Flow-dependent cellular mechanotransduction in atherosclerosis

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Journal of Cell Science 126, 5101–5109

doi: 10.1242/jcs.138313

Summary

Atherosclerosis depends on risk factors such as hyperlipidemia, smoking, hypertension and diabetes. Although these risk factors are relatively constant throughout the arterial circulation, atherosclerotic plaques occur at specific sites where flow patterns are disturbed, with lower overall magnitude and complex changes in speed and direction. Research over the past few decades has provided new insights into the cellular mechanisms of force transduction and how mechanical effects act in concert with conventional risk factors to mediate plaque formation and progression. This Commentary summarizes our current understanding of how mechanotransduction pathways synergize with conventional risk factors in atherosclerosis. We attempt to integrate cellular studies with animal and clinical data, and highlight major questions that need to be answered to develop more effective therapies.

Key words: Endothelium, Fluid shear stress, Vascular biology

Introduction

Atherosclerosis is a chronic inflammatory disease of arteries. Cardiovascular disease is responsible for approximately one out of three deaths in the United States, two thirds of which are attributable to atherosclerosis (Roger et al., 2012). Its initiation and progression involve circulating factors such as low density lipoproteins (LDLs) and triglycerides, inflammatory activation of the cells of the vascular wall, and recruitment of leukocytes. These components interact to initiate a form of sterile inflammation and tissue remodeling, the atherosclerotic plaque. However, a conventional, lipid- and inflammation-based view of this disease fails to account for crucial aspects of its etiology. Atherosclerosis typically develops at arterial branches and sites of curvature that were identified 40 years ago as regions of low and disturbed wall shear stress (Caro et al., 1969; Caro et al., 1971; Glagov et al., 1988) (Fig. 1). Many subsequent studies of atherosclerosis in humans confirmed the preferential location of atherosclerotic lesions at these regions of low and disturbed shear (Schwartz et al., 1991). Continuing work reveals that responses of vascular endothelial cells (ECs) to mechanical stimuli are central to disease initiation and progression. This Commentary will review our current understanding of how endothelial cell responses to fluid shear stress leads to the development and progression of atherosclerosis. After a brief introduction to disease etiology and some basic features of mechanotransduction, we focus on mechanisms by which flow-dependent pathways synergize with conventional risk factors.

Disease etiology

Although the smooth muscle cells that form the medial layer and the fibroblasts that comprise the outer adventitial layer participate in atherosclerosis, the inner endothelial layer largely drives disease initiation. Atherosclerosis-prone regions of artery walls, before any signs of disease, show low, chronic inflammatory activation of the endothelium, with increased activation of NF-κB and expression of leukocyte adhesion receptors (Hajra et al., 2000; Won et al., 2007). These regions also contain increased numbers of immune cells, especially monocytes and macrophages, although dendritic cells, T and B cells are also present (Galkina and Ley, 2009). Atherosclerosis is first visible as fatty streaks consisting mainly of lipid-loaded macrophages and an enlarged smooth muscle layer. These early lesions can progress to larger, more inflamed atherosclerotic plaques. Atherosclerotic lesions result from monocytes that enter the vessel wall, differentiate into macrophages and ingest lipoproteins, developing into so-called foam cells, named for their white, fatty appearance (Stary et al., 1994). Foam cells are highly activated and produce inflammatory cytokines that further activate the endothelium and smooth muscle (van der Wal et al., 1994), thereby creating a positive-feedback loop. Inflammation also leads to the degradation of the elastin-rich internal elastic laminae that separate the layers of smooth muscle from each other and the endothelium. Loss of the elastic laminae further contributes to the proliferation and migration of smooth muscle cells (SMCs) from the media toward chemotactic stimuli in the sub-endothelium. These SMCs synthesize fibrillar collagens that can form a fibrous capsule over the plaque, as well as contribute to the general thickening and stiffening of the artery wall. In parallel, during early stages of plaque development, SMCs that remain in the media become shielded from cyclic stress by the stiff, fibrous plaque and, thus, experience less cyclic stretch during the

