The transcription factor KLF2 in vascular biology

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Chapter 1

General Introduction
Chapter 1

Atherosclerosis

Cardiovascular diseases are the most prevalent causes of mortality and morbidity worldwide today. The increasing prevalence of obesity and diabetics, will only aggravate this in the future. The pathological change of the vessel wall of arteries caused by atherosclerosis is the underlying cause of ischemic heart disease and stroke. Atherosclerosis is believed to initiate before or soon after birth with non-pathological intimal thickening. In the first two decades of life, some of these lesions slowly progress by accumulating lipids, which attract inflammatory cells. These lesions can grow in size over decades, without causing clinically relevant symptoms, mainly because of compensatory outwards remodeling of the arterial wall. Symptoms usually arise around the forth decade, when the arterial lumen is narrowed enough to significantly impede blood flow, causing the downstream tissue to become ischemic, especially during physical exercise. However, acute events like heart attack and stroke can also occur, which are the main cause of morbidity and mortality due to atherosclerosis. These events are caused by the sudden rupture of an advanced atherosclerotic lesion and subsequent occlusion of the artery by a thrombus.

Atherosclerotic lesions initiate with the accumulation of low-density lipoproteins (LDL), which release phospholipids upon oxidation. This causes activation of the overlying endothelium, which then expresses the surface receptors vascular cell adhesion molecule (VCAM) and intracellular adhesion molecule (ICAM) and attracts monocytes, which adhere to and transmigrate through the endothelial layer to differentiate into macrophages in the intima of the artery. These macrophages phagocytose the oxidized LDL, changing into so-called “foam-cells” due to the “foamy” appearance of the intracellular lipid droplets. These “foam-cells” secrete inflammatory cytokines, which not only further activate the local endothelium, but also attract other inflammatory cells like monocytes and T-lymphocytes. This influx further increases the inflammatory burden of the growing lesion and causes migration of smooth muscle cells (SMCs) from the media of the artery, which dedifferentiate and can form a so-called fibrous cap. Next to inflammatory cytokines, macrophages produce matrix metallo-proteinases, which degrade local collagen depositions and destabilize the lesion.

Lipid-laden macrophages can go into necrosis, thereby initiating the formation of a necrotic core. Ischemic areas within the atherosclerotic plaque trigger an angiogenic response resulting in ingrowth of blood vessels from the vaso vasorum. Hemorrhages from these vessels in atherosclerotic plaques deposit erythrocytes in the lesion, further increasing the mass of the necrotic core, which consists of cholesterol crystals and often of calcifications too. Lesions with a small fibrous cap and a large necrotic core are thought to be the unstable plaques that rupture and cause acute clinical symptoms like myocardial infarction or stroke.

Hemodynamics

Despite the systemic nature of the associated risk factors like smoking, diabetes,
Figure 1. Atherosclerosis development correlates with local low shear stress levels. (A) A preparation of the large arterial vasculature, with atherosclerosis prone sites indicated, being a carotid bifurcation, the inner curve of the aortic arch and a branching point of the renal artery from the abdominal aorta development. Adapted from Davies et al. (B) Micro-particles were used to visualize flow patterns in human post mortem coronary arteries. Flow at branching points of the human left main coronary artery is disturbed on one side of the artery and laminar on the other side. Adapted from Asakura et al.
hyperlipidemia and hypertension, atherosclerosis only develops at predisposed sites in the arterial tree.\textsuperscript{8} This is likely due to the local disturbances in blood flow, as these sites are always near bends and bifurcations in the vasculature (Figure 1). Laminar blood flow occurs in straight arteries and near the outer curvatures of bends and exerts a tangential viscous drag on the vascular endothelium, called shear stress. The laminar flow in the straight parts of arteries is unidirectional and pulsatile, due to the cardiac cycle, and reaches shear stress levels of 15 to 70 dynes/cm\textsuperscript{2}.\textsuperscript{9} Near bends and bifurcations, the flow is still pulsatile, but highly turbulent and bidirectional, generating oscillatory shear stress of 0 to 10 dynes/cm\textsuperscript{2}.

