Differential regulation of urokinase-type plasminogen activator expression by fluid shear stress in human coronary artery endothelial cells

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Submitted 16 March 2004; accepted in final form 27 June 2004

Sokabe, Takaaki, Kimiko Yamamoto, Norihiko Ohura, Hideki Nakatsuka, Kairong Qin, Syotaro Obi, Akira Kamiya, and Joji Ando. Differential regulation of urokinase-type plasminogen activator expression by fluid shear stress in human coronary artery endothelial cells. Am J Physiol Heart Circ Physiol 287: H2027–H2034, 2004.—Atherosclerotic plaques preferentially localize at arterial regions exposed to turbulent low-shear flow. Urokinase-type plasminogen activator (uPA) plays a role in vascular remodeling by facilitating smooth muscle cell migration and proliferation in addition to the proteolysis of extracellular matrix, and the expression of uPA is elevated in atherosclerotic lesions. In this study, we analyzed the effects of laminar and turbulent shear stress on uPA expression in cultured human coronary artery endothelial cells. The application of laminar shear stress (1.5 or 15 dyn/cm2) significantly decreased the amount of uPA mRNA as well as the secretion of uPA protein. In contrast, turbulent shear stress (average intensity, 1.5 dyn/cm2) markedly increased uPA gene expression and protein secretion. Laminar shear stress downregulated uPA gene expression transcriptionally and posttranscriptionally; laminar shear stress activated transcription factor GATA6, which binds to a GATA consensus element located between −692 and −687 bp in the uPA promoter, thereby inhibiting uPA gene transcription. Laminar shear stress also accelerated the degradation of uPA mRNA; the half-life of uPA mRNA decreased to about half of the static control’s half-life. Although turbulent shear stress had no effect on the transcription of uPA, it significantly increased uPA mRNA stability; the half-life of uPA mRNA increased by about two times the static control’s half-life. Our results suggest that endothelial uPA expression is flow sensitive and differentially regulated by laminar and turbulent shear stress in vitro. We speculate that this effect may contribute to the local nature of atherosclerosis.

Atherosclerosis is a multifactorial disease involving complex interactions between a wide variety of factors, such as inflammation, serum lipids, glucose tolerance, and genetic background. Atherosclerotic lesions are prone to initially develop at branch points and bifurcations in the arterial vasculature. These regions are characterized by turbulent flow patterns, including flow separation, flow reversal, and low and fluctuating wall shear stresses (mean shear stress less than a few dyn/cm2) (2, 15). In contrast, regions that are exposed to unidirectional laminar flow with a relatively high shear tress (mean shear stress larger than 10 dyn/cm2) are more resistant to atherosclerosis (27). This geometrically defined localization of atherosclerotic lesions strongly suggests that local hemodynamic factors play an important role in the initiation and progression of atherosclerosis.

Shear stress, a biomechanical force generated by flowing blood, acts on endothelial cells (ECs) lining the inner surface of vessels. ECs interpret shear stress as a signal and transmit this signal to the cell interior, leading to cellular responses that involve changes in cell morphology, cell function, and gene expression (5). For instance, when cultured ECs are exposed to shear stress in fluid dynamically designated flow-loading devices, they increase their production of potent vasodilators, like nitric oxide (NO), prostacyclin, C-type natriuretic peptide, adrenomedulin, and many kinds of growth factors; the expression of cell adhesion molecules on their cell surface is also altered. Many of these responses accompany changes in the expression of related genes. Recent studies have revealed that shear stress modulates endothelial gene expression at the transcriptional and/or posttranscriptional levels (1). To date, a large amount of work has been done on laminar shear stress, but only a few studies have examined the responses of ECs to turbulent shear stress.

ECs produce urokinase-type plasminogen activator (uPA), which generates plasmin, potentially initiating a cascade of fibrinolysis in blood vessels. uPA also has other roles in the vessel wall in addition to its involvement in fibrinolysis. uPA can stimulate the migration and proliferation of smooth muscle cells and macrophages (8, 22) directly through its cell surface receptor (uPAR) (28) and indirectly through the activation of metalloproteinases that degrade the extracellular matrix, the generation of plasmin, and the release of growth factors from the extracellular matrix, all of which contribute to vascular remodeling. A number of recent studies in humans and animal models have suggested that uPA plays a role in the initiation and development of atherosclerosis; uPA expression is elevated in ECs, smooth muscle cells, and macrophages in atherosclerotic human aortas, carotid arteries, and coronary arteries (11, 17, 19) and is also increased during neointimal formation in injured rat arteries (4). However, the mechanism responsible for the increase in uPA expression in atherosclerotic lesions remains unclear.

