Intraplaque angiogenesis and therapeutic targeting of angiogenesis

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Chapter 8

General summary and future perspectives

General summary

Atherosclerosis is a progressive disease characterized by the formation of plaques in the intima of major arteries with accumulation of lipids, inflammation, fibrosis, cell death and calcification [1]. The stability of atherosclerotic plaques, rather than the size, is the major determinant for acute clinical implications. When a plaque becomes unstable, it is more likely to rupture, leading to myocardial infarction, stroke and sudden death. Characteristics of unstable plaques are intraplaque angiogenesis and haemorrhage, a large lipid core, high macrophage content and a thin fibrous cap. Over the years, due to cholesterol-lowering drugs, the lifespan and well-being of patients have been significantly improved. However, a large group of patients does not fully benefit from current lipid-lowering strategies and plaque rupture remains the leading cause of acute cardiovascular events [2,3]. Therefore, there is a need for new therapeutic targets to stabilize atherosclerotic plaques and prevent plaque rupture.

Intraplaque angiogenesis is a complex process that depends on the equilibrium between different pro- and anti-angiogenic molecules [4]. Sources of pro-angiogenic signals are hypoxia and inflammation. On one side hypoxia is responsible for the transcription of factors promoting angiogenesis like VEGF-A, in an attempt to create new vessels to repristinate oxygen levels in the plaque. On the other side inflammation is also a strong inducer of angiogenesis as it promotes the synthesis of various angiogenic factors. Beside triggering angiogenesis, several pro-angiogenic molecules can also induce vessel permeability, contributing to the infiltration of leukocytes in the inflammatory core and thereby provoking chronic inflammation [5]. These factors contribute to plaque instability and subsequent rupture.

Because intraplaque neovascularization was shown to have a major causative effect on atherosclerosis and plaque destabilization in humans [6], the aim of the first part of this thesis was to investigate whether inhibition of intraplaque neovascularization might be a promising new therapeutic approach for atherosclerotic plaque stabilization. In the second part of this thesis we focused on a new strategy to increase *in vitro* angiogenesis.

In **chapter 2** we describe the pathological processes associated with angiogenesis in atherosclerotic plaques and illustrate how intraplaque angiogenesis and intraplaque

haemorrhage are strongly correlated with atherosclerotic plaque progression, instability and rupture. We also describe in detail the cellular and molecular mechanisms behind intraplaque angiogenesis. We report that intraplaque hypoxia is the main force driving intraplaque angiogenesis promoting the transcription of pro-angiogenic genes and mediating inflammation by promoting pro-inflammatory cytokine expression and consequently inflammatory cell recruitment. As key players in intraplaque angiogenesis we describe the structure of newly formed vessels as immature and leaky due to incomplete endothelial cell tight junction formation and insufficient and disorganized pericyte coverage. Moreover, we delineate the phenomenon of intraplaque haemorrhage, consisting of extravasation of red blood and inflammatory cells from the leaky neovessels, and its relation with inflammatory mediators, and the subsequent effects of intraplaque haemorrhage on plaque instability, both in experimental models and in humans. Moreover, options to target plaque angiogenesis for imaging and therapeutic purposes are discussed.

Due to the role of hypoxia as the main trigger for intraplaque angiogenesis, in **chapter 3** we hypothesized that plaque reoxygenation would result in decreased intraplaque neovessel formation and therefore reduced intraplaque haemorrhage and inflammation leading to increased plaque stability. To achieve this, we used carbogen gas, a gas that is composed of 95% O_2 and 5% CO_2. For this, we used hypercholesterolemic ApoE3*Leiden mice that underwent vein graft surgery and studied the effect of plaque reoxygenation and its outcome on vessel wall remodeling, intraplaque neovascularization, inflammation and patency. Moreover, since prolonged exposure to high levels of oxygen, hyperoxia, has the risk of generating reactive oxygen species in an amount that is higher than what can be cleared by anti-oxidants, we investigated the effect of reactive oxygen species on the plaque environment *in vivo* and on cultured macrophages *in vitro*. Administration of carbogen gas in an acute short-term setting resulted in a profound reduction of intraplaque hypoxia in murine accelerated atherosclerotic lesions *in vivo*. Long-term treatment with carbogen gas resulted in an increased vein graft patency in ApoE3*Leiden mice, but surprisingly, had no effect on intraplaque hypoxia and intraplaque angiogenesis and haemorrhage. At the same time, long-term carbogen gas treatment resulted in hyperoxia-induced ROS accumulation with subsequent induced transcription of HIF1a gene and increased HIF1a mRNA levels and macrophage apoptosis, probably due to their high oxygen consumption. To study the above-

