The ABC of cholesterol transport

Plösch, Torsten

CHAPTER 8

General discussion

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Cholesterol is the predominant sterol present in the mammalian body. It has indispensable structural and metabolic functions: it determines cell membrane fluidity and is the precursor of bile salts and steroid hormones. Because of its crucial, physiological importance, mammals are able to synthesize cholesterol completely *de novo*, starting with the ubiquitous precursor acetyl-CoA. Mammals are therefore - in principle - independent from dietary intake of cholesterol.

All eukaryotic cells contain sterols and all mammals feed at least in part on other eukaryotes. Therefore, the diet unavoidably contains varying amounts of cholesterol, plant sterols, or sterols specific for other resources. However, only cholesterol is absorbed in a quantitatively important ways in the mammalian intestine, whereas other sterols are only present in trace amount in the body.

Sterols pose a potential threat for the well-being of the organism: accumulation of cholesterol in macrophages, leading to the formation of foam cells, and potentially high plasma LDL levels, are considered a key step in the development of atherosclerosis. Fortunately, the synthesis and breakdown of cholesterol is tightly controlled and several means exist to pharmacologically interfere with these processes, allowing to adjust prevailing plasma cholesterol levels. However, other dietary sterols, particularly plant sterols, could be pro-atherogenic as well. As their concentration obviously cannot be regulated via their synthesis, it must be regulated by controlling their rate of absorption and/or excretion. Work described in this thesis deals with the mechanisms responsible for removal of cholesterol and other sterols from the body.

Sterol transport by enterocytes

Cholesterol approaches the enterocyte mainly in association with mixed micelles, together with bile salts and phospholipids. It has long been thought that it then enters the enterocyte passively, in which further routing would take place intracellularly. Recently, a set of proteins has been identified that is involved in cholesterol uptake.[1,2] The new cholesterol absorption-inhibitor *ezetimibe* has been demonstrated to block a route involving a couple of proteins, including the recently discovered Nieman-Pick-C1-Like-1-protein (NPC1L1) and annexin2/caveolin1.[1-3] Although is has not been shown explicitly, it is likely that this route does not discriminate between cholesterol and plant sterols: ezetimibe also reduces plant sterol absorption in patients with sitosterolemia, who usually absorb huge amounts of plant sterols, and in laboratory animals.[4]

Once inside the enterocyte, the fate of cholesterol markedly differs from that of plant sterols. Cholesterol is efficiently esterified by ACAT2, whereas plant sterols are not.[5-7] This means that cholesterol can be incorporated into chylomicrons and transported to the lymph to become available for the liver and peripheral cells.[8] Plant sterols, in contrast, which are poor

substrates for ACAT2, cannot enter this route and are available for other transport systems.[6,7] Already 34 years ago, Salen *et al.* proposed that esterification is crucial for the difference in intestinal handling between cholesterol and other sterols.[9]

The transport system Abcg5/Abcg8 facilitates plant sterol transport back to the intestinal lumen.[10,11] *Abcg5*$^{-/-}$/*Abcg8*$^{-/-}$ mice have been demonstrated to accumulate plant sterols in their body similar to human patients with sitosterolemia.[12] In this thesis, it is demonstrated that in mice lacking only Abcg5 similar phenomena occur, in favor of the hypothesis that these transporters act as heterodimers. However, cholesterol absorption is unaffected in chow-fed *Abcg5*$^{-/-}$ mice which indicates that the Abcg5/Abcg8 system does not control cholesterol absorption under physiological circumstances.[13]

If gene expression of *Abcg5* and *Abcg8* is increased by activation of the Liver-X-Receptor (LXR), cholesterol absorption is reduced in wild-type, but not in *Abcg5*$^{-/-}$ or *Abcg5*$^{-/-}$/*Abcg8*$^{-/-}$ mice.[14] This can be interpreted as an indication that the heterodimer is, in principle, able to transport cholesterol and to prevent it from entering the ACAT2 route. In addition, in Chapter 3 we provide evidence that the increase in intestinal Abca1 expression upon LXR activation may be the cause of increased plasma plant sterol levels in *Abcg5*$^{-/-}$ mice. Abca1 might thus be able to mediate transport of plant sterols as well as of cholesterol to HDL.[13] A similar observation has recently been published by Field *et al*. in CaCo-2 cells *in vitro*.[15]

