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MOLECULAR BASIS OF MECHANICAL MODULATION OF ENDOTHELIAL CELL MIGRATION

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1. ABSTRACT

Vascular endothelial cells (ECs) play important roles in the regulation of vascular functions. Loss of endothelial integrity can lead to vascular diseases such as stenosis resulting from atherosclerosis. The migration of ECs into wounded area in the vessel wall is required for the restoration of its integrity and functions. EC migration results from a balance of externally applied forces (e.g. shear stress), intracellular forces (e.g., those generated by contractile and cytoskeletal proteins), adhesion force between ECs and extracellular matrix (ECM) proteins, and the force of EC-EC coupling through junction proteins. Shear stress modulates EC migration through the regulation of multiple signaling pathways, gene expression, and the reorganization of cytoskeleton, focal adhesion sites, and cell junctions. Investigations of EC migration under shearing can provide valuable knowledge on vascular remodeling process under physiological and pathological conditions.

2. INTRODUCTION

Cell migration is essential in physiological and pathological processes such as embryo development, atherogenesis, wound healing, tumor invasion and inflammatory responses. ECs play an important role in vascular remodeling during angiogenesis and post-angioplasty re-endothelialization. Although the migration of various cell types may involve different mechanisms, some essential characteristics are shared by most cells, including ECs.

Cell migration is a mechanically integrated molecular process. The physical mechanisms involved in the migration of adherent cells over a planar substratum consists of a sequence of steps, which include: (a) the extension of the leading edge and its adhesion to the substratum, (b) the forward translocation of the cell body, and (c) the release of adhesions at the rear (1-3). Each of these steps involves the exertion of deforming forces on the cell itself to change cell shape and the generation of traction forces on the substratum to provide a foot-hold for the cell to propel the cell forward (1). The molecular mechanisms for force generation in these steps may be different and yet interdependent.

Hemodynamic forces regulate the structure and function of the blood vessel wall (see (4, 5) for review). ECs, being located at the interface between the circulating blood and the blood vessel, are exposed to shear stress, the tangential component of the hemodynamic stresses acting on the vessel wall. Shear stress can regulate many EC functions, including migration, which is an important process in vascular remodeling in physiological states and pathological conditions such as atherosclerosis (see (6, 7) for review) and wound repair (8).

This review is focused on the molecular mechanisms by which mechanical shear stress modulates the migration of ECs. We will discuss the molecular mechanisms that regulate cell migration, the forces generated by migrating cells, and the effects of shear stress on signal transduction, force balance, and gene expression in regulating EC migration.

3. MOLECULAR MECHANISMS OF CELL MIGRATION

3.1. Roles of Integrin-ECM Interactions in Cell Migration

Integrins are a family of more than 20 transmembrane heterodimers composed of alpha and beta subunits with non-covalent association (9). The extracellular domain of integrin binds to specific ligands, including ECM proteins (e.g., fibronectin, vitronectin, and collagen). The cytoplasmic domain interacts with cytoskeletal proteins (e.g., paxillin, talin, vinculin, and actin) and signaling molecules in the focal adhesion sites (e.g., focal adhesion kinase (FAK) and c-Src) (10, 11). Thus, integrins can function as both adhesion receptors and signal transducers to regulate cytoskeletal organization, kinase activities, and gene expression. The unique structural features of integrins enable them to mediate “inside-out” signaling, in which intracellular signals modulate the affinity of integrins for extracellular ligands, and "outside-in" signaling, in which extracellular stimuli induce the intracellular signaling cascade via integrin activation (11) to modulate the kinases in focal adhesion sites (e.g., FAK) and the cytoskeletal proteins (9, 10, 12), as well as the Rho family small GTPases (13, 14), thus affecting cell migration. Different integrins may differentially regulate the signal transduction involved in cell migration. For example, the activation of alpha6/beta1 integrin induces the tyrosine phosphorylation of FAK, but not SHC (15), and integrins beta1 and beta3 preferentially regulate the activities of Rac and Rho, respectively (16). Besides the chemical characteristics of integrins, the strength of integrin-ECM adhesive interaction also plays an important role in regulating cell migration (see Section 4.2).

