions that are responsible for TNF-α activation. However, the modulation of ICAM-1, VCAM-1, and E-selectin by NF-κB may differ in terms of DNA binding affinity or transcriptional activation. De Caterina et al demonstrated that NO can inhibit cytokine-induced expression of VCAM-1, but NOT ICAM-1, in ECs via the inhibition of NF-κB activation. Weber et al indicated that the antioxidants pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC) reduce the TNF-α–induced VCAM-1, but not ICAM-1, expression in ECs by preventing the TNF-α–induced NF-κB mobilization and that these treatments also inhibit the subsequent monocye adhesion to TNF-α–treated ECs. They concluded that the induction of VCAM-1, but not ICAM-1, by TNF-α was controlled by NF-κB. Stangl et al reported that homocysteine, an atherogenic agent, significantly inhibits the TNF-α–induced VCAM-1 and E-selectin expressions in ECs by reducing the TNF-α–induced NF-κB activity, but caused much less pronounced inhibition of the TNF-α–induced ICAM-1 expression. These observations suggest differential effects of NF-κB activation in mediating the cytokine-induced expression of adhesion molecules (ICAM-1 versus VCAM-1) and perhaps in ECs.

In addition, TNF-α may induce NF-κB activation and adhesion molecule expression in ECs by increasing their intracellular reactive oxygen species (ROS) generation. TNF-α and ROS may exert differential effects on ICAM-1, VCAM-1, and E-selectin expressions in ECs through distinct signaling mechanisms. For example, Rahman et al indicated that VCAM-1 and E-selectin share a common redox-sensitive stimulation. They suggested that TNF-α stimulation to ECs increases their intracellular ROS generation, which subsequently induces the VCAM-1 and E-selectin expressions via the activation of NF-κB. Our recent study demonstrated that both preshearing and postshearing and their combination significantly reduced the intracellular ROS levels in ECs induced by TNF-α, as indicated by the reduction of TNF-α–induced superoxide production in ECs subjected to these shearing treatments (data not shown).

It is very likely that the reduction in TNF-α–induced ROS generation by shearing may cause the inhibition of NF-κB activation and consequently contribute to the inhibition of TNF-α–induced VCAM-1 and E-selectin expressions in ECs. However, the TNF-α–induced ICAM-1 expression was augmented, rather than inhibited, by shear stress. Whether this shear stress augmentation of TNF-α–induced ICAM-1 expression resulted from the shear-reduced ROS generation in TNF-α–treated ECs remains unclear. Munoz et al demonstrated that the antioxidant PDTC, which can specifically inhibit oxidant-induced NF-κB activity, increases the ICAM-1 expression in ECs by activation of AP-1. Chen et al demonstrated that the TNF-α–induced expression of VCAM-1 and E-selectin was markedly enhanced by the mitochondrial glutathione depletion, whereas the TNF-α–induced ICAM-1 expression was not affected by this depletion. Roebuck et al found that H2O2 activates ICAM-1 transcription through AP-1/Ets element within the promoter, which is different from the mechanisms involved in the TNF-α–induced ICAM-1 expression. These observations suggest that (1) ROS may play differential roles in modulating ICAM-1 and VCAM-1 and E-selectin expressions in ECs and (2) TNF-α and ROS may exert effects on ICAM-1 expression through distinct mechanisms. The results from these studies may be used to explain the distinct role of shear stress in modulating TNF-α–induced ICAM-1 versus VCAM-1 and E-selectin expressions in ECs. The precise molecular mechanisms by which shear stress regulate cytokine-induced gene expression in ECs warrant further investigation.

In summary, our present study clearly demonstrates that shear stress increases the TNF-α–induced ICAM-1 expression in ECs at both mRNA and surface protein levels, but it decreases the TNF-α–induced VCAM-1 and E-selectin expressions at mRNA and surface protein levels. Furthermore, the effect of shear stress on VCAM-1 and E-selectin expressions has a much longer persistence than that on ICAM-1. Our findings suggest differential roles of shear stress in modulating cytokine-induced adhesion molecule expression in ECs.

