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Effects of Shear Stress on the Distribution of Kindlins in Endothelial Cells

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EFFECTS OF SHEAR STRESS ON THE DISTRIBUTION OF KINDLINS IN ENDOTHELIAL CELLS

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DEDICATION

To my family:

My Mother: Hetty B. Jones, PhD.

My Father: Matthew W. Jones, Sr.

My Siblings: Marko, Tsegaye, Matthew (Jr.), Alonzo, & Carolyne Jones

My Nieces and Nephews: Elias, Jaden, Elias, Alonya, Armani, & Asana Jones

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EFFECTS OF SHEAR STRESS ON THE DISTRIBUTION OF KINDLINS IN ENDOTHELIAL CELLS

Sidney V. Jones

ABSTRACT

Endothelial cells (ECs) are exposed to changes in shear stress under a variety of physiological and pathological circumstances and respond by changing their shape, gene expression and cytoskeletal arrangement. Integrins are central mediators of these mechanotransduction responses of ECs and integrin function is in turn controlled by interaction of their short cytoplasmic tails with cytoplasmic binding partners. Recent studies have demonstrated the importance of the kindlin family members, known cytoplasmic tail binding proteins, as regulators of integrin function. Our lab has previously shown that kindlin-2 (K2) and kindlin-3 (K3) are both expressed and functionally significant for integrin mediated cellular events in ECs.

The conversion of shear stress that realigns ECs in the direction of flow differentially affects the distribution of K2 and K3. This observation was made under shear stress rates of 3 or 10 dynes/cm^2 in cells adherent to fibronectin. K2 is present in

focal adhesion (FA) under static conditions and becomes redistributed into an increase number of FAs with decreased area and length in response to low shear stress. The change in distribution of K2 in response to flow may explain why its colocalization with VE-cadherin was increased in response to flow in a rate dependent manner. K3 changes from its primary and uniform cytosolic distribution to coalesce into discrete punctate patterns. The K3 punctate patterns were assessed for colocalization with clathrin coated vesicles and microtubules, which was significantly increased and decreased, respectively, under low flow. Shear stress induced kindlin-3 punctae formation corresponds to cleavage likely due to shear stress induced calpain activation. Inhibition of calpain prevents the effects of shear stress induced changes in K3 distribution. On ECs adherent to another matrix protein, laminin, kindlin-3 was observed to redistribute into focal adhesions in response to high flow, which is not fully understood.

This report confirms that kindlin-2 and kindlin-3 respond differently to shear stress in ECs and that the shear stress induced protein redistribution is dependent on the magnitude and duration of flow. It also demonstrates how the intensity of flow and extracellular matrix substratum of the EC may be important regulators of the fate of intracellular proteins involved in mechanotransduction. Consequently, this study provides insight into spatiotemporal effects of flow on endothelial cells and suggests that kindlins participate in the adaptation of ECs to mechanical cues.

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ABBREVIATIONS

10D-12H	10 DYNE/CM^2 – 12 HOURS
10D-6H	10 DYNE/CM^2 – 6 HOURS
3D-12H	3 DYNE/CM^2 – 12 HOURS
3D-6H	3 DYNE/CM^2 – 6 HOURS
EC	ENDOTHELIAL CELL
ECM	EXTRACELLULAR MATRIX
FA	FOCAL ADHESION
FN	FIBRONECTIN
GAPDH	GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE
GPCR	G PROTEIN COUPLED RECEPTOR
HUVEC	HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS
ICAM-1	INTRACELLULAR ADHESION MOLECULE-1
K2	KINDLIN-2
K3	KINDLIN-3
LM	LAMININ
NSC	NON-SHEARED CONTROL
PECAM-1	PLATELET ENDOTHELIAL CELL ADHESION MOLECULE-1
VCAM	VASCULAR CELL ADHESION MOLECULE-1
VE-CADHERIN	VASCULAR ENDOTHELIAL- CADHERIN
VEGFR2	VASCULAR ENDOTHELIAL GROWTH FACTOR

CHAPTER I

GENERAL INTRODUCTION

A. SHEAR STRESS.

1. Hemodynamic Properties of Shear Stress-Theoretical Considerations.

Blood flows down a pressure gradient because of its internal fluid dynamics. As a result, its movement is not self-perpetuating and pressure is required for blood to maintain its momentum (1). In the cardiovascular system, the pumping of the heart provides the primary source of this pressure. However, the mechanical properties of the vasculature also influence the efficiency of circulation by affecting blood pressure and volume (1). These properties include the equilibrium between hydrostatic and osmotic pressure, tunica intima vascular permeability, thrombogenicity and inflammatory state, tunica media smooth muscle cell tone, tunica adventia elastin and collagen fibrosity, and venous valve functionality (2). In addition, internal resistance from blood (i.e. hematocrit or viscosity) and external resistance from the vessel wall (i.e. vasodilation or vasoconstriction) also play a role in blood flow perfusion characteristics (3-5). In the latter case, changes in blood vessel diameter and quantity within the tissue are

influential (2, 5). When arterial blood is routed from larger to branch vessels, the flowing blood is distributed into numerous vessels with smaller diameters (6). As a result, the volume becomes divided over a larger cross sectional area (4). Furthermore, the decreased ratio of volume per area causes blood pressure to decrease and results in a decrease in flow rate (7). Therefore, the rate of blood flow is proportional to the vessel diameter and, consequently, inversely proportional to cross sectional area, but ultimately depends on the blood pressure in the context of the region of the vascular tree in which the vessel is located (4, 6, 7).

The balance between blood pressure and vessel configuration determines the hemodynamic properties within a vascular area (8). Normally, the cardiac output manifests a stroke volume, which increases pressure circumferentially in the aorta (8). The systolic increase in blood pressure stretches the vessel wall, where kinetic energy is stored as potential energy in what is called an arterial reservoir (9). The ability for the vessel to expand and to store volume is termed elasticity and compliance, respectively. (10) The vessel wall and the blood apply pressure against each other, and with the development of a pressure gradient within a given length of the vessel, which causes fluid to move, tension, a frictional force, is imparted tangentially across the vessel wall from the fluid flow (11). This hemodynamic property is wall shear stress (12).

The equation for wall shear stress ($\boldsymbol{\tau}_S$) is derived from Newton's Law of Viscosity and Poiseuille's Law of Parabolic Velocity Profile and displayed in **Figure 1A** (7). From the equation, μ is the dynamic viscosity, Q is the volumetric flow rate (more accurately termed "discharge"), and R is the blood vessel lumen radius (7,13). Shear stress is expressed in SI unit Newtons/m^2; which equates to 1 Pascal or 10 dynes/cm^2. The use

of dynes – which 1 dyne equals 10 μN, the amount of force required to accelerate one gram of a substance one centimeter in a second – in the description of shear stress is convenient way to express the frictional force generated from the movement of fluid tangentially across the endothelium lining of the vessel wall as surface tension propagated across the area (in square centimeters) of the vessel wall (4,7, 14).

Wall shear stress differs from fluid shear stress in that the former occurs at the fluid boundary layer and the latter at the central fluid layer (7). The major difference is that the fluid boundary layer involves application of a force along the surface of a definite shape; thereby influencing the symmetry of the central fluid layer according to the shape of the vessel (7). In fact, to the degree the vessel geometry maintains equal pressure on all sides of the fluid, to that degree the boundary fluid layer is preserved, and to that degree the steadiness of the boundary and, consequently, the central fluid layer is maintained (7). To the degree that the tension, and consequently the shear stress, decreases at the blood-vessel wall interface, the compensatory biological responses determine the degree to which the vasculature region becomes remodeled (2).

2. Role of Shear Stress in Vascular Pathobiology.

In my studies, shear stresses of 10 dynes/cm^2 to 3 dynes/cm^2 are used to recapitulate the arterial levels under physiological and pathological conditions, respectively (7). Normal venous and arterial shear stresses exist within ` stress protects the vasculature from the development of vasculopathologies by conferring endothelial cell genotypic resistance to apoptotic stimuli, suppression of reactive oxidative stress

species, prevention of cytokine expression, regulation of vascular permeability, and preservation of basement membrane composition (2). In arterial vessels, altered blood flow patterns are generated at sites where changes in vessel geometry, from straight to curved, cause an increase in collision between the traveling blood and the inner vessel wall (15). In addition, bifurcation sites engender separations between the flowing blood and the outer vessel wall, which causes the fluid boundary layer to become diminished due to a lack of pressure from the vessel wall. This makes the central fluid layer asymmetrical and results in turtuosity in the flow regime that has a linear relationship with the angle and asymmetry of the bifurcation (16). Such interaction generates turbulent flow, as depicted in **Figure 1C**, between the area of the flow separation and reattachment (16). Turbulent flow generates reverse currents which add resistance to forward flow; thereby decreasing flow and causing shear stress to fall below 4 dynes/cm^2 (4,7).

Altered blood flow promotes EC injury that primes the vascular region for atherogenesis (17). When this is the case, endothelial cells lining the vessel wall become arrested in a pro-inflammatory state that promotes EC dysfunction (14). Endothelial cell dysfunction can result in altered maintenance of barrier function, anticoagulant, antithrombotic, and fibrinolytic regulation, changes which are reversible (14). However, under chronic inflammation, endothelial cell dysfunction leads to cell activation, which involves changes in gene expression and protein synthesis (4,18). Interestingly, although the factors which contribute to the development of atherosclerosis are widely distributed within the blood, the localization of plaques

predominate regions of the vasculature where flow regimes are altered, especially bifurcations (12, 16).

Much evidence suggests that endothelial cell injury resulting from pathological shear stress is one of the earliest events contributing to atherogenesis (19). As a consequence, atherosclerosis is characterized by EC activation and subendothelial accumulations of lipids and cholesterol–engorged macrophages, called foam cells (4). At later stages, the clinically silent pathological condition evolves into an overt narrowing of large and medium-sized arteries as a result of plaque formation and calcification (4, 14). It is very important to indicate here that atherosclerosis is the major underlying cause of heart disease, and furthermore, to emphasize that the development of atherosclerosis contributes to many clinical manifestations which depend on the lipid content of the plaque, the fibrotic cap composition, and the plaque location in the vasculature (14). The very formation of plaques creates curvatures in the vessel wall which perpetuate the perturbations of flow characteristics that further injure downstream endothelium and maintain this devastating cycle (20).

Alternatively, if the flow characteristics are high enough in regions of plaque formations, exceeding 70 dynes/cm^2, then the shear stress may erode the plaque. This causes plaque rupture that may result in either a localized thrombus or a mobilized embolism. In both cases, the occurrence is sufficient to contribute to tissue death due to vasooclussion; ultimately causing a stroke, heart attack, or other aberrant vascular functionality (20, 21).

3. Shear Stress Regulates Endothelial Cell Mechanics

The ability of endothelial cells to sense shear stress and convert the mechanical stimuli into biochemical responses is mechanotransduction; a three step process involving mechanotransmission, mechanosensing, and mechanoresponses (22). Although forces may be mechanotransduced between cells, this study focuses on intracellular communications (23). Some mechanotransducers discovered for their roles in EC responses to flow include the cell membrane, ion channels, G-protein coupled receptors, VE-cadherin, cytoskeletal proteins, and integrins (17,24). These coordinate the sensation of flow from the luminal to the basolateral EC surfaces, where adhesive structures mediate responses to flow that may involve changes in cell shape, protein distribution, cell adhesion, metabolic processes, surface protein expression, and gene expression (18, 25). The sensation of force originating at the cell membrane manipulates changes in the membrane microviscosity that alters membrane polarization, membrane fluidity, and cell permeability (26). These changes are associated with changes in membrane lipid composition, intracellular ion concentration, stretch-ion channel activation, and GPCR activation (27). Cytoskeletal networks connect the cell membrane to different regions of the cell and experience rearrangements relative to the force vector applied to the cell surfaces (28). Force-dependent cytoskeletal reorganization provides redistribution of proteins and forces to cell-cell junctions and cell-extracellular matrix junctions, where VE-cadherin and integrins reciprocally regulate the cytoskeletal organization to balance the tension between the cell and the external environment (27). Flow patterns determine the organization of the cytoskeleton, cell-cell junctions, and ECM composition via integrin

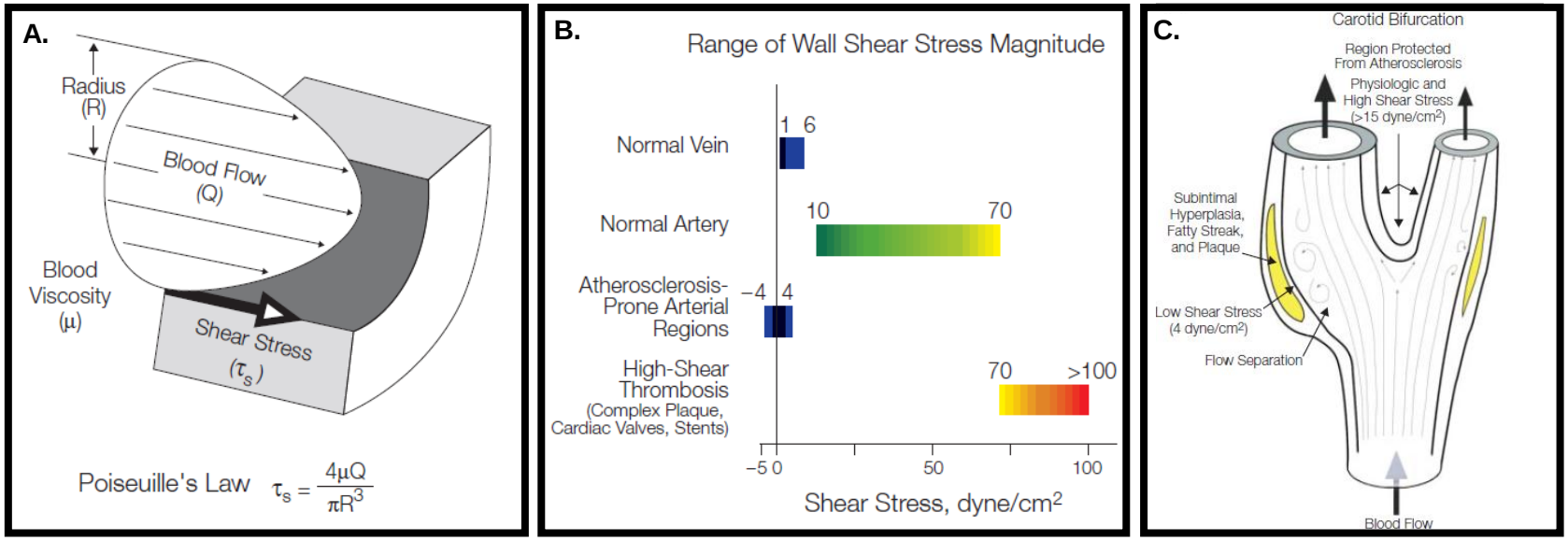


Figure 1. Illustration of Hemodynamic Shear Stress.