3.2. Roles of Cytoskeleton in Cell Migration

Cytoskeleton provides the force and the framework for shape changes, migration, and structural organization of the cell. Actin polymerization is essential for lamellipodial and filopodial protrusions (17). The assembly and disassembly of actin can be modulated by its binding proteins such as actin-related protein (Arp) 2/3 complex, cofilin, gelsolin and profilin. The Arp2/3 complex, which is a nucleating complex for actin polymerization, is concentrated at the leading edge of migrating cells to initiate the nucleation of new actin filaments and the elongation and branching of actin structure by binding to the pre-existing filaments (18-22). Actin filaments also bind to the cytoplasmic domain of integrins through a protein complex that involves talin, vinculin, alpha-actinin, and filamin. Such a complex not only couples integrins to the actomyosin contractile apparatus, but also sequesters the signaling molecules involved in integrin signaling (e.g., FAK, Shc and Crk) (23).

During cell migration, there is a feedback interaction between actin and microtubules (MTs). The dynamics of F-actin creates a polarized gradient of MT instability, and MTs can modulate the actin cytoskeleton to control the direction of cell migration through specific Rho family GTPases (24). MT growth at the cell front may activate Rac1 to induce lamellipodium formation, while inhibition of MT growth decreases lamellipodial protrusion, cell spreading, and cell migration (25-31). MT disruption increases the assembly of actin filaments and contractility in a Rho-dependent manner (29, 32-35), suggesting that MT shortening at the rear of the cell may activate Rho to induce actin contraction and tail detachment. There is also evidence that the MT-plus ends may cause disassembly of focal adhesions at the rear (36, 37).

3.3. Roles of FAK and its Associated Signaling Pathways in Cell Migration

FAK is a cytoplasmic tyrosine kinase that co-localizes with integrins in focal contacts. Integrin binding to ECM induces FAK activation and tyrosine phosphorylation in a variety of cell types (11) to modulate cell motility. Inhibition of FAK by a dominant negative FAK construct results in a decrease in EC motility (38). FAK homozygous knockout in mice is embryonic-lethal, and the cells cultured from these FAK-/- embryos display decreased motility in vitro (39). FAK-null cells lose the ability of mechanosensing of microneedle pushing of ECM and have decreased persistence in their migration (40, 41). In contrast, over-expression of FAK results in increases in cell motility (12) and persistence of cell migration (40). The activation and autophosphorylation of FAK at Y397 upon cell adhesion allows FAK to associate with two other intracellular signaling molecules Src and phosphoinositide-3 kinase (PI3K), via their SH2 domains (12). FAK/Src association triggers downstream signaling events such as phosphorylation of CAS and mitogen-activated protein kinases to mediate cell adhesion and migration (12, 42-46). The rapid and transient activation of ERK2 and JNK1 by laminar shear stress is attenuated by the dominant negative mutant FAK (Y397F) (47), indicating that FAK signaling is critical in the shear stress-induced activation of these MAPK signaling pathways. The phosphorylation of CAS by FAK/Src complex requires binding of CAS to Pro712/715 of FAK and Src to Y397 of FAK (42, 48). Phosphorylation of CAS has been shown to generate multiple binding sites for SH2-domain-containing molecules such as Crk and Nck (48-50). FAK/Cas/Crk pathway can activate Rac through DOCK180, a Rac GTP exchanging factors (GEF), to promote cell migration (51). In parallel to the FAK/Cas pathway, the association of FAK with PI3K has been proposed to activate the lipid kinase activity of PI3K (52), although the specific downstream targets of FAK/PI3K association have not been identified.

3.4. Roles of Rho family Small GTPases in Cell Migration

The Rho family small GTPases Rho, Rac and Cdc42 have specific actions in regulating actin-based cytoskeleton and cell migration (53). While Rac and Cdc42 regulate the formation of membrane ruffles and filopodia, respectively, Rho has been shown to stimulate cell contractility and the formation of actin stress fibers and focal adhesions (54, 55). The activity of Rho family GTPases can be regulated by GEFs, GTPases-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (53). For example, PI3K could stimulate Rac activity through its lipid product phosphatidylinositol 3,4,5-trisphosphate that binds to the PH domain of GEFs such as Tiam1 and Vav (56-59). Recent studies have shown that cytoplasmic p120 catenin, when not associated with cadherins, can form complex with GEF Vav2 or RhoA-GDP to result in increases of Cdc42/Rac activities and a decrease of RhoA activity, respectively (60-62), thus modulating cell migration.

Rho family GTPases modulate cell migration by regulating the remodeling of focal adhesions and actin cytoskeleton. Rho stimulates the formation of focal adhesions (63), and Rac recruits high-affinity integrin alphaV/beta3 to lamellipodia in endothelial migration (64). As a part of outside-in signaling, integrins can regulate the activities of Cdc42, Rac and Rho (29, 65-67). Fibroblasts plated on fibronectin exhibit an early activation of Cdc42 and Rac, and a delayed activation of Rho (29, 66), suggesting that Cdc42/Rac and Rho may be regulated through different mechanisms. FAK phosphorylation leads to an increase of Rac activity (51), but a decrease of Rho activity (68). Syndecan-4, a cell surface proteoglycan, enhances Rho activation and focal adhesion formation (69).