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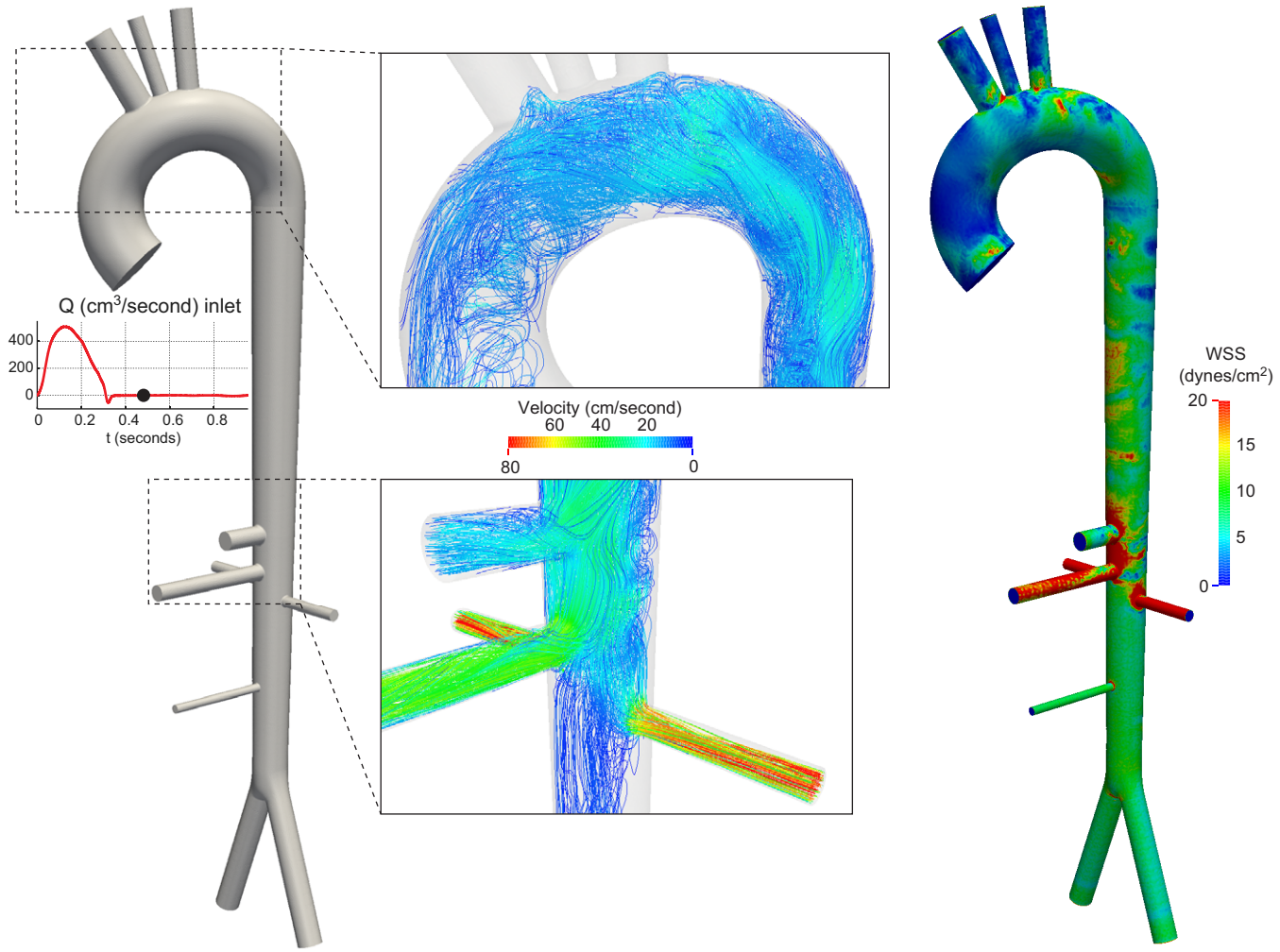

Fig. 1. Illustration of disturbed arterial flow. Schematic illustration of calculated flow patterns (as shown in the graph) in an idealized model of the human aorta. Q inlet refers to flow rate at the entrance to the aorta. The black dot in the graph denotes the specific time during the cardiac cycle to which the streamlines and wall shear stress (WSS) images correspond. Stream lines in the enlargements show velocity color coded according to the key. The enlargement at the top shows the region of disturbed flow at the inner curvature of the aortic arch. The enlarged region at the bottom illustrates disturbed flow at branches off the abdominal aorta. WSS just after the start of diastole is illustrated on the right. Courtesy of Alberto Figueroa and Nan Xiao, King's College London, UK.

cardiac cycle. Deprivation of this essential mechanical stimulation leads to apoptosis of these cells and thinning of the medial smooth muscle layer (Glagov et al., 1987). These effects contribute to outward remodeling of the artery that helps maintain the lumen; however, as the plaque progresses, such outward remodeling ceases to offset the encroachment of the plaque on the lumen, which restricts blood flow. Further exacerbating the situation at later stages, macrophages and smooth muscle cells in the center of the plaque die more rapidly than they can be cleared, creating a necrotic core that further inflames the tissue. If the fibrous cap becomes fragile and suddenly ruptures, a thrombus can form and occlude the artery lumen, resulting in ischemia or necrosis of the tissue downstream of the blockage. Plaque rupture often occurs at sites of modest rather than severe stenosis (the abnormal narrowing of the artery) (Fuster et al., 1992; Hackett et al., 1988; Little et al., 1988). Reasons why and methods to detect individual plaques that are at high risk of rupture are major challenges for current clinical research.