The aforementioned transport system may be involved in a phenomenon described in Chapters 2 and 5. It has been a dogma for many years that the liver is the main organ for removal of excess cholesterol from the organism (see below). However, in several mouse models examined in this thesis, hepatobiliary cholesterol transport does not account for the majority of fecal cholesterol (Chapters 2 and 5). This indicates that the enterocyte might – in addition to its role in cholesterol absorption - have an important role in the *excretion* of cholesterol from plasma into the intestinal lumen, at least under conditions when LXR is activated. The exact mechanisms involved in this process are not yet clear. Possibly, cholesterol is taken up from HDL via the action of a scavenger receptor, *e.g.*, SR-BI, at the basolateral side of the enterocyte. Also the LDL-receptor is expressed in enterocytes, making receptor-mediated endocytosis another option for cholesterol uptake.[16] It seems feasible to suggest that, once cholesterol has entered the enterocyte from plasma, it may be excreted by the action of Abcg5/Abcg8 to the intestinal lumen, especially when these proteins are expressed at high levels induced by pharmacological LXR activation

Hepatobiliary cholesterol excretion

The liver is considered the major excretory organ for cholesterol and an integrative part of the reverse cholesterol transport pathway according to the classical concept.[17,18] Hepatobiliary cholesterol excretion is tightly coupled to that of phospholipids and is stimulated by bile salt excretion.[19] The physicochemical details of this process are still poorly understood. At least three different transport systems are involved in hepatobiliary cholesterol excretion: Mdr2-

P-glycoprotein (Abcb4) for phospholipids, Abcg5/Abcg8 for cholesterol and Bsep (Abcb11) for bile salts.[12,20,21] Mdr2-P-glycoprotein translocates phosphatidylcholine to the outer leaflet of the canalicular membrane putatively leading to the formation of vesicles-like structures into the canalicular lumen.[22,23] This "vesicle" formation is supported by the action of bile salts, which are excreted by the bile salt export pump Bsep.[21] In the absence of phospholipid secretion, the detergent actions of bile salts damage the bile ducts.[20] The Abcg5/Abcg8 complex, finally, promotes cholesterol excretion into bile.[12]

For dissecting the interaction between these processes, knock-out mice for *Mdr2*,[20] *Bsep*,[24] *Abcg5*,[13] *Abcg8*[25] and *Abcg5*/*Abcg8*[12] have been used extensively during the past couple of years. In the absence of Mdr2-P-glycoprotein, hepatobiliary phospholipid excretion is completely disrupted.[20] Based on electronmicrographs, "vesicle" formation at the bile canalicular membrane seems to be absent in *Mdr2*$^{-/-}$ mice.[22,23] Simultaneously, cholesterol excretion is strongly decreased. By infusion of hydrophobic bile salts (*e.g.*, TDCA), cholesterol excretion could be restored in *Mdr2*$^{-/-}$ mice, whereas phospholipid excretion could not.[26] On the other hand, *Abcg5*$^{-/-}$ and *Abcg5*$^{-/-}$/*Abcg8*$^{-/-}$ mice have reduced cholesterol concentrations in gallbladder bile. Phospholipid concentrations in both mouse models are also reduced compared to wild-type littermates.[12,13] The effective excretion rates have been determined in *Abcg5*$^{-/-}$ (Chapter 4 of this thesis) and *Abcg8*$^{-/-}$ mice only.[25] In both models, cholesterol excretion is significantly lowered compared to wild-type mice, but not completely absent. Phospholipid excretion is also disturbed, the reason of which is not yet clear but independent from changes in *Mdr2* expression. Cholesterol output rose in both knockout models to similar extents upon infusion of TDCA, but without ever reaching the level of heterozygous or wild-type mice. *Abcg8*$^{-/-}$ mice became cholestatic upon infusion with TDCA (Kosters *et al.*, unpublished), whereas in *Abcg5*$^{-/-}$ mice bile turned red at infusion rates of 100 nmol TDCA/min and bile flow subsequently started to decrease (Chapter 4).

