Integrin-mediated mechanotransduction requires its dynamic interaction with specific extracellular matrix (ECM) ligands

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The aim of this study is to elucidate the role of integrins in transducing fluid shear stress into intracellular signals in vascular endothelial cells, a fundamental process in vascular biology. We demonstrated that shear stress activates specific integrins in endothelial cells plated on substrates containing the cognate extracellular matrix (ECM) ligands. The shear stress-induced mechanotransduction, as manifested by integrin–Shc association, was abolished when new integrin–ECM ligand interactions were prevented by either blocking the integrin-binding sites of ECM ligands or conjugating the integrins to immobilized antibodies. Our results indicate that the dynamic formation of new connections between integrins and their specific ECM ligands is critical in relaying the signals induced by shear stress to intracellular pathways.

shear stress | endothelial cell | signal transduction

The vessel wall is exposed to hemodynamic forces, including shear stress and mechanical stretch. Vascular endothelial cells (ECs), which are in direct contact with the flowing blood, have been proposed to be the primary sensor of wall shear stress for the transduction of biomechanical stimuli into biochemical responses (see ref. 1 for review). *In vivo* animal experiments indicate that shear stress can modulate EC structure and function, including cell orientation, repair and migration, cytoskeletal reorganization, macromolecular permeability, lipoprotein accumulation, and leukocyte recruitment (see ref. 2 for review). *In vitro* studies using flow channels have shown that shear stress can regulate the expression of many genes and their products in ECs by acting through several signaling pathways (see ref. 3 for review). These include the mitogen-activated protein kinases (MAPKs), e.g., extracellular signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), and the kinases involved in the focal adhesions, e.g., focal adhesion kinase (FAK), Src family kinases, and phosphatidylinositol 3-kinase (PI3K) (4–7).

Mediating both the ''inside-out'' and ''outside-in'' signals, integrins activate MAPKs and the focal adhesion-associated kinases in responses to extracellular stimuli and during cell adhesion to extracellular matrix (ECM) ligands (see refs. 8–10 for review). The involvement of integrins in endothelial responses to shear stress is suggested by the activation of FAK and c-Src (see ref. 11 for review) and by the observation that focal adhesions undergo constant remodeling on the abluminal side of ECs (12). We have previously shown that the shear stress-induction of MAPKs in ECs is at least in part due to the recruitment of the adapter protein Shc to integrins such as $\alpha_v\beta_3$ (13). However, the molecular mechanism by which integrins mediate mechanotransduction is still unknown. In this study, we performed experiments to test the hypotheses that (*i*) the shear stress-induced intracellular signaling is mediated by the interaction of integrins with their specific ECM ligands, and that (*ii*) this signaling process requires the dynamic formation of new connections between the integrins and their specific ligands.

Materials and Methods

Cell Culture and Shear Stress Experiments. Human umbilical vein endothelial cells (HUVECs) were used in all experiments. All cell cultures were kept in a humidified 5% CO_2/95% air incubator at 37°C. HUVECs cultured on 38 mm × 76 mm glass slides to confluence were either kept as static controls or subjected to laminar shear stress in a rectangular flow channel system (14), which was modified to allow the shearing of multiple slides simultaneously. A surface area of 14 cm^2 on the HUVEC-seeded slide, confined by a gasket, was exposed to a fluid shear stress generated by the flow of culture medium over the cells. The pH of the system was kept constant by gassing with 5% CO_2/95% air, and the temperature was maintained at 37°C by keeping the flow system in a temperature-controlled box. The shear stress was 12 dyn/cm^2, which is relevant to the physiological range in human major arteries.

Coating of Slides with Different ECM Proteins. Extracellular matrix proteins human fibronectin (FN), human vitronectin (VN), rat collagen type I (CL), human laminin (LM), and human fibrinogen (FG) (Sigma) were applied to sterile 75 × 38 mm glass slides by adsorption. The concentrations of these matrices were as follows: FN, 5 $\mu g/cm^2$; VN, 0.5 $\mu g/cm^2$; CL, 6 $\mu g/cm^2$; LM, 2 $\mu g/cm^2$; and FG, 5 $\mu g/cm^2$. The ECM proteins were spread as a thin film on the slides and air-dried overnight to allow complete adsorption. The slides were washed three times with PBS, and HUVECs were seeded on the slides in the absence of serum. After 2 h of incubation, the cells were either sheared in the flow channel by using the M199 medium or kept as static controls.

