Laminar Shear Stress Inhibits Vascular Endothelial Cell Proliferation by Inducing Cyclin-Dependent Kinase Inhibitor p21<sup>Sdi1/Cip1/Waf1</sup>

Shigeo Akimoto, Masako Mitsumata, Toshiyuki Sasaguri, Yoji Yoshida

Abstract—Alterations in the functions of vascular endothelial cells (ECs) induced by fluid shear stress may play a pivotal role in both the development and prevention of vascular diseases. We found that DNA synthesis of bovine aortic and human umbilical vein ECs, determined by <sup>3</sup>H]thymidine incorporation, was inhibited by steady laminar shear stress (5 and 30 dyne/cm<sup>2</sup>). This growth inhibition due to shear stress was associated with suppression of cell transition from the G<sub>1</sub> to S phases of the cell cycle. Therefore, we studied G<sub>1</sub>-phase events to find the molecules responsible for this cell cycle arrest. Shear stress inhibited the phosphorylation of a retinoblastoma protein (pRb) and the activity of cyclin-dependent kinase (cdk) 2 and cdk4, which phosphorylate pRb. The level of cdk inhibitor p21<sup>Sdi1/Cip1/Waf1</sup> protein, but not that of p27<sup>Kip1</sup>, increased as a result of shear stress, and the amount of p21 protein associated with cdk2 also increased, although the protein level of cdk2 was unchanged. Shear stress markedly elevated the mRNA level of p21, and this elevation in mRNA faded after the release of cells from shear stress, concomitant with a recovery of DNA synthesis. These results suggest that steady laminar shear stress induces cell cycle arrest by upregulating p21. Derangement of the steady laminar flow may release cells from this inhibition and induce cell proliferation, which, in turn, may cause atherosclerosis through the induction of EC stability disruption. (Circ Res. 2000;86:185-190.)

Key Words: shear stress ■ cell cycle arrest ■ p21<sup>Sdi1/Cip1/Waf1</sup> ■ cyclin-dependent kinases ■ vascular endothelial cell

Medicinal forces have been implicated in the initiation and localization of atherosclerosis. We found that 95% of 268 lipid depositions and 80% of 69 plaques in the aortas of young subjects (aged <39 years) in Japan were initiated within a circle of 5-mm radius, with radii extending from the centers of branch orifices, especially on the proximal side of each orifice. In contrast, the development of atherosclerosis at flow dividers, which are high shear stress regions, was restricted, consistent with other data. We also found that disruption of atherosclerotic plaques on the descending aorta was likely to occur at the distal edges of plaques, where eddies and separation of the bloodstream from the vessel wall occur, resulting in the plaque wall surfaces being exposed to low shear stress. These disruptions are unlikely to occur at the top of plaques, which are exposed to high laminar shear stress. There is evidence that the enhancement of endothelial permeability and of lipid deposition preferentially occurs at turbulent low mean shear stress regions in the arteries. In contrast, a thick glyocalyx and an increase in zonular-type tight junctions between endothelial cells (CEs), which may function as antiatherosclerotic factors, are more often observed in high laminar shear stress regions of the arteries. These in vivo observations were supported by evidence obtained in cultured ECs; ie, high laminar shear stress promotes glycosaminoglycan synthesis, tight junction formation, and the expression of junction-related proteins. These observations suggest that shear stress generated by blood flow may play a pivotal and antipodal role in the induction and progression or prevention of atherosclerosis by changing EC functions.

Several investigations have indicated that the DNA synthesis of ECs preferentially occurs at branch orifices, where atherosclerosis is initiated. Because increased cell division may accelerate endothelial permeability, stabilization of ECs may be achieved and maintained by restricting EC proliferation. Consistent with other researchers, we found that steady laminar shear stress inhibits the DNA synthesis of ECs in vitro. However, the molecular mechanisms of this inhibition are unknown. In the present study, we examined the effects of steady laminar shear stress on cell cycle events during the G<sub>1</sub> and S phases, including the phosphorylation of a retinoblastoma protein (pRb), the activation of cyclin-dependent kinases (cdks), and the expression of cdk inhibitors in cultured vascular ECs.

Materials and Methods

Antibodies

The following antibodies were used: anti-human pRb (XZ-104, G3-245), p21 (6B6), and p27 (G173-524) monoclonal antibodies...
from PharMingen; anti-human cdk2 polyclonal antibody from Upstate Biotechnology; anti-human cdk4 (H-22) and p21 (C-1) polyclonal antibodies from Santa Cruz Biotechnology; and anti-human p27 (57) monoclonal antibodies from Transduction Laboratories.