A. Shear stress occurs at the interface of the fluid and vessel wall. The fluid travels will greater resistance at the wall.

B. Ranges of normal and abnormal wall shear stress magnitudes for veins and arteries.

C. Representation of the changes in fluid profile which occur within the vascular lumen. The velocity vectors of blood entering the vessel is initially laminar with a parabolic front which experiences narrowing at the region of vaso-occlusion in plaque containing vessels. Consequently, alterations in the vessel geometry impose changes in the fluid velocity vectors which become convoluted downstream of plaque obstructions. Although the fluid recovers its laminar velocity vector configuration as it progresses within the straight vessel away from the plaque, the region nearest the plaque experience turbulent and low fluid dynamics. Since flow is proportional to shear stress, these areas subjected to low flow experience low shear stress and contribute to the progression of plaque growth and the dissemination of atherosclerosis within the vessel wall. ***(Figure adopted from Reference*** # 7***)***

mediated functions (27). Since traction forces generated at the EC substratum in response to shear may promote luminal cell surface expression of adhesion molecules such as ICAM and PECAM, integrins may be viewed as the regulators of bi-directional mechanotransduction (29).

B. INTEGRINS.

1. BACKGROUND.

Integrins are heterodimeric transmembrane adhesion receptors present on virtually all cells including ECs (30). There are 24 different integrins which are formed by different combinations of one of 17 alpha subunits with one of eight β subunits (31). Structurally, each integrin subunit possesses a large (80~150 kDa) extracellular domain, a single transmembrane helix of ~20 amino acids, and a cytoplasmic domain of 20~70 amino acids, shown in **Figure 2** (30, 31).

Integrins connect the cytoskeleton within the cell through their cytoplasmic tails and to proteins of the ECM via their ligand binding sites in their extracellular domain (31). By virtue of these interactions, integrins mediate bidirectional signaling across the cell membrane (32-35). As currently envisioned, integrin activation involves a change from resting, low affinity state (a bent conformation), to an intermediate affinity (extended with a closed head piece), and ultimately to a high affinity state (extended with an open head piece) (36). In addition, important in controlling integrin function is their clustering, which allows integrins to engage multivalent ligands, such as extracellular matrix proteins, with high avidity without necessarily changing their affinity

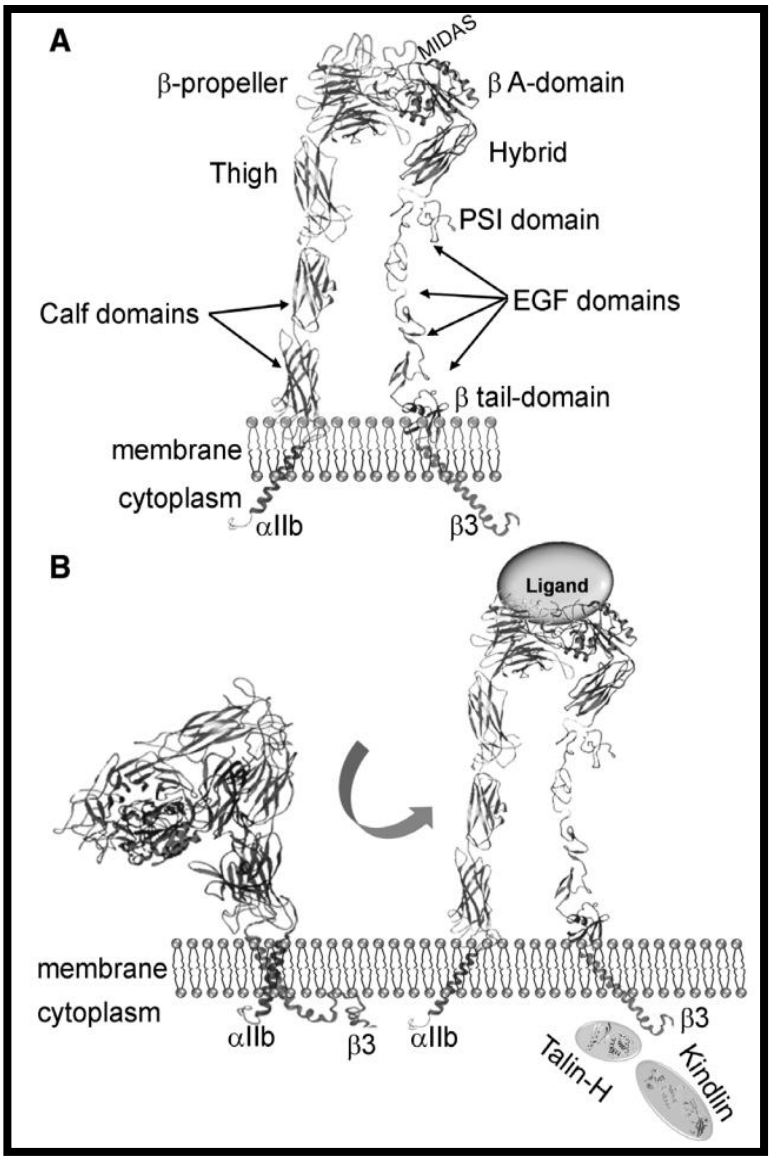


Figure 2. Structural Model of Integrin Heterodimer.

A. Structural model of the domains on the alpha and beta subunits of the heterodimeric integrin receptor.

B. Mechanistic depiction of the conformational change involved in integrin activation. The process includes an unclasping of the membrane proximal tails which instigates extension of the extracellular domain from a bent to an elongated orientation, whereby opening of the headpiece culminates in ligation. Talin-H and kindlin are shown as intracellular proteins involved in the synergistic activation of the integrin via protein-protein interactions with the beta subunit cytoplasmic tail.

state (37). Transmission of tension along the cytoskeleton towards the cytoplasmic tail of integrins culminates in full integrin activation when the extracellular domain of integrin heterodimers becomes fully extended allowing it to recognize its cognate extracellular matrix protein ligand (38).According to the “tensegrity” hypothesis, integrins stabilize the cytoskeletal architecture in a tension-dependent manner (27). Integrins sense distortions in the force balance between the ECM and cytoskeleton and signal events to restore the balance through regulation of the size, composition, and orientation of focal adhesions (39, 40).

Focal adhesions (FAs) are nucleation sites for protein-protein interactions and, as dynamic hubs of signaling, depend on integrin bidirectional signaling to cue the mobilization of proteins to the attachment sites. FAs are not static structures and their composition depends on the on/off rates of proteins for other FA components, including the cytoskeleton, integrin and other FA proteins. FAs are formed under the tensional force generated by the force of myosin on actin and must be bridged by integrins to the force bearing counterparts in the ECM to render the appropriate cell traction to the underlying matrix (41-43). The appropriate cell traction is influenced by the tensional state of the ECM and, through focal adhesion mediated cytoskeletal organization, determines the cell mechanical state (44-48). The mechanical state of the cell determines the cell pre-stress condition, which predetermines the cells’ responsivity to mechanical stimuli, such as shear stress. Ultimately, the cell mechanical state, under pre- and post- stimulated conditions, is regulated by focal adhesions dynamics. Because integrins are adhesion receptors, and integrin activation state is modulated by receptor-ligand interactions, mechanical cues which regulate the affinity between

integrins and their ligands regulate cell mechanics determined by the strength of focal adhesions and integrin mediated cell functions, such as cell migration, cell survival, and changes in cell morphology, and therefore, the cell adaptability to mechanical stimuli, such as resistance to deformation; thus, implicating the importance of integrin function in EC resistance to injury under stress (49, 50). The force balance, therefore, becomes a balance of inside-out and outside-in signaling.

2. Shear Stress Response by Integrin Signaling.

Integrin activation state is not static, but consists of a motion-equilibrium which shifts between bent and extended conformations (51). Since shear stress promotes changes in cytoskeletal organization parallel to the flow direction, cytoskeletal rearrangements mediate force transduction which may regulate integrin activation. The forces transmitted from shear stress, which arrive at focal adhesions by way of cytoskeletal filaments, cause conformational changes in integrins that modulate cell adhesion in an integrin-ligand specific manner (42, 51-55). Whether the integrin-ligand adhesion bond is strengthened (catch bonds) or weakened/broken (slip bonds) in response to flow depends on the effects of ECM rigidity on integrin-cytoskeletal associations and the rate of force transmission. The strength enhancement of catch bonds is due to the prolonged bond lifetimes attributable to the molecular stiffness associated with extended integrin conformations that stabilize the high affinity state in response to high force transmission rates (56). In contrast, slip bonds occur due to low force transmission rates where broken integrin-cytoskeletal linkages stabilize low integrin affinity in areas of low ECM tension that make bond lifetimes small (56). Catch

bonds create synergy between ECM and integrin bond strength attributable to force activation of the head piece (57).

Since the integrin conformation reflects it activation state, and integrin activation promotes regulation of FA strength and composition, the effects of shear stress on integrin conformation also affect on/off rates of protein-protein interactions in FAs. This may explain why the adhesion strength of ECs measured under flow and has been shown to be greatest on fibronectin, followed by vitronectin, laminin, and is weakest on collagen (58). In response to flow, it has been shown that endothelial cells on fibronectin activate $\alpha_v\beta_3$ and $\alpha_5\beta_1$, but inhibit $\alpha_2\beta_1$ in a PKC related manner compared to cells on collagen; which display activation of $\alpha_2\beta_1$, but suppression of $\alpha_v\beta_3$ in a PKA related manner (44, 59-61). The difference in adhesion strength under flow may be explained by the difference in internal focal adhesion dynamics such that forces are transmitted to integrins differently based on the regulation of protein-protein interactions stemming form the cytoskeletal linkages to integrins (62). For example, β_1 integrins are observed to be diffuse, more abundant in nascent adhesions, and experience rearward motion in FAs response to traction forces generated from high myosin activity compared to β_3 integrins which are observed to be concentrated in mature FAs which are immobilized where prominent actin stress fibers are tethered (62). In addition, β_3 integrins have been shown to exhibit physiologically relevant binding to fibronectin in the bent confirmation, which may allow adhesion characteristics in otherwise sterically inhibited FA compositions (63). Although the mechanisms require more investigation, it is clear that forces which enable lateral diffusion of integrins to either reinforce conformation or to relocate integrins into clusters generate cross talk, which promote the recruitment of

integrin binding partners to FAs and enable regulation of unbound integrins by those ligand occupied (64).

Since laminar flow promotes changes in cell alignment to flow, the matrix effects that determine integrin responses in effect determine the appropriate adhesitivity of FAs necessary to allow the cell to move into the flow direction. Thus, aligning force bearing structures in the shear flow force vector that balance the force along the cell surface, relax distinct regions of the cell, reduce asymmetrical surfaces where shear forces are higher, and consequently reduce the stimulatory effects of flow, as in desensitization (65). Ultimately, just as shear stress promotes increased fluidity in the cell membrane, the effects of shear stress which promote depolymerization or dissociation of cytoskeletal structures from FAs increases cytoskeletal fluidization, and the effects of shear stress which promote lateral diffusion of integrins increases FA fluidity (or decreases rigidity) and changes the cells mechanical state to allow changes in shape (65). The degree to which the cell reforms its rigidity depends on the availability of integrin-ligand associations by which integrin anchorage enables equivalent friction in FAs to resist deformation to flow (49). This feature is characterized as the cell viscoelasticity (66). In the larger context, it is important to recognize that in as much as the flow rate imparts distinct bearable force transmission orientations by distinct integrin localities and cytoskeletal associative properties, that in pathological flow settings, where flow is oscillatory, having different directionalities and intensities, it is conceivable that uncontrolled flow characteristics will disorganize the endothelium by disorganizing the cytoskeletal organization, protein distribution, and FA strength (43).

With this emphasis in place, the importance to study the effects of shear stress on the redistribution of integrin binding partners to assess whether they are involved in FA strengthening or weakening on a particular ligand, which come to balance the inside-out and outside-in integrin signaling events responsible for stabilizing the cell mechanical state, those state dependent functions, and effects on vascular biology relevant to the development of atherosclerosis is readily apparent (17).

C. KINDLINS.

1. STRUCTURE AND FUNCTION OF KINDLIN.

Kindlins are a three member family of cytoskeletal proteins recognized for their ability to interact with integrins, (68-70). Kindlins share between 50-60% sequence homology (71). The kindlins exhibit differential tissue expression, which may be explained by the ubiquity of the SP-1 promoter binding site for kindlin-2 and not for the other kindlin paralogs (72). There are structural models available that describe kindlin-1 proteins, as shown in **FIGURE 3** (78). The N-terminal F0 subdomain precedes the F1 (73, 74), kindlin-2 (75-77), and kindlin-3 (78). Kindlins are FERM domain containing adjacent to the F2 subdomain, which is bisected by a PH domain. The F3 subdomain is located at the C-terminal and is involved in binding the β integrin subunit cytoplasmic tail (79). The integrin binding properties of kindlins have been intensively studied for elucidation of regulatory properties relevant to β integrin subunit cytoplasmic including how integrin tail phosphorylation regulates kindlin binding (80). The integrin binding motifs for talin and kindlins are proximal and distal NXXY sequences on the β integrin

