Macrophage activation and cholesterol accumulation in atherosclerosis development

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GENERAL DISCUSSION AND CONSIDERATIONS

VII

Introduction

Atherosclerosis is characterized by the buildup of fatty lesions in the arterial wall. The progression of atherosclerosis is determined by genetic, lifestyle and environmental factors. Advanced atherosclerosis is associated with a high risk of myocardial infarction or cerebral stroke and is one of the main causes of death world-wide.[1]

Commonly, atherosclerotic lesions are formed in areas of the vasculature where blood flow is disturbed. Here, the endothelium can not exert its protective function and expresses surface factors that attract immune cells such as monocytes.[2] Furthermore, the endothelium becomes more permeable and LDL particles can enter the vessel wall.[3] Monocytes that have migrated through the endothelial layer differentiate into macrophages and encounter the LDL particles.[4] At this stage macrophages can differentiate into a number of different subtypes, such as M1, M2 and Mox.[5,6] Mox macrophages are characterized by a low inflammatory potential and attract more immune cells to the lesion.[6] M1 macrophages express iNOS which produces NO, a potent signaling molecule that is pro-atherogenic when present in atherosclerotic lesions.[7] In contrast, M2 macrophages express Arg1 and have wound healing properties that can stabilize the atherosclerotic lesion.[8,9] Arg1 also reduces NO production by metabolizing L-arginine, a substrate required for iNOS function.[10,11] Furthermore, Arg1 metabolites such as L-ornithine can stimulate vascular smooth muscle cell function.[12] VSMCs produce collagen and as such can create a fibrous cap over the atherosclerotic lesion, increasing lesion stability and decreasing the probability of lesion rupture.[13] Both macrophages and VSMCs can take up cholesterol and lipids in the lesion, becoming foam cells.[14,15] The balance between cellular cholesterol production, cholesterol uptake and cholesterol efflux determines the extent of foam cell formation.[14–17] Therefore, cellular cholesterol signaling, particularly in macrophages, is an important factor in atherosclerotic lesion progression. Oxysterol binding protein related proteins (ORPs) are sterol sensors that can regulate cholesterol metabolism and uptake.[18] Macrophage polarization results in the formation of different regions in the atherosclerotic lesion with pro-inflammatory, cytotoxic micro-environments dominated by M1 macrophages, regions rich in VSMCs, M2 macrophages and collagen fibers and a lipid core containing dead cells.[19] In areas rich in M1 macrophages the fibrous cap can degrade, creating an unstable lesion at risk of rupture.

This thesis discusses how alterations in the functionality of the various cell types that make up the lesion affect atherosclerosis. First, the role of endothelial cells in lesion development after placement of a perivascular stent was investigated. Then, the role of macrophage molecules Arg1 and ORP8 in atherosclerosis and

macrophage polarization was studied. Additionally, the expression pattern of the entire ORP family during atherosclerosis development was characterized and correlated with different cell-types important for atherosclerotic plaque development. Finally, the effect of persistent, diet-induced, epigenetic changes on leukocyte function and atherosclerotic plaque formation were characterized.

Discussion

The endothelial layer is essential for maintaining homeostasis of the arterial wall. During stent placement, the endothelial layer in the stented segment of the blood vessel is destroyed, leading to the formation of a lesion. In **chapter 2** the recovery process of the endothelial layer is studied in detail using an advanced surgical model to transplant a stented murine aorta into the carotid artery of a recipient mouse. Endothelial repopulation after stent placement required up to 56 days, in contrast to other studies where complete repopulation in 28 days has been reported.[20,21] In this study, hyperlipidemic apolipoprotein E knockout (apoE KO) mice were used, which have a strongly pro-atherogenic lipoprotein profile. ApoE KO mice display impaired re-endothelialization compared to wild-type mice, providing a closer approximation of the clinical situation.[22] Previous studies have attributed endothelial recovery after stent placement either to infiltration of endothelial cells from the flanking vasculature or to repopulation by endothelial progenitor cells (EPCs) from the bone marrow, although the extent of EPC mediated re-endothelialization varies between publications.[22,23] Here, the majority of endothelial cells responsible for repopulation of the stented artery originated from adjacent, undamaged parts of the aorta, although in some animals surviving endothelial cells from the stented segment also helped to restore the endothelial layer. A bone-marrow transplantation experiment showed a large individual variation in the degree of contribution of EPCs to re-endothelialization, which may explain the disagreement in literature.