mentioned ROS effect on macrophages we mimicked induction of ROS *in vitro* by using the ROS-mimic t-BHP in murine bone marrow derived macrophages and we observed a strong increment in DNA damage and apoptosis. Overall, despite the beneficial effect of hyperoxygenation treatment on vein graft patency, the treatment also induced ROS accumulation and apoptosis. Both ROS accumulation and apoptosis will possibly be detrimental for the plaque environment in this model under the current conditions. This indicates that in order to define potential therapeutic benefits of hyperoxygenation therapy, further research is needed to the define optimal conditions for the treatment of atherosclerosis.

In the signaling cascade following hypoxia and Hif1a stabilization, the recruitment of VEGF-A plays a critical role in promoting angiogenesis via binding to its receptor VEGFR2 and initiating a pro-angiogenic signaling cascade. Therefore, in **chapter 4** we investigated the results of VEGFR2 blockade using DC101 blocking antibody on intraplaque angiogenesis, maturation status of the neovessels, and atherosclerotic lesion size and composition in accelerated atherosclerotic vein graft lesions in ApoE3*Leiden mice. Upon VEGFR2 blockade, we observed a reduction in lesion size in the treated animals when compared to controls. At the same time collagen and smooth muscle cell (SMC) content were increased and macrophage content was decreased, all together pointing to an increased plaque stability. Surprisingly the treatment did not result in a decrease in $CD31^{+}$ neovessels. However, when looking at the maturation status of the vessels we could see that the treated group showed a decrease in intraplaque haemorrhage when compared to the controls. To address this aspect, we looked at the expression of genes that are involved in vessel maturation and found that Ang-2, the vessel destabilizing factor, was decreased upon DC101 treatment. Moreover, we observed an increase in Cx40 mRNA level, involved in inter-endothelial cells connections, as a consequence of VEGFR2 blockade. We used an aortic ring assay to study the effect of VEGFR2 blockade on vessel maturation at a cellular level and found that DC101 treatment increased the pericyte coverage around the endothelial cell layer of the formed neovessels. This study points to vascular maturation as an attractive target to stabilize atherosclerotic lesions. In particular VEGFR2 represents a potential target to induce atherosclerotic plaque stabilization.

Another important growth factor that promotes intraplaque angiogenesis in atherosclerosis is bFGF. In **chapter 5** we study the effect of bFGF blockade on intraplaque angiogenesis, SMC

content and inflammation in accelerated atherosclerotic lesions in ApoE3* Leiden mice that underwent vein graft surgery. To achieve this, we synthesized K5, a small molecule that binds to bFGF and results in bFGF signaling blockade. We found that K5 mediated inhibition of bFGF increases plaque stability via strongly reducing intraplaque angiogenesis and intraplaque haemorrhage. Also, K5 treatment reduced the number of circulating monocytes and decreased the expression of adhesion molecule VCAM-1 and chemoattractant protein Ccl2, together resulting in a decreased macrophage content in the atherosclerotic lesions. It was previously shown that bFGF blockade affects SMC proliferation and migration. Surprisingly we could not observe any effect on SMC in the accelerated atherosclerosis vein graft model nor in the femoral artery cuff model, used to study the isolated effect of K5 on SMC migration and proliferation. We also examined more in depth the effect of K5 on angiogenesis and we found that K5 strongly reduced *in vivo* angiogenesis in a Matrigel plug model. We also demonstrate that K5 is able to impair EC migration, proliferation and tube formation due to a reduced FGFR1 activation *in vitro*. K5 was able to enhance plaque stability via reduced intraplaque angiogenesis and decreased intraplaque haemorrhage. Moreover, it reduced systemic circulating monocytes and decreased macrophages infiltration in the plaque and therefore reduced inflammation in the lesions. Taken together, our results show that K5-mediated bFGF signaling blockade is a promising therapeutic candidate for the treatment of unstable atherosclerotic plaques.

Targeting endothelial cell metabolism has been primarily explored for cancer and other diseases characterized by increased angiogenesis e.g., macular degeneration and inflammatory bowel disease [7-9]. Shifting endothelial cells into a more quiescent state, by targeting enzymes involved in cellular metabolism would potentially slow their proliferation, stabilize the endothelial junctions, and reduce the expression of cellular adhesion molecules [10]. Therefore, in **Chapter 6** we study how the inhibition of transketolase (TKT), a key metabolic enzyme involved in the pentose phosphate pathway (PPP), affects EC and macrophage functions. TKT is a thiamine dependent enzyme in the non-oxidative branch of the PPP that controls nucleotide biosynthesis and energy production. Both ECs and macrophages rely on this metabolic pathway for proliferation [10,11]. Due to the tight relationship between angiogenesis and inflammation in atherosclerosis, we studied the *in vitro* effect of TKT blockade, using a thiamine agonist oxythiamine, on EC and macrophages.