In a series of classical papers, Yousef and colleagues studied the effects of bile salts on biliary lipid composition in rats.[27-30] Upon infusion of hydrophobic bile salts, typically phospholipid secretion declined first, *followed* by decreases in bile flow, bile salt output and cholesterol output. Concomitantly with the decline in phospholipid output, phospholipid composition changed from mainly phosphatidylcholine to more phosphatidylethanolamine and sphingomyelins, which was attributed to partial solubilization of the canalicular membrane.[30] Our data from TDCA-infused *Abcg5*$^{-/-}$ mice, however, differ from the kinetics of the process reported by Yousef: in *Abcg5*$^{-/-}$ mice, the maximum secretory rate for phospholipids and bile salts as well as the maximal bile flow rate were reached *earlier* than that of sterols. This may indicate that in the outer leaflet of the canalicular membrane, sufficient sterols were present - even in the absence of Abcg5 - which could be "dissolved" by hydrophobic micelles. Obviously, this model would argue against a role of Abcg5/Abcg8 as a flippase and would favor a liftase mode of action as proposed by Small.[31] However, further studies are necessary to rule out that the late increase in sterol excretion in the *Abcg5*$^{-/-}$ mice is

caused by hepatic micro-bleedings which could theoretically provide erythrocyte membranes as a source for the sterols measured in bile.

When *LXRα*$^{-/-}$ mice were fed a high cholesterol diet, they excreted a similar amount of cholesterol into bile as their wild-type and heterozygote littermates did (Chapter 4). However, the latter two showed clearly increased hepatic mRNA levels of *Abcg5* and *Abcg8* upon high cholesterol feeding, whereas this response was absent in the *LXRα*$^{-/-}$ mice. Kosters *et al.* have described a correlation between expression of *Abcg5* and *Abcg8* in the liver and excretion rates of cholesterol into bile in mice.[32] However, they also noted some exceptions to this general rule, *e.g.* the diosgenin-fed mouse model,[33] in which this relation did not hold true. The *LXRα*$^{-/-}$ mouse on high-cholesterol diet adds another exception to this list. This again indicates that in particular metabolic situations, Abcg5 and Abcg8 are not rate-controlling for hepatobiliary cholesterol excretion.

Reverse cholesterol transport

Besides the excretory functions of the enterocyte and the hepatocyte, cholesterol flow from the periphery to these excretory organs is of crucial importance to prevent local, potentially harmful, accumulation in peripheral cells. This flow has traditionally been referred to as *reverse cholesterol transport* (RCT) or *centripetal cholesterol flux*.[17,18,34] The first step of reverse cholesterol transport is the transfer of cholesterol from the peripheral cell to pre-ß-HDL, which leads to the formation of mature HDL particles (see Attie *et al.* for review).[35] Epidemiological and experimental studies clearly demonstrated the protective effects of high HDL levels against development of atherosclerosis.[36-40] The underlying explanation was that HDL acts as the sole carrier of cholesterol from the periphery to the liver. In addition, HDL-cholesterol has been advocated as the main source of biliary cholesterol.[41,42] Hence, the picture has emerged that HDL cholesterol is cholesterol on its way to disposal.

The ABC-transporter Abca1, which is defective in Tangier disease, has been shown to be crucial for the formation of HDL.[43-45] It is involved in transfer of cholesterol from the peripheral cell to pre-ß-HDL, although it most likely does not act as a *bona fide* cholesterol transporter.[46] As expected, both patients with Tangier disease as well as *Abca1* knock-out mice are prone to atherosclerosis.[35] However, Groen *et al.* were able to demonstrate that *Abca1*$^{-/-}$ mice do have normal hepatobiliary cholesterol excretion rates.[47] In Chapter 2 of this thesis, we investigated the physiological role of Abca1 in mice treated with the synthetic LXR-agonist T0901317: Abca1 has been shown to be a definite LXR target gene.[48-50] We indeed observed an increased hepatic expression of *Abca1*, a rise in HDL levels and an increased fecal sterol disposal in wild-type mice treated with the LXR agonist.[51] As expected, HDL cholesterol remained completely absent in the *Abca1*$^{-/-}$ mice also after LXR activation. Nevertheless, cholesterol excretion into bile as well as sterol disposal into the feces was undistinguishable from that in wild-type mice. It was concluded that the beneficial effect of HDL cannot be the transport function *per se*. One option would be that HDL contains

substances which protect against atherosclerosis, for example by protecting against oxidation of lipids. Indeed, the HDL receptor (SR-BI) is present in macrophages and could mediate this interaction.[52] Alternatively, HDL levels may only indirectly indicate the activity of Abca1 function in peripheral cells, namely in macrophages. High HDL levels would then only be an indicator of active Abca1 in macrophages, which, in turn, would mean that they are less prone to foam cell formation. The mechanisms by which HDL protects against the development of atherosclerosis, therefore, needs further investigation.

