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In Development ...A New Paradigm for Understanding Vascular Disease

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Abstract

Under physiological conditions, the arterial endothelium exerts a powerful protective influence to maintain vascular homeostasis. However, during the development of vascular disease, these protective activities are lost and dysfunctional endothelial cells actually promote disease pathogenesis. Numerous investigations have analyzed the characteristics of dysfunctional endothelium with a view to understanding the processes responsible for the dysfunction and to determining their role in vascular pathology. The present review adopts an alternate approach: reviewing the mechanisms that contribute to the initial formation of a healthy protective endothelium and on how those mechanisms may be disrupted, precipitating the appearance of dysfunctional endothelial cells and the progression of vascular disease. This approach, which highlights the role of endothelial adherens junctions and VE-cadherin in endothelial maturation and endothelial dysfunction, provides new insight into the remarkable biology of this important cell layer and its role in vascular protection and vascular disease.

Keywords

VE-cadherin; adherens junctions; endothelial dysfunction; postnatal development; aging; developmental origins of vascular disease; atherosclerosis

Normal endothelial function is crucial for maintaining vascular and organismal health. Endothelial cells are key regulators of blood vessel constriction, thrombogenicity, inflammation, permeability, and vascular remodeling. Under normal conditions, the endothelium exerts a protective influence to inhibit these processes and maintain vascular stability and homeostasis. However, during the development of vascular disease, endothelial cells with an altered phenotype, namely 'dysfunctional' endothelium, promote these same processes and contribute to pathological changes in vascular structure and reactivity. Numerous investigations have analyzed and described characteristics of dysfunctional endothelium, with a view to understanding the processes responsible for the dysfunction and to determining their role in the development of vascular disease. The present review will focus on an alternate approach: discussing the mechanisms that contribute to the initial formation of a healthy protective endothelium and on how those continuing mechanisms can

be disrupted contributing to the emergence of dysfunctional endothelial cells and the progression of vascular disease. The approach emphasizes that the protective activity of the vascular endothelium is an active rather than passive function of the endothelium, and that disruption of those active mechanisms can precipitate pathological changes in endothelial and vascular activity.

The Normal Maturation of Protective Arterial Endothelium

The early postnatal period is associated with remarkable changes in arterial structure and function, including the morphology and activity of endothelial cells. The endothelial cells lining fetal or newborn central arteries have a highly unusual phenotype. They have poorly-organized cellular junctions and prominent transcytoplasmic actin stress fibers (Figure 1) (1–8). Although common in cultured cells, native arterial endothelial cells normally lack stress fibers, and the actin cytoskeleton is restricted to a faint cortical network at the cell periphery (1, 4, 9, 10). Endothelial stress fibers are however a prominent feature in vascular disease and in areas susceptible to atherosclerosis (9, 11). Despite high expression levels of endothelial NO synthase (eNOS), endothelial cells lining fetal or newborn arteries have an impaired ability to initiate NO-mediated endothelial dilatation when compared to mature arteries (Figure 2) (7, 12–19). For example, in newborn mice carotid arteries, endothelial stimulation by acetylcholine, thrombin or A23187 was either poorly able or unable to generate endothelium-dependent dilatation (Figure 2) (7, 8, 20). Indeed, thrombin or A23187, which are powerful endothelial secretagogues, actually caused rapid constrictions of newborn arteries that were capable of causing complete occlusion of the arterial lumen (Figure 2) (20). These constrictions were mediated by endothelin-1 (ET-1) generated by the newborn endothelium, and were abolished by antagonizing ET-1 receptors (ET_A or ET_A plus ET_B receptor antagonists), by inhibiting endothelin converting enzyme (ECE) or by endothelial denudation (Figure 2) (20). Indeed, endothelial cells lining newborn central arteries had high expression of ET-1 peptides and rapidly generated and released ET-1 in response to endothelial activation (20). Therefore, endothelial cells lining newborn arteries have highly unusual morphological and functional features, which are reminiscent of dysfunctional endothelium. Indeed, the extent of the impairment in NO-mediated dilatation and intensity of ET-1 mediated constriction of these immature arteries was far greater than was observed during endothelial dysfunction (20, 21).

The unusual structural and functional features of newborn arterial endothelial cells change dramatically during the first few weeks of postnatal life as the cells acquire normal protective features. Morphologically, the actin cytoskeleton transforms from transcytoplasmic stress fibers to formation of a cortical actin network, and the endothelial intercellular connections become more highly organized (Figures 1) (1–8). Thrombin or A23187 no longer evoke endothelium-dependent constriction and instead generate endothelium-dependent dilatation (Figure 2), which is paralleled by a diminution in endothelial expression of ET-1 peptides and a loss in the stimulated generation and release of ET-1 (20). Despite a gradual decrease in endothelial eNOS expression in this immediate postnatal period, there is a dramatic increase in endothelium-dependent NO-mediated dilatation (Figure 2) (7, 20). The emerging endothelium-dependent NO-mediated dilatation

evoked by acetylcholine was associated with increased phosphorylation of eNOS (Ser¹¹⁷⁷) and abolished by inhibition of phosphoinositide-3-kinase (PI3K)/Akt signaling (7).

Signaling through the Rho family of GTPases have divergent roles in regulating endothelial morphology and function. RhoA and its downstream effectors, in particular Rho kinase (ROCK), stimulate endothelial stress fiber formation and attachment to the extracellular matrix, via focal adhesions (22–25). ROCK inhibits myosin light chain (MLC) phosphatase, increases levels of phosphorylated MLCs and facilitates actomyosin interaction. The contractile force generated by this ROCK-dependent actomyosin interaction contributes to disruption of intercellular endothelial junctions (22–26). In contrast, activation of Rac1 via PI3K causes disassembly of stress fibers and focal adhesion complexes and stimulates assembly of a cortical actin network and strengthening of intercellular adhesion (24, 27–31). The divergent effects of PI3K and RhoA/ROCK pathways are also apparent in the regulation of eNOS. ROCK inhibits eNOS activity by suppressing PI3K-dependent activation of Akt and eNOS Ser¹¹⁷⁷ phosphorylation (32, 33). ROCK can also reduce eNOS mRNA stability and promote eNOS protein uncoupling (32, 34, 35). Not surprisingly, endothelial Rho/ROCK signaling is considered to be minimal under normal physiological conditions, but contributes to endothelial dysfunction during the initiation of vascular disease (36, 37).

