The Role of the Macrophage Scavenger Receptor class A in Atherosclerosis

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STELLINGEN BEHORENDE BIJ HET PROEFSCHRIFT
'THE ROLE OF THE MACROPHAGE SCAVENGER RECEPTOR CLASS A IN ATHEROSCLEROSIS'

- Atherosclerose is een inflammatoire ziekte (R. Ross, N. Engl. J. Med. 1999; 340: 115-126).

- Een belangrijke functie van de scavenger receptor is afweer tegen zowel exogeen als endogeen materiaal (M. Krieger, Curr. Opinion Lipid. 1999; 8: 275-280).

- De scavenger receptor beschermt tegen atherosclerose (dit proefschrift).

- Opheldering van de biologisch belangrijke routes leidend tot LDL modificatie is belangrijk om de exacte rol van ge-oxideerd LDL in atherosclerose op te helderen en om nieuwe anti-oxidantia te genereren als therapie tegen atherosclerose (J. Heinecke, J. Clin. Invest. 1999;104: 135-136; D. Steinberg, J. Clin. Invest. 1999; 103: 1487-1488; dit proefschrift)

- Een belangrijk nadeel van de apoE knockout muis als model in het atherosclerose onderzoek is dat deze muis apoE deficient is (Mahley, Science 1988; 240: 622-630; Mahley, Curr. Opnion Lipid. 1999; 10: 207-217; dit proefschrift).

- Om in een muismodel de juiste functie van een eiwit in de atherogenese op te helderen is het noodzakelijk dat experimenten gedaan worden op verschillende atherosclerose gevoelige achtergronden.

- Het is niet juist uit te gaan van het feit dat verhoogde schuimcelvorming *in vitro* resulteert in meer atherosclerose *in vivo* (dit proefschrift).

- De rol van de scavenger receptor in atherosclerose is multifunctioneel en reikt verder dan alleen het mediëren van schuimcelvorming.

- Om de multifunctionele rol van de scavenger receptor in atherosclerose te begrijpen zal toekomstig onderzoek zich moeten richten op het ophelderen van de door de scavenger receptor gemedieerde signaal-transductie routes. (dit proefschrift).

- De meest opwindende uitroep die in de wetenschap wordt gebruikt om nieuwe ontdekkingen aan te kondigen, is niet: 'Eureka, ik heb het gevonden!', maar: 'Dat is raar.....?'. (Isaac Asimov)

- Het dagelijkse treinkrantje 'Spits' is slechts ingevoerd om de vele vertragingen te 'verzachten'.

- Mensen die altijd te laat komen houden niet van wachten

THE ROLE OF THE MACROPHAGE SCAVENGER RECEPTOR CLASS A IN ATHEROSCLEROSIS

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 25 november 1999
te klokke 16.15 uur

door

Menno P.J. de Winther

geboren te Nijmegen
in 1970

Promotiecommissie

Promotor:	Prof.dr. L.M. Havekes
Co-promotor:	Dr. M.H. Hofker
Referent:	Prof.dr. Th.J.C. van Berkel
Overige Leden:	Prof.dr. R. R. Frants Prof.dr. G. Kraal (Vrije Universiteit, Amsterdam) Prof.dr. S. Gordon (Oxford University, Engeland) Dr. K. Willems van Dijk

The studies presented in this thesis were performed at the department of Human and Clinical Genetics of the Leiden University Medical Center and the Gaubius Laboratory of TNO-Prevention and Health, Leiden.
This work was financially supported by the Netherlands Organization of Scientific Research project 901-04-092.

Financial support by the Gaubius Laboratory of TNO Prevention and Health for the publication of this thesis is gratefully acknowledged.

The printing of this thesis was financially supported by the Dr. ir. van der Laar Stichting, Parke-Davis and Hope-Farms.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

LIPID METABOLISM

Cardiovascular diseases are the main cause of mortality in Western society. The majority of these cardiovascular diseases are caused by atherosclerosis. Atherosclerosis is a complex multi-factorial disorder resulting in the formation of focal deposition of lipids, fibrous tissue and blood constituents in the vessel wall. Eventually, this can lead to rupture and thrombus formation causing occlusion of arteries, e.g. myocardial or cerebral infarction (1-3). Several risk factors contribute to the susceptibility to atherosclerosis. One of the main risk factors is elevated cholesterol and/or triglyceride levels in the plasma. Both cholesterol and triglycerides are important for many different cellular processes. Cholesterol is important for the synthesis of cellular membranes, steroid hormones and bile synthesis whereas triglycerides are the major source of energy for the body. These triglycerides can be used either directly by the muscle and other organs or stored as fat in adipose tissues. To transport cholesterol and triglycerides in the circulation, they are packaged into water-soluble lipoprotein particles. These lipoproteins consist of a core of apolar lipids, mainly cholesteryl esters and triglycerides, covered by a surface of polar molecules, primarily free cholesterol, phospholipids and apolipoproteins.

The plasma lipoproteins have been divided in different classes according to their density: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Furthermore, several derivatives have been described, such as the chylomicron and VLDL remnants and intermediate density lipoproteins (IDL). These different classes of lipoproteins not only differ in their density but also in size, electrophoretic mobility, lipid content and apolipoprotein composition.

The lipid metabolism, distributing fat throughout the body, can be divided in three main pathways. The exogenous pathway mediates the uptake of dietary lipids by the body, the endogenous pathways delivers lipids from the liver throughout the body and the reverse cholesterol pathways mediates the redirection of cholesterol from the periphery to the liver(4-7). These pathways are outlined in figure 1 and below.

Dietary fat is absorbed in the intestine and packaged into chylomicrons. These are secreted by the epithelial cells into the lymph and enter the general circulation. The chylomicrons are large triglyceride rich particles containing apolipoprotein (apo) B48, apoAI and apoAIV. Upon entering of the circulation the chylomicrons furthermore acquire apoE, apoCI, apoCII and apoCIII. During circulation the triglyceride core of the particles is hydrolysed by lipoprotein lipase (LPL) on the endothelial cells in the capillaries (8,9). The hereby released free fatty acids are used as energy source by muscles or stored as triglycerides, by adipocytes. Upon hydrolysis the chylomicron particles become smaller and enriched in cholesterol, and

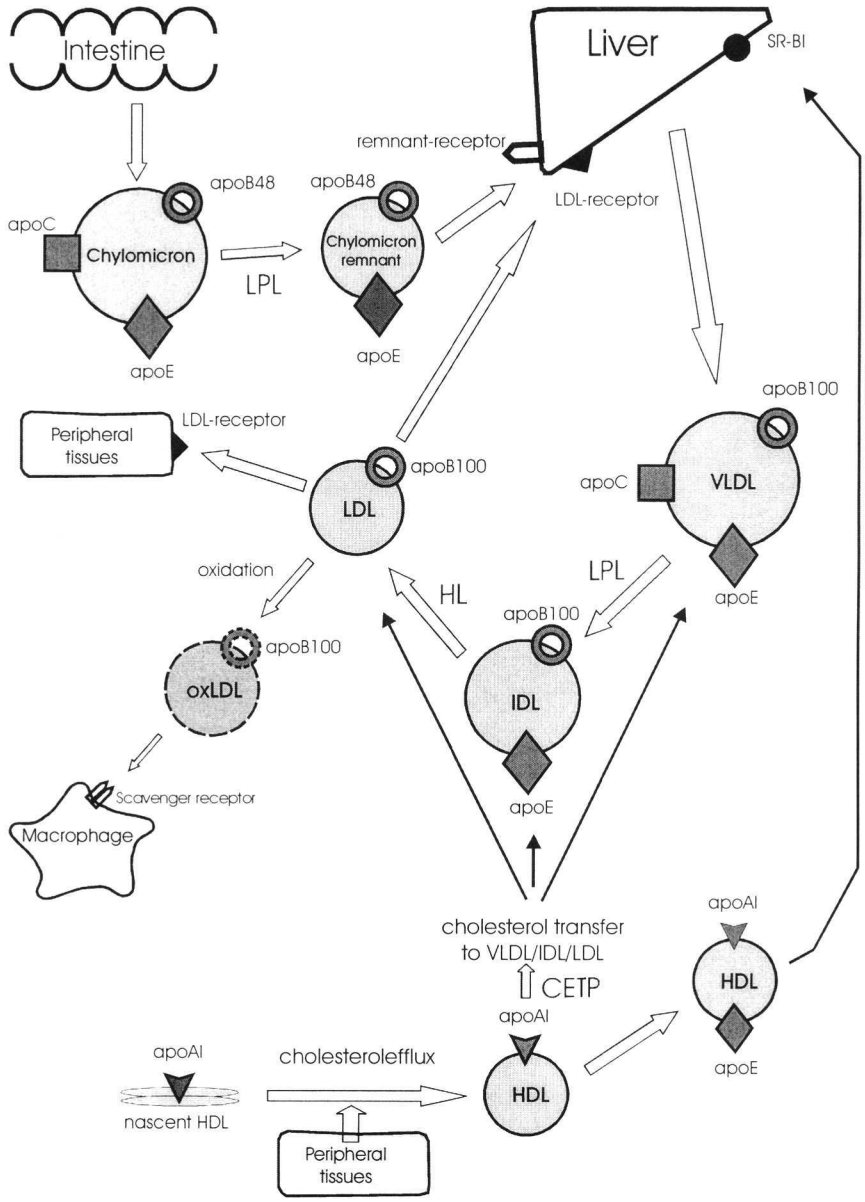

Figure 1: Schematic representation of the lipoprotein metabolism.
HL, hepatic lipase; LPL, lipoprotein lipase; CETP, cholesteryl ester transfer protein

are then called chylomicron remnants. These are rapidly taken up by the liver through apoE specific binding sites.

In the endogenous pathway, hepatic cholesterol and triglycerides are secreted by the liver into the circulation in VLDL particles (10). These nascent triglyceride-rich particles contain apoB100 and acquire apoC and apoE. Initially, the VLDL particles are hydrolysed by LPL resulting in the formation of cholesterol rich VLDL remnants, or IDL particles. Some of the VLDL remnants are taken up by the liver and the rest is further processed, probably involving hepatic lipase (11). Eventually LDL is formed from the VLDL remnants. This is characterised by a loss of triglycerides, phospholipids, apoC and apoE. The resulting LDL is rich in cholesteryl esters and contains apoB100 as the sole apolipoprotein constituent. In humans, approximately two thirds of the plasma cholesterol is present in the LDL fraction. Ultimately, these LDL particles are removed by hepatic or extra-hepatic LDL-receptors, which recognise apoB100 on the LDL surface (12,13).

Finally, cholesterol from extrahepatic tissues can also be transported back to the liver. This process is called the reverse cholesterol pathway. Cholesterol, released by cells, can be taken up by HDL particles, which contain apoAI and apoAII (14). This cholesterol is esterified in the circulation through the enzyme lecithin:cholesterol acyl transferase (LCAT), with apoAI as a co-factor. The HDL cholesterol may then be transported to the liver through three different pathways. First, cholesterol ester transfer protein (CETP) (15) can transfer cholesterol esters from HDL to lower density lipoproteins, such as VLDL, IDL or LDL, which are then taken up by the liver (16). Second, upon circulation, HDL can acquire apoE which mediates the direct uptake of HDL through apoE receptors, such as the LDL-receptor or the LDL-receptor related protein (LRP) (17). Third, cholesterol from HDL particles can be taken up by an HDL specific pathway, the HDL receptor, scavenger receptor BI (SR-BI) (18-21).

Atherosclerosis

Elevated levels of plasma LDL and/or remnant lipoproteins are considered to be unfavourable and predisposes to the development of atherosclerotic disease. Atherosclerosis is a response to injury to the endothelium and smooth muscle cells of the arterial wall. This injury can result from various mediators such as mechanical stress, oxidized LDL (oxLDL), toxins, viruses and bacteria. The response consists of the formation of fibrofatty and fibrous lesions, preceded and coinciding with inflammation.

When plasma levels of VLDL and LDL are elevated these lipoproteins can accumulate in the vascular wall where they get modified, either enzymatically or non-enzymatically. These modifications can be of broad nature, including reactions with lipoxygenases (22,23), superoxide anions (24-27), hydroxyl radicals (28),

peroxynitrite (29,30), haem proteins (31,32), ceruloplasmin (33), hyperchloride (34-36) and myoloperoxidase (37-40). In response to these modified lipoproteins, endothelial cells express recruitment and adhesion factors which recruit circulating monocytes and T-lymphocytes (41-44). Chemotaxis of these leukocytes is mediated by several growth factors. Colony stimulating factors (CSFs) (45), monocyte chemotactic protein-1 (MCP-1) (46), oxLDL (47) and TGFβ (48) can induce monocyte chemotaxis and endothelial transmigration. The initial, low affinity adhesion of the leukocytes to the vascular wall is characterised by their 'rolling' movement along the endothelium. Selectins (L-,E- and P-selectin) mediate this interaction (49). The following high affinity binding of leukocytes and monocytes to the endothelium and extravasation into the underlying intima is mediated by intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), both belonging to the immunoglobin super family (50-52). The cells migrate through the endothelial layer into the intima. The monocytes differentiate into macrophages as a result of the various growth factors and cytokines (including interleukin-1 (IL-1), tumour necrosis factor α (TNFα), interferon γ (IFNγ) and IL2), produced by smooth muscle cells, endothelial cells and other lymphocytes. The now formed macrophages take up the modified lipoproteins, to scavenge it from the vessel wall. Once modified and taken up by the macrophages, the LDL activates the macrophages. But, removal and sequestration of modified LDL are also important parts of the initial protective role of the macrophage in the inflammatory response and minimise the effects of modified LDL on endothelium and smooth muscle cells (3). The uptake of modified LDL can be mediated by various so-called scavenger receptors, such as the macrophage scavenger receptor class A (53-58). These receptors are not downregulated by intracellular cholesterol concentrations, in contrast to for instance the LDL-receptor, and therefore the uptake of modified LDL can result in a massive intracellular deposition of cholesterol (59), stored in the cytoplasm of the macrophages as cholesteryl ester droplets. These fat-laden cells are called foam cells and are the first step in atherogenesis. The accumulation of foam cells in the vessel wall is called the fatty streak, the first stage in atherosclerosis (60).

The diverse array of cytokines, growth factors and other signalling molecules that are locally produced by the macrophages, endothelium and smooth muscle cells attract more cells into the lesion. The smooth muscle cells at the lesion site also proliferate, migrate and secrete extracellular matrix components. This results in the formation of a more advanced fibrous plaque, covered by a dense cap of connective tissue and fibrotic cells. Under the fibrous cap, smooth muscle cells together with fat laden macrophages, T-lymphocytes, necrotic debris, cholesterol crystals and mineralisation can be found. These severe lesions now, partially occlude the vessel at the site where they occur and may impair blood flow. Most of the sudden deaths by myocardial infarcts are due to the rupture or fissure of these lesions, particularly in the margins of the fibrous cap where there are more macrophages, resulting in

haemorrhage into the plaque, thrombosis and total occlusion of the artery.

MOUSE MODELS TO STUDY ATHEROSCLERSOSIS

The detailed analysis of functions of specific genes involved in lipid metabolism and atherosclerosis is often hampered by complex interactions and variations in environmental and genetic factors in humans. Therefore, animal models are a very useful tool. They provide a well defined genetic background and environmental conditions are easily controlled, circumventing the limitations of these studies in man. The mouse is an animal that is easy to breed and it can be modified genetically, easily. However, mouse lipid metabolism differs from that in humans in several aspects, making them very resistant to hyperlipidemia and atherosclerosis. One major difference between mouse and man is that in man the main lipoprotein in the plasma is the pro-atherogenic LDL, whereas in mice it is the anti-atherogenic HDL (61,62). This is probably due to the very efficient clearance of VLDL, IDL and LDL sized particles in mice. However, upon feeding of high fat diets containing cholate, the lipoprotein profile of mice can shift towards the predominance of VLDL/LDL sized particles (63-66). This results also, after several months and especially in the 'athero-susceptible' strains such as C57Bl/6, in the development of atherosclerosis. In addition to the hyperlipidemia induced by severe diets, genetic modification of genes involved in lipid metabolism and atherosclerosis have led to the development of mouse models which can be used to study these processes (reviewed in (67-70)).

In humans, impaired receptor binding of apoE can result in type III hyperlipidemia (HLP), which is associated with the premature development of atherosclerosis (71-74). One of the first generated and most broadly used mouse models to study lipid metabolism and atherogenesis in mice is the apoE deficient mouse (75-77). Due to an impaired clearance of VLDL and IDL these particles accumulate in the plasma of these mice. This results in strongly elevated plasma cholesterol levels, whereas plasma triglyceride levels are only modestly increased. On a regular chow diet these mice have cholesterol levels of approximately 15 mM. When fed a high fat (Western type) diet, containing 0.25% cholesterol, the cholesterol levels increase to about 40 mM. Already on a chow diet, the apoE deficient mice develop atherosclerotic lesions spontaneously. These lesion occur in the aortic root, the coronary arteries and in the entire aorta at branch points of the major arteries in a time dependent manner (75,78). At 8 weeks of age monocyte adhesion to the endothelium is observed, resulting in the first foam cells at week 9. Further progression of atherosclerosis leads to intermediate lesions at week 14 and the most severe, fibrous lesions are first observed at week 19. Feeding the mice a high fat diet, results in a strong acceleration of this process.

The major drawback of studying lipid metabolism and atherosclerosis in apoE

deficient mice is that they lack apoE. ApoE is an important molecule in different processes and complete removal of apoE will dramatically modify several pathways in atherosclerosis. First, several reports show that apoE can mediate the efflux of cholesterol from foam cells in the vessel wall. This was shown by the prevention of atherosclerosis by local expression of apoE in either macrophages or the vessel wall (79,80). In addition, it was shown that transplantation of apoE deficient bone marrow to wild-type mice highly increased atherosclerosis, without effecting plasma cholesterol levels (81). Second, apoE is a ligand for several lipoprotein receptors, including the very low density lipoprotein receptor (VLDLR) and the low density lipoprotein receptor related protein (LRP), which have been described to be expressed in atherosclerotic lesions and may very well play a role in atherogenesis (82,83). Removal of apoE will abolish these uptake pathways. Third, apoE can mediate binding of lipoproteins to extracellular proteoglycans and thereby mediate the retention of lipoproteins in the vessel wall (84). Absence of apoE can change the retention of lipoproteins in the vessel wall, changing their susceptibility to modification and uptake by macrophages.

To overcome these aforementioned drawbacks of the apoE deficient mouse and to be able to study lipid metabolism and atherogenesis in more subtle hyperlipidemic conditions, additional apoE mouse models have been generated. One of such models, frequently used in a broad range of studies is the APOE3Leiden transgenic mouse. APOE3Leiden is a human apoE variant consisting of a tandem duplication of codons 120-126 in the apoE gene (85,86). The presence of a single allele for this mutation results in type III HLP. Mice carrying this dominant variant as a transgene are susceptible to diet induced HLP and atherosclerosis (87-89). On a high fat diet, the APOE3Leiden mice develop plasma cholesterol levels of approximately 35 mM, resulting in atherosclerotic lesions in the aortic arch, the descending aorta and the carotid arteries, varying from fatty streaks containing only foam cells to severe atherosclerotic plaques containing cholesterol clefts, fibrosis and necrotic calcified tissue. In contrast to the apoE deficient mice several functions, including cholesterol efflux and binding to lipoprotein receptors, are still functional (90,91). In addition to the APOE3Leiden mouse models several additional models carrying human variant forms of the apoE gene have been generated and studied in lipid metabolism and atherosclerosis research. These include transgenic mouse models carrying the human apoE variant APOE2 (91-93) and the more rare variant APOE2(142) (94,95) and a knock-in mouse model in which targeting replaced the murine apoE gene by the human APOE2 or APOE3 gene (96).

In addition to the above mentioned models using apoE variants, a mouse model frequently used is the LDL-receptor deficient mouse. In humans, mutations in the LDL-receptor gene cause familial hypercholesterolemia (97,98). Mice homozygously lacking the LDL-receptor, manifest delayed clearance of VLDL, IDL en LDL from the plasma (99). On a chow diet, this does not result in severe hypercholesterolemia and

no pathological features are observed . However, when fed a high fat diet, plasma cholesterol levels increase strongly, resulting in the formation of atherosclerotic lesions (100,101). This makes the LDL-receptor deficient mouse an excellent model to study factors effecting the phenotype of familial hypercholesterolemia.

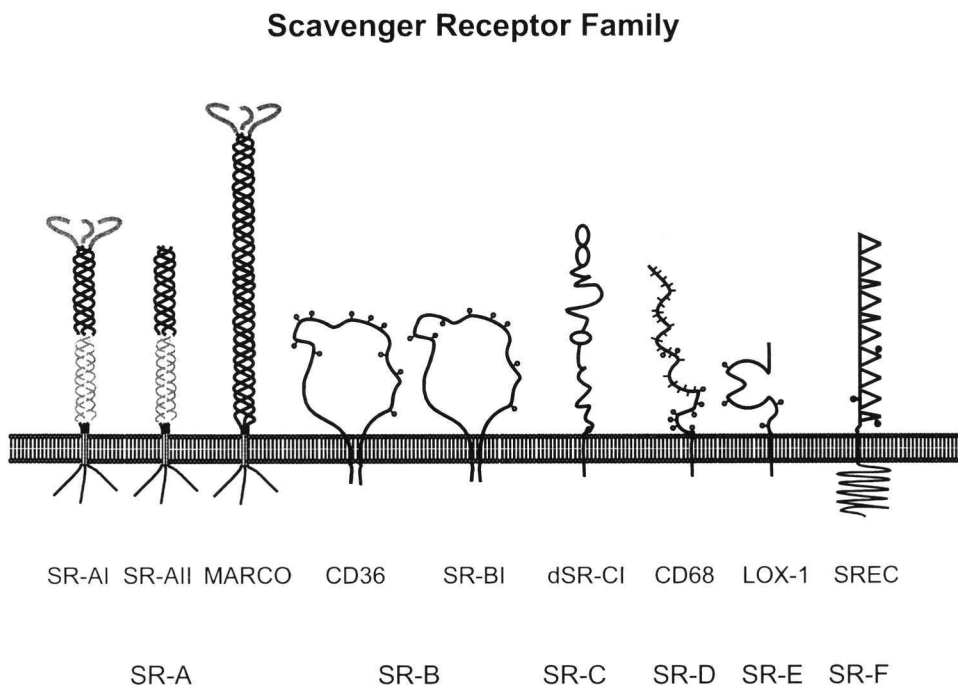

Figure 2: Schematic representation of the scavenger receptor family.
The molecules are not depicted to scale. Adapted from Greaves *et al.* (54).

SCAVENGER RECEPTORS

In 1979 Goldstein and Brown (102) described a receptor activity that could mediate the uptake of acetylated LDL, but not native LDL, by macrophages, resulting in massive accumulation of cholesteryl esters in cultured mouse peritoneal macrophages. This receptor was assigned scavenger receptor, since it mediated the removal of modified LDL from the medium. The scavenger receptor gained importance when oxidized LDL (oxLDL), a physiologically much more relevant form of modified LDL as compared to acetylated LDL, was shown to bind to the scavenger

receptor. Several lines of evidence direct to the important role of the scavenger receptor in atherogenesis: 1) upon incubation with modified LDL expression of the scavenger receptor in cultured macrophages or transfected Chinese hamster ovary cells can lead to the conversion to lipid-laden cells, resembling the foam cells found in atherosclerotic plaques (103); 2) the scavenger receptor is highly expressed in atherosclerotic lesions (104,105); 3) the scavenger receptor is not down-regulated in response to cellular cholesterol loading, as is the case for the LDLR (59); 4) oxLDL has been shown to be present in atherosclerotic lesions (105-107) and intervention with antioxidantia that prevent the oxidation of LDL reduces the progression of atherosclerosis (108-112).

Since the cloning of this first scavenger receptor, the scavenger receptor class A (type I, II) (113-116), a variety of new scavenger receptors have been isolated and characterised (reviewed in (54,57,117-119)). Structurally they greatly differ but they share the feature that they can all bind modified LDL. In addition to the binding of modified LDL the scavenger receptors bind a broad range of other polyanionic molecules and for most scavenger receptors the true physiological relevant ligands remain to be identified. Based on their structure the still growing scavenger receptor family has been divided into several classes, which are depicted in figure 2.

This thesis focuses on the class A scavenger receptor (type I, II). Chapter 2 describes this molecule in more detail and focuses on its multifunctional role in atherogenesis.

SCOPE OF THIS THESIS

In this thesis, studies on the role of the scavenger receptor class A type I/II (SR-A) in atherosclerosis are described. To study this, we generated and analysed several mouse models. Initially, we crossed the SR-A deficient mice generated by Kodama (120) and the APOE3Leiden mice. Atherosclerosis was analysed in APOE3Leiden mice in the absence of the SR-A as compared to APOE3Leiden mice with the SR-A present. Lesion area measurements and plaque composition studies showed that the role of the SR-A is not as straightforward as might be expected based on previous *in vivo* and *in vitro* data in other mouse models. To further extend these studies we started the generation of additional SR-A mouse models. We pursued several strategies (including a CD11b promoter driven cDNA construct) of which the generation of a mouse model overexpressing the entire human SR-A gene (MSR1) using a newly generated 180 kb yeast artificial chromosome (YAC), was most successful. MSR1 transgenic mice were generated and analysed for their overexpression and SR-A function. The next chapter describes the experiments which were performed to study the effect of MSR1 overexpression on atherogenesis. Two different approaches, a cross on an LDLR deficient background and a bone

marrow transplantation to APOE3Leiden mice, showed a protective effect of the SR-A, confirming the results from our cross between SR-A deficient and APOE3Leiden mice. Finally, we show the generation and first results with a mouse model specifically lacking type I SR-A, which will be very useful to establish the exact *in vivo* function of type I SR-A, as compared to type II SR-A. The last experimental chapter describes the effects of two human APOE variants, APOE3Leiden and APOE2, on atherosclerosis and other hyperlipidemia associated pathology.

The results obtained in these studies are discussed in chapter 9. In addition, future perspectives on studies on the role of the SR-A in atherosclerosis will be discussed.

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CHAPTER 2

MACROPHAGE SCAVENGER RECEPTOR CLASS A: A MULTIFUNCTIONAL RECEPTOR IN ATHEROSCLEROSIS.

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(Arterioscler.Thromb.Vasc.Biol., *in press*)

Abstract

In atherogenesis, elevated plasma levels of low density lipoprotein (LDL) lead to the chronic presence of LDL in the arterial wall. There, LDL is modified (e.g. oxidized) and these modified lipoproteins activate endothelial cells which attract circulating monocytes. These monocytes enter the vessel wall, differentiate into macrophages and endocytose the modified lipoproteins through scavenger receptor pathways. This unrestricted uptake, which is not limited by intracellular cholesterol levels, eventually leads to the formation of lipid-filled foam cells, the initial step in atherosclerosis. The macrophage scavenger receptor class A (SR-A) is thought to be one of the main receptors involved in foam cell formation, mediating the influx of lipid into the macrophages. In addition to this role in modified lipoprotein uptake by macrophages, the SR-A has been shown to be important in the inflammatory response in host defence, cellular activation, adhesion and cell-cell interaction. Given the importance of these processes in atherogenesis, these latter functions may prove to make the SR-A a multifunctional player in the atherosclerotic process.

