Macrophage lipid genes in cardiovascular disease

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Chapter 6

General discussion and considerations

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Introduction

Atherosclerosis, the underlying cause of most cardiovascular diseases, is the consequence of lipid deposition in the arterial wall, mainly as a result of high serum cholesterol levels. Currently, the main therapeutic strategy to prevent the progression of atherosclerosis is to reduce serum cholesterol levels by lipid lowering medication, such as statins. Despite impressive progress in the treatment of cardiovascular diseases, atherosclerosis associated clinical events are still a major cause of death in the Western society.[1] This clearly indicates the need for new therapies. Numerous epidemiological studies have established high HDL cholesterol levels to be protective for the development of atherosclerosis. Besides its anti-oxidative and anti-inflammatory functions, HDL plays a crucial role in facilitation of reverse cholesterol transport from macrophages in the arterial wall to the liver and subsequently to the feces.[2]

In this thesis, the bone marrow transplantation technique was used to specifically investigate the importance of macrophage-derived proteins, associated with the removal of lipoproteins and triglycerides, with respect to atherosclerosis. In addition, a myocardial infarction model was used to establish the role of ABCA1 in an acute cardiovascular event.

The role of key reverse cholesterol transport genes in macrophage cholesterol homeostasis and atherosclerotic lesion development

Reverse cholesterol transport is a pathway by which excess cholesterol is transported from macrophages in the arterial wall to the liver for excretion, thereby preventing atherosclerosis.[2,3] The first step in this process involves the efflux of cholesterol from macrophages. This can be facilitated by several macrophage proteins including ABCA1, ABCG1 and apoE.[4,5] As a transmembrane protein, ABCA1 pumps a wide variety of substrates across the cell membrane. It plays an essential role in the maintenance of cellular lipid homeostasis by facilitating the transport of cholesterol and phospholipids towards lipid-poor apoA-I.[6,7] In addition, ABCG1 plays an important role in the efflux of cholesterol from macrophages. ABCA1-mediated cholesterol efflux results in the lipidation of apoA-I, and thus the formation of mature HDL particles, that subsequently act as substrate for ABCG1-mediated transport of cholesterol and phospholipids.[8–10] As a result, combined deletion of macrophage ABCA1 and ABCG1 in mice results in massive foam cell formation in peripheral tissues, coinciding with an attenuated increase in Western-type diet-induced plasma cholesterol levels.[11]

ApoE, secreted by macrophages can also induce cellular cholesterol efflux.[12] Interestingly, apoE enhances the efflux of cholesterol both in the presence and absence of extracellular cholesterol acceptors.[13] In addition, apoE can act as a substrate for ABCA1-mediated cholesterol efflux.[14] Therefore, in this thesis, several studies were performed in order to investigate the (combined) importance of these macrophage-derived cholesterol-related proteins on atherosclerotic lesion development.

In **chapter 3**, LDLr KO mice were transplanted with bone marrow from ABCA1/apoE double KO mice, their respective single knockouts, or WT controls, and challenged with a high-fat/high-cholesterol diet for 6 weeks to induce atherosclerotic lesion development. Macrophage-derived apoE induces efflux of cholesterol, independent of extracellular acceptors.[13] However, no added effect on cholesterol efflux was observed in dKO macrophages compared to ABCA1 deficient macrophages, suggesting that endogenously produced apoE requires ABCA1 to facilitate macrophage cholesterol efflux. In agreement with previous studies,[11,15,16] macrophage ABCA1 deficiency resulted in increased atherosclerotic lesion development, despite a clear reduction in serum cholesterol levels. Single deletion of macrophage apoE led to a comparable increase in atherosclerotic lesion development as single macrophage ABCA1 deletion. Circulating TC levels, however, were over 2-fold higher in apoE KO transplanted animals compared to ABCA1 KO transplanted animals. When adjusted for serum total cholesterol exposure, the pro-atherogenic effects of macrophage ABCA1 deficiency became even more evident. Surprisingly, TC levels in serum were markedly higher upon combined deletion of macrophage ABCA1 and apoE as compared to single

deletion of ABCA1 in macrophages. This thus leads to ABCA1 deficiency – a defect in cholesterol efflux – combined with elevated serum total cholesterol levels. Interestingly, hepatic LPL expression was attenuated in dKO transplanted mice. Since LPL is essential for the lipolysis of TG in lipoproteins, LPL facilitates the clearance of VLDL and chylomicrons.[17] In addition, LPL can also promote LDL[18] and VLDL remnant uptake,[19] independent of apoE-recognizing receptors. The attenuated LPL expression might thus have contributed to the observed increase in serum TC levels compared to ABCA1 KO transplanted animals.

