Cholesterol and phospholipid transporters in atherosclerotic lesion development

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Summary and concluding remarks

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This thesis is focused on the role and interaction of different cholesterol and phospholipid transporters. Cholesterol homeostasis is accomplished via a tightly regulated balance between the intake of dietary cholesterol and *de novo* cholesterol synthesis. A disruption in the cholesterol homeostasis can result in changed plasma cholesterol levels and a modulated risk for the development of cardiovascular disease. Plasma cholesterol levels are the consequence of the complex interaction of gene expression in the liver and peripheral tissues, such as macrophages in the vascular wall.

A detailed description of the regulation of cholesterol homeostasis in macrophages by different ABC-transporters, and the consequences for atherosclerotic lesion development is represented in *Chapter 2*. The excessive accumulation of cholesterol by macrophages is the pathological hallmark of atherosclerosis. Several different receptor-mediated coated-pit endocytosis pathways are implicated in the uptake of cholesterol by macrophages. Macrophages express high levels of scavenger receptors, which bind and internalize modified forms of lipoproteins. Also binding sites for unmodified lipoproteins and lipoprotein remnants, including the LDL receptor (LDLr), LDL receptor-related protein (LRP), VLDL receptor (VLDLr), and proteoglycans are found on macrophages. In addition, (modified) lipoproteins can be taken up by macropinocytosis, a process in which dynamic structures are formed by the closure of lamellipodia at ruffling membranes, that range in size from 0.2 to 5 μm in diameter. Macrophages cannot limit the uptake of cholesterol and therefore depend on cholesterol efflux pathways for preventing their transformation into foam cells. Several ABC-transporters facilitate the efflux of cholesterol from macrophages, including ABCA1 and ABCG1. ABCA1 is implicated in the cholesterol and phospholipid transfer to lipid-poor ApoAI leading to the formation of HDL, while ABCG1 is involved in the efflux of cholesterol and phospholipid to mature HDL. Plasma membranes of eukaryotic cells are asymmetrically composed of lipids, where the external leaflet mainly holds choline containing phospholipids (phosphatidylcholine and sphingomyelin), the inner leaflet is mainly composed of amine containing glycerophospholipids (phosphatidylserine and phosphatidylethanolamine). This membrane lipid asymmetry is maintained by two different classes of transporters: the flippases, which translocate the aminophospholipids, and the flippases which translocates lipids form inside to outside of the membrane. Several members

of the ABC-transporter super familiy are recognized as floppases. ABCB4 is a selective floppase for phophatidylcholine which also affects membrane asymmetry implicating an important role in cellular endocytotic pathways. We propose that in addition to the generally accepted role of these ABC-transporters in the prevention of foam cell formation by induction of cholesterol efflux from macrophages, they also influence the macrophage endocytotic uptake.

In *chapter 3* we explore the role of bone marrow derived ABCB4 on cardiovascular disease by means of transplantation of ABCB4 deficient bone marrow into the LDL receptor deficient (LDLr KO) mice. Systemic disruption of ABCB4 in mice results in a virtual absence of phospholipids in bile and a strongly impaired biliary cholesterol secretion, indicating that ABCB4 plays an essential role in cellular lipid efflux. Since macrophages express ABCB4 mRNA and the fact that ABCB4 functions as a phospholipid and cholesterol transporter at other sites of the body, it is likely that macrophage ABCB4 plays a role in atherosclerotic lesion development. Chimeras were created that specifically lack ABCB4 in bone marrow derived cells, including macrophages. Atherosclerotic lesion development was induced by feeding a high-cholesterol diet. Serum cholesterol levels were significantly lower in mice reconstituted with ABCB4 knock out bone marrow, as a result of reduced VLDL and LDL cholesterol levels. Despite the lower serum cholesterol levels, ABCB4 deficiency in bone marrow derived cells resulted in a 1.8-fold increase in lesion size. In vitro foam cell formation was increased in the absence of ABCB4, possibly due to a 2-fold increase in the association of modified LDL, while the efflux of cholesterol was unaffected. Bone marrow derived ABCB4 thus has an important anti-atherosclerotic function probably by limiting macrophage foam cell formation. These findings support the proposition in *chapter 3* for a role of ABCB4 in macrophage endocytotic uptake.

