Laminar Shear Stress Upregulates Integrin Expression
Role in Endothelial Cell Adhesion and Apoptosis

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Abstract—Laminar shear stress exerts important effects on endothelial cell (EC) function and inhibits apoptosis of ECs induced by various stimuli. The mechanism by which hemodynamic forces, such as shear stress, are transduced into cellular signaling is still not known. Located at the cell surface, integrins, which are required for cell adhesion and cell survival, are potential mechanotransducers. Therefore, we investigated the effect of shear stress on integrin expression in ECs. Shear stress time-dependently increased the mRNA expression of the fibronectin receptor subunits α5 and β1 with a maximum at 6 hours (283±41% and 215±27% of control, respectively). In addition, the protein levels of the fibronectin receptor subunits α5 and β1 were enhanced with a maximum at 12 hours of shear stress exposure (343±53% and 212±38% of control, respectively). The shear stress–induced upregulation of integrins is independent of nitric oxide. Furthermore, we confirmed the enhanced functional activity of α5β1 integrin expression by FACS analysis. As a functional consequence, human umbilical vein ECs, which were preexposed to shear stress, revealed a significantly increased attachment (178±10% of static controls) and a more pronounced extracellular signal–regulated kinase 1 and 2 activation in response to cell attachment. Finally, we demonstrated that shear stress requires RGD-sensitive integrins to mediate its antiapoptotic effect. Taken together, these results define a novel mechanism by which shear stress may exert its atheroprotective effects via upregulation of integrins to support EC adhesion and survival. (Circ Res. 2000;87:683-689.)

Key Words: integrins ■ shear stress ■ endothelial cells ■ gene expression

The interaction of cells with the extracellular matrix is mediated by integrins. Integrins, a large family of heterodimeric transmembrane receptors, are composed of α and β subunits. Currently, at least 16 α and 8 β subunits have been identified.1 Each αβ combination has specific binding and signaling properties. For example, the α5β1 integrin binds to fibronectin, and laminin is the ligand for αvβ3, whereas other integrins are capable of binding multiple ligands.2 Thus, the αvβ3 integrin is known to interact with vitronectin, fibronectin, and von Willebrand factor. Adherent cells anchor via integrins to the matrix, which is essential to maintain the survival of the cells.3,4 Thus, various integrins, including α5β1 or αvβ3, prevent apoptosis of endothelial cells (ECs).5–7 Although the exact mechanism of the antiapoptotic effect is not clear, it has been suggested that antiapoptotic integrin signaling involves the activation of the prosurvival kinase Akt or the mitogen-activated protein (MAP) kinase extracellular signal–regulated kinase (ERK) 1/2.5–10 Furthermore, activation of the integrin α5β1 was shown to transcriptionally upregulate antiapoptotic genes, such as Bcl-2.5 Besides their role in cell survival, integrins are necessary for cell migration, a process essential for angiogenesis or reendothelialization.7,8,11

The laminar blood flow (shear stress) is a hemodynamic force, which acts on the luminal site of the vessel wall. Because of their unique location, ECs are continuously exposed to shear stress, which modulates gene expression and cellular structure and function.12 Briefly, shear stress regulates gene expression of various proteins, including vasoactive substances (eg, nitric oxide [NO] synthase and endothelin-1), growth factors (eg, transforming growth factor-β and platelet-derived growth factor), adhesion and chemotactant molecules (eg, intercellular adhesion molecule-1, vascular cellular adhesion molecule-1, and monocyte chemotactant protein-1), coagulation factors (eg, tissue factor), proto-oncogenes (eg, c-fos, c-jun), and antioxidant enzymes (eg, superoxide dismutase).13 Furthermore, laminar shear stress completely inhibits apoptosis of ECs in response to various stimuli,14 demonstrating the potent atheroprotective effects of shear stress to preserve the integrity of the endothelium. The mechanisms by which hemodynamic forces such as shear stress are transduced into cellular signaling are still not known. Located at the cell surface, integrins are possible candidates for the transduction of hemodynamic forces into biochemical signals.15–17 For example, Muller et al18 showed that shear stress–induced vasodilation in coronary arteries could be blocked with RGD peptides that inhibit the binding of integrins to their ligands.2 Moreover, shear stress–mediated ERK1/2 activation and Akt
phosphorylation, which are important survival signals in ECs, depend on integrin binding to the extracellular matrix. Although compelling evidence suggests that integrins play an important role as mechanotransducers, there are no data concerning the regulation of integrin subunits by laminar shear stress in ECs.