Acknowledgments

This work was supported in part by grants ME-090-PP-I3, ME-091-PP-I3, and ME-092-PP-02 from the National Health Research Institutes, Taiwan, R.O.C.

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Arterioscler Thromb Vasc Biol. published online November 13, 2003;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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METHODS

**Endothelial Cell Culture.** ECs were isolated from fresh human umbilical cords by means of the collagenase perfusion technique [1]. The cell pellet was resuspended in a culture medium consisting of medium 199 (M199, Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). ECs were grown in Petri dishes for 3 days and then seeded onto glass slides (75 by 38 mm, Corning, NY) pre-coated with fibronectin (Sigma, St. Louis, MO) to reach confluence (~ 1-2 × 10^5 cells/cm²). The culture medium was then exchanged with a medium that was identical to the previous medium except that it contained only 2% FBS, and the cells were further incubated for 24 h prior to the experiment.

**Flow Apparatus.** The slide with cultured ECs was mounted in a parallel-plate flow chamber, which has been characterized and described in detail elsewhere [2]. The chamber was connected to a perfusion loop system, kept in a constant-temperature controlled enclosure, and maintained at pH 7.4 by continuous gassing with a humidified mixture of 5% CO₂ in air. The osmolality of the perfusate was adjusted to 285-295 mOsm/kg H₂O during the perfusion. The flow channel width (w) was 1 cm, and the channel height (h) was 0.025 cm. The fluid shear stress (τ) generated on the ECs was estimated to be 20 dynes/cm², using the formula, \( \tau = \frac{6\mu Q}{wh^2} \), where \( \mu \) is the viscosity of the perfusate and \( Q \) is the flow rate. The static control cells subjected to the same time protocol in terms of incubation media and other conditions as the experimental cells, except that they were not subjected to flow.

**Experimental Procedure.** To test the regulatory effect of pre-shearing on the TNF-α-induced ICAM-1, VCAM-1, and E-selectin gene expressions in ECs, EC monolayers were first incubated in a static condition or exposed to flow for 10 min, 1 h, 4 h or 24 h, and...
then treated with TNF-α (100 U/mL) for 4 h under static condition. In addition, shear stress was applied to ECs either simultaneously with the addition of TNF-α or at 1 h, 2 h, or 3 h after the TNF-α addition to study the effect of post-shearing on the TNF-α-induced gene expression in ECs. The combinatory effect of pre-shearing and post-shearing on the TNF-α-induced gene expression was studied by subjecting ECs to flow for 24 h before treating with TNF-α for 4 h under the identical flow condition. The EC mRNA and surface protein expressions of ICAM-1, VCAM-1, and E-selectin were determined by Northern blot analysis and by indirect immunofluorescence using flow cytometry, respectively. The promoter constructs containing the promoter region of ICAM-1, VCAM-1, or E-selectin and the reporter gene luciferase were used to determine the transcriptional activities of these adhesion molecules. To examine whether the shear stress modulation of ICAM-1, VCAM-1, and E-selectin is dependent on changes in the stability of their mRNA, ECs were exposed to flow or left under static condition for 4 h before and 4 h after TNF-α addition and further 0, 0.5, 1, and 2 h in the presence of actinomycin D (10 µg/mL; Sigma). To analyze the reprogramming of the TNF-α-induced ICAM-1, VCAM-1, and E-selectin gene expressions in pre-sheared ECs after removal of the shear stress, ECs which had been pre-sheared for 24 h were either immediately treated with TNF-α for 4 h or subjected to static incubation for 1 h, 6 h, or 24 h before treating with TNF-α for 4 h. Moreover, nuclear protein extracts were obtained from the experimental cells to investigate the effect of shear stress on the TNF-α-induced nuclear factor (NF)-κB-DNA binding activities in the nucleus by using the electrophoretic mobility shift assay (EMSA). Cell viability was assessed by a trypan blue dye exclusion test and there were no differences in the viability of the cells following different treatments.
RNA Isolation and Northern Blot Analysis. Total RNA was isolated from the ECs by the guanidium isothiocyanate/phenol-chloroform method as described previously [3]. The RNA (10 µg/lane) was separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran, Schleicher & Schuell Inc., Germany) by using a vacuum blotting system (VacuGene XL, Pharmacia, Piscataway, NJ). The ICAM-1, VCAM-1, and E-selectin cDNA probes were the products of reverse transcriptase-polymerase chain reaction (RT-PCR) by using the respective sense and antisense primers. Primer sequences were designed as: ICAM-1 sense (5′-GAC-TGG-ACG-ACA-GGG-ATT-GT-3′) and ICAM-1 antisense (5′-ATT-ATG-ACT-GCG-GCT-GCT-ACC-3′; product length, 290 base pairs [bp]) [4], VCAM-1 sense (5′-GGA-AGT-GGA-ATT-AAT-TAT-CCA-A-3′) and VCAM-1 antisense (5′-CTA-CAC-TTT-TGA-TTT-CTG-TG-3′; product length, 441 bp) [4, 5], and E-selectin sense (5′-G-TGA-ACC-CAA-CAA-TAG-GCA-A-3′) and E-selectin antisense (5′-CAG-GTG-AAG-TTG-CAG-GAT-GA-3′; product length, 705 bp). After hybridization with the 32P-labeled ICAM-1, VCAM-1, or E-selectin cDNA probe, the membrane was washed with 1× saline-sodium citrate (SSC) containing 1% sodium dodecylsulfate (SDS) at room temperature for 30 min and then exposed to X-ray film (Kodak X-Omat-AR, Rochester, NY) at -70°C. Autoradiographic films were scanned and the results were analyzed by using a densitometer (Computing Densitometer 300S, Molecular Dynamics, Sunnyvale, CA).

