

The role of blood flow in determining the sites of atherosclerotic plaques

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Abstract

Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of lipids and inflammatory cells along the inner walls of arteries, and is an underlying cause of cardiovascular disease. Atherosclerotic lesions develop predominantly at branches, bends, and bifurcations in the arterial tree because these sites are exposed to low or disturbed blood flow, which exerts low/oscillatory shear stress on the vessel wall. This mechanical environment alters endothelial cell physiology by enhancing inflammatory activation. In contrast, regions of the arterial tree that are exposed to uniform, unidirectional blood flow and experience high shear stress are protected from inflammation and lesion development. Shear stress is sensed by the endothelium via mechanoreceptors and is subsequently transduced into biochemical signals resulting in modulation of proinflammatory signaling pathways. In this article, we address the molecular mechanisms behind the spatial localization of vascular inflammation and atherosclerosis, with particular focus on studies by our own group of two key proinflammatory signaling pathways, the mitogen-activated protein kinase pathway and the nuclear factor-kappa-B pathway.

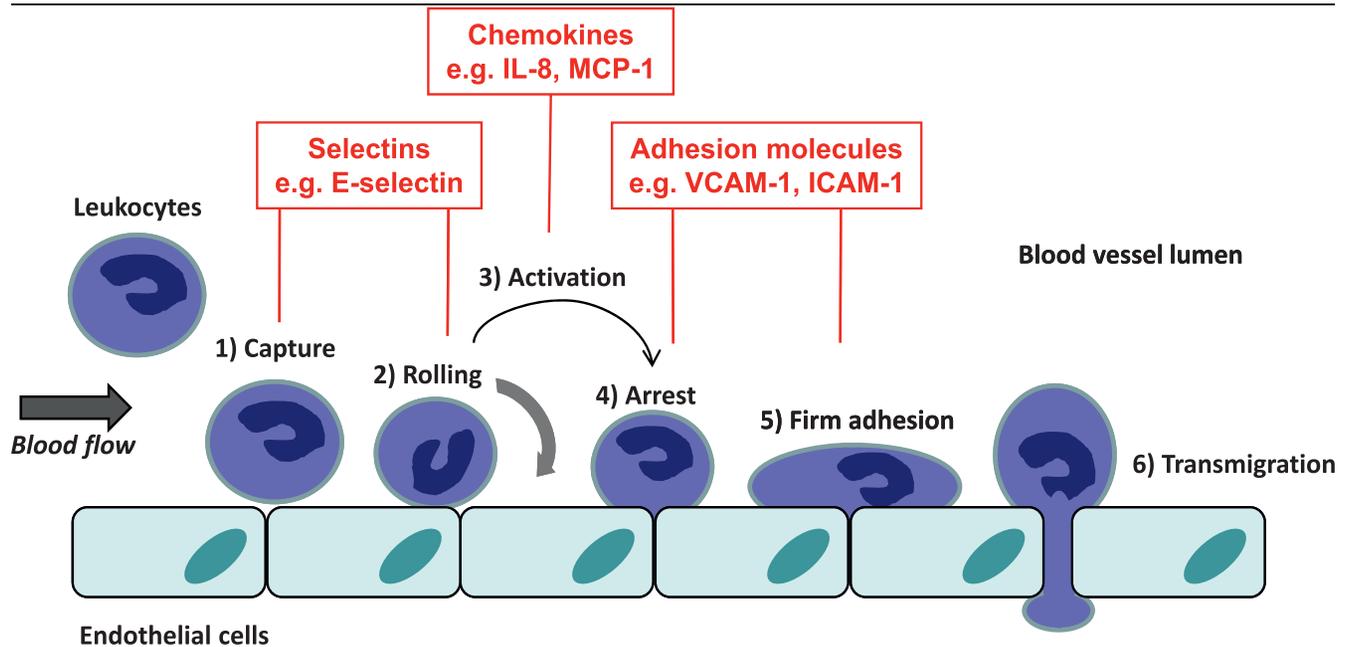
Introduction and context

Cardiovascular disease accounts for the greatest number of deaths worldwide and currently presents a major burden to health services and society. This burden is set to increase as obesity and diabetes, two major risk factors for the disease, reach epidemic levels. Most resources are currently directed towards surgical intervention and management of advanced cardiovascular disease, but clearly prevention would be preferable. Encouraging lifestyle changes is expected to play a major role in slowing disease progression; however, greater understanding of the disease at the molecular level is of critical importance in informing future treatment strategies and aiding disease prevention.