Therefore, we examined whether shear stress regulates integrin expression. For the first time, we demonstrate that shear stress modulates the expression of integrin subunits on mRNA and protein levels. Furthermore, the enhanced functional activity of integrins is confirmed by FACS analysis. Specifically, shear stress induces an upregulation of the fibronectin receptor \( \alpha_5 \) and \( \beta_1 \) subunits. As a functional consequence, human umbilical vein ECs (HUVECs), which were preexposed to shear stress, revealed a significantly increased attachment and a more pronounced ERK1/2 activation in response to cell attachment. Finally, we demonstrate that integrins are required to mediate the apoptosis-inhibitory effect of shear stress. Taken together, these results define a novel mechanism by which shear stress mediates and intensifies its atheroprotective effects via upregulation of integrins as potential mechanotransducers.

**Materials and Methods**

**Cell Culture**

Pooled HUVECs were purchased from Cell Systems/Clonetics and were cultured and exposed to laminar fluid flow in a cone-and-plate apparatus as previously described. A constant shear stress of 15 dyne/cm² was used in all experiments to simulate physiological levels of shear stress. N°-Monomethyl-L-arginine (LMA; Alexis) and the synthetic peptide GRGDNP (GIBCO-BRL) or the neutralizing integrin \( \alpha_5 \) and \( \beta_1 \) antibodies (Dianova and GIBCO-BRL) were preincubated for 30 minutes before shear stress exposure.

**Atlas cDNA Expression Array**

Total RNA was isolated as described and radioactively labeled with a mixture of gene-specific primers. The differential gene expression was analyzed by hybridization with 588 cDNAs immobilized on a membrane according to the instructions of the manufacturer (Clontech). After a high-stringency wash, the expression profile was quantified by phosphor imaging.

**Western Blot Analysis**

For the detection of integrin expression, HUVECs (4.0×10⁵ cells) were lysed in buffer as previously described. For determination of the phosphorylated form of ERK1/2, the lysis buffer was supplemented with phosphatase inhibitors as described. The protein content of the samples was determined according to the Bradford method. Western blots were performed using antibodies directed against integrins \( \alpha_5 \) and \( \beta_1 \) (1:2500; Transduction Laboratories), phospho-ERK1/2, or ERK1/2 (1:2000; Biolabs) or actin (1:1000; Boehringer Mannheim).

**Flow Cytometry**

HUVECs were washed with PBS and detached with 1 mmol/L EDTA (pH 7.4) for 20 minutes at 37°C. After centrifugation the cell pellet was suspended in 100 µL PBS/10% FCS and incubated for 30 minutes at 4°C with 10 µL FITC-conjugated \( \alpha_5 \) or \( \beta_1 \) integrin antibodies (Dianova). Subsequently, cells were fixed with 4% paraformaldehyde in PBS and analyzed by flow cytometry using FACS Calibur (Perkin Elmer; CellQuest software). All experiments were performed with standardized instrumental settings (FL1-H [fluorescence], voltage 498, and amp gain 1.16).

**Cell Adhesion Assay**

After exposure to shear stress for 24 hours, HUVECs were washed with PBS and detached with 1 mmol/L EDTA (pH 7.4) in PBS for 20 minutes at 37°C. After centrifugation the cell pellet was suspended in endothelial cell basal medium complete medium in the presence or absence of PD98059 (10 µmol/L; Biomol), RGD peptides (GRGDNP; 0.5 mmol/L), or neutralizing antibodies. Identical numbers of cells were placed onto uncoated or fibronectin-coated culture dishes and incubated for 20 minutes at 37°C. Adherent cells were counted by 2 independent blinded investigators, or cells were lysed for Western blot analysis.