The unusual morphology and function of newborn endothelium is mediated by heightened activity of endothelial Rho/ROCK signaling (8). Endothelial cells lining newborn carotid arteries have increased levels of phosphorylated MLCs, which is associated with the prominent actin stress fibers, compared to more mature arteries (8). Acute inhibition of Rho or ROCK in isolated newborn arteries reduced the levels of phosphorylated MLCs and reorganized the endothelial actin cytoskeleton: the transcytoplasmic stress fibers were reduced and filamentous actin was restricted to a cortical actin network in the cell periphery (Figure 1) (8). Inhibition of Rho or ROCK also markedly increased endothelial NO-mediated dilatation to acetylcholine in newborn carotid arteries (Figure 2) (8). However, in more mature arteries, inhibition of Rho or ROCK had no effects on endothelial morphology or function (8). Therefore, acutely inhibiting Rho and ROCK signaling in newborn endothelial cells mimics the structural and functional maturation process of neonatal endothelial cells. Heightened activity of Rho/ROCK signaling in newborn endothelium, including in combination with reduced NO activity, may also contribute to increased expression and activity of ET-1 (20, 38).

Increased Rho/ROCK activity in newborn endothelial cells may reflect an unusual sub-endothelial matrix in newborn arteries. Native endothelial cells normally reside on a matrix composed primarily of laminin and collagen IV (39). However, the subendothelial matrix in fetal or newborn arteries is enriched in fibronectin, with endothelial stress fibers connecting via focal adhesion complexes to fibronectin fibrils (3, 5, 6, 40). As endothelial cells mature during the immediate postnatal period, they reorganize their underlying matrix, decreasing the levels of fibronectin and increasing laminin and collagen IV (1, 3, 4, 40). Fibronectin promotes activation of RhoA but not Rac1, whereas laminins enable persistent activation of Rac1 and suppression of RhoA (39, 41). As a result, endothelial adhesion to fibronectin stimulates formation of stress fibers and strong focal adhesions, which is mediated by Rho/ROCK signaling (41). Increased deposition of fibronectin may also contribute to increased

Rho/ROCK signaling and the presence of actin stress fibers in dysfunctional endothelium during vascular disease (9, 42–44). Additional mechanisms may contribute to Rho/ROCK signaling under both conditions, including increased generation of reactive oxygen species (ROS) (7, 15, 45–48)

The neonatal maturation of arterial endothelium therefore appears to be associated with a change in endothelial signaling from heightened Rho/ROCK activity in newborn to increased PI3K/Akt/eNOS signaling in maturing endothelium (7, 8). Although Rho/ROCK can inhibit endothelial NO activity through multiple mechanisms, a key inhibitory step appears to be activation of PTEN, which counteracts PI3K activity and reduces Akt activity (33, 49, 50). Therefore, the emerging activity of PI3K/Akt/eNOS signaling in the immediate postnatal period may reflect a diminution in Rho/ROCK and PTEN signaling. This possibility is also evident from the ability of Rho/ROCK inhibition to mimic the process of developmental endothelial maturation (8).

The unusual phenotype of endothelial cells lining fetal and newborn central arteries including prominent expression, production and contractile activity of ET-1, the presence of stress fibers and a provisional fibronectin matrix, minimal NO-mediated dilatation, and heightened Rho/ROCK signaling, is highly reminiscent of dysfunctional endothelial cells that contribute to vascular disease (7, 8, 20). Indeed, eNOS may be uncoupled in neonatal endothelial cells and be associated with increased production of ROS (7, 15, 45, 46). This unusual endothelial phenotype may reflect a developmental requirement for embryonic and fetal growth, including a role for ET-1 in the development of the heart and great vessels (51–53). Presumably, the fetal and newborn cardiovascular system are protected from this unusual endothelial phenotype because of concomitant immaturity in other systems, including inflammatory and thrombotic processes. However, the presence of this immature endothelial phenotype in an adult setting would be expected to promote pathological effects within the cardiovascular system. Therefore, mechanism(s) responsible for initiating and maintaining the protective maturation of arterial endothelium are likely to be of fundamental importance to vascular health.

Endothelial adherens junctions are formed by engagement and clustering of VE-cadherin (CD144) molecules on adjacent endothelial cells (Figure 1). This clustering of VE-cadherin stimulates and supports the assembly of a macromolecular signaling complex that regulates endothelial morphology and function, including inhibition of RhoA/ROCK signaling and amplification of PI3K/Akt signaling (27, 54–59). The width and complexity of endothelial adherens junctions increases during the early postnatal period, consistent with increased clustering of VE-cadherin (Figure 1) (7). To determine whether this might contribute to endothelial maturation, neonatal arteries were incubated with a function-blocking antibody to VE-cadherin, which inhibits VE-cadherin clustering and so disrupts adherens junctions (7). Blocking VE-cadherin clustering inhibited the emergence in neonatal arteries of acetylcholine-induced increases in endothelial NO-mediated dilatation and eNOS phosphorylation, suggesting that increased VE-cadherin and junctional signaling contributes to endothelial maturation (7). This would be consistent with increased clustering of VE-cadherin at adherens junctions acting to reduce Rho/ROCK signaling and amplify PI3K/Akt signaling. The early postnatal maturation of central arterial endothelium occurs in the face of

dramatic increases in blood pressure but with relatively stable blood flows in these central arteries (4, 60, 61). Indeed, morphological maturation of the endothelium, including reorganization of the actin cytoskeleton, was proposed to reflect the hemodynamic effects of increasing pressure (4). Remarkably, when isolated newborn mice carotid arteries were exposed to a transient increase in transmural pressure, which was equivalent to mean blood pressure of a 1 week old mouse, the width and complexity of adherens junctions were increased, mimicking the maturation process (Figure 1) (7). Likewise, the transient increase in transmural pressure dramatically amplified endothelial NO-mediated dilatation to acetylcholine in newborn arteries, which was associated with increased phosphorylation of eNOS (Ser¹¹⁷⁷) and was abolished by inhibition of PI3K/Akt signaling (Figure 2) (7). The function-blocking antibody to VE-cadherin markedly reduced responses evoked by the transient increase in pressure (Figure 2) (7). Therefore, adherens junctions appear to be involved in a novel endothelial mechanotransduction process, with stretch or developmental increases in blood pressure causing increased clustering of VE-cadherin (7). Indeed, in cultured endothelial cells, tugging forces stimulate the growth and stabilization of adherens junctions (62). These results suggest that increasing pressure in developing arteries, through a stretch-mediated increase in clustering of VE-cadherin at endothelial adherens junctions leads to amplification of PI3K-Akt signaling, emergence of eNOS signaling and NO-mediated dilatation in the immediate postnatal period. Increased clustering of VE-cadherin at adherens junctions and VE-cadherin-dependent signaling therefore appears to be of fundamental importance for the protective maturation of arterial endothelium and for actively maintaining vascular homeostasis and stability (7, 63, 64).