Endothelial or neointima coverage of the stent struts is an important parameter for complete re-endothelialization, as delayed vessel healing is often accompanied by incomplete strut coverage.[20,24] However, we showed that use of paclitaxel coated stents have an inhibitory effect on endothelial cell repopulation, despite complete stent coverage by neointima formation. This indicates that the proliferation-inhibiting drug coated on the stent inhibits endothelial cell growth. Furthermore, in agreement with literature strut coverage alone is insufficient for endothelial repopulation.[25] Despite a reduction in endothelial coverage compared to bare metal stents, paclitaxel coated stents reduced neo-intima formation at 28 days after deployment. However, incomplete re-endothelialization leaves the vessel segment at risk to adverse effects as the protective function of the endothelial layer is

lacking.[26] Therefore, a method of limiting neo-intima formation that is driven by improved endothelial cell function would be preferable. In this chapter, we provide proof that improving endothelial function by increasing endothelial Nitric Oxide (NO) indeed leads to a reduction in neo-intima formation comparable to the effect of paclitaxel eluting stents. Placement of bare stents in GTP cyclohydrolase 1 (GCH) overexpressing (Tg) apoE KO mice, which have increased NO production, resulted in decreased neo-intima formation and improved re-endothelialization. Decreased neointima formation could be due to improved endothelial cell function, such as an improved survival or proliferative capacity.[27,28] In line, endothelial cells isolated from GCH-Tg apoE KO mice displayed increased NO production, providing a viable mechanism for enhanced endothelial function. Although previous studies have attempted to increase endothelial cell function or endothelial progenitor cell recruitment, these have been hampered by aspecific side-effects.[29–31] Here, we show that a genetic endothelial cell-specific intervention that improves endothelial cell function and repopulation reduces neo-intima formation after stent placement. The studies clearly indicate that inhibition of endothelial repopulation has adverse effects on the recovery of the vascular wall after injury, and that improved endothelial function is an important target for new therapies.

NO is a potent signaling molecule that has a number of positive effect on endothelial cell function.[32] In contrast, NO production by macrophages in the atherosclerotic plaque is considered to be cytotoxic and pro-inflammatory.[33,34] In **chapter 3** the role of Arginase 1 (Arg1), a macrophage molecule important for NO production, is studied.[10,11] Arg1 function limits inducible Nitric Oxide Synthase (iNOS) mediated production of NO by competing with iNOS for the substrate L-arginine. Using L-arginine, Arg1 produces L-ornithine, a precursor for molecules that promote cell proliferation and collagen formation. We show that macrophages deficient in Arg1 have an altered response to acetylated low-density lipoprotein (acLDL) induced lipid loading. A reduction in expression of the M1 macrophage marker iNOS was observed, while the expression of the M2 marker FIZZ-1 was increased in acLDL loaded Arg1 KO bone-marrow derived macrophages (BMDMs) when compared to WT macrophages. M2 macrophages have a reduced inflammatory potential compared to M1 macrophages, and display wound healing properties.[35,36] Furthermore, Arg1 KO BMDMs display increased expression of apoE, which stimulates macrophage cholesterol efflux, reduces inflammation in macrophages and inhibits lesion development.[37–40] Unlike WT BMDMs, Arg1 KO BMDMs did not exhibit a reduction in SREBP-1 expression upon acLDL loading. SREBP-1 is a regulator for cellular lipid metabolism, but increased expression SREBP-1 can also result in increased cholesterol production and foam cell formation.[41,42] In line, we found increased foam cell formation in the acLDL loaded Arg1 KO BMDMs compared to the WT BMDMs. Next, bone marrow (BM) from Arg1 deficient mice was transplanted into hyperlipidemic LDL receptor (LDLr) KO mice for 9 weeks.