SR-A structure, function and expression

In 1979 Brown and Goldstein (1) first described a binding site on macrophages that mediated the uptake and degradation of acetylated low density lipoprotein (LDL) and produced massive intracellular cholesterol deposition. This binding site was initially referred to as the acetylated LDL receptor and later became the macrophage scavenger receptor. Currently, it is known that this receptor belongs to a large family of scavenger receptors consisting of at least 6 classes (table I (2)) all mediating the uptake of modified LDL. This paper will focus on the macrophage scavenger receptor class A type I and II (SR-A). This multifunctional receptor has been shown to bind a broad variety of ligands. Based on binding and competition studies it was shown that these ligands are generally poly-anionic macromolecules, including (1) acetylated LDL, oxidized LDL, maleylated or glycated bovine serum albumin, (2) polyribonucleotides (poly G and poly I; but not poly A, T or C), (3) polysaccharides including lipopolysaccharide (LPS), lipoteichoic acid (LTA) (both surface components of bacteria), dextran sulphate and (4) anionic phospholipids, such as posphatidylserine (3-8).

The molecular characterisation of the SR-A began with the cloning of the bovine SR-A in 1990 (9,10). It was shown that the SR-A is a trimeric transmembrane glycoprotein consisting of six distinct domains (fig.1). The collagen-like domain has been shown to be the site for receptor interaction with modified lipoproteins (11-14). This collagen-like domain contains a lysine cluster which forms a positively charged groove which specifically interacts with the negatively charged ligands. The gene

Table I: The members of the scavenger receptor family, their celltypes of expression, putative functions and ligands.

Class/Name	Celltype	Function	Major ligands
Class A			
SR-A I/II/III	macrophages	Uptake of modified LDL, innate immunity, adhesion	AcLDL, oxLDL, AGE, LPS, LTA, apoptotic thymocytes
MARCO	Spleen macrophages	Innate immunity ?	AcLDL, bacteria
Class B			
CD36	Platelets, monocytes, macrophages	Fatty acid transporter, uptake of apoptotic cells	OxLDL, apoptotic cells
SR-BI (CLA-I)	Adrenals, liver, gonads	Cholesterol transport	HDL, oxLDL
Other			
CD68 (macrosialin)	Macrophages	unknown	OxLDL, apoptotic cells
SR-C	Embryonic insect macrophages	Uptake of apoptotic cells	AcLDL
SREC	Endothelial cells	Unknown	AcLDL, oxLDL
LOX-1	Endothelial cells	Unknown	OxLDL

OxLDL, oxidized LDL; acLDL, acetylated LDL; LPS, lipopolysaccharide; LTA, lipoteichoic; AGE, advanced glycation endproducts.

coding for the six different domains contains 11 exons and spans approximately 80 kb in humans (15). Two variants of the SR-A (type I and type II) are generated by alternative splicing of the same gene. Type I SR-A is encoded by exon 1-8 and 10-11 and contains an 110 aa long C-terminal cystein rich domain. Type II SR-A is encoded by exon 1-9 and lacks the cystein rich domain (fig.1). Recently, a third transcript was described which acts in a dominant negative manner. This variant, resembles type I SR-A but skips exon 10, thereby deleting part of the cystein domain (16). The exact function of the cystein rich domain has not been elucidated yet. However, specific regulation of the type I and II isoforms has been described (17-19).

Freshly isolated human monocytes express low amounts of SR-A mRNA, but it is highly upregulated during differentiation to macrophages. This increase in expression is mainly observed for type I SR-A. However, both types have been shown to be present in atherosclerotic lesions (20). Since the SR-A type I has been described as a receptor containing a cystein rich domain, a new family of genes has emerged, all containing a scavenger receptor cystein rich (SRCR) domain. A function has been proposed only for one of these genes. CD6, containing three SRCR domains, has been shown to be the ligand for the leukocyte adhesion molecule CD166 (21) indicating the involvement of the SRCR domain in cell-cell interaction. For the SR-A type I and II no differences have been shown in ligand interaction between both isoforms. Recently, we have generated a mouse model specifically lacking type I SR-A. This mouse may give new insights in the *in vivo* function of the cystein rich domain of type I SR-A.

The expression of the SR-A is mainly confined to macrophages including Kupffer cells, alveolar, splenic, thymic and many other tissue macrophages (20,22,23). It is also expressed on endothelial cells lining the liver and adrenal sinusoids (20,22), and on high endothelial cells of postcapillary venules in the lymph nodes (24). Immunohistochemical studies could not detect SR-A expression in the endothelium of the aorta in cow(23), mouse(22) and human(25). Therefore, it is likely that aortic endothelium does not express SR-A. Different reports have shown the presence of the SR-A on smooth muscle cells, both *in vitro* and *in vivo* in atherosclerotic lesions (26-28) although others did not find SR-A expression on the smooth muscle cells and found it to be restricted to the macrophage foam cells in the lesions (29,30).

Several different motifs in the promoter of SR-A are required for the expression of SR-A in macrophages. Positive transcriptional control of SR-A has shown to be mediated by a domain for PU.1/Spi-1, a macrophage- and B-cell specific transcription factor belonging to the ets domain family and by a domain binding AP-1 family members and a distinct sub-set of ets family members, which include c-Jun, JunB and ets2 (31). Especially the AP-1 domain has been shown to be of great importance since several AP-1 sites have been found in the SR-A promoter area. These elements induce SR-A transcription in monocytes in response to macrophage colony-stimulating factor (M-CSF) (31-34), probably through protein kinase C activity (26,33). The high expression in atherosclerotic lesions and foam cells is also attributed to these transcriptional elements as was shown in transgenic mice using a reporter gene driven by different SR-A promoter elements (35).

The expression levels of SR-A in macrophages are influenced by several different cytokines (table II). Both tumor necrosis factor-α and interferon-γ are produced locally in atherosclerotic lesions and inhibit SR-A activity on macrophages by transcriptional and post-transcriptional regulation (36,37). M-CSF and granulocyte macrophage colony-stimulating factor (GM-CSF) are upregulators of murine SR-A

expression (34,38,39). However, another group reported a downregulation of SR-A expression by GM-CSF(18) , so the effect of this factor is not completely clear, yet. Two growth factors which are expressed in lesions, platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) were shown to decrease and increase, respectively, SR-A expression in monocyte derived macrophages(40-42). Recently, it was shown that peroxisome proliferator-activated receptor-γ (PPAR-γ), a regulator of macrophage activation, inhibits the expression of SR-A (43).

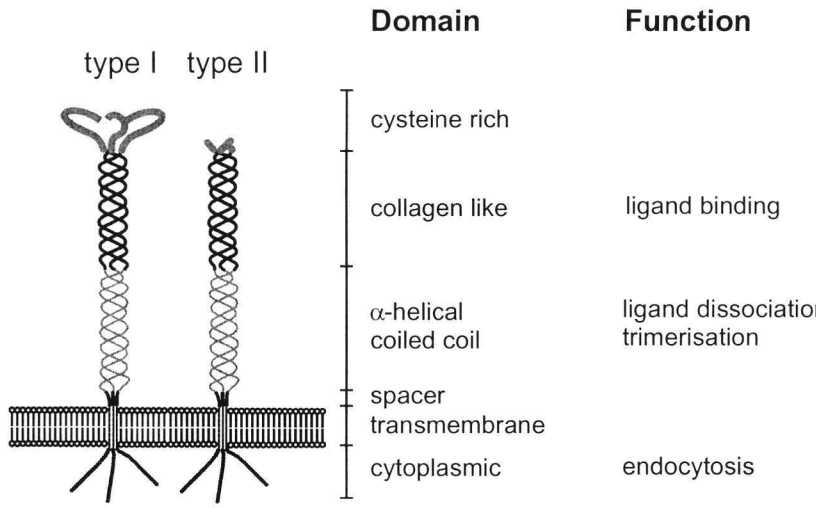

Figure 1: Structure of the SR-A type I and II with the six domains and their functions.

Table II: The effects of different cytokines and growth factors on macrophage SR-A expression.

Cytokine/growth factor	Effect on macrophage SR-A expression
TNF-α	↓
IFN-γ	↓
M-CSF	↑
GM-CSF	↑
TGF-β	↓
PDGF	↑

TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; M-CSF, macrophage colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; TGF-β, transforming growth factor-β; PDGF, platelet derived growth factor.

SR-A IN ADHESION AND CELL-CELL CONTACT

In 1993 Fraser and colleagues (44) isolated a monoclonal antibody (2F8) that inhibited the calcium independent adhesion of RAW264 macrophages to tissue culture plastic. They showed that this monoclonal antibody was directed against the murine SR-A and this was the first evidence that SR-A could mediate cellular adhesion and thereby function in the r ecruitment and retention of mononuclear phagocytes to tissues, such as atherosclerotic lesions. The adhesion observed was dependent on the presence of serum, indicating that SR-A adhesion activity needs a serum component to function. In more elaborate studies it was shown that 2F8 could also block the adherence of macrophages to tissue sections. RAW264 macrophages adhere in an EDTA resistant fashion to spleen, lymph node, liver, thymus, gut, skin and lung tissue sections. This adhesion could be completely blocked by 2F8 in all tissues except liver and gut (22,44-46). Again, this shows that the SR-A may be important for the recruitment and retention of macrophages to tissues, although it has not been possible to demonstrate that SR-A can mediate macrophage migration *in vivo* using 2F8 antibodies. This problem may be attributable to the very rapid clearance of 2F8 from the circulation which prohibits these *in vivo* experiments. Recently Robbins and co-workers (47) transfected HEK293 cells with the human SR-A. Although this cell line poorly adheres to tissue culture plastic, after transfecting these cells with the human SR-A adherence to plastic and glass is largely improved.

In the thymus immature thymocytes often undergo apoptosis during selection and are removed by phagocytosis by thymic macrophages. The SR-A was shown to be an important macrophage receptor involved in the recognition and uptake of these apoptotic thymocytes. *In vitro*, thymic macrophages from SR-A deficient mice showed an approximate 50% reduction in the uptake of apoptotic thymocytes (48-50). The phagocytotic uptake of normal thymocytes was not changed (50). In addition, Yokota *et al.* (51) demonstrated the cell adhesion properties of the SR-A by showing that the SR-A could mediate the adhesion of activated B cells to CHO cells stabely expressing SR-A type I or type II.

Non-enzymatic glycation of arterial basement membrane proteins occurs during normal ageing and at an accelerated rate in diabetic patients. With time, these glucose adducts form advanced glycation end (AGE) products. These AGEs are taken up by cells through SR-A pathways (52-56). El Khoury and colleagues (57) showed that macrophages can adhere to surfaces coated with glucose-modified basement membrane collagen IV through their SR-As. These findings indicate a potential role of SR-A in the accelerated atherogenesis found in diabetes i.e. the SR-A promotes the adhesion of macrophages to glucose modified basement membrane proteins in the arterial wall. On top of this, it has been shown that ligation of AGEs to SR-A stimulates macrophages to secrete proinflammatory cytokines and growth

factors and could thereby enhance the attraction of monocytes and inflammation at the lesion site (58-62).

SR-A IN HOST DEFENCE, INNATE IMMUNITY AND CELLULAR ACTIVATION

In the course of the characterisation of the binding specificity of the SR-A, many different ligands, in addition to the modified lipoproteins, have been discovered. These include maleylated bovine serum albumin, polyribonucleotides like poly I and poly G, but not poly A, T or C, polysaccharides e.g. dextran sulphate, anionic phospholipids like phosphatidylserin and other negatively charged molecules like polyvinyl sulphate (1,3-6) . All these ligands share the property that they are polyanionic, but not all polyanionic molecules are ligands for the SR-A. Extension of the search for new ligands for SR-A resulted in the identification of lipid A and its precursors lipid IVA as molecules that can bind to the SR-A (7). Lipid A is a constituent of lipopolysaccharide (LPS), present on the surface of Gram-negative bacteria, that stimulates macrophage activation and causes endotoxic shock. Binding and uptake of LPS by macrophage cell line RAW264 is mediated by the SR-A. *In vivo*, the liver uptake of LPS could be blocked by SR-A ligands. More recently, it was shown that SR-A can also bind to Gram-positive bacteria, including *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus aureus*, *Enterococcus hirae* and *Listeria monocytogenes* (8). Competition studies using lipoteichoic acid (LTA), a surface molecule of these Gram-positive bacteria, showed that this was recognised by the SR-A. With the establishment of the SR-A knockout mice, the *in vivo* role of SR-A on LPS and infection has been investigated. Suzuki et al. (63) have found that SR-A deficient mice show an increased susceptibility to infection with *Listeria monocytogenes* or herpes simplex virus type-1. Haworth and co-workers (64) extended these investigations to a model with acquired immunity using a viable bacillus Calmette Guerin (BCG) infection model. They showed that after BCG infection, SR-A knockout mice are much more susceptible to LPS induced endotoxic shock. These mice produce more TNFα and interleukin-6 and show an increased mortality in response to LPS. This increased mortality could be blocked by administration of anti-TNFα antibodies prior to the LPS challenge. An explanation for this increased sensitivity to LPS induced endotoxic shock might be a changed balance between LPS receptors that do trigger the release of cytokines (e.g. CD14) and receptors that mediate binding and uptake of LPS but do not directly mediate an inflammatory response (e.g. SR-A). The exact nature of the increased susceptibility of SR-A knockout mice to completely different pathogens as the bacteria and viruses remains unclear but may indicate an even broader involvement of the SR-A than just the binding of LPS and LTA.

OxidizedLDL (oxLDL) and lysophosphatidylcholine (lysoPC) are able to induce the growth of macrophages. It has been shown that oxLDL has this mitogenic effect but not acetylated LDL. Treating acLDL with phospholipase A_2, however, markedly increased its mitogenic activity, concomitant with a 75% conversion of its phospholipids to lysoPC (65). The mitogenic effect of lysoPC containing modified LDL was shown to be mediated by uptake through the SR-A, since macrophages from SR-A deficient mice showed a strong decrease in cell growth in response to oxLDL (66). More recently it was shown that macrophage growth induced by oxLDL is mediated through the uptake via the SR-A, followed by protein kinase C activation and subsequent secretion of GM-CSF. This GM-CSF secretion was strongly reduced in SR-A deficient macrophages (67). In the atherosclerotic lesion, this GM-CSF production in response to oxLDL may play a very important role in priming macrophage growth, in conjunction with other cytokines. In addition to this proliferative role for the SR-A, more recently SR-A expression was shown to confer resistance of macrophages to apoptosis(68). In both differentiated THP-1 monocytes and Chinese hamster ovary cells, expression of SR-A increased the resistance to G-protein coupled apoptosis. This anti-apoptotic effects of the SR-A can greatly effect apoptosis in atherosclerotic lesions.

Other activation markers are also affected by oxLDL. Recently, it was shown that PPAR-γ, a modifier of macrophage activation, is regulated by both colony stimulating factors (M-CSF and GM-CSF) and oxLDL (43,69,70). These two factors, both present in atherosclerotic lesions, were shown to stimulate PPAR-γ expression in primary macrophages and monocytic cell lines. The activation of PPAR-γ was shown to inhibit NF-κB activation and thereby reduce the inflammatory response. SR-A expression is affected by PPAR-γ, and oxLDL uptake through SR-A modifies GM-CSF production by macrophages (67). Some papers also reported a modifying effect of the activation response to LPS by oxLDL through SR-A (71,72). How these processes are exactly intertwined is not yet clear.

In conclusion, it can be said that different SR-A ligands, including oxLDL, modify macrophage activation through, so far unknown, pathways thereby greatly affecting the atherosclerotic process in the vessel wall.

SR-A IN FOAM CELL FORMATION AND ATHEROSCLEROSIS

Atherogenesis is induced by elevated levels of atherogenic lipoproteins, such as LDL and very low density lipoprotein, in the blood. This leads to the accumulation of LDL in the intima where it becomes modified. This modified LDL activates the endothelial cells lining the vessel wall, attracting monocytes from the circulation, which subsequently will adhere to the endothelial cells, cross the endothelial layer to enter the media, differentiate into macrophages and eventually become foam cells.

These foam cells are characterized by a massive accumulation of cholesterolesters (73) resulting from the unrestricted uptake of modified lipoproteins, such as oxidized LDL, through scavenger receptor pathways. The SR-A was first identified as a binding site specific for acetylated LDL on macrophages that mediates the formation of foam cells (1). Overexpression of the SR-A in Chinese hamster ovary (CHO) cells resulted in foam cell formation after incubation with acetylated LDL (74). We found that isolated peritoneal macrophages from a recently generated mouse model overexpressing the human SR-A gene, also show an increased foam cell formation and cholesterolester accumulation after incubation with acetylated LDL(75). Furthermore, the SR-A has been shown to be highly expressed in atherosclerotic lesions (76,77) whereas the SR-A ligand oxidized LDL is present in plaques (77-79). Based on these results the SR-A was assigned an important role in atherogenesis (80-82).

The relevance of the SR-A in atherosclerosis was completely established when SR-A knockout mice were generated (63). Resident peritoneal macrophages from SR-A deficient mice show an 80% reduction in acetyl LDL degradation and a 30% reduction in oxidized LDL degradation. However, the *in vivo* clearance by the liver of both forms of modified LDL was not changed (63,83,84). This observation is in line with the results of Van Berkel *et al.* (85) showing that 70% of the injected dose of acetylated LDL was cleared by the liver endothelial cells in both SR-A deficient and wildtype mice, indicative for the fact that for this process other receptors are the main players. Future research into these receptors is needed to elucidate the main endothelial receptors involved in modified LDL clearance. It should be noted however, that uptake of modified LDL by the liver for clearance and by macrophages in the subintimal space in the process of atherosclerosis, are two completely different processes that do not necessary share any receptor pathways.

To study the effect of MSR deficiency on atherosclerosis, the SR-A knockout mice have been crossed with different mouse strains susceptible to atherosclerosis. On an apolipoprotein E (apoE) deficient background, SR-A deficiency resulted in a moderate increase in plasma cholesterol levels coinciding with a strong decrease (60%) in lesion area development (63). However, on a low density lipoprotein receptor (LDLR) deficient background, SR-A deficiency resulted in a 20% lower plasma cholesterol levels and only a 20% reduction in atherosclerosis(86). We have bred the SR-A knockout mice on an APOE3Leiden transgenic background. These mice carry a dominant variant of the human APOE gene resulting in hypercholesterolemia and sensitivity to diet induced atherosclerosis (87,88). In these mice, absence of the SR-A resulted in the development of more severe lesions, as judged by their cellular composition (89). This means that in the APOE3Leiden mice, SR-A deficiency actually enhanced atherosclerosis.

Table III: The different atherosclerosis experiments performed using different SR-A mouse models.

SR-A genotype	Atherosclerotic model	Effect on cholesterol	Effect on atherosclerosis
SR-A $^{-/-}$	Apoe$^{-/-}$	↑	↓↓
SR-A $^{-/-}$	Ldlr$^{-/-}$	↓	↓
SR-A $^{-/-}$	APOE3Leiden	-	↑
MSR1 transgene	Ldlr$^{-/-}$	-	↓

Shown are the SR-A model, the atherosclerotic background and the effects of the SR-A on cholesterol levels and atherosclerosis both as compared to SR-A wildtype mice.

To extend the *in vivo* studies on SR-A, additional models have been generated. Wölle *et al.* (90) generated a mouse model with hepatic overexpression of the SR-A. The bovine SR-A type I cDNA was cloned behind the mouse transferrin promoter which resulted in overexpression of the SR-A in hepatocytes. They showed that the SR-A can prevent diet induced hyperlipidemia through a reduction in apoB containing lipoproteins. In addition, high levels of SR-A overexpression increased HDL cholesterol levels, decreased cholesteryl esters in the liver and increased the fecal bile acid flux, all on a high fat diet. This shows that the SR-A can effect lipid levels in the blood, although the hepatocytes are not the natural site of SR-A expression in the liver. Overexpression of SR-A in the liver Kuppfer cells, its natural site of expression, will have more subtle effects. Recently, we generated a mouse carrying the human SR-A gene region as a 180 kb transgene(75). Macrophages from these humane SR-A transgenic mice (MSR1 mice) show a high expression of the human SR-A. This results in an increased degradation of acetylated and oxidized LDL, *in vitro*. Moreover, these macrophages show an enhanced foam cell phenotype after incubation with acetylated LDL. However, when crossed on an LDLR deficient background the MSR1 transgenic mice showed a decreased atherosclerosis. Similar results were obtained by Thierry and Teupser (91,92). They used a completely different approach by generating two rabbit strains, from one parental strain, by selecting for a high and a low susceptibility to diet induced atherosclerosis. This strategy was maintained during more than 10 generations of breeding. They showed that the rabbits with a low susceptibility to atherosclerosis have elevated SR-A expression levels as compared to rabbits that have a high susceptibility.

Thus, several different atherosclerosis experiments do not confirm each other. The results are outlined in table II. Between the different mouse models with absence of the SR-A opposing effects of the SR-A on atherosclerosis development have been observed. In addition, these models do not all confirm the results with animals overexpressing the SR-A. This shows that, beside the uptake of modified lipoproteins, the other functional properties of the SR-A probably effect

atherogenesis. Some research is focussing on the use of SR-A agonists or antagonists as possible drugs against atherosclerosis(93). However, the broad range of functional properties of the SR-A should be taken into account when considering this. Blocking the SR-A using antagonists will not just simply block lipid uptake and thereby reduce atherosclerosis, as is clear from our results using the SR-A deficient mice on an APOE3Leiden background(89).

Conclusion

Figure 2 outlines the atherosclerotic process and the many steps in which the macrophage SR-A participates. In three processes the SR-A may be a player.
First, the invasion of monocyte derived macrophages into the lesion area is the first process in which the SR-A may play a role. In response to the activation of the endothelium, circulating monocytes adhere to the vessel wall and enter the lesion area. There, they differentiate into macrophages. The adherence and interaction of these macrophages with other cells in the plaque, such as the endothelial cells, the smooth muscle cells or other macrophages may be mediated through the SR-A, as is strongly suggested by *in vitro* data. Furthermore, uptake or removal of apoptotic cells from the atherosclerotic lesion may also be mediated by the SR-A. In addition to this direct cell-cell interaction, the SR-A may also mediate binding of macrophages to (AGE modified) extracellular membrane molecules, which have also been shown to be present in the plaque. Whether this adhesion function of the SR-A in atherogenesis will turn out pro- or anti-atherogenic, is not clear, yet.

Second, the SR-A may modulate activation of macrophages in the plaque. In response to activating, macrophages secrete a repertoire of cytokines which will act on the endothelial cells, smooth muscle cells and macrophages itself, present in the lesion. Binding and uptake of modified LDL and AGE modified proteins have been shown to modulate the activation of macrophages, which may also be true in atherosclerotic lesions. The different SR-A ligands present in the plaque, can modify the activation of macrophages, thereby changing the cytokines produced in the plaque. This will result in a change in activation and inflammation profile of such a lesion. The anti-apoptotic effect of SR-A expression, may modulate the rate of apoptosis in the lesion, effecting the development of the plaque. Again, these modifications of macrophage activation through SR-A pathways can turn out positive or negative for atherogenesis. On top of this modification of macrophage activation through SR-A ligands, changing the cytokines produced in a lesion can also effect SR-A expression itself, as a sort of feedback loop.

Third, the SR-A does play an important role in the foam cell formation itself. The modified LDL present in the plaques is taken up through the SR-A resulting in an accumulation of lipid droplets in macrophages, leading to foam cell formation. Modification of SR-A will influence this process and change lesion formation. Most of

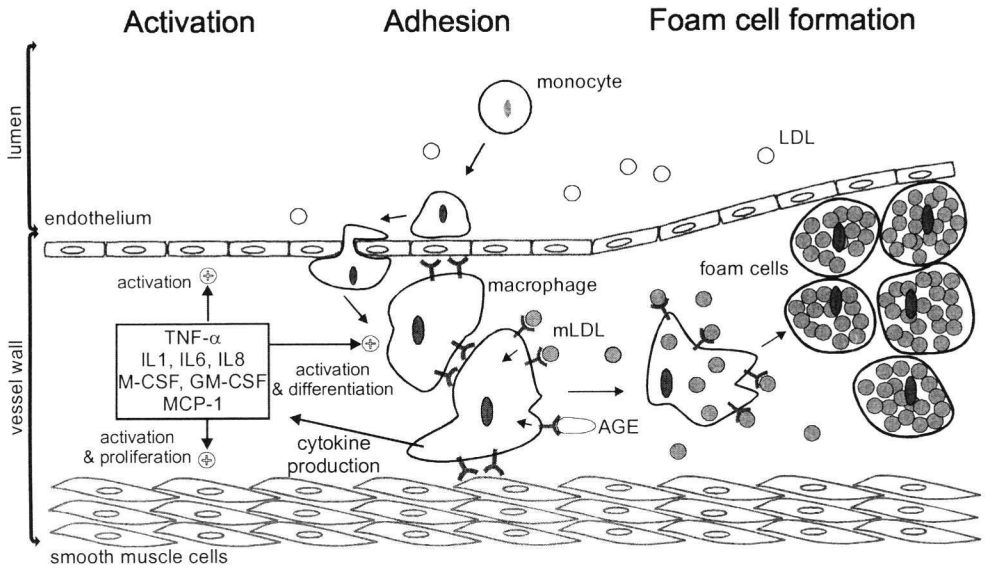

Figure 2: Atherosclerotic lesion formation with the different processes in which the SR-A may play an important role.
Indicated are adhesion and cell-cell interaction of macrophages in the lesion, activation of the lesion through macrophage derived cytokines modulated by the SR-A and foam cell formation in which the SR-A mediates the unrestricted uptake of mLDL by macrophages. Endothelial cells are red, smooth muscle cells blue and the SR-A is depicted in green. mLDL, modified LDL; AGE, advanced glycation endproducts; TNF-α, tumour necrosis factor-α; IL, interleukin; GM-CSF/M-CSF, macrophage granulocyte/macrophage-colony stimulating factor; MCP-1, monocyte chemotactic protein-1.

the *in vitro* data suggest that overexpression of the SR-A, enhances the accumulation of fat in the cells and thus the SR-A should be a pro-atherogenic factor. However, one can also envision, very early in the process of atherogenesis, removal of modified lipoproteins from the vessel wall to be beneficial because this may reduce inflammation at the lesion area.

The multifunctional nature of the SR-A is clearly illustrated by its involvement in cell adherence, activation and foam cell formation. All these processes are involved in the development and progression of atherosclerosis. This likely explains the diverging outcomes of atherosclerosis experiments using different animal models. SR-A deficient mice showed a reduction in lesion area when crossed on an apoE or LDLR deficient background but showed an enhancement of lesion development when crossed on an apoE3Leiden transgenic background. Mice overexpressing the human SR-A showed a reduction in lesion area when crossed on an LDLR deficient background. An anti-atherogenic effect of the SR-A was also found in rabbit strains expressing a high level of SR-A.