Role and Regulation of VE-cadherin and Adherens Junctions in Arterial Endothelial Cells

VE-cadherin-dependent signaling at endothelial adherens junctions promotes barrier function, amplifies NO activity, inhibits apoptosis, maintains endothelial differentiation, and inhibits inflammatory activation including inhibiting leukocyte extravasation and the expression of inflammatory mediators (Figure 3) (7, 55, 63, 65, 66). These effects reflect the assembly of a signaling complex in response to junctional clustering of VE-cadherin (27, 54–56, 67). Adherens junctions are stabilized by the interaction of VE-cadherin with catenins including β -catenin, which along with α -catenin connects the adhesion complex to the actin cytoskeleton, and p120 catenin, which inhibits VE-cadherin internalization (27, 65). The clustering of VE-cadherin and β -catenin at adherens junctions recruits additional proteins that stabilize the junctions including Rac1 along with regulators that increase or maintain Rac1 activity (Tiam1, Vav, IQGAP1), and P190RhoGAP, which inhibits RhoA (27, 54–56, 59, 65). Rac1 stimulates assembly of a cortical actin network and maintains junctional stability, whereas RhoA/ROCK promotes actin stress fiber formation and inhibits adherens junctional stability (24, 27, 67). VE-cadherin and β -catenin also interact with and regulate the activity of additional signaling proteins and pathways including PI3K/Akt, growth factor receptors including for VEGF, several non-receptor tyrosine kinases and phosphatases, and additional small G-proteins such as Rap1 (63, 68, 69).

VE-cadherin appears to be a crucial and versatile mediator of endothelial responses to hemodynamic forces. Shear stress stimulates interaction between VE-cadherin, PECAM1 (CD31) and VEGFR2, which contributes to flow-induced activation of eNOS (70, 71). This shear stress mechanotransduction pathway is thought to initially involve PECAM-1 with subsequent transactivation of VEGFR2, leading to VEGFR2-dependent activation of PI3K/Akt, increased eNOS (Ser¹¹⁷⁷) phosphorylation and NO production (70–75). Although PECAM-1^{-/-} arteries have reduced dilatation to shear stress, responses to acetylcholine are normal, suggesting this proposed pathway may be specific for shear stress (76). Within this model, VE-cadherin:β-catenin acts merely as a scaffolding intermediary to facilitate the transactivation of VEGFR2 (72, 73, 77). Indeed, shear stress-induced signaling does not actually require junctional localization of VE-cadherin or PECAM-1 and is not inhibited by VE-cadherin function blocking antibodies (65, 71, 78). This role is therefore distinct from the activity of VE-cadherin in mediating pressure-induced and developmental maturation of endothelial dilatation in neonatal arteries, which required junctional localization of VE-cadherin (7). In addition to facilitating VEGFR2-dependent activation of PI3K/Akt signaling, VE-cadherin can directly activate PI3K/Akt signaling independently of VEGFR2 (55–57). Moreover, this direct effect is dependent on VE-cadherin clustering at adherens junctions and is inhibited by function-blocking antibodies to the protein (55–57). Interestingly, in cultured endothelial cells, shear stress activates Akt in a VEGFR2-dependent manner, whereas pulsatile stretch activates Akt independently of VEGFR2 (71, 73–75, 79). This latter effect is likely mediated by clustering and activation of VE-cadherin-dependent signaling at adherens junctions. Therefore, stretch and shear stress activate distinct signaling pathways in endothelial cells, both of which are dependent on VE-cadherin.

Endothelial adherens junctions are a primary target during inflammatory responses: inflammatory cells and mediators disrupt endothelial adherens junctions by initiating tyrosine phosphorylation of VE-cadherin and its binding partners, causing dissociation of the signaling complex (Figure 3) (27, 55, 63, 68, 80, 81). Although tyrosine phosphatases, including vascular endothelial protein tyrosine phosphatase (VE-PTP) and SHP2, interact with VE-cadherin and β-catenin to stabilize the junctions, inflammatory stimuli cause dissociation of phosphatases from the complex (55, 82, 83). Src tyrosine kinase is primarily responsible for tyrosine phosphorylation of VE-cadherin (27, 81, 84, 85). Inflammatory stimuli activate and increase the association of Src with VE-cadherin and adherens junctions (81, 85). Once phosphorylated, VE-cadherin is ubiquitinated, then internalized via clathrin-mediated endocytosis for lysosomal degradation (81, 86). Inflammatory mediators can also disrupt adherens junctions by causing proteolysis of VE-cadherin, in particular its extracellular domain (68, 87, 88). Disruption of endothelial adherens junctions by inflammatory mediators reduces their protective influence in endothelial cells and increases endothelial permeability, inflammatory cell extravasation, and the vascular and organismal sensitivity to inflammatory stimuli (Figure 3) (89–91). Reduced activity of endothelium-derived NO and increased activity of Rho/ROCK as a result of diminished VE-cadherin signaling would be expected to amplify this pro-inflammatory activity.

Junctional disruption can also contribute directly to the activity and expression of inflammatory mediators. Once freed from its interaction with VE-cadherin, β-catenin can act

as a transcriptional co-regulator by translocating to the nucleus and binding to TCF4 (Figure 3) (92). Although free β -catenin is normally targeted for degradation by a destruction complex, numerous mechanisms enable β -catenin to escape degradation and translocate to the nucleus (25, 55, 92). β -catenin:TCF4 transcriptional activity increases expression of key pathological and inflammatory mediators including the renin angiotensin system (angiotensinogen, renin, angiotensin converting enzyme, AT1 receptors), ET-1, TNF α , IL-6, iNOS, fibronectin, COX-2, MMP2, MMP9 (Figure 3) (93–99). Complementing the actions of these mediators, the disassembly of junctional signaling complexes may directly contribute to endothelial oxidant stress. Rac1 has conflicting roles within the endothelium. VE-cadherin clustering at adherens junctions causes Rac1 to localize at cell junctions, where it stimulates assembly of a cortical actin network, maintaining junctional stability and vascular integrity (27, 54–56). However, Rac1 also contributes to activation of NADPH oxidase (NOX1, NOX2) and increased ROS production (100). Disruption of adherens junctions causes Rac1 to dissociate from the VE-cadherin complex (54, 56), and can increase ROS activity (101), potentially by enabling Rac1 to contribute to NOX activity. Therefore, junctional disruption by inflammatory mediators can amplify inflammatory activity and pathological signaling by contributing to generation of additional inflammatory mediators and oxidant stress (84, 85, 102, 103).