In addition to correlating plaque location with regions of low and disturbed flow, more recent studies have established a direct causal connection. Hypercholesterolemic mice develop atherosclerotic plaques that share many features with human disease and provide a valuable model system. In these mice, surgical introduction of regions of low or disturbed flow into previously normal arterial segments triggers plaque formation (Cheng et al., 2006; Nam et al., 2009). Conversely, surgically converting a region of disturbed flow into one with high laminar flow can induce plaque regression even in the presence of high cholesterol (Stephanie Lehoux, McGill University, personal communication). It was initially hypothesized that low shear stress promotes atherosclerosis through increased mass transport at regions where residence times of solutes and cells was longer (Caro et al., 1971), but experimental studies revealed an opposite relationship (Caro and Nerem, 1973; Fry, 1969; Sill et al., 1995; Tarbell, 2003). How active sensing of physical forces by ECs leads to inflammatory activation of artery walls and progression to atherosclerosis is a subject of current research.

Endothelial responses to shear stress

EC responses to fluid-induced shear stress have most often been studied *in vitro* by acute application of flow to naive cell monolayers, which, although non-physiological, provides a convenient approach for understanding mechanisms (Frangos et al., 1985). Application of shear stress to ECs elicits several responses on a time scale of seconds, including activation of potassium channels (Hoger et al., 2002; Olesen et al., 1988), kinases of the Src family (Jalali et al., 1998; Takahashi and Berk, 1996) and vascular endothelial growth factor receptor 2 (VEGFR2) tyrosine kinase (Jin et al., 2003), and release of nitric oxide (Andrews et al., 2010; Corson et al., 1996). On times scales of minutes to tens of minutes, flow activates MAP kinases (Berk et al., 1995), Rho family GTPases (Tzima et al., 2002; Tzima et al., 2003; Wojciak-Stothard and Ridley, 2003), integrins (Tzima et al., 2001), NF-κB, p21 activated kinase and Jun N-terminal kinase (JNK) (Hahn et al., 2011; Orr et al., 2007; Tzima et al., 2002). Over longer times (hours to days), ECs express distinct sets of genes downstream of these signaling pathways (Dai et al., 2004; Davies, 2008).

Different flow patterns induce distinct endothelial phenotypes. Normal, high arterial shear stress (either steady or forward pulsatile shear stress) stimulate anti-proliferative, anti-inflammatory and anti-thrombotic gene expression, thereby inducing an atheroprotective phenotype (Nigro et al., 2011). By contrast, low flow or flow with reversal or other changes in direction (so-called disturbed flow) induces pro-inflammatory and pro-thrombotic genes and increases both proliferation and apoptosis (Hahn and Schwartz, 2009). Interestingly, flow also influences the elongation and alignment of ECs in the direction of flow, both *in vivo* and *in vitro*. Failure to align *in vivo* is a hallmark of an atherosclerosis-prone region (Davies, 2008). *In vitro*, cells align in high laminar shear but not in low or disturbed shear (Davies et al., 1986). Alignment has been proposed to alter the transmission of forces from the fluid shear stresses to the ECs and thus might be an adaptive mechanism (Davies and Barbee, 1994). Indeed, it is striking that onset of laminar flow *in vitro* triggers nearly the same events and acts through the same mechanisms as disturbed flow, albeit only transiently (Hahn and Schwartz, 2009). As the cells align, proliferative and inflammatory pathways are downregulated, whereas these pathways remain activated in disturbed flows. Recent work revealed that cell alignment in the flow direction is crucial for adaptation. Flow that is perpendicular to the axis defined by the shape of the cell and cytoskeletal organization strongly activates the inflammatory NF-κB pathway, whereas flow parallel to that axis activates NF-κB weakly and stimulates the anti-inflammatory eNOS–nitric-oxide pathway (Wang et al., 2013). Together, these responses provide two complementary mechanisms that can account for the non-random localization of atherosclerotic plaques.

Mechanosensors of endothelial shear stress

Considerable effort has focused on elucidating the primary mechansensors for fluid shear stress. An early hypothesis was that the force from fluid shear stress was large enough to increase tension at key stress points throughout the cell (Davies and Barbee, 1994). However, calculation of the force imparted by shear stress indicates that this force is approximately two orders of magnitude less than the forces that are exerted by a cell on the matrix. [According to Barbee and colleagues (Barbee et al., 1995), the topology of the endothelial cells increases applied force from laminar flow by around 40% above the level exerted on a flat surface. For an average arterial level of shear stress (1.5 Pa), the actual average force on the cell would therefore be increased to ~2 Pa. According to Balaban and co-workers (Balaban et al., 2001), forces at focal adhesions for fibroblasts are ~5000 Pa. Assuming the adhesions occupy 5% of the surface, the forces from adhesions spread over the whole cell would be 250 Pa. Traction forces that originate in myosin are therefore ~100 times larger than the forces from moderate physiological shear stress on the cell.] A more recent report determined that the forces within the cytoskeleton that are induced by exposing ECs to long-term shear stress were nearly one order of magnitude larger than the value required to passively balance the force from shear stress (Hur et al., 2012). Taken together, these observations suggest that the response of ECs to shear stress cannot be explained as a passive response to applied force (e.g. a global deformation of the cell), but rather is instead an active signaling response that is initiated by one or more mechanosensors attuned to the smaller forces of shear stress.