Perspectives

Mechanisms of regulation

As eluded to in some detail in this thesis, cellular cholesterol homeostasis is a result of the integrated actions of proteins involved in synthesis, transport and breakdown of cholesterol. Quite early, pharmaceutical means have been developed to interfere with these three types of protein-dependent processes in various organs to lower blood cholesterol levels in patients with hypercholesterolemia.

Cholesterol synthesis can be efficiently reduced by treatment with *statins*.[53] Statins are inhibitors of HMG-CoA reductase, an important rate-controlling enzyme in the cholesterol synthesis pathway.[54] Statins are frequently prescribed drugs in our Western society.[55] However, not all patients react upon statin treatment with the desired reduction of plasma cholesterol levels. Possibly, the non-responders have underlying causes of the hyper-cholesterolemia which are less dependent on the endogenous synthesis rate, for instance when synthesis is low *a priori*. This may be the case in subjects with a relatively high intestinal cholesterol absorption efficacy.

Intestinal cholesterol transport was thought to be the target of plant sterols which are added to dietary products to interfere with cholesterol absorption (see ref. 56 for review). These products have been demonstrated to efficiently lower cholesterol absorption: In a study with normolipidemic, male, healthy subjects, cholesterol absorption was reduced by 50 to 75 % upon a diet containing mainly sitostanol or sitostanol esters.[57] In parallel, LDL-cholesterol level were reduced by 8 to 22 %. However, the mechanism of action of plant sterols is still obscure. It was speculated that plant sterols interfere with cholesterol micellisation in the intestinal lumen and thereby impair absorption. Alternatively, it has been proposed that plant sterol competitively block the uptake transporter for cholesterol. However, a third mechanism is possible which goes beyond the protein level (see below).

An effective manner to interfere with cholesterol absorption is provided by the recently discovered drug *ezetimibe*. Ezetimibe is effective in reducing blood cholesterol levels, especially when combined with statins, in hypercholesterolemic subjects.[3] It putatively inter-

feres with the uptake of cholesterol via the Niemann-Pick-C1-like-1 protein (Npc1l1)-mediated pathway, although no direct interaction of ezetimibe with Npc1l1 has been demonstrated so far.[1] Ezetimibe has also been shown to target a complex formed from annexin 2 and caveolin 1 in enterocytes.[2] The relationship between the annexin 2-caveolin 1 route and Npc1l1 needs further investigation.

Finally, acceleration of cholesterol turnover is a consequence of therapy with bile salt-binding resins like cholestyramine. Cholestyramine binds bile salts in the intestine, inhibits their re-uptake and therefore interrupts their enterohepatic circulation.[58] As a consequence of de-repressed hepatic bile salt synthesis, more cholesterol will be used for bile salt synthesis, erecting a relative depletion of cholesterol in hepatic cells. These cells will adopt by increasing bile salt synthesis as well as the expression of the LDL-receptor on their surface, leading to reduction of plasma LDL-cholesterol levels.

All these pathways are regulated, in part, at the level of gene expression. Consequently, it seems feasible to interact with cellular cholesterol homeostasis by influencing transcription factors involved in the regulation of important, specific genes. A key regulator of cholesterol homeostasis is LXR.[59] General activation of LXR in mammals by synthetic LXR agonists leads to increased HDL levels, increased hepatobiliary cholesterol excretion and reduced intestinal cholesterol absorption.[13,14,50,51] Hence, LXR is a prime target for drug development.[60-62]