The build up of atherosclerotic plaques within the arterial tree is the underlying cause of most forms of cardiovascular disease, including coronary artery disease and stroke.

Atherosclerosis is a lipid-driven chronic inflammatory disease in which inflammatory cells, lipids, and extracellular matrix (ECM) accumulate within the artery wall to form plaques which reduce, and may eventually shut down, blood flow through the artery, with devastating consequences for the tissue or organ it supplies. A critical step in lesion formation is the oxidative modification of circulating low-density lipoprotein (LDL) by reactive oxygen species. Oxidized LDL promotes the recruitment of leukocytes from the bloodstream to the arterial wall by triggering the expression of adhesion proteins, chemokines, and other proinflammatory molecules on endothelial cells (such as vascular cell adhesion molecule-1 [VCAM-1] and E-selectin, illustrated in Figure 1). ECM remodeling and alterations in ECM composition during atherogenesis can stimulate proliferation and migration of smooth muscle cells and promote proinflammatory signaling, contributing to the development of the

Figure 1. Leukocyte adhesion and transmigration



Proinflammatory signaling results in the increased expression of adhesion molecules such as E-selectin on endothelial cells, which facilitates “capture” of the leukocytes to the vessel wall. Chemokines secreted by endothelial cells activate leukocyte integrins, a process that promotes firm adhesion between leukocytes and endothelial cells via integrin-adhesion molecule interactions. Adherent leukocytes subsequently transmute through the endothelium to the underlying tissue. E-selectin, endothelial selectin; ICAM-1, intercellular adhesion molecule-1; IL-8, interleukin 8; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1.

plaque [1]. There is also evidence that endothelial cell apoptosis plays a role in plaque formation, potentially by increasing the permeability of the arterial wall to LDL [2].

Although a wide range of global risk factors such as age, high cholesterol, and obesity significantly increase the risk of developing atherosclerosis, plaque formation occurs predominately at specific sites within the arterial tree, suggesting a critical role for local factors within the vasculature. Atherosclerotic plaques are mainly found at arterial bifurcations and branch points and areas of vessel curvature whereas straight unbranching segments of artery are generally spared. Several molecular mechanisms that influence the localization of plaque formation have been described recently and will be explored within this article.

It was recognized by Caro *et al.* [3] in 1969 that sites of lesion development were associated with regions of altered blood flow, leading to the suggestion that flow-regulated mechanical forces could play a key role in lesion development. Two hypotheses emerged to account for this effect: the mass transport theory and the shear stress theory of atherosclerosis (depicted in Figure 2).

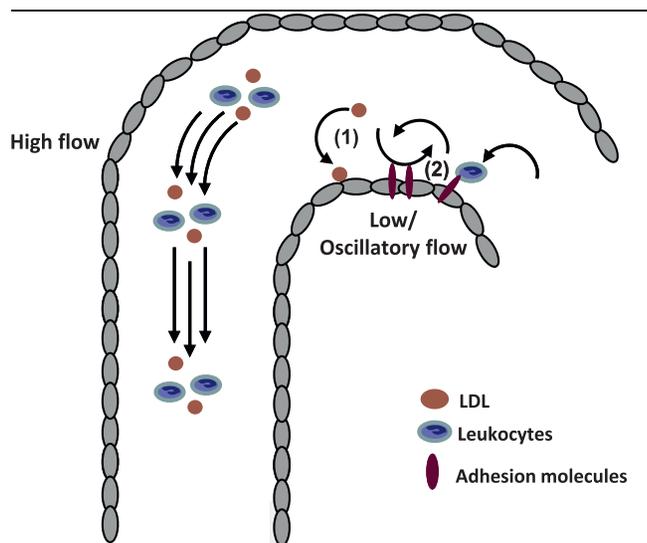
The mass transport theory of atherosclerosis