In conclusion, we propose the SR-A to play an important role in many processes in atherogenesis, some of which are pro- and others are anti-atherogenic depending on the local factors mediating atherosclerosis. The net result of the balance between these pro- and anti-atherogenic properties of the SR-A will be determined by the dominance of these processes that drive atherogenesis, in a specific situation. It may be clear that additional work is necessary to fully appreciate the role of the SR-A in the different processes underlying atherogenesis.

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CHAPTER 3

SCAVENGER RECEPTOR DEFICIENCY LEADS TO MORE COMPLEX ATHEROSCLEROTIC LESIONS IN APOE3LEIDEN TRANSGENIC MICE.

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(*Atherosclerosis 144 (1999); 315-321*)

Summary

Apolipoprotein (apo) E3Leiden is a dysfunctional apoE variant associated with familial dysbetalipoproteinemia in humans. Transgenic mice carrying the APOE3Leiden gene develop hyperlipidemia and are highly susceptible to diet-induced atherosclerosis. An early step in atherosclerosis is foam cell formation, which is thought to result from the unrestricted uptake of modified lipoproteins by macrophages. To investigate the role of the macrophage scavenger receptor type I and II (SR-A) in this process, APOE3Leiden transgenic mice were crossed onto a SR-A deficient background and the development of atherosclerosis was examined. In view of recent results with apoE deficient mice (Suzuki *et al.* Nature 1997; 386; 292-296), absence of the SR-A in APOE3Leiden mice was expected to lead to a reduction of atherosclerosis. In our study we compared APOE3Leiden / SR-A deficient mice (E3L SR-A$^{-/-}$) to APOE3Leiden / SR-A wild-type mice (E3L SR-A$^{+/+}$). These animals were fed an atherogenic diet for 10 weeks. Quantification of the lesion area showed no significant difference between E3L SR-A$^{-/-}$ and E3L SR-A$^{+/+}$ mice although there was a trend towards the development of larger lesions in the E3L SR-A$^{-/-}$ mice. All lesions were typed according to their cellular composition. In both male and female E3L SR-A$^{-/-}$ mice, significantly more severe lesions developed as compared to E3L SR-A$^{+/+}$ mice. These results indicate that the effect of SR-A deficiency on atherogenesis may depend on the presence or absence of apoE.

Introduction

ApoE is one of the structural components of very low density lipoproteins (VLDL) and serves as a ligand in the uptake of these particles from the circulation by the liver. Mutations in apoE may lead to an accumulation of remnant lipoproteins in the circulation and cause Familial Dysbetalipoproteinemia (FD). The previously described APOE3Leiden mutation is associated with a dominant inheritance of FD (1,2). Transgenic mice carrying the APOE3Leiden gene are hyperlipidemic and highly susceptible to diet induced atherosclerosis(3,4).

An early step in atherogenesis is the formation of foam cells in the vessel wall. These foam cells are lipid loaden macrophages and are thought to be the result of the unrestricted uptake of modified lipoproteins by scavenger receptors (5-7). The first identified and best characterized scavenger receptor, thusfar, is the macrophage scavenger receptor class A type I and II (SR-A) (8-10). The SR-A is a trimeric membrane glycoprotein which binds a broad range of negatively charged ligands, including oxidized low density lipoproteins (oxLDL). Recently, evidence is accumulating for an important role of the SR-A in the atherosclerotic process (11-15). Expression of SR-A in CHO cells was shown to result in cholesterol accumulation

after incubation with modified lipoproteins (16). This uptake leads to the formation of foamy cells similar to the foam cells found in atherosclerotic plaques. Additionally, high intracellular levels of oxidized lipoproteins have been demonstrated in foam cells in atherosclerotic plaques (17). Recently, a mouse model deficient for SR-A has been generated (15). With these mice, it was shown that on an apoE deficient background, absence of SR-A leads to a reduction in plaque size.

To study the role of the SR-A in an atherosclerosis model where apoE is present we used the apoE3Leiden mouse model. Atherosclerosis in this model has been extensively studied (4,18). In this mouse model and other transgenic models, the atherosclerotic process occurs by typical subsequent stages of development similar to those described extensively for humans (19). We studied these processes in APOE3Leiden in the presence or absence of the SR-A (E3L SR-A$^{+/+}$ vs. E3L SR-A$^{-/-}$). Surprisingly, after the mice had been fed an atherogenic diet, in E3L SR-A$^{-/-}$ mice, we found no reduction in lesion area as compared to E3L SR-A$^{+/+}$ mice. In contrast, in the absence of the SR-A there was a trend to an increase in lesion area and the lesions we observed were of a more complex nature.

Methods

Animals

Homozygous SR-A knockout mice ((129xICR)F_2 intercross) (15) were crossed with mice of line #2, carrying the human APOE3Leiden and the human APOC1 genes (crossed back on C57Bl6 for 11 generations) (20). Offspring from this cross was intercrossed to yield the mice used in these experiments. Experimental groups consisted of apoE3Leiden transgenic animals without the SR-A (E3L SR-A$^{-/-}$) and apoE3Leiden transgenic animals with the SR-A (E3L SR-A$^{+/+}$).

Male and female mice, 10 weeks of age, were put on a high fat, high cholesterol diet containing 1% cholesterol and 0.5% cholate diet (diet N (21), Hope Farms, Woerden, The Netherlands) for 10 weeks. After this period the mice were sacrificed for analysis.

Lipid and lipoprotein analysis

Blood samples were taken from the tail vein after a four-hour fast. Total serum cholesterol and triglyceride concentrations (without free glycerol) were measured enzymatically using commercial test kits 236691 from Boehringer Mannheim GmbH, Mannheim, Germany and test kit 337-B from Sigma, St Louis, USA, respectively.

Tissue preparation and sectioning of the aortic root.

Mice were sacrificed and the heart was removed, cutting the aorta just above the atria. The heart was bisected perpendicular to the heart axis, just below the atrial tips. The base of the heart was taken for analysis and quick-frozen perpendicular on a piece of liver in liquid nitrogen. For histochemistry, the heart was sectioned on a cryostat, starting within the heart and working in the direction of the aortic arch as described by Paigen *et al.* (21). Once the atrioventricular valves were identified and the media was visible, 8 μm sections were taken and mounted on gelatinized slides. Sections were collected until the valves disappeared. For oil red O (lipid) staining 6 sections with an 80 μm interval were taken. For toluidine blue and acid phosphatase (macrophages) and for immunohistochemistry 3 sections with a 180 μm interval were used.

Quantification of the lesion area

For atherosclerosis quantification, six oil red O stained sections with an 80 μm interval were used. Using computer-aided morphometry (Kontron-Videoplan, Zeiss, Germany) total area of the lesions was measured. From the six sections the average lesion area per section was calculated.

Immunohistochemistry and characterization of the lesions

Cryostat sections of the aortic root were fixed in acetone for 10 minutes and air-dried for at least 30 minutes. After washing in 10 mM phosphate buffered saline (PBS, pH 7.4) the sections were incubated with a primary antibody, either MOMA-2 (22) for macrophages or ERTR-7 (23) for fibroblasts. This was followed by a peroxidase-conjugated second step antibody for 30 minutes. After washing again, the peroxidase actvity was demonstrated with 3,3'-diaminobenzidine-tetrahydrochloride (Sigma). Negative controls included omission of the primary antibody. One slide was stained with toluidine blue after acetone fixation and one slide was stained with oil red O without acetone fixation. All lesions, were characterized by one investigator, who was blinded to the presence or absence of the SR-A, using toluidine blue, oil red O, MOMA-2 and ERTR-7 stained sections. Characterization was performed as described previously (4) with some modifications. Briefly, lesions were typed, in three catagories. The first is the fatty streak, consisting only of foam cells. The second category, is the mild plaque with foam cells and mild fibrosis. In the third category, the severe plaque, the media is involved and the plaque consists of foam cells, cholesterol clefts, necrosis and calcification. For the three types of lesions the frequencies in the groups were calculated.

Statistics

All values are presented as means ± S.D. Statistical significance was tested using the t-test for lesion areas and the χ^2 test for frequency distribution of the categorization of the lesions.

RESULTS

Serum lipid and apoE3Leiden levels

APOE3Leiden mice (line #2) were crossed with SR-A deficient mice to generate E3L SR-A$^{+/+}$ and E3L SR-A$^{-/-}$ mice. After ten weeks of the atherogenic diet all animals were hypercholesterolemic (Table 1). In the female mice, plasma levels of cholesterol, triglycerides, apoE and body-weight were similar in the E3L SR-A$^{-/-}$ and E3L SR-A$^{+/+}$ groups. These values are similar to data previously obtained from apoE3Leiden mice (4). In the male mice, plasma levels of triglycerides and apoE and weight were the same. Unexpectedly, plasma cholesterol levels in male mice were lower in the E3L SR-A$^{-/-}$ as compared to the E3L MSR$^{+/+}$ group (Table 1).

Table I: Serum lipid, human apoE levels and body-weights after 10 weeks dietary treatment

	Female		*male*	
	E3L SR-A$^{+/+}$	E3L SR-A$^{-/-}$	E3L SR-A$^{+/+}$	E3L SR-A$^{-/-}$
cholesterol (mM)	40.7±13.3	41.1±8.7	37.7±28.3	19.1±4.3
triglycerides (mM)	1.37±1.2	1.42±1.5	2.53±2.9	1.65±1.2
apoE3Leiden(mg/dl)	42±13	41±11	36±18	26±12
body-weight (g)	28±3	28±3	35±4	37±6
n =	10	12	8	8

Quantification of lesion area

In all four groups, atherosclerosis was quantified in the aortic root area. Surprisingly, both male and female APOE3Leiden mice show an increased lesion size in the absence of the SR-A (fig.1). In male E3L SR-A$^{+/+}$ and E3L SR-A$^{-/-}$ mice, the lesion area was 44,000 ± 25,000 μm^2 and 60,000 ± 47,000 μm^2, respectively. In female mice, lesion size was larger then in male mice. Lesion areas were 72,000 ± 46,000 μm^2 in E3L SR-A$^{+/+}$ mice and 134,000 ± 87,000 μm^2 in E3L SR-A$^{-/-}$ mice. Due to the large standard deviations, the differences between SR-A$^{-/-}$ and SR-A$^{+/+}$ mice in both male and female groups did not reach statistical significance.

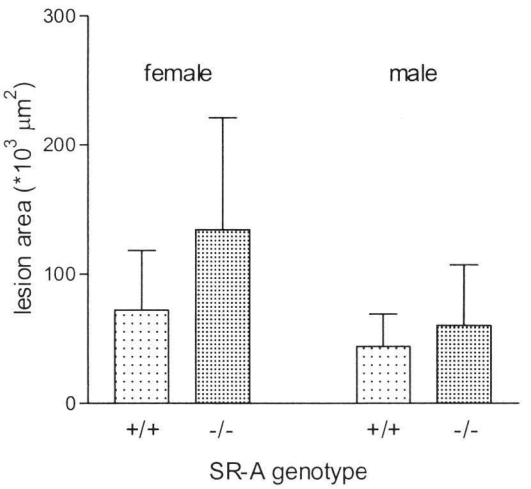

Figure 1: Mean lesion area (± S.D.) in female and male apoE3Leiden mice with (+/+, open bars) or without (-/-, hatched bars) SR-A.
Difference between groups did not reach statistical significance (p=0.47 for male mice and p=0.057 for female mice).

Histological characterisation of the lesions

To further investigate the atherosclerotic lesions in the mice, sections from the aortic root were examined histologically. Lesions were typed into three categories, adapted from a method described previously (4). Examples of the three categories are shown in figure 2. All three categories were present in all four groups but with different frequencies. To compare the types of lesions found in the mice, the number of each type of lesions was counted and presented as a percentage of the total number of lesions found in a group of mice (Fig. 3). In male E3L SR-A$^{+/+}$ mice 80% of the lesions, were fatty streaks, consisting only of foam cells. In contrast, in the male E3L SR-A$^{-/-}$ group there was a clear shift towards developing more severe lesions. Here, the most commonly found lesion was the mild plaque (53%) characterized by the presence of a fibrotic cap on the foam cells while only 35% of the lesions were fatty streaks.

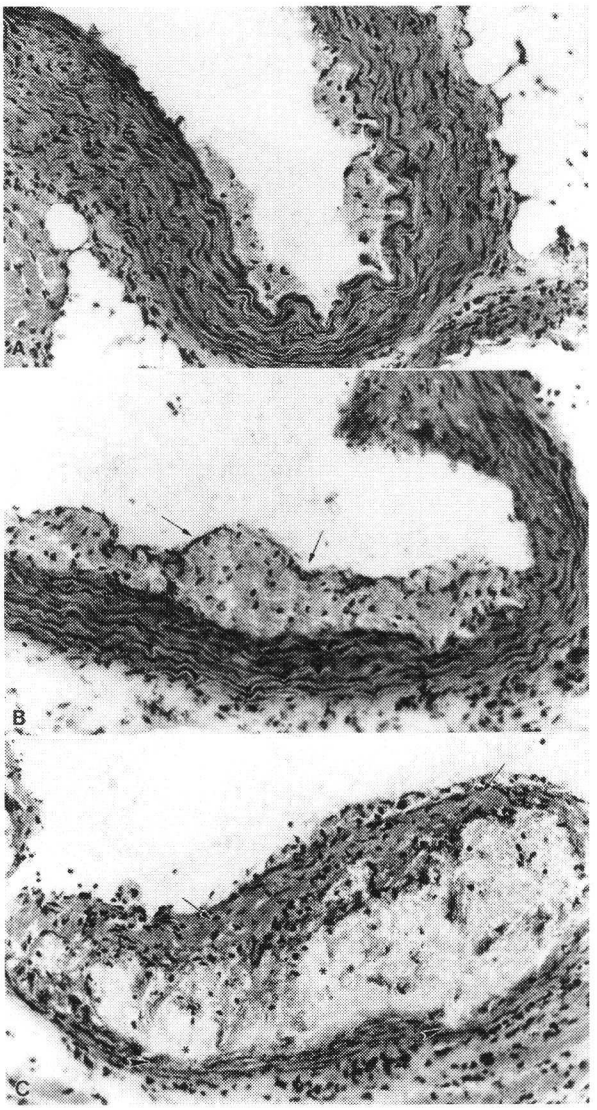

Figure 2: Representative photographs of the three stages observed in the apoE3Leiden transgenic mice.

A. stage I: fatty streak composed of foam cells. B. stage II: mild plaque with fibrotic cap (arrow). C. stage III: severe plaque with fibrotic cap (arrow), media damage (arrowhead) and necrosis (asterisk).

In female mice the lesions found were more severe then in the male mice (Fig.3). In the female E3L SR-A$^{+/+}$ group the most abundant type of lesion was the mild plaque (59%), while only 11% of the lesions had progressed to severe plaques. In the female E3L SR-A$^{-/-}$ group there was a substantial increase in the number of severe plaques (34%), with extensive fibrosis, necrosis and intima damage.

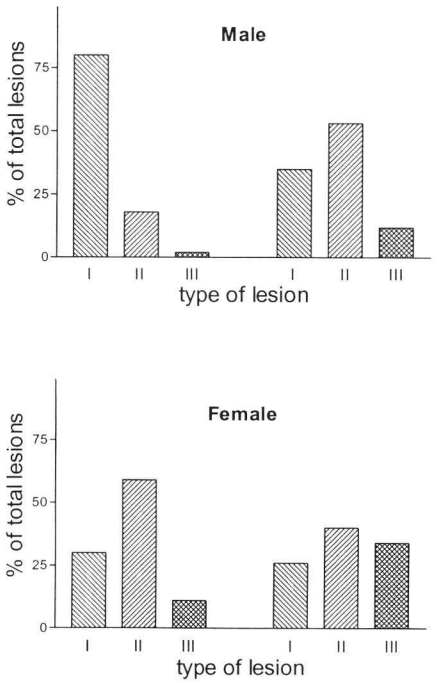

Figure 3: Lesion catagorization in female and male mice with (left, E3L SR-A$^{+/+}$) or without (right, E3L SR-A$^{-/-}$) SR-A.
All lesions were typed according to the catagories in figure 1 and expressed as a percentage of the total number of lesions found in a group. Lesion distribution differed between groups with $p<0.001$ for both male and female groups.

Discussion

In this paper, we describe the effects of SR-A deficiency on atherosclerosis in the APOE3Leiden mouse. This mouse model carries a dysfunctional variant of human APOE as a transgene, resulting in a high susceptibility to diet-induced atherosclerosis. Recently, it has been demonstrated that SR-A deficiency in the hyperlipidemic and atherosclerosis-prone apoE knock-out (E$^{-/-}$) mouse leads to a decrease in atherosclerosis as determined by lesion area (15). We quantified the progression of atherosclerosis both by measuring lesion area and by assigning lesion severity based on cellular composition. Surprisingly, there was no decrease in lesion area in E3L SR-A$^{-/-}$ as compared to E3L SR-A$^{+/+}$ mice. In contrast, there was even a trend towards increased lesion area in E3L SR-A$^{-/-}$ mice. In addition, there were significantly more severe lesions in E3L SR-A$^{-/-}$ mice. Thus, our data show that the effects of SR-A deficiency on APOE3Leiden mice are clearly different from the

effects of SR-A deficiency on apoE$^{-/-}$ mice.

Examination of the cellular composition of the lesions may reveal information that is not readily detected by lesion area measurements. Progression of lesions is not always accompanied by an increase in size. More advanced lesions often show changes in their cellular composition, such as an increase in fibrotic cells, calcification or involvement of the media. These changes are clear characteristics of progression but are not accompanied by an increase in lesion area. Hence, examination of the lesions on cellular composition will give additional information on the rate of progression of atherosclerosis.

The effect of SR-A deficiency on the development of atherosclerosis seems to be determined by the presence or absence of apoE. Evidence is accumulating for a role for apoE in several processes directly involved in atherogenesis. One of these processes may be mediating cholesterol efflux from the vessel wall. Experiments using a macrophage specific promoter (24) or an arterial wall specific promoter (25) to generate transgenic mice expressing apoE, showed that local expression of apoE can reduce atherosclerosis. Alternatively, transplantation of apoE$^{-/-}$ bone marrow to wild-type mice resulted in a 10 fold increase in atherosclerosis, without effecting serum cholesterol levels (26). This experiment shows that the absence of apoE from macrophages leads to enhanced foam cell formation. These data are all consistent with apoE mediating cholesterol efflux from the vessel wall. It is obvious that this process will be disturbed in the apoE deficient mice. However, since apoE is present in the macrophages of APOE3Leiden mice, cholesterol efflux is not likely to be affected in this mouse model.

A major role of apoE is to serve as a ligand for lipoprotein receptors which may be involved in cellular cholesterol accumulation, including the very low density lipoprotein receptor (VLDLR) and the low density lipoprotein receptor related protein (LRP). Both are expressed in lesions and can play an important role in foam cell formation (14,27). By removing the ligand for these receptors, as is the case in apoE deficient mice, the relative contribution of the SR-A to foam cell formation will increase. In the mouse model we have used, apoE is present. We have shown that lipoproteins from the APOE3Leiden mouse can still bind to the VLDLR (28) and the LRP (29) thereby serving as a route for foam cell formation. Thus, compared to the apoE deficient mouse, the APOE3leiden mouse has additional pathways available to mediate cellular cholesterol accumulation. Another difference between our experiments and the experiments performed with the apoE deficient mice is the use of cholate in the diet we used to mediate hypercholesterolemia. However, it is unclear how this addition would effect atherosclerosis. Considering the important role of apoE as mentioned above, we believe that apoE is the major atherosclerosis determining difference between both experiments.

Apparently, the cumulative result of a disturbed apoE mediated influx and efflux in apoE deficient mice leads to a pathological role for the SR-A in

atherogenesis. However, when apoE mediated influx and efflux are operational, such as in the APOE3Leiden mouse, the SR-A has a beneficial effect on atherogenesis. In line with this hypothesis, others have observed a decrease in atherosclerosis susceptibility at increased levels of SR-A expression in the rabbit (30-32). Thus, under normal conditions, the major function of the SR-A may be a beneficial one by scavenging modified lipoproteins from the vessel wall and thereby preventing damage as a consequence of these reactive lipoproteins. However, during hypercholesterolemia, the chronic supply of modified and unmodified lipoproteins will irreversibly lead to foam cell formation and atherosclerosis. Thus, in the presence of apoE, the SR-A will delay atherogenesis. In the absence of apoE, however, the SR-A may be the major route for foam cell formation. This process is aggravated by the absence of apoE mediated cholesterol efflux.

Atherosclerosis is the result of numerous different interacting processes. This complexity is clearly illustrated by the contrasting effects of SR-A deficiency on apoE-/- and APOE3leiden mice. To further elucidate the role of the SR-A in atherogenesis, we have generated additional mouse models over-expressing the SR-A gene in all natural tissues. These mice will be cross bred both with mice lacking apoE and mice transgenic for APOE3Leiden to gain additional insight in the interaction between apoE and the SR-A in atherogenesis.

ACKNOWLEDGEMENTS:

This research was supported by the Netherlands Organisation for Scientific Research (project #900-504-092). Marten H. Hofker is an established investigator of the Dutch Heart Foundation.

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CHAPTER 4

CENTROMERIC AND NONCENTROMERIC ADE2-SELECTABLE FRAGMENTATION VECTORS FOR YEAST ARTIFICIAL CHROMOSOMES IN AB1380.

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(*Genome Res.* 7 (1997); 6; 657-660)

Abstract

We have constructed a set of fragmentation vectors for the truncation of either the centromeric or the noncentromeric end of YACs containing a human DNA insert. These vectors carry ADE2 or HIS5 as the selectable marker, enabling direct use in AB1380, the host strain of most publicly available YAC libraries. Centromeric fragmentation vectors for AB1380 have not been reported previously; the noncentromeric vectors show high frequencies of fragmentation.

Introduction

Yeast artificial chromosomes (YACs) consist of a large (50-2500 kb) DNA insert, flanked on both sides by a yeast selectable marker and a telomere, and on one side by a yeast centromere. An important application of YACs lies in the mapping of the human genome and the isolation of human disease genes. YAC contigs now cover nearly the entire human genome (1), but the depth of these contigs is often not sufficient to allow high-resolution ordering of markers. Because many candidate regions for disease genes have been allocated to YAC contigs still comprising several megabases, improvement of the resolution of these contigs is highly desirable. More recent, complementary resources in contig generation are bacterial artificial chromosome (BAC) and P1-derived artificial chromosome (PAC) libraries. Although valuable in the ultimate generation of sequence-ready genomic regions, they preclude the delineation of the available YAC contig information in the megabase range, as the assembly of BAC/PAC contigs is a de novo endeavor. YAC fragmentation, that is, the creation of YACs with deletions from one end (2), is an attractive method for obtaining more refined mapping while optimally using existing knowledge. Compared to the common route of constructing cosmid contigs, YAC fragmentation is a simple and quick alternative. Furthermore, YAC fragmentation provides the possibility of discarding the noninteresting parts of a YAC, which is useful for subsequent studies like the production of well-defined restriction maps, subcloning, gene searches, and gene characterizations, and as an attractive tool to focus YAC-derived transgene studies.

YAC fragmentation is based on the presence of sequences in the YAC insert homologous to a part of the fragmentation vector, which further contains a selectable marker, a telomere, and a centromere if the centromeric arm is to be targeted. Common repeats like the long interspersed repetitive element (LINE) and, in particular, Alu are attractive candidates for this purpose, as they occur frequently in YACs with a human

insert. The series of fragmentation vectors described by Pavan et al. (3) enable the construction of YAC fragmentation panels in a his3 background. However, all publicly available YAC libraries, like the Centre d'Etude du Polymorphisme Humain (CEPH), Imperial Chemical Industries PLC (ICI), and Imperial Cancer Research Fund (ICRF) libraries (4-6), have been constructed in yeast strain AB1380, which is not his3. Therefore, YACs must first be transferred to a his3 background, through meiosis or kar1 transfer (7), before these vectors can be used. To bypass this problem, we have adapted existing fragmentation vectors with the ADE2 gene and/or CEN6, thereby creating a set of vectors that can be used directly in AB1380 to fragment YACs from both ends.

RESULTS

For fragmentation of YACs from libraries in AB1380 a vector with a suitable selectable marker is required. AB1380 has the genotype MATa ura3 trp1 ade2, his5, lys2-1, can1-100, allowing the use of ADE2, HIS5, or LYS2, as the URA3 and TRP1 genes are in use for selection of the YAC and the ile and thr mutations are not characterized. We chose the ADE2 gene and inserted it into the fragmentation vectors pBP108 and pBP109, containing the Alu target sequence in opposite orientations (3). The resulting plasmids pBP108/ADE and pBP109/ADE (Fig. 1A) were tested for their ability to create fragmentation panels on YACs from different parts of the human genome (see Table I and below). As a result of the fragmentation, YACs lose the URA3 gene while acquiring the ADE2 gene, which is selected for on media lacking adenine. Replica plating to media lacking uracil showed that the majority of the transformants (80%-97%) had the correct Ade+Ura phenotype, indicating a high recombination rate at the Alu repeats of the YAC. The remaining colonies had not become uracil dependent, which might be explained by integration of the pBP108(109)/ADE vectors elsewhere in the genome, by maintenance as a circular plasmid, or by the presence in some of the cells of two YACs, one original and one fragmented. Pulsed-field gel electrophoresis (PFGE) analysis of a fragmentation panel derived from YAC 939_h_7, showed that all Ade+Ura colonies contained correctly retrofitted YACs, ranging in size from ~150 to 2300 kb. Figure 2 shows a selection of fragmented YACs, ordered by size. The panel was analyzed with several rare-cutting restriction endonucleases and no inconsistencies were found (8).