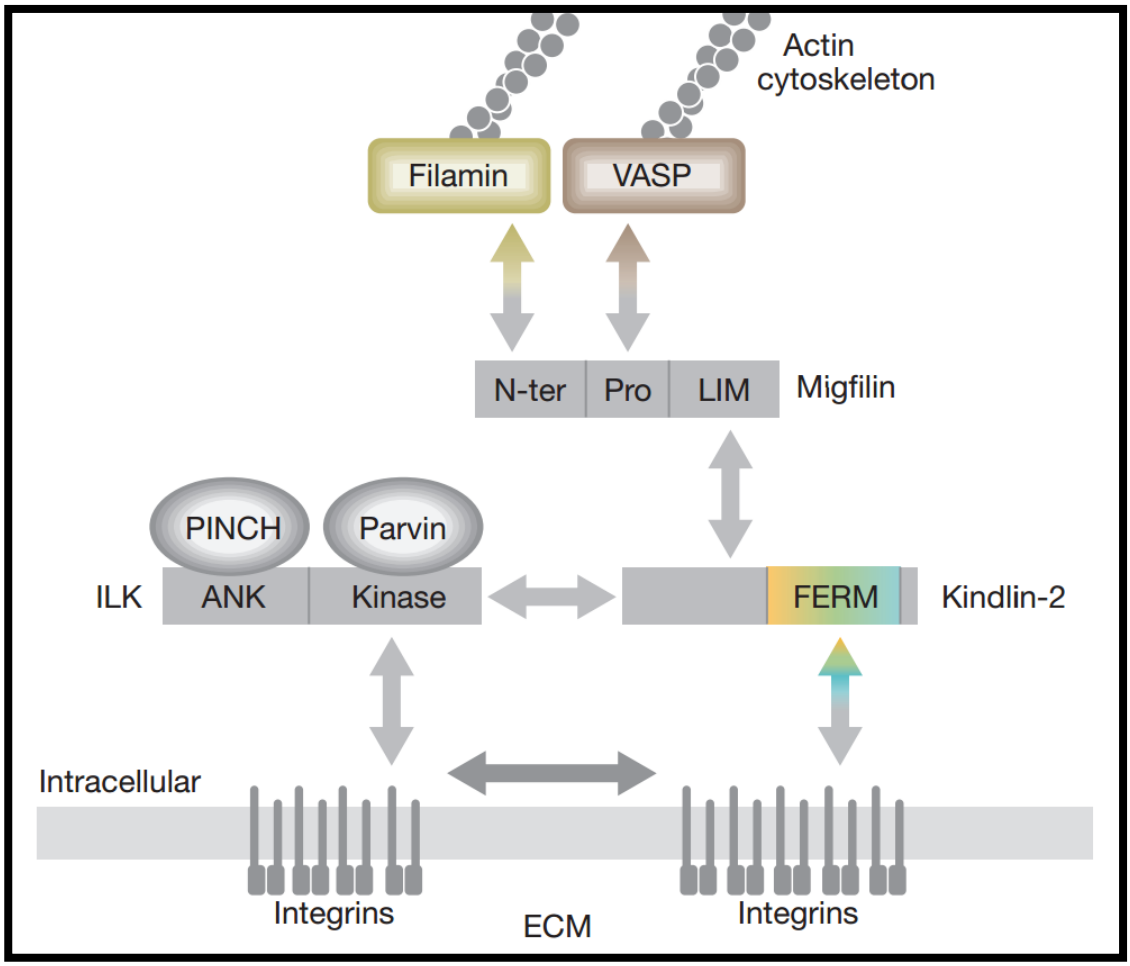


Figure 3. Kindlins Mediate Linkage Between Integrins and the Cytoskeleton.

Kindlin-2 has been observed to be important for cell morphology. Reduced levels of kindlin-2 impair changes in cell shape in ECs (67). Kindlin-2 is displayed exhibiting multivalent binding properties with migfilin at the N-terminal region and the cytoplasmic tail of the integrin β subunit via its C-terminal region. The association with migfilin enables cross-linking with the actin cytoskeleton via filamin-migfilin interactions. Ultimately, changes in f-actin cytoskeletal organization are coupled with changes in focal adhesion dynamics via complex protein-protein interactions of this variety.

subunit, respectively (69). The FERM domain of talin is located on its N-terminus and lacks the unique PH domain present in kindlins (69).

Most often, studies of talin and kindlin have emphasized their roles as integrin co-activators and as linker of integrins to the actin cytoskeletal network, but recent work has provided insight into other associations between kindlin and β-catenin, sorting nexin 17, as well as to the plasma membrane, an interaction which also has proven to be important for its regulation of integrin function (81).

2. Involvement of Kindlins in Integrin Activation

Kindlins have not been shown to possess enzymatic activity, but rather mediate signal amplification via their binding to and their recruitment of other proteins to integrins (71,82). As a result, kindlins enhance integrin recognition of ligands present in the extracellular environment (71,82). In general, a defect or deficiency in kindlins impairs integrin mediated cell functions required for normal human health. Kindlins are encoded by three different genes: KIND-1, KIND-2, and KIND-3 (83,84). Kindlin gene pathologies relevant to human health give rise to Kindler syndrome, a skin and intestinal disease caused by KIND-1 deficiency, and leukocyte adhesion deficiency-III, a syndrome associated with bleeding, susceptibility to infections and bone abnormalities caused by KIND-3 deficiency (69,82,85). The clinical significance of KIND-2 deficiency has not been discovered in humans; however, in mice, KIND-2 deficiency is embryonically lethal (71).

Kindlins cooperate with talin in promoting integrin activation and both are required for productive integrin activation; i.e., kindlins and talin are integrin co-

activators. NMR spectroscopy of kindlin demonstrated their PH domains are essential for mediating their interaction with phosphoinositdes, which mediate their recruitment to the cell membrane and facilitates their co-activator function (75). The functional data showed the presence of positively charged IP4, of PIP3, binding pocket in kindlin-2 PH-domain is responsible for anchorage to inner cell membrane and enhanced function (75, 76). The crystal structure of kindlin-2 PH domain (76) demonstrated consistency with the NMR solution structure and revealed a conformational transition in the PH domain occurs upon IP4 binding. This change involves moving Phe382 and Lys393 of β1 and β2 loops, respectively, toward the center of the binding pocket upon interaction with IP4 (76). In addition to this property, the solution structure of kindlin-2 F0 subdomain, located on the molecule's N-terminus, also displayed binding properties to PIP2 with preference for PIP3 (77). It is noteworthy that the role of PI3K activation in integrin activation involves conversion of PIP2 to PIP3 which also enhances kindlin-2 membrane association (75). The high sequence homology of the PH and N-terminal domain in kindlins suggests this is a mechanism for membrane association is shared by all kindlins (75).

Interestingly, talin lacks a classical PH domain, but also depends on phosphoinositide binding via its F2 subdomain within its FERM domain for integrin activation; refer to **Figure 4** (75). Thus, the molecular details of association of kindlins and talin with internal membranes differ and their associations are important in the role of stabilizing the integrin in an activated state (75). In addition, the integrin binding partners' membrane associations may also destabilize the association of the

cytoplasmic tail of the integrin with the membrane, which would also enhance integrin activation (75).

Furthermore, the integrin activation state is differentially regulated by kindlin-3 and talin whereby talin is sufficient to induce integrin extension, but requires kindlin-3 to promote activation of the integrin head piece (36). Since the activation of the head piece increases the molecular stiffness of integrins, the role of kindlin-3 in activating the head piece suggests the recruitment of kindlins to the cytoplasmic tails provides reinforcement to the force bearing capacity of integrin containing structures (36). A study of the conformational changes in kindlin-3 upon integrin binding demonstrated talin is has a more compact structure than kindlins due to the absence of a PH domain (78). The authors suggested the elongation of both kindlin and talin are important for forming a ternary complex, which depends on the β integrin subunit cytoplasmic tail, maintaining integrins in the activation state (78). Therefore, transmission of forces from shear stress is likely to mediate conformational changes in kindlins that enhance integrin activation, thus raising interest in kindlin/integrin/membrane mechanochemistry (90). This finding is supported by numerous studies defining the role of kindlins in integrin mediated functions, such as cell migration, which require force generation (91-94).

3. Roles of Kindlin in Endothelial Cells.

There has been an extensive interest in kindlins as evidenced by recent reviews in high impact journals including *Nature, Science, EMBO, Blood* (33,41,93). The focus of this work is the role of kindlins in endothelial cells. There is ample evidence that

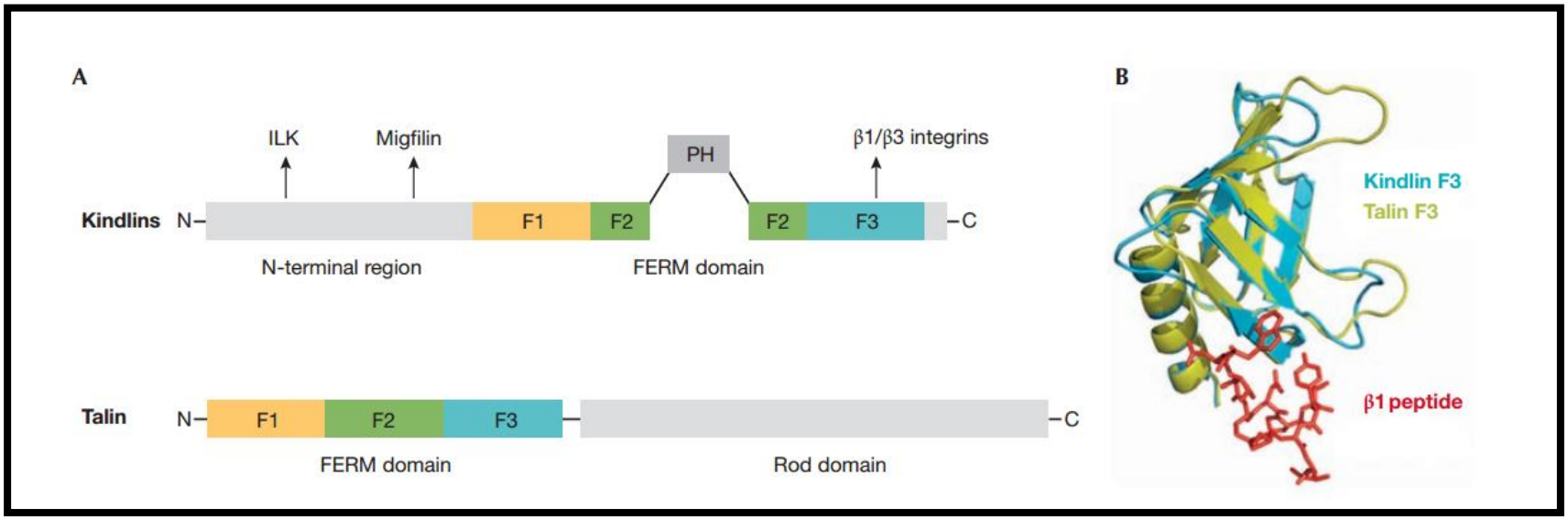


Figure 4. Domain Structure and Sequence Alignment of Talin and Kindlin.

The F3 region of FERM (four-point-one, ezrin, radixin, moesin) domain contains phosphotyrosine-like binding fold resembling that of talin and is involved in binding to the cytoplasmic tails of integrin β subunits (86). Kindlins initially gained interest among researchers of integrin function because they resemble talin, an essential regulator of integrin function (87). Interactions between talin and kindlins with the short cytoplasmic tail of the beta subunit of integrins occur at two distinct non-overlapping NXXY motifs and the simultaneous interaction of talin and kindlins act in a synergistic manner to optimally promote integrin activation (88). The consequence of kindlin enhancement of integrin activation, a shift from a low to a high affinity state for binding their matrix ligands, leads ultimately to increased connectivity between the cytoskeleton and the extracellular environment (89).

kindlin-2 is present and plays an important role in EC responses (82,93). Our lab has recently demonstrated that kindlin-3, previously thought to only to be expressed in hematopoietic cells, is present in EC as well and that K2 and K3 may have different functions in EC (67).

Kindlins have a role in integrin activation. In human umbilical vein endothelial cells (HUVECs), individual knockdown of kindlin-2 and kindlin-3 blunted cell functions dependent on $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin activity, respectively (67). EC which have reduced kindlin-2 levels do not spread, migrate or form vessel-like tube structures on β_3 integrin ligands as efficiently as EC with normal levels of kindlin-2 (33,67). This is not surprising since these functions require changes in cell shape. This emphasizes the importance of kindlins for integrin activation and that kindlins are indispensable for critical cellular functions. Until now, there are no data considering the effects of shear stress on the protein levels or organization of kindlin-2 or kindlin-3 in HUVECs. These issues are considered in this study.

D. CALPAIN

1. Calpain Cleaves Kindlin-3, and Cleavage is Important for Interaction with Integrin

Calpain 1 (μ-calpain) cleaves kindlin-3 at Y373 in platelets, leukocytes, and endothelial cells by. Kindlin-3 cleavage in HUVECs can be stimulated by PMA (protein kinase C activator), calcium ionophore A23187 (inducer of calpain activity), ionomycin, and thapsigargin (a mobilizer of intracellular calcium). Kindlin-3 cleavage may also

occur in unstimulated cells (95). K3 cleavage weakens cell adhesion, but enhances cell migration. K3 becomes resistant to cleavage by calpain with Y373N mutation (95). The interaction between K3 and the integrin β subunit usually occurs transiently in an integrin activation-dependent manner (95). However, the Y373N mutant enables K3 to associate with β subunit in an integrin activation-independent manner (95,96). Inhibition of calpain I via calpeptin prevents cleavage of kindlin-3 and promotes the same effects on integrin adhesion and migration as the K3 Y373N mutation, that is enhances cell adhesion, but reduces cell migration. Inhibition of calpain cleavage of kindlin-3 may delay dissociation of kindlin-3 from β subunit integrin CTs (95). Calpain cleaves various integrin associating molecules such as talin, FAK, paxilin, and even the β_3 integrin subunit itself (95). Calpain cleavage of kindlin-3, talin, and FAK destabilize focal adhesions, which enhances migration; the opposite occurs upon calpain cleavage of paxilin (95).

2. Shear Stress Activates Calpain Proteolytic Activity in EC.

Shear stress promotes calpain proteolytic activity in a shear rate dependent manner (97). This process can occur downstream of PI3K function with dependence on PIP2 (98). Shear stress promotes calpain cleavage of talin. Talin cleavage by calpain is considered to be the rate limiting step, prerequisite to zyxin-mediated mechanosensor responses (99). Since calpain cleaves kindlin-3, and kindlin-3 cleavage is associated with integrin activation, and it is understood that integrin activation is enhanced in a shear stress rate-dependent manner, our studies will investigate whether shear stress induced calpain activation promotes cleavage of kindlin-3.

E. RATIONALE OF THE WORK

This work is based on ongoing work at the Lerner Research Institute at the Cleveland Clinic dedicated to identifying the molecular events that lead to vascular pathologies, including atherosclerosis. This research project is of importance because it provides information regarding cellular signal transduction mechanisms in response to shear that are generally poorly understood. Scientifically, this work seeks to develop a fundamental understanding of how kindlins help to mediate the response of ECs to shear. Clinically, this report addresses the molecular events that may contribute to several cardiac diseases in which EC respond to changes in shear such as during atherosclerosis, thrombosis and restenosis; which encompass the leading cause of death in the US.

Several different shear flow assays have been used to investigate integrin involvement in endothelial cell response to flow (100-102). Although it is known that kindlins are involved in linkage between the cytoskeleton and integrins and disruption of the linkage inhibit ECs responsiveness to flow (32-34,70); as yet no papers have considered the importance of kindlins and their localization in response to shear flow. Therefore, the relationships between shear responses of ECs, integrins and kindlins warrant investigation.