One possible explanation for the accumulation of atheromatous material at sites of low or disturbed blood flow is that an increased rate of uptake of bioactive substances (e.g., LDL, oxygen, nitric oxide) from the blood stream into the vessel wall results from the increased length of time the blood is in contact with the vessel wall in such areas, particularly in areas of flow stagnation. This idea is supported by a wealth of experiments in animals that have demonstrated greater uptake of labeled macromolecules such as albumin in regions of low blood flow that are susceptible to the development of atherosclerosis [4]. Low or disturbed blood flow might also enhance atherogenesis due to altered transport of leukocytes from the bloodstream to the endothelial surface.

The shear stress theory of atherosclerosis

The endothelium is in direct contact with flowing blood and hence it is constantly exposed to the mechanical forces that the blood exerts. The drag force exerted by flow on the vessel wall, called shear stress, is a critical factor in maintaining endothelial function and varies with time, magnitude, and direction, according to vascular pulsatility and anatomy. Relatively straight, unbranched arteries are exposed to uniform, unidirectional flow and

Figure 2. Atherosclerotic plaques occur at sites of low/disturbed blood flow



Atherosclerotic lesions form predominantly at regions of the arterial tree exposed to low/disturbed blood flow such as branches, bends, and bifurcations, whilst regions exposed to unidirectional high flow are spared. Two theories have been proposed that account for this localization: **(1)** the mass transport theory, where atherogenic material such as low-density lipoprotein (LDL) and leukocytes have better access to the arterial wall in areas of low flow or stagnation; and **(2)** the shear stress theory, where shear stress (mechanical drag) is sensed by endothelial cells, resulting in an altered phenotype. Low/oscillatory shear stress primes endothelial cells for inflammation by inducing adhesion molecule expression.

experience relatively high shear stress (time-averaged mean value approximately $150 \mu\text{N}/\text{cm}^2$; equivalent to $15 \text{ dynes}/\text{cm}^2$). By contrast, at vessel bifurcations and bends, the flow is disturbed and changes direction during the cardiac cycle resulting in relatively low and/or oscillatory shear stress (time-averaged mean $<50 \mu\text{N}/\text{cm}^2$; equivalent to $<5 \text{ dynes}/\text{cm}^2$) [5].

Atherosclerosis preferentially develops in regions of flow disturbance and separation, where shear stress is significantly lower in magnitude and exhibits directional variations [3]. Thus low shear stress provides predisposition for atherogenic transformation by contributing to endothelial dysfunction whereas high shear areas might shield against atherosclerosis by enhancing endothelial protection.

For shear stress to influence endothelial cell function, the cell must be able to sense, and then convert, mechanical forces into biochemical signals that alter physiology. A mechanosensory complex on the endothelial cell surface has been identified as an initial transducer of mechanical forces consisting of the adhesion molecules PECAM-1

(platelet endothelial cell adhesion molecule-1), which is the mechanosensor, and VE-cadherin and VEGFR-2 (vascular endothelial growth factor receptor-2), which, once activated, stimulate a host of downstream signaling pathways. Mechanotransduction via this trimolecular complex mediates integrin activation, which causes elongation and alignment of cells in the direction of flow, characteristic of cells in high-shear, protected regions [6]. This is an adaptive response that redistributes and reduces the local mechanical load experienced by the cell, reducing subsequent injury, and is dependent on anchoring of endothelial cells to the ECM via integrins. High laminar shear stress induces rapid conformational activation of integrins leading to remodeling of endothelial attachment sites and increased binding to the ECM, triggering cytoskeletal rearrangement and cell alignment [7].

High shear stress is anti-inflammatory, antiproliferative, antiapoptotic, antithrombotic, and also protective against intracellular endoplasmic reticulum stress [8], which collectively may confer some degree of protection from lesion formation. Here, we focus on the molecular mechanisms that underlie the modulation of inflammatory activation by shear stress.