After high fat, high cholesterol Western-type diet (WTD) feeding for 9 weeks, Arg1 KO BM recipients had a decreased amount of circulating leukocytes and decreased spleen weight. Surprisingly, the decrease in circulating leukocytes appeared to be driven by a decrease in $CD19^+$ B-cells. The contribution of B-cells to the pathogenesis of atherosclerosis is unclear and there are conflicting reports in literature.[43] Also in our model it is not clear what the effect of the reduction in circulating $CD19^+$ B-cells is. Spleen weight and splenocyte content was also reduced in the Arg1 KO BM recipient mice. The reduction however could not be attributed to a single cell type. Notably, there was a slight increase in $CD4^+$ T-cells in spleen. This is not unexpected, as T-cells require L-arginase to function and L-arginine deprivation by Arg1 expressing macrophages suppresses T-cell proliferation.[44,45] In line with the observed increase in foam cell formation *in vitro*, peritoneal cells in Arg1 KO BM recipients also display an increased amount of foam cells. There was no difference in blood cholesterol between the groups to explain the increase in peritoneal foam cells. Surprisingly, despite the observed effects on circulating blood cells and foam cell formation, macrophage Arg1 deficiency did not result in any changes in plaque size or plaque morphology. It has previously been reported by Wang et al. that lentiviral-mediated overexpression of Arg1 applied locally to the site of plaque development resulted in increased VSMC proliferation, reduced inflammation and increased plaque stability.[12] However, while our study is focused on Arg1 expressed on bone marrow derived cells, the method used in the Arg1 overexpression study of Wang et al. resulted in increased expression of Arg1 in VSMCs and endothelial cells as well as in macrophages. Increased expression of Arg1 in endothelial cells results in decreased production of NO by the endothelium, which can lead to endothelial activation and increased atherosclerotic plaque formation.[46] Furthermore, increased Arg1 on VSMCs results in a reduced inflammatory potential.[47] These conflicting effects of Arg1 expression seriously complicate the interpretation of the results on atherosclerosis. In the current study we found differences in the macrophage response to lipid loading and in macrophage foam cell formation as well as a reduction in circulating $CD19^+$ B cells, but no changes in atherosclerotic plaque formation or morphology, suggesting that the role of macrophage Arg1 could be of less importance than endothelial or VSMC expressed Arg1.

Chapter 4 focuses on the role of another macrophage molecule, oxysterol binding protein 8 (ORP8), on macrophage function and atherosclerosis development. The ORP family comprises 12 proteins closely related to the oxysterol binding protein (OSBP). Each family member is capable of binding to cholesterol and oxysterols. However, there is a large variety in function between the different family members, ranging from phagocytosis, cholesterol trafficking and cellular signalling.[48–53] We demonstrate that ORP8 is expressed in collar-induced plaques in mice, and that ORP8 expression correlates with the expression of macrophage marker CD68. This