We found that TKT is abundantly present in human atherosclerotic lesions, specifically in EC and macrophages. TKT blockade resulted in reduced EC proliferation and migration of HUVEC *in vitro*. More interestingly we found TKT to be upregulated in macrophages with a pro-inflammatory phenotype (M1 macrophages) when compared to resting M0 macrophages. Upon TKT blockade the mRNA expression of pro-inflammatory and pro-angiogenic cytokines in M1 macrophages was reduced when compared to untreated M1 macrophages. Surprisingly we found that this reduction in pro-angiogenic molecules had a functional effect. HUVEC stimulated with supernatant from oxythiamine-treated M1 macrophages acquired a decreased migratory ability when compared to cells stimulated with supernatant from untreated M1 macrophages. These preliminary *in vitro* results show that TKT blockade can be an interesting target to reduce angiogenesis and inflammation in atherosclerosis.

In the second part of this thesis we examined the effect of bis(maltolato)oxovanadium(IV) (BMOV) on *in vitro* angiogenesis. VEGF-A binding to VEGFR2, induces the activation of the receptor and its phosphorylation at different tyrosine sites that triggers the initiation of the signaling cascade leading to the promotion of angiogenesis. Each tyrosine site promotes different cellular responses among which, cell permeability, proliferation and migration. The phosphorylation of these residues is tightly regulated. In this aspect, protein tyrosine phosphatases (PTPs) dephosphorylate VEGFR2 receptor or its downstream signaling enzymes, resulting in decreased angiogenesis. In **chapter 7** we examine the effect of PTPs blockade on VEGFR2 induced angiogenesis on *in vitro* cultured HUVEC using BMOV, a nonselective tyrosine phosphatase inhibitor. Based on our finding that HUVEC produce a basal amount of endogenous VEGF-A and this results in activation of VEGFR2 and subsequently low amount of angiogenesis, in this chapter we hypothesized that upon endogenous VEGF-A receptor activation, BMOV would enhance *in vitro* angiogenesis. Moreover, we hypothesized that exogenous VEGF-A addition would enhance the effect of BMOV resulting in increased VEGFR2 activation and subsequent angiogenesis. We found that BMOV alone strongly increases endothelial cell migration, proliferation and tube formation. Additionally, it stimulates the formation of mature neovessels, lined by EC and covered by pericytes, in an ex vivo aortic ring assay. Moreover, it increases the number of these newly formed vessels when compared to untreated control cultures. To unravel the molecular signaling involved in the observed effect on angiogenesis, we studied the BMOV-induced activation of VEGFR2 in HUVEC. Upon BMOV

treatment, the phosphorylation of the tyrosine residue Y951 was increased when compared to control as well as the phosphorylation of the downstream enzyme p38MAPK. Interestingly the ERK1/2 pathway was not activated by BMOV treatment indicating that the phosphorylation tyrosine residue Y1175 was not altered. In the *in vitro* assays performed we found that BMOV and VEGF-A do not work in a synergistic way in increasing angiogenesis. In fact, in the cell proliferation, tube formation and aortic ring assay the pro-angiogenic effect of BMOV resulted to be higher than its effect when co-administered with exogenous VEGF-A. Our results show that BMOV-mediated inhibition of PTPs is therefore a new promising strategy to induce and stimulate angiogenesis.

Future perspectives

Altogether, the association of intraplaque neovessels and their dysfunction with unstable plaque phenotype presents several therapeutic opportunities for the prevention of plaque rupture. In this thesis we investigated new potential angiogenic targets for the treatment of unstable atherosclerotic plaques and new therapeutic angiogenic targets. Several new therapies have emerged to treat high-risk patients. PCSK9 monoclonal antibodies and inclisiran have been successfully used to reduce cholesterol risk, and canakinumab, a monoclonal antibody against interleukin-1β, was used to reduce plaque inflammation [12-14]. Despite these major advances in cardiovascular research, plaque rupture remains the leading cause of acute events [15]. Therefore, additional therapies aimed at reducing atherosclerotic plaque rupture and its complications are needed.