In the present study, we examined the effect of shear stress on the expression of uPA in human coronary artery ECs (HCAECs). Cultured HCAECs were exposed to laminar and turbulent shear stress in in vitro flow systems, and changes in the expression of uPA at both protein and mRNA levels were examined. The precise molecular mechanism for the shear

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First published July 1, 2004; doi:10.1152/ajpheart.00260.2004.
stress-mediated regulation of uPA gene expression was also investigated.

MATERIALS AND METHODS

Cell cultures. HCAECs were obtained from Clonetics and grown on a 1% gelatin-coated tissue culture flask in medium 199 (M199) supplemented with 15% FBS, 2 mmol/l L-glutamine (GIBCO), 50 µg/ml heparin, and 30 µg/ml EC growth factor (Becton Dickinson). Bovine ECs were isolated from the descending thoracic aorta of a bovine fetus by brief collagenase digestion and cultured in M199 containing 15% FBS, 2 mmol/l L-glutamine, 50 µg/ml penicillin, and 50 µg/ml streptomycin. The cells were harvested and used for four to seven passages.

Flow-loading apparatus. A parallel plate-type apparatus was used to apply laminar shear stress to the cells, as previously described (14). Briefly, one side of the flow chamber consisted of a 1% gelatin-coated glass plate on which the cultured ECs rested, the other side was a polycarbonate plate, and these two flat surfaces were held 200 µm apart by a Teflon gasket. The intensity of the shear stress (τ; in dyn/cm²) acting on the EC layer was calculated using the formula τ = 6μQ/a²h, where μ is the viscosity of the perfusate (in Poise), Q is the flow volume (in ml/s), and a and h are the cross-sectional dimensions of the flow path (in cm).

A cone-plate-type apparatus was used to apply turbulent shear stress to the cells, as described by Garcia-Cardena et al. (7). The apparatus consists of a stainless steel cone driven by an electric motor and a stage that holds a 10-cm diameter culture dish with a glass plate inserted at the bottom of the dish. Rotation of the cone forces the fluid between the cone and glass plate to flow concentrically, exposing cells attached to the 1% gelatin-coated glass plate to fluid shear stress. We analyzed the nature of flow in the cone-plate-type apparatus by visualizing flow with polystyrene particles and a high-speed video camera and by numerical simulation by means of a finite-element method. Under the conditions under which laminar flow occurred, the flow streamlines formed concentric circles. As the cone angle and rotation rate increased, strong secondary flows occurred: the fluid adjacent to the rotating cone flowed radially toward the periphery of the cone, and the fluid close to the stationary plate flowed radially in the opposite direction, which caused turbulent flow and flow reversal. The numerical simulations showed that under turbulent flow conditions, shear stress was not distributed uniformly on the EC monolayer at the bottom of the dish and that the concentric flow velocity profile was distorted by the secondary flow, indicating that the flow was unsteady and chaotic. The turbulent flow in our apparatus has features similar to the flow found at arterial bifurcations, that is, flow reversal and unsteady low shear stress.

τ (in dyn/cm²) acting on the EC layer was calculated using the formula τ = μωα², where ω is the angular velocity of the cone and α is the cone angle (in radians). The shear fluid stress was constant over the entire plate surface. The modified Reynolds number (R) was used to determine the appropriate experimental conditions required to induce turbulent flow and was calculated using the formula R = r²ω²/τ, where r is the radial distance from the apex of the cone and v is the kinematic viscosity of the fluid (23). With the use of this parameter, the flow was predicted to be turbulent at R > 4. In the present experiments, we used a 5° cone and a rotational velocity of 120 rpm. Because R is proportional to the radial dimension, turbulent flow was established at radii ≥ 2.4 cm, which corresponded to an R > 5 and represented an average shear stress intensity of 1.5 dyn/cm². Thus the cells for the turbulent experiments were harvested from only the outer portion of the glass plate (≥2.4 cm).