Importantly, ABCA1/apoE dKO transplanted mice exhibited a large induction of the pro-inflammatory cytokines IL-6 and TNFα in the liver. In addition, IL-6 levels in the circulation were highly increased in these animals, indicating an enhanced inflammatory status. All together, combined deletion of macrophage ABCA1 and apoE resulted in a defect in cholesterol efflux and, compared to ABCA1 KO transplanted mice, elevated serum total cholesterol levels, and enhanced systemic and hepatic inflammation, together resulting in the observed augmented atherosclerotic lesion development. Despite the beneficial function of ABCG1 in cellular cholesterol efflux, differential results have been published on the role of macrophage ABCG1 in atherosclerosis.[20–22] In agreement with its protective role in cellular cholesterol efflux, macrophage ABCG1 has been reported to play an anti-atherogenic role.[21,23] In contrast, studies showing a pro-atherogenic role for macrophage ABCG1 suggested that ABCG1 KO macrophages are either more prone to undergo apoptosis,[20] or exhibit increased ABCA1 expression and apoE secretion.[22] The effect of combined deficiency of macrophage ABCG1 and apoE on atherosclerotic lesion development is described in **chapter 4**.

Deletion of macrophage ABCG1 or apoE resulted in a comparable effect on cholesterol efflux towards HDL, whereas a larger attenuation of cholesterol efflux to HDL was observed in dKO macrophages. Two-way ANOVA analysis indicated that the effects of macrophage ABCG1 and apoE on HDL-mediated efflux were independent of each other. Similar results were obtained with respect to atherosclerotic lesion development in these mice. Single ABCG1 or apoE KO transplanted mice showed a 1.4-fold increase in atherosclerotic lesion development as compared to WT transplanted controls, whereas their dKO transplanted littermates revealed a 1.9-fold increase. Furthermore, a clear significant inverse relation between HDL-mediated efflux and the size of atherosclerotic plaques was apparent. The independent effects of both genes on atherosclerotic lesion development was further strengthened by the fact that no compensatory upregulations of apoE or ABCG1 protein expression were observed in bone marrow-derived macrophages from single ABCG1 KO and apoE KO transplanted animals, respectively. The specific role of ABCG1 in atherosclerosis was further defined in **chapter 5**. Total body ABCG1/LDLr dKO mice were used to assess the effect of ABCG1 deficiency on different stages of atherosclerotic

lesion development. In this study, ABCG1 was shown to be able to induce, as well as attenuate atherosclerotic lesion development. Feeding ABCG1/LDLr dKO mice a Western-type diet for 10 weeks resulted in a significant increase in early atherosclerotic lesion size. However, a significant reduction in more advanced lesions was observed after 12 weeks of Western-type diet feeding, indicating that the effect of ABCG1 deficiency on atherosclerotic lesion development in LDLr KO mice depends on the stage of atherogenesis.

In order to validate this observation, all studies on the role of ABCG1 in atherosclerotic lesion development were compared using correlation analysis, including data from eight published studies, the current study, and one unpublished study of our group. The correlation analysis revealed that the fold increase/decrease in atherosclerotic lesion size of ABCG1 KO mice compared to ABCG1 WT mice is highly correlated (R=0.92) with the atherosclerotic lesion size. Based on these data, ABCG1 is primarily protective in early atherosclerotic lesions as its deficiency causes an impaired cholesterol efflux to HDL, resulting in increased atherosclerotic lesion development. In more advanced atherosclerotic lesions, however, the role of ABCG1 in atherogenesis switches from anti- to pro-atherosclerotic, since the persistent impaired cholesterol efflux from ABCG1-deficient macrophages is likely to induce accumulation of (oxy)sterols. This leads to enhanced apoptosis and/or other compensatory mechanisms, and subsequently decreased atherosclerotic lesion size. A previous study on the role of ABCG1 in atherosclerosis suggested a correlation between serum cholesterol levels and the fold increase/decrease in atherosclerotic lesion size compared to ABCG1 WT mice, whereby at about 900 mg/dL serum cholesterol a switch from the protective function of ABCG1 to lesion formation was noticed.[21] Upon inclusion of recent studies, as well as the studies described in this thesis, again a high correlation between the fold increase/decrease in atherosclerotic lesion size and total serum cholesterol is observed.