Fragmentation vectors for centromeric fragmentation of YACs have been described (3, 9). However, as these vectors carry the HIS3 marker, they do not allow direct fragmentation of AB1380 YACs. For the construction of more convenient CEN fragmentation vectors, we cloned CEN6 into pBP108/ADE and pBP109/ADE (see

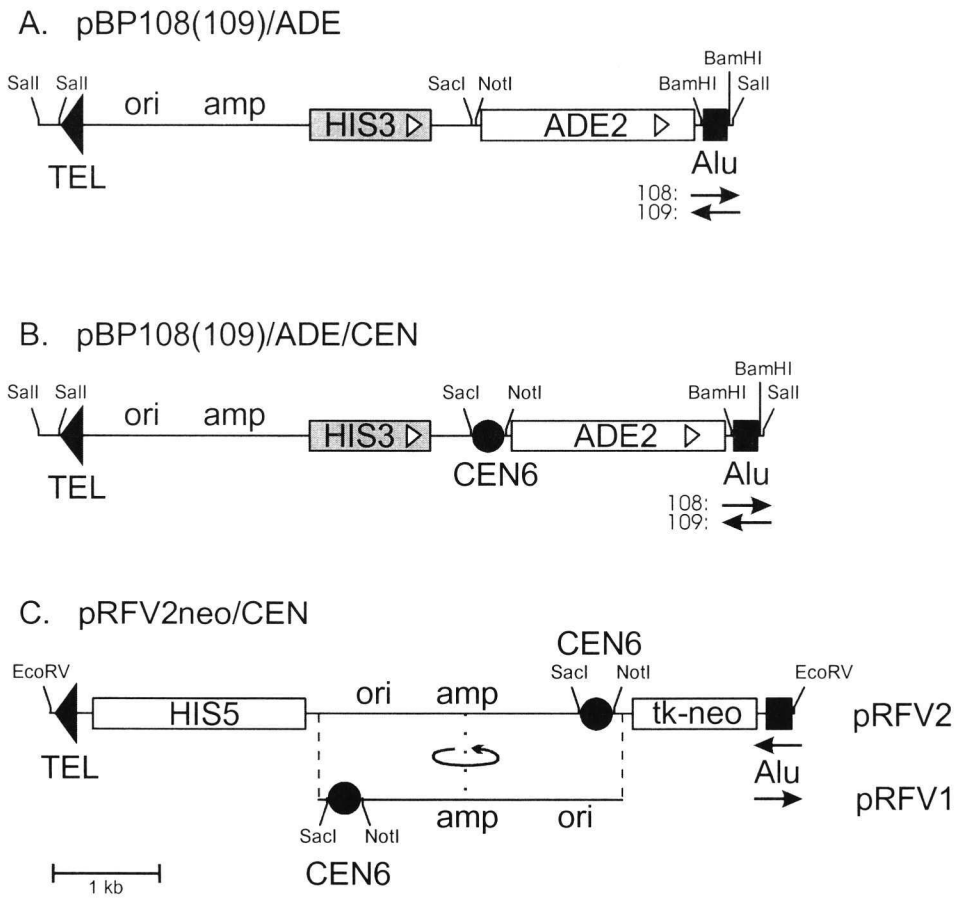

Figure 1: Schematic representation of the new fragmentation vectors, linearized between the Alu and telomere (TEL) fragments.

(CEN6) Centromere from yeast chromosome VI; (tk-neo) thymidine kinase promoter coupled to the neomycin gene conferring G418 resistance to mammalian cells. Relevant restriction sites are indicated. The open arrowheads within HIS3 and ADE2 boxes indicate the transcriptional direction.

above) as well as into pRFV2neo (10). The resulting CEN plasmids (Fig. 1B,C) were used to fragment two overlapping YACs, 766_a_12 (1050 kb) and 932_e_9 (1400 kb). The results indicate that both pBP108/ADE/CEN and pBP109/ADE/CEN truncate YACs efficiently: ~25%-40% of the colonies had lost the TRP1 gene from the centromeric YAC arm and had become shorter, judged by PFGE and Southern blotting (Table 1). pRFV2neo/CEN was also used successfully: All six fragmentation YACs tested had

become shorter and hybridized to a CEN6 probe as well as to an Alu probe (data not shown). If the ultimate aim of fragmentation is transferring the YAC into mammalian cells, this vector directly supplies a marker (neoR) for selection.

Table I. YAC Fragmentation Frequencies of pBP108 (109)/ADE, pBP108 (109)/ADE/CEN, and pRFV2neo/CEN.

Vector		YAC	No. of colonies[a]	No. of correct phenotype/no. tested[b]	No. truncated YACs/no. tested by PFGE
A	pBP108/ADE	939_h_7	200-300	29/30 (97%)	29/29 (100%)
	pBP109/ADE	939_h_7	200-300	31/37 (84%)	31/31 (100%)
	pBP108/ADE	960_a_5	21	20/21 (95%)	20/20 (100%)
	pBP109/ADE	960_a_5	43	21/23 (91%)	21/21 (100%)
B	pBP108/ADE/CEN	766_a_12	408[c]	47/100 (47%)	44/44 (100%)
	pBP109/ADE/CEN	766_a_12	481[c]	38/100 (38%)	32/32 (100%)
	pBP108/ADE/CEN	932_e_9	931[c]	26/100 (26%)	23/24 (96%)
	pBP109/ADE/CEN	932_e_9	940[c]	22/100 (22%)	08/09 (89%)
C	pRFV2neo/CEN	766_a_12	600-700	53/149 (36%)	06/06 (100%)

[a]Total number of colonies found on different spreads of the same transformation mixture per 5 µg of vector. [b]Number of correct phenotype: Ade+ Ura (A); Ade+ Trp (B); and His+ Trp (C). Number tested: Number of colonies transferred manually or by replica plating to a plate selecting for the targeted YAC arm; upon loss of the original YAC arm, colonies become Ura (A) or Trp (B and C). [c]16%-38% of the colonies were red; these were not analyzed further.

Compared to the HIS5 and LYS2 fragmentation vectors for the noncentromeric arm of YACs, pBP108/ADE2 and pBP109/ADE2 give a high percentage of correctly fragmented YACs [80%-97% vs. 21%-46% (10) and 27%-49% (11). However, no direct comparisons have been performed. Both vectors have been used successfully to obtain a detailed rare-cutter restriction map of a 2300-kb region on the X chromosome (8). Two other groups have also obtained truncated YACs using these plasmids (G. Williams and M. Cruts, unpubl.).

We believe that these vectors are a valuable addition to the existing sets of fragmentation vectors, allowing direct and efficient truncation from both ends of YACs directly in AB1380, thus alleviating the need of an additional time-consuming step, that is, swapping the YACs through meiosis or kar1 transfer to another genetic background. All vectors are available upon a mailed request to J. den Dunnen.

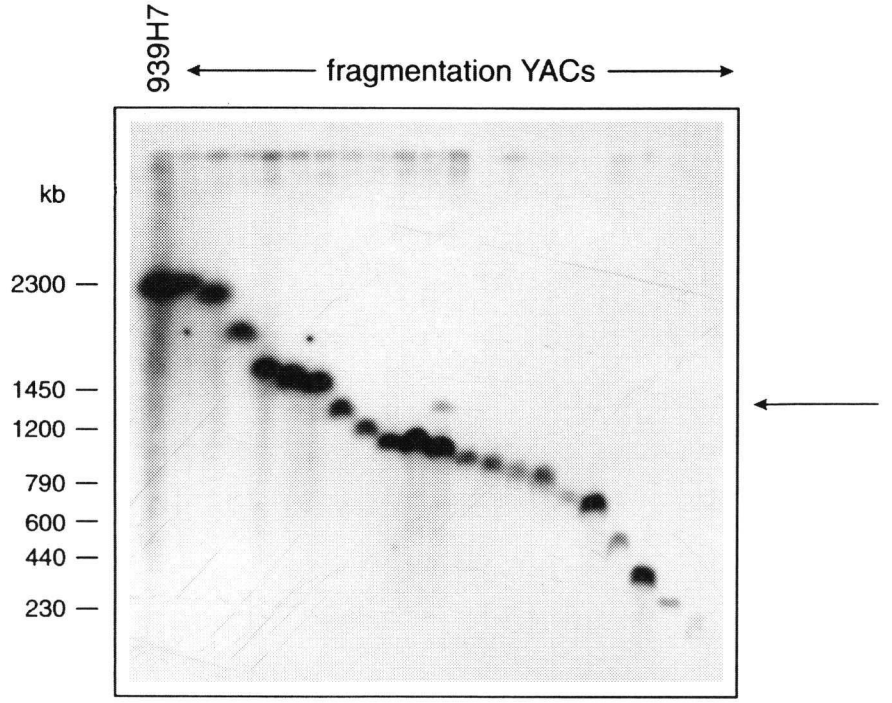

Figure 2: YAC fragmentation panel of YAC 939_h_7 made using pBP108/ADE2 and pBP109/ADE2. Fragmentation YACs were ordered by size (deduced from an initial gel) and separated on a PFGE gel, which was blotted and probed with a 270-bp Alu fragment (isolated from pBP108 by BamHI digestion). The conditions used were 0.6% SeaKem Gold agarose, 1× PFG buffer (0.045 M Tris, 0.045 M boric acid, 0.2 mM EDTA at pH 8.3), for 44 hr, at 100 V with 20-500 sec pulse time. The extra band indicated by the arrow may be the result of an impure YAC clone.

METHODS

Vector Constructions

Using partially filled-in fragments, a 2.0-kb BglII fragment from pASZ11 (12), containing the ADE2 gene, was cloned into the XbaI site of pBP108 and pBP109 (3). Both ADE2 transcriptional orientations were obtained. The initial fragmentation experiments showed that the vectors in which ADE2 transcription is in the direction of the Alu fragment gave slightly better results and were therefore used in the fragmentation experiments and for the subsequent addition of CEN6. For the construction of pBP108(109)/ADE/CEN, CEN6 was amplified by PCR from pLA433 (13), using the following primers: 5-

AAGGAAAAAAGCGGCCGCGTTATGGAACCTGTCG-3 and 5-GGAAGGAAGAGCTCTTTCGTGTCGGTCGTCC-3 (NotI and SacI sites are underlined), and cloned between the NotI and SacI sites of pBP108(109)/ADE and pRFV2neo (10).

Yeast Transformation and Analysis of Fragmented YACs

The Alkali Cation Kit of BIO101 was used for all fragmentation experiments. Before transformation, vectors were linearized with SalI (the pBP derivatives) or EcoRV (pRFV2neo/CEN). YACs were grown in SD+ medium [synthetic dextrose media containing 8 grams/liter of Bacto yeast nitrogen base (YNB), 20 grams/liter of dextrose, 55 mg/liter of adenine, 55 mg/liter of tyrosine, 14 grams/liter of Bacto casaminoacids]. After transformation, cells were plated on SD media [SD without casaminoacids, but supplemented with arginine (20 mg/liter), methionine (20 mg/liter), isoleucine (30 mg/liter), phenylalanine (50 mg/liter), leucine (60 mg/liter), valine (150 mg/liter), histidine (20 mg/liter), tryptophan (20 mg/liter), uracil (20 mg/liter), lysine (30 mg/liter)]. For the pBP vectors, cells were spread on SD plates lacking adenine and tryptophan (when truncating from the noncentromeric end of the YAC) or adenine and uracil (truncating from the centromeric end), whereas for pRFV2neo/CEN SD plates lacking histidine and uracil were used. After 3-4 days at 30°C, colonies were either manually transferred or replica plated to SD plates lacking uracil or tryptophan (for fragmentations of the URA3 and TRP1 arm, respectively) to screen out the false-positive colonies resulting from integration in the yeast genome or circular maintenance of the fragmentation plasmid. Agarose plugs were made from colonies with the correct genotype and subsequently analysed by pulsed field gel electrophoresis.

ACKNOWLEDGEMENTS

We thank Dr. S. Pestka for kindly providing pRFV2neo and Dr. P. Philippsen for pLA433.

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CHAPTER 5

MACROPHAGE SPECIFIC OVEREXPRESSION OF THE HUMAN MACROPHAGE SCAVENGER RECEPTOR IN TRANSGENIC MICE, USING A 180 KB YEAST ARTIFICIAL CHROMOSOME, LEADS TO ENHANCED FOAM CELL FORMATION OF ISOLATED PERITONEAL MACROPHAGES.

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(*Atherosclerosis, in press*)

Summary

Macrophage scavenger receptors class A (MSR) are thought to play an important role in atherogenesis by mediating the unrestricted uptake of modified lipoproteins by macrophages in the vessel wall leading to foam cell formation. To investigate the *in vivo* role of the MSR in this process, a transgenic mouse model expressing both isoforms of the human MSR was generated. A 180 kb yeast artificial chromosome (YAC) containing the human MSR gene (MSR1) with 60 kb and 40 kb flanking sequence at the 5' and 3' end, respectively, was obtained by reducing the size of a 1050 kb YAC by homologous recombination. This 180 kb YAC was microinjected into mouse oocytes. In the resulting transgenic mice, high levels of mRNA for both type I and type II human MSR1 were detected in peritoneal macrophages and trace levels in other organs, known to contain macrophage-derived cells. Using an antibody against the human MSR, the Kupffer cells in the liver were shown to contain the MSR protein. *In vivo* clearance of acetyl-LDL was not changed in the MSR1-transgenic mice. However, *in vitro* studies using peritoneal macrophages from the transgenic mice showed a two-fold increased degradation of acetyl-LDL and cholesterolester accumulation concomitant with a four-fold increase in foam cell formation, as compared to wild-type macrophages. Thus, macrophage specific overexpression of the MSR may lead to increased foam cell formation which is one of the initial and crucial steps in atherogenesis.

Introduction

The formation of foam cells in the subendothelial space of the vessel wall is a hallmark of early atherogenesis. These foam cells are macrophages characterized by massive accumulation of cholesterol esters (1). They are thought to result from the unrestricted uptake of oxidatively modified lipoproteins, such as oxidized LDL, through scavenger receptors (2-4).

The first scavenger receptor which was cloned and characterized, was the macrophage scavenger receptor class A (MSR). The MSR is a trimeric membrane glycoprotein consisting of six structural domains including the collagen-like domain, which is involved in ligand binding (5). Two isoforms, type I and type II, are produced from a single gene through alternative splicing of mRNA (6,7). In the type II MSR, the 110 amino acid long C-terminal cysteine-rich domain of type I is replaced by a C-terminus of 6 residues (6,8). The human MSR gene (MSR1) is located on chromosome 8, band p22 (6) and spans about 80 kb.

Recently, a mouse model has been generated which is deficient in the MSR (9). On an apolipoprotein (apo) E-deficient background these mice showed a 60% reduction in the development of atherosclerosis. Interestingly, these mice also

showed an increased susceptibility to infection with *Listeria monocytogenes* or herpes simplex virus type-1 (9). Additional *in vitro* experiments demonstrate that the MSR can be involved in cell adhesion (10). These results predict a major role for the MSR in both atherosclerosis and host defense.

In humans, the MSR1 may be important in the same processes. Individuals with increased levels of MSR activity have been described (11). Despite the fact that these patients are normolipidemic, they show planar xanthomas which are formed by foam cells. Macrophages from these patients have a changed phenotype, characterized by an increased cell adhesion and maturation. Moreover, it has been shown that within the human population the activity of the MSR can vary considerably (12). These observations predict that the MSR may play a major role in atherosclerosis in humans.

To explore these observations in an animal model, we generated MSR overexpressing mice. In these mice, the MSR1 gene is expressed in a macrophage specific pattern. *In vitro*, macrophages from these MSR1-transgenic mice, show a two-fold increase in MSR activity as compared to the wild-type situation, leading to increased cholesterol ester accumulation and increased foam cell formation after incubation with acetyl-LDL.

Experimental Procedures

Generation of the YAC transgenic mice

To generate transgenic mice carrying the human MSR gene, yeast artificial chromosome (YAC) clone 766_a_12 was obtained from the Centre d'Etude Polymorphisme Humaine (CEPH) library (13). This YAC was 1050 kb in size and southern blot analysis using several restriction enzymes proved the MSR gene region to be present, intact and of the expected size (6). The MSR gene was located near the non-centromeric YAC arm (Fig. 1a). To decrease the chance of introducing, in addition to the MSR1, other genes located on the 1050 kb YAC, the size of the YAC was reduced using vector arm replacement by recombination with human DNA specific repetitive sequences (Alu-repeats). This fragmentation was done from the vector arm carrying the centromeric sequences (CEN4) as described earlier (14) using the centromeric fragmentation vector pBP108/ADE/CEN. Resulting YACs were analyzed by pulse field gel electrophoresis (PFGE). Yeast DNA was run through a 0.6 % Seakem Gold (FMC Bioproducts, Rockland, ME) agarose gel in 0.5 x TBE for 44 h, at 100 V with 20-100 sec pulse time, at 4°C. DNA was blotted to Hybond N+ (Amersham, UK) and hybridized with an Alu-type repetitive sequence probe to determine the size of the YACs using the yeast chromosomes as size markers. The fragmentation yielded many different-sized YACs (Fig. 1b), ranging in size from slightly smaller than the original size of 1050 kb to the smallest YAC being 80 kb. Southern analysis of a 180 kb YAC revealed the presence of the complete MSR1 gene (80 kb) located almost in the middle of the YAC with 60 kb 5' and 40 kb 3' flanking sequence (Fig. 1c). This YAC, designated yHSR1, was expected to contain all regulatory elements required for a natural *in vivo* expression pattern in the mouse.

For gel purification of yHSR1 YAC DNA, agarose plugs were made at a final concentration of $2.5 * 10^9$ cells / ml using the method described previously (15). Thirty plugs with in total $7.5 * 10^9$ yeast cells were run on a 0.6% Seakem Gold agarose gel in 0.5 x TBE buffer for 43 hours, at 100 V with 10-

50 sec pulse time at 4°C. Strips of 1 cm wide were cut from each side of the preparative gel and stained with ethidium bromide to locate the YAC position in the gel. Using the stained parts as indicators, a slice 0.5 cm wide, containing the YAC was cut from the unstained part of the gel and equilibrated in 1 x TAE for 2 x 30 min. In addition a gel slice of the 220 kb yeast chromosome band was used. Both slices were embedded, turned 90° into a regular 0.5 % multipurpose agarose gel in 1 x TAE. A cutout 1 cm^2 hole in front of the slices was filled with 4% NuSieve GTG (FMC Bioproducts) in 1 x TAE. This gel was run overnight at 50 V at 4°C. The yeast chromosome containing part of the gel was removed and stained with ethidium bromide to check for DNA migration. The analogous part of the YAC lane was cut from the 4% NuSieve GTG gel and equilibrated in agarase buffer (0.2 mM EDTA, 7.5 mM TrisCl pH 6.5, 100 mM NaCl) at 50°C for 1 hour. The agarose slice was placed in a 1.5 ml Eppendorf tube and heated to 70°C for 20 min. The melted agarose sample was equilibrated at 45°C for 10 min and 5 ml of Gelase (0.2 U/µl, Epicentre, Madison, WI) was added. The agarose was digested at 45°C for 1 hour, 3 ml of Gelase was added and digestion continued for another hour. Next, the sample was placed on ice and was dialyzed (dialysis filter Spectra/Por MWCO: 3,500; Spectrum Medical Industries Inc., Houston, TX) overnight against injection buffer (0.2 mM EDTA, 7.5 mM TrisCl pH 7.5, 100 mM NaCl) at 4°C. The sample was spun for 5' at 13.000 rpm to remove remaining debris, supernatant was removed and 10 µl was run on a PFGE to check the integrity and concentration of the YAC preparation. The DNA was diluted to 2 ng/ml and used to generate transgenic mice according to standard procedures (16).

Analysis of transgenic off-spring

DNA isolated from the tail tip from 14 possible founders were tested by PCR using four sets of primers. Sequences were: set1 (centromeric YAC arm) 5'-AAGGAAAAAAGCGGC-CGCGTTATGGAACCTGTCG-3' and 5'-GGAAGGAAGAGCTCTTTCGTGTCGGTCGTCC-3' ; set2 (non-centromeric YAC arm) 5'-CTTGAGATCGGGCGTTCGACTCGC-3' and 5'-CCGCACCTGTGGCGCCGGTGATGC-3'; set3 (MSR1 promoter) 5'-CTTTGCATCTCTCAAAGACCG-3' and 5'-TTAGCCTCAAACACAAAACACG-3'; set4 (3'end of MSR1) 5'-GAAGATGCTGGAGTCACTTGC-3' and 5'-TGGAGCCAATTACTGGTATGC-3', all forward and reverse primers, respectively (Fig. 1c). Two founder mice were shown to be positive for all four PCRs. These mice (MSR1-transgenic mice) were bred to generate two transgenic lines (line 2 and 3). Mice backcrossed 2 times were used for further analysis

Macrophage isolation

One ml of 4% thioglycolate was injected intraperitoneally in YAC transgenic and nontransgenic littermates. After four days, the peritoneum was flushed with 8 ml of ice-cold PBS.

RNA analysis

Total RNA was isolated from thioglycolate-elicited macrophages, liver, kidney, spleen, heart, lungs, thymus and brain using the RNA Instapure System (Eurogentec s.a., Seraing, Belgium). To study the induction of MSR1 *in vitro,* RNA was isolated from resident or thioglycolate-elicited macrophages or thioglycolate-elicited macrophages cultured with and without phorbol 12-myristate 13-acetate (PMA) or acetyl-LDL for 20 h. RNA samples (10 µg per lane) were separated by electrophoresis through a denaturing agarose gel (1 % w/v) containing 7.5% formaldehyde and transferred to Hybond N+ according to manufacturer's recommendations. Blots were subsequently hybridized with a ^{32}P-labeled probe of human MSR cDNA (exons 4-6) and mouse 18S rRNA at 52°C in a solution containing 50% formaldehyde. The signal was visualized with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Immunohistochemistry

Organs from YAC transgenic and nontransgenic littermates were obtained and fixed using 2%

PLP (2% paraformaldehyde, 0.05 M lysine and 0.075 M periodate) for 20 hours. After fixation, samples were embedded in paraffin and 4 μm sections were cut. Sections were stained for human MSR using the Histomouse-SP Kit (Zymed, San Francisco, CA) with polyclonal antiserum (αhMSR), which was a generous gift from Dr. T. Kodama and Dr. M. Honda (both from Tokyo University). The first antibody incubation was carried out overnight at 4°C. Other incubations were performed according to the Histomouse-SP Kit manual. Negative controls consisted of incubation with pre-immune serum as first antibody step or the omission of the first antibody.

LDL isolation and modification

Human low density lipoprotein (LDL) was isolated by ultracentrifugation as described previously (17). LDL was acetylated with acetic anhydride (18) and checked by agarose gel electrophoresis (Paragon Lipoprotein Electrophoresis kit, Beckman Instruments). Acetyl-LDL was radiolabeled with ^{125}I by the iodine monochloride method (19).

Macrophage culture

Isolated peritoneal macrophages were washed with medium A (DMEM supplemented with 10% fetal calf serum, 50 units/ml penicillin and 50 mg/ml streptomycin), counted and plated at 0.5 x 10^6 cells/well in 24-well culture plates. After 2 hours, the cells were washed with medium A to remove nonadherent cells. To study MSR1 induction thioglycolate-elicited macrophages were cultured for 20 h in medium A containing PMA (100 nM) or AcLDL (20 μg/ml).

In vitro degradation of acetyl-LDL

Degradation of ^{125}I labeled acetyl-LDL was determined after 3 hour incubation at 37°C with 0-100 μg/ml of ^{125}I-labeled acetyl-LDL either in the presence or absence of 20 times excess of unlabeled acetyl-LDL, as described previously (20).

In vivo clearance of acetyl-LDL

The *in vivo* clearance of acetyl-LDL was studied under saturated conditions. Three YAC transgenic mice and three nontransgenic littermates were injected intravenously with 200 μl PBS containing 200 μg acetyl-LDL (consisting of 1 * 10^5 cpm ^{125}I labeled acetyl-LDL) and 0.1% bovine serum albumin. At 1, 2, 4, 6, 8 and 10 min. after injection, 50 μl blood samples were taken from the tail using heparinized microcappilary tubes. Radioactivity was determined in 10 μl serum samples. After 10 min., the mice were sacrificed and organs were removed and analyzed for radioactivity. Clearance was calculated as a percentage of the injected dose using a plasma volume of 0.045% of the body weight.

In vitro foam cell formation

Freshly isolated peritoneal macrophages were cultured on coverslips for 20 hours with various concentrations of acetyl-LDL. Subsequently, cells were fixed with 4% formaldehyde, stained with oil red O and counterstained with hematoxilin. From three areas of the coverslips, a minimum of 200 cells were counted and designated foam cell or non-foam cell, judged by their oil red O stain. Foam cell formation was calculated as a percentage of the total number of cells. To examine the cholesterol ester accumulation, macrophages (1 * 10^6 cells/well) were plated on 6-wells plates and incubated overnight without or with 25 μg/ml AcLDL after which the cellular lipid accumulation was examined as described previously (21).

RESULTS

Generation of YAC transgenic mice and expression analysis

To generate transgenic mice overexpressing the human MSR1 gene (22), YAC yHSR1 was generated as described in experimental procedures and used for oocyte injection. Two transgenic mice were obtained (line 2 and line 3). From these mice various tissues were examined by Northern blotting for expression of the MSR1 gene using a cDNA probe spanning exons 4-6 of the human MSR1 gene (Fig. 2). The highest expression was observed in thioglycolate-elicited peritoneal macrophages. The observed transcripts were 4 kb and 3.2 kb in size, representing mRNA for type I and type II MSR, respectively (23). The type II transcript was the most prominent one. In addition, several other organs showed low expression. The wild-type mice showed no signal with this probe. Of the two lines generated, one showed low expression of the human MSR1 and the other showed high expression of the human MSR1 (data not shown). The reason for this difference was not investigated. All further experiments described in this paper were performed using the high expressor mouse (line 3). To study the expression of MSR1 in response to different stimuli, resident and thioglycolate-elicited peritoneal macrophages were isolated and cultured for 20 h in the presence of PMA or AcLDL (Fig. 2b). Resident peritoneal macrophages showed very low expression of MSR1 as compared to thioglycolate-elicited macrophages. Culturing thioglycolate-elicited macrophages withhout stimulus did not change MSR1 expression. However, expression was increased by either PMA and AcLDL.

Demonstration of the human MSR protein in tissue macrophages

Kupffer cells are macrophages present in the liver, expressing moderate levels of MSR (24,25). To demonstrate the human MSR protein in the Kupffer cells of MSR1-transgenic mice, liver sections were stained using a polyclonal anti-human MSR antibody (αhMSR). Kupffer cells in livers from yHSR1 transgenic mice showed clear positive staining with αhMSR (Fig. 3a), whereas livers from wild-type mice showed no staining (Fig. 3b). Incubation of liver sections from MSR1-transgenic mice with pre-immune serum or with omission of the first antibody-step gave no signal (data not shown), providing additional evidence that the signal for the human MSR was specific. Macrophage specific expression was also confirmed by showing expression in perivascular macrophages (Mato cells) in the choroid plexus (Fig. 3c) and in macrophages in the renal capsule (Fig. 3d).