In most cases, the responses to shear stress are specific to ECs, indicating that endothelial-specific proteins are involved in mechanotransduction. Many different components have been proposed to be mechanosensors of shear stress, including cell–cell junctions (Tzima et al., 2005), heterotrimeric G-proteins (Gudi et al., 2003), primary cilia (Hierck et al., 2008), caveolae (Yu et al., 2006), integrins (Jalali et al., 2001), the glycocalyx (Pahakis et al., 2007), intermediate filaments (Helmke et al., 2000), the nucleus (Deguchi et al., 2005; Tkachenko et al., 2013), ion channels (Barakat, 1999) and the actin cytoskeleton (Osborn et al., 2006). Although all of these components might contribute in some way, establishing the primary components that mediate conversion of force into biochemical information has been difficult, because proteins could be required for responses without directly mediating mechanotransduction. The many candidate mechanosensors have been discussed in previous reviews (Davies, 2009; Hahn and Schwartz, 2009; Lehoux and Tedgui, 2003; Wang and Thampatty, 2006; White and Frangos, 2007) and further discussion is beyond the scope of this article. However, two of these candidate mechanotransducers stand out as being relevant to atherosclerosis.

A complex of VE-cadherin, PECAM-1 and VEGFR2 at cell–cell junctions is probably the best-studied mechanotransducer (for a detailed review, see Conway and Schwartz, 2012). These proteins are required for activation of NF-κB and downstream inflammatory events that are induced by flow, as well as the long-term realignment of ECs in the direction of shear stress (Tzima et al., 2005). Recent work to further dissect distinct roles for these proteins focused on the small GTPase Rac1, whose activation by shear stress is essential for production of reactive oxygen and activation of NF-κB (Tzima et al., 2002). Interestingly, Rac1 activation is polarized toward the downstream edge of the cell relative to the flow direction, which mediates endothelial cell alignment. Recent work, however, showed that Rac1 activation by flow requires PECAM-1, acting through the Rac GEF VAV2; however, its spatial polarization requires a distinct GEF, TIAM1 (Liu et al., 2013). Flow induces TIAM1 association with VE-cadherin and the Par3–Par6–aPKC polarity complex at the downstream edge of the cell. Surprisingly, this role for TIAM1 does not require GEF activity but is required for coupling of Rac1 with its effector, the

NADPH oxidase complex and production of reactive oxygen (Liu et al., 2013). These data point toward a highly intricate polarization mechanism involving a non-canonical function for TIAM1 in conjunction with membrane receptors and polarity proteins.

PECAM-1 appears to be the true mechanosensor, supported in part by evidence that force applied to PECAM initiates signals in a similar manner to flow (Osawa et al., 2002; Chiu et al., 2008; Collins et al., 2012; Tzima et al., 2005). VE-cadherin and VEGFR2 are also essential for signaling through this pathway but do not act as direct transducers (Tzima et al., 2001; Tzima et al., 2005). Recent application of a fluorescence-based tension sensor (Grashoff et al., 2010) to PECAM showed that flow triggers the rapid application of force to this molecule (Conway et al., 2013), supporting its role as a mechanotransducer. By contrast, force measurements across VE-cadherin detected a decrease in tension across this molecule after flow (Conway et al., 2013). Deletion of PECAM in hypercholesterolemic mice reduces atherosclerosis in the aortic arch, supporting its role in this disease (Goel et al., 2008; Harry et al., 2008; Stevens et al., 2008). However, the effects of PECAM at other atherosclerosis-prone sites were unclear, possibly because it has other functions apart from mechanotransduction. Furthermore, single-nucleotide polymorphisms in the human PECAM gene are linked to early atherosclerosis and increased cardiovascular disease (Novinska et al., 2006). These polymorphisms were found to affect PECAM tyrosine phosphorylation and leukocyte transmigration (Bayat et al., 2010; Elrayess et al., 2004), suggesting that they can affect the intracellular signaling of PECAM. Taken together, available evidence clearly implicates PECAM in both disturbed flow-induced endothelial activation *in vitro* and in atherosclerosis *in vivo*; however, further work to separate mechanotransduction from other functions of PECAM are required to clarify these effects.