ELISA. The amount of uPA released by the ECs was assayed using commercially available ELISA kits (Biopool). Briefly, 50 µl of perfusate were incubated on a microplate coated with anti-human monoclonal antibody against uPA for 2 h. After being washed with detergent, the uPA conjugate was added and incubated for 1 h. The

color reagent was then incubated for 20 min. Absorbance at 492 nm was measured using a microplate reader (Bio-Rad), and the concentration of uPA in each sample was determined by comparison with the standard curve.

Real-time PCR analysis. Real-time PCR was performed using a Smart Cycler (Cepheid) with an Ex Taq R-PCR version (Takara), SYBR green 1 (BioWhittaker), and the following primer pairs: 5'-GCCCTGTGAGATCTCGTTCAAGGAGGC-3' and 5'-CAGG-CCATTCTTCTTCTGAGTACCTGC-3' for uPA, and 5'-ACA-TCATCGCTTCCTACTG-3' and 5'-AGTGGGTGTCGTGTCGTAAGATC-3' (gapDH).

Nuclear run-on assay. A nuclear run-on assay was performed using a previously described method (14). Briefly, nuclear extracts obtained from HCAECs were reacted in reaction buffer containing [α-32P]UTP, and the 32P-labeled RNA was extracted. Plasmids containing human uPA or a human GAPDH fragment (Clontech) were spotted onto nylon membranes, and the DNA was hybridized to the radiolabeled RNA. Autoradiograms were obtained using a GS363 Molecular Image System (Bio-Rad).

Luciferase assay. Reporter plasmids containing the human uPA promoter linked to the luciferase gene (a kind gift from Dr. Soichi Kojima, The Institute of Physical and Chemical Research, Wako, Japan) were used for the transcription assay. The following deletion constructs were generated: −2.3 kbp, 2.3 kbp (−2,345 to +32) of the uPA upstream region cloned into pGL2-basic; −1.7 kbp, 1.7 kbp (−1,737 to +10) of the uPA upstream region cloned into pGL2-basic with XbaI and HindIII; −782 luc, 782 bp (−782 to +10) of the uPA upstream region cloned into pGL2-basic with HindII and Hind III; −537 luc, 537 bp (−537 to +10) of the uPA upstream region cloned into pGL2-basic with EcoRV and HindIII; and −150 luc, 150 bp (−150 to +10) of the uPA upstream region cloned into pGL2-basic with BamHI and HindIII.

Three constructs were transfected into bovine ECs using LipofectAMINE PLUS (GIBCO). The pRL-TK vector (Promega) was cotransfected to normalize the transfection efficiency. After 24 h, the cells were either incubated under static conditions or exposed to shear stress for 12 h; luciferase activity was then determined using a Dual-Luciferase reporter assay system (Promega) and a luminometer (Berthold).

Site-directed mutagenesis. Mutations were generated at a consensus binding site (TTATCA) for a known transcription factor, GATA, between bp −692 and −687 from the transcription start site in the uPA gene promoter using a Quick Change site-directed mutagenesis kit (Stratagene). Briefly, PCR was performed using −782 luc as the template, two synthetic oligonucleotide primers containing specific mutations (5'-AACCCCAATTCCTTGAAGCTGC-3' and 5'-GACAGGCGTTCAAGGAGTTTGTT-3'), and PfuTurbo DNA polymerase.

Electrophoretic mobility shift assay. An electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts or cytoplasmic extracts obtained from HCAECs, as previously described (13). A 30-mer oligonucleotide containing a GATA consensus element with a sequence of 5'-AACCCCAATTCCTTGAAGCTGC-3' was labeled using T4 polynucleotide kinase and [γ-32P]ATP. The binding reactions between the radiolabeled oligonucleotides and the nuclear extracts were allowed to proceed, and the reaction mixtures were separated using 4.7% polyacrylamide gel electrophoresis.