Shear stress regulates endothelial activation

Studies of cultured endothelial cells have revealed that prolonged unidirectional high shear stress suppresses proinflammatory activation and leukocyte recruitment in response to an inflammatory stimulus, for example, tumor necrosis factor- α (TNF- α) [9,10]. This observation is supported by in vivo studies where endothelial cells at low shear, atherosusceptible sites express proinflammatory markers. In contrast, proinflammatory signaling is suppressed in regions of the arterial tree that are protected from atherosclerosis and exposed to high shear stress [11-14].

Understanding the mechanisms by which high shear stress reduces proinflammatory signaling is an area of active research. In vitro and in vivo studies have revealed a plethora of genes that are altered by shear stress, albeit with a wide disparity between studies, which can be attributed to the different cell types studied and variation in magnitude, direction, and duration of shear stress. Nevertheless, two key proinflammatory signaling pathways: the mitogen-activated protein kinase (MAPK) pathway and nuclear factor- κ -B (NF- κ B) pathway have been identified as being differentially regulated at protected and susceptible sites of the arterial tree [12,15,16]. It is likely that both contribute to inflammation and lesion development at susceptible sites since several proinflammatory genes

implicated in atherosclerosis (e.g., the genes encoding VCAM-1, E-selectin, and IL-8) require the activation of both pathways for transcription to occur. High laminar shear stress suppresses inflammation in part by modulating or inhibiting NF- κ B and MAPK signaling, and the underlying mechanisms are discussed in this article.

The MAPK Pathway

The MAPKs are a group of highly conserved serine/threonine protein kinases that play an important role in many cellular processes including apoptosis, proliferation, and inflammation. The three well characterized MAPK pathways are the c-Jun N-terminal kinase (JNK), the p38, and the extracellular signal-regulated kinase (ERK) pathways. The JNK and p38 pathways are preferentially activated by inflammatory cytokines and stress, whereas the ERK pathway is preferentially activated by growth factors. Typically, activation of MAPK pathways is initiated by proinflammatory mediators via activation of a cell surface receptor, activating a chronological phosphorylation cascade. Activation of JNK and p38 requires the activity of upstream MAPK kinases (MAPKKs, e.g., MKK3 and MKK6) and MAPK kinase kinases (MAPKKKs, e.g., apoptosis signal-regulating kinase-1 [ASK-1] and TGF- β activated kinase-1 [TAK-1]). Active, phosphorylated forms of JNK and p38 activate transcription factors belonging to the activating protein-1 (AP-1) superfamily (including c-Jun and activating transcription factor-2 [ATF2]), which drives transcription of proinflammatory genes such as VCAM-1. Proinflammatory MAPK activation has been linked to the development of atherosclerotic lesions, as increased levels of phosphorylated nuclear ATF-2 have been observed in human atherosclerotic plaques [16]. We have also found increased activities of JNK and p38 at atherosusceptible sites in murine aorta along with increased expression of VCAM-1, indicative of a proinflammatory phenotype [14].

Proinflammatory signaling also induces MAPK phosphatase-1 (MKP-1), a negative regulator of the MAPK pathway that inactivates p38 and JNK by removing phosphate groups, which may serve to limit the inflammatory response. It is clear that shear stress is an important factor in determining the expression pattern of MKP-1 because exposing cultured cells to unidirectional high shear stress produced a marked induction of MKP-1. We also demonstrated that MKP-1 dampens proinflammatory signaling since silencing MKP-1 function in cultured endothelial cells increased p38 activation and enhanced VCAM-1 expression in response to shear stress. Deletion of MKP-1 using an MKP-1 knockout mouse also revealed increased activity of JNK and p38 and increased expression of VCAM-1 in protected regions of the mouse