The effects of Rho family GTPases on cell migration are mediated by multiple downstream signaling molecules that regulate actin dynamics (53). Rho controls the actin cytoskeleton through the downstream effectors p160ROCK and mDia proteins (70). P160ROCK, a Rho-associated protein kinase, regulates both actin filament assembly and actinomyosin contractility (53, 71, 72). P160ROCK promotes F-actin accumulation by activating LIM-kinases (LIMK), which phosphorylate and inactivate the actin filament depolymerizing factor cofilin (73-75). P160ROCK also enhances the activity of myosin light chain (MLC) kinase in SMCs through Ca^{2+} sensitization (76, 77), and increases actinomyosin contractility and stress fiber formation (78). mDia proteins, in concert with p160ROCK, collaboratively induce organized stress fibers (70, 79). The Rho-mediated actin dynamics plays a major role in generating the pulling force on ECM during cell migration (80, 81).

At the leading edge of the cell, Rac can activate Wiskott-Aldrich syndrome protein (WASP)-family proteins (e.g., SCAR1, WAVE) to recruit the Arp2/3 complex in the lamellipodia and induce actin nucleation (82-84). In addition, Cdc42 activates N-WASP and profilin for filopodia formation (85-87). Knocking out WAVE2 has been shown to impair lamellipodia formation and EC migration in angiogenesis (88). Rac and Cdc42 also decrease the activity of actin-severing proteins such as gelsolin and cofilin. Rac activation triggers uncoupling of actin and gelsolin to promote actin polymerization (89), and knocking out gelsolin in fibroblasts decreases membrane ruffling (but not filopodia formation) and cell motility (90). Activation of Rac and Cdc42 also activates PAK1 and LIMK, and increases cofilin phosphorylation, thus inhibiting actin depolymerization (73, 74, 91). These results suggest that cofilin is a common target of Cdc42, Rac and Rho in the regulation of actin polymerization.

3.5. Roles of Cell Junctions in Cell Migration

During EC migration, cell junctions undergo dissociation and reorganization to allow EC movement. The cell-cell junctions in the vascular endothelium primarily consist of adherens junctions (AJs), and there are also gap junctions (GJs) and tight junctions (TJs) (92-94). The organization of cell-cell junctions requires adhesion molecules such as cadherins (AJ), occludin (TJ), and connexins (GJ). These adhesion molecules are connected to actin filaments via linker proteins such as catenins (AJ) and ZO-1 (TJ). VE-cadherin belongs to the calcium-dependent cadherin family and is the major and endothelium-specific cadherin at AJs in ECs (94). Inhibition of homotypic interactions of VE-cadherins by using monoclonal antibodies to their extracellular domain of VE-cadherin disrupts the organization of EC junctions (95-97). VE-cadherin has an extracellular domain that mediates the cell-cell adhesion, a transmembrane region, and a short cytoplasmic domain that interacts with beta-catenin, plakoglobin (gamma-catenin), and p120 catenin. Beta-catenin and plakoglobin, but not p120, bind α-catenin, which mediates the linkage of the cadherin-catenin complex to the actin cytoskeleton (93, 94). p120 binds to the juxtamembrane region of the cytoplasmic domain and modulates cadherin clustering independent of beta-catenin or plakoglobin (94, 98). Increases of tyrosine phosphorylation of beta-catenin, plakoglobin, p120 and VE-cadherin has been shown to be associated with a decrease in cell-cell adhesion (99-104). The dynamics of cell junctions can be controlled by signaling events, including those mediated by integrin signaling. Integrins (e.g.alphaV/beta3, alpha5/beta1, and alpha2/beta1), FAK, Shc, and vinculin are located at cell junctions, and Src can mediate the tyrosine phosphorylation of p120 and beta-catenin to destabilize AJs (99, 105-108).

In addition to strengthening cell-cell adhesion, AJ complexes of cadherin-catenins also regulate intracellular signaling, e.g., by controlling the cytoplasmic levels of catenins. When released into the cytosol, β-catenin and plakoglobin can translocate into the nucleus and modulate gene expression (109). It has been shown that cytoplasmic p120 catenin, when not associated with cadherins, can form complex with GEF Vav2 or RhoA-GDP, resulting in an increase of

Cdc42/Rac activity and a decrease of RhoA activity, respectively (60, 61), thus modulating cell migration.