Reporter Gene Construct, Transfection, and Luciferase Assay. The E-selectin promoter construct was a gift from Dr. P.E. DiCorleto (Case Western Reserve University School of Medicine). This construct (E-selectin-Luc) contains the luciferase reporter gene driven by an E-selectin promoter sequence (540 bp 5′ to the transcription initiation start site) [6]. The ICAM-1 promoter construct (ICAM-1-Luc) contains 850 bp of ICAM-1 5′-flanking DNA linked to the firefly luciferase reporter gene of plasmids pGL2 (Promega Inc., Madison,
Wisconsin) [7]. For construction of the VCAM-1 promoter chimera (VCAM-1-Luc), the VCAM-1 genomic clone [8] was fused into yT&A cloning vector (ECOS Inc., Taiwan) and digested with KpnI and BglII restriction enzymes. The resulting fragments (2296 bp) were cloned with T4 DNA ligase (Quick Ligation Kit, New England BioLabs Inc., Beverly, MA) into the corresponding sites of the luciferase reporter gene of plasmids pGL2. The orientation of the insert was determined by DNA sequencing. The fragments of ICAM-1, VCAM-1, and E-selectin promoters contain various locations of binding sites for the transcriptional factors such as AP-1, AP-3, NF-κB, C/EBP, and Ets [6, 8, 9]. DNA plasmids, purified by Wizard Maxipreps DNA purification system (Promega Inc.), were transfected into bovine aortic ECs (BAECs) at their 60% confluence level by using the lipofectamine method (GIBCO-BRL). The pSV-β-galactosidase plasmid, which contains a β-galactosidase (β-gal) gene driven by SV40 promoter and enhancer, was cotransfected to normalize the transfection efficiency. After transfection, cells were incubated with Dulbecco’s modified eagle medium (DMEM, GIBCO) containing 10% FBS overnight and then seeded onto slides. The medium of the cultured BAECs was exchanged with medium that was identical to the previous medium except that it contained only 0.5% FBS, and the cells were further incubated overnight before being subjected to shear stress and TNF-α treatment. Luciferase activity was measured by using the BioteC assay system (Promega Inc.). β-gal activity was assayed by adding the substrate o-nitrophenyl-β-D-galactopyranoside to 20 μL of cell lysate and incubating at 37°C before recording at 420 nm.