High shear stress induces an atheroprotective, endothelial phenotype, while turbulent blood flow causes low or oscillatory shear stress, which is associated with endothelial dysfunction and atherosclerosis development. This was first demonstrated by the group of Glagov, who elegantly showed that an inverse correlation between shear stress and atherosclerosis development exists.\textsuperscript{10} Much later, with the development of the first mouse models for atherosclerosis, lack of shear stress was actually shown to be the cause of atherosclerosis development.

**Figure 2. Atherprone versus atheroprotected regions of the vasculature have distinctive endothelial phenotypes.**

Near the bifurcation of a rabbit aorta that branches into the two iliac arteries, endothelial actin cytoskeleton on the inner side of the bifurcation is aligned in the direction of the local flow and forms typical “stress” fibers (A) and (C). (B) At the outer curve, where flow is irregular and atherosclerosis develops, the actin cytoskeleton is only present at the lateral sides of the ECs. Adapted from Kim et al.\textsuperscript{16}
formation. In one of these models, developed in our country, a silastic cuff is placed around the carotid artery, thereby narrowing the lumen and causing an increase in blood flow and wall shear stress in the part of the artery that is inside the collar. This region is then protected from atherosclerosis formation in ApoE−/− mice and atherosclerosis actually develops just proximal of the collar, where shear stress is lowered. The causal relationship between shear stress and atherogenesis was recently studied in more detail by artificially applying different flow regimes using a tapered cuff. Using this mouse model, low unidirectional shear stress was shown to induce a vulnerable plaque phenotype, with a small fibrous cap and a large lipid pool, while low oscillatory shear stress caused a more stable plaque, consisting of a large fibrous cap and little lipid content.

Mechanosensing

Endothelial cells (ECs) form the inner lining of the vasculature, thereby being the only cells subjected to (blood flow-generated) shear stress. It has therefore been hypothesized that ECs possess receptors for shear stress, also termed mechanosensors. The exact molecular composition of these mechanosensors is not known, but most likely involves the simultaneous actions of several sensory systems. Among the proposed sensory mechanisms are G protein-coupled receptors, receptor tyrosine kinases, the glycocalyx, cilia, caveolae, Ca++ channels, integrins, focal adhesions, the actin cytoskeleton and intercellular junctions. Through activation of these systems and subsequent intracellular signal propagation, shear stress signals ultimately lead to alterations in protein activity and transcriptional changes in the nucleus.

One of the apparent morphological changes induced by shear stress is alignment of the ECs in direction of the flow. This is most likely caused by the concomitant formation of stress fibers. These fibers consist of actin polymers and run parallel with the flow direction and between focal adhesions along the basal membrane. Next to providing cellular structural integrity, stress fibers also function in the shear stress sensory complex. Stress fiber formation is also accompanied by activation of RhoA and phosphorylation of focal adhesion kinase (FAK) at tyrosine residue 397.

Krüppel-like factor 2

One of the transcription factors that elicit shear stress-induced transcriptional changes is Krüppel-like factor 2 (LKLF, KLF2). KLF2 was first identified to induce single-positive T-cell survival and quiescence. In ECs, KLF2 was found to be specifically induced by laminar shear stress. In accordance with these findings, KLF2 expression in vivo was found to correlate with expected local shear stress levels. KLF2 expression is required for normal blood vessel formation and vascular tone as the KLF2−/− mouse has an embryonically lethal phenotype. This was shown to be caused by a lack of vascular tone and vessel stability.
Figure 3. KLF2 expression is induced specifically by shear stress and correlation with high shear stress (i.e. atheroprotected) regions in vivo.