4. FORCES GENERATED BY MIGRATING CELLS

4.1. Protrusion Forces for Membrane Extension

As mentioned above, the initial step of cell migration is the extension of the cell membrane. It is generally agreed that this extension step is powered by actin polymerization at the leading edge, where the protrusion force generated by actin assembly extends the plasma membrane against its mechanical resistance and the compression force imposed by the environment (1, 3, 110). The role of actin in membrane extension is supported by the finding that the polymerization of actin within a lipid vesicle can dramatically deform the lipid bilayer (111-113). In addition, the rate of new actin polymerization can be correlated with that of membrane extension in migrating fibroblasts (114). The available evidence indicates that myosin motors are not involved (or not necessary) in membrane extension. Thus, mutant cell lines defective in certain types of myosin molecules exhibit some defects in locomotion, but not in membrane extension (115-118).

In contrast to the large number of techniques devised for measuring forces generated by individual motor proteins (such as myosin and kinesin), there is little quantitative experimental work on forces produced by an individual growing actin filament (110). It is assumed that the chemical energy liberated from the polymerization and elongation of actin filaments is converted into a pushing mechanical force against the opposing pressure exerted by the membrane, and hence the protrusion force could be deduced from the thermodynamics of protein polymerization (1, 110). There is a considerable amount of literature on this topic, and theoretical analysis suggests that regional actin polymerization alone is an adequate energy source for membrane extension (1, 2, 110).

4.2. Cell-Substratum Adhesion and De-adhesion Forces

Migrating cells have a dynamic adhesive interaction with the substratum. As a migrating cell forms new adhesions at the leading edge and releases those at the rear, the main cell body maintains a certain degree of adhesion with the substratum to withhold dispersive forces; for example, the adhesion of ECs to the blood vessel wall needs to be strong enough to resist the constant fluid shearing that results from blood flow. The strength of cell-substratum adhesion is a major factor determining whether a cell can migrate on a given substratum or not, as well as the speed when migration does occur (2, 119, 120). A weak adhesion force is not sufficient to stabilize new adhesion contacts between the protruding lamellipodium and the substratum, whereas a strong adhesion force may impede the release of adhesion contacts at the rear; as a result, the migration speed is maximal at an intermediate level of adhesion strength. Such a relation was predicted in theory (121) and has been found in several cell-substratum systems (120, 122).

In most cell types, adhesion is primarily mediated by cell surface receptors such as integrins. The adhesion strength of the reversible binding of integrins to their ECM ligands is affected by several factors, including ligand concentration, receptor density, and receptor-ligand binding affinity, as well as the level of cytoskeletal-association proteins at receptor-cytoskeleton linkages (2, 119, 123). Adhesion forces can be estimated by subjecting adherent cells to mechanical dispersions, such as centrifugation or flow; detachment occurs when the dispersive force (centrifugal or fluid shearing force) exceeds the adhesion force. These methods are simple, convenient, and commonly employed for estimating adhesion forces. More sophisticated adhesion assays have been developed in which atomic force microscopy or optical tweezers are used to determine the adhesion strength of individual cells to substrata with detailed spatial information such as comparison between the leading edge and the tail of a cell (124-126). Microfluidic devices with high-throughput have been developed for studying cell adhesion and cell mechanics in response to shear stress and biochemical stimulation (127).

The de-adhesion during cell migration may be mediated by a myosin II-driven contractile mechanism (mechanical release) and/or by enzymatic or chemical factors (1, 3). These two mechanisms may work in concert: contraction may supply the force required to break the bonds, while biochemical mechanisms may determine whether a linkage is preferentially broken (123). Experimental evidence of a role for myosin II-driven contraction includes: (a) myosin II has been found to localize preferentially at the rear of migrating fibroblasts (128), and (b) amoebae deficient in myosin II have a decreased motility, which is exaggerated on substrata with greater adhesiveness that makes rear-detachment the rate-limiting step (117, 129).

4.3. Traction Forces Exerted by the Migrating Cell on the Substratum

Cell migration involves cell-substratum interactions, which can be described by a vector field of traction forces. In effecting a net migration, an adherent cell must push or pull against the substratum while crawling over it, and such physical interactions result in the traction force generated by the cell on the substratum (1, 3). There is evidence suggesting that traction forces result from the contraction of the actin-myosin cytoskeleton and are transmitted to the substratum through cell-substratum contact sites. The substratum, in turn, exerts a counter force on the cell via the same transmission sites, propelling the translocation of the cell body (1-3, 130). Thus, the traction force is closely associated with the intracellular contractile force and cell-substratum adhesion structures.