is in line with data from literature showing that ORP8 is expressed by macrophages in human atherosclerotic plaques.[52] ORP8 deficient BMDMs were generated and differentiated to M1 and M2 macrophages using LPS and IL-4, respectively. LPS stimulated macrophages lacking ORP8 have reduced expression of the M1 marker IL-6 and decreased excretion of pro-inflammatory cytokines IL-6 and TNFα. Reduced expression of these factors by macrophages can reduce the inflammatory reaction inside the atherosclerotic plaque.[19,54–56] Furthermore, expression of FIZZ-1 in macrophages stimulated towards the M2 phenotype was decreased in the absence of ORP8. FIZZ-1 is expressed by macrophages as they first infiltrate the atherosclerotic plaque and is an M2 macrophage marker associated with an anti-inflammatory phenotype.[57] Furthermore, expression of ATP binding cassette A1 (ABCA1) was reduced in differentiated and control macrophages lacking ORP8. ABCA1 is important for efflux of cholesterol to lipid poor apolipoprotein A-I (apoA-I), which reduces cellular cholesterol load and leads to the formation of mature HDL particles.[58] Next, ORP8 deficient bone marrow was transplanted into LDLr KO mice, which were left to recover for 8 weeks and were then fed WTD for 6 and for 9 weeks. After 9 weeks of WTD feeding the ORP8 KO recipients had a more pro-atherogenic lipoprotein profile, displaying increased VLDL cholesterol and triglycerides.[59] No differences were found in the amount of circulating leukocytes. There was a small increase in the amount of peritoneal monocytes which was not accompanied by a change in peritoneal macrophages. Interestingly, the amount of peritoneal foam cells was decreased, but in the presence of lowered ABCA1 expression. Despite the decrease in ABCA1 mRNA expression *in vitro* in BMDMs and *in vivo* in peritoneal leukocytes no difference could be found in macrophage cholesterol efflux of BMDMs to either apoA-I or HDL. In line, previous studies on full body ORP8 KO mice have found differences in ABCA1 mRNA expression that did not translate into differences in protein expression or cholesterol efflux, and it has been reported that ABCA1 RNA expression is not a reliable indicator for protein expression.[60,61] Despite the observed increase in pro-atherogenic VLDL cholesterol, a decrease in atherosclerotic plaque size was observed after 9 weeks of WTD feeding and a trend towards a decrease at 6 weeks. Furthermore, plaque macrophage content was augmented, while there was no difference in plaque collagen, plaque apoptosis or adventitial T-cells. Overall results of the studies described in chapter 4 indicate that macrophages deficient in ORP8 have an altered polarization profile and inflammatory potential, leading to reduced plaque formation despite an increase in circulating VLDL cholesterol.

ORP8 is part of the ORP family, which counts 12 members in total. **Chapter 5** describes the expression pattern of all members of the ORP family. mRNA expression of the ORP family membes was measured in kidney, liver, thymus, spleen, lymph node and aortic arch samples of WT and apoE KO mice. Interestingly, in both WT nad apoE KO mice all ORPs displayed higher than average expression

in kidney tissue, identifying this as an important organ for ORP function. Although little research has been done into renal oxysterols and ORPs, levels of oxysterols can be induced in chronic kidney failure as a result of increased oxidative stress, indicating a potential important role for renal oxysterol metabolism.[62,63]

Expression of ORPs in spleen and lymph node was below average, whereas expression in liver, thymus and aortic arch varied widely between the different ORP family members. Expression of ORP3, ORP5 and ORP9 was high in aorta samples. ORP8 expression in aortic arch was strongly increased in samples from apoE mice fed WTD. This increase correlated with an increase in expression of the macrophage marker CD68.[52] Neither expression of CD68 or ORP8 correlated with iNOS or FIZZ-1 expression, indicating that neither M1 or M2 macrophage accumulation in the aorta of the apoE KO mice contributed to the increase in ORP8 expression. In contrast, expression of the Mox marker HO-1 had a strong linear relationship with both the CD68 and the ORP8 signal, indicating infiltration of ORP8 expressing Mox macrophages in the aorta's of apoE KO mice. Macrophage HO-1 is a strong protective factor against oxidative stress in the atherosclerotic plaque as well as in the liver. [64,65] When HO-1 expression in the liver was investigated, a linear correlation between HO-1 and CD68 was found. This signal did not correlate with expression of ORP8, possibly due to interference by high ORP8 expression of hepatocytes.