Therefore, under normal physiological levels of cholesterol, the role of ABCG1 in atherogenesis is likely to be protective. Furthermore, since higher serum cholesterol levels are associated with a more rapid development of atherosclerotic lesions, this correlation is probably also a direct effect of the stage of atherosclerotic lesion development.

Role of macrophage ATGL in atherosclerosis

Triglyceride stores are the most important energy reserves in vertebrates. Dysfunctional lipolysis affects energy homeostasis and may contribute to the pathogenesis of diseases like obesity and insulin resistance. Adipose triglyceride lipase (ATGL) is mainly expressed in adipose tissue.[24] However, ATGL is also expressed at high levels in macrophages and foam cells in atherosclerotic lesions,[25] raising the possibility that it might impact atherosclerosis.

In **chapter 6**, the specific role of ATGL in macrophages, as well as the consequences of macrophage TG accumulation for atherogenesis was examined. Hereto, LDLr KO mice were transplanted with bone marrow from ATGL KO mice or WT littermates.

Although lack of ATGL in bone marrow-derived cells had no significant influence on serum lipid levels, distinct TG loading was observed in BMDM of ATGL KO transplanted mice. Surprisingly, despite increased macrophage TG concentrations, atherosclerotic lesion formation in the aortic root was highly attenuated in ATGL KO transplanted mice. The observed reduction in atherosclerotic lesion formation coincided with increased apoptosis, which might limit lesion cellularity and the progression of early lesions in these animals.[26,27] Depending on the stage of lesion development, increased apoptosis can either enhance,[28] or suppress[29,30] atherosclerotic lesion development. In agreement, ATGL deficiency in macrophages was recently shown to result in enhanced apoptosis involving mitochondrial dysfunction and activation of the mitochondrial apoptosis pathway.[31] Leukocytes play an essential role in all stages of atherosclerotic lesion development,[32–34] whereby the amount of predominantly neutrophils and monocytes are positively correlated with atherosclerotic lesion development.[35,36] Importantly, we found highly decreased numbers of circulating leukocytes in Western-type diet fed ATGL KO transplanted LDLr KO mice. Remarkably, the LSK stem cell population in bone marrow from ATGL KO transplanted mice was decreased compared to WT controls. LSK cells are the most primitive hematopoietic stem cells, which can give rise to all mature cell types found in the circulation. The attenuated LSK population might therefore have resulted in a reduction of leukocyte-producing progenitor cells. In addition, plasma MCP-1 levels were drastically reduced in ATGL KO transplanted animals. Since MCP-1 is an important chemoattractant for mononuclear cells,[37] the observed reduction most likely attributed to the observed attenuation in circulating leukocytes.

Altogether, the absence of ATGL in macrophages resulted in reduced susceptibility to diet-induced atherosclerosis in LDLr KO mice due to decreased infiltration of less inflammatory macrophages. Based on these data, macrophage ATGL plays a significant role in atherogenesis independent of its expression in other tissues. A high intracellular TG content in macrophages is therefore, at least under these conditions, protective against lesion development, making macrophage ATGL a putative novel target to attenuate early atherogenesis when the removal of apoptotic cells is still functional.