DNA was synthesized using a complementary oligonucleotide containing 56 nucleotides from the human uPA 3'-untranslated region (3'-UTR), including an AU-rich motif (positions 2,144–2,199). Transcription of this AU-rich motif segment was performed using T3 RNA polymerase in the presence of [α-32P]UTP. The cytoplasmic extracts were incubated with [32P]RNA, and the reaction mixture was electrophoresed using 15% SDS-PAGE and analyzed using an image analyzer.

AJP-Heart Circ Physiol • VOL 287 • NOVEMBER 2004 • www.ajpheart.org
Fig. 1. Effects of shear stress on urokinase-type plasminogen activator (uPA) mRNA levels in human coronary artery endothelial cells (HCAECs). Total RNA was isolated from HCAECs that had been either exposed to laminar or turbulent shear stress for the indicated period of time or maintained as a static control, represented by time 0; the samples were then analyzed using real-time PCR. A and B: time courses for the reductions in uPA mRNA caused by laminar shear stress (15 and 1.5 dyn/cm² in A and B, respectively). C: time course for the increase in uPA mRNA caused by turbulent shear stress (1.5 dyn/cm²). All values are means ± SD of 5 separate experiments. *P < 0.01 vs. static control.

Fig. 2. Effects of shear stress on the release of uPA protein by HCAECs. The amounts of uPA in the perfusates obtained from HCAECs that had been cultured under static conditions or exposed to laminar or turbulent shear stress for 1, 3, 6, 12, 24, or 48 h were measured using an ELISA. A and B: time courses for the reductions in uPA release caused by laminar shear stress (■, 15 dyn/cm²; ▼, 1.5 dyn/cm²). ○, Basal release under static conditions. C: time course for the increase in uPA release caused by turbulent shear stress (●, 1.5 dyn/cm²). The results are presented as means ± SD of 3 separate samples. *P < 0.01 vs. static control.
mRNA stability assay. HCAECs were treated with 5 μg/ml actinomycin D (Wako) after either being incubated under static conditions or being exposed to laminar or turbulent shear stress (1.5 dyn/cm²) for 12 h; changes in the concentration of uPA mRNA were then measured using competitive PCR, as previously described (14).

Statistical analysis. All results are expressed as means ± SD. Statistical significance was evaluated by ANOVA and a Bonferroni adjustment applied to the results of a t-test, performed with SPSS software (SPSS). P values < 0.01 were regarded as statistically significant.

RESULTS

Laminar flow decreases uPA gene expression in HCAECs, whereas turbulent flow increases uPA gene expression. HCAECs were exposed to laminar or turbulent shear stress for 1, 3, 6, 12, 24, or 48 h, and changes in the uPA mRNA levels were analyzed by real-time PCR. Shear stress affected both HCAEC morphology and orientation. Static control cells were polygonal in shape and did not exhibit any orientation. Some of the cells subjected to low laminar shear stress (1.5 dyn/cm²) became elongated, and their long axis was aligned with the direction of flow, whereas all of the cells subjected to high shear stress (15 dyn/cm²) became even more elongated and were aligned with the direction of flow. By contrast, the cells exposed to turbulent shear stress (1.5 dyn/cm²) did not undergo any elongation or exhibit any orientation.

Laminar shear stress transiently decreased expression of the uPA gene: the uPA mRNA level began to decrease after 3 h of exposure to a laminar shear stress of 15 dyn/cm² and continued to decrease over time, reaching its lowest level 6 h after exposure, after which the uPA mRNA level gradually returned to the level in the static control (Fig. 1A). Similar temporal changes in the uPA mRNA level occurred in response to a low laminar shear stress of 1.5 dyn/cm², although the lowest expression level was reached 12 h after exposure (Fig. 1B). The reasons for the difference at 12 h after exposure and the lack of dose dependency, however, are unknown. The differences in the remodeling of the HCAEC monolayer, i.e., alterations in cell shape and orientation between low and high laminar shear stress, may be related to the lack of dose dependency. By contrast, turbulent shear stress markedly increased expression of the uPA gene: the uPA mRNA level began to increase 6 h after the start of turbulent flow and continued to increase, peaking at approximately three times the level in the static control (at time 0) after 24 h of exposure to turbulent shear stress, and was slightly lower at 48 h after exposure (Fig. 1C).