Since deficiencies in kindlins results in pathologies linked to integrin adhesion defects in mice and humans (69,71), completion of this aim will provide insights into the importance of kindlins for endothelial cell function under flow conditions. We anticipate that under the relatively short term conditions of our flow experiments, that changes in

kindlins will be reflected in changes of distribution and not changes in expression levels. We have seen that kindlin-3 does undergo time dependent changes in distribution and degradation in response to shear and we intend to explore this further. Since kindlins are important for integrin function, the effects of shear stress on the distribution of individual kindlins in HUVECs may be investigated further by modulating the substratum to which particular integrin binding may be controlled (37).

In summary, we now know that kindlins are important regulators of integrin function and that integrins are central mediators of mechanotransduction. However, the relationship between the kindlins and flow is not known. Thus, the research in this study addresses a significant gap in our knowledge and will seek to determine if the kindlins participate in the response of ECs to shear. Therefore, completion of this goal will allow us to characterize the physiological significance of the effects of shear stress on the distribution of individual kindlins in endothelial cell function.

CHAPTER II

MATERIALS AND METHODS

1. CELL CULTURE.

Research on vascular biology events that occur in humans involving coagulation, thrombosis, inflammation, and atherosclerosis has been accomplished by retrieval of various cell types from relevant human tissue. Isolation of endothelial cells cultured from the vein of human umbilical cords was first described by Jaffe et al. in 1973 and second by Gimbrone et al. in 1974 (103, 104). Genotypic and phenotypic variations exist for ECs derived from large vessels relative to those harvested from microvascular EC layers (105). HUVECs are genotypically similar to ECs in large vessels (105). Since atherosclerosis occurs in large and medium sized vessels, HUVECs are used as a cell model for studying the effects of shear stress on the distribution of proteins in ECs that may be involved in atherogenesis in this study. HUVECs acquired from the DiCorleto Lab of the Lerner Research Institute (LRI) were cultured to 80% confluency on glass slides prior to experiments and were incorporated in the shear stress assay within 12 hours. Slides were cleaned with 70% ethanol, rinsed, and coated with either 10 µg/mL solution of fibronectin or laminin, 20 µg/mL fibrinogen or collagen, or 2.5 µg/mL vitronectin overnight at 4°C. These matrix proteins will be used to evaluate the distribution of kindlin-2 and –3 under static conditions.

2. SHEAR STRESS ASSAY.

To simulate the effects of blood flow, the Steamer® parallel plate laminar flow device manufactured by FlexCell Int.® of North Carolina, USA was used to perfuse MCB131 media with 1000IU penicillin/streptomycin and 0.5% BSA over HUVECs plated on glass slides with a cell growth area of 1.875 cm^2 (106). The device setup and components are described in **Figure 5** (106). Two shears, 3 and 10 dynes, corresponding to low shear in atherosclerotic vessels and high shear rates in arteries will be applied for times of 0, 1.5, 3, and 6, and 12 hours. Under these conditions, the distribution of kindlin-2 and kindlin-3 in under flow will be assessed and compared to static conditions.

3. IMMUNOFLUORESCENCE AND REAGENTS.

Static or post-sheared endothelial were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X 100, blocked with 4% horse serum and labeled with primary antibody from either Millipore: mouse anti-kindlin-2 (#MAB2617), Invitrogen: mouse anti-zyxin (#39-6000), Molecular Probes: mouse anti-alpha-tubulin (#A1126), Abcam: Anti-VE-cadherin FITC (#ab33321), BD Transduction Laboratories: mouse anti-clathrin heavy chain (#610499) and mouse anti-CD61 (#340715), or our lab: a rabbit antibody raised against kindlin-3 a unique peptide sequence (67). Alexa Flour species specific secondary antibodies conjugated to fluorescent dyes were used to label the primary antibodies.

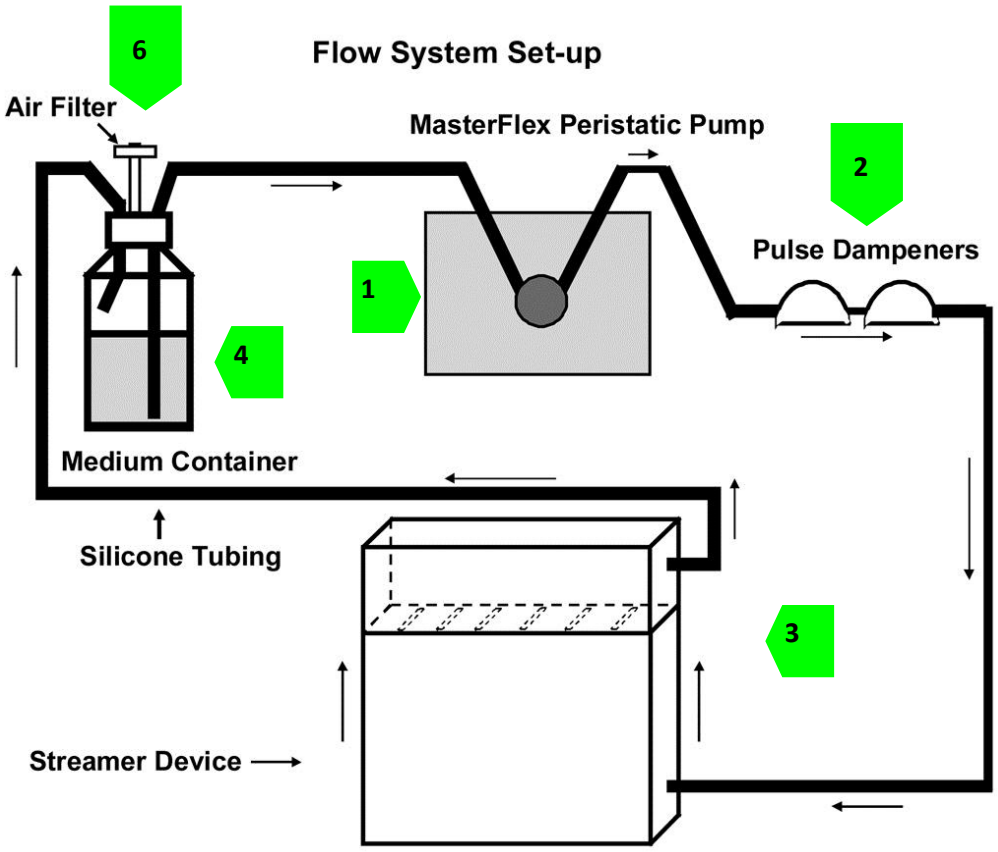


Figure 5. Shear Stress Device Setup

The components of the system are: 1) peristaltic pump and silicon tubing, 2) pulse dampeners that trap air bubbles and absorb the pulsatility of the fluid exiting the pump, 3) 6-chambered Streamer® device, 4) fluid reservoir, and 5) Hepa filter for diffusion of carbon dioxide. The shear rates available with this device range from 0.00 to 185.99 dynes/cm^2. The pump is programmed using software from National Instruments that offers automated flow regimes such as shear stress gradients or bidirectional flow. The entire setup fits into an incubator, which allows us to maintain physiological conditions

(37°C and 5% CO_2). When separated from the pump, the apparatus can be autoclaved for sterility. *(Adopted from Reference #106)*

4. CONFOCAL MICROSCOPY.

Images were collected using the Leica TCS-SP Spectral Laser Scanning Confocal Microscope equipped with four lasers for excitation and three photomultiplier tube detectors. This setup permitted visualization of multi-labeled cellular components simultaneously for comparison of the three-dimensional relationships between them.

5. IMAGE ANALYSIS.

With the image captured, we then upload the pictures into an image processing program, typically NIH ImageJ, to compare the quantity and localization of proteins of interest relative to other labeled cellular components. Quantification was achieved by automated calculation of the Pearson Correlation Coefficient (PCC) of the image pixels (107). Briefly, the PCC (r_p) represents the quality of the linear relationship between the pixels in an image compiled from images of two separate channels (107). The linearity is evaluated from a scatter plot which displays the distribution of the pixels. The intensity of pixels from the green and red channel is used as the x-coordinate and y-coordinate, respectively. This approach is based on the measurement of the intensity (0-255) of pixels adjacent to a reference pixel (5). Pixels are considered to be part of the same structure when above the threshold intensity of the reference pixel (5). Therefore, the structures in an image with pixel intensities above a selected threshold are evaluated for their nearness to the linear regression and scored within a range of -1 to 1 (1= colocalize completely, 0 = no colocalization , or -1 = negative correlation) based on the sum of the intensities at a particular coordinate. (107)

6. Western Blot Analysis of Kindlins.

SDS-PAGE separation and western blot detection and densitometric scanning are used to examine the levels of the individual kindlins and their degradation in response to stress using antibodies listed above. GAPDH is used as a loading control for the gels. Shear stress can influence the turn over of proteins via activation of specific proteolytic enzymes that cleave expressed proteins (47). Consequently, the extent to which proteolysis affected kindlin protein expression in HUVECs was quantified.

CHAPTER III

RESULTS

1. Validation Of Endothelial Cell Alignment With Flow.

We first analyzed the effects of shear stress on cell morphology and actin organization of our cultured HUVECs to validate that the cells are responding appropriately to flow. HUVECs in confluent monolayers were exposed to a shear stress of 3 dynes/cm^2 (108). We found that the cells responded to shear stress by increasing the alignment of their actin filaments, visualized by staining with phalloidin, an F-actin specific reagent (14), in a temporal manner. For example, compared to cells under static conditions, cells exposed to flow for 6 hours displayed a significant 9.2% ($p<0.005$) increase in endothelial cell alignment within ± 10° of the flow direction (**FIGURE 6**) (108). Cell alignment was calculated from the ratio of the angle of the long axis of the cell and the horizontal, as described (109). These results indicate we have valid methods for inducing expected changes in ECs in response to flow and will be able investigate changes in kindlin distribution in the context of EC response to flow. When shear stress imparts tension onto ECs, the cytoskeleton and focal adhesions change in size and composition to manage the altered force distribution (101,110).

For comparison, we examined zyxin, a cytoskeletal associated molecule known to reorganize with shear, and integrin β subunits (111). In our system, we next determined that 3 dynes/cm^2 could be employed to induce changes in zyxin localization from focal adhesions to actin filaments at 6 hours (**FIGURE 7A2-F2**). Integrins have been probed for mechanosensitivity at higher shear rates, but not at 3 dynes/cm^2 (100). Indeed, we found, unlike zyxin, neither β_3 integrin nor β_1 integrin (not shown) were displaced from focal adhesion sites (**FIGURE 7A1-F1**). Thus, our findings provide molecular validation that our system and approach are capable of replicating reported responses of endothelial cell behavior under flow conditions.

2. KINDLIN-2 AND KINDLIN-3 HAVE DIFFERENT STATIC DISTRIBUTIONS ON VARIOUS ECM.

In agreement with previous reports (33), kindlin-2 has been observed to localize in focal adhesions unlike kindlin-3, which is broadly distributed throughout the cytoplasm and along the cell periphery. The previous report demonstrated the distribution of kindlins in HUVECs allowed to spread on vitronectin, fibronectin, collagen and fibrinogen. In this report, we report an additional examination of the distribution of kindlin-2 and kindlin-3 on laminin. On this β_1 integrin ligand, we observe kindlin-2 to be localized within focal adhesions and kindlin-3 to remain unrestricted in its cytoplasmic distribution (**FIGURE 8**). In endothelial cells, β_1 and β_3 integrins have distinct localization within FAs, which enable their association with distinct actin networks, and since kindlin-2 localizes in FAs it therefore demonstrates association with similar actin

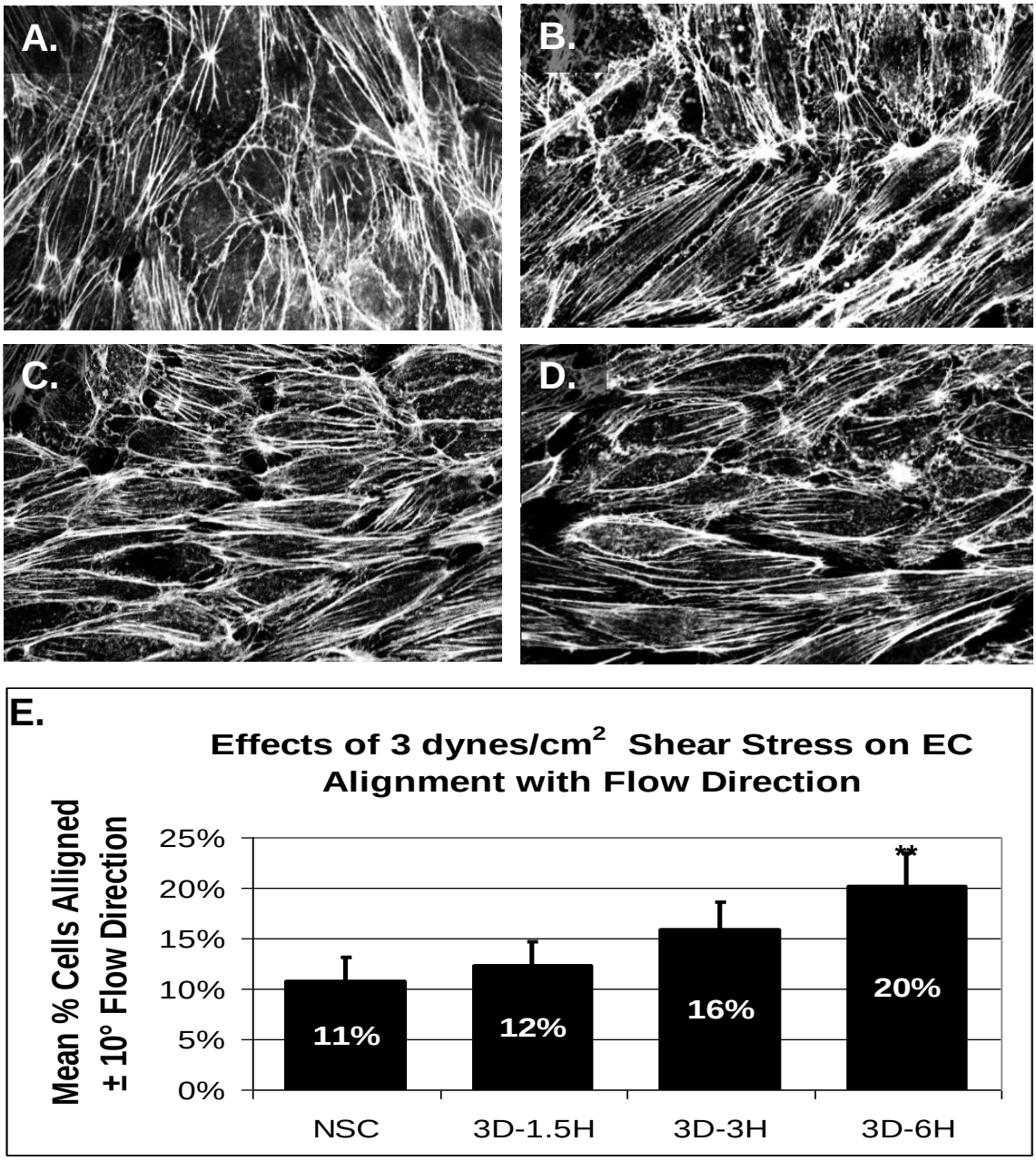


FIGURE 6. Cytoskeletal Reorganization Induced by 3 dynes/cm^2 Shear Stress.