Disruption of endothelial adherens junctions and release of β -catenin is also a key initial step in endothelial mesenchymal transition (EndoMT or EndMT), the trans-differentiation of endothelial cells to a mesenchymal cell phenotype (Figure 3) (104–108). The process is characterized by a progressive loss in endothelial proteins (e.g. VE-cadherin, von Willebrand Factor) and acquisition of mesenchymal markers (e.g. smooth muscle α -actin, SM22, FSP-1) (107, 109, 110). TGF β and Smad2/3 signaling are considered central mediators of EndoMT and cause increased expression of transcriptional regulators (Snail1, Snail2/Slug, Twist) that suppress VE-cadherin and upregulate mesenchymal genes (92, 104, 107, 109–111). β -catenin is also a dynamic transcriptional mediator of EndoMT, increasing expression of Snail1, Snail2, and Twist, and amplifying the activity of other transcription factors including Smads (92, 104, 111). EndoMT-derived cells have a highly invasive and proliferative phenotype, with high fibrogenic activity and marked production of collagens (I and III) and fibronectin (104–107, 112, 113). Indeed, EndoMT can contribute to intimal lesions, interstitial fibrosis and microvascular rarefaction (104–107, 110, 112–118).

Uncontrolled disruption of endothelial adherens junctions can therefore have severe consequences for vascular health (Figure 3). Such disruption can not only reduce protective signaling and amplify the activity of pathological and inflammatory mediators, it can also threaten the stability and viability of endothelial cells and the blood vessel wall (64, 65).

Adherens Junction Disruption, Endothelial Dysfunction and Vascular Disease

The neonatal period appears to be a crucial transition phase for increased engagement and signaling of VE-cadherin at endothelial adherens junctions, resulting in endothelial maturation and full expression of endothelial protective activity (7). This protective process

continues into adulthood with VE-cadherin and adherens junctions promoting vascular stability and homeostasis (Figure 3) (64, 65). Therefore, interruption of this process in developing arteries or its reversal in mature arteries could be equivalent to enabling the retention or re-emergence of the newborn endothelial phenotype, and would be expected to promote endothelial and vascular destabilization and progression of vascular disease. Although this concept is likely valid for numerous vascular diseases, the following examples are selected to highlight the potential role of endothelial junctional disruption on pathological processes initiated in an adult setting (Vascular Aging), in a developmental setting ('Developmental Origins of Vascular Disease'), and in the complex combined setting of Atherosclerosis, which begins in childhood and progresses throughout our lifetime.

The Aging Arterial System

Old age is associated with a structural and functional deterioration of the arterial system that contributes to organ dysfunction and progression of cardiovascular disease. Endothelial dysfunction is a key contributor to this process of vascular aging (122, 123). Aging arterial endothelial cells are severely compromised: their production of protective NO is reduced, their barrier function is impaired, they are highly susceptible to apoptosis, and they have a prominent pro-inflammatory phenotype, including production of pathological mediators such as ROS, angiotensin II (ANGII), cytokines (e.g. TNF α , IL-6), and ET-1 (21, 123–140). Aging endothelial cells are therefore caught in a cycle of chronic stress, both producing and responding to pathological mediators, and their resulting frailty is considered an important instigator of vascular aging (123, 141).

A key aspect of vascular aging is central arterial stiffening, which results from increased fibrotic and inflammatory activity that transforms the arterial wall into a less compliant structure. Elastic central arteries normally expand during systole to store a portion of the heart's stroke volume, which is then released during diastole to support continuous organ blood flow (142–145). This process is facilitated by a partial reflection of the pulse wave, which normally returns during diastole to augment central diastolic pressure (142–145). However, in aging, remodeling of central arteries reduces their compliance and increases pulse wave velocity, causing the reflected wave to return during systole and resulting in summation of the incident and reflected waves, which augments central pulse or systolic pressure (142–145). This disruption in central arterial dynamics can cause systemic vascular dysfunction and precipitate or exacerbate cardiovascular diseases including hypertension (142–145).

Aging central arteries have prominent intimal fibrous lesions with deposition of poorly-distensible proteins, collagen (I, III) and fibronectin, and degradation of highly-distensible elastin fibers (127, 134, 137–139, 146–149). Stiffness is further enhanced by increased fragility and reduced compliance of aging elastin fibers and by increased crosslinking and resilience of collagen and fibronectin matrices (127, 150). The expression of key inflammatory and fibrotic mediators is localized predominantly to endothelial cells and intimal smooth muscle cells (SMCs), and appears to be coordinated by local generation of ANGI (135, 137, 139, 140, 148, 149, 151, 152). ANGI-dependent increases in fibrotic (e.g. TGF β), inflammatory (e.g. MCP-1, TNF α) and proteolytic activity (e.g. MMP2, MMP9) by

endothelial and intimal SMCs drive the remodeling process by sustaining inflammatory stress, by promoting the secretory and invasive phenotype of intimal SMCs and by degrading elastin (135, 137, 139, 140, 148, 149, 151–153).

We have observed that endothelial adherens junctions are severely compromised in aging arteries with internalization and degradation of VE-cadherin (22–24 month old compared to 3–4 month old F344 rat aorta and tail arteries) (Figure 1) (154, and unpublished observations). Endothelial NO-mediated dilatation evoked by acetylcholine was significantly decreased in old compared to young arteries, although responses to an NO donor were not affected by aging. A function blocking antibody to VE-cadherin, which blocked clustering of the protein at adherens junctions, inhibited endothelium-dependent dilatation to acetylcholine in young arteries, but did not affect the already reduced response to the agonist in old arteries (154). These results are consistent with the structural disruption of endothelial adherens junctions and suggest that the functional activity of VE-cadherin and adherens junctions are diminished in aging arteries. Interestingly, in the presence of the VE-cadherin function blocking antibody, we no longer observed any significant difference between dilatation to acetylcholine in young and aged arteries (154). Therefore, the disruption in activity of VE-cadherin and adherens junctions in aging arteries can account entirely for the aging-induced impairment in endothelial dilatation to acetylcholine.