Anti-angiogenic therapeutics for the treatment of atherosclerosis have not yet entered clinical trials, although promising results have been found in preclinical animal models. However, what we have learned from the treatment of other pathologies in which uncontrolled angiogenesis plays an important role, like cancer or neovascular ocular diseases, is that one of the major challenges in anti-angiogenic therapies is the fine tuning in finding the right dosage of the compound used. In fact using too high dosages could have vessel disruption as a consequence with increased extravasation of erythrocytes and inflammatory cells while a low anti-angiogenic treatment could potentially normalize the neovessels by increasing their pericyte coverage and reducing their leakiness, resulting in a more stable environment (Fig.1) [16]. In the cancer field, the results of different preclinical studies support the beneficial effects of tumor vascular normalization and show that the normalized

vasculature is characterized by vessels that are less leaky with a more normal basement membrane and enhanced coverage by pericytes [17-19]. Moreover, clinical data from a phase I trial comparing a low dose versus a high dose of bevacizumab, an antibody against VEGF, in patients with rectal cancer are consistent with the vascular normalization hypothesis. Therapy with 5 mg/kg Bevacizumab resulted in vessel normalization while the higher dose of 10 mg/kg induced dose-limiting toxicities and excessive disruptions of the vessels [20,21]. In the context of age-related macular degeneration and diabetic retinopathy, in which neovascularization and vascular leakage, including haemorrhage, are major causes of visual loss, it was previously shown that therapeutic antagonism of VEGF results in inhibition of both retinal and choroidal neovascularization, as well as a reduction in vascular permeability [22]. Furthermore, recent clinical trials have shown that treatment with anti-VEGF therapies can improve vision in these patients, probably due to the fact that anti-angiogenic therapy also normalizes and stabilizes immature vasculature in the eye [23-25].

Since it has been shown that pathologically formed vessels in tumors, ocular diseases and atherosclerosis are very similar between each other due to their abnormal structure and leakage of red blood cells [26] and that vascular normalization resulted in improved quality of angiogenesis in tumor and ocular diseases, it is very likely that this therapy could also be successful in patients with advanced unstable atherosclerotic plaques that present intraplaque angiogenesis and haemorrhage.

In advanced plaques, with immature neovessels penetrating into the growing atherosclerotic lesion with high levels of intraplaque haemorrhage, normalization of neovessels by anti-angiogenic molecules should aim at preventing or decreasing leakage of erythrocytes that contribute to plaque progression by stabilizing the neovessels [16,23] (Fig.1). This could be achieved by improving the formation of the basement membrane, increasing the number of cell-cell junctions and/or developing a more mature layer of covering pericytes around the endothelial cells. The resulting less leaky, less haemorrhagic neovessels might then stabilize the plaque microenvironment by delivering intraplaque oxygen and nutrients and therefore alleviating hypoxia [16] (Fig.1). On the other hand, high doses of anti-angiogenic agents are aimed to completely eradicate the abnormal neovessels, but at the same time they could also harm the vasculature of normal tissues. Moreover, blocking intraplaque angiogenesis as a whole could also result in increased hypoxia. In fact, the complete elimination of intraplaque

neovessels would not resolve the intraplaque lack of oxygen and by consequence would result in an even higher hypoxia and stimulation of angiogenesis and inflammation, and therefore a more unstable and prone to rupture plaque phenotype. Another aspect to take into account is that by disrupting intraplaque neovessels, high doses of anti-angiogenic agents could also increase the leakiness of the vessels and cause an increment in intraplaque haemorrhage. In this perspective the right dosage should be aimed at partially reduce the number of intraplaque neovessels and improve their maturation in order to decrease intraplaque haemorrhage rather than entirely eradicate them.