Detection of Specific Integrin–Ligand Binding by using Monoclonal Antibodies that Recognize Only the Ligand-Occupied Integrins. Sterile glass slides were coated with FN or VN. HUVECs were plated on the slides in the absence of serum for 2 h and then

Abbreviations: CL, rat collagen type I; EC, endothelial cell; ECM, extracellular matrix; FG, fibrinogen; FN, fibronectin; HUVEC, human umbilical vein endothelial cell; JNK, c-Jun N-terminal kinase; LM, laminin; VN, vitronectin; pAb, polyclonal antibody.

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exposed to shear stress (12 dyn/cm^2) for 5 or 30 min, or kept as static controls. The cells were fixed with 3% paraformaldehyde-PBS for 30 min, permeabilized in 0.2% Triton X-100 in PBS for 5 min, and blocked with 10% normal goat serum. Ligand-occupied β_1 and β_3 integrins were detected by using HUTS-21 (2 μg/ml) and LIBS-6 (5 μg/ml), respectively, followed by FITC-conjugated goat anti-mouse IgG (1:100). The images were acquired by using a Bio-Rad 1024 MRC laser scanning confocal imaging system.

Detection of the Association of $\alpha_v\beta_3$ with Shc. Sheared or static HUVECs on glass slides coated with FN, VN, CL, or LM were lysed in a buffer containing 50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4, and 10 μg/ml leupeptin. Equal amounts of cell lysates were incubated with 2 μg of the anti-$\alpha_v\beta_3$ monoclonal antibody (mAb) LM609 (Chemicon) conjugated with protein A-Sepharose beads (Amersham Pharmacia). The precipitated immunocomplexes were washed with the lysis buffer, separated with 10% SDS/PAGE, and transferred to a nitrocellulose membrane for immunoblotting. The membrane was blocked with 5% BSA, followed by incubation with an anti-Shc polyclonal antibody (pAb) (Transduction Laboratories, Lexington, KY) in Tris-buffered saline with 0.05% Tween-20 containing 1% BSA. The bound primary antibodies were detected by using a goat anti-rabbit IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology) and the enhanced chemiluminescence (ECL) detection system.

Blocking of Unbound Sites of Fibronectin and Vitronectin. Sterile glass slides were coated with either FN or VN. HUVECs were then seeded on these slides in the absence of serum and allowed to adhere for 2 h. The cells were incubated with 20 μg/ml of various blocking and nonblocking antibodies specific for the binding sites for FN and VN. The blocking antibodies were: 16G3, which blocks both the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ binding sites for FN; 3B8, which blocks only the $\alpha_5\beta_1$ binding sites of FN but not its $\alpha_v\beta_3$ sites; and 661, which blocks only the $\alpha_v\beta_3$ binding sites of VN. The nonblocking antibodies used as controls were 11E5, which is specific for FN, and 443, which is specific for VN.

Coating of Slides with Antibodies Against $\alpha_v\beta_3$. Plastic slides were used instead of glass slides in these experiments to reduce the nonspecific binding of integrins to parts of the surface not coated with the antibody. Sterile slides were coated with secondary goat anti-mouse antibody (50 μg/ml; Sigma, 1:1000 in PBS). After rinsing twice with PBS, the slides were incubated with 10 mg/ml of BSA for 1 h to block the unbound sites. The slides were then coated with LM 609 anti-$\alpha_v\beta_3$ mAb (1:1000 in PBS) for 1 h. After rinsing the slides twice with PBS and once with M199 medium, HUVECs were seeded on the antibody-coated slides in the absence of serum and allowed to adhere for 2 h before shear stress experiments.

JNK Kinase Activity Assays. To assay JNK activities of HUVECs on FN, FG, or LM 609, the cells were lysed in a lysis buffer (25 mM Hepes, pH 7.4, 0.5 M NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 50 mM NaF, 10 mM Na_3VO_4, and 2 mM β-glycerophosphate). JNK1 was immunoprecipitated with anti-JNK1 mAb (Santa Cruz Biotechnology) and protein A-Sepharose beads. Two micrograms of GST-c-Jun(1–79) fusion protein and 10 μCi (1 Ci = 37 GBq) of γ-[^{32}P]ATP (ICN) in 30 μl kinase assay buffer (25 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml leupeptin, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, and 25 μM ATP) were added to each immunocomplex pellet for kinase reaction at 30°C for 20 min. The phosphoproteins were separated by SDS/PAGE, and the gels dried for autoradiography.