Confocal micrographs showing F-actin labeled with phalloidin in endothelial cells exposed to a shear stress of 3 dynes/cm^2. (A) Static cells and (B–D) cells stimulated with shear stress for 1.5, 3, and 6 hours, respectively. The flow direction is from left to right. (E) Quantitation of endothelial cells aligned within ± 10° of the direction of flow represented as the average and (error being standard deviation) of three independent experiments. [**p<0.005: relative to non-sheared control].

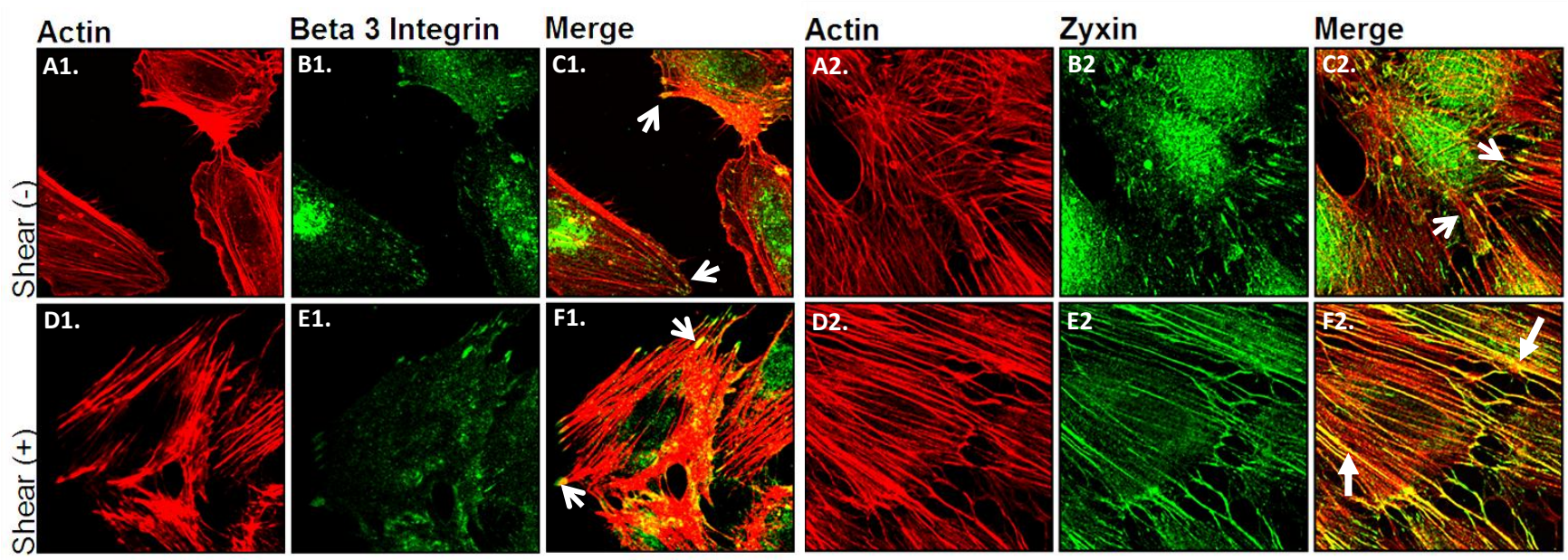


FIGURE 7. Focal Adhesions are Sites of Mechanosensation.

HUVECs spread on FN were stained for β_3 integrin subunit, zyxin, and F-actin. Cells were imaged under static and post flow conditions. The flow direction is from left to right. β_3 integrin subunit stained focal adhesions (A-C1) remain present in these adhesion structures (D-F1) unlike zyxin (A-C2), which redistributes to the actin cytoskeletal filaments in response to flow (D-F2). The yellow intensities shown in merged images depict regions where colocalization between actin filaments and focal adhesion proteins where filaments terminate (➔) or along the filaments (➔). These experiments provide evidence that at 3 dynes/cm^2, 6 hours is sufficient to promote cellular responses which involve mechanotransduction to FA sites.

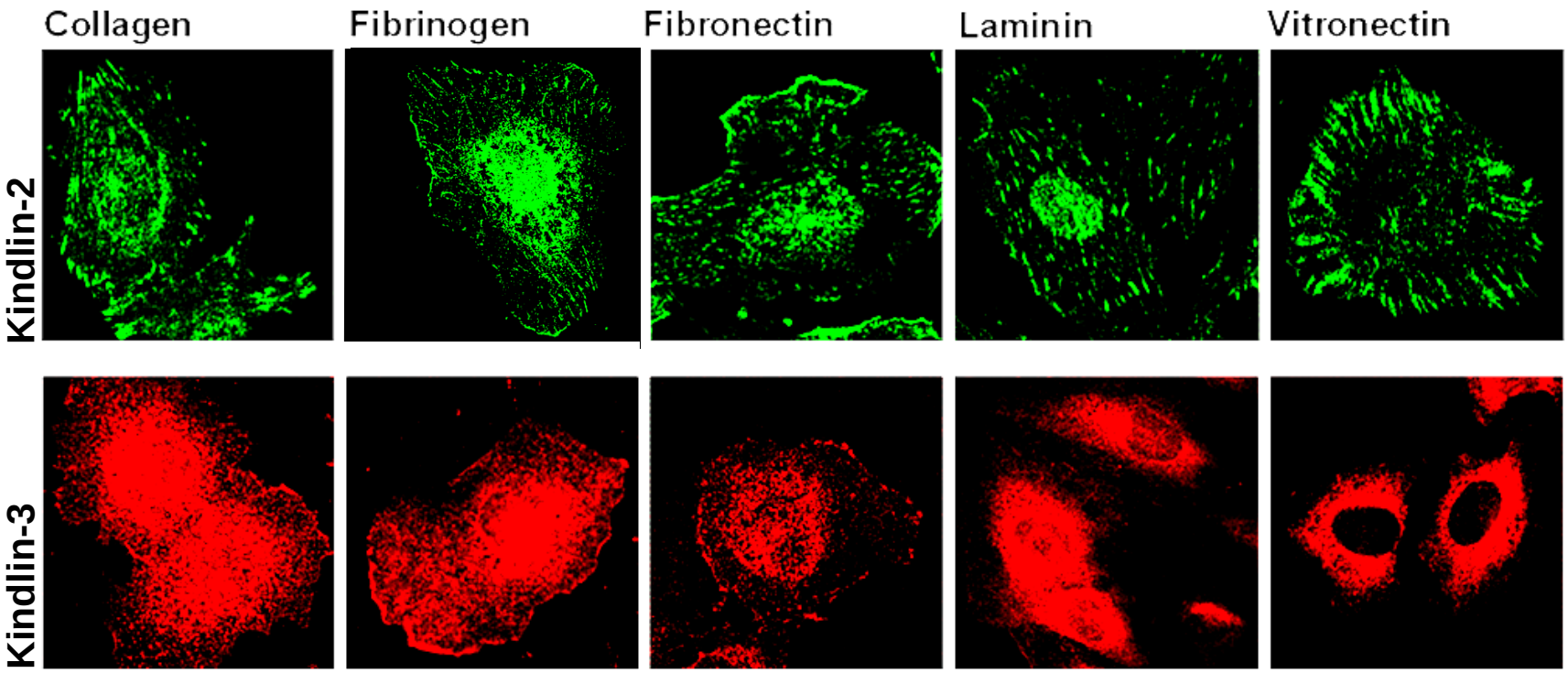


FIGURE 8. The Distribution of Kindlin-2 is Different than Kindlin-3 in HUVECs under Static Conditions.

HUVECs spread on different integrin ligands demonstrate that the distribution of kindlin-2 and kindlin-3 is different and that this difference is not changed by the selection of the β_3 or β_1 ECM ligands under static conditions. Kindlin-2 is localized mostly to focal adhesions and kindlin-3 has a broad cytoplasmic perinuclear distribution with concetrated localization at the cell periphery. Although both kindlins are known to associate with the primary adhesion receptors for each of the presented integrin ligands in other cells, ECs coexpression of kindlin-2 and -3 suggests they possess functional heterogeneity such that kindlin-3 association with integrins in focal adhesions may require cellular adaptations to modulate the prevailing determinants of its distribution otherwise.

networks as these integrins; however, not kindlin-3: which does associate with β_3 integrins in platelets. These results indicate integrin-ligand complexes in static EC do not allow kindlin-3 to associate with FAs and may require conformational changes in the integrin cytoplasmic tails in response to flow.

3. KINDLIN-2 REDISTRIBUTION UNDER FLOW.

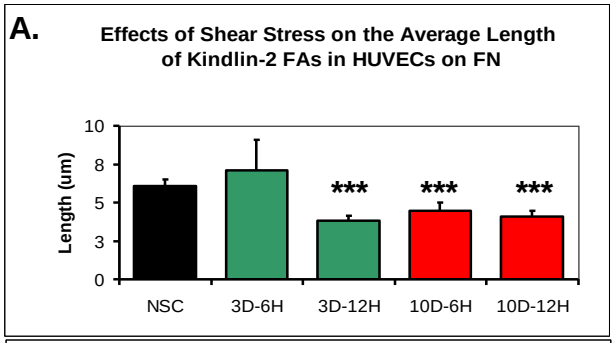
The staining of sheared and non-sheared cells with an antibody revealed that kindlin-2 behaves similarly to β_3 integrin and vinculin, a well-accepted marker of focal adhesions (111), in that it remains distributed in adhesion structures in response to flow. Quantitation of the effects of flow on the morphology of K2 FAs is summarized in **TABLE 1**. Briefly, significant decreases in the average length of K2 FAs in response to shear stress conditions of 3 dynes/cm^2 at 12 hours, and 10 dynes/cm^2 at 6 and 12 hours occurred compared to static controls and cells treated with 6 hours of 3 dynes/cm^2 shear stress (**FIGURE 9A**). No significant differences in K2 FA length were observed between 3 dynes/cm^2 at 12 hours, 10 dynes/cm^2 at 6, or 12 hours. These results suggest that shear stress promotes a decrease in K2 FAs length that occurs sooner at higher shear stress rates than at low shear stress.

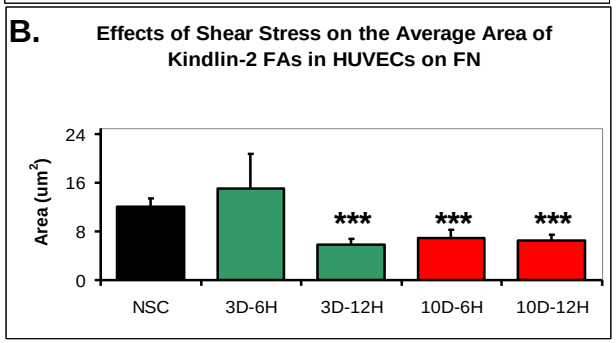
The average area of K2 FAs also showed decreases in response to flow in the same pattern as the K2 FAs length (**FIGURE 9B**). The values are also listed in **TABLE 1**. Significant decreases ($p < 0.005$) in the average area of K2 FAs in response to shear stress conditions of 3 dynes/cm^2 at 12 hours, and 10 dynes/cm^2 at 6 and 12 hours occurred compared to static controls and cells treated with 6 hours of 3 dynes/cm^2 shear stress (**FIGURE 9B**). There were no significant differences in the area of K2 FA staining

between 3 dynes/cm^2 at 12 hours or 10 dynes/cm^2 at 6 or 12 hours. These results suggest that shear stress promotes a decrease in K2 FAs area, which takes longer at lower shear stress rates than at high shear stress.

The quantity of K2 FAs/cell displayed a rate dependent change in response shear stress. Exposure to 3 dynes/cm^2 for 6 hours promoted a significant increased ($p<0.05$) in the number of K2 FAs/cell compared to static control levels, but returned to control levels after 12 hours at 3 (**FIGURES 9C**). Interestingly, after 6 hours of 10 dynes/cm^2 shear stress, the number of K2 FAs/cell very significantly decreased ($p<0.005$) compared to static controls, yet slightly increased after 12 hours. Representative images of the changes in K2 focal adhesion length, area, and count/cell area are provided in **FIGURE 9D1-3**. This study demonstrates for the first time that kindlin-2 exhibits a tunable mechanical response that is shear rate intensity dependent. The K2 FAs under 10 dynes/cm^2 after 12 hours display more organization in the flow direction than FAs from static and 3 dynes/cm^2 sheared FAs.

