KLF2, a critical modulator in vascular disease
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Link to publication

Citation for published version (APA):
van Thienen, J. V. (2009). KLF2, a critical modulator in vascular disease

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General Discussion
The research described in this thesis is aimed at providing novel insights into the pathogenesis of atherosclerosis, specifically by detailed studies of the role of the transcription factor KLF2 and its functions in endothelial cells and macrophages. Functional properties of KLF2 in the inflammatory process of atherogenesis were studied, as well as the molecular pathways that regulate its expression in conditions reflecting both function and dysfunction of the cells in which it is expressed. These research efforts are ultimately directed at devising novel pharmacotherapeutic strategies that may aid in mitigating the development of vascular disease.

**Functional properties of endothelial KLF2: regulation of vascular tone and inflammation**

The initiation of atherogenesis is importantly determined by the presence of turbulent bloodflow, causing lowered shear stress on the endothelial monolayer. The mainstay of atherosclerosis development in low-shear regions of the vasculature is endothelial dysfunction. Vasomotor function is regulated strictly by the endothelial monolayer through intricate communication between blood-borne mediators and the vascular smooth muscle cells that effectuate vessel tone. Hypertension is the most common contributor to the risk factor profile that determines the initiation of atherogenesis. It has been well documented that adequately targeting hypertension by pharmacological treatment may contribute greatly to prevention strategies in cardiovascular disease. Chapter 2 highlights the important contribution of KLF2 to the proper regulation of vessel tone in the shear-exposed endothelium, demonstrating its potential in mediating shear-induced endothelial function. On balance, KLF2 seems to exert vasodilating, i.e. anti-hypertensive actions, through regulation of major determinants of vessel tone (eNOS, ACE, endothelin-1). A murine model of endothelial cell-specific knockout of KLF2 resulted in high-output cardiac failure that could only be corrected by the catecholamine phenylephrine, indicating the existence of a delicate balance of vasoconstrictive and vasodilatory mediators controlled by KLF2. To adequately control hypertension, the use of multiple anti-hypertensives is often necessary. The control exerted by KLF2 on multiple determinants of vessel tone, may offer the possibility of devising a pharmacological intervention that is effective by the use of only a single anti-hypertensive.

The endothelial inflammatory response importantly contributes to flow turbulence-mediated atherogenesis. Current prevention strategies for diminishing atherosclerosis development are mainly based on cholesterol-lowering drugs, anti-hypertensives and anti-coagulants. In these strategies, the inflammatory components that initiate atherosclerosis development are not efficiently targeted. Notwithstanding, the anti-inflammatory properties of statins have become an important pathophysiological explanation for their pleiotropic effects. Although clinical significance of these effects is often difficult to establish, cholesterol-independent actions of statins have led to clinical trials aimed at evaluating their efficacy of statins in multiple auto-immune diseases. Chapter 3 illustrates the crucial role of KLF2 in mediating these pleiotropic effects in the endothelium. However, the data presented establish
that fluid shear stress confers an even more potent anti-inflammatory response, indicating that pharmacological anti-inflammatory therapy may indeed be amenable to improvement, should it become possible to more efficiently target increased KLF2 expression. Exercise-induced increases in shear may also augment the expression of anti-inflammatory, anti-thrombotic and anti-oxidant proteins \textit{in vivo}. As a testament to the increased inflammatory status of patients who develop atherosclerosis, increased levels of Cell Adhesion Molecules (CAMs) and inflammatory mediators like MCP-1 and TNF-\(\alpha\) have been well documented. It has been shown that physical exercise reduces levels of inflammatory markers like TNF-\(\alpha\) and that it is the most important physiological stimulus of nitric oxide (NO)-mediated vasodilation. Findings presented in Chapter 3 demonstrate that especially shear-induced increases of KLF2 result in anti-inflammatory and anti-thrombotic gene expression, in the presence of inflammatory mediator TNF-\(\alpha\), may provide the molecular basis for the importance of exercise-induced NO-release and suppression of the inflammatory response.

\textbf{Molecular mechanisms of endothelial inflammatory modulation by KLF2}

Studies into the pathways that regulate endothelial inflammatory activation have lead to the analysis of myriad regulatory networks. In the literature, NF-kB is the prime transcription factor implicated in atherogenesis, however it is known to exert protective actions as well. It has been shown that NO diminishes inflammatory gene expression by inducing and stabilising the NF-kB inhibitor I\(\kappa\)B\(\alpha\). Furthermore, it has been demonstrated that NF-kB is primed for activation in endothelium that is exposed to decreased shear. Given these data, it is not surprising that KLF2 was documented to counteract some of the pro-inflammatory actions of NF-kB. However, data presented in Chapter 4 strongly support an important role for prolonged shear stress-induced KLF2 in the repression of chronic inflammatory activation, by preventing nuclear translocation of the AP-1 constituent ATF2, while NF-kB activation may be reserved for facilitating the acute inflammatory response. Thus, modulation of the basal inflammatory status of endothelium is possible, without sacrificing the vital capability of endothelial cells to respond to acute inflammatory stimuli via NF-kB activation. These properties make KLF2 even more interesting as a potential target for therapeutic intervention in vascular inflammatory states.