(A) Micro-array analysis was performed on human umbilical vein endothelial cells (HUVECs) stimulated with shear stress, cytokines or growth factors as well as other (non-endothelial) cells. Shown is the panel of genes that was specifically induced by shear stress. Adapted from Dekker et al.19

(B-F) In situ hybridization for KLF2 on vessel specimens indicated that KLF2 is highly expressed by ECs on the outer curve, where shear stress is high. On the inner curvature, KLF2 expression levels are markedly lower. NI indicates neo-intima. Adapted from Dekker et al.23
leading to heart-failure and intra-embryonic bleeding, respectively. Interestingly, both the complete systemic knock-out of KLF2 and the endothelial-specific KLF2 gene ablation do not prevent initial EC development, but rather impair recruitment and migration of SMCs. However, the endothelial-specific KLF2 null mouse is a complete pheno-copy of the total KLF2 null mouse and the SMC-specific ablation of KLF2 resulted in no apparent phenotypic change. Furthermore, KLF2 was shown to be essential for the shear stress-mediated induction of endothelial nitric oxide synthase (eNOS) and Thrombomodulin (THBD) as well as the shear stress-mediated inhibition of adrenomedullin (ADM) and endothelin (EDN1).22, 23

The signaling pathway responsible for induction of KLF2 has recently been elucidated and comprises the mitogen activated protein kinases (MAPKs) MAPK kinase 5 (MEK5) and extracellular-signal-regulated kinase 5 (ERK5) and the transcription factor myocyte enhancer binding factor 2 (MEF2).24 Interestingly, these MAPKs have also been found to be essential for proper endothelial function.25, 26 In addition, MEF2C is a critical mediator in vascular

**Figure 4. Statins induce KLF2 transcription through inhibition of Rho.**
Statins inhibit HMG-CoA reductase, which is the rate-limiting enzyme for cholesterol biosynthesis. This leads to a depletion of the cellular pool of geranylgeranyl pyrophosphate, which is essential for Rho function. KLF2 is inhibited by Rho and NFκB, but activated by shear stress. Adapted from Jain and Ridker.34
development and MEF2A loss of function mutations have been found to be associated with cardiovascular disease.\textsuperscript{27, 28} Next to transcriptional induction, KLF2 protein levels are also increased by shear stress through mRNA stabilization by inhibition of phosphoinositide-3-kinase (PI3K).\textsuperscript{29} Inhibition of KLF2 transcription occurs in the presence of inflammatory signals like tumor necrosis factor α (TNFα) or interleukin 1 β (IL1β). This is due to increased nuclear factor κ B (NFκB) activity, which recruits histone deacetylases to the KLF2 promoter, thereby inhibiting the transcriptional activity of MEF2.\textsuperscript{30}

The expression of KLF2 has been shown to be limited to the hematopoietic lineage, which consists of ECs and leukocytes. In T-cells, for instance KLF2 promotes cell survival and quiescence as well as migration from the thymus.\textsuperscript{18, 31} Recently, KLF2 was also described to be a marker gene for undifferentiated embryonic stem cells (ESC), but to be repressed upon differentiation.\textsuperscript{32} Similarly, in monocytes, which contain high levels of KLF2, KLF2 has been described to inhibit activation and differentiation into macrophages, which have low levels of KLF2.\textsuperscript{33}

**HMG-CoA reductase inhibitors (Statins)**

Known pharmacological inducers of KLF2 are the 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors, also known as statins.\textsuperscript{34} Commonly used for their cholesterol lowering effects, statins are known to induce pleiotropic, beneficial effects in ECs, which were found to depend on KLF2 induction.\textsuperscript{35} One of the downstream effects of inhibiting cholesterol synthesis is attenuation of the essential geranylgeranyl pyrophosphate modification of RhoA (Figure 4). This inhibition of RhoA activity was found to be essential for induction of KLF2 transcription by statins. Another Rho inhibitor, C3 exotoxin, which is produced by pathogens to inhibit inflammatory activation in the host, was also found to induce KLF2 expression, while exogenous activated RhoA was shown to inhibit KLF2 expression.\textsuperscript{36}

In the context of atherosclerosis, the Rho pathway has been successfully modulated to inhibit atherogenesis. Inhibition of Rho-kinase (ROCK), the activated downstream kinase of RhoA, with the specific inhibitor Y-27632, was shown to inhibit experimental atherogenesis in LDLR\textsuperscript{-/-} mice.\textsuperscript{37} And in a clinical setting, treatment with Fasudil, which also inhibits ROCK, improved endothelial function in coronary artery disease (CAD) patients.\textsuperscript{38, 39}