**Exposure to Shear Stress**

Bovine aortic ECs (BAECs) and human umbilical vein ECs (HUVECs) were plated on plastic slides or polyester sheets, as described previously.12 Flow experiments were performed according to a method similar to that described previously.13 A confluent monolayer of ECs in a parallel-plate flow chamber was exposed to steady laminar shear stress. Control ECs were grown in the same manner as the sheared ECs and were placed into fresh medium before being maintained in an incubator.

**DNA Synthesis**

After being exposed to shear stress, ECs were incubated in culture medium containing 2 μCi/mL [3H]thymidine for 2 hours. After the cells were treated with trichloroacetic acid, they were dissolved with 0.1 mol/L NaOH, and the incorporated radioactivity was counted and normalized by cellular protein, which was measured in parallel samples according to the method of Lowry.

**Flow Cytometry**

BAECs (1×10^6) were pelleted and fixed with 70% ethanol. After the cells were digested with RNase, the DNA was stained with propidium iodide and then analyzed with a flow cytometer.

**Immunoprecipitation and Western Blotting**

HUVECs were lysed in a lysis buffer containing 10 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L NaVO₃, 50 mmol/L NaF, 20 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40, followed by centrifugation. The supernatant was reacted with an antibody for 1 hour at 4°C. Immunoprecipitates recovered by protein A– or protein G–conjugated Sepharose beads were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and then incubated with an antibody for 2 hours. Immunoblots developed with a peroxidase-conjugated secondary antibody were visualized with enhanced chemiluminescence reaction reagents (Amersham) and analyzed with the public domain NIH image program.

**cdk Assay**

The immunoprecipitates, which were isolated from cells by reaction with an antibody for cdk2 or cdk4, were reacted with glutathione S-transferase–fused murine pRb carboxyl terminal (GST-Rb, Santa Cruz Biotechnology) in a kinase reaction buffer in the presence of 50 μmol/L cold ATP and 3.7 MBq/mL [γ-32P]ATP. The radiolabeled pRb, fractionated with SDS-PAGE, was quantified with a BAS-2000 Bio Image analyzer.

**Northern Blotting**

The cDNA for p21 was obtained from human vascular smooth muscle cells as described previously.14 Extraction of total RNA, electrophoresis, and transfer onto nylon membranes were performed as previously described.15 Blots were hybridized with cDNA probes for 16 hours at 50°C and were visualized and quantified with a BAS-2000 Bio Image Analyzer.

**Statistical Analysis**

The results are expressed as mean±SD of the number of observations (n=3 to 5 at each point) and analyzed with a 1-factor ANOVA, after confirming variance homogeneity by use of the Bartlett test. An expanded Materials and Methods section is available online at http://www.circresaha.org.

---

**Results**

**Effects of Shear Stress on DNA Synthesis**

There was no obvious detachment of ECs subjected to shear stress (Figure 1a, 24-hour exposure). The DNA synthesis of BAECs significantly decreased after exposure to 30-dyne/cm² shear stress (Figure 1b). This decrease was first evident 2 hours after the start of cell exposure to shear stress, reached 40.4% of the static control level after 4 hours, and remained at this low level for at least 24 hours. An inhibitory effect on DNA synthesis was also observed with 5-dyne/cm² shear stress (4 and 12 hours) in BAECs (Figure 1c). However, no significant inhibition was observed at 1-dyne/cm² shear stress (Figure 1d), suggesting that there is a threshold of growth inhibition due to steady laminar flow between 1- and 5-dyne/cm² shear stress in certain populations of BAECs. The DNA synthesis of HUVECs also decreased significantly, to 38.6%,...
17.9%, and 7.9% of the static control level, after exposure to 30-dyne/cm$^2$ shear stress for 6 hours and 5-dyne/cm$^2$ shear stress for 4 and 12 hours, respectively (Figures 1b and 1c). However, unlike BAECs, 1-dyne/cm$^2$ shear stress also significantly inhibited the DNA synthesis of HUVECs with 4 and 12 hours of exposure (Figure 1d), suggesting that the threshold in HUVECs may be set at a lower level than that in BAECs. This diversity in response to flow may represent a species difference (bovine versus human) or a vascular difference (artery versus vein or aorta versus umbilical vessel).