Identifying the exact mechanism by which transduction of the biomechanical signals exerted by shear stress are translated into an effective anti-inflammatory response, as described above, has proven difficult. A model that has arisen in literature is the tensegrity model of shear-transduction, in which the cell itself, as well as its contacts with neighbouring cells and the subcellular matrix, serves as the operative mechanosensor. This model involves an intricate network of communication between integrins, matrix proteins, the cytoskeleton, and signal transduction by MAPKines. Chapter 5 outlines the involvement of KLF2 in such a network, in which a negative feedback mechanism is established to operate through cytoskeleton-dependent MAPK signalling involving RhoA, a GTPase known to...
participate in integrin-mediated mechanosensing. These findings provide evidence for the existence of a regulatory network that enables KLF2 to create a robust and controllable cellular response to diverse stimuli. The involvement of VE-cadherin in regulating KLF2 expression, as presented in Chapter 6, further illustrates the important link toward signalling involving cytoskeletal proteins. Upon vascular injury, the role of KLF2 in counter-balancing the inflammatory response appears to be controlled by VE-cadherin expression, as KLF2 expression is importantly diminished upon restoration of cell-cell contacts in the new endothelial monolayer. The involvement of VE-cadherin in a mechanosensory complex, that is able to mediate shear-responses, again links the regulatory pathways that determine KLF2 expression to the influence of biomechanical forces. Taken together, the studies presented in this thesis further elucidate the crucial molecular link between mechanotransduction of laminar shear stress and the concomitant anti-inflammatory cellular responses governed by KLF2.

**KLF2 in macrophage inflammatory signalling.**

Given the well-documented anti-inflammatory properties that statins may convey to monocytes/macrophages, studies described in Chapter 7 led to the hypothesis that KLF2, in analogy to its induction of a quiescent endothelial state, may function as a molecular switch between an activated and more quiescent phenotype of monocytes/macrophages. The results indeed showed that KLF2 mediates some of the important pleiotropic anti-inflammatory effects of statins. However, further detailed analyses of the KLF2-mediated macrophage transcriptome, as described in Chapter 8, only partially corroborated this hypothesis. Contrary to expectations, a clear indication for a KLF2-induced phenotypic drift towards an anti-inflammatory (M2) state of differentiation could not be established. Given the partial, but distinct, influence of KLF2 on LPS-induced inflammatory activation, the involvement of TLR4-dependent signalling was hypothesized, which would indicate use of inflammatory signalling also elicited by microbial pathogens. Indeed, the modulation of KLF2 expression by microbial pathogens has been described, linking TLR-mediated signalling to KLF2 regulation. TLR4 is known to be expressed on macrophages in both human and murine lipid-rich atherosclerotic plaques. Like TLR4, its adapter protein MyD88 has been linked to atherosclerosis, as MyD88 null mice display reduced atherosclerosis, based on defective recruitment of macrophages upon inflammatory stimuli. Although the macrophage content of atherosclerotic plaques in mice overexpressing macrophage KLF2 was significantly increased, the presented *in vitro* data may primarily point towards MyD88-independent signalling. Downstream inflammatory signalling occurring through this pathway involves the chemokines CCL5, IP-10 and Interferon-β (IFN-β). As a consequence, this may indicate involvement the IFN-β response, which was recently shown to be enhanced in insufficient arteriogenesis, a process also known to depend on influx of monocytes/macrophages and their TLR-mediated inflammatory activation in the vessel wall.
Perspectives

The studies described in this thesis add to the amassing evidence that demonstrates KLF2 to be a major molecular regulator in inflammatory vascular disease, by importantly determining cellular functions in multiple cell types involved in atherosclerosis. Its beneficial effects on endothelial function may not only contribute to preventing focal atherosclerosis but may also prevent endothelial dysfunction provoked by systemic disease risk factors, as illustrated by studies that demonstrate systemic mediators of diabetes to induce endothelial dysfunction through inhibition of signal transduction driving KLF2 expression. These findings further substantiate the notion that not only focal, but also systemic KLF2-mediated improvement of endothelial function may mitigate risk factors predisposing to atherosclerosis.

Data pertaining KLF2 function in macrophages presented herein, illustrate that the inflammatory balance may easily be disturbed, leading to unwanted effects, as has also been shown in studies on the effects of NF-kB inhibition in macrophages, which led to increased atherosclerotic plaque size. This further supported a function for NF-kB in the resolution of inflammation. These caveats are also stressed by reports showing bacterial pathogens, employing toxins, to augment KLF2 anti-inflammatory effects during infection. Herein lays the danger of unwanted side-effects such as impaired immune responses to bacterial or viral pathogens.

Notwithstanding, current evidence showing that, in the endothelium, KLF2 is the transcription factor that counter-balances the inflammatory responses mediated by AP-1 and NF-kB, makes it a most interesting target for pharmacological intervention. Here too, past research efforts have taught us that there is a delicate balance between pro- and anti-inflammatory mechanisms, but our increasing knowledge about the regulation of endothelial KLF2 expression may eventually enable us to safely and effectively use its potential to protect the vessel wall from inflammation and thereby further decrease the burden of atherosclerotic disease.

Reference List