Since the mechanosensation of shear stress requires continuity of force transmission from the cell surface to the cell-ECM site through the cytoskeleton, it is not clear whether the transient changes in the number of K2 FAs/cell arise from changes in F-actin polymerization or FA dynamics. Furthermore, flow induced changes in K2 FA quantity may implicate conformational changes in the confirmation of kindlin-2 binding partners or kindlin-2 itself. Therefore, the mechanism of the changes in K2 FAs in response to flow is not clear. However, the change in K2 FAs after 6 hours suggests the transmission of shear forces to FAs transiently increases the recruitment of kindlin-2 to





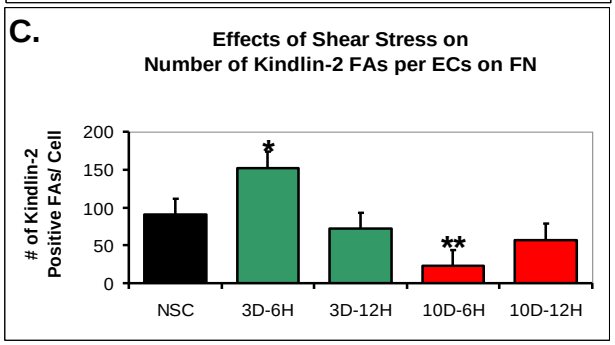


TABLE 1. Summary of Effects of Flow on Kindlin-2 Focal Adhesion Morphology

SHEAR RATE	DURATION	LENGTH (μm)	AREA (μm^2)	#FAs/CELL
Non-sheared Control	0 hours	6.05 ± 0.45	12.16 ± 1.28	90.28
3 dynes/cm^2	6 hours	7.10 ± 2.00	15.03 ± 5.69	152.33
3 dynes/cm^2	12 hours	3.83 ± 0.33	5.90 ± 0.87	71.79
10 dynes/cm^2	6 hours	4.45 ± 0.55	6.93 ± 1.37	22.77
10 dynes/cm^2	12 hours	4.08 ± 0.39	6.48 ± 1.00	57.11

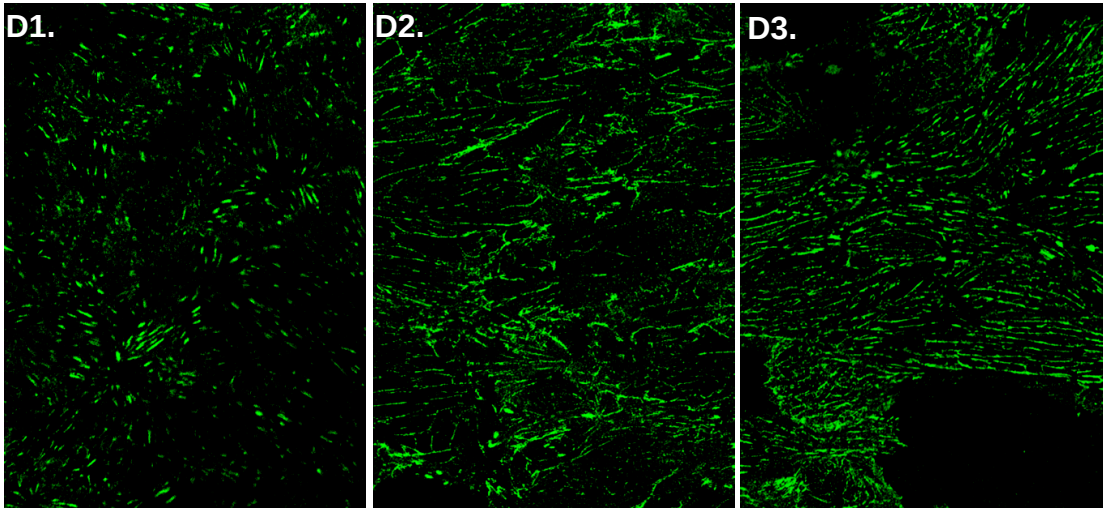


FIGURE 9. Shear Stress Affects the Morphology and Distribution of Kindlin-2 in Focal Adhesions.

The (A) average length, (B) average area, and (C) ratio of FAs per cell area were quantified analyzing the confocal micrographs using ImageJ software from NIH and are summarized **TABLE 1**. Representative images of HUVECS non-sheared or sheared for 12 hours at 3 or 10 dynes/cm^2, respectively, are shown with the direction of flow from left to right (D1-3). [*p<0.05, **p<0.005, ***p<0.0005: relative to non-sheared control].

FAs under low flow, but transiently decreases the recruitment of K2 to FAs under high flow.

I also investigated involvement of kindlin-2 in cell-cell junctions and whether this relationship might be affected by shear stress on fibronectin. To investigate this property, the cell junction marker VE-cadherin was stained in HUVECs to map its colocalization with kindlin-2. **FIGURE 10** shows that VE-cadherin significantly colocalize with kindlin-2 in static HUVECs and increases after 6 hours of shear stress of 10 dynes/cm^2, but not 3 dynes/cm^2. Using Pearson's Correlation Coefficient (PCC) to quantify the colocalization of kindlin-2 in VE-cadherin positive cell junctions, a very significant ($p<0.0005$) increase in PCC was measured between static (0.624 ± 0.0479) and 10 dynes/cm^2 sheared (0.737 ± 0.0543) ECs; an approximately 16.5% increase. The ratio between the mean fluorescent intensity of kindlin-2 in the junctions versus total kindlin-2 was calculated for these experiments to validate the increase in colocalization occurs because of the redistribution of kindlin-2 from FAs. Although colocalization did not increase after 6 hours of 3 dyne/cm^2 shear stress, under these conditions, there was a significant ($p< 0.05$) 3% increase in the staining of kindlin-2 at cell-cell junctions compared to static conditions. The mean fluorescent intensity of kindlin-2 staining in VE-cadherin positive cell junctions increased very significantly ($p<0.0005$) after 6 hours of 10 dynes/cm^2 stimulation by 11.25% compared to static conditions. Cell-cell junctions also experience force-dependent adhesion strengthening by mechanisms physically similar to FAs. The redistribution of kindlin-2 to cell-cell contacts may implicate kindlin-2 as a cell-cell

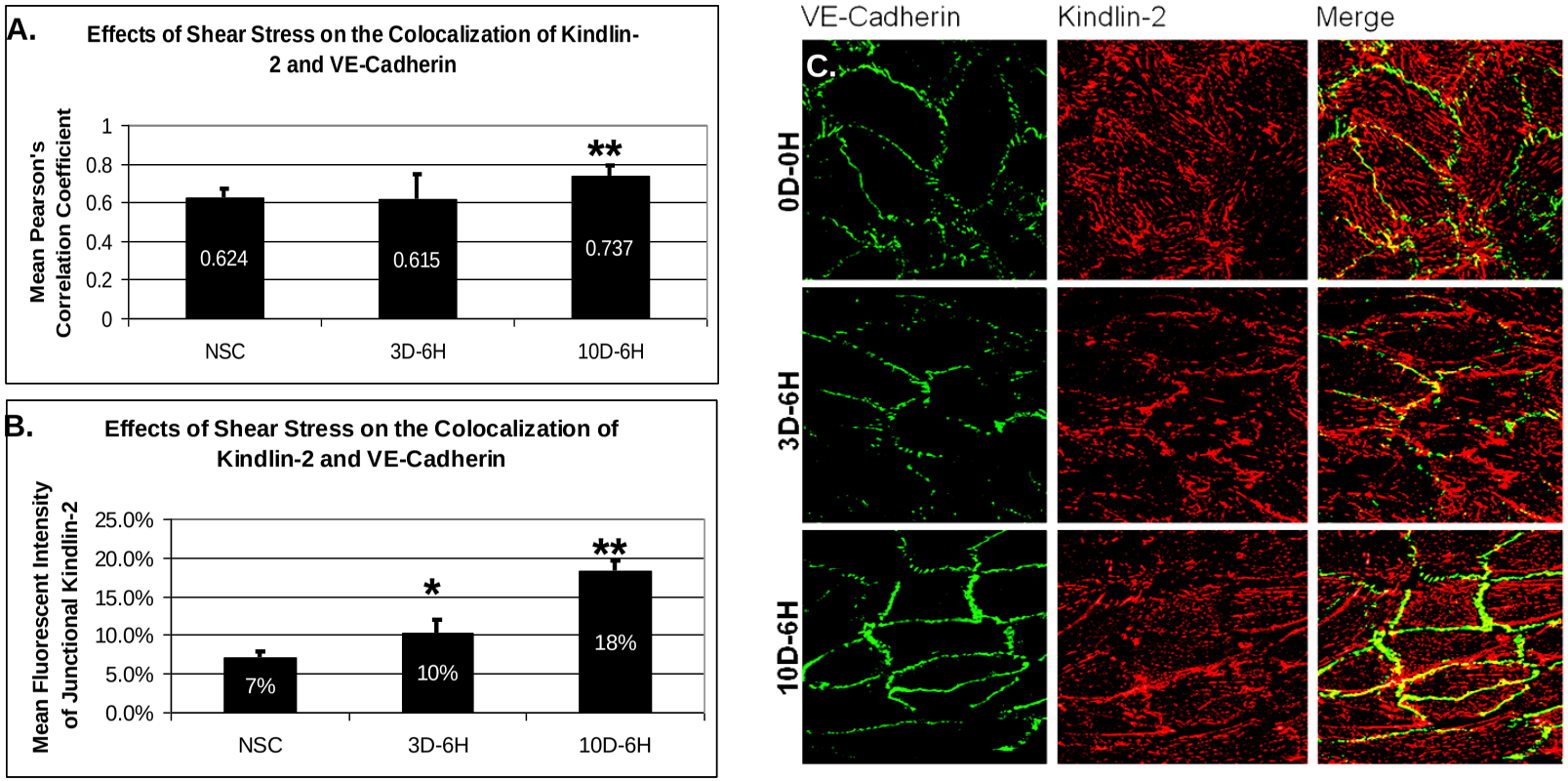


FIGURE 10. Shear Stress Increases the Colocalization of Kindlin-2 and VE-Cadherin.

(A) Pearson's correlation coefficient and (B) Percentage of Mean fluorescent intensity of Kindlin-2 within junctions relative to total cell kindlin-2 were calculated from confocal micrographs of HUVECs shows the increase of colocalization is dependent on the effects of shear stress in an intensity dependent manner. (C) Colocalization between Kindlin-2 and VE-cadherin is shown for HUVECs under static, low and high flow conditions on fibronectin, respectively. [* $p < 0.05$ relative to NSC, ** $p < 0.0005$ relative to NSC and 3D-6H].

junction reinforcement protein. Kindlin-2 localization to junctions may be important for EC barrier function required for to prevent vascular permeability.

4. KINDLIN-3 REDISTRIBUTION UNDER FLOW

In non-sheared cells, kindlin-3 is present in a predominately cytoplasmic distribution where its staining is concentrated proximal to the nucleus and in membrane extensions. In response to shear, the distribution of kindlin-3 changes dramatically such that it is detected in the cytoplasm in discrete punctate patterns (**FIGURE 11A1-2**). The mean fluorescent intensity was measured and showed cells exposed to flow resulted in a 62.7% decrease in mean fluorescence intensity of kindlin-3 staining after 6 hours of 3 dynes/cm^2 shear stress. The mean fluorescence intensity of kindlin-3 in cells before and after flow changed from $3.73*10^7$ to $1.49*10^7$ in arbitrary units (**FIGURE 11B**). The percentage of HUVECs with kindlin-3 punctate pattern increased after 3 hours of 3 dynes/cm^2 shear stress to 25% and to 72% after 6 hours (**FIGURE 11C**).

In addition, shear stress was found to induce degradation of kindlin-3. A novel, lower molecular weight form of kindlin-3 band was detected as early as 3 hours by western blot (**FIGURE 11D)**. Gels were scanned, loading variations were noted, and corrections were made based on the amount of GAPDH quantified. Accordingly, there was about 50% increase of the kindlin-3 band, lane 3 of **Figure 11D**. However, scanning intensity of the degradation product was increased 17.8 fold compared with non-sheared conditions. Thus, shear induced degradation of kindlin-3. This was still prominent at 6 hours in that the expression of the degradation kindlin-3 band was half as much as at 3 hours. The molecular weight of the degradation band was ~37 kDa;

which was consistent with literature reports of the effects of calpain cleavage on K3 (95).

Since it is reported in the literature that calpain is activated by shear stress, we examined if shear stress induced changes in kindlin-3 was associated with calpain activity (97). Our study indicates for the first time that the K3 cleavage in response to shear stress was prevented by pre-treating HUVECs with 1µM calpain inhibitor. The prevention of kindlin-3 cleavage was monitored by western blot (**FIGURE 12A-B**) and immunofluorescence (**FIGURE 12C**). The western blot shows that with the presence of calpain inhibition, the shear stress-induced kindlin-3 degradation product was reduced by 14% compared to cells sheared without calpain inhibition. Since calpain inhibition may nonspecifically affect actin organization and FA formation, kindlin-2 and actin were stained to ensure these cells functioned normally (112).

The punctae of kindlin-3 were evaluated for potential association with vesicle marker proteins via immunofluorescent based colocalization experiments, which involved shearing HUVEC monolayers and staining for clathrin. Kindlin-3 displayed some colocalization (PCC = 0.576) with clathrin prior to shear stress application and this relationship was modestly increased (~11%, $p = 0.04$) after shear stress (PCC = 0.638) induced conversion of kindlin-3 into its post shear form (**FIGURE 13**). In comparison, the kindlin-3 and microtubules do not colocalize, static (PCC = 0.317), and this was not changed by shear, post shear (PCC = 0.297). Clathrin and microtubules participate in the focal adhesion disassembly (113). It may be speculated that the changes in colocalization of kindlin-3 involves its re-compartmentalization from the cytoskeletal to

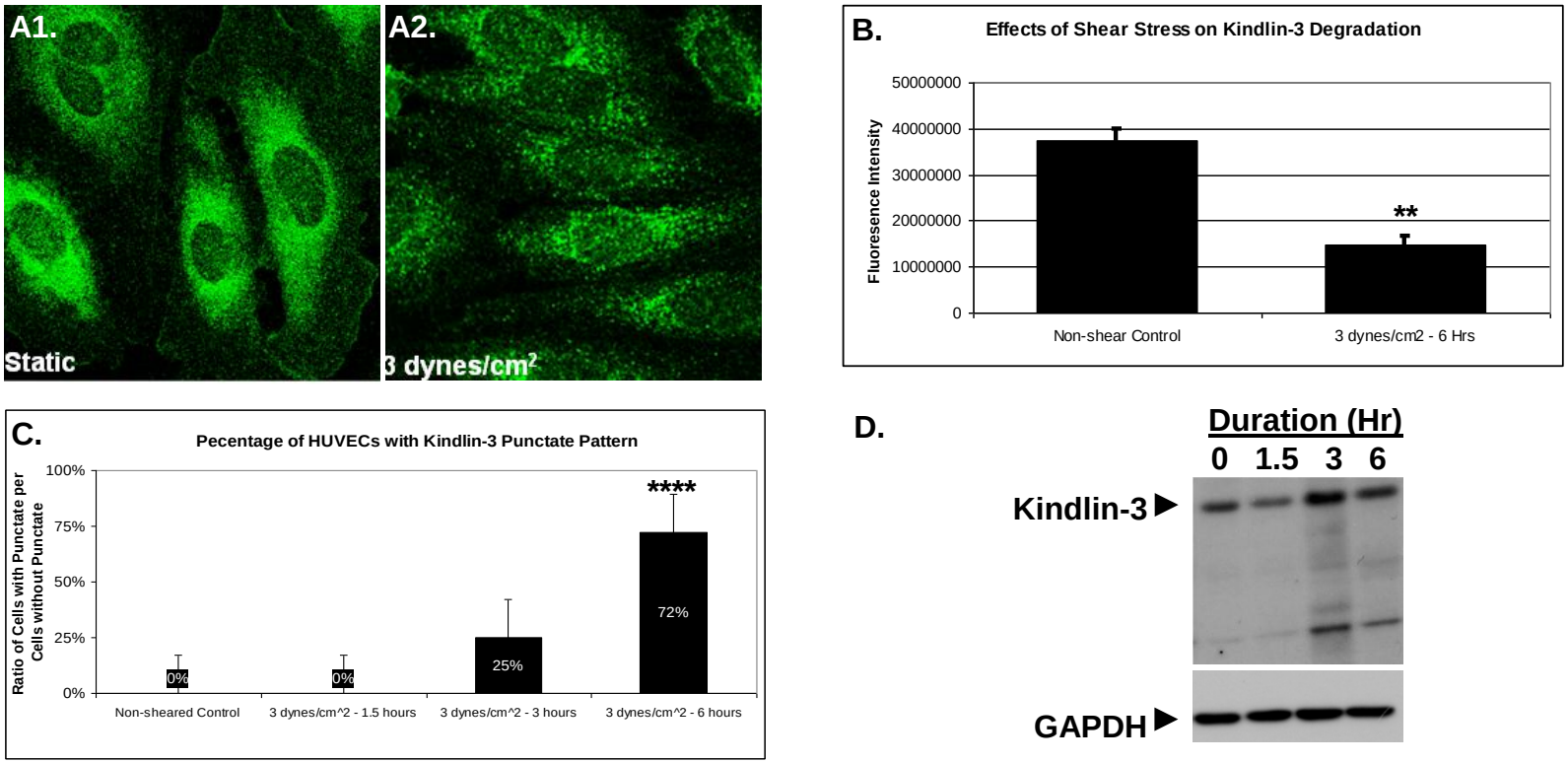


FIGURE 11. Low Shear Stress Promotes Redistribution and Degradation of Kindlin-3 in HUVECs on FN.