There is also evidence for the participation of primary cilia in atherosclerosis. These structures are well known to sense fluid flow by bending (Van der Heiden et al., 2011). Primary cilia are disrupted by high shear *in vitro* (Iomini et al., 2004) and absent in most regions of arteries (Van der Heiden et al., 2006; Van der Heiden et al., 2008), which makes it unlikely that they are required for transducing the anti-atherosclerotic effects of high laminar shear. However, they are detectable in a fraction of the ECs in regions of low and disturbed flow (Van der Heiden et al., 2006; Van der Heiden et al., 2008): precisely the regions that are susceptible to atherosclerosis. Endothelial cells containing cilia show a stronger response to shear stress *in vitro* than those without cilia (Hierck et al., 2008), supporting a role in mechanotransduction. Interestingly, polycystin 1 and polycystin 2 proteins, whose mutations cause polycystic kidney disease, localize to primary cilia and mediate sensing of urine flow by kidney epithelial cells (Deane and Ricardo, 2012). Polycystins are also involved in flow stimulation of nitric oxide production in endothelial cells (AbouAlaiwi et al., 2009). A fraction of patients with mutations in polycystin genes are hypertensive at very early ages, well before they show signs of kidney dysfunction, and frequently die of vascular complications (Ecder and Schrier, 2009). The exact connection between these two sets of observations is unclear, because the former implicate primary cilia in pro-inflammatory signaling, whereas the latter point to anti-inflammatory signaling. Nevertheless, both *in vitro* and *in vivo* data implicate polycystins or other components of primary cilia as candidates for one or more pathways of flow sensing in endothelia during atherogenesis.

Role of matrix remodeling

The inflammatory effects of disturbed flow on the ECs are strikingly dependent on the underlying extracellular matrix (ECM). In unperturbed vessels, this matrix is comprised primarily of basement membrane proteins, such as collagen IV and laminins. However, atherosclerosis-prone regions of arteries show a subendothelial deposition of fibronectin even in wild-type mice (Orr et al., 2005). Fibronectin staining increases in hypercholesterolemic mice, and fibrin also appears as the plaques progress. *In vitro*, application of disturbed flow to EC monolayers increases both fibronectin gene expression and matrix assembly, which depend on PECAM (Feaver et al., 2010). Thus, a PECAM-dependent pathway also drives the remodeling of the basement membrane.

This matrix remodeling appears to be important for inflammatory activation. Flow activates NF-κB, JNK and p21-activated kinase (PAK) in ECs that are plated on fibronectin or fibrinogen, whereas these pathways are suppressed in cells that are plated on laminin or collagen IV (Orr et al., 2006; Hahn et al., 2009; Orr et al., 2005). These signaling molecules are organized hierarchically, with PAK determining the matrix-specific activation of NF-κB and JNK (Orr et al., 2008). Studies show that deletion of various isoforms of fibronectin in hypercholesterolemic mice decreases atherosclerosis, demonstrating the *in vivo* relevance (Babaev et al., 2008; Rohwedder et al., 2012; Tan et al., 2004). Interestingly, deletion of plasma fibronectin in mice also leads to thinner fibrous caps, typical of the vulnerable plaques that are susceptible to rupture (Rohwedder et al., 2012). This result illustrates the complex roles that basement membrane remodeling and inflammation play in this disease, and cautions against interventions in processes that are not well understood. In humans, a polymorphism in the collagen IV gene was identified as a risk factor for arterial disease (Schunkert et al., 2011), which is consistent with a role for matrix proteins in this disease.

The cAMP-protein kinase A (PKA) pathway was identified as the key upstream regulator of matrix-specific inflammation in ECs. This pathway is stimulated by flow in cells plated on basement membrane proteins but not fibronectin (Funk et al., 2010). PKA functions by activating eNOS and triggering the production of nitric oxide (NO) and activation of protein kinase G, which then inactivates PAK through phosphorylation on an inhibitory site, serine 20 (Yurdagul et al., 2013). PAK regulates the junctional integrity of ECs, as well as flow activation of NF-κB and JNK (Hahn et al., 2011; Orr et al., 2007; Orr et al., 2008), suggesting that PAK is a central player. However, the suppression of NO production in cells plated on fibronectin has even broader implications. NO is a crucially important anti-inflammatory, anti-thrombotic molecule. Loss of its production mediates so-called endothelial dysfunction, the inability to trigger vessel dilation in response to parasympathetic or mechanical stimuli, which is closely linked to artery disease (Tousoulis et al., 2012). In summary, the interaction between ECs and their associated ECM appears to play a central role in atherogenesis. The available data reveal a PECAM-dependent mechanotransduction pathway that governs basement membrane remodeling, inflammatory activation of the endothelium and endothelial function or dysfunction – all of which are mediated by a complex network of signaling pathways (see Fig. 2).