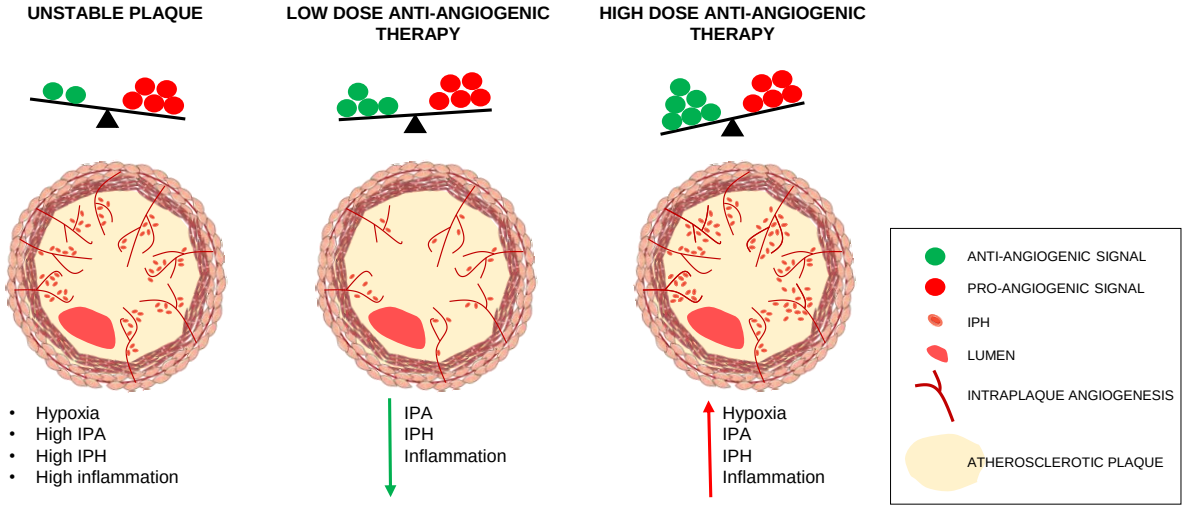

Figure 1. Hypothesis of intraplaque neovessels normalization. Schematic representation of anti-angiogenic treatment in atherosclerosis. **(Left panel)** Atherosclerotic plaque with an unstable phenotype in which pro-angiogenic signals are increased. In unstable plaques hypoxia, high intraplaque angiogenesis and haemorrhage and elevated inflammation are present. **(Middle panel)** Effect of low dose anti-angiogenic therapy on intraplaque environment. More stable plaque with decreased intraplaque angiogenesis, haemorrhage and inflammation. **(Right panel)** High dose anti-angiogenic therapy results in vessel disruption that causes increased hypoxia, intraplaque angiogenesis, haemorrhage and inflammation resulting in more unstable plaque phenotype.

In this thesis we showed this effect in both **chapter 4 and 5**. VEGFR2 blockade on *in vivo* accelerated atherosclerotic lesions in ApoE3*Leiden mice resulted in a decrease intraplaque haemorrhage induced by an increase vessel maturation, possibly due to increased stability of endothelial cells junctions and increased pericyte coverage. bFGF blockade in the same murine model resulted in decreased number of neovessels and intraplaque haemorrhage, and therefore increased lesion stability.

As we learned from the cancer field, another great challenge in anti-angiogenic therapies is that anti-angiogenic therapies often have transient effects as there are multiple compensatory mechanisms that take over [6]. Future strategies for the long-term treatment

of atherosclerosis should take this aspect into account. One possible approach to overcome this problem might be to use a combined therapy that targets not only one single growth factor but two or more. For example, it would be interesting to see the long-term effect of K5 treatment (**chapter 5**) combined with VEGFR2 blockade treatment (**chapter 4**). Another future strategy could be to combine anti-angiogenic factors with anti-inflammatory treatment. Another approach might be targeting cell metabolism. Li et al. proposed that if endothelial cell metabolism is targeted, the blood vessel will no longer be able to grow, regardless of how many pro-angiogenic signals are still present [10]. Based on this rationale, cellular metabolism is considered as the engine of the cell and if targeted and impaired, the endothelial cell would be deprived of energy and therefore would not be able to functionally respond to the pro-angiogenic growth factor signaling. The aim of this kind of therapy should not be to completely shut down cellular metabolism, because that would almost inevitably lead to cellular death but rather shifting the cells in a more quiescent and less proliferative state. In cancer studies, inhibition of the master regulator of glycolysis PFKFB3 decreased glycolysis in pericytes, thereby impairing their migration and proliferation, while increasing quiescence and adhesiveness [27]. This led to a tighter layer of pericytes covering the endothelial cells and contributed to the maturation and normalization of the tumor vasculature [27]. Due to the importance of neovessels and their maturation state in atherosclerosis, targeting cellular metabolism in advanced atherosclerotic plaques may have the added advantage of structurally stabilizing intraplaque neovessels by affecting not only the endothelial cells but also the pericytes. Since we showed in **chapter 6** that TKT blockade reduces endothelial cells and macrophage proliferation and reduces pro-angiogenic and pro-inflammatory cytokines production by M1 macrophages, it would be interesting to evaluate the effect of TKT blockade on vessel maturation.

In conclusion, this thesis presents further understanding in the role of intraplaque angiogenesis and haemorrhage in atherosclerosis. Furthermore, the studies included in this thesis identified new potential angiogenic therapeutic targets. Further research will show if these targets can successfully be used in patients suffering from cardiovascular disease.

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