The effects of 3 dynes/cm^2 were monitored at 1.5, 3, and 6 hours and were compared with static conditions. (A) Representative images showing the distribution of kindlin-3 in (A1) static and (A2) sheared HUVECs on FN. (B) The mean fluorescent intensity of kindlin-3 staining was compared in static and sheared HUVECS from three independent experiments. (C) The percentage of HUVECs which displayed the kindlin-3 punctate pattern in response to flow was quantified for the different time points of shear exposure. (D) Western blot of lysates from static and sheared HUVECs shows kindlin-3 becomes cleaved in response to shear stress. [**p<0.005, ****p<0.0001 relative to non-sheared control].

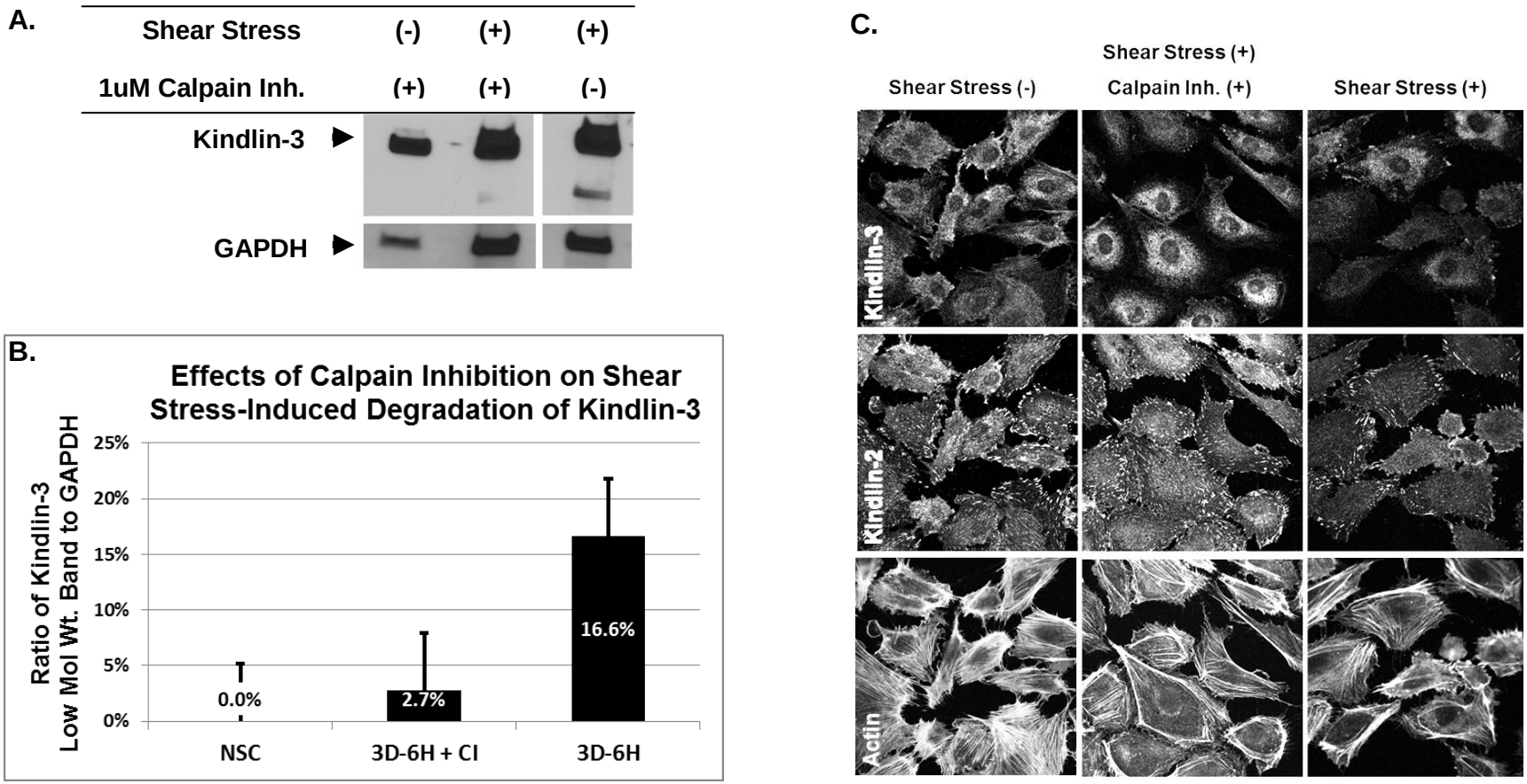


FIGURE 12. Calpain Inhibition Reduces Cleavage of Kindlin-3 in Response to Prolonged Low Shear Stress in HUVECs on Fibronectin

(A) Western blot showing shear induced cleavage kindlin-3 cleavage fragment is prevented in cells pretreated with calpain inhibitor before shear stress. (B) Quantitation of the amount of kindlin-3 fragment produced relative to the total kindlin-3 normalized to GAPDH levels. (C) Immunofluorescent staining of kindln-3, kindlin-2, and actin from the same ECs. Cells were either static or sheared, with or without calpain inhibitor.

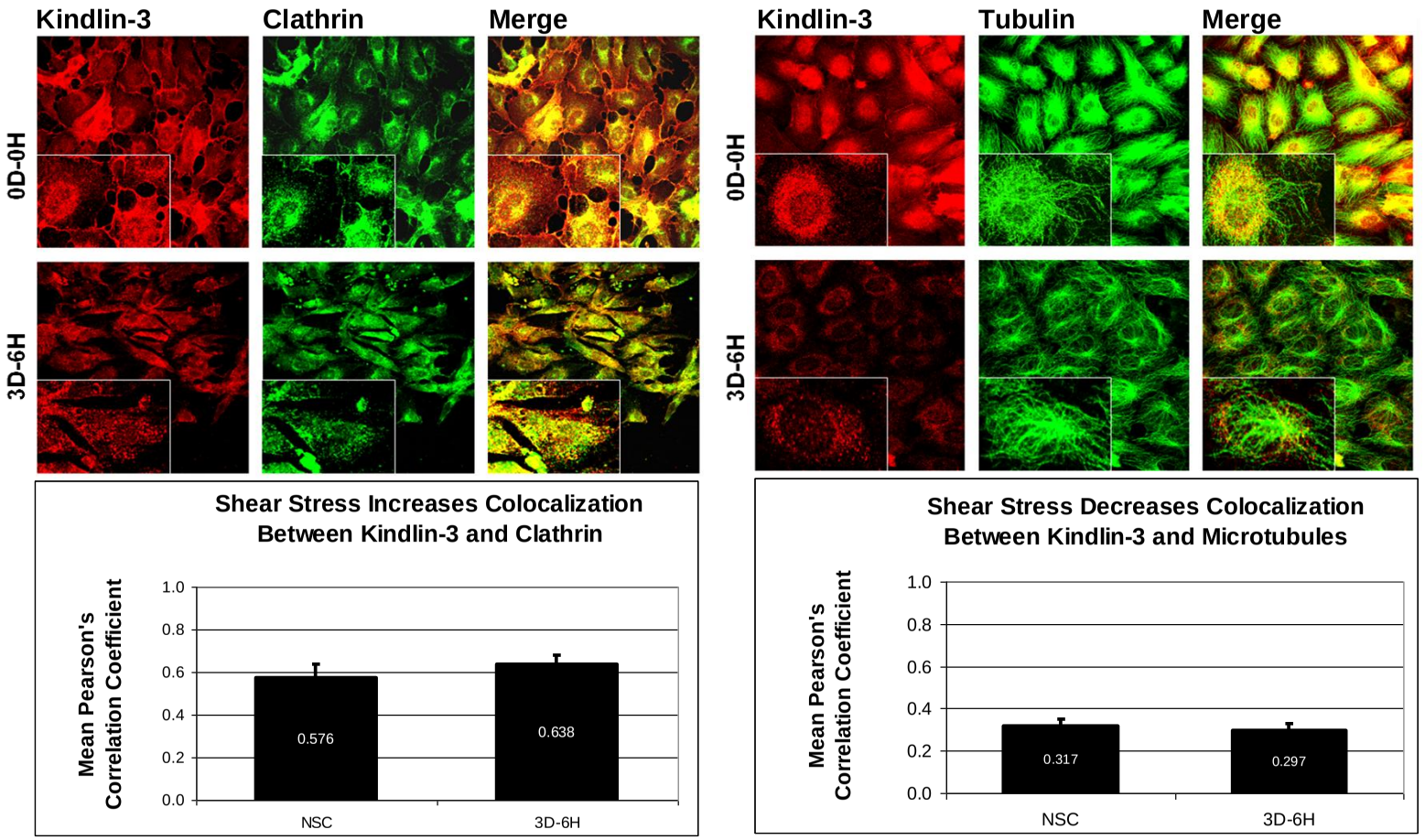


FIGURE 13. Quantitation of Colocalization between Kindlin-3 and Clathrin or Microtubules.

HUVECs attached to fibronectin were exposed to low for 6 hours to induce punctate pattern formation of kindlin-3 which was assessed for colocalization with either clathrin (A, B) or microtubules (C,D).

the cytoplasmic subcellular fraction, which may contribute to the microtubule/clathrin/integrin FA disassembly in a yet recognized manner (113).

5. Effect Of Atheroprotective Flow Regime On Kindlin-3 Distribution In ECs.

Our experiment revealed HUVECs plated on laminin display migratory phenotypes under non flow conditions. They present leading edges where more β_3 integrin subunits cluster. **FIGURE 14** shows under static conditions, kindlin-3 and β_3 integrin subunit do not colocalize on laminin or fibronectin. On laminin kindlin-3 is localized in the perinuclear distribution as noted in ECs on fibronectin; however, the peripheral membrane staining is less noticeable. In static cells, the β_3 integrin subunit is localized in the focal adhesion at the leading edge of the cells. In response to flow, kindlin-3 sustains predominate perinuclear staining on fibronectin, but is observed for the first time to localize in focal adhesions. Merges are provided to show that the kindlin-3 focal adhesions are not restricted only to β_3 integrin containing focal adhesions, but does colocalize in some of them. The rare occurrence of kindlin-3 in FA was observed at 1 hour in response to 10 and 12 dynes/cm^2. More time or greater shear rates may further enhance this effect. It is not clear whether the localization of kindlin-3 to FAs is due to changes in the conformational state of K3, from biosignaling from integrin-ligand complex mediated FA compositional changes, or other phenomena. However, the force induced recruitment of K3 to FA in ECs under high flow on laminin suggests K3 is a strong FA protein. More work is needed to determine the physiological significance of K3 FA localization in EC function relevant to atherogenesis.

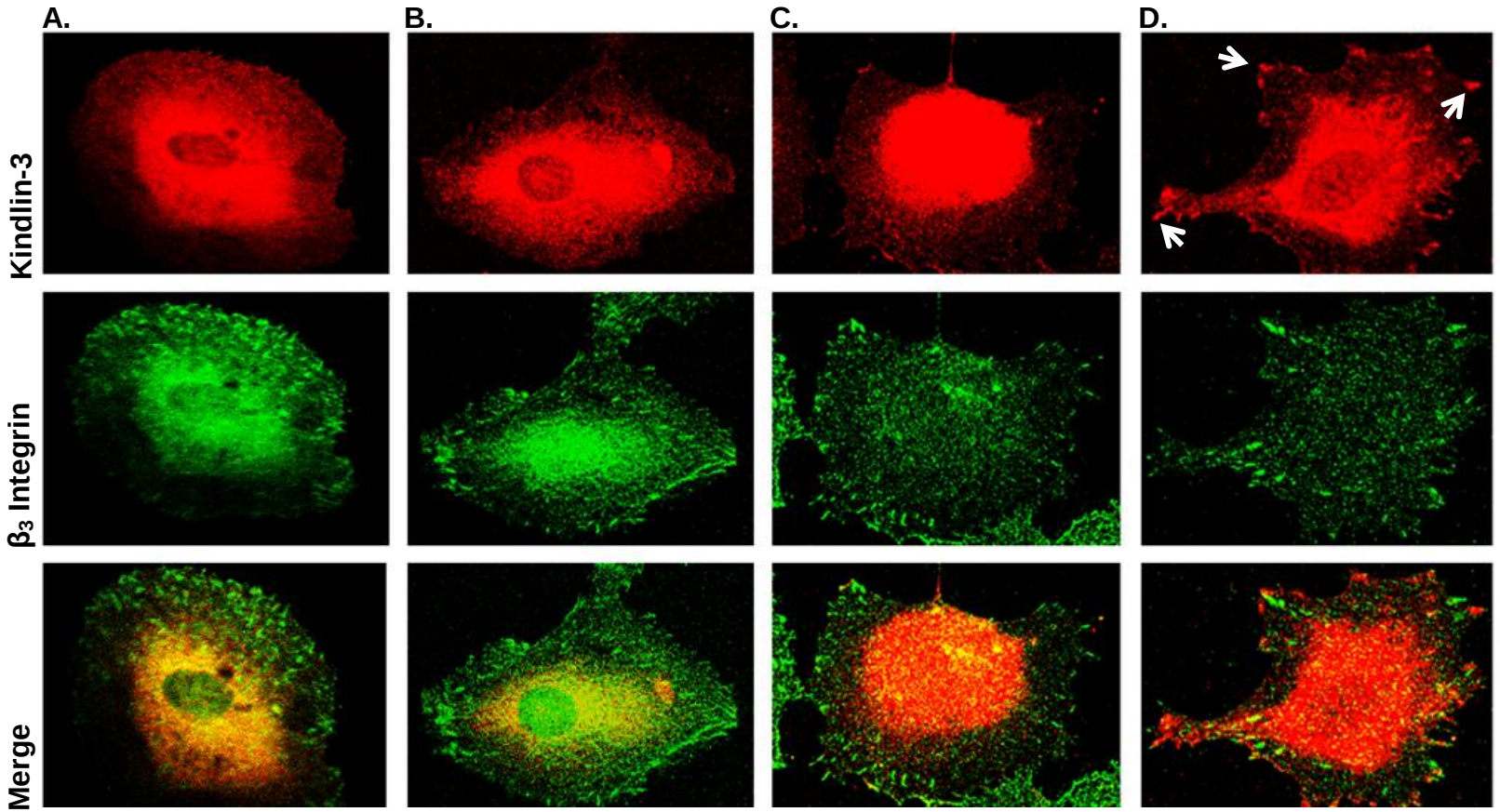


FIGURE 14. Shear Stress Promotes the Redistribution of Kindlin-3 into Focal Adhesions in HUVECS on Laminin, but not Fibronectin.