In addition to contributing to a diminution in endothelial NO-mediated dilator activity, an aging-induced disruption in endothelial adherens junctions could contribute to other aspects of endothelial and vascular dysfunction present in aging arteries, including increased endothelial permeability and apoptosis, and increased activity of inflammatory mediators. Indeed, β -catenin/TCF4 transcriptional activity increases expression of key pathological mediators, including the renin angiotensin system (angiotensinogen, renin, angiotensin converting enzyme, AT1 receptors), ET-1, TNF α , IL-6, iNOS, fibronectin, COX-2, MMP2, MMP9 (93–99), that are increased in aging endothelium and contribute to vascular aging (Figure 3) (132, 137, 141, 148, 151). Although transcriptional activity of β -catenin is minimal in normal arterial endothelium (25, 95, 155), it may contribute to cellular and organismal aging (156–159). β -catenin and β -catenin/TCF4 can interact with and regulate the activity of other transcriptional factors, including FOXO1 and NF κ B (57, 92, 111, 160). NF κ B activity is known to be increased in aging endothelial cells and is considered a key mediator of their inflammatory activity (161–164). During oxidant stress, β -catenin can switch from TCF4 to FOXO leading to expression of FOXO-dependent genes, which may also contribute to vascular aging (157, 158).

The intimal lesion of aging central arteries is formed by fibrogenic SMCs (127, 141). When cultured in vitro, ‘SMCs’ from aging arteries have increased proliferative, migratory and secretory capacity compared to ‘SMCs’ from young arteries, including increased ROS production, increased NF κ B activity, and increased expression of inflammatory and fibrotic mediators (135, 137–141, 148, 149, 151, 153, 164–167). In the intact artery, the increased expression of inflammatory and fibrotic mediators mostly localize to endothelial and intimal ‘SMC’-like cells (135, 137, 139, 140, 148, 149, 151), and these cells are considered to play a dominant pathological role in aging arteries promoting the remodeling and stiffening of the arterial wall. In contrast to expanding intimal SMCs, the mature contractile SMCs in the

media appear to have reduced viability in aging arteries (134, 139, 141). The intima of aging arteries should present a highly permissive environment for EndoMT. The expression and activity of TGF β 1 is increased in aging endothelial and intimal 'SMCs' (137, 149). Combined with the disruption of adherens junctions and release of β -catenin, aging endothelial cells are likely exposed to synergistic activation of the EndoMT process, which could contribute to vascular and interstitial fibrosis and to microvascular rarefaction in aging. Indeed, EndoMT-derived cells rather than traditional SMCs may be responsible for the intimal lesions and increased stiffness of aging central arteries.

Therefore, an aging-induced disruption of endothelial adherens junctions and VE-cadherin-dependent signaling could be a major driving force behind the process of vascular aging.

Atherosclerosis

Atherosclerotic lesions reflect focal expansion of the arterial intima by vascular and immune/inflammatory cells, extracellular matrix proteins, native and modified lipids, and cellular debris (206–209). Lesions develop preferentially at sites of disturbed blood flow, such as arterial branches or curvatures, where normal high laminar shear stress is distorted by flow separation and directional changes, resulting in low and reciprocating shear stress (209–213). Although exposure of cultured endothelial cells to prolonged normal shear stress is anti-inflammatory and atheroprotective, disturbed or atheroprone shear stress stimulates expression of inflammatory and thrombotic mediators (44, 209–213). Atheroprone shear stress increases transcriptional activity of NF κ B, which is considered a master regulator of the atherosclerotic process, resulting in increased endothelial expression of its target genes including inflammatory mediators ICAM-1, VCAM-1 and MCP-1 (monocyte chemoattractant protein) (42, 44, 214, 215). Likewise, NF κ B activity and expression of these inflammatory mediators is increased in atherosclerosis-prone regions of the vasculature, and occurs before lesion formation suggesting they are very early events in the atherosclerotic process (44, 214, 215). Disturbed blood flow patterns can therefore sustain local inflammatory activity that provides the basis for atherosclerotic lesion development (44, 209–213). The atherosclerotic process begins in childhood and progresses through adolescence and young adulthood into middle age (119–121). As described above, the early postnatal maturation of arterial endothelial cells is associated with reorganization of the actin cytoskeleton from transcytoplasmic stress fibers to a cortical actin network and with increased organization and clustering of VE-cadherin at adherens junctions (7, 8). These interdependent processes enable protective maturation of the endothelium (7, 8). However, in arterial regions prone to atherosclerosis, endothelial cells retain transcytoplasmic stress fibers and have poorly organized adherens junctions (1, 4, 95, 213, 216). An interruption in endothelial maturation and disruption of adherens junctions and VE-cadherin-dependent protective signaling in these lesion-prone sites is likely a key driving force for the initiation and progression of the atherosclerotic process (Figure 4).

Arterial regions prone to atherosclerosis are sites of increased endothelial proliferation and increased endothelial permeability to macromolecules including low density lipoprotein (LDL) (209, 210, 212, 217), both effects consistent with reduced junctional clustering of VE-cadherin (Figure 4). Indeed, atheroprone shear stress disrupts adherens junctions leading