These findings indicate that laminar and turbulent shear stress have opposite effects on uPA gene expression in HCAECs.

![Fig. 3. Effects of shear stress on the transcription of the uPA gene in HCAECs. A: nuclear run-on assay. Nuclei (1 × 10⁷ cells) were harvested from HCAECs that had either been incubated under static conditions (static) or exposed to laminar or turbulent shear stress (1.5 dyn/cm²) for 12 h; transcription was allowed to continue in the presence of [α-32P]UTP. Labeled RNA was extracted and used as a probe against immobilized plasmids containing cDNA inserts for human uPA, human β-actin (positive control), and the plasmid pGL-2 (negative control). Laminar shear stress markedly suppressed uPA gene transcription, whereas turbulent shear stress had no effect. Neither laminar nor turbulent shear stress affected the transcription of the β-actin gene. B: results of quantitative densitometry analysis. The ratio of the density of uPA in cells exposed to laminar shear stress was significantly lower than that in static control cells. All values are means ± SD of 3 separate experiments. *P < 0.01 vs. static control.](http://ajpheart.physiology.org/)

![Fig. 4. Effects of shear stress on the transcription of the human uPA gene. Bovine ECs transfected with reporter plasmids containing the human uPA promoter linked to the luciferase gene (~2.3 k luc, ~1.7 k luc, ~782 luc, ~537 luc, ~150 luc, or ~782 mut) were exposed to shear stress (1.5 dyn/cm²) for 24 h, and changes in luciferase activity were measured using a luminometer. Values are means ± SD of data from 8 separate coverslips. The horizontal axis represents the percentage of the luciferase activity of ~2.3 k luc under static conditions. Laminar shear stress significantly decreased the luciferase activity of cells transfected with ~2.3 k luc, ~1.7 k luc, and ~782 luc but not of cells transfected with ~537 luc and ~150 luc. Turbulent shear stress did not significantly change the luciferase activity of cells transfected with any constructs. A mutation in the GATA consensus sequence located between ~692 and ~687 bp abolished the laminar shear stress-induced reduction in luciferase activity in cells (~782 mut). *P < 0.01.](http://ajpheart.physiology.org/)
Laminar flow decreases the release of uPA by HCAECs, whereas turbulent flow increases uPA release. HCAECs were exposed to laminar or turbulent shear stress for 1, 3, 6, 12, 24, or 48 h, and the concentration of uPA protein in the perfusion medium was determined using an ELISA. The HCAECs in this experiment tended to release uPA; the uPA concentration increased throughout the first 24 h and then leveled off (Fig. 2). Laminar shear stress decreased the release of uPA; compared with the basal level of release, a significant decrease was observed after 12, 24, and 48 h (Fig. 2A and B). In contrast, turbulent shear stress markedly increased the release of uPA; an increase of more than twofold the level of basal release was observed at 3, 6, 12, 24, and 48 h after exposure to the turbulent shear stress (Fig. 2C).

Laminar flow downregulates the transcription of the uPA gene in HCAECs. A nuclear run-on assay was performed to determine whether shear stress affects the transcription of the uPA gene. Laminar shear stress markedly decreased the transcription of the uPA gene in HCAECs compared with that in the static control, whereas turbulent shear stress had no effect (Fig. 3A). Neither laminar nor turbulent shear stress changed the transcription level of the β-actin gene. Densitometry of individual spots of interest revealed that the density of the uPA mRNA signal in the cells exposed to laminar shear stress decreased to around 60% of that in the static controls (Fig. 3B).

A luciferase assay was also performed to confirm the effect of shear stress on the transcription of the uPA gene. The luciferase activity of cells exposed to laminar shear stress was markedly lower than that of the static control cells, whereas the luciferase activity of cells exposed to turbulent shear stress did not change significantly compared with that of the static control cells (Fig. 4, −2.3 k luc). This indicates that laminar shear stress downregulates the transcription of the uPA gene in HCAECs, whereas turbulent shear stress does not affect the transcription of the uPA gene.