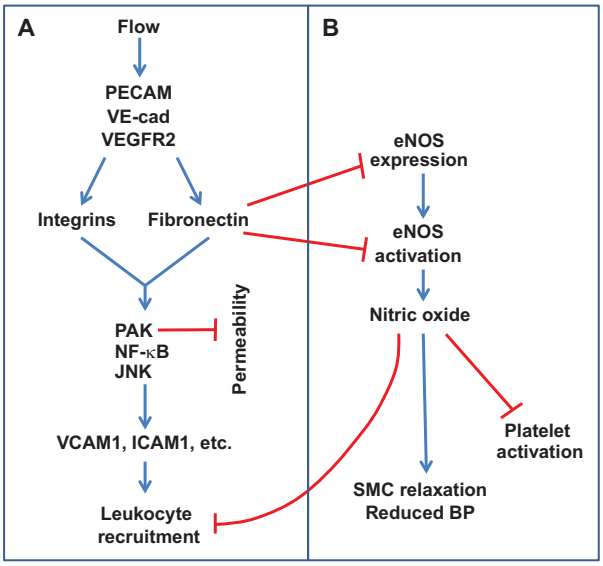

Fig. 2. Initiation of atherosclerosis by disturbed flow. (A) Inflammatory signaling. Fluid shear stress activates the PECAM–VE-cadherin–VEGFR2 pathway, which activates integrin signaling. Fluid shear also increases the expression of fibronectin and induces the assembly of the fibronectin matrix. Fibronectin switches integrin signaling to pro-inflammatory pathways, resulting in activation of NF-κB, PAK and JNK among others. (B) Anti-inflammatory signaling. Fibronectin also suppresses the flow activation of eNOS and production of nitric oxide (NO), thus inhibiting a major anti-inflammatory pathway. As a result, endothelial cells increase their expression of the leukocyte recruitment receptors ICAM-1 and VCAM-1, and a variety of cytokines. The combination of these effects results in the recruitment of monocytes and other leukocytes to the vessel wall. NO also decreases smooth muscle cell (SMC) contraction to reduce blood pressure (BP), and inhibits platelet reactivity, which also suppresses atherogenesis.

Interactions with systemic risk factors

What we have described so far represents mainly early, biomechanical events that occur in every arterial wall, indeed, even in atherosclerosis-resistant wild-type mice. So, what else is required for the development of atherosclerotic lesions? Atherosclerosis is promoted by clinical risk factors, such as high LDL and VLDL, cholesterol, low HDL, hypertension and diabetes. Although our understanding of their contribution is highly incomplete, we can propose mechanisms through which flow patterns and systemic risk factors synergize in the formation and progression of plaques.

Lipoproteins

From the perspective of the lipoprotein field, the earliest event in atherogenesis is the entry and retention of LDLs and other cholesterol-rich lipoproteins into the vessel wall (Sima et al., 2009). Fluid shear stress patterns can affect lipoprotein transport through the endothelium by multiple mechanisms. First, flow stimulates the paracellular permeability of endothelial tissue through activation of PAK, which stimulates the opening of EC cell–cell junctions (Orr et al., 2007). This occurs through both VE-cadherin phosphorylation and endocytosis, and through myosin-dependent contractility (Gavard and Gutkind, 2006; Orr et al., 2007). Flow can also increase the transport of LDL receptors through endocytosis (Sprague et al., 1987), although the effect of different flow patterns on this pathway has not been compared. Once LDL has passed through the endothelial barrier, its retention within the vessel wall is also thought to be important. Retention is thought to occur mainly through the binding of LDL to negatively charged species, especially proteoglycans (Chait and Wight, 2000). There is currently no evidence that flow patterns specifically regulate the content or charge of proteoglycans within the vessel wall, although, as discussed above, laminar and disturbed flow differentially control other aspects of matrix remodeling (Feaver et al., 2010). Thus, biomechanical stimulation is likely to facilitate an increase in lipoproteins in the arterial wall through effects on endothelial permeability and perhaps changes in the composition of the ECM.

A second potential synergy involves the modification of lipoproteins. LDL can undergo a number of modifications, including oxidation, nitration, glycation, acetylation, aggregation and proteolysis, to generate species that are more atherogenic than native LDL (Miller et al., 2010). Oxidized LDL (oxLDL), which can be produced through multiple enzymatic and non-enzymatic mechanisms, is the best-studied species. OxLDL has cytokine-like properties and stimulates inflammatory pathways in ECs, smooth muscle cells and macrophages (Mitra et al., 2011). Modified LDLs are also potentially immunogenic and might stimulate adaptive immunity, leading to the generation of autoantibodies and to activated T cells that contribute to inflammation (Ketelhuth and Hansson, 2011). Disturbed shear stimulates reactive oxygen species (ROS) production by ECs in part through activation of the NADPH oxidase (reviewed by Jo et al., 2006), which oxidizes the retained LDL. Disturbed flow also reduces the expression of Mn-superoxide dismutase, which contributes to elevated ROS (Ai et al., 2008). Interestingly, this study also showed that cells and regions of artery walls under disturbed flow have increased nitrotyrosine modification of LDL. This adduct forms when NO from eNOS and superoxide from NADPH oxidase react to form peroxynitrite, which modifies tyrosine residues on proteins. Thus, the evidence suggests that disturbed flow will promote the modification of locally accumulated LDL to more atherogenic species.