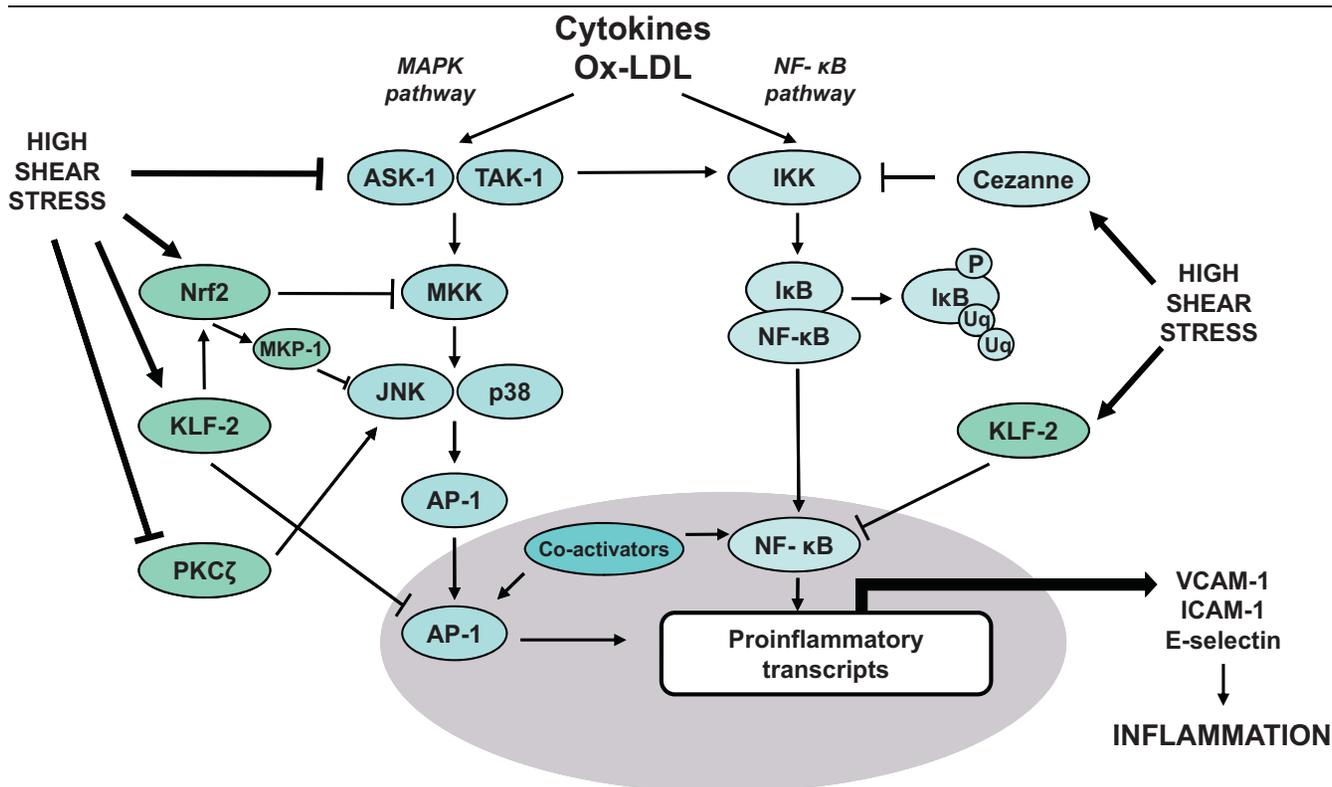
aorta. Our findings present a novel molecular mechanism contributing to the spatial distribution of vascular inflammation and atherosclerosis, where high shear stress protects arteries from inflammation by inducing persistent endothelial expression of MKP-1, which suppresses the activities of p38 and JNK [14].

Other reports have identified several other intermediaries in the JNK/p38 MAPK pathway that are negatively regulated by shear stress and that may also serve to dampen proinflammatory signaling in regions of high shear stress. In cultured cells, for example, exposure to high laminar shear stress reduces the activation of ASK-1 (a MAPKKK) by promoting association with its inhibitory binding protein, depicted in Figure 3 [17]. High shear stress also reduces JNK activity by blocking cleavage of protein kinase C epsilon (PKC ζ) [18,19]. In addition, the influence of shear stress on JNK activation may be influenced by the nature of the subendothelial matrix [20], underlying the complex interplay of flow-regulated and inflammatory signaling pathways.