In recent years, several methods have been developed to characterize cellular traction forces by

measuring the deformation of the elastic substratum. This approach was first developed by Harris and colleagues, using silicone rubber film as the cell culture substratum, which wrinkles in response to cellular traction forces (131). This method is qualitative and has limited spatial resolution due to the complexity of the relationship between wrinkles and forces (130, 132). Modified methods include the uses of nonwrinkling silicone polymers embedded with particles (133) and silicone elastomers imprinted with micropatterns of dots or lines generated by lithography (134). Particles and micropatterned dots/lines are used as markers of deformation, allowing the construction of detailed vectorial maps of traction forces (134-136).

Wang and Dembo have used a flexible substratum made of acrylamide and bis-acrylamide embedded with fluorescent latex beads to determine traction forces (137, 138). The polyacrylamide substratum has a more controllable and optimum elastic property and, when covalently coated with ECM proteins, provides a more physiological environment for cell adhesion than the silicone sheets. The flexibility of the material can be easily adjusted to match the level of traction forces for specific cell types (130, 132, 138). With the aid of numerical algorithms for solving inverse problems (130, 137, 139), traction forces can be estimated from the mechanical properties of the elastic substratum and the bead displacements (139). An optical flow algorithm is used to obtain bead displacement maps, which are then converted into maps of traction forces. This approach, called the "traction force microscopy", produces traction force maps with higher spatial and temporal resolution than the silicone-based methods (140). The application of traction force microscopy to study the traction forces generated by migrating fibroblasts and endothelial cells has shown that tractions are organized in a radial pattern, with the backward and forward tractions located in the advancing front and trailing back, respectively (138). The forces are greatest near the leading edge of the cell. The forces in the posterior region, which are stronger than those in the central/nuclear region of the cell, reflect the passive resistance to the active pulling forces in the front generated by actin-myosin contractions (138, 141). Using this method and immunofluorescent labeling of focal adhesions, it has been found in migrating cells that nascent, small adhesion structures (which form preferentially at the front) exert stronger traction forces than mature, large adhesion structures under the central/nucleus region (142). In stationary non-motile cells, however, studies using micropatterned silicone elastomers show that the traction force increases with the size of the focal adhesions (134). Thus, the relationship between the focal adhesion size and the traction force may vary with experimental conditions, including cell type, cell motility, and the methods used to determine traction force.

The above methods use continuous, flexible substrata in which strain (or deformation) propagates across the substratum and decreases as a function of distance from the source of stress. Such approaches require sophisticated computational analysis for the derivation of traction forces (130). Several investigators have developed microfabrication-based strategies that use micrometer-sized flexible cantilevers or posts for probing cellular traction forces. In these strategies, the adherent cells exert forces on flexible cantilevers to cause their bending. The resulting strains are confined to individual cantilevers, and hence forces can be easily calculated from the displacement and spring constant of the cantilevers (143, 144). These methods circumvent the computational problem and provide well-defined geometry, though the surface topology of the cantilevers may exert some effects on cell adhesion and migration through contact guidance (130).

4.4. Spatial Asymmetry of Force Distribution

A mechanical asymmetry in the cell-substratum interactions and the maintenance of overall directionality are critical aspects in cell movement (2, 145). Because the Arp2/3 complex is concentrated at the leading edge, the actin-driven protrusion force is selectively generated at the advancing front, but not the tail, of the cell (110, 146-149). The polarity of a migrating cell that forms new adhesion contacts with the substratum preferentially at the advancing front is attributed to the spatial distribution of cell surface receptors and spatial variations of receptor-ligand binding affinity and actin polymerization (2, 3, 119). The resulting adhesive imbalance between the front and rear cell-substratum contact sites leads to a corresponding spatial variation in traction forces. As the traction force integrated over the area of the leading lamellipodia is greater than that in the smaller tail region, the front invariably wins the "tug-of-war" over the rear of the cell, thus giving rise to a net forward movement of the cell and detachment at the rear.

5. EFFECTS OF SHEAR STRESS ON SIGNAL TRANSDUCTION AND FORCE BALANCE IN ENDOTHELIAL CELL MIGRATION

5.1. Effects of Shear Stress on Cell Migration/Wound Healing

EC migration plays important roles in wound healing, angiogenesis and embryonic vasculogenesis. Under pathological conditions such as atherosclerosis and denudation injury following angioplasty and bypass grafting, the loss of endothelial integrity leads to EC dysfunction and thrombosis (150, 151). The recovery of endothelial integrity and vascular wall homeostasis requires the healing process that involves EC migration at the wound edges. At the microcirculatory level, EC migration is critical for vascular assembly in native and engineered tissues to promote the healing of damaged tissue and the survival of tissue-engineered vascular grafts. Since ECs are constantly subjected to fluid shear stress due to blood flow, it is important to understand EC migration under flow condition.