The local accumulation of modified LDL can contribute to the local recruitment and activation of leukocytes in the plaque through several mechanisms. First, oxLDL binding to endothelial cells through the lectin-like receptor for oxLDL (LOX-1) is pro-inflammatory, increasing expression of chemokines, such as MCP-1, interleukin 8 and CXCL2, and thereby stimulating monocyte binding (Li and Mehta, 2000; Mattaliano et al., 2009). Indeed, deletion of LOX-1 inhibits atherosclerosis in mouse models, providing clear *in vivo* evidence for its contribution to atherogenesis (Mehta et al., 2007). Second, modified LDLs are readily taken up by monocyte or macrophages. This is mediated by scavenger receptors, such as SCA1 and CD36, which activate inflammatory signals (Collot-Teixeira et al., 2007; Dunn et al., 2008). OxLDL also enhances progression of monocytes into macrophages and foam cells, in part through receptor signaling and in part because of its effects of lipid loading (Badimon et al., 2011). Consequently, LDL modification contributes to atherogenesis in two ways. First, it induces a switch in the binding specificity of LDL from the LDL receptor to these various scavenger receptors that induce distinct signals upon binding. Second, LDL uptake through these receptors circumvents the homeostatic mechanisms by which LDL-receptor-mediated uptake is decreased by cholesterol loading

(Miller et al., 2010). Interestingly, endothelial expression of LOX-1 is strongly stimulated by the onset of shear (Murase et al., 1998). These results suggest another mechanism by which flow can locally stimulate oxLDL binding, uptake and inflammatory signaling. Taken together, flow can modulate the effects of high LDL by enhancing its transport into and retention in the vessel wall, its oxidation or other modifications and the subsequent interactions that increase local inflammation.

Diabetes

Patients with diabetes suffer from atherosclerosis at dramatically increased rates, and vascular complications are the major source of mortality for diabetics (Roger et al., 2012). Two potential mechanisms for this synergy can be suggested. First, elevated levels of blood glucose lead to advanced glycosylation end product (AGE) modification of ECM proteins. These adducts bind to RAGE (receptor for AGE) on endothelial and other cells, which triggers oxidative stress and immune activation (Basta et al., 2004). Second, high glucose levels stimulate gene expression of fibronectin and matrix assembly (Cagliero et al., 1988; Lin et al., 2002). Indeed, diabetic mouse and primate models, and human specimens exhibit greatly increased fibronectin staining of vascular basement membranes that is no longer restricted to regions of disturbed flow (Cherian et al., 2009; Rincon-Choles et al., 2012; Spirin et al., 1999). As discussed above, fibronectin appears to have a significant role in the immune activation of ECs. These data therefore suggest that fibronectin mediates increased inflammatory activation of the endothelium. Interestingly, diabetic atherosclerosis is often diffuse, with weaker restriction to the usual atherosclerosis-prone regions (Vavuranakis et al., 1997; Vigorita et al., 1980). The extensive deposition of fibronectin could contribute to this effect. It will be interesting to test these ideas in mouse models, for example, by using genetic deletion of specific fibronectin isoforms (Babaev et al., 2008; Rohwedder et al., 2012; Tan et al., 2004).

Hypertension

Hypertension is an additional risk factor that appears to interact with disturbed flow to accelerate atherosclerosis. Several models for possible synergistic effects have been advanced. First, stretching ECs along their cytoskeletal axis activates JNK, an inflammatory pathway, whereas stretching perpendicular to the actin stress fibers does not (Kaunas et al., 2006). As mentioned above, ECs in atherosclerosis-resistant regions of vessels are aligned in the direction of flow along the vessel, whereas cells in atherosclerosis-prone regions are poorly aligned. Thus, circumferential stretch will only activate JNK and promote inflammatory activation in the atherosclerosis-prone regions where ECs are misaligned. Second, it has been proposed that during the cardiac cycle, the relative phases of the stretch and flow components is crucial, becoming out of phase at regions of disturbed flow (Dancu et al., 2004). However, these hypotheses remain to be tested *in vivo*.

Disturbed flow, diabetes, hypertension and hyperlipidemia are all associated with ROS production (Cai and Harrison, 2000). The balance between oxidant production and anti-oxidative protective mechanisms is crucial, because an excess of ROS results in oxidant stress. In this way, multiple sources of ROS might act synergistically by exceeding the antioxidant capacity of the arterial tissue. Thus, adding diabetes or hypertension to already disturbed flow can tip the balance, resulting in local oxidant stress. One effect of ROS is due to the very rapid reaction of superoxide with NO to form peroxynitrite. This reaction reduces NO availability, thereby suppressing its beneficial vasodilatory, anti-inflammator and anti-thrombotic effects, and, moreover, producing peroxynitrite, which is itself an oxidative species that has deleterious effects.

A model summarizing the interactions among biomechanical activation of the endothelium, circulating lipoproteins, and pro-oxidant stresses and how they could combine to generate lesions is presented in Fig. 3.