**Cell Cycle Arrest by Shear Stress**

Flow cytometric analysis for the DNA content in BAECs indicated that the cell populations in the S and G2/M phases progressively decreased after the cells were subjected to shear stress (30 dyne/cm$^2$). The percentage of cells in the S phase was reduced to 10.4% compared with the static control value of 13.1% after 6 hours and was reduced to 9.7% compared with the static control value of 12.3% after 12 hours. Similarly, the G2/M population was 15.7% versus 21.8%, 14.0% versus 20.7%, and 9.1% versus 17.9% in sheared and control cells after 2, 6, and 12 hours, respectively. This reduction was accompanied by an increase in the percentage of cells in the G0/G1 phase (Figure 2). Similar results were obtained after 24 hours (n=5, data not shown). These data indicate that shear stress inhibits the DNA synthesis of ECs via inhibition of the cell transition from the G0/G1 to the S phase of the cell cycle.

**Inhibition of cdk Activities by Shear Stress**

To elucidate the mechanism underlying cell cycle arrest induced by shear stress, we examined whether the cdk-pRb system, which regulates the G1/S transition, participated in this mechanism.

As shown in Figure 3, a hyperphosphorylated form of pRb, which moves more slowly than the hypophosphorylated form in SDS-polyacrylamide gels, decreased 12 hours after the start of exposure to shear stress (30 dyne/cm$^2$) in HUVECs. Therefore, shear stress was considered to inhibit a cell cycle event upstream from pRb phosphorylation. Thus, we analyzed the effects of shear stress on the activity of cdk2 and cdk4, which are responsible for the phosphorylation of pRb. The phosphorylation of GST-Rb by both cdk2 (Figure 4a) and cdk4 (Figure 4b) in HUVECs was greatly decreased by shear stress (30 dyne/cm$^2$) within 2 and 4 hours, respectively.
Induction of cdk Inhibitor p21 by Shear Stress

The activities of cdk's are controlled by the cdk inhibitor proteins, which include Cip/Kip family members, such as p21\textsuperscript{Sdi1/Cip1/Waf1} and p27\textsuperscript{Kip1}, and Ink4 family members, such as p15\textsuperscript{Ink4b} and p16\textsuperscript{Ink4a}.\textsuperscript{15} The Cip/Kip family universally inhibits several cdk's, whereas the Ink4 family specifically inhibits cdk4 and cdk6. Because shear stress inhibits both cdk2 and cdk4, we wondered whether shear stress stimulates the expression of the Cip/Kip family inhibitors.

As shown in Figure 5a, the p21 protein level increased 2 hours after the start of exposure to shear stress (30 dyne/cm\textsuperscript{2}) and maintained a high level for 12 hours, with an almost 2.2-fold increase over that of the control. To measure the amount of cdk2-associated p21, immunoprecipitates from an anti-cdk2 antibody were blotted with an anti-p21 antibody. The protein level of the cdk2-associated p21 clearly increased after the exposure of HUVECs to shear stress (30 dyne/cm\textsuperscript{2}) for 4 and 12 hours (Figure 5b), although the total amount of cdk2 itself was unchanged by shear stress (Figure 5c). In contrast to p21, the p27 protein did not increase as a result of shear stress (30 dyne/cm\textsuperscript{2}) (Figure 5d).

Shear stress (30 dyne/cm\textsuperscript{2}) increased the level of p21 mRNA quite quickly in HUVECs (Figure 6a). This expression was first evident after 15 minutes and became maximal 4 hours after the start of exposure to shear stress. It was maintained at a high level for 12 hours, similar to the protein level, with an almost 2.3-fold increase over that of the control (Figure 6b). The induction of p21 mRNA expression was also observed with lower shear stress. With 5-dyne/cm\textsuperscript{2} shear stress, HUVECs expressed p21 mRNA within 30 minutes and maintained a high level for 12 hours (Figure 6c).
For further confirmation of the linkage between shear stress–induced growth inhibition and p21 expression, we measured the DNA synthesis and p21 mRNA expression after releasing the cells from shear stress. When we released the cells from shear stress (30 dyne/cm²) after 6 hours of exposure and incubated them in a static condition for various periods up to 12 hours, the DNA synthesis of the cells recovered to almost the control level within 6 hours after the cessation of shear stress. Simultaneously, the p21 mRNA level, which had increased after 6 hours of exposure to shear stress, decreased during the same period of incubation (Figure 7).