In subsequent studies we have found that nuclear factor erythroid 2-related factor (Nrf2), an antioxidant (protective) transcription factor, is preferentially activated by high shear stress and may be atheroprotective via negative regulation of the MAPK pathway [21]. In unstimulated cells, Nrf2 is suppressed by binding to kelch-like ECH-associated protein-1 (Keap-1), which targets it for ubiquitination and degradation by the proteasome. Upon stimulation, this interaction is disrupted, leading to dissociation from Keap-1. This results in stabilization and accumulation of Nrf2 and translocation to the nucleus where Nrf2 can induce the expression of many antioxidant genes (e.g., those encoding heme oxygenase-1, glutathione S-transferase, and ferritin).

Given the important role of oxidative mechanisms in vascular inflammation, we recently studied the role of Nrf2 in regulating endothelial activation. We found that Nrf2 is activated in regions of the mouse aorta exposed to unidirectional high shear stress and protected from atherosclerosis. Only inactive cytoplasmic forms of Nrf2 could be detected in the inner curvature, an area of low/oscillatory shear stress and susceptible to inflammation and lesion formation. In mice lacking Nrf2, both p38 activity and VCAM-1 expression were significantly increased in the protected region, suggesting that Nrf2 blocks proinflammatory signaling in regions of high shear stress. Parallel *in vitro* studies confirmed this, where application of unidirectional high shear stress to cultured cells significantly enhanced expression and nuclear activity of Nrf2 with a simultaneous reduction in VCAM-1 expression. We propose that Nrf2 suppresses

Figure 3. Inflammatory signaling pathways that are regulated by high shear stress



Both the mitogen-activated protein kinase (MAPK) and nuclear factor-kappa-B (NF-κB) signaling pathways are active at sites of the arterial tree exposed to low/oscillatory shear stress and are stimulated by proinflammatory cytokines leading to inflammation and lesion development. At sites of high shear stress, these signaling pathways are inhibited at several levels with a consequent inhibition of inflammation. AP-1, activating protein-1; ASK-1, apoptosis signal-regulating kinase 1; ICAM-1, intercellular adhesion molecule-1; IκB, inhibitory κB; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; KLF-2, Krüppel-like factor-2; MKP-1, MAPK phosphatase-1; MKK, MAPK kinase; Nrf2, nuclear factor erythroid 2-related factor; Ox-LDL, oxidized low-density lipoprotein; P, phosphorylation; PKCζ, protein kinase C epsilon; TAK-1, transforming growth factor (TGF)-β activated kinase 1; VCAM-1, vascular cell adhesion molecule-1; Uq, ubiquitin.

endothelial activation by targeting the MAPK signaling pathway at two levels: by reducing MKK3/6 signaling to p38 and by enhancing the activity of MKP-1. Expression levels of these intermediaries are not altered, rather Nrf2 facilitates increased activity by influencing their redox state; Nrf2 promotes an antioxidant environment that favors the reduced form of MKP-1, which has greater catalytic activity [21]. Thus our studies reveal that Nrf2 is a key determinant of the spatial differences in inflammation and atherosclerosis. Interestingly, genetic deletion of Nrf2 did not completely restore proinflammatory activation at the protected site, suggesting that Nrf2 works in concert with other anti-inflammatory molecules to inhibit inflammation at sites of high shear stress.