GATA consensus element is essential for the laminar flow-induced downregulation of uPA gene transcription. A deletion analysis was performed to localize the cis-acting regions within the uPA promoter that are responsible for the laminar shear stress-induced downregulation of uPA gene transcription. Transfection with deletion constructs −2.3 k luc, −1.7 k luc, or −782 luc resulted in a marked decrease in luciferase activities in response to laminar shear stress, whereas transfection with construct −537 luc or −150 luc abolished the responsiveness to laminar shear stress (Fig. 4). Transfection with the various deletion constructs did not significantly change the luciferase activity in response to turbulent shear stress. These findings indicate that the cis-acting regions are located between the −782 and −537 bp in the 5′-flanking region of the human uPA gene.

To further localize the cis-acting regions, we performed an EMSA, in which nuclear extracts from static or shear-stressed cells were incubated with 1 of 15 different radiolabeled oligonucleotides synthesized from various sequences located in the −782- to −537-bp region upstream of the transcription initiation site of the uPA gene. An oligonucleotide bearing the GATA consensus element also abolished the band for the protein-DNA complex. Laminar shear stress enhanced the band of the protein-DNA complex (lanes 2 vs. lane 9). The addition of an antibody against GATA6 completely abolished the band for the protein-DNA complex seen in static control cells (lane 8) and shear-stressed cells (lane 15). The mutation in the GATA consensus sequence also abolished the band for the protein-DNA complex (lanes 17 and 19). All experiments were repeated 3 times, with similar results.

Fig. 5. Effects of shear stress on the binding of transcription factors to the GATA consensus element. EMSA was performed to examine the complexes formed by oligonucleotides (oligo) bearing the GATA consensus element (lanes 1–15) or mutated elements (lanes 16–20) and nuclear extracts from static cells (lanes 2–8) or laminar shear-stressed cells (lanes 9–15). Lanes 1 and 16, no nuclear extracts; lanes 3, 10, 18, and 20, addition of relevant unlabeled oligonucleotides as a competitor in 500-fold excess; lanes 4–8 and 11–15, addition of antibodies against GATA; lanes 4 and 11, GATA1; lanes 5 and 12, GATA2; lanes 6 and 13, GATA3; lanes 7 and 14, GATA4; lanes 8 and 15, GATA6. The arrow indicates the shifted band representing the protein-DNA complex. Laminar shear stress enhanced the band of the protein-DNA complex (lane 2 vs. lane 9). The addition of an antibody against GATA6 completely abolished the band for the protein-DNA complex seen in static control cells (lane 8) and shear-stressed cells (lane 15). The mutation in the GATA consensus sequence also abolished the band for the protein-DNA complex (lanes 17 and 19). All experiments were repeated 3 times, with similar results.
transcription factor GATA consensus element (−692 to −687 bp) was able to form distinct complexes with nuclear protein derived from either static or shear-stressed cells (Fig. 5). Laminar shear stress markedly increased the quantity of the protein-DNA complex, indicating that the GATA binding site may function as a shear stress-responsive element (SSRE).

To test whether the GATA binding site is critical for the uPA gene response to laminar shear stress, we constructed a chimeric gene with site-specific mutagenesis at the GATA binding site and assayed the luciferase activity in bovine ECs transfected with the mutant. The mutant abolished the uPA gene’s response to laminar shear stress (Fig. 4, −782 mut).

Transcription factor GATA6 is involved in the laminar flow-induced downregulation of uPA gene transcription. To establish the identity of the nuclear protein that binds to the GATA consensus element, we used antibodies to GATA1, -2, -3, -4, and -6 peptides. The antibody for GATA6, but not the antibodies for GATA1, -2, -3, or -4, completely inhibited the formation of the protein-DNA complex under static and laminar flow conditions (Fig. 5). These findings indicate that GATA6 is involved in both the basal transcription and the laminar shear stress-induced downregulation of uPA gene transcription in HCAECs.