Confocal micrographs showing static and sheared HUVECs immunofluorescently labeled for detection of the distribution of kindlin-3 and β_3 integrin subunit on fibronectin (A-B) and laminin (C-D), respectively. The images were merged to demonstrate the localization of these of kindlin-3 in FAs at the cell periphery from which β_3 integrin positive FAs arise centripetally.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Due to the complex nature of the vascular system, the effects of flow on the vessel wall vary depending on the vessel composition and distance from the heart (4). To simulate specific vascular endothelial cell microenvironments, a number of specific parameters may be varied. These parameters include the shear applied, flow directionality (i.e. unidirectional, reversed flow; laminar or oscillatory), flow magnitudes (i.e. above or below physiological), exposure times, fluid composition (i.e. serum free or media content; +/- growth factors), extracellular matrix substrates and its pliability, and endothelial cell confluence (7,100,101). Since shear stress is a physical stimulus, many studies rely on observations of spatial changes in cell morphology as a determinate of temporal EC responsiveness to flow (102). Depending on the selected flow conditions and cellular environment, cells may not display gross morphological transformations after completely realigning in the direction of flow; however, biochemical events may still occur over time and be probed to assess endothelial cell responses to shear stress (107-109,111,114).

In vascular biology, the change in extracellular matrix proteins beneath the endothelial cell monolayer influences the development of atherosclerosis (115,116). Normally, the endothelium is underlined primarily with collagen and laminin, where $\alpha_2\beta_1$ integrin is the primary mediator of interaction with the former ligand and $\alpha_6\beta_1$, the latter (48). However, in atherosclerotic plaques the subendothelial ECM becomes reconstituted with increased deposition of fibronectin and fibrin(ogen) $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins play more dominant roles in substrate adhesion (100). The molecular consequences of the subendothelial matrix transition include increased expression of cytokines, reactive oxidative species, which activate NFkB, ICAM-1, VCAM-1, and further modification of the matrix composition by proteolysis mediated by matrix metalloproteinases (44,117-119). Therefore, the atherogenic state of the endothelium is influenced by shear stress, which is in turn regulated by ECM composition, integrin engagement, and ECM tensional state (54,55).

Our study used low flow, 3 dynes/cm^2, on fibronectin to create an atherogenic environment to examine endothelial cell function. Physiological flow, 10 dynes/cm^2, and normal basement membrane protein, laminin, were used in some experiments to examine rate and matrix dependent EC responses, respectively. We verified that the selection of 3 dynes/cm^2 was sufficient to promote responses in known markers of mechanosensitive proteins, zyxin and filamentous actin. In response to shear stress, zyxin redistributed from focal adhesions to actin filaments and actin reorganized in parallel to the flow direction, which was used to quantify cell alignment to flow (111). In this system, β_3 integrin and β_1 integrin remained present in focal adhesions in response to shear stress, unlike zyxin. These observations indicated that our apparatus and

parameters were capable of stimulating cellular responses that confirm force transmission was occurring. Knowing that kindlin-2 was present in focal adhesions under static conditions and that kindlin-3 might change its position to focal adhesions under flow, we felt that our system had the sensitivity and potential to identify and detect changes in the distribution of the kindlins.

The contributions this study has made to the body of knowledge regarding the effects of shear stress on endothelial cell function include determinations that: 1) shear stress affects kindlin-2 FA length, area, and number per cell, 2) shear stress increases the colocalization of kindlin-2 with VE-cadherin by redistribution of kindlin-2 into cell-cell junctions, 3) kindlin-3 is redistributed in response to shear stress into a punctate pattern, 4) shear stress induced kindlin-3 punctate pattern exhibits increased colocalization with clathrin, but does not increase colocalization with microtubules, 5) shear stress induced punctate formation and cleavage of kindin-3 is mediated by calpain and both are prevented by calpain inhibition, and 6) kindlin-3 localizes to FAs in ECs sheared under high flow on laminin.

Mechanosensation is followed by mechanotransmission of forces to cell structures including FAs where mechanoresponses regulate the cell mechanical state. The balance between intracellular and extracellular tension is regulated by adhesion sites at the cell-cell junction and cell-ECM interface. The transmission of forces along actin filaments induces conformational changes that permit compositional changes in adhesion sites to become modulated to provide the cell with the most appropriate strength characteristics to adapt to the mechanical environment. In response to shear stress, the cytoskeletal reorganization involves the realignment of actin filaments and

FAs in the direction of flow as well as enlargement of cell-cell junctions. When shear stress is low, a higher frictional drag is imparted on the EC surface than under high shear stress and consequently generates different cellular responses. Our observations implicate kindlin-2 as a weak focal adhesion protein because it is displaced from FAs in response to low force; characteristic of a slip bond. This is based on a reduction in kindlin-2 FA length, area, and quantity in response to flow. Coincidentally, the observed increase in localization of kindlin-2 in cell-cell junctions positively stained for VE-cadherin implicates kindlin-2 in cell-cell junction reinforcement, although we have not shown this relationship biochemically, we have shown an increase in colocalization between kindlin-2 and VE-cadherin by immunofluorescence. Changes in actin organization occur upstream of changes in kindlin-2 distribution and suggests the decrease in kindlin-2 FA number at 6 hours at low flow and the increase in kindlin-2 FA number at 6 hours at high flow are induced by force induced changes in organization. However, the amount of kindlin-2 FAs at high and low flow after 12 hours were similar to static levels which suggest feedback signals from changes in integrin activation states regulate the recruitment of kindlin-2 to FAs for FA stability when the cell mechanical state has adapted to the mechanical environment.

The mechanosensititvity of kindlin-2 in FAs can be characterized as inversely proportional to flow rate, whereby a decrease in flow promotes an increase FA size on fibronectin. The data suggests a transient role in FA reinforcement under acute low flow stimulation, where FA size is unchanged, followed by a reduction in kindlin-2 FA involvement. The transient increase in kindlin-2 FAs per cell may involve the mobilization of kindlin-2 into focal adhesions for integrin activation that would allow the

cell to generate traction forces necessary to resist the deformation of the cell morphology. Since flow applies more friction to the cell surface at lower flow rates, due to an increase in surface tension, the increase in kindlin-2 FAs may signify the importance of kindlin-2 in mediating force transmission to integrins from cytoskeletal rearrangements. This is speculated to occur by capacity for kindlin-2 to bridge to cytoskeletal linkage between filamentous actin and integrins. By this association, propagation of force through kindlin-2 may promote specific activation of integrin high affinity conformation that increases the number integrin-ligand bonds. Consequently, since integrin-ligand bonds are required for tuning the cell adhesion strength to appropriate levels of traction forces, the increase in kindlin-2 FAs in response to the mobilization of the surface tension along the cell surface may enable the cell to apply equal surface tension at the substratum (14, 44, 46). This is partially supported by the observation that high flow promoted a significant transient reduction in kindlin-2 FAs which may be because of a frictional slip that may occur in response to the reduction in surface tension at the flow onset (119). This is because static fluid imparts a higher surface tension, and therefore pressure, than flowing fluid on the cell surface. Thus, the higher the onset flow rate, the higher the resulting pressure drop across the cell surface, and ultimately the cell is transiently destabilized in a manner that enables polarization of the cell shape into the flow direction (64).

Many studies describe cell polarization as an event in cell migration, an integrin mediate process, which is attributable to the redistribution of actin, PIP2, talin, and integrins to the leading edge (120). Rac1 also is recruited in a polarized manner at the leading edge of migrating cells and this protein has been shown to be important in

kindlin function and may require exploration in the context of kindlin responses to shear stress (121-127).

Exogenous factors may also cue this polarization including VEGF (128, 129). Shear stress has been cited as VEGF mimetic and may involve stimulation of similar pathways involved in cell alignment to flow that occur during cell migration (130, 131). The effects of shear have been mapped and shown to vary with the degree of confluency since the presence of cell-cell adhesions provide cells with force redistribution patterns different from cells with subconfluent monolayers (132). When subconfluent, actin stress fibers and focal adhesion components were observed to be centripetally redistributed until reaching an orientation parallel to the flow direction in response to shear stress (132). In another example, it has been shown that β_3 integrins are involved in mechanotransduction responses that promote localization of β_3 integrins to the cell periphery and this is opposed to the role of β_1 integrins which accommodate a centralized cellular localization.

Furthermore, β_3 integrin activation stimulates VEGFR2 function, a well known participant in endothelial cell mechanosensory complex with VE-cadherin and PECAM-1. Since our study shows that K2 FAs are reduced in size in response to flow and that K2 staining is increased in junctions, thus increasing colocalization with VE-cadherin, it is conceivable that shear stress causes redistribution of kindlin-2 from FAs to cell-cell junctions through mechanisms that trigger cytoskeletal reorganization from the initiation of β_3 integrin activation. One such overlapping signaling event that may confirm the systematic relationship between junctions and integrin signaling is the activity of PI3K. As mentioned above, PI3K activity enriches the membrane with PIP3, which enhances

membrane recruitment of kindlin-2, and enhances integrin activation. Interestingly, shear stress activation of PI3K occurs downstream of VEGFR2 transactivation that depends on VE-cadherin mediated association with PECAM. β_3 integrin has been shown to interact with PECAM and VEGFR2 (48). Kindlin-2 likely toggles through conformations along the cell membrane at the regions of the cell where the localization of forces regulate its affinity for its binding partners, and thereby its distribution may be determined in response to force that are integrated by FA and cell junction dynamics. Since forces applied to cell-cell junctions was demonstrated to promote growth of VE-cadherin positive junctions in a manner involving Rac1, specific tugging experiments may be used to assess the changes in kindlin-2 distribution (133).

Until 2010, kindlin-3 was not known to be present in ECs. Since then, kindlin-3 binding partners in HUVECs have been unnoticeable until now. The transition of kindlin-3 into a punctate pattern in response to flow occurred at 3 hours and was significantly increased after 6 hours under low flow. In addition, cleavage of kindin-3 occurred in response to flow that trended with the occurrence of the punctate pattern. We show that this flow induced redistribution and cleavage of kindlin-3 was associated by calpain proteolytic activity. By blocking calpain activation, the production of the kindlin-3 punctate pattern and cleavage was prevented. Shear stress induced calpain cleavage of kindlin-3 is integrin activation-dependent (90). Shear stress activates integrin activation and our experiments implicate kindlin-3 cleavage as a biomolecular reporter of shear stress induced integrin activation in ECs.

It addition, kindlin-3 punctate pattern formation was shown to increase colocalization with clathrin. In this way kindin-3 presents functional homology with

kindlin-2. On laminin, kindlin-3 also exhibited functional homology with kindlin-2 in that kindlin-3 was observed to localize in FAs in response to high flow, which did not occur on fibronectin. The difference in kindlin-3 distribution in a matrix dependent manner implicates receptor-ligand dependent regulation of kindlin-3 distribution in a flow induced manner. The increase of kindlin-3 FA localization in response to increase mechanical force application is characteristic of catch-bond properties; where by the recruitment of kindlin-3 to FAs may represent a role in FA reinforcement. The mechanism of kindlin-3 FA formation is a not well understood. Since this is the first time it has been shown that kindlin-3 exists as a FA protein in ECs, future work will be needed to elucidate specific approaches to induce more kindlin-3 FA formation and to determine the physiological significance of the shear stress induced changes which may be important for understanding the role of EC function in the development of vascular pathologies involving altered blood flow.

Thus, my work has shown that kindlin-2 and kindlin-3 function differently in response to mechanical stimuli and demonstrates they belong to different categories of the mechanotransductome of shear stress responsive proteins in ECs. Based on their different responses to flow that changes their distributions within focal adhesions, kindlin-2 may be involved in slip bonds, whereas kindlin-3 may function in catch bonds. The function of these stress induced characteristics is not fully known. Since kindlins may interact with several force responsive cell structures, including the cell membrane, actin cytoskeleton, and integrin short cytoplasmic tails, it is anticipated that this work will provide insight into how to investigate the mechanobiology of the endothelium using kindlin as mechanosensors of the flow response. Endothelial cells provide the primary

protection against atherogenesis and are the initial site of injury targeted for atherosclerosis (134). Cells behave differently under stress. We now know kindlins do also. Kindlins may serve as therapeutic targets for regulation of integrin-mediated functions that contribute to improved vascular health via targeting desired endothelial cell mechanics in vascular regions where flow regimes demand modifications. Furthermore, future work should emphasize determining how specific protein expression levels of kindlin and kindlin mutants provide tunable regulation of cell mechanics that depend on interactions between kindlins and force bearing structures in the cell. The accomplishment of this goal would likely provide mechanisms of engineering flow-resistant endothelium-coated stents that graft artificial sustainability in regions with natural vulnerability to plaque formation and rupture.

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