The NF-κB pathway

The transcription factor NF-κB has been closely linked with cardiovascular health and disease due to its control of multiple processes including immunity, inflammation,

cell survival, differentiation and proliferation, and regulation of cellular responses to stress, hypoxia, stretch, and ischemia. NF-κB signaling in endothelial cells typically leads to the expression of genes that induce recruitment of inflammatory cells to the vessel wall, including ICAM-1 (intercellular adhesion molecule-1), VCAM-1, E-selectin, and cytokines such as TNF-α and IL-1. NF-κB is composed of two subunits, with the p50-p65(RelA) heterodimer most abundant in endothelial cells. In the absence of a stimulus, NF-κB is bound to an inhibitory molecule, IκB, which masks its nuclear localization sequence and sequesters it in the cytoplasm. NF-κB is activated by several signaling pathways that cause it to be released from IκB, mostly via activation of an IκB kinase (IKK), which phosphorylates IκB, leading to its ubiquitination and subsequent degradation by the proteasome. Once the heterodimer is released, the nuclear localization sequence permits translocation to the nucleus and binding to target sequences on the genome. NF-κB may also itself be

phosphorylated, which facilitates DNA binding and transcription, but its activity is tightly regulated by negative feedback loops that limit the duration of nuclear localization and hence transcriptional activity. This relies on NF- κ B-dependent transcription of I κ B α , which contains a nuclear export sequence, and by binding to NF- κ B serves to remove it from the nucleus. Also, activation of the deubiquitinating enzymes A20 and Cezanne by proinflammatory signaling molecules blocks IKK activation and prevents release of NF- κ B from I κ B [22,23].

Active NF- κ B has been identified in atherosclerotic plaques and early lesions along with many NF- κ B regulated molecules, indicating that it is instrumental in atherogenesis. One significant study demonstrated reduced inflammation and plaque formation in atherosclerosis-prone cholesterol-fed mice lacking endothelial expression of IKK γ , supporting the idea that NF- κ B-mediated proinflammatory gene expression plays a causal role in lesion development [24]. Studies in "healthy" mice and pigs revealed that the expression of NF- κ B subunits and regulatory components (p65, I κ B α , and I κ B β) is significantly increased in regions of the aorta exposed to low shear stress compared to high shear stress [11,12]. Expression was, however, restricted to the cytoplasm, indicating that NF- κ B was inactive. This suggests that cells exposed to low shear stress are primed for activation upon challenge with a proinflammatory stimulus due to an increased expression of NF- κ B subunits [11]. Studies in pigs also showed a reduced expression of negative regulators that switch off NF- κ B activity (e.g., Cezanne) at the atherosusceptible site [12]. We have subsequently shown that the expression of Cezanne is shear-sensitive and is increased by unidirectional laminar shear stress [22].

NF- κ B transcription factors have the potential to exert dual functions since they can be both antiapoptotic (protective) and proinflammatory. Our own studies, using a parallel plate flow chamber, demonstrated that high laminar shear stress enhanced NF- κ B-mediated induction of several antiapoptotic genes, such as *BCL2*, *A20* (*TNFAIP3*), *A1* (*BCL2A1*), and *MnSOD* (*SOD2*), whilst simultaneously dampening proinflammatory activation in response to a proinflammatory stimulus, TNF- α [10]. We therefore conclude that high shear stress can influence the function of NF- κ B by affecting the balance of induction of inflammatory and antiapoptotic genes. Thus endothelial cells at low shear regions are prone to inflammatory activation via multiple mechanisms that influence both the expression and activity of NF- κ B.

Numerous studies have shown a specific induction of Krüppel-like factor-2 (KLF-2) in response to laminar

shear stress and have placed KLF-2 as a key regulator of shear-responsive transcripts and a mediator of the atheroprotective effects of high shear stress [15,25,26]. KLF-2 has been shown to attenuate endothelial activation and leukocyte migration in cultured endothelial cells by inhibiting the expression of proinflammatory genes such as those encoding VCAM-1 and E-selectin. The suppressive effects of KLF-2 on inflammatory activation were attributed to its ability to sequester critical coactivators of NF- κ B, cyclic AMP response element-binding protein (CBP) and p300, thereby reducing NF- κ B mediated transcription [15].