to formation of gaps and increased endothelial permeability (95, 213, 215). The disruption in the endothelial barrier and the subendothelial deposition and modification of apoB-containing lipoproteins, predominantly LDL, is considered an important early amplification step in the atherosclerotic process (206–208). Modified lipids, as with atheroprone shear stress, cause inflammatory activation of the endothelium reducing the activity of protective mediators such as NO and increasing activity of inflammatory mediators including NF κ B, ROS, VCAM-1, MCP-1 and MCSF (macrophage colony stimulating factor) (206–209). Indeed, a high cholesterol diet strongly amplifies the inflammatory activity of the endothelium at atherosclerosis-prone regions (44). Inflammatory mediators increase recruitment of monocytes, and stimulate their subsequent survival and differentiation to macrophages (206–209). The transmigration of leukocytes including monocytes across the endothelium is critically dependent on disruption of adherens junctions (80, 218, 219). As with atheroprone shear stress, oxidized lipids derived from modified LDL disrupt endothelial adherens junctions, which is mediated by a ROS-dependent activation of Src and increased tyrosine phosphorylation and internalization of VE-cadherin (Figure 4) (84, 220). Likewise, subendothelial oxidized LDL increases the transendothelial migration of monocytes, which is associated with decreased expression of VE-cadherin and reduced intensity of adherens junctions (219). Monocyte adhesion to endothelial cells also triggers disruption of adherens junctions, which is mediated by a VCAM-1 and ICAM-1-dependent increase in Src activity and tyrosine phosphorylation of VE-cadherin (Figure 4) (80, 218). Therefore, transmigration augments subsequent monocyte transmigration and recruitment to the lesion-prone site (221). Although these subendothelial monocyte/macrophages attempt to clear the modified LDL particles from the subendothelial space, they become engorged with lipids, are unable to leave and are transformed into foam cells (206–209). The evolving atherosclerotic lesion reflects a coordinated interaction between vascular, immune and inflammatory cells, with continual remodeling of the atheroma resulting in formation of a lipid-rich necrotic core comprising extracellular lipid droplets and cellular debris that is capped by a subendothelial fibrous layer of SMCs and extracellular matrix, which provides stability to the plaque (206–209).

Consistent with studies in cultured endothelial cells, a high cholesterol diet increases degradation of VE-cadherin, further disrupting endothelial adherens junctions and increasing endothelial permeability in atherosclerosis-prone regions (88). Several cellular mechanisms have been proposed to explain this increased degradation of VE-cadherin. The expression of ADAM10 and ADAM15 are increased in atherosclerosis and may contribute to extracellular cleavage of VE-cadherin and to Src-mediated phosphorylation and internalization of VE-cadherin, respectively (87, 222, 223). Miyazaki and colleagues demonstrated a clear role for m-calpain in causing cleavage of VE-cadherin and disruption of endothelial adherens junctions in cholesterol-fed mice (88). Expression of m-calpain was selectively increased in the endothelium of human atherosclerotic lesions, in cholesterol-fed mice, and in endothelial cells exposed to modified or oxidized LDL (88). Pharmacological or molecular inhibition of m-calpain prevented VE-cadherin degradation and disruption of adherens junctions in cholesterol-fed LDLR^{-/-} and ApoE^{-/-} mice (88). The proteolysis likely reflected VE-cadherin degradation by m-calpain following its internalization in clathrin-enriched domains (224). Inhibition of m-calpain suppressed atherosclerosis in cholesterol-fed mice, acting to

reduce endothelial hyperpermeability, monocyte/macrophage infiltration into the vascular wall, and activity of inflammatory mediators including NF κ B, ICAM-1, VCAM-1 and E-selectin (88). This confirms that disruption of endothelial adherens junctions may contribute importantly to endothelial inflammatory activity and to the initiation and progression of atherosclerotic lesions (Figure 4).

In addition to disrupting endothelial adherens junctions, atheroprone shear stress increases the nuclear accumulation and transcriptional activity of β -catenin, resulting in increased expression of β -catenin target genes, including fibronectin, IL-8 and cyclin D1 (Figure 4) (95). Inhibition of β -catenin transcriptional activity blocked expression of these mediators, and also markedly reduced the activation of NF κ B by atheroprone shear stress (95). This reflects the key inflammatory activity of fibronectin in endothelial cells (44, 215). Indeed, molecular inhibition of fibronectin expression reduced the ability of atheroprone shear stress to increase NF κ B activity and to increase expression of inflammatory mediators including MCP-1 and E-selectin (Figure 4) (42). These in vitro results are consistent with in vivo analyses. Inhibiting fibronectin in vivo reduces inflammatory activity and leukocyte infiltration into the vessel wall (42, 225). Moreover, fibronectin is preferentially deposited beneath the endothelium of human atherosclerotic plaques and in atherosclerosis-prone regions of control and ApoE^{-/-} mice (42, 44, 215). In ApoE^{-/-} mice, the endothelial fibronectin expression is paralleled by increased endothelial nuclear translocation and transcriptional activity of β -catenin (95). These effects appear to be early events in the atherosclerotic process (44, 95). Therefore, activation of a key regulator of the atherosclerotic process, NF κ B, and the resulting endothelial inflammatory activity that sustains the atherosclerotic process, is in turn dependent on disruption of endothelial adherens junctions (Figure 4).

Because of the important role of adherens junctions in maintaining a stable endothelial phenotype, the degradation of VE-cadherin, disruption of endothelial adherens junctions and increased transcriptional activity of β -catenin in atherosclerosis-prone regions would be expected to activate EndoMT at these sites (Figures 3 and 4). Indeed, cells expressing endothelial and mesenchymal marker proteins, a characteristic feature of EndoMT, are present in the neointima of human atherosclerotic plaques (226, 227). Furthermore, lineage tracking of endothelium-derived cells demonstrated that a significant number of intimal fibroblast-like cells in ApoE^{-/-} mice are derived from EndoMT (227). EndoMT-derived cells are also present in the porcine vasculature in regions susceptible to atherosclerosis, but not in atherosclerosis-resistant areas with normal flow patterns (226). Indeed, exposure of endothelial cells to atheroprone shear stress stimulates EndoMT and increases expression of mesenchymal marker proteins (228), whereas normal laminar shear stress increases resistance to EndoMT (226). Likewise, oxidized LDL amplifies EndoMT, whereas native LDL does not (229). Production of extracellular matrix by Endo-MT-derived mesenchymal cells might be expected to contribute to expansion of the atherosclerotic cap and stabilization of the plaque. However, EndoMT was increased in unstable compared to stable human atherosclerotic lesions and an increased number of EndoMT-derived cells were present in ruptured versus non-ruptured plaques (227). Indeed, there was an inverse relationship between cap thickness and the amount of EndoMT-derived cells within the plaque (227). The phenotype of EndoMT cells may be determined by the nature and strength of the

inciting stimulus, with EndoMT-derived cells in atherosclerotic lesions having increased expression of MMPs resulting in degradation of the cap and plaque destabilization (227). So far, the focus has been on TGF β as a potential mediator of EndoMT in atherosclerosis (227, 228). However, it is highly likely that disruption of adherens junctions and transcriptional activity of β -catenin plays a key role in the process.