Further understanding of the role of ABCA1 in cardiovascular disease

Deficiency of leukocyte ABCA1 on the LDLr KO background leads to increased atherosclerosis, despite largely attenuated cholesterol levels.[38] Interestingly,

these mice also showed elevated leukocyte counts in the circulation,[39] and accumulation of macrophages in the peritoneal cavity, liver, and spleen.[38] This indicates that leukocyte ABCA1, in addition to its role in cholesterol efflux, exerts regulatory functions in the recruitment of inflammatory cells to the periphery. The spleen is the largest lymphoid organ in the body, producing antibodies, facilitating phagocytosis and being capable of eliminating foreign antigens.[40,41] Moreover, it functions as an important monocyte reservoir.[42] In order to investigate the possible interplay between the spleen and leukocyte ABCA1 with respect to the development of atherosclerosis, in **chapter 7**, ABCA1 KO bone marrow was transplanted into LDLr deficient recipient mice, which were subsequently either splenectomized or underwent a sham operation. Leukocyte ABCA1 KO mice fed WTD showed highly induced concentrations of MCP-1 and KC (murine IL-8) in the serum. IL-8 is one of the most potent chemoattractants for neutrophils.[43,44] Accordingly, leukocyte ABCA1 KO mice exhibited increased neutrophil presence in the spleen. Upon splenectomy, ABCA1 KO transplanted animals exhibited blood neutrophilia as compared to WT splenectomized controls. Surprisingly, leukocyte ABCA1 deficieny or splenectomy alone did not alter circulating neutrophil concentrations.

The spleen is also associated with systemic immune responses in which it is the principal organ responding to antigens such as oxLDL. In addition, the titers of anti-oxLDL antibodies in serum have been suggested to play an anti-atherogenic role.[45] However, no differences in anti-oxLDL antibody serum titers were observed upon splenectomy. Moreover, whereas leukocyte ABCA1 KO mice displayed attenuated serum TC levels, TC concentrations remained unaffected after splenectomy. In contrast, splenectomized mice did show enhanced atherosclerotic lesion development as compared to sham operated controls. Moreover, splenectomized ABCA1 KO transplanted animals exhibited an additional increased lesion development. These results evidently show that leukocyte ABCA1 deficiency resulted in decreased TC levels, increased inflammation, and lipid and neutrophil accumulation in the spleen. However, the observed splenic alterations induced by leukocyte ABCA1 deficiency did not alter anti-oxLDL antibody levels, nor played a significant role in atherosclerotic lesion development as evidenced by splenectomy.

The importance of ABCA1 in cellular cholesterol transport became clear when mutations in the ABCA1 gene were discovered to cause Tangier disease; a rare inherited disorder characterized by a severe reduction in HDL.[46] HDL has been proposed to exert multiple anti-atherogenic and anti-inflammatory functions beyond cellular cholesterol efflux, including the modulation of lymphocyte activity,[47] endothelial function,[48] and anti-oxidative mechanisms.[48] Interestingly, low plasma HDL is associated with a long-term unfavorable prognosis in patients who have recovered from a MI, suggesting detrimental effects of low plasma HDL

on post ischemic myocardial function. To investigate the effects of low plasma HDL after MI, in **chapter 8**, ABCA1 KO mice were subjected to MI by ligation of the left anterior descending coronary artery. To our surprise, ABCA1 deficiency attenuated cardiac ischemia after a myocardial infarction. Thus, although ABCA1 has an important protective role in atherosclerosis, it has detrimental effects on cardiac function after MI. The presence of ABCA1 in isolated hearts did not contribute to the observed effects, indicating that the smaller myocardial infarction area was caused by secondary, ABCA1-mediated effects. Although MI did not affect leukocyte numbers in control mice, ABCA1 KO mice displayed an induction of circulating leukocytes after MI. Most leukocyte subtypes are generally associated with increased inflammation and subsequent cardiovascular disease. B- and T-lymphocytes, however, exhibit protective effects upon acute cardiovascular events,[49–51] thereby attenuating adverse effects on cardiac function after MI.
ABCA1 is considered to be an important therapeutic target to treat atherosclerosis based on its protective effects. However, it is of great importance to further identify the possible adverse effects of ABCA1 upregulation on cardiac recovery after MI.