It is well known that EC migration can be modulated by environmental factors through different

mechanisms such as chemotaxis (152-154) and haptotaxis (155), but the effect of shear stress on EC migration has been characterized only recently. Work by Ando et al. has demonstrated that low levels of shear stresses (0.3-1.7 dyn/cm^2, in a cone-and-plate setup) enhance EC wound closure, and that ECs at wounding edge downstream to the flow migrate against flow direction into the wounding area with even greater degrees of cell migration and proliferation (156). At high shear stresses (>10 dyn/cm^2), however, ECs extend lamellipodia and migrate at a faster rate in the flow direction (8, 157-160). Additional studies are needed to compare the effects of high and low shear stresses on EC migration. When an EC monolayer is wounded along the direction of the flow, shear stresses between 3 and 20 dyn/cm^2 increase wound closure rate (161), suggesting that shear stress may promote cell migration through mechanisms other than directional lamellipodial protrusion. For example, shear stress can reduce cell-cell adhesion, thus facilitating the migration of ECs into the wound area (159, 162, 163). Shear stress has been shown to cause cell junction dissociation in plakoglobin-depleted ECs (164) and to induce the remodeling of subendothelial matrix proteins (165, 166). These shear-induced remodeling of cell junctions and ECM may facilitate cell migration. In addition to the magnitude of shear stress, different flow patterns have differential effects on EC migration. Tardy et al. have shown that ECs tended to migrate away from the area with high shear stress gradient (167). Hsu et al. have shown that laminar flow enhances EC migration to a greater extent than disturbed flow (159).

5.2. Effects of Shear Stress on Signaling Events in Cell Migration

The studies on EC monolayers and subconfluent cells have provided insights into the molecular mechanisms of shear stress-induced EC migration. *In vitro* studies with flow chambers have shown that shear stress induces the remodeling of the EC monolayer, with cell alignment in the flow direction and an increase of stress fibers (168-171). The alignment of cells and actin stress fibers in EC monolayers involves signal transduction through intracellular calcium release, tyrosine kinases, MT integrity, and signaling mediated by Rho/p160ROCK, Rac, Cdc42, p38MAPK and PAK (172-178). However, non-confluent ECs migrating in the flow direction have less stress fibers with no obvious alignment in comparison to ECs in confluent monolayer (160). These differences may be attributed to the relative lack of constraint for the non-confluent ECs to migrate, in contrast to the constraints in EC monolayers due to the cell-cell interactions though junctions. By monitoring green fluorescence protein (GFP)-tagged actin in ECs, we and others have shown that shear stress induces lamellipodial protrusion and actin polymerization in the flow direction within minutes, and increases actin dynamics during cell migration (160, 179). Concurrent to the remodeling of actin structure, shear stress induces remodeling of MTs and intermediate filaments (180, 181). The MT dynamics under flow is required for Rac activation and polarized EC migration (182). The microtubule organization center reorients to the downstream side of the nucleus relative to flow direction, and this process is regulated by Cdc42 (183).

Concomitant with the cytoskeleton remodeling under flow, shear stress induces physical and chemical changes at focal adhesions. Shear stress increases the size and decreases the number of focal adhesions in both non-confluent ECs and confluent EC monolayers (160, 184). In contrast to the relatively stable focal adhesions in confluent ECs, the shear-induced clustering of focal adhesions in non-confluent ECs is transient and is followed by the disassembly of focal adhesions to allow cell migration. Spatially, shear stress induces a polarized distribution of alphaV/beta3 integrin at the upstream end of the ECs in a monolayer (185), while it induces polarized focal adhesions at the downstream end of the non-confluent ECs (160).

By using integrin-Shc association as a readout, Chen et al. have shown that shear stress induces a sustained activation of integrins over several hours (186), and this requires the dynamic binding of matrix proteins with their specific integrin receptors such as alpha5/beta1 and alphaV/beta3 (187). Using an antibody that recognizes activated alphaV/beta3, Tzima et al. have shown that shear stress can directly increase integrin affinity for matrix binding (176). In addition to posttranslational activation of integrins, shear stress increases the expression of integrin alpha5/beta1 to enhance cell migration (188). Besides integrins, cell surface heparan sulfate proteoglycans (HSPG) can also mediate EC adhesion and migration. Disruption of HSPG decreases EC adhesion and enhances EC migration speed, but blocks the directional migration of ECs under flow, suggesting that HSPG may play a role in sensing the direction of shear stress (189).