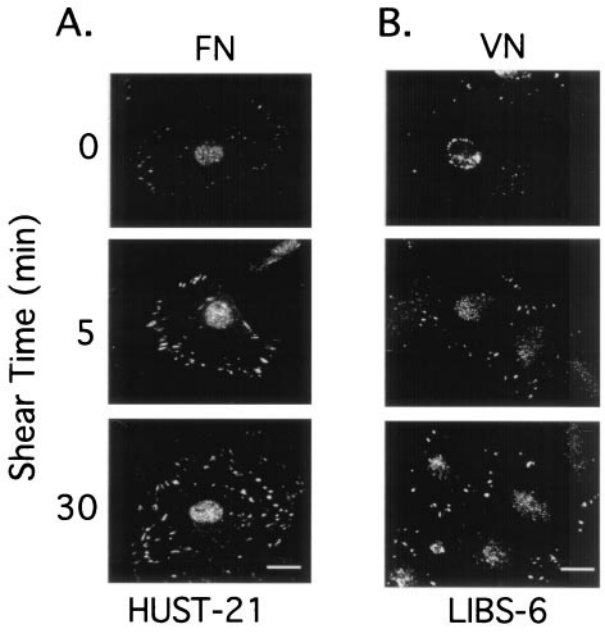

Fig. 1. Shear stress increases integrin occupancy on VN and FN. Glass slides were coated with in FN (5 μg/cm^2; *A*) and VN (0.5 μg/cm^2; *B*). HUVECs were plated on these coated slides in the absence of serum for 2 h and then exposed to shear stress (12 dyn/cm^2) for 5 and 30 min or kept as static controls. The cells were fixed with 3% paraformaldehyde in PBS for 30 min, permeabilized in 0.2% Triton X-100-PBS for 5 min, and blocked with 10% normal goat serum. (*A*) The occupied β_1 integrin was revealed by immunostaining using HUTS-21 mAb. (*B*) The occupied β_3 integrin was revealed by LIBS-6 mAb. These mAbs were recognized by FITC-conjugated goat anti-mouse IgG (1:100).

Results

Shear Stress Increases Integrin Binding to Specific ECM Ligands. We investigated the effects of shear stress on integrin–ligand binding in HUVECs by using mAbs that recognize only integrins with the ligand-occupied conformation. HUVECs were plated on FN (ligand for $\alpha_5\beta_1$ and $\alpha_v\beta_3$) or VN (ligand for $\alpha_v\beta_3$), and then exposed to shear stress. Fig. 1 shows the confocal images of the basal layers of the Z-section of ECs. Staining with HUTS-21, which binds specifically to the ligand-bound β_1 integrin (15, 16), showed that shear stress caused an increase in ligand binding of the β_1 integrin in HUVECs plated on FN (Fig. 1*A*). Staining with LIBS-6, which binds specifically to the ligand-bound β_3 integrins (17, 18), revealed that shear stress caused an increase in ligand binding of the β_3 integrin in HUVECs plated on VN (Fig. 1*B*).

Shear Stress-Induced Integrin Signaling is ECM-Specific. Shear stress induces a sustained association of integrins, e.g., $\alpha_v\beta_3$, with Shc in ECs (13). We tested the roles of ECM proteins in the shear stress-activation of integrins, using their association with Shc as readout. HUVECs were plated on FN, VN, CL, or LM (ligand for $\alpha_6\beta_1$). The cells were then sheared and their lysates immunoprecipitated with an integrin-specific mAb (LM609 anti-$\alpha_v\beta_3$ or S3-41 anti-$\alpha_6\beta_1$) followed by immunoblotting with an anti-Shc pAb. Fig. 2*A* shows that shear stress induced $\alpha_v\beta_3$–Shc association in cells plated on FN (lane 2) or VN (lane 8), but not on CL (lane 4) or LM (lane 6). In contrast, shear stress caused $\alpha_6\beta_1$–Shc association only in cells plated on LM, but not on FN, VN, or CL (Fig. 2*B*). These results indicate that shear stress-