Considerations

Cardiovascular disease in humans develops over decades, and clinical manifestations often only occur as a result of ruptured, advanced atherosclerotic plaques. Although the use of animal experiments enables us to induce and investigate atherosclerotic lesion development in for example mice and rats, it has proven to be challenging to induce ruptures in established atherosclerotic lesions in these animal models.
The lack of these so-called "plaque rupture animal models" makes it difficult to extrapolate findings to the human situation. On the other hand, the possibility of controlled induction of atherosclerotic lesion formation in (genetically engineered) mice has resulted in a powerful tool to identify key proteins and mechanisms behind the onset and progression of atherosclerosis. Over the last decades, these efforts have resulted in an extensive amount of data, which have increased our insight in the mechanism of atherosclerotic lesion development, and more importantly also provided numerous promising targets for therapeutic intervention.
One of the earliest success stories resulting from basic cardiovascular research is the development of statins, which inhibit HMG-CoA reductase, the first step in cholesterol synthesis.[52] By their action on the liver, statins lower circulating cholesterol levels and thereby reduce the risk of clinical events from cardiovascular disease. More recently, CETP was believed to be a new promising target. Inhibiting the action of CETP in genetically engineered CETP-expressing mouse models (mice naturally do not express CETP) resulted in highly favorable

changes in lipid profile, including increased HDL levels and decreased VLDL/LDL levels. The subsequent phase III clinical trial, however, was prematurely stopped because patients treated with the CETP inhibitor torcetrapib had a 25% increased chance to suffer from major cardiovascular events and a 40% increased chance to die from cardiovascular causes as compared to patients treated with placebo.[53] Despite the favorable changes in lipid profile, torcetrapib also caused severe off-target effects. In addition, very recently, a phase III clinical trial with another CETP inhibitor, dalcetrapib, was stopped due to a lack of clinically meaningful efficacy.[54] These results indicate that the current CETP inhibitors do not result in additional lowering of cardiovascular risk on top of statins. Importantly, it also demonstrates that the pathways of the underlying basic research have to be studied extensively and interpreted with great care.

The research described in this thesis mainly focuses on the role of important lipid-related genes on macrophages. Macrophages play a crucial role in the onset and progression of atherosclerotic lesions in the arterial wall by engulfing pro-atherogenic lipoproteins. The subsequent transformation of macrophages into lipid-rich foam cells - considered the initial step in the development of atherosclerosis - underlines the importance of macrophage lipid homeostasis.

Macrophage reverse cholesterol transport is a pathway by which excess cholesterol from peripheral tissues, including macrophages, is transported back to the liver for excretion, and thus of potential interest for the prevention of atherosclerosis.[2,3] Induction of cholesterol efflux from macrophage foam cells in atherosclerotic lesions, the first step in reverse cholesterol transport, is therefore considered an attractive approach to prevent lesion development or even evoke regression of atherosclerotic lesions. ABCA1, ABCG1 and apoE play pivotal roles in macrophage reverse cholesterol transport.[5,55] Interestingly, expression of these proteins can be enhanced upon pharmacological LXR activation.[56] Of note, systemic application of LXR agonists also induces off-target effects in liver, including increased lipogenesis and production of TG.[57] However, specific targeting of LXR agonists to macrophages inside the atherosclerotic lesion might be a potential new therapeutic strategy for inhibiting atherosclerotic lesion progression of induction of regression of established lesions.

Another, somewhat underrated part of macrophage lipid homeostasis is the storage and metabolism of TG. The rate limiting enzyme in this process is ATGL, responsible for the catabolism of TG into DG and FFA. Disruption of ATGL in mice results in premature death.[58] However, mice lacking ATGL in all tissues except cardiac muscle have a normal life expectancy,[59] indicating that TG accumulation specifically in cardiac muscle has detrimental effects. Surprisingly, accumulation of TG in macrophages causes apoptosis and attenuates atherosclerotic lesion development in leukocyte ATGL KO mice. In early atherosclerotic lesions, macrophage apoptosis results in attenuated progression or even regression of

the lesion.[29,60] On the other hand, apoptosis of macrophages in advanced lesions can lead to increased inflammation and vulnerable atherosclerotic lesions.[61,62] In addition, differential stages of atherosclerotic lesion are present throughout the arterial tree.[63] It is therefore difficult to regard macrophage apoptosis as a possible target against atherosclerosis.

In conclusion, modulating macrophage lipid homeostasis may provide an attractive possibility for future drug design in the field of atherosclerosis. However, due to the complexity of the disease and difficulties concerning the extrapolation of advances in basic cardiovascular research, further investigations are necessary before these potential new drug candidates can be applied in clinical research.

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