The progression of atherosclerosis is influenced by a large number of factors, such as local changes in endothelial activation and inflammatory status in the vessel wall and systemic changes in leukocyte numbers and activation status.[66,67] In **chapter 6** persistent changes in leukocyte activation induced by changes in diet were investigated. Long-term WTD feeding in bone marrow induced a reduction of DNA methylation of transcription factor Pu.1, which is important for macrophage maturation and differentiation.[68–70] DNA methylation is an epigenetic mechanism that results in reduced transcription of heavily methylated gene regions.[71] Furthermore, DNA methylation patterns are very persistent and can even be inherited.[72,73] To investigate the effect of bone marrow DNA methylation on atherosclerosis, we transplanted bone marrow (BM) from mice that had been fed WTD or control chow diet for 45 weeks into LDLr KO mice. Although the recipient mice were fed only chow diet, the mice receiving the WTD BM displayed increased atherosclerotic plaque formation. BM isolated from WTD BM recipient mice showed decreased methylation in both Pu.1 and IRF8. There were no differences in methylation of Tal-1, required for a wide range of hematopoietic cells or Notch-1, required for T cell development.[74,75] Decreased methylation could result in increased transcription of the methylated gene. In line, an increase in circulating F4/80+ monocytes was found, corresponding with an increased expression of the activation markers CD14 and CD86 on splenic monocytes and macrophages, respectively. CD14 is an inflammatory marker, and when CD14

expression is reduced production of the M1 macrophage markers TNFα and iNOS is decreased.[76,77] Similarly, CD86 is a marker for macrophage activation and co-stimulatory potential for T-cells.[78] We propose that diet induced changes in DNA methylation of macrophage transcription factors contributed to the increased activation status of splenic macrophages and higher numbers of circulating monocytes, which led to increased atherosclerosis. Importantly, we show that the pro-atherogenic effect of WTD feeding on DNA methylation patterns in the bone marrow persists long after the dietary challenge is withdrawn. This suggests that specific targeting of the epigenome using dietary or pharmacological interventions might contribute to current treatment strategies for atherosclerosis.

Considerations

Atherosclerosis is a disease that progresses over a time span of decades. In humans, the first fatty streaks can commonly be seen around the age of 20 years.[79] However, clinical manifestations usually occur when patients have passed 50 years of age.[79] During this period of disease progression, the atherosclerotic lesions become increasingly complex, subject to the interactions between a number of cell types such as endothelial cells, macrophages and VSMCs.[3,80] At the early stage of plaque development, healthy endothelial function is crucial to prevent LDL particles from entering the vessel wall and become oxidized. If the endothelium becomes activated, it attracts patrolling monocytes towards the site of activation and in doing so facilitates the start of an inflammatory reaction which results in the formation of a fatty streak. Numerous studies have identified the importance of a healthy endothelial layer to preventing the formation of atherosclerotic lesions.[81,82] Some clinical interventions with beneficial effects on endothelial function have been reported, notably the effect of fluvastatin on endothelial health. Fluvastatin treatment improves vascular tone of patients by promoting endothelial relaxation.[83,84] Furthermore, circulating levels of endothelial adhesion molecules P-selectin and ICAM are reduced after fluvastatin treatment.[85] However, it is not clear how much of the beneficial effect on endothelial function can be attributed to a direct effect on endothelial cells and how much is due to the systemic lowering of cholesterol and inflammation.

The clinical need to improve endothelial function is evident in the case of percutaneous coronary intervention. Significant amounts of atherosclerotic plaque formation in coronary arteries can lead to occlusion of the blood vessels and a limited blood supply to the heart. Blood flow can be improved by the placement of a perivascular stent, however this procedure leads to profound damage to the endothelium.[21] The resulting loss in endothelial coverage is the major factor leading to neointima formation; the development of a lesion at the location of

stent placement, which can lead to re-occlusion of the stented coronary. To combat neointima formation, drug eluting stents (DESs) have been developed which slow cell growth. However, these DESs also reduce re-endothelialization, thereby limiting their effectiveness.[86] Newer generation stents have tried to overcome this inherent problem, for instance by capturing circulating endothelial progenitor cells to promote endothelial growth.[87] In this thesis, we show that neointima formation can be reduced by directly improving endothelial function, thereby bringing to question the need to coat stents with growth-limiting drugs. Endothelial repopulation after stent placement was greatly improved in mice expressing increased levels of GTP cyclohydrolase 1 (GCH), which led to enhanced function of endothelial Nitric Oxide Synthase and ultimately to increased NO production, which is a key molecule mediating endothelial function.[88] This finding demonstrates that a therapeutic strategy of rapid re-endothelialization could be successful in minimizing neo-intima formation. The presence of NO reduces vascular tone and has an anti-thrombotic and anti-inflammatory effect on endothelial cells. Both eNOS and its family member iNOS require L-arginine to produce NO, however Arg 1 also requires L-arginine as a substrate. In line, Arg1 expression on endothelial cells reduces endothelial function and increases hypertension by limiting NO production.[89,90]