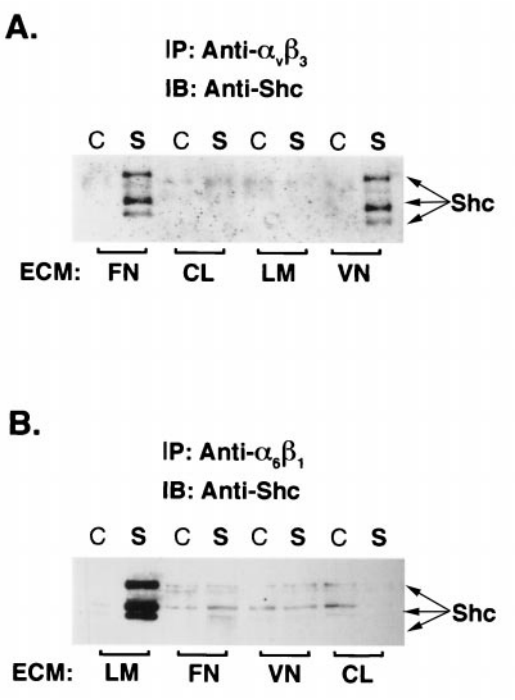

Fig. 2. Shear-induced integrin–Shc association is ECM-dependent. Glass slides were coated with FN (5 $\mu g/cm^2$), CL (6 $\mu g/cm^2$), LM (5 $\mu g/cm^2$), or VN (0.5 $\mu g/cm^2$). HUVECs were seeded on the slides in the absence of serum, and exposed to shear stress (12 dyn/cm^2) for 30 min or kept as static controls. (*A*) The cell lysates were subjected to immunoprecipitation (IP) with LM609 anti-$\alpha_v\beta_3$, followed by immunoblotting (IB) with anti-Shc pAb. Each pair of lanes represents static control (C) and sheared (S) samples of cells plated on the ECM indicated. $\alpha_v\beta_3$–Shc association is demonstrated by coimmunoprecipitation in the sheared, but not static, HUVECs on FN and VN. (*B*) $\alpha_6\beta_1$–Shc association is demonstrated by coimmunoprecipitation in the sheared HUVECs on LM only. Gels shown are representatives of three independent experiments.

induced mechanotransduction depends on the specific integrin–ECM interaction.

A Dynamic Integrin–Ligand Interaction Is Essential for Mechanotransduction. The constant remodeling of focal adhesions on the abluminal side of ECs (12) suggests that dynamic integrin–ligand connections may be essential for mechanotransduction. To test this hypothesis, HUVECs were plated on FN or VN. After waiting 2 h to allow the completion of cell spreading and adhesion formation, we blocked the unoccupied FN and VN molecules by adding the appropriate mAbs (19, 20) before the application of shear stress. The addition of these mAbs would prevent the formation of new integrin–ligand connections in response to shear stress without affecting the existing connections. Fig. 3*A* shows that shear stress caused $\alpha_v\beta_3$–Shc association in HUVECs on FN in the absence of any antibody (lane 2), in the presence of the nonblocking mAb 11E5 (lane 4), or in the presence of 3B8 (lane 6), which blocks the $\alpha_5\beta_1$-binding sites (but not the $\alpha_v\beta_3$-binding sites). In contrast, 16G3, which blocks both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ sites, attenuated the shear stress-induced $\alpha_v\beta_3$–Shc association (lane 8). Similarly, Fig. 3*B* shows that shear stress caused $\alpha_5\beta_1$–Shc association in HUVECs on FN either without mAb (lane 2) or with the nonblocking mAb 11E5 (lane 4), but not when treated with the $\alpha_5\beta_1$-blocking mAbs 3B8 or 16G3 (lanes 6 and 8). Fig. 4*C* shows that shear stress caused $\alpha_v\beta_3$–Shc association in HUVECs on VN when treated with the nonblocking mAb 443 (lane 2), but that this association was inhibited when treated with mAb 661, which occupies the available $\alpha_v\beta_3$-binding