**Detection of Apoptosis**

Cell culture dishes were centrifuged (10 minutes at 700g), fixed in 4% formaldehyde, and stained with DAPI. Five hundred cells were counted by 2 independent blinded investigators, and the percentage of apoptotic cells per total number of cells was determined.

**Statistical Analysis**

Data are expressed as mean±SEM from at least 3 independent experiments. Statistical analysis was performed by t test. For serial analyses (time-dependency), ANOVA was performed.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

**Results**

**Shear Stress Modulates mRNA and Protein Expression of Integrins**

To investigate the effect of shear stress on mRNA expression of integrins, HUVECs were exposed to laminar shear stress (15 dyne/cm²) for 6 and 12 hours. Total RNA was isolated and used in an Atlas cDNA Expression Array, which allowed the analysis of differential gene expression of various integrins. Shear stress time-dependently increased the expression of the integrin subunits \( \alpha_5 \) and \( \beta_1 \), which are the preferential receptor for fibronectin in HUVECs, with a maximum at 6 hours of shear stress exposure (Figures 1A and 1B). In addition, the integrin subunit \( \alpha_5 \) (the receptor for fibronectin, collagen, and laminin) and the laminin receptor subunit \( \alpha_5 \) are slightly upregulated in response to shear stress (Figures 1C and 1D). In contrast, mRNA expression of integrin \( \alpha_5 \), the vitronectin receptor, was not affected by shear stress (12 hours; 95±27% of static control).

To confirm the data obtained with the cDNA expression array, protein expression of fibronectin receptor \( \alpha_5 \) (integrin \( \alpha_5 \)) and fibronectin receptor \( \beta_1 \) (integrin \( \beta_1 \)) was analyzed by Western blotting. As shown in Figure 2A, integrin \( \alpha_5 \) was time-dependently upregulated with a maximum of protein expression within 12 to 18 hours of shear stress exposure. Similar to the effects of shear stress on mRNA expression, prolonged application to shear stress led to an enhanced integrin \( \beta_1 \) protein expression with a maximum at 12 to 18 hours (Figure 2B). The expression of the integrin subunits \( \alpha_5 \) and \( \beta_1 \) remained elevated ≥2-fold up to 48 hours of shear stress exposure (data not shown). Moreover, the effects of shear stress were dose-dependent, as illustrated in Figure 2C.

**Shear Stress Induces the Cell Surface Expression of Integrins**

Because the presence of integrin mRNA and protein does not necessarily establish its biological function, we further determined the regulation of integrin \( \alpha_5 \) and \( \beta_1 \) by shear stress in
HUVECs using FACS analysis (Figure 3). In accordance with the findings of the Atlas cDNA Expression Array and Western blot analysis, exposure of ECs to laminar shear stress for 24 hours enhanced cell surface expression of integrin $\alpha_5$ (Figure 3A) and integrin $\beta_1$ (Figure 3B). Thus, the shear stress–induced upregulation of integrin mRNA and protein results in an increased cell surface expression of integrins, which can mediate cell-matrix interactions.

**Mechanisms of Shear Stress–Induced Upregulation of Integrin $\alpha_5$ and Integrin $\beta_1$: Role of NO and Paracrine Growth Factors**

Murohara et al demonstrated that inhibition of endothelial NO synthase (eNOS) by the L-arginine analog N-nitro-L-arginine methyl ester in HUVECs inhibited surface expression of integrin $\alpha_5$, which plays an important role in EC survival and angiogenesis. Previous studies have demonstrated that physiological levels of shear stress induced upregulation of eNOS followed by an increase of NO release. Therefore, we examined the role of NO in shear stress–induced upregulation of integrins $\alpha_5$ and $\beta_1$ by Western blot analysis. HUVECs were incubated for 12 hours with sodium nitroprusside (SNP) or 1-propanamine,3-(2-hydroxy-2-nitroso-1-propylhydrazino) (PAPA NONOate) (50 $\mu$mol/L), each of which is an NO donor. Incubation with SNP or PAPA NONOate for 12 hours led to a significantly enhanced expression of integrins $\alpha_5$ and $\beta_1$, similar to the effects obtained with 12 hours of shear stress exposure (Figure 4; data not shown).