**Transforming growth factor β signaling**

One of the effects of disturbed shear stress on endothelium is signaling through the transforming growth factor β (TGF-β) pathway.\textsuperscript{40} TGF-β signaling can occur in many cell-types, each of which responds differently to TGF-β depending on the specific cellular context. For example, TGF-β signaling in macrophages, smooth muscle cells and T-lymphocytes is deemed atheroprotective, as it was found to be associated with a decrease in inflammation and vulnerable plaque formation in atherosclerosis.\textsuperscript{41, 42} TGF-β signaling in endothelium
however promotes apoptosis and increases permeability through the MAPK p38. Furthermore, TGF-β induces the endothelial oxidized-LDL receptor OLR1, plasminogen activator inhibitor 1 (PAI-1) and monocyte chemotactic protein 1 (MCP-1), all of which are considered pro-atherogenic.

TGF-β signaling occurs through a heteromeric complex of type I and type II TGF-β receptors. ECs express one type II receptor and two type I receptors, activin receptor-like kinase (ALK) 5 and ALK1. Stimulation with TGF-β leads to phosphorylation of either ALK1, which results in phosphorylation of receptor-regulated Smads (R-Smads) Smad1 and Smad5, or ALK5, which leads to phosphorylation of R-Smads Smad2 and Smad3. Phosphorylated R-Smads then translocate to the nucleus with Smad4, where gene-expression is regulated through binding to Smad binding elements. Known attenuators of TGF-β signaling are the inhibitory Smads (I-Smads) Smad6 and Smad7, which compete with R-Smads for association with the type I TGF-β receptor, thereby inhibiting phosphorylation of R-Smads. Moreover, Smad7 induces degradation of the type I receptor by recruiting ubiquitinases, is specifically expressed in ECs and known to be induced by shear stress.

**Mitogen activated protein kinase signaling**

Parallel to signaling through the Smad protein cascade as discussed above, TGF-β signaling has also been described to occur through MAPKs. MAPK signaling consists of basically four distinct signaling routes with complex interactions and is a pro-inflammatory signaling pathway implicated in atherosclerosis. These four canonical routes comprise the externally regulated kinase (ERK) 1/2, Jun NH2-terminal kinase (JNK), p38 and ERK5 pathways (Figure 5). Mitogen-activated protein kinases (MAPK) are a family of Ser/Thr protein kinases, which act in a consecutive three-stage activation cascade. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs, MAP2Ks), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKks, MAP3Ks). The MAP3Ks are activated by interaction with the family of small GTPases and/or other protein kinases, connecting the MAPK signaling cascade to cell surface receptors or external stimuli.

In case of TGF-β signaling, MAPKs like JNK and p38 act through activation of activator protein 1 (AP-1), which consists of a homo- or heterodimer of members of the Jun, Fos, musculoaponeurotic fibrosarcoma oncogene homolog (MAF) or activated transcription factor (ATF) families and acts as a co-factor for the Smad-signaling cascade. Next to TGF-β, TNF-α and other inflammatory cytokines are also known to activate the p38 and JNK MAPK signaling routes. The ERK1/2 and ERK5 signaling pathways are generally considered to have anti-inflammatory and protective effects in ECs. Indeed, as discussed above, KLF2 expression is induced through activation of the ERK5 pathway.
Oxidative stress