Many integrin-mediated signaling pathways are activated by shear stress. Shear stress transiently activates FAK, Src, Shc, PI3K, p130Cas (CAS) and MAPK in EC monolayers (47, 186, 190-194). Inhibition of Shc, PI3K and ERK has been shown to decrease EC migration under flow (188). Using GFP-FAK to monitor the subcellular molecular dynamics of focal adhesions under flow, we have hound that shear stress causes new focal adhesions to form at the leading edge of migrating ECs in the flow direction following lamellipodial protrusion (160). Shear stress also enhances the disassembly of pre-existing focal adhesions. The number of focal adhesions decreases significantly in ECs migrating under flow.

Rho family GTPases play important roles in the remodeling of actin cytoskeleton and EC migrations in response to shear stress. Direct assay of Rho GTPase activity in confluent bovine aortic ECs (BAECs) has demonstrated that shear stress (~12 dyn/cm^2) transiently activates Cdc42 and Rac and that it induces a transient decrease followed by an increase of Rho activity (176, 178, 183, 195). We have found that shear stress transiently increases the activities of Cdc42, Rac (Shiu,

Li, and Chien unpublished data) and Rho (196) in non-confluent BAECs. A recent study has also shown that shear stress (~3 dyn/cm^2) transiently increases the activities of Cdc42, Rac and Rho in subconfluent human umbilical vein ECs (178). It has been shown that cell junctions can modulate Rho activity (61, 197). By using fluorescence resonance energy transfer assay, Tzima et al. have shown that the shear stress-induced cdc42 and Rac activities polarize in the flow direction (183, 195). The polarization and migration speed of ECs are regulated by Rho GTPases. Rac and Rho are required for polarized migration under flow (178, 182). While the inhibition of Rac and Cdc42 decreases migration speed under flow (178, 182), inhibition of Rho has been found to either decrease (159) or increase EC migration under flow (178). The discrepancy in results on Rho GTPase may depend on experimental conditions such as the magnitude of shear stress, the degree of cell confluency, and the method of measurement.

5.3. Effects of Shear Stress on Forces Generated during Cell Migration

Although the shear-induced directional protrusion of lamellipodia in ECs has been studied extensively (47, 159, 160, 178, 184, 198), there is a lack of direct, quantitative measurement of the physical forces involved in this process under shearing. It is possible that shear stress may affect the direction and magnitude of actin-driven protrusion forces for membrane extension by regulating the localization and nucleating activity of the Arp2/3 complex (110). Sato and colleagues have found that ECs have a decreased membrane deformability after exposure to shear stress (199-201). It is possible that ECs migrating under flow generates larger protrusion forces to overcome the EC membrane resistance to deformation. The actin-driven pushing force for the barbed end of a growing actin filament has not yet been measured directly in any cell type, but can be deduced from thermodynamics of protein polymerization. Thus, total protrusion strength can be deduced if the number of growing F-actin ends at the lamellipodium margin is known (110). Using standing-wave fluorescence microscopy and image-based photometry to measure the thickness of the ultra-thin lamellipodium and its F-actin density in migrating fibroblasts, respectively, Abraham and colleagues have estimated the maximum pressure that actin assembly can generate at the leading edge to be in the order of 103 dyn/cm2 for a 10 micrometer-wide lamellipodium with a thickness of 100~200 nanometer (202). Thus far, such measurements have only been done in the fibroblasts, and there is no equivalent experiment on ECs.

There is a wealth of experimental data showing that shear stress induces extensive remodeling of focal adhesions with directional preference in ECs (159, 160, 178, 184, 198, 203), but little is known about the change, if any, in the EC-substratum adhesion strength under flow. The strength of individual integrin-mediated adhesion sites in fibroblasts has been found to increase upon local application of external forces (e.g., by optical traps or glass needles); such force-induced adhesion reinforcement is through a strengthening of receptor-cytoskeleton linkages (204, 205). It is likely that shear stress may have a similar effect on ECs. It is reasonable to expect that the shear-enhanced adhesion strength may protect ECs against dispersive forces from flow; indeed, our preliminary study has found an increased resistance to flow detachment (with high shear stresses) in ECs after pre-exposure to a physiological level of shearing forces (Shiu YT, Li S, and Chien S; unpublished results). Furthermore, a strengthened adhesion structure can sustain transmission of larger contractile forces to the substratum for propelling cell movement (121).