Turbulent flow increases uPA mRNA stability, whereas laminar flow decreases mRNA stability. Actinomycin D chase experiments were performed to examine whether shear stress affects the stability of uPA mRNA. The amount of uPA mRNA decreased as the exposure time to actinomycin D increased, but the rate of decrease was markedly lower in the cells exposed to turbulent shear stress than in the static control cells (Fig. 6). In contrast, the rate was significantly higher in the cells exposed to laminar shear stress than in the static control cells. The estimated half-life of uPA mRNA (means ± SD, n = 4) was 2.2 ± 0.2 h in static control cells, 1.1 ± 0.3 h in cells exposed to laminar shear stress, and 4.4 ± 0.2 h in cells exposed to turbulent shear stress. These findings indicate that turbulent shear stress increases uPA mRNA stability, whereas laminar shear stress accelerates uPA mRNA degradation.

We also searched for AU-binding factors in HCAECs that specifically bind to the AU-rich region in the 3’-UTR of uPA mRNA. An EMSA showed two proteins with a molecular mass of around 40 kDa and 45 kDa (arrows) in the cytoplasmic extracts formed distinct complexes with the AU-rich motif (Fig. 7). Turbulent flow increased the binding activity of the 40-kDa AU-binding factor was increased by turbulent shear stress. All values are means ± SD of 4 separate experiments. *P < 0.01 vs. static control.

### Fig. 6. Effects of shear stress on uPA mRNA stability. HCAECs were either incubated under static conditions or exposed to laminar or turbulent shear stress (1.5 dyn/cm² for 24 h) and then treated with actinomycin D for 1, 2, 3, 4, or 5 h. Competitive PCR was then performed to quantitate the changes in uPA mRNA levels after treatment. Turbulent shear stress enhanced the stabilization of the uPA mRNA, whereas laminar shear stress accelerated the degradation of the mRNA. All values are means ± SD of 4 separate experiments. *P < 0.01 vs. static control.

### Fig. 7. Effects of shear stress on the binding activity of AU-binding factors. A: lane 1, no extracts; lanes 2 and 3, extracts from static control cells; lanes 4 and 5, extracts from cells exposed to laminar shear stress (1.5 dyn/cm² for 24 h); lanes 6 and 7, extracts from cells exposed to turbulent shear stress (1.5 dyn/cm² for 24 h); lanes 3, 5, and 7, addition of relevant unlabeled oligonucleotide as a competitor in 500-fold excess. Two proteins with molecular masses of around 40 kDa and 45 kDa (arrows) in the cytoplasmic extracts formed distinct complexes with the AU-rich motif. B: results of quantitative densitometry analysis. The binding activity of the 45-kDa AU-binding factor was significantly decreased by laminar shear stress, whereas the binding activity of the 40-kDa AU-binding factor was increased by turbulent shear stress. All values are means ± SD of 4 separate experiments. *P < 0.01 vs. static control.
tein, whereas laminar flow decreased the binding activity of the 45-kDa AU-binding protein.

DISCUSSION

The present study demonstrated that endothelial uPA expression is flow sensitive and differentially regulated by laminar and turbulent flow. Turbulent flow significantly increases the amount of uPA mRNA in HCAECs as well as the secretion of the uPA protein, whereas laminar flow decreases uPA gene expression and protein secretion. These opposite effects seem to be caused by the differences in the dynamic characteristics of the flow (i.e., laminar vs. turbulent shear stress), because equivalent average stress intensities (1.5 dyn/cm²) were used for both applications of shear stresses. These observations are consistent with those of several reports indicating that ECs show differential responses to distinct fluid mechanical stimuli. For instance, turbulent shear stress, but not laminar shear stress, increases DNA synthesis in bovine aortic ECs (6). Laminar shear stress increases the mRNA level of endothelial NO synthase (eNOS) in human umbilical vein ECs (HUVECs), leading to an increase in NO release, whereas turbulent shear stress has no effect (18). Turbulent shear stress, but not laminar shear stress, also decreases endothelial electrical resistance and permeability to macromolecules in bovine aortic ECs (20). Recent studies using DNA microarrays or a PCR-based differential display have revealed striking differences in the number of kinds of endothelial genes that respond to laminar or turbulent shear stress (3, 7, 26). These findings suggest the existence of a mechanism by which ECs can differentially sense and respond to laminar or turbulent shear stress.