Circulating lipid-poor ApoAI takes up the excess of both cholesterol and phospholipids from peripheral cells and from the liver, to grow into a mature HDL particle, which is the facilitator of the reverse cholesterol transport (RCT). This process brings cholesterol back from the peripheral sites to the liver. After delivery of the HDL cholesterol esters to the liver, the excess cholesterol can be removed from the body via bile by excretion into the faeces.
ATP-binding cassette transporter 1 (ABCA1) mediates cellular cholesterol efflux from the liver and macrophages, while SR-BI in theliver selectively takes up cholesteryl esters from HDL while it can also mediate cholesterol efflux to HDL, especially in macrophages. The role

of ABCA1 and SR-BI in reverse cholesterol transport is reviewed in *chapter 4*. Both transporters are highly conserved in evolution. The highest expression of SR-BI is found in organs important in cholesterol metabolism (liver), and organs important in steroidogenesis (adrenal, ovary and testis). The highest expression of ABCA1 is found in liver, placenta, lung and adrenal glands. While SR-BI mediates the selective uptake of cholesteryl esters from HDL, ABCA1 interacts with lipid-poor ApoAI to form nascent HDL. The role of SR-BI in lipoprotein metabolism became evident from murine adeno-viral hepatic overexpression studies, which resulted in a virtual disappearance of plasma HDL. Complete disruption of SR-BI in mice resulted in an increase in total plasma cholesterol levels, due to the accumulation of abnormally large HDL particles. This clearly demonstrates the importance of SR-BI for the delivery of cholesteryl esters to the liver in mice. There are several indications that SR-BI plays an anti-atherogenic role in the formation of atherosclerosis. Moderate hepatic overexpression protects against atherosclerotic lesion development. Conversely the atheroprotective effects of SR-BI are lost when the turnover of HDL cholesterol is impaired as a result of a reduction in SR-BI expression. Bone marrow transplantation studies have shown that macrophage SR-BI reduces the development of advanced lesions in LDLr KO and ApoE KO mice. In contrast, the development of small fatty streak lesions in LDLr KO mice is facilitated by macrophage SR-BI. It thus appears that macrophage SR-BI is pro- or anti-atherogenic, depending on the stage of lesion development. The importance of ABCA1 in reverse cholesterol transport was first established through the discovery of mutations in the human ABCA1 gene that where found to be the underlying genetic defect causing Tangier disease. Murine models confirmed these findings. Targeted disruption of ABCA1 resulted in a virtual absence of HDL cholesterol, while overexpression of ABCA1 increased HDL levels. Although in the classical view of reverse cholesterol transport, ABCA1 in peripheral cells, including macrophages, is thought to initiate HDL formation, specific deletion of ABCA1 on macrophages does not lead to decreased circulating HDL levels. This in contrast to specific hepatic ABCA1 deletion, suggesting that the liver itself mediates lipidation of HDL proteins. Humans heterozygous for mutations in ABCA1 are more susceptible to coronary artery disease. The atheroprotective effects of ABCA1 are confirmed in several animal models. Overexpression of ABCA1 in ApoE KO mice reduces the susceptibility to atherosclerotic lesion development. Furthermore, bone marrow transplantation studies demonstrated that disruption of ABCA1 on macrophages increases atherosclerosis in mice, even though macrophage ABCA1 does contribute only little to circulating HDL cholesterol levels.

Since ABCA1 and SR-BI play an essential role at either end of the RCT pathway, it is conceivable that ABCA1 and SR-BI might act synergistically in this process. To study the RCT process under conditions in which both of these key mediators are absent, ABCA1xSR-BI double knockout mice were generated by cross-breeding. The characterization of the resulting double deficient mice is described in *chapter 5*. Combined ABCA1/SR-BI deficiency resulted in a decrease in serum HDL cholesterol levels. The double KO mice thus resemble the single ABCA1 KOs with respect to their lipid phenotype, indicating that the virtual absence of HDL cholesterol levels in absence of ABCA1 leaves no substrate for SR-BI. Despite the dramatic reduction in serum HDL cholesterol levels, no effect of combined ABCA1/SR-BI deficiency was observed on the hepatic cholesterol content of these mice. In addition, no effect of combined ABCA1/SR-BI deficiency was observed on the bile salt and phospholipid content of the bile. The cholesterol content of the bile, however, was reduced in the double knockout mice. Similar results have previously been shown for the single SR-BI KO mice, indicating that in this respect the ABCA1/SR-BI double KO mice resemble the SR-BI single KOs. Finally, no effect of combined ABCA1/SR-BI deficiency was observed on the fecal excretion of neutral and acidic sterols as previously observed in the single KOs.