First-generation LXR agonists, *e.g.*, T0901317, are efficient in achieving the three goals described before.[50,63] However, LXR activation also enhances transcription of *SREBP-1c* and *FAS*, leading to increased *de novo* lipogenesis, hepatic steatosis, increased VLDL production and elevated plasma triglyceride levels in mice.[64,65] These side-effects render them unsuitable for therapeutic interventions. Two strategies exist to circumvent these problems: targeting to specific tissues or reducing the side-effects in all tissues by designing gene-specific agonists. Combination therapy may also be worthwhile. Efficacy of the latter approach has been tested by co-administration of mice with T0901317 and the PPARα agonist Wy14643.[66] The PPARα agonist induces beta oxidation; hence, the elevation of plasma triglyceride levels as induced by T0901317 was attenuated. In contrast and somewhat surprising, hepatic steatosis was not prevented in this model. Obviously, efforts must be made to achieve an effective combination of drugs to counterbalance the side-effects. Also “weaker” LXR agonist ("LXR modulators") are currently being investigated which are thought to activate lipogenic genes less than cholesterol transport genes.[67]

Targeting to specific tissues is the second, interesting option for preventing negative side effects. If one could target an LXR agonist specifically to macrophages, cholesterol efflux towards HDL via Abca1 could theoretically be enhanced. This would not have a measurable effect on HDL levels, as macrophages contribute only marginally to overall HDL formation. However, it would affect a key step in the development of atherosclerotic plaques in a desirable way.

Another potentially promising approach would be the specific activation of LXR in enterocytes, leading to increased expression of *Abcg5* and *Abcg8* and, consequently, reduced cholesterol absorption from the intestine. This approach has been used recently by Kaneko and co-workers.[68] These authors developed LXR agonists that are closely related to sterols and are therefore substrates of Abcg5 and Abcg8 on their own. Accordingly, these ligands are effectively taken up by the enterocyte to activate LXR, leading to increased expression of *Abcg5* and *Abcg8*. Subsequently they are excreted back into the intestinal lumen. The increased level of Abcg5/Abcg8 leads to enhanced efflux of cholesterol to the intestinal lumen, thereby reducing net absorption.

Hydrodynamic injection of siRNA as a new tool for liver specific gene knock-down: potential applications

A major problem associated with studies concerning ABC-transporter function in cholesterol transport is related to the fact that the relevant genes *Abcg5*, *Abcg8* and *Abca1* are expressed at high levels in both enterocytes and hepatocytes.[10,69,70,71] Therefore, it is difficult to evaluate the relative contribution of these two organs, for instance in development of hypercholesterolemia, in *in vivo* studies. Consequently, one has to find ways to influence their expression in tissue-specific ways.

One suitable way would be the use of agonist or antagonist which are tissue-specific, for example the agonist described above.[68] However, such agonists are not yet freely available and antagonists to reduce gene expression have not yet been reported. Another efficient means would be to generate tissue-specific knockout mice or tissue-specific overexpressors. These are elegant models, but their development is laborious, time-consuming and expensive.

Adenoviral overexpression is efficient to reach high expression levels predominantly in the liver (see, for example, refs. 72-74). It is a well-defined method, which was also applied during the course of the projects described in this thesis (data not shown). Using currently available systems, it is nevertheless time-consuming to produce sufficient quantities of virus particles for *in vivo* application.[74,75] In addition, in the European Union it requires extensive permission to work with genetically modified viruses.

During the last couple of years, a new technique for the transient expression of a transgene in mouse liver has become available. This technique is based on the injection of "naked" plasmid DNA with high pressure into the tail vein of the mouse. It is commonly referred to as *hydrodynamic injection* or *high-volume-injection*.[76,77] In short, the endotoxin-free DNA is dissolved in buffer; the volume of the buffer has to be 1/10 of the body weight of the mouse. Subsequently, the solution is injected into the tail vein of the mouse within 10 seconds under isoflurane anesthesia. The solution will approach the heart via the *vena cava inferior*. As the volume injected exceeds the cardiac output rate, it will be forced to the organs connected to the vena cava in a retrograde way. Amongst these, the liver is the organ with the most expandable structure; therefore, the majority of the solution will reach the liver.[78,79] The mechanism by which DNA then enters the hepatocyte is not known in detail. Possible mecha-

nisms include both receptor-mediated endocytosis and pore formation, referred to as *hydroporation*.[80,81] The transgene is predominantly expressed in the liver, with average transfection rates of approximately 40 % of the hepatocytes.[77] The duration of expression largely depends on the properties of the transgene and its promoter. The method has been successfully applied in our lab using marker genes (data not shown) and will putatively be an easy, fast way of transgene expression in mouse liver in the near future.