Anton Horrevoet's group have shown that KLF-2 may also reduce proinflammatory gene expression by inhibiting the MAPK pathway. They demonstrated that KLF-2 inhibits nuclear localization of the ATF-2 transcription factor thereby reducing transcription from AP-1 promoter sites. Similarly, knockdown of KLF-2 with small interfering RNA caused a marked reduction in proinflammatory gene expression in cells without flow [16]. Subsequent studies revealed that KLF-2 inhibits the phosphorylation of JNK, an effect that is dependent on the actin cytoskeleton [27]. Work from this group has also shown that KLF-2 is able to potentiate the atheroprotective effects of flow-induced Nrf2 by enhancing its nuclear translocation and activation [28], indicating that these central regulators of the anti-inflammatory effects of shear stress may work in synergy to modulate the transcriptome.

Implications for therapeutic strategies to prevent or treat atherosclerosis

It is clear that both the MAPK pathway and the NF- κ B pathway play a significant role in endothelial activation at atherosusceptible sites and that high shear stress is able to suppress activation of these pathways and protect against lesion development (summarized in Figure 3). KLF-2 and Nrf2 have been identified as central transcriptional regulators of the physiological response to shear stress and are of major interest since pharmacological manipulation could potentially be used to reduce vascular inflammation at atherosusceptible sites and hence slow or prevent lesion formation to reduce morbidity of cardiovascular disease. To this effect, we recently demonstrated that sulforaphane, a naturally occurring organic compound that acts as an antioxidant and is derived in high quantities from broccoli, watercress, and other green vegetables, has atheroprotective actions. We found that sulforaphane, at a relatively low concentration, can reduce inflammatory activation of endothelial cells at atherosusceptible sites by increasing Nrf2 activity via inhibition of Keap-1 [21]. Thus sulforaphane is an example of a targeted therapeutic

intervention that enhances expression of protective, anti-inflammatory transcripts at atherosusceptible sites and may, in the future, have clinical utility for the prevention or treatment of cardiovascular inflammation. Since sulforaphane can be found in abundance in many green vegetables, this finding is consistent with the concept that eating broccoli and other green vegetables can lower the risk of atherosclerosis.

Several groups have recently studied the effects of statins (cholesterol-lowering drugs) on endothelial physiology and observed that statins, independently of their lipid-lowering actions, can suppress numerous inflammatory genes via the induction of KLF-2 [29,30]. Interestingly, exposure of endothelial cells to laminar shear stress enhanced the responsiveness of endothelial cells to statins, whereas exposure to disturbed blood flow had the opposite effect [31]. These data highlight the influence of mechanical forces on vascular responses to pharmacological agents and suggest that statins may preferentially influence high-shear regions of the arterial tree.

Conclusions

Genome-wide approaches are now required to further define the molecular mechanisms that control the spatial distribution of vascular inflammation and atherogenesis. Analysis of the endothelial transcriptome at sites of low/oscillatory and high shear stress will reveal specific transcripts that are differentially regulated in response to shear stress and could, in time, be targeted for pharmacological manipulation. This approach will also shed light on the mechanisms by which shear stress modulates endothelial apoptosis, proliferation, and thrombogenicity, all of which contribute to the pathogenesis of atherosclerosis. Better understanding of these mechanisms will enable targeted therapeutic interventions that promote a protective phenotype in atherosusceptible regions in order to slow or even halt the progression of cardiovascular disease.

Abbreviations

AP-1, activating protein-1; ASK-1, apoptosis signal-regulating kinase-1; ATF-2, activating transcription factor-2; CBP, cyclic AMP response element-binding protein; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; E-selectin, endothelial selectin; ICAM-1, intercellular adhesion molecule-1; I κ B, inhibitory κ B; IKK, I κ B kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; Keap-1, kelch-like ECH-associated protein-1; KLF-2, Krüppel-like factor-2; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; MKP-1, MAPK phosphatase-1; MnSOD, manganese superoxide dismutase; NF- κ B, nuclear factor-kappa-B;

Nrf2, nuclear factor erythroid 2-related factor; PECAM-1, platelet endothelial cell adhesion molecule-1; PKC ζ , protein kinase C epsilon; TAK-1, transforming growth factor (TGF)- β activated kinase-1; TNF- α , tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1; VE-cadherin, vascular-endothelial cadherin; VEGFR-2, vascular endothelial growth factor receptor-2.

Competing interests

The authors declare that they have no competing interests.

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