Therefore, the status of endothelial adherens junctions likely plays a key role in the initiation and progression of the atherosclerotic process. Junctional disruption would contribute to a breakdown in the endothelial barrier at atherosclerosis-prone regions, enabling the subendothelial accumulation of atherogenic lipids, increased inflammatory activity and increased monocyte transmigration at these sites and contribute to formation and expansion of the atherosclerotic plaque. Furthermore, junctional disruption and release of transcriptionally active β -catenin will amplify endothelial inflammatory activity and lead to destabilization of the plaque via formation of EndoMT-derived cells, precipitating clinically significant and life threatening events (Figure 4).

Developmental Origins of Vascular Disease

The concept that adult disease can be influenced by events occurring in utero was first popularized by David Barker. Epidemiological studies by Barker and others demonstrated that compared to infants born ‘appropriate for gestational age’ (AGA), those born ‘small for gestational age’ (SGA) are at increased risk for developing cardiovascular and metabolic diseases (168–170). This led to the ‘Barker hypothesis’: that a suboptimal environment or poor nutrition during fetal or neonatal development predisposes an individual to develop cardiovascular and metabolic diseases later in life. The theory has received considerable support from animal models demonstrating that intrauterine growth restriction (IUGR) causes pathological changes in adult offspring (170).

The vascular pathological changes resulting from intrauterine stress have similarities to vascular aging, highlighting the possibility that it may mimic premature aging of the cardiovascular system. Indeed, Barker originally observed that systolic, but not diastolic, blood pressure was inversely related to birth weight (171). Numerous studies have since confirmed that birth weight or size has a greater impact on systolic compared to diastolic blood pressure (172–175). Indeed, compared to AGA youth, those born SGA have increased pulse wave velocity, Augmentation Index (measure of premature wave reflection) and central arterial stiffness (172, 174, 176–179).

The neonatal period is likely to be a crucial stage for understanding pathogenic mechanisms initiated by intrauterine stress. For example, endothelial dilator dysfunction is evident in SGA infants soon after birth (180), earlier than pathological changes in blood pressure or metabolic processes (168, 169, 181–184). Martyn and Greenwald originally proposed that intrauterine stress might disrupt the crucial neonatal synthesis of elastin resulting in a lifelong reduction in the protein and increased arterial stiffness (185). Elastin is long-lived protein and the immediate postnatal period represents a crucial period for its synthesis (1, 185–188). Certainly, in adult offspring from stressed pregnancies, central arteries have markedly reduced elastin and increased collagen content, with a concomitant increase in arterial stiffness and an increase in immature dedifferentiated SMCs (189). Furthermore,

transient inhibition of angiotensin activity in the early postnatal period prevents the subsequent development of cardiovascular disease resulting from intrauterine stress (190, 191).

As with endothelial cells, SMC maturation or differentiation also increases in the immediate postnatal period, with a concomitant reduction in immature SMCs (1, 187, 192–198). Interestingly, immature or dedifferentiated SMCs have increased capacity to synthesize collagens and fibronectin, which contribute to arterial stiffness, whereas mature SMCs have increased ability to generate elastin, which increases arterial compliance (199). Endothelial cells promote the maturation of SMCs, and stimulate these mature differentiated SMCs to express elastin (200–203). Therefore, disruption of postnatal endothelial maturation could result in inefficient SMC maturation, with retention of immature SMCs, an imbalance in structural proteins (including reduced elastin) and arterial stiffness. Interestingly, EndoMT may be involved in generating vascular mesenchymal cells during arterial development (105, 204), and failure of endothelial adherens junctions to fully engage in the early postnatal period could amplify EndoMT and increase the activity of immature SMCs.

We have observed that intrauterine stress disrupts the protective maturation of central arterial endothelial cells occurring in the early postnatal period (205, and unpublished observations). The normal emergence of endothelial-dependent NO-mediated dilatation to acetylcholine, and which is dependent on VE-cadherin clustering at adherens junctions, was disrupted in mice models of IUGR (205). This was associated with a failure of endothelial adherens junctions to expand in size and complexity during the immediate postnatal period, suggesting that the normal increased clustering of VE-cadherin at adherens junctions is impaired by intrauterine stress (205).

By delaying or preventing the protective maturation of arterial endothelial cells in the early postnatal period, intrauterine stress could lead to the retention of an inappropriate endothelial phenotype and could disrupt the normal maturation of the arterial wall. The developmental origins of adult vascular disease likely reflects the continued presence of an abnormal endothelial and vascular phenotype, rather than a sudden triggering of pathological activity later in life.

Amplifying Endothelial Adherens Junctions as a Therapeutic Approach in Vascular Disease

Adherens junctions appear to function as an amplification nexus for endothelial signaling, both in terms of protective and pathological signaling. For example, PI3K/Akt signaling increases VE-cadherin clustering and stabilization of adherens junctions, which in turn augments PI3K/Akt signaling and protective endothelial activity. Likewise, pathological and inflammatory stimuli specifically target endothelial adherens junctions, with subsequent junctional disruption leading to additional pathological and inflammatory changes in endothelial and vascular structure and function (Figures 3 and 4). Indeed, targeted strengthening or protection of endothelial adherens junctions, by interrupting such pathological amplification, should be a powerful therapeutic approach to treating vascular disease.

Amplifying the activity of endothelial adherens junctions has already emerged as an innovative approach to treat pathological inflammatory processes (89, 230). Molecular strategies to increase VE-cadherin clustering at adherens junctions reduced inflammation-induced increases in endothelial permeability and inflammatory cell extravasation (83, 231, 232). Likewise, increased VE-cadherin clustering (via Robo4/Slit signaling) decreased vascular inflammation, tissue injury and mortality caused by LPS or infection (89, 233). The role of endothelial adherens junctions was confirmed by demonstrating that the protective effects were inhibited by a function blocking antibody to VE-cadherin (89). Several factors act as protective vascular mediators by increasing the activity of endothelial adherens junctions. For example, angiopoietin-1 (Angpt1) or sphingosine 1-phosphate (S1P) S1P1 receptor agonists (or Robo4/Slit signaling) increase VE-cadherin clustering at endothelial adherens junctions, and prevent phosphorylation and internalization of VE-cadherin and the junctional disruption occurring in response to inflammatory mediators (24, 29, 81, 89, 230, 234–238). Increasing VE-clustering at endothelial adherens junctions can also inhibit the transcriptional activity of β -catenin, presumably by restricting its ability to translocate to the nucleus (57, 239, 240). As a result, this approach should reduce β -catenin-dependent processes such as amplification of inflammatory activity and EndoMT. Protecting and amplifying endothelial adherens junctions inhibit increases in permeability and leukocyte extravasation, reduce endothelial cell apoptosis, and protect tissues from injury and reduce mortality in inflammatory models (234–238, 241–246).