Using the traction force microscopy technique described above, our laboratory has found that migrating ECs generate stronger traction forces under flow and that this shear-induced response is abolished by a specific inhibitor of the Rho-associated kinase p160ROCK (196). Under flow, the traction force integrated over the entire cell body is greater in magnitude than under static conditions; the largest regional increases in traction magnitude tend to occur along the edge of the advancing front and the area of detachment also has a significant increase in traction force. These findings suggest that shear stress enhances the frontal forward-pulling force and posterior retraction force, thereby increasing EC migration speed under flow.

6. EFFECTS OF SHEAR STRESS ON ENDOTHELIAL CELL GENE EXPRESSION IN REGULATING CELL MIGRATION

One of the consequences of shear-induced signal transduction is the modulation of expression of molecules involved in cell migration. Shear stress has been shown to regulate expression of many genes in ECs (Reviewed by Davies (7); Chien et al. (206)). The conventional Northern blotting or RT-PCR techniques have been used to demonstrate that shear stress increases the transcripts of multiple growth factors, such as PDGF-A and B (207, 208), basic fibroblast growth factor (b-FGF) (209), heparin-binding epidermal growth factor-like growth factor (HBEGF) (210), and transforming growth factor-beta ?TGF-beta) (211). These growth factors may serve as potential chemotactic factors to regulate EC migration.

The recent advent of the DNA microarray technology has provided a more powerful and efficient approach to study the differential expression of a large number of genes simultaneously (see (212-214) for review). Several groups, including ours, have applied the DNA microarray technology for relatively comprehensive studies of the shear stress-regulation of gene expression profiles (215-219). Hundreds of genes have been identified to be modulated by shear stress using the high-throughput microarray technology, including many cytoskeleton- and ECM-related genes are relevant to the regulation of EC migration. The cytoskeleton-related genes that are up-regulated by shear stress include spectrin, myosin, beta–actin,

desmin, etc., and those down-regulated include actin capping protein, plakophillin 4, and alpha-tubulin etc. nonmuscle myosin heavy chain B, cytokeratin 4 and 2E, cytotactin, etc (215-217). In addition, shear stress decreases the expression of several focal adhesion-related genes, such as integrin alpha1, alpha7b, and beta3, zyxin related protein ZRP-1 (215, 216). Shear stress also significantly modulates several genes related to EC junctions and ECM, such as gap junction alpha4 protein, cadherin precursor (11 and 43), Connexin (40 and 43), laminin (alpha4 and beta1), collagen (4 and 11) (215-218). Shear stress causes the up-regulation of matrix metalloproteinase (MMP) 1 and 8, and down-regulation of MMP 7 and 14 (215, 218). In general, laminar shear stress caused more cytoskeleton/ECM/junction-related genes to be down-regulated than up-regulated. It has not been established how these gene regulations contribute to the remodeling of EC cytoskeleton during migration. The microarray results provide clues for selecting the candidate genes for future investigations of the potential mechanisms for shear-regulated EC migration.

7. CONCLUSIONS AND PERSPECTIVES

The ECs in the vascular tree are constantly subjected to shear stress. Shear stress regulates EC functions through multiple sensing mechanisms, leading to the activation of signaling networks, which in turn regulate gene expressions and functional responses, including migration. EC migration plays an important role in vascular remodeling in many physiological processes and pathological conditions. In contrast to the extensive literature on the modulation of EC migration by chemical factors, there is relatively little information on the effects of flow. Vascular ECs are constantly subjected to flow dynamics, which is known to affect EC migration. As summarized in this review, in vitro studies on cultured ECs in flow channels have allowed us to analyze the molecular mechanisms by which cells convert the mechanical inputs into biochemical events, which eventually lead to modulations of EC migration. Investigations on the forces exerted by the migrating EC on its underlying ECM and the neighboring cells during EC migration in the presence of external shear force can help us to understand the contributions of mechanical forces to the regulation of cell migration. EC migration can be viewed as a result of balance of forces, i.e. the external forces applied to the cell, intracellular forces (which are related to signaling, contractile proteins, cytoskeleton remodeling, and gene expression), intercellular forces (junction protein interactions), and forces due to cell-ECM interaction. The elucidation of the roles of these forces in EC migration, together with their molecular correlates, will generate new information on the mechanisms of EC migration at the molecular, cellular, and multi-cellular levels and provide important insights into the fundamental mechanisms of cell migration in physiological conditions and pathological states.

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