In vitro degradation of acetyl-LDL

Acetyl-LDL is a specific high affinity ligand for the MSR (26). Degradation of acetyl-LDL *in vitro* was analyzed using thioglycolate-elicited peritoneal macrophages from MSR1-transgenic mice and non-transgenic littermates. Saturation curves were

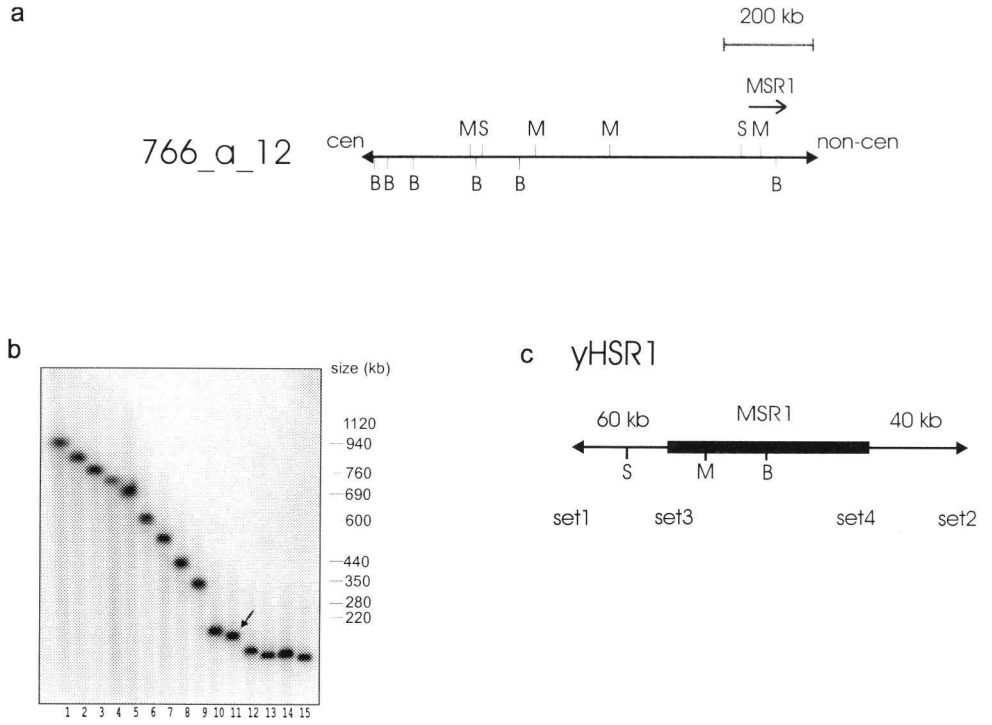


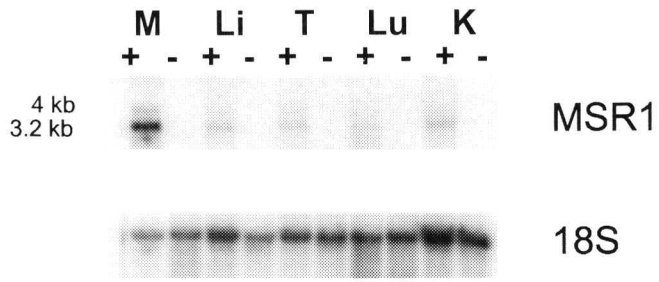
Fig.1: Generation of YAC yHSR1.
A restriction map of YAC 766_a_12 was made using Mlu I (M), BssH II (B) and Sfi I (S). The centromeric (CEN) and non-centromeric (NON-CEN) vector arms are indicated (a). Several YACs resulting from the fragmentation of YAC 766_a_12 were ordered to size, electrophorized, blotted and hybridized with an alu probe. Lane 1 contains the original YAC 766_a_12, lane 11 contains YAC yHSR1 (arrow). Sizes indicated are yeast chromosomes in kilobases (kb)(b). The resulting YAC yHSR1 contains the MSR1 gene with 60 kb 5' flanking and 40 kb 3' flanking sequence. Also indicated are the locations of the primer sets used for transgene confirmation (c).

generated using increasing concentrations of ^{125}I-labeled acetyl-LDL. Maximum receptor-mediated degradation of acetyl-LDL by macrophages from MSR1-transgenic mice was shown to be almost twice as high as compared to the wild-type macrophages (Fig. 4).

In vivo clearance of acetyl-LDL

No difference was observed in the clearance rate of acetyl-LDL between MSR1-transgenic mice and non-transgenic littermates (Fig. 5). After 10 minutes, the mice were sacrificed and the tissue distribution of the label was determined. At that time, 65% of the label had disappeared from the blood and no difference in label distribution in both groups was observed (data not shown).

a.



b.

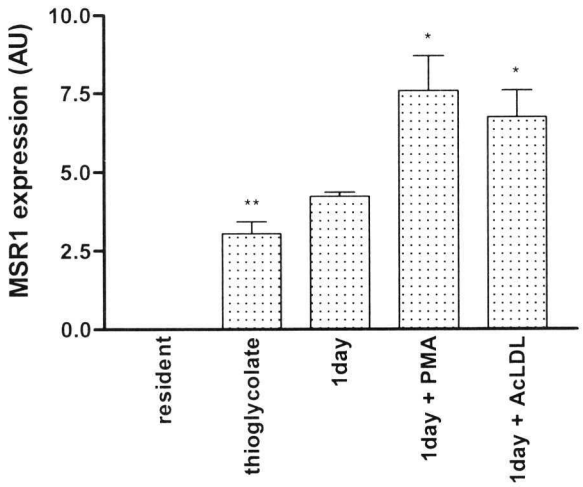


Fig. 2: Expression of MSR1.
(a) Expression of MSR1 in MSR1-transgenic mice (+) and nontransgenic littermates (-) was analyzed. Northern analysis was performed on 10 μg of total RNA from thioglycolate elicited peritoneal macrophages (M), liver (Li), thymus (T), lungs (Lu) and kidney (K) using probes for the human MSR1 mRNA (MSR1) and mouse 18S rRNA (18S). Sizes were determined using an RNA ladder. (b) Expression of MSR1 in freshly isolated resident and thioglycolate-elicited peritoneal macrophages (left two bars) and in thioglycolate-elicited peritoneal macrophages cultured without addition (1 day), with PMA (100 nM)(1day + PMA) or acetyl-LDL (20 μg/ml)(1day + AcLDL) for 20 h. (** $p<0.01$ difference from resident macrophages, * $p<0.05$ difference from 1 day untreated, students t-test)

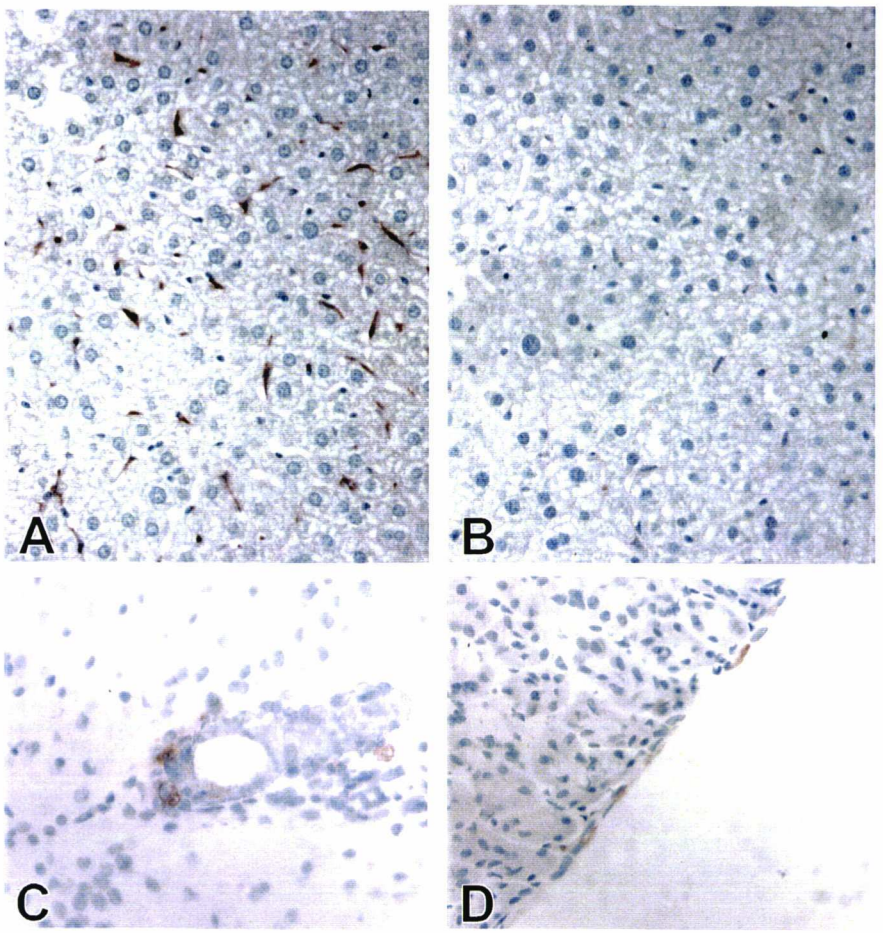


Fig. 3: Demonstration of human MSR in tissue macrophages of MSR1-transgenic mice.
Liver sections from MSR1-transgenic mice (a) and wildtype mice (b) were stained with an anti-human MSR antibody (see Experimental procedures). Other tissue macrophages, such as perivascular macrophages in the brain (c) and macrophages in the renal capsule (d) were also positive in the MSR1 transgenic mice.

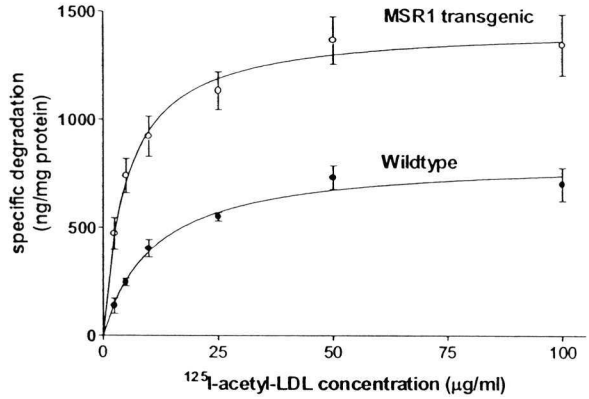


Fig. 4: Degradation of acetyl-LDL by peritoneal macrophages from MSR1-transgenic mice and nontransgenic littermates.
To determine MSR activity in MSR1-transgenice mice, thioglycolate elicited peritoneal macrophages were incubated with increasing concentrations of ^{125}I-labeled acetyl-LDL at 37°C. After 3 hours, degradation was determined (see Methods). Macrophages from MSR1-transgenic mice (O) were compared with macrophages from nontransgenic littermates (●) as controls. Values are the mean ± SD of 3 samples and are representative for three experiments. Differences between MSR1 and wildtype macrophages were significant ($p<0.01$, students t-test)

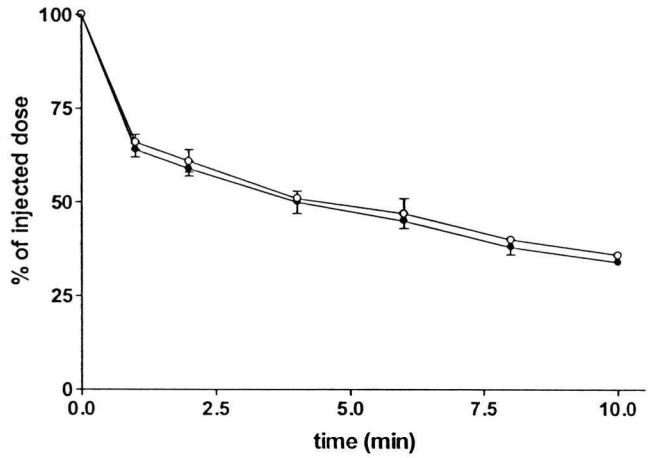


Fig. 5: *In vivo* clearance of acetyl-LDL
MSR1-transgenic mice (O) and nontransgenic littermates (●) were injected with 200 µl PBS containing 200 µg ^{125}I-labeled acetyl-LDL and 0.1 mg/ml bovine serum albumin. At indicated timepoints, blood samples were taken and serum content of ^{125}I-acetyl-LDL was measured and calculated as a percentage of the injected dose. Values are the mean ± SD of three mice and are representative of two experiments.

In vitro foam cell formation and cholesterolester accumulation

Macrophages from MSR1-transgenic mice were analyzed for *in vitro* foam cell

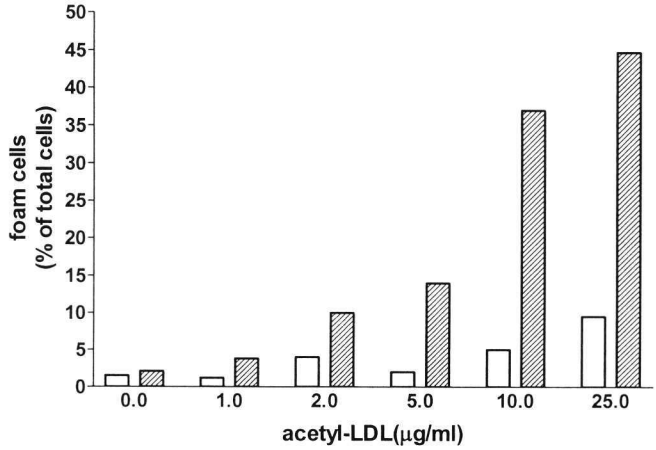
formation upon incubation with acetyl-LDL. The number of cells having a foam cell appearance, indicated by positive oil red O staining, was expressed as a percentage of the total number of cells (Fig. 6a). With increasing concentrations of acetyl-LDL, more foam cells appeared, whereby macrophages from MSR1-transgenic mice were much more prone to convert into foam cells than macrophages from wild-type mice. At this highest acetyl-LDL concentration (25 μg/ml), the amount of cholesterolesters that had accumulated in macrophages from MSR1 transgenic mice was twice as high as compared to wild-type macrophages (20.24 ± 4.27 and 9.19 ± 1.45 respectively, $p<0.05$)(Fig. 6b). No differences were found in cellular cholesterol or triglyceride levels between the two groups (data not shown).

Discussion

Here we describe a new mouse model expressing high levels of the human MSR in macrophages from all tissues investigated. The DNA construct to generate this model was a 180 kb YAC. Since the human MSR1 locus spans 80 kb, an additional 100 kb was included to provide regulatory sequences. This construct resulted in expression according to a natural pattern, including a bone fide regulation of alternative splicing. Macrophages from MSR1 transgenic mice expressed two mRNA species, of 4 and 3.2 kb. These represent type I and type II human MSR1 mRNA, respectively (23). The type II MSR transcript was the most prominent of both types. This confirms previous findings, which described that in human monocyte-derived macrophages MSR type II mRNA was much more abundant than type I mRNA (27). Expression of the MSR1 gene was detected in different tissues by Northern blotting. These results were confirmed by examining tissue sections with an antibody specific for the human MSR showing macrophages to be responsible for the expression. In the livers from MSR1 transgenic mice, the Kupffer cells were highly positive, as was expected from previous findings in both cow (24) and mouse (25). In the MSR1 transgenic mice additional tissues were examined. Macrophages (such as perivascular macrophages in the brain and macrophages in the renal capsule) were positive for the human MSR confirming the natural expression pattern of the transgene.

Expression of the transgene could be highly induced by treating MSR1 transgenic mice with thioglycolate. While in resident peritoneal macrophages MSR1 expression was virtually absent, thioglycolate-elicited macrophages showed high expression levels. This is in contrast to the mouse MSR, where expression in thioglycolate-elicited macrophages was only 70% higher as compared to resident

a.



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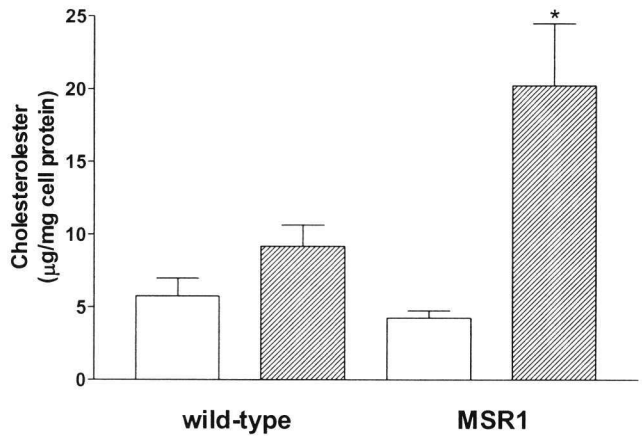

Fig. 6. *In vitro* foam cell formation and cholesterolester accumulation.
Macrophages from MSR1-transgenic mice (hatched bars) and nontransgenic littermates (open bars) were incubated with increasing concentrations of acetyl-LDL for 20 hours. Next, cells were stained with oil red O and the number of oil red O positive cells was counted as a percentage of the total number of cells (a). Macrophages from MSR1-transgenic mice (MSR1) and nontransgenic littermates (wild-type) were incubated without acetyl-LDL (open bars) or with 25 μg/ml acetyl-LDL (hatched bars) and after 20h cholesterolester accumulation was determined as described in methods. In MSR1 macrophages cholesterolester accumulation was higher as compared to macrophages from non-transgenic littermates (* $p<0.05$, students t-test) (b).

macrophages (data not shown). Treatment of thioglycolate-elicited macrophages with PMA and acetyl-LDL resulted in a further increase of expression. PMA has previously been shown to activate protein kinase C (PKC) resulting in increased MSR1 expression (28,29) and recently it was shown that acetyl-LDL could upregulate mouse MSR in murine RAW cells. We show that the MSR1 transgene is subject to the same regulation as has been described for the MSR1 gene in it's natural genomic environment.

Clearance studies have shown that intravenously injected acetyl-LDL is rapidly taken up by the liver (30). Whereas overexpression of the bovine MSR cDNA in mouse liver resulted in an increased *in vivo* catabolism of acetyl-LDL (31), we found no effect on the clearance rate of acetyl-LDL in our MSR1-transgenic mice. This difference could be attributed to the ectopic expression of the bovine cDNA in parenchymal cells, in contrast to our mice expressing the MSR1 exclusively in Kupffer cells of the liver.

Our acetyl-LDL clearance results indicate that other receptors are involved in the clearance of acetyl-LDL and experiments using MSR knock-out mice have confirmed this possibility (9,32). Kodama's group (9) and others (32) found no reduction in the clearance rate of acetyl-LDL and oxidized LDL in MSR-deficient mice. They attributed this to the presence of other oxidized LDL receptors involved in acetyl-LDL clearance. The relative importance of these other receptors in modified lipoprotein metabolism remains unclear.

Finally we have obtained evidence of increased activity of the human MSR in our MSR1-transgenic mice, *in vitro*. The maximum degradation of acetyl-LDL was almost doubled in macrophages from MSR1-transgenic mice, indicating that increasing levels of MSR can, indeed, affect cellular metabolism of modified lipoproteins, *in vitro*. Previously it was shown, *in vitro,* that overexpression of the MSR in Chinese hamster ovary cells leads to highly enhanced foam cell formation (33). We showed that the same is true for macrophages derived from our transgenic mice overexpressing the MSR1 gene with the native regulatory elements. Cholesterolester accumulation was doubled in macrophages from MSR1 transgenic mice. Moreover, almost four times more foam cells were formed in macrophages from MSR1-transgenic mice compared to wild-type macrophages when the cells were incubated with 25 mg/ml acetyl-LDL. This emphasizes that changing levels of MSR can modulate foam cell formation.

Due to the complex function of the MSR as a multifunctional receptor and adhesion molecule, the role of the MSR in atherogenesis is unlikely to be straightforward. The normal function of the MSR could be to prevent the build-up of lipid deposits in the intimal space of the vessel wall, by scavenging modified atherogenic lipoproteins. However, when the supply of modified lipoproteins increases in hyperlipidemia, the MSR may stimulate atherogenesis by supporting the change of macrophages into foam cells. In addition, the MSR may be able to

mediate macrophage migration into the plaque area. Recent experiments have shown that MSR deficiency protects against atherosclerosis in apoE deficient mice. This is in accordance with our *in vitro* result with macrophages from MSR1-transgenic mice where MSR overexpression enhances foam cell formation. There are, however, other reports which show a different effect of MSR levels on atherosclerosis. We found that on an apoE3Leiden background MSR deficiency enhances atherosclerosis. Others have described that in rabbits which are less sensitive to diet-induced atherosclerosis the scavenger receptor levels are increased as compared to rabbits that show a high susceptibility to diet-induced atherosclerosis (34,35). These experiments underscore that another role of the MSR, in addition to mediating foam cell formation, might be the scavenging of modified lipoproteins from the circulation or the vessel wall. In this process the MSR would act as an atherosclerosis-reducing factor. We think that the present MSR1 overexpressing mouse model may prove to be a useful model for investigating the role of the MSR *in vivo*. Experiments are in progress to investigate the effects of overexpression of MSR1 on atherosclerosis. The MSR1-transgenic mice have been crossed with apoE-deficient, LDLR-deficient and apoE3Leiden-transgenic mice to evaluate MSR function in different atherosclerosis models with their own specific characteristics, representing different forms of hyperlipidemia found in humans. Besides its role in atherosclerosis the MSR also plays a role in the removal of apoptotic cells (36,37), cell adhesion and migration (10), host defense (9) and possibly Alzheimer's disease (38). MSR1-transgenic mice will prove a useful model to study these phenomena.

FOOTNOTES

Abbreviations used in this paper:
MSR, macrophage scavenger receptor class A; YAC, yeast artificial chromosome; LDL, low density lipoprotein; PFGE, pulse field gel electrophoresis.

Acknowledgements:
We gratefully acknowledge the technical assistance of J. van der Boom, L. van Vark and P. van Gorp. We thank Dr. T. Kodama and Dr. M. Honda from RCAST, Tokyo University, for sending us the polyclonal antiserum for the human MSR. Dr. Marten Hofker is an Established Investigator of the Netherlands Heart Foundation.

Keywords:
macrophage scavenger receptor, yeast artificial chromosome, acetyl-LDL, transgenics

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CHAPTER 6

OVEREXPRESSION OF THE HUMAN MACROPHAGE SCAVENGER RECEPTOR CLASS A REDUCES ATHEROSCLEROSIS IN BOTH LOW DENSITY LIPOPROTEIN RECEPTOR DEFICIENT MICE AND APOE3LEIDEN TRANSGENIC MICE.

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Abstract

An early step in atherogenesis is the formation of foam cells in the vessel wall accompanied by an inflammatory response. The macrophage scavenger receptor class A (SR-A) is thought to be involved in both these processes. To elucidate the role of the SR-A in the pathogenesis of atherosclerosis we have generated transgenic mice overexpressing the human SR-A gene (MSR1). These mice were crossed onto a low density lipoprotein receptor deficient background and fed a high fat diet for 10 weeks. After this period lesion size in the proximal aorta was measured. The effect of MSR1 overexpression on diet-induced atherosclerosis was determined without or with correction for cholesterol exposure. Interestingly, atherosclerosis was significantly reduced in the MSR1 overexpressing group. Correcting for individual cholesterol exposure even decreased the p-value of the observed difference. To study the effect of MSR1 overexpression on a different athero-susceptible background and to exclude non-macrophage specific effects of MSR1 overexpression we transplanted bone marrow from MSR1 mice and wildtype littermates to APOE3Leiden transgenic mice. After 8 weeks of a high fat diet, atherosclerosis in the mice that had received MSR1 bone marrow appeared to be reduced, as compared to mice that had received wildtype bone marrow. This difference reached statistical significance when individual cholesterol exposure of the mice was taken into account. The anti-atherogenic role of the SR-A cannot be explained by the effect of overexpression on foam cell formation, which is enhanced, as previously shown. To investigate the effect of SRA overexpression on the activation of macrophages, the response to lipopolysaccharide (LPS) was measured. The MSR1 macrophages showed a reduction in their activation in response to LPS, as measured by nitric oxide production. These data indicate that additional SR-A expression reduces atherosclerosis, potentially by modifying the response of macrophages to activation signals in the plaque.

Introduction

Atherogenesis can be induced by elevated plasma levels of atherogenic lipoproteins, such as low density lipoproteins (LDL). This leads to the accumulation of lipoproteins in the intima where it becomes modified. Modified LDL activates the endothelial cells lining the vessel wall and attracts monocytes from the circulation, which subsequently will adhere to the endothelial cells, cross the endothelial layer to enter the intima, differentiate into macrophages and eventually become foam cells. These foam cells are characterised by a massive accumulation of cholesterolesters (1) resulting from the unrestricted uptake of modified lipoproteins, such as oxidized LDL. The SR-A was first identified as a receptor, specific for acetylated LDL, on macrophages that mediates the formation of foam cells (2). Overexpression of the

SR-A in Chinese hamster ovary cells resulted, after incubation with modified LDL, in foam cell formation (3). Furthermore, the SRA has been shown to be highly expressed in atherosclerotic lesions (4,5) and indeed the SR-A ligand, oxidized LDL, is present in plaques (5-7). Based on these results, the SRA was assigned an important role in atherogenesis (8-10).

The role for the SR-A in atherosclerosis was further studied in SR-A knockout mice (SR-A$^{-/-}$) (11) cross-bred onto a pro-atherogenic background. The absence of SR-A in apolipoprotein E deficient (*apoe*$^{-/-}$) mice, led to a decreased lesion area development (-60%) (11). Furthermore, on a low density lipoprotein receptor deficient (*ldlr*$^{-/-}$) background, SR-A deficiency led to a modest decrease in atherosclerosis (-20%) (12). However, using an APOE3Leiden transgenic background, we showed that SR-A deficiency actually enhanced atherosclerosis (13).

These results show that the role of the SR-A in atherogenesis may not be as straightforward as was initially expected. Experiments using additional atherosclerosis susceptible mouse models are therefore required to further delineate the role of the SR-A in atherogenesis. We have generated a mouse model with a highly increased level of SR-A expression (14). In this mouse, we introduced a 180kb yeast artificial chromosome covering the entire human SR-A gene (MSR1) and 100kb flanking sequence. This construct gives a macrophage specific overexpression of the human SRA. The present paper, describes the effects of SR-A overexpression on atherogenesis in two different athero-susceptible mouse models, *ldlr*$^{-/-}$ mice and APOE3Leiden transgenic mice. In both models, overexpression of the SR-A reduced atherosclerosis by approximately 30% indicating that the SR-A can, depending on the genetic background, reduce the development of atherosclerosis.

METHODS

Mice.

The generation of the MSR1 mice has been described elsewhere (14). These mice carry a 180kb yeast artificial chromosome covering the human SRA gene (MSR1) and 60kb and 40kb flanking at the 5' and 3' region. MSR1 mice were crossed back onto a C57bl6/J background and generation N3 mice were used. Male MSR1 mice were crossed with female low density receptor deficient (*ldlr*$^{-/-}$) mice. Resulting *ldlr*$^{+/-}$-MSR1 male mice were again crossed with female *ldlr*$^{-/-}$ mice, yielding the *ldlr*$^{-/-}$-MSR1 and *ldlr*$^{-/-}$ littermates used in this paper. For atherosclerosis experiments 10 weeks old female *ldlr*$^{-/-}$-MSR1 (n=17) and *ldlr*$^{-/-}$ (n=12) mice were fed a high fat diet (Hope Farms, Woerden, The Netherlands), containing 15% cacao butter, 0.25% cholesterol, 40.5% sucrose, 10% corn starch, 1% corn oil and 5.95% cellulose, by weight.

For the second atherosclerosis experiments, bone marrow transplantation was performed as described elsewhere (15,16). Bone marrow from three MSR1 transgenic mice and their wildtype littermates was used as donor. As recipients 10 weeks old female APOE3Leiden mice were used (17). Four weeks after transplantation, the mice (10 MSR1 transplanted and 9 wt transplanted) were put on a high fat diet containing 15% cacao butter, 1% cholesterol, 40.5% sucrose, 10% corn starch, 1% corn oil and 4.7% cellulose and 0.5% cholate, by weight.

All mice were maintained in a specific pathogen-free facility with a 12-h light/dark cycle and given free access to food and water.