**Immunofluorescence with Flow Cytometry.** The expressions of ICAM-1, VCAM-1, and E-selectin on the surface of ECs were measured by indirect immunofluorescence using flow cytometry. Subsequent to shear stress and TNF-α treatments, ECs were washed with M199 three times, detached with Versene buffer containing EDTA, and centrifuged. Each sample
(4 × 10^5 cells) was washed with PBS containing 0.5% BSA and resuspended in 0.2 mL PBS containing mouse monoclonal antibody (mAb, R&D, Minneapolis, MN) to ICAM-1, VCAM-1, or E-selectin at a saturating concentration (20 mg/L). After incubation at 4°C for 30 min, the cells were centrifuged at 1500 rpm for 5 min and washed twice with PBS to remove the unbound antibody. The ECs were then incubated with anti-mouse IgG (Cappel, West Chester, Pennsylvania) conjugated with FITC for 30 min at 4°C. After two final washes in PBS, the cells were resuspended in 0.5 mL PBS containing 10% FBS and assayed within 1 h. Fluorescein-labeled cells (~ 1.0 × 10^4 cells/sample) were analyzed with the flow cytofluorometer (FACScan, Becton Dickinson). Cells incubated with FITC-conjugated antibody alone were used as negative controls.

**Electrophoretic Mobility Shift Assay (EMSA).** To prepare nuclear protein extracts, ECs were washed with cold PBS and then immediately removed by scraping in PBS. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were resuspended in cold buffer A (containing, in mmol/L, KCl 10, ethylenediamine tetraacetate [EDTA] 0.1, dithiothreitol [DTT] 1, and phenyl methylsulfonyl fluoride [PMSF] 1) for 15 min. The cells were lysed by adding 10% Nonidet P40 (NP-40) and then centrifuged at 6000 rpm to obtain pellets of nuclei. The nuclear pellets were resuspended in cold buffer B (containing, in mmol/L, 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid [HEPES] 20, EDTA 1, DTT 1, PMSF 1, and NaCl 400), vigorously agitated, and then centrifuged. The supernatant containing the nuclear proteins was used for the EMSA or stored at -70°C until used.

Double-stranded consensus oligonucleotides (5′AGT-TGA-GGG-GAC-TTT-CCC-AGG-C3′; Promega Corp., Madison, WI) containing the DNA binding site for NF-κB were end-labeled with [γ-32P]ATP. The extracted nuclear proteins (10 µg) were incubated with 0.1 ng 32P-labeled DNA for 15 min at room temperature in 25 µL binding buffer containing 1 µg
poly(dI-dC). In the antibody supershift assay, antibodies to NF-κB subunits, i.e., p50 and p65 (1 µg, Santa Cruz Biotechnology), was incubated with the mixture for 10 minutes at room temperature followed by the addition of the labeled probe. The mixtures were electrophoresed on 5% nondenaturing polyacrylamide gels. The gels were dried and imaged by autoradiography.

**Statistical Analysis.** Results are expressed as mean ± SEM. Statistical significance was determined by using Student’s t-test for two groups of data and one-way analysis of variance (ANOVA) followed by the Scheffe’s test for multiple comparisons. The level of statistical significance was defined as p < 0.05 from 3 or 4 separate experiments.