However, incubation of eNOS with LNMA (1 mmol/L) did not reduce the shear stress–mediated upregulation of integrins $\alpha_5$ and $\beta_1$ (Figures 4A and 4B). These data indicate that NO is sufficient to induce integrin expression. However, shear stress in addition activates a second NO-independent pathway leading to upregulation of integrin expression.

Because shear stress is known to stimulate the release of growth factors, one may speculate that it can induce integrin expression in a paracrine manner. However, conditioned medium from shear stress–exposed HUVECs (24 hours) did not increase integrin expression (Figure 5).

**Shear Stress Induces HUVEC Adhesion via Upregulation of Integrins**

Having demonstrated that shear stress stimulates the expression of integrins $\alpha_5$ and $\beta_1$, we examined the effect of integrin upregulation on EC adhesion. Therefore, HUVECs were exposed to laminar shear stress for 24 hours, detached, and replated on cell culture dishes. As shown in Figure 6A, ECs preexposed to shear stress exhibited a significant increase in the number of adherent cells after replating (178±10% of static controls). Similar results were obtained when ECs were replated on plastic dishes coated with fibronectin (data not shown). In contrast, incubation of preexposed ECs with the MAP/ERK kinase (MEK) inhibitor PD98059 (10 $\mu$mol/L) abrogated the shear stress–stimulated increase in EC adhesion (Figure 6A). Moreover, RGD peptides (GRGDNP; 0.5 mmol/L) or neutralizing antibodies against integrins $\alpha_5$ and $\beta_1$ prevented shear stress–induced increase in cell adhesion after replating (Figure 6B), whereas control peptides (GRGESP; 0.5 mmol/L) had no effect (data not shown).

**Figure 1.** Shear stress increases the mRNA expression of integrins. Total RNA was isolated from HUVECs exposed to laminar shear stress (15 dyne/cm$^2$) for 6 and 12 hours. The differential gene expression of various integrin subunits was analyzed using an Atlas cDNA Expression Array. Shown are effects of shear stress on mRNA expression of fibronectin receptor $\alpha_5$ (A), fibronectin receptor $\beta_1$ (B), integrin $\alpha_5$ (C), and integrin $\alpha_6$ (D). Data were quantified by phosphor imaging and are expressed as integrin mRNA/GAPDH mRNA compared with static control (mean±SEM; n=3).

**Figure 2.** Shear stress enhances protein expression of integrins $\alpha_5$ and $\beta_1$. A and B, Time-dependency. HUVECs were exposed to laminar shear stress (15 dyne/cm$^2$) for 6, 12, and 18 hours, and protein levels of integrin $\alpha_5$ (A) and integrin $\beta_1$ (B) were detected by Western blot analysis. C, Dose-dependent effect of shear stress on integrin expression (18 hours of incubation). Actin reprobes served as loading control. Integrin expression was densitometrically analyzed and normalized to actin. Representative blots of 4 independent experiments are shown.
demonstrating a specific role of integrins in shear-stimulated cell adhesion.

We further determined the effect of shear stress preexposure on ERK1/2 activation in replated ECs. As assessed by Western blot analysis using a phosphospecific ERK1/2 antibody, ERK1/2 phosphorylation was significantly enhanced in replated ECs, which were preexposed to shear stress for 24 hours, compared with static controls (206±77%; Figure 6A). In contrast, preexposure of ECs to shear stress for 20 minutes did not increase cell adhesion after replating (data not shown). These data indicate that long-term exposure to shear stress stimulates the ability of ECs to adhere to the matrix. Furthermore, HUVECs, which were preexposed to shear stress, revealed a more pronounced ERK1/2 activation in response to cell attachment.