Lipid analysis.

Blood samples were taken from the tail vein after a four-hour fast. Total serum cholesterol and triglyceride concentrations were measured enzymatically using commercial test kits (kit no. 236691 from Boehringer Mannheim GmbH, Mannheim, Germany and kit 337-B from Sigma, St Louis, USA, respectively). For the analysis of the distribution of cholesterol over the different lipoprotein fractions pooled samples were analyzed on the Smart-system (Pharmacia, Uppsala, Sweden) with a Superose 6 column as described previously (15,16).

Atherosclerosis analysis.

After 10 (*ldlr*$^{-/-}$) or 8 (APOE3Leiden) weeks of a high fat diet the mice were sacrificed and the heart was removed, cutting the aorta just above the atria. The heart was bisected perpendicular to the heart axis, just below the atrial tips. The tissues were fixed in 4% formaldehyde and embedded in paraffin. 5 μm sections were cut and once the atrioventricular valves were identified and the media was visible, sections were collected until the valves disappeared. From the valve area, 3 sections were used for measuring lesion area using computer-aided morphometry (Kontron-Videoplan, Zeiss, Germany). The average lesion area per section was calculated from these three sections. In addition, the degree of atherosclerosis was expressed as lesion area corrected for cholesterol exposure. This approach was justified by previous work showing that atherosclerotic lesion area and cholesterol levels correlate linear over a large range of plasma cholesterol values. This was demonstrated for both the APOE3Leiden transgenic (18) and *ldlr*$^{-/-}$ mice (19). Variation in cholesterol levels might introduce an additional factor affecting lesion area development. Therefore, we analysed the lesion area measurements by dividing them by the plasma cholesterol levels. The lesion area per millimolar cholesterol was used to get a more accurate value of the effects of MSR1 overexpression on atherogenesis in the individual mice.

Macrophage isolation.

1 ml of sterile Brewers thioglycollate broth (4.5%, w/v) was injected peritoneally. After 4 days, macrophages were isolated by washing the peritoneum with 8 ml of ice-cold sterile phosphate buffered saline. After isolation, cells were washed with R10 (RPMI 1640 medium containing 10% fetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin) and counted.

In vitro macrophage activation.

Macrophages were plated in 96-wells plates at $1*10^5$ cells per well. After adherence cells were washed with R10 and incubated in RPMI 1640 with 5% fetal calf serum without phenol red for 48 hours with or without the addition of 10 ng/ml lipopolysaccharide (LPS, Re595; Sigma). Next, nitric oxide production was quantified by measuring nitrite in the medium using Greiss reagent (1% (w/v) sulfanilamide and 0.1% (w/v) naphtylethylenediamine dihydrochloride in 2.5% phosphoric acid) and Sodium-nitrite as standard.

Statistics.

Data are reported as mean ± S.D. The students t-test was used for calculating significance of differences between means, $p<0.05$ was accepted as statistically significant.

RESULTS

The generation of the MSR1 transgenic mice has been described elsewhere (14). These mice were crossed onto an *ldlr*$^{-/-}$ background. Two groups consisting of *ldlr*$^{-/-}$-MSR1 mice and *ldlr*$^{-/-}$ littermates were fed a high fat diet for 10 weeks resulting in severe hyperlipidemia (table I).

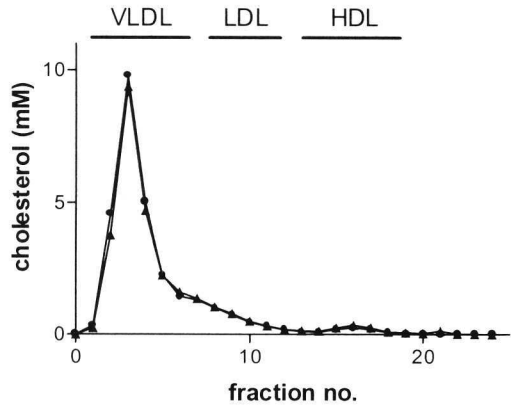
Table I: Plasma cholesterol values after 10 weeks (ldlr$^{-/-}$ and ldlr$^{-/-}$-MSR1) or 8 weeks (wt→APOE3Leiden and MSR1→APOE3Leiden) high fat diet, as described in methods.

Genotype	Plasma cholesterol (mM)
Ldlr$^{-/-}$	49.3 $\pm$ 10
Ldlr$^{-/-}$-MSR1	51.5 $\pm$ 10
wt → E3L	31.3 $\pm$ 12
MSR1 → E3L	37.4 $\pm$ 5

The mice had plasma cholesterol levels of 49.3±10 and 51.5±10 in the *ldlr*$^{-/-}$-MSR1 and *ldlr*$^{-/-}$ groups respectively. Triglyceride levels did not differ between the two groups (data not shown). FPLC profiles of plasma lipoproteins showed no difference in the distribution of cholesterol between the different lipoprotein fractions (Fig. 1A). Cholesterol mainly accumulated in the very low density lipoprotein (VLDL) sized fractions. After 10 weeks of the high fat diet the mice were sacrificed and atherosclerosis was determined by measuring lesion size in the valve area of the aortic root (Fig. 2). In both groups severe atherosclerosis, with necrosis and cholesterol clefts, had developed (Fig. 2A and 2B). Measuring the lesion size revealed that in the *ldlr*$^{-/-}$-MSR1 group atherosclerosis was decreased as compared to the *ldlr*$^{-/-}$ controls (304,257 ± 126,167 versus 412,691 ± 130,100 μm^2, respectively, -27%, $p<0.05$)(Fig. 2C). Additionally, these differences were more significant ($p=0.01$) when correcting for cholesterol exposure (Fig. 2D).

To investigate the effects of increased SR-A expression in the MSR1 transgenic mice to a different athero-susceptible background, we performed a bone marrow transplantation to APOE3Leiden transgenic mice. In addition, this technique also excludes non-macrophage effects of the MSR1 overexpression. MSR1 mice and wildtype littermates were used as donors and APOE3Leiden transgenic mice as recipients. PCR analysis of bone marrow after transplantation confirmed the replacement of the recipient bone marrow with that of the donor (data not shown). Four weeks after transplantation the mice were put on a high fat diet. This resulted in a hyperlipidemia in both groups (APOE3Leiden mice that had received MSR1 bone marrow: MSR1→E3L; and APOE3Leiden mice that had received wildtype bone

A.



B.

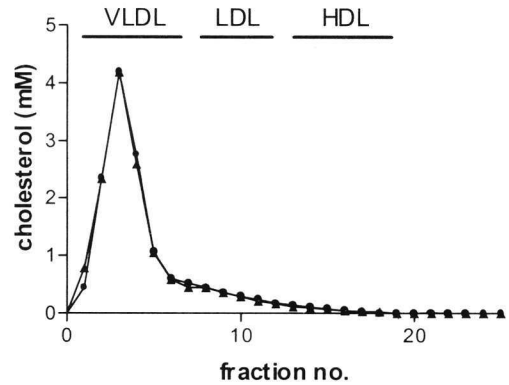


Figure 1: Lipid profiles of (A) *ldlr*$^{-/-}$ (triangles) and *ldlr*$^{-/-}$-MSR1 (circles) mice and (B) wt→APOE3Leiden (triangles) and MSR1→APOE3Leiden (circles) after 10 and 8 weeks of a high fat diet. Indicated are cholesterol values.

marrow: wt→E3L)(table I). The MSR1→E3L appeared to have slightly higher plasma cholesterol levels but this difference was not significant. Distribution among the lipoprotein fractions also did not differ (Fig. 1B). Atherosclerosis was determined in the valve area of the aortic root. The atherosclerotic lesions that had developed after 8 weeks consisted mainly of foam cell filled lesions (Fig. 3A and 3B). Measuring lesion size revealed that in the MSR1→E3L group atherosclerosis appeared to be reduced as compared to the wt→E3L group (56,940 ± 34,950 and 75,510 ± 33,580 μm^2, respectively)(Fig. 3C), although this difference did not reach statistical significance. However, correcting for cholesterol exposure did reveal that the decrease in atherosclerosis was statistically significant (-35%, $p<0.05$) (Fig. 3D).

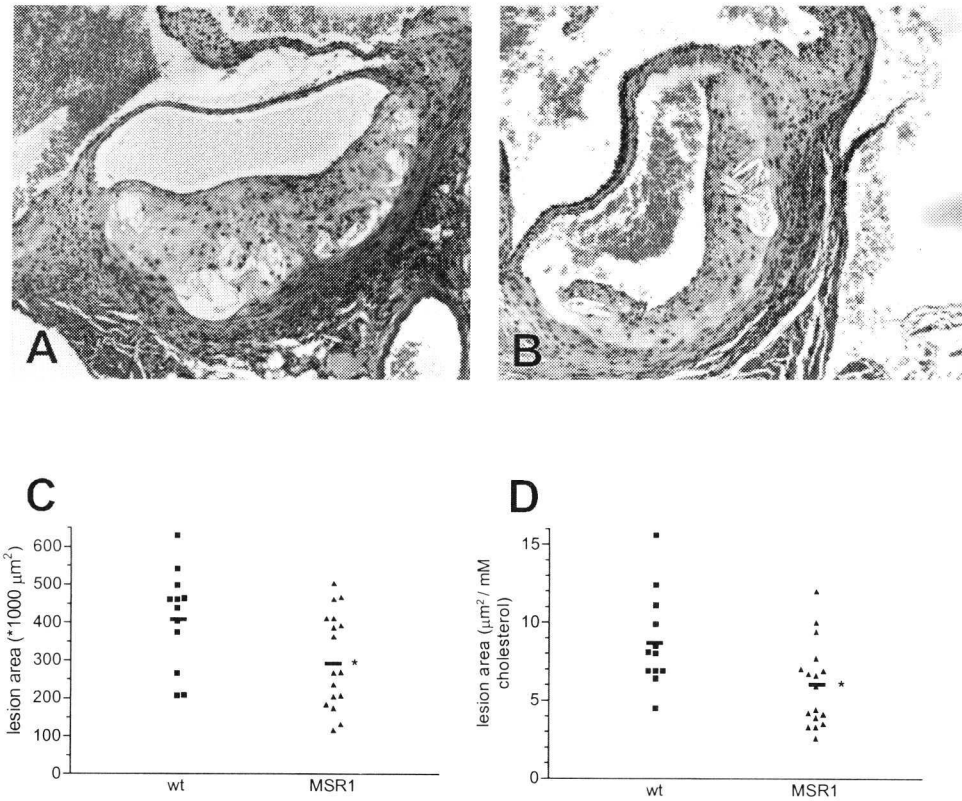


Figure 2: Atherosclerosis after in *ldlr*$^{-/-}$ and *ldlr*$^{-/-}$-MSR1 mice after 10 weeks of high fat diet.
Shown are representative lesions from *ldlr*$^{-/-}$ mice (A) and *ldlr*$^{-/-}$-MSR1 mice (B). The lesion area is shown in (C) and corrected for cholesterol exposure in (D). Data are individual mouse values, lines indicate group averages and * indicates $p<0.05$.

In addition to its role in the uptake of modified LDL the SRA is also known to bind the bacterial surface molecule lipopolysaccharide (LPS), and SRA deficient mice have a highly increased susceptibility to bacteria induced septic shock (20). We tested if the activation of macrophages was changed. For this, we examined whether the macrophage response to LPS was changed due to MSR1 overexpression. In response to LPS macrophages become activated as indicated by an increase in nitric oxide secretion. Thioglycollate elicited macrophages were incubated with 10 ng/ml LPS and subsequently nitric oxide production was quantified (Fig.4). MSR1 macrophages showed a decrease in their activation in response to LPS as compared to macrophages from their wildtype littermates. After 48 hours the wildtype macrophages had produced 56.4 ± 1.7 mM nitrite, whereas MSR1 macrophages had produced 42.0 ± 1.0 mM. These data demonstrate the SR-A overexpression reduces

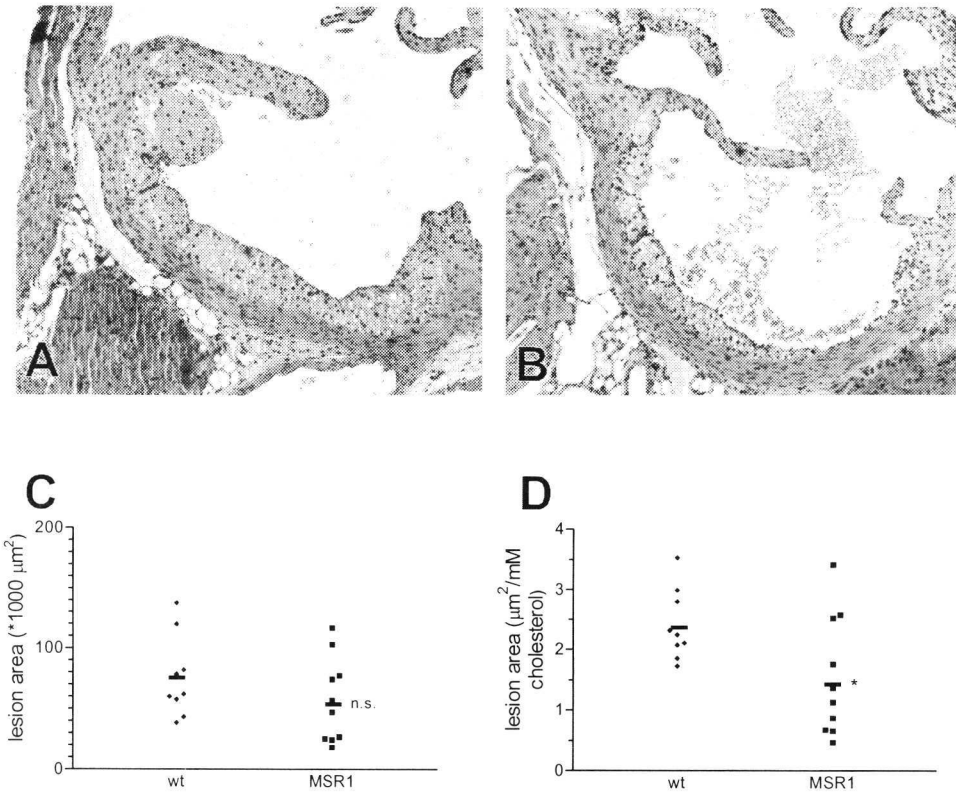


Figure 3: Atherosclerosis in wt→APOE3Leiden and MSR1→APOE3Leiden bone marrow transfer mice after in 8 weeks of high fat diet.
Shown are representative lesions from wt→APOE3Leiden mice (A) and MSR1→APOE3Leiden mice (B). The lesion area is shown in (C) and corrected for cholesterol exposure in (C). Data are individual mouse values, lines indicate group averages and * indicates $p<0.05$; n.s. non-significant.

the activation of macrophages in response to LPS, as measured by nitric oxide production.

Discussion

In the present report we show that macrophage specific overexpression of the SRA reduces the development of atherosclerotic lesions. We used the MSR1 mouse model, overexpressing the human SR-A through a construct containing the entire human SR-A gene (MSR1) and 100 kb flanking sequence. These mice express the human SR-A according to the natural expression pattern due to the presence of the natural promoter, enhancer and silencer elements. In two independent models for

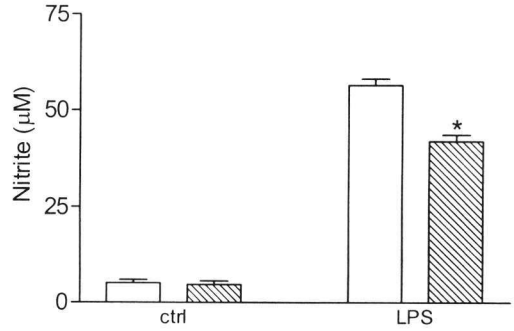


Figure 4: Activation of wildtype (open bars) and MSR1 macrophages (hatched bars) in response to LPS.

Thioglycollate elicited peritoneal macrophages were untreated (ctrl) or treated with 10 ng/ml LPS (LPS) for 48 hours and nitric oxide production was quantified by measuring nitrite as described in methods. Data are means ± s.d. and * indicates $p<0.01$

atherosclerosis we show that overexpression of the human SR-A gene leads to a reduction of atherosclerotic lesion development. One possible explanation is the changed capacity of the macrophage to mediate an inflammatory response, as was demonstrated by the fact that increased SR-A activity made the MSR1 macrophages less responsive to activation by LPS. We conclude that overexpression of SR-A has the ability to protect against atherosclerosis.

The SR-A is thought to mediate the initial step in atherosclerosis: the unrestricted uptake of modified lipoproteins by macrophages in the vessel wall, resulting in foam cell formation. Initial findings were in line with this hypothesis. Atherosclerosis experiments performed with the SRA knockout mice ($SRA^{-/-}$) on a $apoe^{-/-}$ deficient background (11) showed a 60% reduction in the absence of the SR-A, indeed. Subsequently, it was shown on a $ldlr^{-/-}$ background the SRA deficiency led to a 20% reduction in lesion formation, although coinciding with a 20% decrease in plasma cholesterol levels (12). These data already indicated that the role of the SR-A in atherosclerosis might be dependent upon the type of atherogenic background. We showed, recently that on an APOE3Leiden transgenic background, SRA deficiency leads to an increase in the development of atherosclerotic lesions (13). Thus the role of the SRA in atherosclerosis development is not as straightforward as might be expected from its facilitating role in mediating foam cell formation in macrophages. To further elucidate the role of the SR-A in the pathogenesis of atherosclerosis we generated the MSR1 overexpressing mice (14).

In the present study we analysed the effects of MSR1 overexpression in two different mouse models. The $ldlr^{-/-}$ mouse model is a model for familial hypercholesterolemia and develops high cholesterol levels and atherosclerosis due to the absence of the low density lipoprotein receptor (21,22). The APOE3Leiden

mice carry a dominant defective APOE variant, in humans associated with familial dysbetalipoproteinemia, leading to susceptibility to diet induced hypercholesterolemia and atherosclerosis (17,18,23). In both models we observe that overexpression of SRA reduces atherosclerotic lesion formation.

The present results and our previous paper (13) demonstrated that the SRA can act anti-atherogenic. Others have also shown that high expression of SR-A correlated with low susceptibility to diet induced atherosclerosis. Teupser and Thierry (24,25) generated two rabbit strains, from one parental strain, by selecting for high and low susceptibility to diet induced atherosclerosis. They showed that rabbits with a low susceptibility had strongly elevated levels of SRA activity as compared to rabbits with a high susceptibility. Furthermore, a recent study showed that monocyte derived macrophages from healthy old subjects displayed higher SRA activity as compared to macrophages from young individuals, indicating that high SRA expression also in the human population might be beneficial (26).

To explain the protective effect of the SR-A, alternative functions of the SR-A, in addition to facilitating foam cell formation, should be taken into account. The SR-A may play an important role in three processes which can effect atherogenesis. First, evidence has accumulated for a role of the SR-A in adhesion. The SR-A can mediate the adhesion of cells to artificial substrates, such as tissue culture plastic (27), to modified proteins, such as modified collagen (also found in lesions) (28) and to other cells in different organs (29). This adhesive property may effect macrophage behaviour in the lesion by changing its migratory capabilities. This can also result in an increased capacity of the macrophage to scavenge modified lipoproteins from the vessel wall, thereby reducing the inflammatory action of modified LDL. Uptake of oxLDL from the vessel wall into macrophages, removes it from the intima. As a result of this, oxLDL cannot get extensively modified and it can no longer exert its inflammatory action on the cells present in the intima. Second, as we also show in this paper, SR-A expression might modify the activation of macrophages by LPS which can influence lesion progression. Increased expression of SR-A enhances the uptake of LPS through non-signalling pathways (SR-A mediated removal) as compared to signalling pathways (CD14 mediated response). As a consequence of this, SR-A deficient mice are more susceptible to endotoxic shock (20). This may also be true for other activating components, present in the atherosclerotic lesion. How these different processes are exactly intertwined and effect macrophage activation is not clear, yet.

Finally, it has been described recently that the uptake of oxLD by the SR-A mediates the antigen presentation of oxLDL derived peptides on macrophages (30). This can mediate the production of anti-oxLDL antibodies, which have been described to protect against atherosclerosis in rabbit atherosclerosis models (31,32). Thereby, one could speculate that if the SR-A plays a role in the production of

antibodies against oxLDL, overexpression of the SR-A enhances this production and thereby protects against atherosclerosis.

At present we cannot discriminate between the aforementioned possibilities and further mechanistic studies in the various animal models will be needed. However, we have clearly shown that overexpression of SR-A reduces atherosclerosis, both in APOE3Leiden and LDL-receptor deficient mice, and can therefore be considered, at least in these atherogenic models as a protecting factor against atherosclerosis.

Acknowledgements

This work was supported by the Netherlands Heart Foundation and the Netherlands Foundation of Scientific Research (project nr. 901-04-092). Marten H. Hofker is an Established Investigator of the Netherlands Heart Foundation..

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CHAPTER 7

A MOUSE MODEL TO STUDY THE FUNCTION OF TYPE I MACROPHAGE SCAVENGER RECEPTOR CLASS A IN ATHEROSCLEROSIS.

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Abstract

The macrophage scavenger receptor class A (SR-A) is a multifunctional receptor involved in several processes, including foam cell formation, host defense and cellular adhesion. Two isoforms are expressed (type I and type II) which are generated from a single gene by alternative splicing. Type I and type II SR-A differ in their C-terminal domain. Type I contains a scavenger receptor cystein rich (SRCR) domain, which is lacking in type II. Specific regulation for both isoforms has been described. However, no significant difference in binding characteristics has been found and so far the in vivo function of the SRCR domain remains unclear. To study the exact role of the type I SR-A with the SRCR domain we generated an isotype specific knockout. Exon 10, specifically used by type I SR-A of the murine SR-A gene was replaced by a neomycine resistance gene using homologous recombination in embryonic stem cells. Using these embryonic stem cells type I knockout mice were generated. RT-PCR confirmed the absence of type I mRNA in homozygous type I deficient mice. Association and degradation of both acetylated LDL, an SR-A specific ligand, and oxidized LDL was not changed in type I deficient macrophages. This type I knockout mouse model will provide a useful tool to study the function of the two isoforms of the SR-A, in vivo.

Introduction

The macrophage scavenger receptor class A (SR-A) is a trimeric integral membrane glycoprotein that has been implicated in foam cell formation, host defense and cellular adhesion. The SR-A binds a broad range of polyanionic ligands. These include modified lipoproteins, such as oxidized and acetylated low density lipoprotein (LDL), maleylated and glycated bovine serum albumin, polyI and polyG and polysaccharides including lipopolysaccharide (LPS) and lipoteichoic acid (LTA)(1-4).
The SR-A consists of six distinct domains of which the collagen-like domain has been shown to be the site for receptor interaction with modified lipoproteins(5). The gene coding for the six different domains contains 11 exons and spans approximately 80kb in humans(6) and 60 kb in mice(7). Two variants of the SR-A (type I and type II) are generated by alternative splicing of the same gene. Type I SR-A is encoded by exon 1-8 and 10-11 and contains an 110 aa long C-terminal cystein rich domain. Type II SR-A is encoded by exon 1-9 and lacks the cystein rich domain. Recently, a third transcript was described which acts in a dominant negative manner. This variant, resembles type I SR-A but skips exon 10, thereby deleting part of the cystein domain (8). The exact function of the cystein rich domain has not been elucidated yet. However, specific regulation of the type I and II isoforms has been described (9-11). Freshly isolated human monocytes express low amounts of SR-A mRNA, but it is highly upregulated during differentiation to macrophages. This increase in expression

is mainly observed for type I SR-A. However, both types have been shown to be present in atherosclerotic lesions (12). Since the SR-A type I has been described as a receptor containing a cystein rich domain, a new family of genes has emerged, all containing a scavenger receptor cystein rich (SRCR) domain. A function of the SRCR domain has been proposed only for one of these genes, CD6, containing three SRCR domains. It was shown to be the ligand for the leukocyte adhesion molecule CD166 (13) indicating the involvement of the SRCR domain in cell-cell interaction. For the SR-A type I and II no differences have been shown in ligand interaction between both isoforms. Chinese hamster ovary cells stably expressing type I or type II SR-A showed similar binding characteristics for acetylated LDL, polyl and maleylated bovine serum albumin. However, competition studies revealed some differences in LPS binding. LPS was able to completely compete acetyl LDL from type II transfected cells but only partly from type I transfected cells. This indicates that LPS binds better to type II SR-A as compared to type I SR-A.

Despite these differences in type I and II expression, regulation and to some extend binding characteristics, the exact *in vivo* function remains unclear. Therefore, we have generated a mouse model specifically lacking type I SR-A. We replaced the type I specific exon 10 by a neomycin cassette, resulting in absence of type I expression. This did not change modified LDL degradation.

METHODS

Generation of SR-A typeI$^{-/-}$ mice.

A murine genomic DNA clone was isolated from a lambda library. The 10kb linearized targeting vector was electroporated into IB10 embryonic stem (ES) cells, as described previously. G418-resistant colonies were screened by Southern blotting using probe S5 (Fig. 1). 1 out of 68 G418 resistant clones had undergone the desired homologous recombination. The positive clone was injected into B6 blastocysts and chimeric male offspring were mated to B6 females. Mice carrying the mutation in the heterozygous state were inter-crossed to produce homozygous typeI knockout (typeI$^{-/-}$) mice.

RT-PCR.

To confirm absence of type I mRNA RT-PCR was performed. For this RNA was isolated from macrophages from wildtype, heterozygous and homozygous type I mice using RNA Insta-pure (Eurogentec) according to standard procedures. Subsequently, cDNA was transcribed using random primers and reverse transcriptase (Life technologies) according to manufacturors instructions. PCR was performed using this cDNA as template. Primers for PCR were: PCR1 (SR-A type II): 5'-CTGGACCCCAAGGTGAAAAG-3' and 5'-AAGGGTCTTGCCCCAATATG-3'; PCR2 (SR-A type I, exon10): 5'- CTGGACCCCAAGGTGAAAAG-3' and 5'-CTTGTCCAAAGTGAGCTCTC-3'; PCR3 (SR-A type I, exon 11): 5'-CTGGACCCCAAGGTGAAAAG-3' and 5'-AAGTACAAGTGACCCCAGCA-3', all forward and reverse primers, respectively.

Macrophage isolation.

1 ml of sterile Brewers thioglycollate broth (4.5%, w/v) was injected peritoneally. After 4 days,

elicited macrophages were isolated, washing the peritoneum with 8 ml of ice-cold sterile phosphate buffered saline. After isolation cells were washed with R10 (RPMI 1640 medium containing 10% fetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin) and counted.

Association and degradation studies.

Human low density lipoprotein (LDL) was isolated by ultracentrifugation as described previously (14). LDL was acetylated with acetic anhydride or oxidized with $CuSO_4$ for 20 hours, as described elsewhere (15). Acetylated LDL and oxidized LDL was radiolabeled with ^{125}I (16). Macrophages were used at $5*10^5$ cells per well in 24-wells plates. Association and degradation of ^{125}I labeled modified LDL was determined after 3 hour incubation at 37°C as described previously (17).