Conclusions and future challenges

Cells integrate information from many sources, including soluble factors, cell–cell and cell–matrix adhesions, and mechanical stimuli to determine cell behavior and patterns of gene expression. In complex tissues that are made up of multiple cell types, the behavior of individual cells is integrated more broadly, with cells communicating with each other to determine the behavior of the composite tissue. Seen this way, atherosclerosis is a disease in which local mechanical forces, the soluble milieu and genetic background interact to determine the inflammatory remodeling of the vessel wall. We have attempted to present atherogenesis from this perspective, focusing on how mechanical forces interact with other variables to determine disease progression and outcome.

Thus, local disturbances in fluid shear stress patterns lead to local inflammatory activation of the endothelium, with remodeling of the ECM and concomitant influx of leukocytes. These events are essentially universal and, in the absence of other risk factors, mainly benign. However, systemic risk factors synergize with these biomechanical preconditions to exacerbate inflammation and trigger sustained inflammatory remodeling. Fatty streaks form at early stages of atherosclerosis, which can

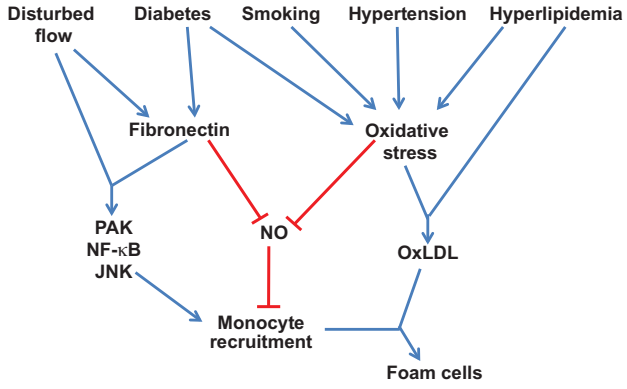

Fig. 3. Interaction of disturbed flow with conventional risk factors. Endothelial activation and leukocyte recruitment to regions of disturbed flow synergize with other factors that promote atherosclerosis. High glucose levels in diabetes patients upregulate fibronectin expression, thus promoting NF-κB and inhibiting NO production. Increased endothelial permeability and ECM remodeling promote the entry and retention of low density lipids (LDLs) in the vessel wall. In addition, diabetes, smoking, hypertension and hyperlipidemia all increase reactive oxygen species (ROS). These provide a further inflammatory stimulus; they promote oxidization of LDLs (oxLDL) and react with NO to decrease its availability. Monocyte or macrophages that are recruited through the pathway illustrated in Fig. 2 ingest oxLDL and are activated to foam cells.

progress to true atherosclerotic lesions. Restriction of the vessel lumens by plaques then creates regions of disturbed flow downstream of the narrowing and results in propagation of the lesion in the downstream direction (Smedby, 1997). Narrowing of vessels also creates regions of very high shear stress at the upstream edge of the plaques, which may induce endothelial erosion or contribute to plaque rupture (Cicha et al., 2011). In either case, subsequent thrombosis can further narrow the vessel, resulting in unstable angina, or in the case of complete occlusion, in myocardial infarction (Falk et al., 1995). Although many questions remain, the view put forth here (summarized in Fig. 3) concerning development of atherosclerosis encompasses a wide range of *in vitro*, animal model and clinical observations.

Many crucial questions at the cellular level remain to be addressed. For example, what additional mechanosensors are required to exert the full range of EC responses to flow? In particular, it is currently a mystery how cells sense flow direction as opposed to its magnitude, an important question given the relationship between cell alignment and inflammatory activation (Wang et al., 2013). ECs can also sense specific frequency components of the oscillatory flow patterns at atherosclerosis-prone regions (Feaver et al., 2013). Although PECAM-1 is required for the responses through this pathway, how cells sense the temporal patterns of flow dynamics is unknown. Finally, we need a thorough analysis of the interaction of flow pathways with other features of the atherosclerotic microenvironment in order to determine how inflammatory remodeling can be suppressed without interfering with beneficial remodeling processes. For example, how can we inhibit inward remodeling without blocking the outward remodeling that maintains vessel patency? How can we stimulate plaque regression without destabilizing fibrous caps? How can we inhibit plaque formation without affecting flow-dependent remodeling that promotes collateral artery formation? Development of successful strategies for intervention will require us to answer these questions by developing a detailed understanding of the cell biology of vascular cells and their environment.

Acknowledgements

We thank Jay Humphrey for critical reading of the manuscript and Alberto Figueroa for generously providing the image for Fig. 1.

Funding

Work in our laboratory is supported by the United States Public Health Service [grant number HL75092 to M.A.S.]; the National Institutes of Health [grant number RO1 HL75092]; and an American Heart Association postdoctoral fellowship to D.E.C. Deposited in PMC for release after 12 months.

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