In conclusion, systemic combined deficiency of SR-BI and ABCA1 establish the dominant effect of ABCA1 on serum cholesterol levels, while for biliary cholesterol the SR-BI gene seems to be more crucial. Thus, ABCA1 is the primary determinant for cellular cholesterol efflux and the formation of HDL, while SR-BI is mainly involved in the delivery of HDL cholesterol to the liver and the secretion of cholesterol into the bile.

In order to address more specifically the combined function of ABCA1 and SR-BI in macrophages of the arterial wall, we subsequently performed a bone marrow transplantation in LDLr KO mice using ABCA1/SR-BI dKO mice as donors. In *chapter 6* the results of this study on the role of bone marrow derived ABCA1 and SR-BI in atherosclerotic lesion development are presented. The local formation of HDL is initiated by the cholesterol efflux to lipid-poor apoAI via ABCA1, while HDL subsequently might mediate the efflux of cholesterol via SR-BI. Deletion of ABCA1 in bone marrow derived cells resulted in enhanced atherosclerosis in LDLr KO mice, demonstrating the atheroprotective role of ABCA1 in bone marrow derived cells. As also indicated above, disruption of SR-BI in bone marrow derived cells showed a dual role for this transporter in atherosclerotic lesion development. The aim of the study described in *chapter 6* was to determine the putative synergistic role of bone marrow derived ABCA1 and SR-BI in atherosclerotic lesion development. Hereto, chimeras were created that specifically lack ABCA1 and SR-BI in bone marrow derived cells,

including macrophages, by performing a bone marrow transplantation study using LDLr KO mice as recipients. Atherosclerotic lesion development was induced by feeding a high-cholesterol Western-type diet for 10 weeks. Under these conditions, combined deletion of ABCA1 and SR-BI in bone marrow significantly decreased serum cholesterol levels. Despite the lower serum cholesterol levels, ABCA1/SR-BI deficiency in bone marrow derived cells resulted in a 2.3-fold increase in lesion size, compared to wild type transplanted animals. In addition, the lesion size in the dKO transplanted animals was also significantly larger as compared to single ABCA1 KO or SR-BI KO transplanted mice (1.4-fold and 1.5-fold, respectively). This study shows the independent additive roles of ABCA1 and SR-BI in bone marrow derived cells in the formation of atherosclerotic lesions, demonstrating that a dual upregulation of the genes will form the most attractive therapeutic approach.

Statins are the most commonly used therapeutic compounds to prevent atherosclerotic lesion development. Use of statins is based on the fact that statins are inhibitors of de novo cholesterol synthesis resulting in a decrease of serum cholesterol levels, thereby preventing the uptake of native and oxidatively circulating modified LDL by macrophages. Cholesteryl ester transfer protein (CETP) transfers cholesteryl esters from HDL to VLDL and LDL, thereby playing an important role in cholesterol metabolism. The development of CETP inhibitors, like Torcetrapib (Pfizer Inc, New York, NY) represented a novel approach for raising HDL cholesterol and reducing LDL cholesterol levels, thereby altering atherogenesis. (1). However, recent clinical trials demonstrated that Torcetrapib increased blood pressure and had no effect on decreasing the progression of coronary atherosclerosis. The dramatic failure of clinical trials evaluating the cholesterol ester transfer protein inhibitor Torcetrapib has led to considerable doubt about the value of raising high-density lipoprotein cholesterol (HDL-C) as a treatment for cardiovascular disease (2). With the discovery of cholesterol efflux mechanisms via SR-BI or the ABC-transporters a new therapeutic era arises. The ABC-transporters influence macrophage cholesterol homeostasis via the generally accepted efflux pathways, however, there is also a role for these transporters in endocytotic pathways, and thus atherosclerotic lesion development as is shown in *chapter 3*. Both scavenger receptors and ABC-transporters are recognized as of great influence in cholesterol homeostasis, since both are implicated in the reverse cholesterol transport. In *Chapter 5* it becomes evident that there are different roles for both proteins. ABCA1 is the primary determinant for cellular cholesterol efflux and the formation of HDL, while SR-BI is mainly involved in the delivery of HDL cholesterol to the liver and the secretion of cholesterol into the bile. More specifically

there is no synergistic function for SR-BI and ABCA1 in atherosclerotic lesion formation, although combined deletion of both ABCA1 and SR-BI in macrophages increases foam cell formation, as is shown in *Chapter 6*.

In conclusion, the modulation of cholesterol metabolism and as a consequence the treatment or prevention of atherosclerotic lesions should not be a one target quest, but rather be a multiple transporter alteration.

References

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