RESULTS

Generation of SR-A typeI$^{-/-}$ mice.

To gain insight in the *in vivo* role of type I SR-A we generated typeI$^{-/-}$ mice by targeting exon 10 of the murine SR-A locus (Fig. 1a). A neomycin resistance gene replaced exon 10 after targeting via homologous recombination in ES cells. Correct homologous recombination resulted in the introduction of an additional *Eco*RI site (present in the neomycine gene). The targeted locus could be detected by Southern hybridisation using probe S5 (Fig 1b). The wildtype locus detected by S5 was 7.5 kb in size, whereas the targeted locus was 5.5 kb in size. The resulting typeI$^{-/-}$ mice were apparently healthy and bred normally.

SR-A expression in typeI$^{-/-}$ mice.

To confirm the absence of type I mRNA in typeI$^{-/-}$ mice RT-PCR was performed. RNA was isolated from thioglycolate elicited peritoneal macrophages. cDNA was was generated as described in methods. To detect isoform specific expression three different primer sets were developed (Fig.2). Set 1 detects type II cDNA(259 bp, primers in exon 6 and 9), set 2 detects type I cDNA (408 bp, primers in exon 6 and 10) and set 3 also detects type I cDNA (537 bp, primers in exon 6 and 11). As can be seen from figure 2 type II mRNA was present in wildtype, heterozygous and homozygous macrophages. PCR2 could not detect typeI mRNA in homozygous knockout macrophages. Finally, PCR3 detected a band of the correct size in wildtype and heterozygous macrophages, while in homozygous knockout macrophages the PCR resulted in a faint band, smaller then the expected size. This fragment was the result of abbarant splicing from exon 8 to exon 11 (confirmed by sequencing, not shown) in typeI$^{-/-}$ mice.

Association and degradation of acetylated LDL and oxidized LDL.

The degradation of 10 ug/ml acetylated LDL and oxidized LDL after 3 hours incubation with thioglycolate elicited peritoneal macrophages from typeI$^{-/-}$ and wildtype mice was determined. As can be seen from figure 3, both association and

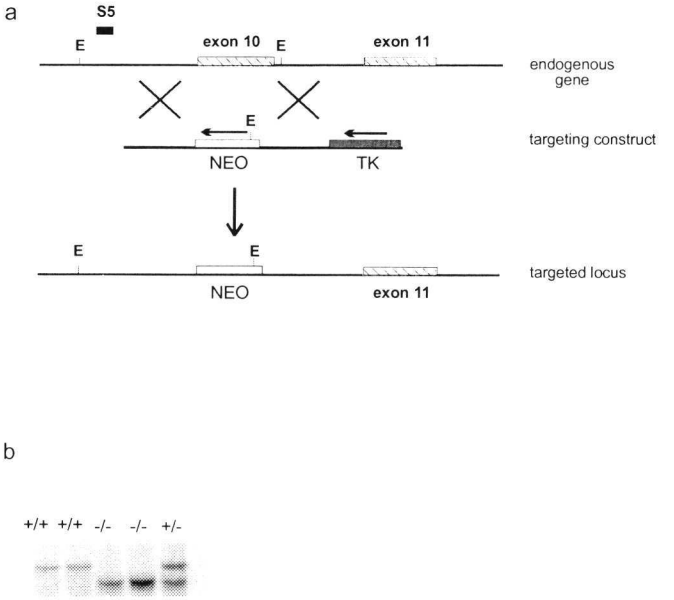

Figure 1: Targeted disruption of exon 10 of the murine SR-A gene.
a. Partial restriction maps of the wildtype SR-A locus, the targeting vector and the mutant allele. Exon 10 is disrupted by the replacement with a neomycin gene (NEO, positive selective marker), introducing an additional EcoRI restriction site. The thymidine kinase gene (TK) was used for negative selection. The external probe S5 was used for Southern blot analysis. E: EcoRI restriction site b. Southern blot analysis of DNA isolated from wildtype (+/+), heterozygous (+/-) and homozygous (-/-) knockout mice after restriction with EcoRI and hybridisation with probe S5. The wildtype allele is 7.5 kb in size, whereas the targeted allele is 5.5kb in size.

degradation of acetylated LDL was not changed. Association and degradation of oxidized LDL appeared slightly increased in typeI$^{-/-}$ macrophages but this difference was not significant.

Discussion

In this paper we describe the generation of an SR-A typeI specific knockout mouse model. This was generated by targeted replacement of the typeI specific exon10, by a neomycin resistance gene. Absence of SR-A typeI in typeI$^{-/-}$ mice did not change the association and degradation of both acetylated LDL and oxidized LDL by peritoneal macrophages.

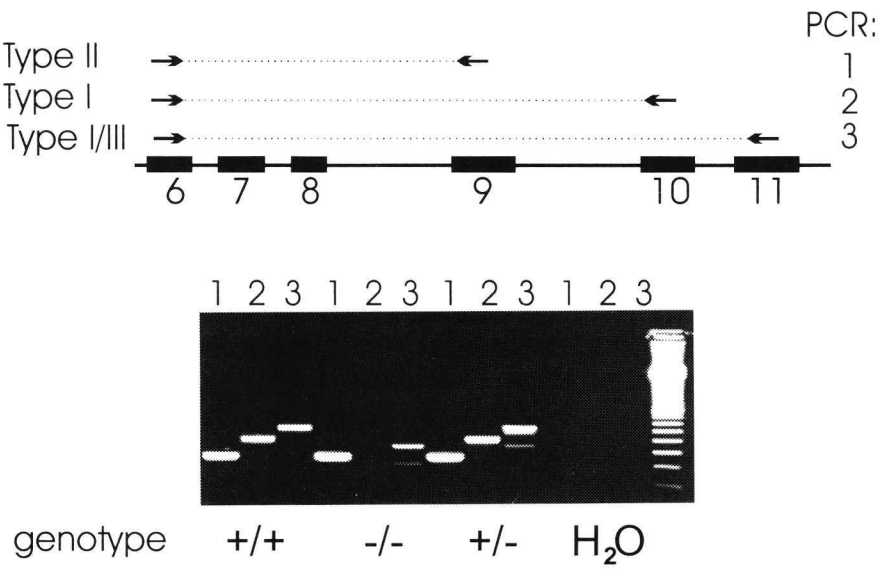

Figure 2: Primer localisation (a) and RT-PCR (b) performed on RNA isolated from macrophages from wildtype (+/+), heterozygous (+/-) and homozygous ($^{-/-}$) knockout mice.
Primer set 1 detects type II cDNA, set 2 detects type I cDNA (using exon 10) and set 3 detects type I cDNA (using exon 11). The latter also detects the SR-A splice variant, skipping exon 10, also described as type III SR-A.

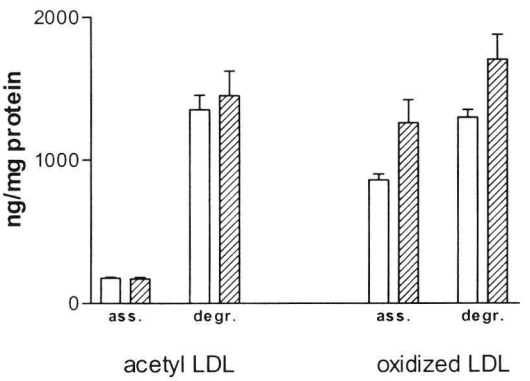

Figure 3: Association and degradation of acetyl LDL (left) and oxidized LDL (right).
Wildtype (open bars) and SR-AI$^{-/-}$ (hatched bars) macrophages were incubated with 10 ug/ml of either acetyl LDL or oxidized LDL and association and degradation was determined as described in methods. No significant differences were detected between wildtype and SR-AI$^{-/-}$ macrophages

The exact difference in function between type I and type II SR-A has never been studied thoroughly. Ashekenas and collegues(18) studied structure and binding properties of murine type I and type II SR-A. They used the macrophage-like cell line P388D1, which predominantly expresses type II SR-A, and chinese hamster ovary (CHO) cells transfected with either murine type I or type II cDNA. Studying competition of binding of acetyl LDL by other SR-A ligands, no differences were found in binding to typeI or type II for acetyl LDL, poly I and maleylated bovine serum albumin. However, LPS could only compete 60% of the bound acetyl LDL from type I expressing CHO cells, whereas it could completely compete acetyl LDL binding to type II expressing CHO cells. This indicates that LPS has a higher affinity for type II SR-A, as compared to type I SR-A. This was also shown for bovine type I and type II SR-A.

The expression and regulation of both SR-A isoform has also been the subject of several publications. Naito et al. (12) used isotype specific antibodies but found no difference in expression pattern in several human tissues, including atherosclerotic lesion. Others confirmed equal staining of atherosclerotic lesions for type I and type II SR-A(19). Using quantitative RT-PCR, type II mRNA was shown to be the most abundant isoform in cultured human monocyte derived macrophages(11). They found a range of 10-130 copies of type I mRNA per cell and 30-640 copies per cell for type II mRNA. In addition, Van der Kooij et al.(10) also showed the predominance of type II mRNA in human monocyte derived macrophages. They also found that monocytes predominantly express type II. Upon differentiation to macrophage, expression of type II slightly decreases and type I increases, still resulting in type II being the predominantly expressed isoform. Finally, Geng and co-workers showed that upon differentiation of human monocytes to macrophages, type I is specifically upregulated. From all these finding it can be concluded that both isoforms are expressed in most tissue macrophages but type I is specifically upregulated during differentiation from monocyte to macrophage.

Our experiments, examining the association and degradation of both acetyl LDL and oxidized LDL demonstrate the lack of function of type I SR-A in these processes in isolated peritoneal macrophages. The low amount type III mRNA, which has been described as a dominant negative SR-A isoform and is generated by splicing from exon 8 to exon 11 (8), which we detected in the typeI$^{-/-}$ macrophages, did not effect this. Two possible reasons can explain the lack of difference in degradation of acetyl LDL and ox LDL. First, it is possible that type I SR-A is normally not highly expressed on thioglycollate elicited peritoneal macrophages, as has been shown for the murine macrophage-like cell line P388D1(18). Type I deficiency will then not results in a change of phenotype. Second, it has been shown, as mentioned earlier, that acetyl LDL and oxidized LDL have equal binding affinities to type I and type II SR-A(18,20). In this respect, type II SR-A can completely take over type I function if protein levels are high enough. It is not clear, yet, if in our mice absence of

type I SR-A results in an upregulation of type II SR-A to establish equal levels of total SR-A activity.

In conclusion, this SR-A type I deficient mouse model will prove very useful to study the in vivo relevance of both SR-A isoforms.

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CHAPTER 8

DIFFERENTIAL EFFECTS OF APOE3LEIDEN AND APOE2(158) ON FOAM CELL FORMATION BUT NOT ON ATHEROGENESIS IN APOLIPOPROTEIN E / LOW DENSITY LIPOPROTEIN RECEPTOR DOUBLE KNOCKOUT MICE.

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Abstract

The role of apolipoprotein E (apoE) mutants in atherogenesis and organ specific pathology was examined in mice deficient for both the low density lipoprotein receptor (*ldlr*) and the endogenous *apoe* gene and expressing moderate levels of either human APOE2 or APOE3Leiden. These mice have similar lipid levels (30-40 mM cholesterol on a chow diet) and are an excellent tool to investigate the human apoE specific effects. At twelve weeks of age all mice had severe atherosclerosis but no differences were found between the three groups. In all three groups complete necroscopy was performed at the age of 8-10 months. Microscopical evaluation revealed that the mice differed in other pathology, including xanthomas, glomerulosclerosis, myocardial degeneration, lesions in the brain and pericentral macrovesicular steatosis in the liver. The xanthomas were equally present in both transgenic groups but were more severe in the *apoe*$^{-/-}$-*ldlr*$^{-/-}$ group. The other phenomena were most severe in the *apoe*$^{-/-}$-*ldlr*$^{-/-}$ and APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ groups and less in the APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice. We conclude that, in the *apoe-ldlr* double knockout mice, APOE3Leiden expression can only reduce some aspects of the phenotype, while APOE2 expression is more capable of rescuing the hyperlipidemia associated pathology.

Introduction

Apolipoprotein E (apoE) is one of the major constituents of circulating lipoproteins, such as chylomicrons and very low density lipoprotein (VLDL) remnants. It serves as a ligand in the receptor mediated uptake of these particles. Mutant forms of apoE may lead to an impaired clearance of remnant lipoproteins from the blood by the liver. This condition is known as Familial Dysbetalipoproteinemia (FD) or Type III hyperlipoproteinemia which is a genetic disorder in the lipid metabolism characterized by hypercholesterolemia and hypertriglyceridemia (1,2). Mutations in the APOE gene can behave as a dominant or recessive trait. The recessive inheritance pattern occurs in FD patients carrying the APOE2(Arg-158→Cys) mutation. Another variant is the rare APOE3Leiden mutation (a 7-amino acid tandem repeat of residues 120-126) which behaves as a dominant trait (3). Both the recessive and dominant mutations of APOE are influenced by additional environmental and genetic factors, which modulate the severity of the disease.

A clinical feature often associated with hyperlipoproteinemia is the occurrence of xanthomas, an accumulation of fat-laden histiocytes. In about 50% of the FD patients, tuberous xanthomas are observed on the elbows and hands (4). In addition, some patients also develop tendinous lesions.

Several reports have shown that, in mouse models, apoE deficiency can result in xanthoma formation. Interestingly, two reports (5,6) showed that cholesterol

feeding results in atypical xanthoma formation. Xanthomatous lesions were observed in various tissues with a predilection for subcutaneous and peritendinous tissues. More recently, Walker *et al.*(7) showed that aged *apoe* deficient mice develop xanthomatous lesions in the brain. A mouse model for Familial Hypercholesterolemia (FH), the low density lipoprotein receptor (*ldlr*) deficient mouse, has also been shown to develop xanthomatous lesions. On a cholesterol diet these mice develop xanthomas in the skin (8). More recently, a knock-in mouse model was generated (9). Replacement of the mouse apoE gene with the human APOE2 variant resulted in spontaneous atherosclerosis and when fed a high fat diet these mice developed xanthomas as well.

In addition, evidence is accumulating for an important role of apoE in atherosclerosis. It has been shown that local apoE production by macrophages in the vessel wall greatly effects atherogenesis in mouse models (10-13). Macrophage specific expression of apoE, through specific promoters or bone marrow transplantation, reduces the severity of atherosclerosis. This is probably due to an enhanced cholesterol efflux, mediated by macrophage derived apoE. Absence of apoE, specifically in macrophages, highly accelerated atherogenesis (11).

We evaluated the role of two FD associated apoE mutants in atherogenesis and in organ specific pathology associated with hyperlipidemia, such as xanthoma formation. Mice deficient in both the *ldlr* and *apoe* and carrying the APOE2 transgene, the APOE3Leiden transgene or no transgene, were examined. We used *apoe* deficiency to be able to study the effects of both the dominant APOE3Leiden and recessive APOE2 variants. In addition, the ldlr was removed to obtain equal plasma cholesterol levels in all three groups

We found that, on an *apoe-ldlr* deficient background, expression of the APOE variants does not reduce atherosclerosis. However, lipid depositions in other organs, such as the liver, kidney, heart and brain, differed between the three groups. In general, the APOE2 variant can rescue some aspects of the hyperlipidemia associated pathology in the $apoE^{-/-}$-$ldlr^{-/-}$ deficient mice, while the APOE3Leiden transgene is much less capable.

EXPERIMENTAL PROCEDURES

Mice

Transgenic mice carrying the human APOE3Leiden and E2(158) gene have been described previously (14). These mice were crossed with *apoe* deficient and *ldlr* deficient mice to yield three groups. All animals are deficient for *apoe* and the *ldlr*. The first group does not have a transgene, the second carries the APOE3Leiden transgene (APOE3Leiden) and the third carries the APOE2(158) transgene (APOE2). All mice were fed a regular breeding chow diet containing 6.2% fat.

Lipid and apoE measurements

Mice were fasted for four hours and blood samples were taken through tail-bleeding. Total serum cholesterol and triglyceride levels were measured enzymatically using commercially available kits: no.236691 (Boehringer Mannheim, Mannheim, Germany) and no.337-B (Sigma Chemicals Co., St. Louis, MO.). Human apoE concentrations were measured by sandwich ELISA as described previously (15).

Atherosclerosis

Groups of 5-6 female mice were sacrificed at the age of 12 weeks. The heart was removed, cutting the aorta just above the atria and bisected perpendicular to the heart axis, just below the atrial tips. The base of the heart was taken for analysis and quick-frozen perpendicular on a piece of liver in liquid nitrogen. The heart was sectioned on a cryostat, starting within the heart and working in the direction of the aortic arch as described by Paigen *et al.* (16). Once the atrioventricular valves were identified and the media was visible, 8 μm sections were taken and mounted on gelatinized slides. Sections were collected until the valves disappeared. For atherosclerosis quantification 3 toluidine stained sections were used. Using computer-aided morphometry (Kontron-Videoplan, Zeiss, Germany) total area of the lesions was calculated. Lesion were qualified as described previously (17) using oil red O and toluidine stained sections.

Histological analysis

Groups of 3-8 male mice were sacrificed at 8-10 months of age. Complete necroscopy was performed. Tissues were fixed in 4% neutral-buffered formaldehyde, processed and embedded in paraffin. Three μm sections were routinely stained with hematoxylin-eosin.

RESULTS

Lipid analysis and atherosclerosis

Cholesterol and triglyceride levels did not differ between both human APOE transgenic groups but were lower in the *apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice without transgene (table I). Human apoE levels were higher in APOE2 mice. No differences were observed in lesion areas between all three groups at 12 weeks of age (fig. 1). In addition, the cellular composition of the lesions was examined. Here also, no differences were observed (data not shown).

Table I: Serum lipid and human apoE levels in *apoe*$^{-/-}$-*ldlr*$^{-/-}$, APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ and APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice.

	Apoe$^{-/-}$-*ldlr*$^{-/-}$	APOE3Leiden *Apoe*$^{-/-}$-*ldlr*$^{-/-}$	APOE2 *Apoe*$^{-/-}$-*ldlr*$^{-/-}$
Cholesterol (mM)	30.4 ± 3.0	37.0 ± 4.0	39.1 ± 7.1
Triglycerides (mM)	0.5 ± 0.1	5.9 ± 2.6	9.6 ± 7.0
apoE (mg/dl)	n.a.	19.9 ± 6.3	28.8 ± 2.2
N	5	6	5

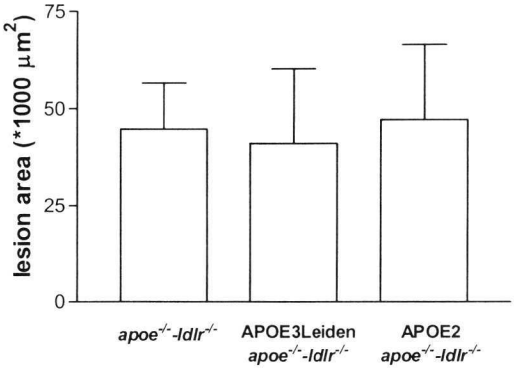

Figure 1: Atherosclerosis in *apoe*$^{-/-}$-*ldlr*$^{-/-}$, APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ and APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice. Lesion area was determined at the age of 12 weeks as described in the methods. Values are mean ± S.D.

Pathology

Mice were used at the age of 8-10 months for the pathological examination. Lipid levels in these mice were approximately equal to those used for the evaluation of atherosclerosis at 12 weeks of age. Accumulation of fat-laden macrophages in various tissues were the most pronounced pathological lesions observed in the three groups. An overview of the pathology observed and the differences between the three groups is given in table II. The heart showed myocardial degeneration in the *apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice and the APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice, characterized by intramyocardial fat droplets, muscle necrosis and fibroblast proliferation (fig. 2a). Moreover, foam-cells were observed perivascular of the small coronary capillaries (fig. 2b). These lesions were never observed in the APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice (Table II). All animals showed perivascular foam cells in glomeruli of the kidneys. In addition, the mesangium was most severe thickened in the APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice with scattered necrotic cells in the glomeruli (table II and fig. 3a). In all mice the renal capsule showed moderate xanthomatous lesions with cholesterol clefts, foam cells and multinucleated giant cells (fig. 3b). All *apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice, 2 out of 5

Table II: Pathology in *apoe*$^{-/-}$-*ldlr*$^{-/-}$, APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ and APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice.

	Apoe$^{-/-}$-*ldlr*$^{-/-}$	APOE3Leiden *Apoe*$^{-/-}$-*ldlr*$^{-/-}$	*APOE2 Apoe*$^{-/-}$-*ldlr*$^{-/-}$
Myocardial degeneration	+	+	-
Pericentral hepatocellular lipid deposition	++	+++	+/-
Xanthomas (typical)	++	+	+
Glomerulosclerosis	+	++	+

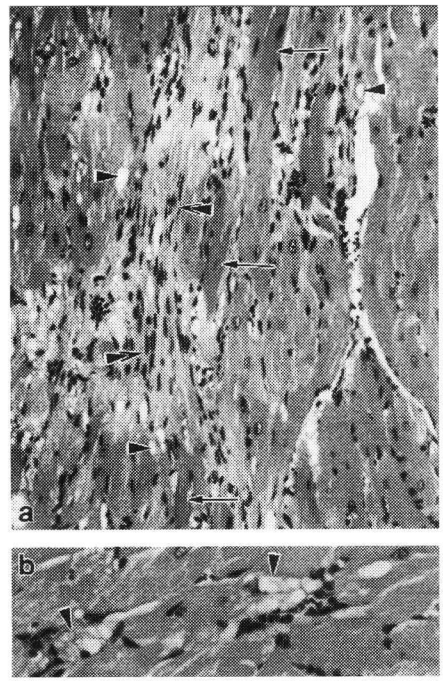

Figure 2: Heart of an APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mouse. (a) Myocardial degeneration (arrows) with intracellular lipid droplets (arrowheads) and fibroblast proliferation (double arrowheads); x250. (b) Foam cells (arrowhead) perivascular of coronary capillaries; x400. These photographs are representative for all APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ as well as the *apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice. HE staining.

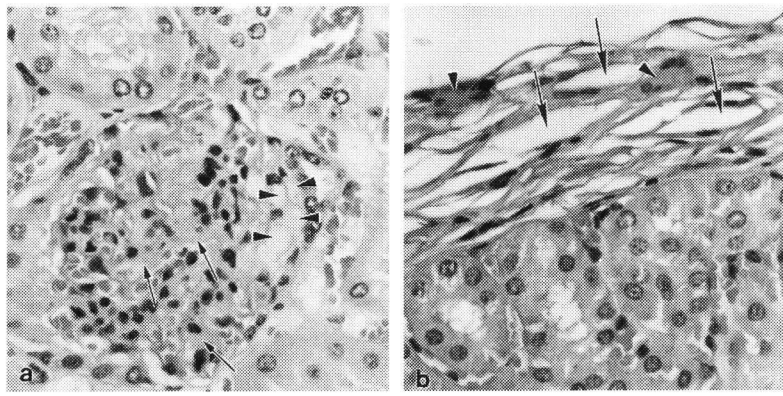

Figure 3. (a) Glomerulus in a kidney of an APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mouse. Note the foam cells (arrowhead) and the mesangium thickening (arrows). (b) Xanthomatous lesion in renal capsule of the kidney of an *apoe*$^{-/-}$-*ldlr*$^{-/-}$ with cholesterol clefts (arrows) and giant cells (arrowheads). This photograph was representative for all mice. HE staining; x400

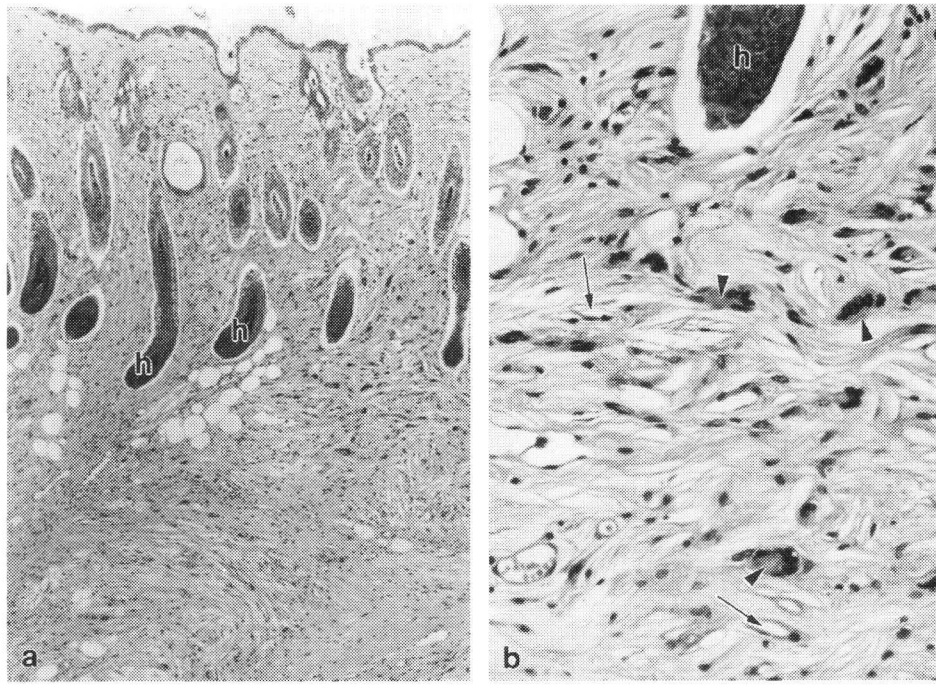

Figure 4. (a) Xanthoma in the dermis of the skin of an APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mouse; x50. (b) Higher magnification of the xanthomatous lesion with cholesterol clefts (arrows), giant cells (arrowheads) and necrosis (double arrowheads); x400. h: hairshaft. This photograph was representative for the mice with these xanthomatous lesions. HE staining.

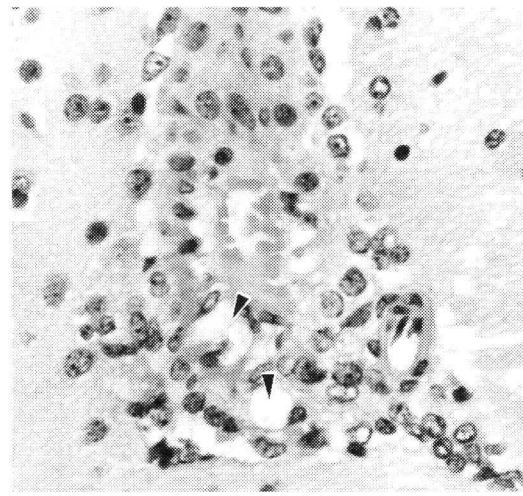

Figure 5. Foamcells (arrowheads) at the origin of the choroid plexus in an APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mouse. HE staining; x400. This photograph was representative for the mice with these lesions.

