Modulation of Atherothrombotic Factors: Novel Strategies for Plaque Stabilization

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Summary

Atherosclerosis is still one of the main causes of death in Western society and is characterized by clinical outcomes as myocardial infarction and stroke. The actual cause of these sudden events is rupture of an atherosclerotic plaque, commonly formed in the larger arteries of the human body during life. During the development of an atherosclerotic plaque, a lipid core grows increasingly bigger under a thin fibrous cap. At some point, when the balance between lipid core size and fibrous cap strength is disturbed, the cap ruptures, most often in the shoulder region of the plaque. When the highly thrombogenic content of the plaque is exposed to the blood circulation, the coagulation cascade is activated and an occluding thrombus can be formed. In this thesis, we aimed to modulate the atherosclerotic plaque towards a more stable phenotype, hereby preventing the plaque to rupture. Secondly, we tried to identify new targets that can have plaque destabilizing effects or increase the risk of atherothrombosis. This thesis was divided in two parts, the first part describing the influence of matrix and cell homeostasis on atherosclerotic plaque stability, the second part the determination of the role of inflammation in atherosclerotic plaque development and its effect on advanced lesions.

In Chapter 2, the effect of the serine protease inhibitor (serpin) Serp-1, a protein derived from the myxoma virus, was determined on atherosclerotic lesion development and on plaque progression in collar-induced carotid artery lesions of western-type diet fed ApoE$^{-/-}$ mice. Serp-1 has been shown to affect urokinase-type Plasminogen Activator (uPA) and its receptor, thereby modulating the coagulation and the fibrinolytic system. The protease uPA is present and active in atherosclerotic tissue, hereby reducing plaque stability. In this study, we demonstrate that continuous infusion of Serp-1 for 4 weeks indeed inhibited plaque development and stabilized the plaque phenotype, as we found a reduction in macrophage content and a highly significant increase in collagen content. Also on advanced lesions, Serp-1 administration led to a stabilization of the collar-induced atherosclerotic plaques, which was demonstrated by an inhibition of plaque development and an increased smooth muscle cell and collagen content.

Apoptosis in the atherosclerotic plaque contributes significantly to the destabilization of the lesions. Apoptosis of vascular smooth muscle cells could lead to cap rupture, whereas apoptosis of endothelial cells might induce plaque erosion. Macrophage apoptosis has a dual role in plaque stabilization. On one hand, macrophages produce the extracellular matrix degrading enzymes, the MMPs. Inhibition of the production of these MMPs might reduce matrix degradation and increase plaque stability. However, on the other hand, apoptotic macrophage residues partly consist of apoptotic bodies which contain high amounts of Tissue Factor (TF), the main activator of the extrinsic coagulation pathway. In Chapter 3, the role of two cross-class serpins Cytokine response modifier A (CrmA) and Serp-2 were assessed in different animal models of either atherosclerotic or neointimal lesion development. CrmA and Serp-2 are both inhibitors of Interleukin-1β Converting Enzyme (ICE), also known as caspase-1, and Granzyme B. ICE and Granzyme B are two of the enzymes involved in the induction of cellular apoptosis. In this chapter, it was demonstrated that especially Serp-2, and not CrmA or two reactive center loop mutants of Serp-2, were able to inhibit lesion formation after aortic transplantation or injury in two rat models. Also, Serp-2 inhibited collar-induced atherosclerotic lesion development as well as spontaneous aortic root atherosclerosis of western-type diet fed $ApoE^{-/-}$ mice. *In vitro* studies revealed that Serp-1 was a potent inhibitor of the cytotoxic T-lymphocyte (CTL) mediated induction of apoptosis of T-cells through inhibition of perforin, which acts together with Granzyme B to mediate this CTL induced apoptosis.

The lipid content of the atherosclerotic plaque highly determines the phenotype of the plaque. Lipid core size together with fibrous cap thickness decides the clinical outcome of a plaque rupture. One of the components present in human atherosclerotic lipid core samples is lysophosphatidic acid (LPA), one of the most thrombogenic lipids. In Chapter 4, we investigated whether LPA also accumulates in atherosclerotic lesions of LDL receptor deficient mice. Indeed, the LPA content in collar-induced atherosclerotic plaques of these mice increased significantly during atherosclerotic lesion development, being similar in advanced lesions as the LPA amount retrieved from human carotid artery samples. Secondly, we determined if the intracellular LPA metabolism during plaque development was altered. During atherosclerotic plaque progression, we found that mRNA levels of some of the LPA producing enzymes, like cPLA2IVa and PLD_3 were significantly upregulated and that one of the LPA degrading enzymes, LPAATα, was downregulated in advanced lesions. These data might explain the accumulation of LPA in advanced plaques. Also, LPA signal transduction was altered due to different mRNA expression levels of the LPA receptors during plaque progression. Although these data have to be verified by *in situ* hybridization to locate the specific cell type responsible for the mRNA expression of the enzymes and also by protein expression analysis, we have identified several target genes that can contribute to either plaque

stabilization or destabilization by modulation of the thrombotic LPA content of the plaque.