Although endothelial NO is a strong atheroprotective factor, NO produced by macrophages in the atherosclerotic lesion is pro-atherogenic.[7,91,92] Macrophages are highly plastic cells, being able to differentiate into functionally distinct subtypes. Pro-inflammatory M1 subtypes strongly express iNOS and produce NO. Anti-inflammatory M2 macrophages express Arg1 and produce factors that promote collagen formation and VSMC proliferation.[92] The reciprocal relationship between Arg1 and iNOS in macrophages is one of the major determinants of macrophage differentiation and function. Differentiated macrophages strongly influence the micro-environment. M1 macrophages have cytotoxic properties and are located at sites in the atherosclerotic lesion that are vulnerable to rupture.[5] In contrast, M2 macrophages are located more deeply in the lesion.[14,19] Mox macrophages are characterized by a lower inflammatory potential compared to M1 macrophages but also by impaired phagocytotic capabilities compared to M2 macrophages. It was postulated that Mox macrophages promote a chronic, low level of inflammation that attracts cells to the lesion, while lacking the phagocytotic capacity to clear apoptotic cells and the associated cytotoxic material.[6] Although there is clear therapeutic potential in manipulating macrophage differentiation locally, currently too many factors in the process of macrophage differentiation are poorly understood. In addition, it is becoming increasingly clear that even after polarization, macrophages remain highly adaptable, further complicating the association between specific subtypes and clinical indications.[6,35] This thesis demonstrates that macrophages lacking ORP8 have a reduced expression of

macrophage polarization markers, indicating that ORP8 deficient macrophages are more likely to remain naïve. Mice transplanted with ORP8 KO bone marrow had reduced atherosclerotic lesion formation, despite having a more pro-atherogenic lipoprotein profile. We attribute this to the observed reduction in macrophage differentiation, resulting in a diminished production of inflammatory factors by macrophages. The study described in chapter 6 of this thesis also illustrates the importance of macrophage activation in atherosclerosis. We show that mice that received BM from donors that have been subjected to long-term WTD challenge had a strongly increased number of macrophages and more macrophage activation, leading to increased atherosclerotic lesion formation. This illustrates that macrophage activation can be a strong driving factor for atherosclerosis and that increasing our understanding of macrophage subsets could lead to novel therapeutic strategies.

One such strategy could be manipulation of the leukocyte epigenome. Changes in the epigenome, such as altered DNA methylation patterns, have already been identified as strong markers for atherosclerosis.[93,94] As DNA methylation patterns are persistent, changes in DNA methylation enacted by smoking or high cholesterol could maintain even after the causal factor is removed, i.e. the patient stopped smoking or achieved a lower blood cholesterol. As this thesis demonstrates, pro-atherosclerotic DNA methylation patterns can potentially contribute to atherosclerosis formation independently of other pro-atherosclerotic factors. This could lead to a deeper understanding of how our diet affects our health and the consequences certain dietary choices bring.

Current therapies for patients with atherosclerosis are targeted primarily on reducing blood cholesterol, but this fails to prevent a large number of cardiovascular events.[95] In order to identify new therapeutic strategies for treating atherosclerosis, it is important to study the interactions between the cells that make up the atherosclerotic lesion. This thesis describes the role of endothelial cells and macrophages during lesion formation, as well as the expression patterns of sterol sensors in vascular smooth muscle cells present in the lesion. These new insights contribute to our understanding of atherosclerosis open up new avenues of atherosclerosis research.

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