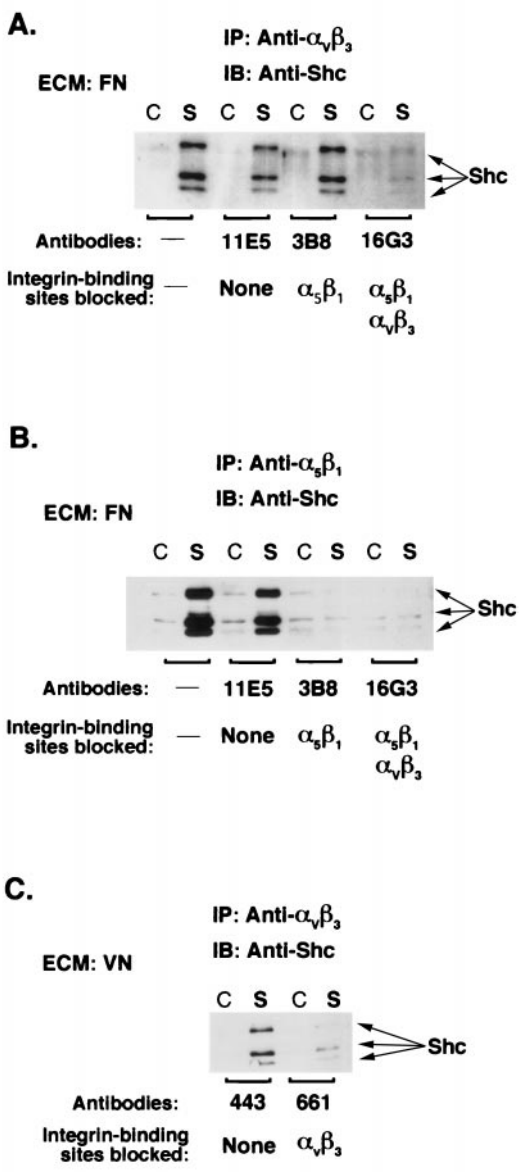

Fig. 3. Dynamic interaction with matrix proteins is essential for shear-induced $\alpha_v\beta_3$–Shc association. (*A*) HUVECs were allowed to adhere to FN-coated slides for 2 h in the absence of serum and then subjected to the following treatments: Lanes 1 and 2, untreated samples; lanes 3 and 4, incubated with 20 μg of the nonblocking 11E5; lanes 5 and 6, incubated with 3B8, which blocks the $\alpha_5\beta_1$, but not the $\alpha_v\beta_3$, binding sites; lanes 7 and 8, incubated with 16G3, which blocks both the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ binding sites. HUVECs were either sheared (S) or kept as static control (C). The cell lysates were subjected to IP with LM609, and the immunoprecipitated $\alpha_v\beta_3$ was subjected to IB with an anti-Shc pAb. (*B*) Experiments were similar to Fig. 3*A*, except that anti-$\alpha_5\beta_1$ mAb 1950 was used for IP. (*C*) Experiments were similar to Fig. 3*A*, except that HUVECs were seeded on VN-coated slides and incubated with either the nonblocking antibody 443 (lanes 1 and 2) or antibody 661 (lanes 3 and 4), which blocks the $\alpha_v\beta_3$ binding sites of VN. Gels are representative of three separate experiments.

sites on VN (lane 4). These results provide evidence in support of our hypothesis that the activation of mechano-sensitive integrins requires the formation of dynamic new connections with ECM ligands.

Plating cells on either antibodies or ECM ligands induces similar events of cell spreading and signaling; but integrin–antibody binding is generally insensitive to the affinity modulation that regulates integrin–ligand binding (21, 22). Hence,

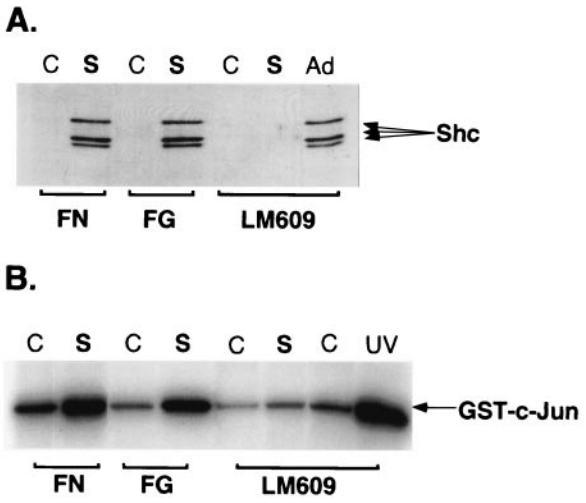

Fig. 4. Shear-induced $\alpha_v\beta_3$–Shc association and JNK activation were inhibited when HUVECs were seeded on slides coated with LM609 anti-$\alpha_v\beta_3$ mAb. The slides for lanes 1 and 2 were coated with FN (5 $\mu g/cm^2$) and those for lanes 3 and 4 were coated with FG (5 $\mu g/cm^2$). The slides for the remaining lanes were first coated with an anti-mouse IgG followed by LM609. HUVECs were seeded on the slides and allowed to adhere for 2 h (lanes 1–6) or 30 min (lane 7). (*A*) The seeded HUVECs were kept under static condition (C) or subjected to shear stress (S). Lane 7 (Ad) served as a positive adhesion control to show that $\alpha_v\beta_3$–Shc association occurred in static HUVECs at 30 min after onset of adhesion, but not at 2 h (lane 6). The cell lysates were subjected to IP with LM609, followed by IB with an anti-Shc pAb. (*B*) HUVECs were kept as static controls (C), subjected to shear stress (S), or exposed to UV irradiation of 80 J/m^2 for 5 min (UV, lane 8). The cells were lysed and JNK kinase activity was assayed by using GST-c-Jun as the substrate. Gels are representative of three separate experiments.