Shear Stress Requires RGD-Sensitive Integrins to Mediate Its Antiapoptotic Effect

Physiological levels of laminar shear stress completely prevent apoptosis of human ECs in response to a variety of stimuli, including tumor necrosis factor α, oxidized LDL, and angiotensin II.14,19 Several studies suggest that integrins are important for cell survival.3,5–7,25 Therefore, we examined the role of integrins in the antiapoptotic effect of shear stress using RGD peptides, which compete with the matrix, namely fibronectin and vitronectin, for integrin interactions.2,18 As shown in Figure 7, incubation of ECs with RGD peptides (GRGDNP; 0.5 mmol/L) for 18 hours potently induced apoptotic cell death (316±87% of control) as assessed by morphological analysis of fluorescence-stained nuclei, whereas control peptides (GRGESP; 0.5 mmol/L) had no effect (data not shown). Exposure of ECs to shear stress could not inhibit RGD peptide-induced apoptosis (326±61% of control), indicating that shear stress requires RGD-sensitive integrins to mediate its antiapoptotic effect. Coincubation of ECs with RGD peptides and a specific caspase-3 inhibitor (Ac-Asp-Glu-Val-Asp-aldehyde [AcDEVD-CHO]; 100 μmol/L) led to a decrease of apoptosis (183±55%), demonstrating the involvement of the caspase cascade in apoptosis induction by RGD peptides.

Discussion

The data of the present study demonstrate that shear stress upregulates the expression of integrins in ECs. Moreover, preexposure of ECs to laminar shear stress profoundly enhances the attachment of the cells to the matrix. The integrins α5β1 and αvβ3 play a central role in EC migration and angiogenesis and support EC survival.21,25,26 The biological effects induced by shear stress require an intact integrin signaling. Thus, shear stress–induced stimulation of the
protein kinase Akt, which mediates the activation of the eNOS, depends on the integrin pathway.\textsuperscript{10,27} Consistent with this finding, shear stress–induced stimulation of blood vessel relaxation is prevented by RGD peptides, which block \( \alpha_5 \) and \( \alpha_v \beta_3 \) integrins.\textsuperscript{2,18} Furthermore, an emerging body of evidence suggests that integrins are involved in shear stress–induced activation of focal adhesion kinase and MAP kinases.\textsuperscript{10,28–30} The present study extends these observations by demonstrating that shear stress additionally upregulates integrin expression, which may importantly contribute to the long-term effects of shear stress on EC biology.

Shear stress mainly affects the expression of the fibronectin receptor subunits \( \alpha_5 \) and \( \beta_1 \). Adhesion of HUVECs to fibronectin was shown to promote EC survival via activation of the p52Shc adapter protein, which in turn recruits the Grb2-mSOS complex to the membrane and thereby activates the MAP kinase pathway.\textsuperscript{21} In line with these findings, the inhibition of the integrin pathway by blockade with RGD peptides prevented the apoptosis-suppressive effect of shear stress. Preexposure of ECs to shear stress results in an enhanced adhesion of ECs after replating. Moreover, activation of the ERK1/2 MAP kinase was more pronounced in replated cells, which were preexposed to shear stress. As it has also been demonstrated that short-term exposure of ECs to shear stress for 20 minutes enhances adhesion-induced ERK1/2 phosphorylation probably by activation of additional intracellular signaling pathways,\textsuperscript{17} we also investigated the effects of short-term shear stress exposure on EC adhesion. However, after 20 minutes of preexposure, no increase in EC adhesion was detectable after replating, which indicated that the long-term shear stress–induced integrin expression may be required for the enhanced cell adhesion. Palecek et al\textsuperscript{31} demonstrated that an increase in integrin \( \alpha_5 \) expression results in a dose-dependent enhancement of cell adhesion and migration. Thus, the integrin upregulation induced by shear stress might well explain the augmentation of cell attachment.