As atherosclerosis is considered as a chronic inflammatory disease, we hypothesized that immunosuppression could have a beneficial effect on atherosclerotic plaque development. The possibility of described side-effects like nephrotoxicity, which is often problematic in patients that are treated with immunosuppressive drugs after transplantation, led us to investigate the effect of a low dose of the immunosuppressive drug FK506 on atherosclerotic plaque development and on plaque progression of collar-induced atherosclerotic lesions in western-type diet fed $ApoE^{-/-}$ mice (Chapter 5). Mice received 0.05 mg/kg/day of FK506 by means of continuous infusion during 4 weeks, either from 1 week or 5 weeks after collar placement. FK506 did inhibit atherosclerotic plaque development and blocked plaque progression completely. Especially on advanced lesions, FK506 had a plaque stabilizing effect, mainly by increasing the vSMC content and decreasing the necrotic core size. Also on spontaneous plaque formation in the aorta of $ApoE^{-/-}$ mice, FK506 reduced the plaque size. FK506 blood concentration in the mice showed to be sufficient to inhibit the transcription factor NFAT, which normally drives the expression of pro-inflammatory cytokines as IFNγ and IL-2.

The adventitia of atherosclerotic lesions also contributes to atherosclerotic plaque development, as increased inflammation of the adventitia correlates with the progression of atherosclerosis. Interestingly, the amount of adventitial mast cells was also found to correlate with disease development and especially in ruptured lesions the amount of adventitial mast cells was significantly increased. In Chapter 6, we describe the effects of activated atherosclerotic mast cells on the morphology of collar-induced advanced atherosclerotic plaque of $ApoE^{-/-}$ mice. In this study, we show that in mice which had an increased amount of activated adventitial mast cells, the incidence of intraplaque hemorrhage was significantly increased. This increase in hemorrhage was in part caused by an enhanced rate of macrophage apoptosis in the plaque, which could *in vitro* be inhibited by specific mast cell protease inhibitors and by the H_1-receptor antagonist triprolidine. In addition, increased vascular leakage was caused by degranulation of mast cells, which resulted in increased amounts of inflammatory cells in the plaque at the site of the neovessels and in hemorrhage in the plaque. Erythrocytes in the plaque contribute to plaque destabilization by deposition of cholesterol from their membranes, thereby increasing the lipid core size. Interestingly, administration of the mast cell stabilizer cromolyn during the activation of the mast cells completely prevented the observed hemorrhages and plaque destabilization, which suggests that mast cell stabilizing therapy could be useful in the prevention of acute clinical events.

In Chapter 7, we describe the development of a novel technique, which gives the opportunity to increase the speed and efficiency of studies on the role of macrophage genes in atherosclerosis. Bone marrow transplantation

is a widely used approach, although donor mice have to be generated with a transgene or knockout phenotype, which can be time-consuming or even impossible when deletion of the target gene leads to embryonic lethality. In this study we evaluated the possibility of using lentivirally transduced bone marrow for transplantation and use short hairpin RNA (shRNA) sequences to create a “knockdown” of a target gene. We used CC-Chemokine Receptor 2 (*CCR2*), which was previously shown to be involved in migration of leukocytes to sites of inflammation, as target gene to proof our concept. We designed a shRNA sequence targeting CCR2, which showed to be highly efficient in downregulation of CCR2 mRNA and protein levels. After transduction of bone marrow with either control lentivirus or virus containing the shCCR construct, lethally irradiated recipient mice were transplanted with this bone marrow. At least until 7 weeks after transplantation, the CCR2 mRNA expression was 70% reduced in the shCCR2 mice. Migration of leukocytes in these mice was silenced to levels of $CCR2^{-/-}$ bone marrow transplanted mice, while the mice displayed a $CCR2^{+/+}$ phenotype. The speed and efficiency renders this strategy very valuable for addressing the role of other leukocyte genes in inflammatory disorders.

In conclusion, this thesis describes the plaque stabilizing effects of different protease inhibitors and of the immunosuppressive drug FK506. Also, the accumulation of the thrombogenic lipid LPA in atherosclerotic lesions and its metabolism during atherogenesis was described. Furthermore, we have analyzed the contribution of adventitial mast cells to plaque destabilization and thrombogenicity. These studies have provided new therapeutic entries in the prevention of plaque destabilization, plaque rupture followed by occluding thrombus formation and acute clinical events. Finally, a new research model using bone marrow that had been *ex vivo* transduced with a shRNA construct containing lentivirus was developed, which displayed similar knockdown characteristics *in vivo* as the actual knockout mouse. This model can be applied to efficiently determine the role of leukocyte genes in inflammatory disorders, which renders this technique very valuable for future research on the role of particular leukocyte genes in atherosclerosis.