to further test our hypothesis, studies were conducted on HUVECs plated on slides coated with antibodies rather than the ECM ligands. Plating of HUVECs on the anti-$\alpha_v\beta_3$ mAb LM609 initially triggered the association of $\alpha_v\beta_3$ with Shc at 30 min (Fig. 4*A*, lane 7), but this association diminished at later times (lane 5), as in the case of HUVECs on FN or FG, which are both ECM ligands for $\alpha_v\beta_3$ (lanes 1 and 3). The application of shear stress induced $\alpha_v\beta_3$–Shc association in HUVECs plated on FN or FG (lanes 2 and 4), but not in HUVECs plated on the LM609 mAb (lane 6). These results indicate that shear stress can activate the integrins when they are sufficiently mobile to make dynamic new connections, but not when they are firmly attached to the mAb.

In addition to using $\alpha_v\beta_3$–Shc association as readout, we also performed experiments to examine the role of dynamic integrin-ligand connections in the shear-induced JNK activation, which is downstream to $\alpha_v\beta_3$–Shc association (5, 13). Fig. 4*B* shows that the shear stress-activation of JNK was prominent in HUVECs on FN or FG (lanes 2 and 4), but minimal in HUVECs on LM609 (lane 6). UV irradiation did induce JNK activation in HUVECs on LM609 (Fig. 4*B*, lane 8), indicating that JNK activity was still inducible in cells plated on the antibody. These results are in agreement with those on integrin–Shc association (Fig. 4*A*).

Discussion

Our results provide evidence for the following conclusions. First, the mechanotransduction in ECs in response to shear stress requires the activation of integrins by their specific ligands. More importantly, we showed that this activation requires the dynamic formation of new integrin–ligand connections. The evidence for the first conclusion on the specificity of the shear-induced integrin–ligand association is based on two types of findings: (*i*) Shear stress increases integrin binding to specific ligands, as demonstrated by using mAbs that recognize only the ligand-binding conformation of integrins; (*ii*) shear stress increases integrin–Shc association only in ECs plated on the appropriate ECM ligand.

The requirement of the dynamic formation of new integrin–ligand connections in the shear-induced mechanotransduction is a significant finding. This conclusion is based on the results of two types of experiments aimed at preventing the formation of such new connections, namely, (*i*) blocking the unoccupied ECM ligands with mAbs and (*ii*) plating the ECs on antibodies instead of ligands. Both procedures inhibited the shear-induced intracellular signaling, including integrin–Shc association and JNK activation.

The report that focal adhesions on the abluminal side of ECs undergo dynamic, local reorientation without a noticeable change in the total attachment area (12) is in concert with the hypothesis that the integrin connections to the substratum undergo dynamic breakage and new formation. Cell adhesion and spreading on matrix induces integrin activation and intracellular signaling as a result of integrin–ligand interaction. With time, this adhesion-induced integrin activation subsides and these static cells reach a new equilibrium. The application of shear stress perturbs this equilibrium and initiates integrin–ligand interaction and integrin activation to balance the shear force acting on the cell until another new equilibrium is attained. Thus, the dynamic disengagement and formation of connections between integrins and their ligands in the substratum are features common to cellular responses to adhesion and to shear stress.

A recent study (23) indicated that vascular endothelial growth factor receptor (VEGF-R) may be involved in integrin–Shc association. To test whether VEGF-R plays a role in the shear-induced integrin–Shc association, we probed the integrin–Shc immunocomplex with anti-VEGF-R (flk-1) and anti-PDGF-Rα. Neither receptor tyrosine kinase was present in the integrin–Shc immunocomplex (data not shown). Therefore, it is unlikely that the shear-induced integrin–Shc association is mediated through RTKs such as VEGF-R and PDGF-R.

In summary, our results have shown the critical role played by integrins, through their specific and dynamic connections with ECM ligands, in transmitting the mechanically initiated signals into the cell to trigger the intracellular signal transduction pathways for the modulation of gene expression and cellular functions.

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