On the side of the artery where shear stress is high, KLF2 is expressed and atherogenesis is inhibited, ECs exhibit potent anti-oxidant capacity, which acts as a cytoprotective determinant. Production of reactive oxygen species (ROS) is a naturally occurring phenomenon in ECs exposed to shear stress. The main ROS produced is superoxide, which is transformed into hydrogen peroxide, a process catalyzed by superoxide dismutases, or to peroxynitrite through a reaction with nitric oxide, which is produced by nitric oxide synthases like eNOS. At low levels, ROS act as important mediators of signal transduction, but too much oxidative stress is harmful. Therefore, cells utilize antioxidant enzymes for protection against this detrimental oxidative stress. These enzymes are regulated through a common transcription factor binding site in their promoters, the antioxidant response element (ARE). Heterodimers of the nuclear factor erythroid 2-like 2 (NFE2L2, Nrf2) and small MAF proteins bind the DNA sequence of the ARE in response to oxidative stimuli. This is realized through the ROS-mediated modification of Keap1, the chaperone protein that keeps Nrf2 in the cytoplasm or targets Nrf2 for degradation, which results in dissociation of the Keap1-Nrf2 complex and nuclear translocation of Nrf2. It was recently demonstrated that shear stress activates Nrf2 and induces ARE-dependent gene expression.

Figure 5. MAPK signaling consists of four major pathways. A simplified scheme of the MAPK signalling cascades shows that MAPK kinases (MAPKK) are phosphorylated by MAPKK kinases (MAPKKK) in four distinct routes. Many of the MAPK routes have crosstalks with other (MAPK) signalling routes. Adapted from http://www.cellsignal.com/.
Figure 6. Atherosclerosis development correlates with local low shear stress levels. After endothelial injury (1), the vessel wall is repaired/re-endothelialized through migration/proliferation of neighbouring ECs (2) and/or incorporation of circulating endothelial progenitor cells (3), which differentiate into ECs or promote ingrowth of ECs in a paracrine manner. Adapted from Dimmeler and Zeiher.67
After vascular injury, the endothelial monolayer can be repaired via multiple mechanisms (Figure 6). The “classical” mechanism is through migration and proliferation of neighboring ECs. Another mechanism by which ECs are replenished, is incorporation of endothelial progenitor cells (EPCs) in the endothelium. EPCs are circulating bone marrow–derived cells, that can home to sites of neovascularization and differentiate into ECs or promote angiogenesis by secreting paracrine factors. Furthermore, EPCs were shown to be mononuclear cells with both endothelial and monocytic features. Stromal cell derived factor-1 (SDF1), vascular endothelial growth factor (VEGF), granulocyte monocyte colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and Epo have been identified as factors promoting the mobilization of EPCs from the bone marrow. EPCs need to migrate and adhere to sites where endothelial repair is required. This process is known as homing and depends on numerous stimuli. For example SDF-1 is required for chemotaxis towards these sites, while integrins are essential for the adherence of EPCs. Shear stress is one of the factors that play a role in the differentiation of EPCs to ECs.

In terms of cardiovascular disease, an inverse correlation was found between the number of circulating EPCs and risk for cardiovascular events. Furthermore, EPC function was found to be impaired in diabetic and CAD patients. Similarly, statins, the most common drugs used to treat CAD patients, have been shown to induce mobilization from the bone marrow and improve EPC function, as a pleiotropic effect.

Aim of this thesis

This thesis aims to identify the role of KLF2 in the shear stress-mediated atheroprotective phenotype of ECs. It has been known for decades that atherosclerosis develops focally, a phenomenon which has been linked to local blood flow disturbances. However, the underlying mechanism has long been elusive. The identification of KLF2 as a shear-responsive transcription factor that is absent from atheroprone vascular regions yielded the hypothesis that this transcription factor is responsible for the atheroprotective endothelial phenotype induced by shear stress. Moreover, the recent finding that the most often prescribed drug-class in the Western world, the statins, induce transcription of the KLF2 gene, which was also found to be essential for the statin-mediated beneficial transcriptional changes in ECs, underscores the importance of this transcription factor for EC homeostasis. This thesis describes the role of KLF2 in ECs and EPCs in the context of cardiovascular disease, which was investigated using lentiviral overexpression and siRNA-mediated knock-down in combination with other state-of-the-art techniques like micro-arrays and protein arrays. Given the recently proposed pivotal role of KLF2 as a potential atheroprotective factor, a more detailed insight into its regulation and functions is needed to be able to translate these basic insights into beneficial treatment modalities for patients suffering from cardiovascular disease.
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