**RESULTS**

**Effects of Shear Stress on Stability of ICAM-1, VCAM-1, and E-selectin mRNA.** To investigate whether the modulation of ICAM-1, VCAM-1, and E-selectin mRNA expressions by shear stress is dependent on changes in the stability of mRNA, we examined the mRNA stability of these adhesion molecules in the presence or absence of shear stress. After treatment of ECs with TNF-α for 4 h, the cells were either exposed to shear stress of 20 dynes/cm² or left under static condition for 0.5, 1, and 2 h in the presence of actinomycin D. The mRNA expressions of ICAM-1, VCAM-1, and E-selectin were examined by Northern blot analysis, as described in Methods. As shown in Figure II (please see [http://atvb.ahajournals.org](http://atvb.ahajournals.org)), the mRNA stability of ICAM-1 (Figure IIA), VCAM-1 (Figure IIB), and E-selectin (Figure IIC) was not influenced by shear stress treatment, as compared with static controls. Because the shear stress modulation of ICAM-1, VCAM-1, and E-selectin is dependent on the length of shearing time, we also examined the mRNA stability under conditions where shear stress application was started 4 h before TNF-α stimulation and
continued until RNA sampling. In this measurement, actinomycin D was added 4 h after TNF-α addition, and total RNA was collected at 0, 0.5, 1, and 2 h after actinomycin D addition. As expected, the normalized ICAM-1 mRNA level in sheared ECs at time 0 (just before actinomycin D was added to the medium) was higher than that of the cells without shearing (Figure IIIA, please see http://atvb.ahajournals.org), but the VCAM-1 (Figure IIIB) and E-selectin (Figure IIIC) mRNA levels in sheared ECs were lower than those in the cells without shearing. The declining pattern of mRNA levels of these adhesion molecules in sheared ECs was similar to that of the cells without shearing. Thus, the mRNA stability of ICAM-1, VCAM-1, and E-selectin after TNF-α stimulation was not affected by shear stress treatment.

REFERENCES


**ON LINE FIGURE LEGENDS**

**Figure I.** Combinatory effects of pre-shearing and post-shearing on TNF-α-induced ICAM-1 (A), VCAM-1 (B), and E-selectin (C) gene expressions in ECs. ECs were kept as control (C) or treated with TNF-α (100 U/mL) for 4 h (TNF). Shear stress application (20 dynes/cm²) was started 24 h before TNF-α stimulation and continued for 4 h until RNA sampling (S24+TNF+S4). The mRNA expressions in these ECs were determined using Northern blot analysis, as described in Methods. Data are presented as a percentage change (relative to static controls) in band density, normalized to 18S RNA levels and shown as mean ± SEM from three independent experiments. *p < 0.05 vs static control ECs. #p < 0.05 vs TNF-α-treated ECs without shearing.

**Figure II.** Effects of actinomycin D on TNF-α-induced expression of ICAM-1 (A), VCAM-1 (B), and E-selectin (C) mRNA in ECs in the presence or absence of shear
stress. After treatment of ECs with TNF-α (100 U/mL) for 4 h, cells were either left static or exposed to shear stress of 20 dynes/cm² for 0.5, 1, and 2 h in the presence of actinomycin D (10 µg/mL). The mRNA expression of ICAM-1, VCAM-1, and E-selectin was examined by Northern blot analysis, as described in Methods. Data are presented as a percentage change (relative to static controls) in band density, normalized with respect to corresponding 18S RNA levels and shown as mean ± SEM from three independent experiments.

Figure III. Effects of shear stress on stability of ICAM-1 (A), VCAM-1 (B), and E-selectin (C) mRNA in ECs. ECs were left static or exposed to shear stress (20 dynes/cm²) for 4 h before and 4 h after TNF-α (100 U/mL) addition and further 0, 0.5, 1, and 2 h in the presence of actinomycin D (10 µg/mL). The mRNA expression of ICAM-1, VCAM-1, and E-selectin was examined by Northern blot analysis, as described in Methods. Data are presented as a percentage change (relative to static controls) in band density, normalized with respect to corresponding 18S RNA levels and shown as mean ± SEM from three independent experiments.
(A) ICAM-1 mRNA Level (% of Control ECs)

(B) VCAM-1 mRNA Level (% of Control ECs)

(C) E-selectin mRNA Level (% of Control ECs)

Time after Actinomycin D Addition (h)

Static Shear

0 0 0 0 0 0
100 100 100 100 100 100
200 200 200 200 200 200
300 300 300 300 300 300
400 400 400 400 400 400

C 0.5 1 2 0.5 1 2 0.5 1 2 (h)

ICAM-1

VCAM-1

E-selectin

18 S
(A) ICAM-1 mRNA Level (% of Control ECs)

(B) VCAM-1 mRNA Level (% of Control ECs)

(C) E-selectin mRNA Level (% of Control ECs)

Time after Actinomycin D Addition (h)