Discussion

In the adult aorta, the average turnover rate of ECs is very low (<0.8% per day). An exception was observed in the area surrounding the branch orifices, where flow separation and a vortex occur, and 1.3% of these ECs were found to be in the S phase of the cell cycle. In tissue cultures, consistent with our data, it has been shown that the exposure of ECs to steady laminar flow decreases proliferation, whereas turbulent flow stimulates proliferation. When a flow-disturbing bar was placed in a parallel-plate flow chamber and cell division was monitored, cell division increased in the restricted area of flow separation with a high shear stress gradient (just behind the obstacle) but not in the area where shear stress recovered to its nondisturbed value with uniform laminar flow (1.5 mm downstream from the obstacle). Together with our other results, these data suggest that blood flow has antipodal effects on EC growth, namely, inhibition of growth with steady laminar flow and a stimulation of growth with disturbed flow.

In the present study, steady laminar shear stress blocked a cell cycle event that occurred before entry into the S phase, because the accumulation of the cell population in the G2/M phase after exposure to shear stress was accompanied by a decrease in the number of cells in the S phase as well as a decrease in the G2/M population. These results suggest that laminar shear stress plays a key regulatory role in the inhibition of proliferation, in association with maintaining cells in the quiescent G0/G1 phase of the cell cycle. Furthermore, the derangement of blood flow (such as at branch orifices) may release cells from this inhibition and induce cell proliferation, which may in turn disturb the stability of ECs.

How is the shear stress signal transmitted to the EC nuclei, leading to G0/G1 arrest in the cell cycle? Many mRNAs, second messengers (for a review, see References 19 and 20), and transcription factors, which could be involved in the signal transduction of EC proliferation, have been shown to be induced by shear stress. However, almost none of them has been shown to be directly responsible for the mechanisms by which shear stress–dependent growth arrest is induced. The importance of cdk2 and cdk4 activation and the subsequent phosphorylation of pRb in the G1 to S transition has been emphasized in a variety of cells (for a review, see Reference 22). The phosphorylation of pRb is required to release E2F transcription factors from the pRb/E2F complex; these transcription factors then activate the expression of genes required for initiating DNA synthesis. The activities of cdk are negatively regulated by several cdk inhibitor proteins. p27 binds to several cdk proteins, including cdk2 and cdk4, to inhibit their activities.

In the present study, we have demonstrated for the first time that steady laminar shear stress induces p21 expression and enhances cdk2-p21 binding, concomitant with the suppression of both cdk2 activation and pRb phosphorylation, causing cell cycle arrest in ECs. Because cdk4 activity was also suppressed by shear stress, p21 may also inhibit cdk4-mediated pRb phosphorylation.

On the other hand, the level of p27 was not altered by shear stress. Therefore, the contribution of p27 to the shear stress–induced inhibition of cdkks appeared to be small. However, this does not rule out the possibility of the involvement of p27, because the manner in which p27 inhibits cdkks may be different from that of p21. In epithelial cells, transforming growth factor-β (TGF-β) elevates the expression of the cdk4/cdk6-specific inhibitor p15 and induces the release of p27 from cdk4 and cdk6. This release increases the binding of p27 to cdk2, which leads to cell cycle arrest. TGF-β is also induced by shear stress in ECs. Therefore, the possibility remains that TGF-β induced by shear stress shifts p27 from cdk4 and cdk6 to cdk2 and inhibits cdk2 activity. To study this possibility, we exposed HUVECs to shear stress.
(30 dyne/cm²) for 8 hours in the presence of either a neutralizing antibody for TGF-β1 (10 ng/mL) or tetraethylammonium (3 mmol/L), a K⁺ channel blocker that inhibits shear stress–induced TGF-β expression.²⁶ Neither of these affected the inhibition of DNA synthesis by shear stress (data not shown), suggesting that TGF-β may not be involved in the mechanotransduction of shear stress–mediated growth inhibition. The upstream signal transduction of p21 expression, involved in the growth inhibition of ECs induced by shear stress, remains to be determined.

Acknowledgments

This study was supported in part by a grant-in-aid from the Ministry of Education, Science, and Culture, the Ministry of Science and Technology Agency, and the Ministry of Health and Welfare of Japan. The authors are grateful to Drs Akihiko Hashi (Yamanashi Medical University) and Katsuhiko Teramoto (Yamanashi Prefectural Central Hospital) for providing the human umbilical cords.

References


Laminar Shear Stress Inhibits Vascular Endothelial Cell Proliferation by Inducing Cyclin-Dependent Kinase Inhibitor p21 Sdi1/Cip1/Waf1
Shigeo Akimoto, Masako Mitsumata, Toshiyuki Sasaguri and Yoji Yoshida

Circ Res. 2000;86:185-190
doi: 10.1161/01.RES.86.2.185

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/86/2/185

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2000/02/01/86.2.185.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/