Laminar shear stress downregulated the transcription of the uPA gene in HCAECs. A luciferase assay utilizing reporter constructs containing different lengths of the human uPA promoter identified a shear-sensitive binding site located between −783 and −537 bp in the uPA promoter. The EMSA data showed that this site is a GATA consensus sequence that specifically binds the GATA6 transcription factor. Reporter constructs in which the GATA binding site was mutated were unresponsive to shear stress. These results indicate that GATA6 and its binding site play an essential role in the laminar shear stress-induced downregulation of uPA gene transcription. A number of genes known to respond to shear stress, including endothelin-1, eNOS, platelet endothelial cell adhesion molecule-1, and lectin-like oxidized LDL receptor-1, contain GATA binding sites in their promoters. However, shear-sensitive transcription factors and binding sites vary among genes. For instance, shear stress upregulates the transcription of the platelet-derived growth factor (PDGF)-B chain gene in ECs through the binding of NF-κB to the sequence GAGACC, which was the first sequence to be designated as a SSRE (10, 21). Similarly, activator protein (AP)-1 and the TPA-responsive element (TRE), Sp1 and a GC-rich region, and Egr-1 and its binding site are involved in the responses of genes encoding monocyte chemotactic protein-1 (25), tissue factor (16), and PDGF-A chain (9), respectively. We previously reported that AP-1 and TRE mediate the shear-induced downregulation of vascular cell adhesion molecule-1 gene transcription (12) and that Sp1 and its binding site are essential for the inhibitory effect of shear stress on purinoceptor P2X4 gene transcription in ECs (13).

Our previous study demonstrated that shear stress regulates endothelial gene expression not only transcriptionally but also posttranscriptionally. Laminar shear stress increased the expression of the granulocyte/macrophage colony stimulating factor gene in HUVECs, and this process was then mediated by posttranscriptional mRNA stabilization (14). The present study revealed that turbulent and laminar shear stress exert opposite effects on uPA mRNA stability; turbulent shear stress increased mRNA stability, whereas laminar shear stress decreased mRNA stability. Relatively little is known about the regulatory mechanisms of mRNA stability. A few mRNA stability determinants have been identified, including AU-rich sequences in the 3′-UTR of mRNAs and mRNA-binding proteins. Recently, uPA gene expression in human lung epithelial cells has been shown to be regulated at the posttranscriptional level by a cis-trans interaction between a 65-nucleotide sequence of the uPA mRNA 3′-UTR and a 30-kDa uPA mRNA-binding protein (uPA mRNABp) (24). Using a gel-shift assay, we searched for the presence of mRNA-binding proteins that interact with the 65-nucleotide sequence at 2,055–2,119 bp or the AU-rich region at 2,144–2,199 bp in the uPA mRNA 3′-UTR. Proteins that bind to each of these regions were observed, but the quantity of the uPA mRNABp did not change in response to laminar or turbulent shear stress (data not shown). The binding of a cytoplasmic 45-kDa protein to the AU-motif decreased in response to laminar shear stress, whereas the binding of a 40-kDa protein increased in response to turbulent shear stress. However, whether these proteins are actually involved in shear stress-induced changes in uPA mRNA stability has not yet been determined. Clarification of uPA mRNA stability determinants would provide useful information for understanding the molecular mechanism by which ECs differentially respond to turbulent and laminar shear stress.

Turbulent low-shear flow is considered to be a local biomechanical risk factor for atherosclerosis. To date, however, its role in the pathogenesis of atherosclerosis remains unclear. Increasing evidence suggests that uPA is involved in the formation and progression of atherosclerotic lesions; uPA expression is increased in the vessel wall of humans as well as in animal models of atherosclerosis. The present study, showing the different effects of laminar and turbulent shear stress on endothelial uPA expression, may help to explain these observations. Turbulent low-shear flow may contribute to the pathogenesis of atherosclerosis by increasing endothelial uPA production. Further study is needed to elucidate the ultimate impact of shear stress-dependent uPA production in ECs on the pathogenesis of atherosclerosis in vivo.

GRANTS

This study was partly supported by grants-in-aid for Scientific Research, for Scientific Research on Priority Areas, and for Special Coordination Funds for Promoting Science and Technology from the Japanese Ministry of Education, Culture, Sports, Science and Technology and by a research grant for cardiovascular diseases from the Japanese Ministry of Health and Welfare.

REFERENCES