Approaches to increase the activity of endothelial adherens junctions in vascular disease may actually be ‘replacement therapy’. For example, S1P and Angpt1 are local endogenous promoters of vascular stability (242, 247). Indeed, laminar shear stress increases production of S1P from endothelial cells (247), which might contribute to stabilization of adherens junctions (213). Likewise, S1P is a constituent of high density lipoprotein (HDL) and a key mediator of HDL’s anti-inflammatory effects on the endothelium, with loss of S1P contributing to ‘HDL dysfunction’ in atherosclerosis (248–250). Inflammatory stimuli increase endothelial expression of alternate S1P receptors, such as S1P2 that counter the stabilizing influence of S1P1 (50, 244, 251), and the Angpt1 antagonist Angpt2, which contribute to inflammatory responses (242, 252). These disruptive processes can also be upregulated during the development of vascular disease (253, 254). Interestingly, a very early feature of the atherosclerotic process is a selective impairment in endothelial signaling mediated by the pertussis toxin-sensitive Gi-protein (255–258). S1P1 dependent effects to stabilize endothelial adherens junctions are mediated by this Gi-protein (28, 259). The early dysfunction in endothelial Gi-protein signaling may therefore reflect activity of an alternate signaling pathway (e.g. S1P2), which inhibits this protective pathway.

Amplification of endothelial adherens junctions and VE-cadherin-dependent signaling may contribute to the protective effects of existing vascular therapeutic agents. For example, independently of lowering LDL levels, statins have direct, protective effects on endothelial cells that likely contribute to their therapeutic efficacy. These so called ‘pleiotropic’ effects are mediated predominantly by inhibition of Rho/ROCK signaling (108, 260, 261). Statins prevent disruption of endothelial adherens junctions by inflammatory mediators or adherent monocytes by preventing the tyrosine phosphorylation of VE-cadherin and thereby blocking monocyte transmigration (262). This protective effect is mediated by inhibition of Rho/

ROCK signaling, which is an upstream regulator of Src and VE-cadherin tyrosine phosphorylation (262). Statins also inhibit the process of EndoMT (108), which is consistent with protection and stabilization of endothelial adherens junctions.

Therapeutic amplification of VE-cadherin signaling and endothelial adherens junctions should therefore be a powerful mechanism to restore protective endothelial signaling and vascular stability, and prevent the initiation and progression of vascular disease (Figures 3 and 4).

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Figure 1.

Representative LSM fluorescent images highlighting the morphology of native arterial endothelial cells. In **A** and **B**, images present the changes occurring in the actin cytoskeleton (**A**, phalloidin in red) and adherens junctions (**B**, VE-cadherin in red) of endothelial cells lining mouse arteries during the early postnatal period. Carotid arteries were isolated from newborn (postnatal day 1 or P1) and P7 mice. In **A**, the endothelial actin cytoskeleton displayed prominent stress fibers in P1 arteries, but had significantly reduced intensity and was restricted to a cortical actin structure delineating cell borders in P7 arteries. In P1 arteries, Rho inhibition (P1 C3, C3 transferase) reduced the appearance of endothelial stress fibers, and the actin cytoskeleton was restricted to a cortical network in the cell periphery. In **B**, there was reduced staining of VE-Cadherin and organization of endothelial adherens junctions in P1 compared to P7 arteries. If P1 arteries were exposed to a transient increase in transmural pressure (P_{TM}) (50 mmHg, 60 mins) (P1-50), the intensity and complexity of VE-cadherin and adherens junctions were increased compared to arteries maintained at a P_{TM} of 20 mmHg (P1-20). Panel **C** presents VE-cadherin staining in the endothelial cells lining young (4 months) and old (22 months) rat tail arteries. Old age caused marked disruption of endothelial adherens junctions, which was associated with internalization of VE-cadherin. Images in **A** and **B** are taken from Flavahan et al, 2013 (7) and Flavahan and Flavahan 2014 (8). Images in **C** are unpublished observations.

Figure 2.

Regulation of endothelium-dependent responses to acetylcholine (**A**) or to calcium ionophore A23187 (**B**) in mice carotid arteries in the immediate postnatal period. Arteries were isolated from postnatal day 1 (P1, newborn), P7 and P21 mice, and analyzed in a microperfusion system at a transmural pressure (P_{TM}) of 20 mmHg, the mean blood pressure of P1 mice. Functional responses are expressed as a percentage of the baseline diameter of the arteries (BD), and presented as means $\pm$ SEM. To observe dilatation or constriction, arteries were initially constricted with the thromboxane receptor agonist U46619 (U4). **A**: In P1 arteries, acetylcholine caused significant endothelium-dependent dilatation only at the highest dose tested, but caused markedly increased dilatation in P7 and P21 arteries (top left panel). In P1 arteries, the minimal dilator responses to acetylcholine were dramatically increased by inhibition of Rho signaling (P1 C3, C3 transferase) (top right panel) or by transiently increasing P_{TM} to 50 mmHg (60 mins), corresponding to the mean blood pressure of P7 mice (middle left panel). This effect of increased pressure to amplify the dilator response to acetylcholine was prevented by a function blocking antibody to VE-cadherin (compared to a control antibody) (middle right panel). **B**: A23187 caused endothelium-dependent constriction in P1 arteries (bottom left panel), but endothelium-dependent dilatation in P21 arteries (bottom right panel). Combined antagonism of endothelin ET_A and ET_B receptors (BQ123 plus BQ788) abolished constriction to A23187

in P1 arteries, but had no effect on the dilatation to A23187 in P21 arteries. Data taken from Flavahan et al, 2013 (7), Flavahan and Flavahan 2014 (8). and Chang et al 2016 (20).

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Figure 3. Schematic representation of endothelial adherens junctions, with protective activity of normal active junctions (right) and the potential pathological effects of disrupted junctions (left). The red underlined mediators and processes reflect potential effects induced by disruption of adherens junctions.

Figure 4. Schematic representation highlighting the potential importance of adherens junction disruption in the atherosclerotic process. Abbreviations not defined in manuscript text: LSS, laminar shear stress; P_{TM}, transmural pressure; KLF, Kruppel-Like Factor.