Taken together, given that EC adhesion was mediated by a RGD-dependent pathway and the activation of ERK1/2 after adhesion was suggested to be mediated by \( \beta_1 \) integrin,\textsuperscript{17,28} the enhanced integrin expression induced by shear stress most likely facilitates binding of the ECs to the matrix, which promotes ERK1/2 activation. This conclusion is also supported by our finding that inhibition of ERK1/2 by PD98059 eliminated the enhanced attachment capacity of replated ECs.

The mechanism by which the integrin subunits are upregulated remains unclear. The data of the present study, demonstrating that exogenous NO can upregulate integrins, are in line with the findings by Murohara et al,\textsuperscript{22} who demonstrated that inhibition of NO synthase decreases the expression of the integrins \( \alpha_5 \) and \( \beta_1 \). The study by Murohara et al,\textsuperscript{22} however, only revealed an effect of NO on the cell surface expression of the fibronectin receptor, whereas the absolute protein levels were unchanged. These findings are in accordance with our results.

Figure 4. Shear stress–induced upregulation of integrins \( \alpha_5 \) and \( \beta_1 \) is independent of NO. HUVECs were incubated for 12 hours with the NO donor SNP (50 \( \mu \)mol/L) or exposed to laminar shear stress (15 dyne/cm\textsuperscript{2}) for 12 hours. LNMA (1 mmol/L) was preincubated for 30 minutes before shear stress exposure. A, Integrin \( \alpha_5 \) and integrin \( \beta_1 \) protein expression was determined by Western blot analysis. The blot was then reprobed with actin to confirm equal loading. A representative blot of 4 independent experiments is shown. B, Integrin \( \alpha_5 \) and integrin \( \beta_1 \) protein expression was quantified by densitometric analysis of the Western blots from 4 independent experiments (data are normalized against actin and expressed as mean±SEM; \( n=4 \)).

Figure 5. Effect of conditioned medium. HUVECs were exposed to laminar shear stress or kept under static conditions for 24 hours. Then, conditioned medium was removed, added to static cultures, and again incubated for 24 hours. Integrin \( \alpha_5 \) (A) and \( \beta_1 \) (B) expression was detected by Western blot analysis. A representative blot of 3 independent experiments is shown.
showing that the α5 subunit is not regulated by shear stress on the mRNA level. However, although exogenous NO appears to be sufficient to upregulate the integrin subunits α5 and β3, shear stress–induced endogenous NO synthesis does not seem to be required for the increase of integrin expression. Thus, blockade of NO synthesis did not prevent shear stress–stimulated integrin expression, suggesting that shear stress can use an additional pathway beyond NO to enhance integrin expression. Moreover, conditioned medium of shear stress–induced HUVEC does not influence the expression of integrin subunits α5 and β3, thus excluding the possibility that shear stress induces integrin expression in a paracrine manner via the release of growth factors, which are known to modulate integrin expression. However, shear stress can also directly stimulate tyrosine phosphorylation of growth factor receptors and, for example, activate the VEGF receptor Flk-1 even in the presence of neutralizing VEGF antibodies. Therefore, one may speculate that other growth factor–induced signaling events are triggered by shear stress in a similar manner. Thus, shear stress may directly activate downstream signaling pathways and thereby mimic growth factor stimulation.

The survival of ECs is critical for the maintenance of blood vessel integrity and angiogenesis. Moreover, EC apoptosis may contribute to the initiation of atherogenesis. The attachment of ECs to the matrix, which is mediated by integrins, thereby plays an essential role in maintaining EC survival. Thus, shear stress–induced upregulation of integrins may enhance the stimulation of integrin signaling and thereby promote EC survival. Moreover, given that integrins are also required for EC migration, one may speculate that shear stress may accelerate reendothelialization, eg, after balloon denudation, by enhancing cell migration via integrin upregulation.

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