A second new technique is currently revolutionizing modern cell biology. *RNA interference* (*RNAi*) is an efficient way to transiently knock-down the expression of a target gene (see ref. 82 for review). Its molecular background is specific breakdown of mRNA which is induced upon the recognition of double-stranded RNA of the same sequence by a cellular system, *dicer*. Two efficient systems for delivering double-stranded RNA to the cell are of special interest for our field of research. The first is the use of *small interfering RNA* (*siRNA*) which is chemically synthesized, the second is viral expression of siRNA. Commercially available siRNA can be directly used to transfect cells and therefore silence gene expression *in vitro*.[83] Moreover, this siRNA can be applied to mice by using the hydrodynamic injection technique (see above).[84] This provides a simple means to reduce hepatic expression of a target gene *in vivo*. In addition, systems are available were siRNA is transcribed from a viral genome, for example an adenovirus or a retrovirus.[85,86] This combines the high efficacy of viral delivery with the specificity of RNAi. Both viral delivery as well as hydrodynamic injection of synthetic siRNA will provide new possibilities for research on tissue-specific effects of genes involved in cholesterol homeostasis.

A couple of research questions can be approached by the hydrodynamic injection method. For example, one could study if reconstitution of Abcg5 in *Abcg5*$^{-/-}$ mice with plasmid DNA carrying an *Abcg5* construct would increase hepatobiliary cholesterol and phospholipid excretion even in the presence of high concentrations of plant sterols in the canalicular membrane. Furthermore, in a couple of models heterozygous mice show sufficient expression to perform a particular process, whereas this process is completely absent in knockout mice. Here, it could be desirable to reduce gene expression to levels at which the encoded protein becomes limiting. This could putatively be reached by hydrodynamic injection of siRNA. The hydrodynamic injection of siRNA will therefore be of importance for a couple of future projects in our field of research.

Regulation of cholesterol metabolism in the fetus and early in childhood: a new field with broad perspectives

The importance of cholesterol for the organism is most obvious during ontogenesis, when the growing embryo and fetus requires large amounts of cholesterol for its rapidly growing cell mass. Hence, both the embryo and the fetus synthesize relatively large quantities of cholesterol. In addition, a maternal supply of cholesterol to the embryo is plausible. Besides its role as a structural membrane constituent and hormone precursor, cholesterol is critically involved in pattern formation in early development. A number of proteins with basal functions in pattern formation, the so called *hedgehog proteins*, must be covalently bound to

cholesterol in order to become active (see Farese and Herz[87] for review). In this respect, a shortage of cholesterol can create major disturbances with devastating consequences for body organization. Furthermore, cholesterol is indispensable for brain development as a part of myelin. Consequently, the developing brain has a huge demand of cholesterol.[88] From this point of view it becomes clear that mutations which disrupt cholesterol synthesis or distribution to the developing embryo lead to major defects or even intrauterine death.

One major genetic aberration has been described which is compatible with life, the *Smith-Lemli-Opitz syndrome*.[89] In Smith-Lemli-Opitz syndrome, 7-dehydrocholesterol reductase activity is impaired which leads to abnormal low cholesterol synthesis and accumulation of the precursor 7-dehydrocholesterol.[90] The Smith-Lemli-Opitz syndrome is characterized by mental retardation, microcephaly, and pattern defects as misplaced thumbs and congenital cardiac abnormalities. Similar defects are seen when cholesterol synthesis is inhibited pharmacologically during embryogenesis in experimental animals.[91] Mutations in hedgehog genes have been shown to have lead to anomalities comparable to those seen in Smith-Lemli-Opitz syndrome.

In desmosterolosis, a defect in 24-dehydrocholesterol reductase leads to accumulation of desmosterol and deleterious effects in humans.[92,93] In *24-dehydrocholesterol reductase* knock-out mice, the murine model of desmosterolosis, only minor developmental changes occur.[94] This discrepancy can putatively be explained by the fact that in mice maternal cholesterol can reach the embryo and fetus, whereas this is not the case in humans.[87,95] Therefore, maternal cholesterol fulfills the crucial role in the mouse embryo.

Besides its synthesis, cholesterol transport is also of crucial importance for the embryo and fetus, at least in mice. Mutations in the genes encoding *ApoB*,[96,97] *Srbi*,[98] or *Abca1*[99] lead (partially) to intrauterine death in mice, but not in human. Obviously, the different environment of the rodent and the human embryo is of importance for cholesterol homeostasis: whereas the human embryo and fetus is connected to the maternal circulation solely via the placenta, the yolk sac plays an additional role in rodents for adequate nutrition.[87,95] This adds an extra layer of complexity to the unraveling of human and murine embryonic cholesterol homeostasis.

It is obvious that large shortages in cholesterol availability during embryogenesis have devastating effects. But what about small, more subtle variations? During the last couple of years it has become clear that the nutritional status of both the unborn fetus and the newborn have implications for adulthood. In principle, both a shortage and an excess of cholesterol could have negative implications for the health status of the individual.

An *excess* of cholesterol may predispose the fetus to the development of atherosclerosis later in life (see refs. 100 and 101 for review). It has been demonstrated that maternal hyper-cholesterolemia leads to fatty streak formation, *i.e.*, pre-atherosclerotic lesions, already *in utero*.[102] Furthermore, the size of atherosclerotic lesions in normocholesterolaemic children under 14 years was increasing faster in children of hypercholesterolaemic mothers.[103] Therefore, exposure to high levels of cholesterol in fetal life is most likely a risk factor for the development of cardiovascular disease in the adult.

In analogy, intrauterine *starvation* has been proposed to result in increased risks for the development of cardiovascular disease as well. The so-called *Barker hypothesis* states that fetal undernutrition, measurable as underproportionate fetal growth, predisposes for cardiovascular disease later in life.[104-106] Barker proposed that during a critical period of intrauterine life, programming takes place which fixes the level at which cholesterol – and other important substances - is maintained later in life. Until now, this hypothesis has been underlined with a wide array of epidemiological data. Classically, historical hospital documents have been analyzed for data on births weight for term babies and then these data were compared with the medical records for the adults. Results from studies from different countries show, for example, that the so-called *small for gestational age* (sga) babies have a higher risk for cardiovascular mortality and noninsulin-dependent diabetes later in life (summarized in ref. 106).

However, these epidemiological data indicate the existence of correlations, but do not reveal causal relationships. Until now, no mechanistical model has been proposed to explain these phenomena. In principle, the time has come to combine the molecular parts to a more compelling picture of the metabolic situation in the embryo. It has been speculated that increased expression of the SREBPs is responsible for the accelerated synthesis of cholesterol in fetal tissues.[95] However, time- and tissue-specific expression of this transcription factor has not been defined so far. The PPARs have been demonstrated to be expressed in the fetus in a time-dependent way.[107] The role of these and of other regulators of cholesterol homeostasis (or of lipid homeostasis in general) has not been elucidated in detail. But, as discussed above with the LXR target Abca1, some of their target genes play a role in development. The placenta has a high cholesterol synthesis rate on its own, but not much is known about its regulation. Finally, a number of ABC-transport proteins is expressed at high levels in the placenta, which makes it an interesting target for further projects within the framework of this research line.

Concluding remarks

In our society, morbidity and mortality from cardiovascular diseases is becoming an increasingly severe problem. Overnutrition and a sedentary lifestyle lead to obesity and hypercholesterolemia already in a high percentage of children, who will be the future patients with atherosclerosis-associated diseases.[108-112] Certainly, preventive educational programs have to be developed to adjust and prevent this process. However, also therapeutic options have to be improved to ameliorate hypercholesterolemia.

It seems that the major tools are now in ours hands. The most important metabolic pathways have been outlined and we have some clue about their regulation. Clearly, intracellular handling of cholesterol needs more emphasis, as well as the regulation of cholesterol homeostasis during development. However, the current situation allows to identify promising targets for cholesterol-lowering therapies at a molecular level and to develop effective means to prevent cardiovascular disease in the future.

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