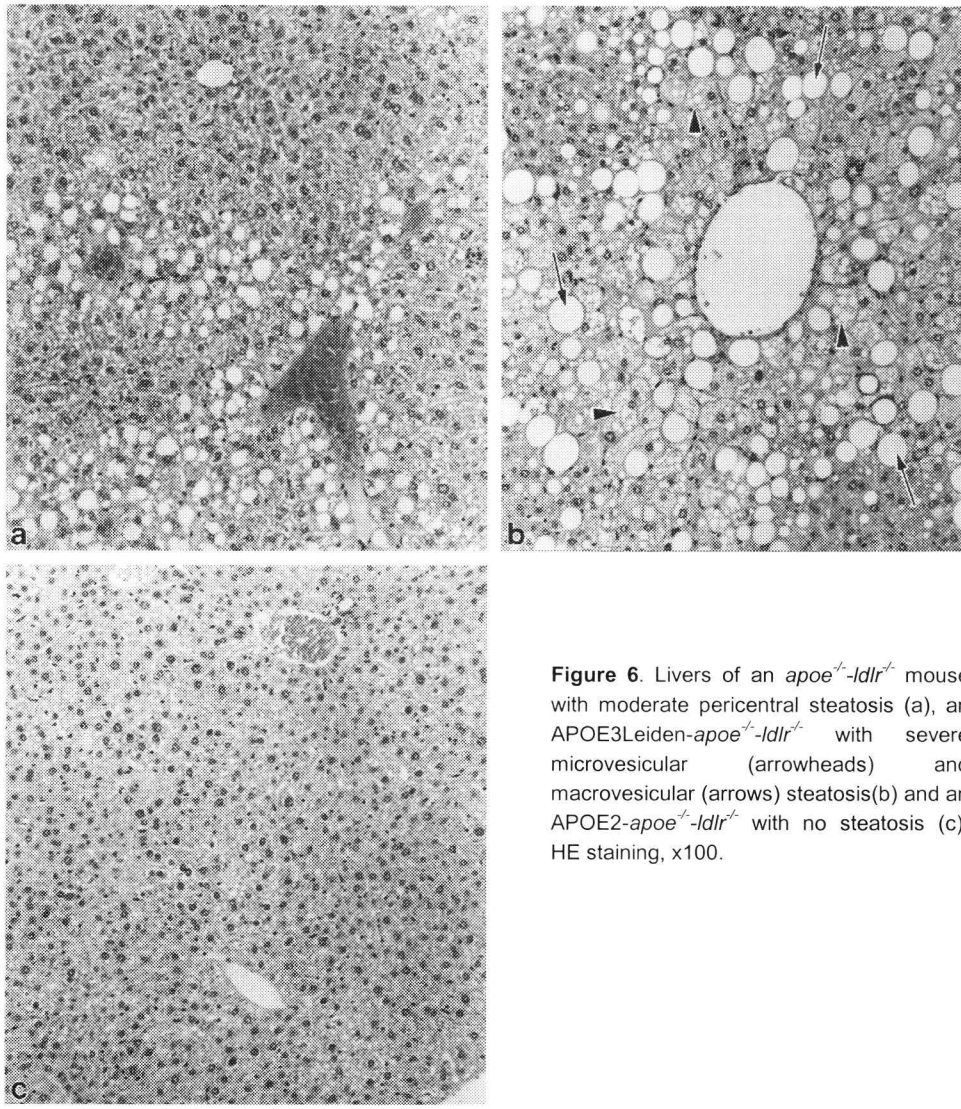

Figure 6. Livers of an *apoe*$^{-/-}$-*ldlr*$^{-/-}$ mouse with moderate pericentral steatosis (a), an APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ with severe microvesicular (arrowheads) and macrovesicular (arrows) steatosis(b) and an APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ with no steatosis (c); HE staining, x100.

APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice and 3 out of 8 APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice showed extensive xanthomatous lesions in the axillary region of the skin with cholesterol clefts, multinucleated giant cells and necrosis (fig. 4, table II). Some animals also had periarticular and peritendinous xanthomatous lesions. In the brain, perivascular foam cells were observed in 2 out of 3 *apoe*$^{-/-}$-*ldlr*$^{-/-}$, 2 out of 5 APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice, and one of the APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice at the origin of the choroid plexus (fig. 5). This APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mouse showed a xanthomatous lesion subependymal in

the roof of the third ventricle. Finally, in the liver the *apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice showed moderate pericentral macrovesicular steatosis (fatty liver) (fig. 6a), while in the APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice severe micro- and macrovesicular steatosis was observed (fig. 6b). The APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice showed no steatosis with exception of two animals which showed only very mild macrovesicular steatosis (fig. 6c, Table II).

Discussion

In this paper, we show that mice deficient for apoE and the LDLR develop pathological lesions in response to the overt hyperlipidemia. Expression of APOE2 and APOE3Leiden is only partially able to reduce the severity of some of the lesions. None of the apoE variants could attenuate the development of atherosclerosis. In *apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice that carried the transgene APOE2(158) or APOE3Leiden, the atherosclerotic lesion area was similar as in the control groups. In addition, no differences were found in the cellular composition of the atherosclerotic lesions. However, differences were found on close pathological examination of other organs of the mice at 8-10 months of age. Xanthomatous lesions in the skin developed in all mice. However, the extent of these xanthomas in the mice expressing APOE2 or APOE3Leiden was decreased as compared to the *apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice. Glomerulosclerosis was most severe in APOE3Leiden mice as compared to APOE2 and nontransgenic mice. Furthermore, the APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice had less severe foamy infiltrations in the heart and no lipid depositions in the liver in comparison to the other two lines (table II). Thus, for these lesions APOE2 was able to rescue the phenotype, while APOE3Leiden was not. Generally spoken, the pathological lesions, apart from the xanthomas, are most severe in the *apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice, then the APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice and relatively mild in the APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice.
Recent experiments using *apoe*$^{-/-}$ mice have shown an important role for macrophage derived apoE in atherogenesis. It was described that transplantation of bone marrow from apoE deficient mice to wild-type mice resulted in a 10-fold increase of atherosclerosis, without effecting serum cholesterol levels (11). Other experiments using arterial wall expression (12) or a macrophage specific promoter (10) to drive apoE expression, showed a strong reduction in the extend of atherosclerosis. These data illustrate that local expression of apoE in macrophages does effect the atherosclerotic process. This is probably due to the fact that apoE mediates the efflux of cholesterol from macrophages. We have confirmed macrophage expression of apoE in both our APOE3Leiden and APOE2 transgenic lines, by Northern hybridization (data not shown). In addition, recent *in vitro* experiments have shown that both APOE3Leiden and APOE2 are able to mediate the efflux of cholesterol from lipid laden macrophages at a rate equal to wild-type macrophages (M. van Eck, personal communication). The double knockout mice are

hyperlipidemic and have equal serum cholesterol levels. Thus, these mice provide us the opportunity to evaluate the effects of two APOE variants on hypercholesterolemia associated pathology. Since macrophages from both transgenics express APOE, we expected that both transgenes would attenuate atherogenesis. However, in our mice we see no effect of the presence or absence of APOE in double knockout mice on atherosclerosis. Probably, this is attributable to the fact that lesions develop through a continuous process of influx and efflux of lipid. In our mice cholesterol levels are high, resulting in a massive influx of cholesterol into the macrophages in the vessel wall giving severe atherosclerosis. How the expression of the APOE3Leiden or APOE2 transgene effects the cellular influx is, presently, not clear. The enhanced efflux mediated by the APOE transgenes is not able to modify the cholesterol deposition resulting from the influx. The net result is that in the presence or absence of APOE on a double knockout background no difference in the lesions is observed.
The xanthomas in the skin of our mice are also composed of lipid laden macrophages. Both for the apoE deficient and ldlr deficient mice the formation of xanthomas has been described (5,6,8). We tested if the APOE3Leiden or the APOE2 variant could attenuate this. Indeed, in our mice the APOE variants are able to diminish xanthoma formation caused by the absence of both apoE and the ldlr, since both APOE3Leiden and APOE2 show a reduced xanthoma formation. In this process, the apoE mediated efflux may be effective in reducing the macrophage derived lesion formation. In other processes in which macrophages are involved, glomerulosclerosis and fatty deposits in the heart and brain, the efflux had a less clear effect and differences observed could not be solely explained by apoE mediated efflux.

The mice we examined differ strongly in their lipid deposition in the liver. In this deposition many different processes may be involved. For instance, production of lipoprotein particles may be disturbed resulting in an increased accumulation of lipid in the hepatocytes. Previously, it was shown that on an *apoe*$^{-/-}$ background the production of VLDL particles as measured by triglyceride production is impaired in APOE3Leiden and normal in APOE2 mice(14). However, in our mice both APOE3Leiden and APOE2 were not able to rescue the production of VLDL. Hepatic VLDL triglyceride production was the same in *apoe*$^{-/-}$*-ldlr*$^{-/-}$ mice without or with the APOE3Leiden or APOE2 transgene (K. Willems van Dijk, personal communication). Thus, the difference in hepatic lipid deposition is not caused by differences in VLDL production. Other disturbances in uptake, intracellular processing and efflux are possibly involved. The APOE3Leiden transgenic mice do show deposits of apoE in the liver (unpublished observations). This may disregulate cellular function and result in the lipid deposition observed.

From our data it is clear that absence of both apoe and the ldlr results in severe lipid lesions throughout the body. Expression of two human apoE variants can only partially rescue this. The effect of APOE3Leiden and APOE2 is different in

different organs probably due to different effects of these APOE variants on the cellular lipid metabolism in the various organs.

Acknowledgements

This work was supported by the Dutch Heart Foundation and the Netherlands Organization for Scientific Research. M. Hofker is an established investigator of the Dutch Heart Foundation.

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CHAPTER 9

DISCUSSION AND FUTURE PERSPECTIVES

DISCUSSION AND FUTURE PERSPECTIVES

Atherosclerosis is the major factor contributing to mortality due to cardiovascular diseases. It is characterised by the accumulation of foam cells in the vessel wall and accompanied by an inflammatory response. These foam cells result from the unrestricted uptake of modified lipoproteins, through scavenger receptor pathways. The SR-A was the first identified scavenger receptor that could mediate the formation of foam cells.

The exact in vivo relevance of the SR-A was first studied when the SR-A deficient mice were generated (1). However, the results of experiments examining the effect of SR-A deficiency on atherosclerosis were not as straightforward as might be expected from the previous in vitro en in vivo data. On an apoE deficient background, absence of SR-A resulted in a 60% reduction in atherosclerosis, as deduced by lesion area measurements in the proximal aorta. Taken into account that this reduction was accompanied by an increase in plasma cholesterol level, the effect was a huge and completely in line with the general hypothesis of the SR-A mediating foam cell formation by uptake of modified LDL and thereby being a pro-atherogenic factor. However, when crossed onto an LDL-receptor background the results were somewhat different (2). Absence of the SR-A again resulted in a decrease in lesion formation. However, this moderate effect coincided with a 20% decrease in plasma cholesterol levels. Taken into account the dependence of lesion formation on plasma cholesterol levels one could state that in this mouse model the effect of absence of the SR-A was virtually absent.

THE SR-A CAN ACT ANTI-ATHEROGENIC

Based on the results from Kodamas group, absence of the SR-A in APOE3Leiden transgenic mice was expected to decrease atherosclerosis. To our surprise absence of the SR-A did not decrease lesion area formation in APOE3Leiden mice. In contrast, there was even a trend towards an increase in lesion area. Upon close examination of the lesions we found that, in addition to a trend towards an increase in lesion area, the lesions in the SR-A deficient mice were more severe as compared to mice with the wildtype SR-A allele. Thus, in our experiments the SR-A turned out to exert an anti-atherogenic property.

To expand the possibilities to investigate the role of the SR-A in atherosclerosis in vivo, we generated a mouse model with high level expression of the human SR-A gene (MSR1) according to its natural pattern. To accomplish this we used a 180 kb yeast artificial chromosome (YAC) carrying the 80 kb MSR1 gene located in the middle.

Transgenic mice showed a natural expression pattern, i.e. we found expression in isolated macrophages and demonstrated expression in different tissue macrophages as well. The overexpression enhanced the metabolism of modified LDL and increased in vitro foam cell formation in isolated peritoneal macrophages. Previous experiments using SR-A overexpression were mostly performed in Chinese hamster ovary cells, using cDNA constructs (3). We now demonstrated that under its natural promoter and enhancer elements, overexpression of the SR-A in macrophages can indeed enhance foam cell formation. How the overexpression would effect atherosclerosis was assessed in two atherosclerotic mouse models. First, the MSR1 transgenic mice were crossed onto a LDLR-receptor deficient background. Mice overexpressing MSR1 had smaller lesions as compared to mice without the MSR1 transgene. Second, bone marrow from wildtype and MSR1 transgenic mice was isolated and transplanted to lethally irradiated APOE3Leiden mice. Again, in the mice that had received the bone marrow with MSR1 overexpression, lesions appeared to be smaller and thus the SR-A protected against atherosclerosis. Since macrophage foam cell formation was decreased, these observations completely uncouple the in vitro foam cell formation from the actual in vivo atherogenesis, as these two parameters did not correlate in our experiments. To explain the anti-atherogenic effects of the SR-A, additional SR-A functions may be important. Interestingly, we found that the activation of MSR1 macrophages by LPS, as measured by nitric oxide production, was changed as compared to wildtype macrophages, which may contribute to anti-atherogenic effects of the SR-A.

AN ANIMAL MODEL TO STUDY TYPE I SR-A FUNCTION

The SR-A occurs mainly in two isoforms, type I and type II, generated by alternative splicing of the same gene. They differ in their C-terminal domain. Type I contains a cystein-rich domain, which is lacking in the type II SR-A. Despite their structural difference no significant differences in binding properties of several SR-A ligands have been found between type I and type II. However, specific regulation of type I and II has been described. In general, type I appears to be upregulated upon macrophage differentiation, although most macrophages co-express type I and type II SR-A. To be able investigate the function of type I SR-A versus type II SR-A we generated type I specific knockouts. *In vitro* studies showed that SR-AI$^{+/+}$ and SR-AI$^{-/-}$ peritoneal macrophages exerted equal degradation and association of both oxidized and acetylated LDL. Absence of a difference in this respect suggests that SR-AI is absolutely required in these processes in peritoneal macrophages. To establish the effects of absence of SR-AI on atherogenesis, atherosclerosis experiments should be performed.

These could be done by performing bone marrow transplantation to APOE3Leiden mice. This mouse model gives the unique possibility to study the role of SR-AI and SR-AII in atherogenesis and other SR-A related processes, *in vivo*.

Modifications of LDL

Our experiments using several different mouse models clearly show that the SR-A can act anti-atherogenic. But the question arises what oxidative modifications to lipoproteins and which uptake routes are really of major importance in atherogenesis. Several lines of evidence now indicate the important role of oxLDL in atherosclerosis. LDL isolated from atherosclerotic lesions, but not from healthy vessels, strongly resembles in vitro oxidized LDL (4,5). Epitopes that are characteristic for oxLDL can be demonstrated in atherosclerotic lesions by immunohistochemistry (6-8). Both atherosclerotic lesions and serum contain autoantibodies against oxLDL (9-11). Finally, several experiments in animal models have shown the anti-atherosclerotic effects of anti-oxidative treatments (12,13).

However, oxLDL found in atherosclerotic lesions is not a homogenous population of particles. It contains a wide range of LDL particles modified to a different extent. Two different kinds of oxLDL can be discriminated. The biological properties of both modified forms of LDL are not identical. First, minimally oxidized LDL (mmLDL) can occur. These particles can be defined by the presence of only mild lipid oxidation, proteins are still unaffected. mmLDL will affect the expression of inflammatory mediators, but it does not cause lipid accumulation in cells. It has been described to induce macrophage colony stimulating factor (MCSF) (14) and monocyte chemotactic protein-1 (MCP1) (15). In the more extensively modified LDL (oxLDL), lipid peroxidation is more extensive and apoB is modified as well. OxLDL is cytotoxic to various blood cells and highly chemotactic to circulating monocytes (16). Additionally oxLDL can result in uptake through scavenger pathways, that will lead to foam cell formation.

The forms of oxLDL can arise through the action of a broad range of molecules. These include, lipoxygenase, locally expressed nitric oxide synthase and myoloperoxidase. In addition to these molecules, LDL can get modified by more specific enzymes, such as sphingomyelinase and secretory phospholipase A_2. These enzymes mediate the formation of mmLDL by conversion of native phospholipids present in LDL to more biologically active phospholipids (17). Additionally, secretory phospholipase A_2 makes LDL more susceptible to more extensive oxidation by other enzymes (18), such as lipoxygenases, and sphingomyelinase can cause LDL aggregation (19). Recently, it was demonstrated that increased expression of secretory phospholipase A_2 enhances

atherosclerosis in mice (20), although this effect may be, at least partly, due to effects on plasma lipoprotein levels. In more advanced lesions auto-oxidation of LDL may occur in the necrotic areas, which abundantly contain extracellular lipids and metal ions.

RECEPTORS INVOLVED IN ATHEROGENESIS

The various modifications of LDL, will have different effects on the uptake route of the resulting oxidized LDL. Copper oxidized LDL can mediate foam cell formation and is a good ligand for the SR-A. Oxidation of LDL by myeloperoxidase also results in the formation of a form of LDL that can mediate foam cell formation (21). However, uptake of myeloperoxidase modified LDL does not involve the SR-A. Other receptors are probably involved. These may include other members of the scavenger receptor family, such as CD36, CD68 (in mouse macrosialin) or LOX-1. All receptors can bind oxLDL but it is not clear to what extend differently modified forms of LDL will bind. Very recently, CD36 deficient mice came available (22). They show a strong reduction in binding of oxLDL. Atherosclerosis experiments showed that absence of CD36 in apoE knockout mice strongly reduced lesion formation, indicating a significant role for CD36 in atherogenesis. However, CD36 deficient mice also showed increased levels of HDL, which is known to protect against atherosclerosis. Thus, the exact relative contribution of CD36 in the vessel wall on lesion formation, remains uncertain. Originally LOX-1, also a member of the scavenger receptor family, was cloned from bovine endothelial cells. Recently, it was shown that LOX-1 is expressed in human atherosclerotic lesions by endothelial cells, macrophages and smooth muscle cells, indicating that this receptor may also be involved in foam cell formation in the vessel wall (23).

In addition to these scavenger receptors, it should be noted that several receptors recognising unmodified lipoproteins are also present in lesions, including the LRP, VLDLR and LDLR (24-26). The LRP and VLDLR are not down-regulated by intracellular cholesterol levels and could thereby mediate foam cell formation. Thus, these receptors might be able to contribute to the atherosclerotic process. Again, the relative contribution of these receptors is remains unclear. Future experiments using LRP or VLDLR deficient mice or bone marrow from LRP or VLDLR deficient mice should give more insight whether these receptors contribute to atherosclerosis

From these data, it should be obvious that atherogenesis involves a broad range of LDL modifications and uptake routes. Within an atherosclerotic lesion a variety of modifications can alter the biochemical properties of LDL. These modifications are likely to occur through several different enzymatic and non-enzymatic pathways. The resulting modified lipoproteins might be taken up by different receptors, of which their relative

significance is still unclear. Furthermore, the major atherogenic effects of modified LDL may not be by mediating foam cell formation but due to its pro-inflammatory property.

ANTI-ATHEROGENIC PROPERTIES OF THE SR-A

Interestingly, but the SR-A is a multi-functional receptor and can protect against atherosclerosis. To explain this protective effect of SR-A several SR-A functions should be taken into account. First, uptake of modified LDL can be beneficial because intracellular modified LDL is less harmful then extracellular LDL. Modified LDL in the early atherosclerotic lesion can exert its pro-inflammatory action on different celltypes (14,15). This will increase the inflammation, attract more cells to the lesion and enhance the progression of atherosclerosis. In addition, it can become more modified and thereby become even more harmful. Uptake of modified LDL by macrophages through the SR-A, removes these harmful substances from the vessel wall. In addition, the SR-A can affect the migration of the macrophage in the plaque via its role in cellular adhesion (27). SR-A may thereby enhance the ability of macrophages to remove debris. Thus, scavenging of modified LDL can be beneficial and reduce the progression of the atherosclerotic process (28). In addition, the SR-A has been shown to be involved in the uptake of modified proteins for presentation to antigen-specific T cells (29). Several papers have also shown that immunisation of rabbits using modified LDL protects against atherosclerosis (30,31). Therefore, one could assume that this protective effect of immunisation requires, at least to some extend, SR-A, again making the SR-A beneficial in atherogenesis. Finally, several molecules present in the plaque are known inflammatory mediators, including modified LDL and AGE modified proteins. It is unknown which uptake routes for these molecules lead to their inflammatory action. Uptake of modified LDL by the SR-A may be a pathway resulting in less activation as compared to other uptake routes, as has been described for LPS (32). Again, this can reduce inflammation in the early atherosclerotic lesion and reduce the progression of atherosclerosis. Future research is needed to establish the exact relevance, in humans, of the SR-A in atherogenesis. However, our results show that the SR-A is not always a pro-atherogenic factor.

FUTURE FOCUS

Recently, some work is focussing on the design of new molecules that can act as SR-A antagonists (33), to use as drugs against atherosclerosis. These are tested on their ability to prevent uptake of modified LDL, *in vitro*. However, we clearly show that

the uptake of modified LDL and the formation of foam cells in vitro, do not correlate with atherogenesis in vivo. These two actually opposed in our results using the MSR1 overexpressing mice. Future research should focus on the aforementioned functions of the SR-A to understand why the SR-A can sometimes act pro- and other times anti-atherogenic, and how to control this.

The broad range of modifications occurring in the atherosclerotic lesion and their relative role in atherogenesis should be delineated. These modifications might be tested for their uptake routes by macrophages and/or other cells. This could be done using in vitro modified LDL isolated from co-cultures with cells overexpressing the different modifying enzymes, such as myeloperoxidase. The uptake routes (i.e. receptors) resulting in the activation pathways stimulated by the different forms of LDL should be examined. For this, isolated cells from mice deficient or overexpressing the variety of receptors possibly involved could be used. Not much is known if and how the SRA elicits signal transduction pathways by the uptake of ligands, such as modified LDL. Only for LPS it has been shown that uptake through SR-A does not result in signalling while binding of LPS to other receptors (such as CD14) does result in signalling and cellular activation (32). In the atherogenic process, in which modified LDL is regarded as one of the main inflammatory mediators, the pathways by which modified LDL activates cells are crucial in understanding atherogenesis. It is known that the activation of endothelial cells and macrophages in atherosclerotic lesions involves the transcription factor NF-kB. Activated NF-kB is crucial for the expression of several pro-inflammatory mediators, such as cytokines and adhesion molecules. Whether the activation of NF-kB involves the SR-A and/or other routes is unknown and should be studied in future work. The focus should also be on the investigation of the inflammatory gene expression activated by the SR-A in vivo. To study this SR-A deficient or MSR1 transgenic mice could be evaluated during very early atherogenesis, using antibodies against a variety of activation markers.

In summary, the translation of in vitro results to in vivo atherogenesis remains difficult. A good focus for future research should be the role of differently modified lipoproteins in the inflammatory response involved in atherosclerotic lesion development.

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SUMMARY

SUMMARY

In the present thesis we have investigated the role of the SR-A in the development of atherosclerosis. Atherosclerosis is the major factor contributing to mortality due to cardiovascular diseases. It is characterised by the accumulation of fat-laden macrophages in the vessel wall, initiated and accompanied by a strong inflammatory response. These so called foam cells result from the unrestricted uptake of modified lipoproteins from the vessel wall, through scavenger receptor pathways. The SR-A was the first identified scavenger receptor that could mediate the formation of lipid-laden cells in vitro, resembling the foam cells present in the atherosclerotic lesion. Expression of SR-A in transfected Chinese hamster ovary cells results in the formation, after incubation with acetylated LDL, of foam cells. Furthermore, the SR-A is highly expressed in atherosclerotic lesions and the SR-A ligand oxLDL has also been demonstrated in lesions. Based on these in vitro and in vivo results the SR-A was assigned an important role in atherogenesis.

To further explore the exact role of the SR-A in atherogenesis the research described in this thesis was performed.

Based on the previous results obtained by Kodamas group using the SR-A deficient mice in combination with the apoE-knockout and LDL-receptor knockout mice, absence of the SR-A in APOE3Leiden transgenic mice was expected to decrease atherosclerosis. In chapter 3 we compared both male and female APOE3Leiden mice with and without the SR-A. After 10 weeks high fat diet, lesion area was measured and the lesions were examined morphologically. To our surprise absence of the SR-A did not decrease lesion area formation. In contrast, there was even a trend towards an increase in lesion area. We examined the lesions in all mice more closely and categorised them according to their severity. Three categories were made. Type I lesions, the fatty streak, only contained foam cells; in type II lesions, the mild plaque, also fibrotic cells were present and type III lesions, the severe plaques, showed much fibrosis, media damage and necrosis. When examining the lesions we found that, in addition to the observed increase in lesion area, the lesions in the SR-A deficient mice were more severe as compared to mice with the wildtype SR-A allele. These results clearly showed that in the APOE3Leiden transgenic mice absence of the SR-A does not decrease atherosclerosis but actually enhances atherosclerosis. In our experiments the SR-A turned out to act as an anti-atherogenic property.

To expand the possibilities to investigate the role of the SR-A in atherosclerosis *in vivo*, we generated a mouse model overexpressing the SR-A. To accomplish this we

wanted to generate a natural overexpression mouse model, using the complete human SR-A gene (MSR1) with its natural genomic environment. This restricted us to the use of large DNA vectors since the MSR1 region alone is already 80 kb in size. Screening of literature and genomic libraries resulted in the isolation of two large (>1 Mb) yeast artificial chromosomes (YAC) that carried the MSR1 gene. On YAC 766_a_12 the MSR1 gene was located near one vector arm. The use of a 1 Mb YAC for oocyte injection would give handling problems and increase the possibility of introducing additional genes. Therefore, we decided to down-size this YAC. To accomplish this a new set of fragmentation vectors was designed (Chapter 4). These vectors can be used to recombine with alu-repeats dispersed throughout the human DNA, thereby introducing new and selectable vectors arms. In this way the YAC could be shortened to the desired size.

In chapter 5, using these vectors, a new YAC of 180kb (yHSR1) was generated with the MSR1 gene approximately in the middle. Transgenic mice showed a natural expression pattern, i.e. we demonstrated expression in isolated macrophages and in different tissue macrophages as well. In addition, regulation of the MSR1 gene in macrophages from MSR1 transgenic mice appeared to be normal. The overexpression enhanced the metabolism of modified LDL by isolated peritoneal macrophages. Degradation of acetylated LDL was approximately 2-3 fold increased in MSR1 macrophages as compared to wildtype macrophages. When incubated overnight with acetylated LDL more foam cells developed, as was measured by oil red O staining, and more cholesteryl esters accumulated in the macrophages. Previous experiments using SR-A overexpression were mostly performed in Chinese hamster ovary cells, using cDNA constructs. We now demonstrated that under its natural promoter and enhancer elements, overexpression of the SR-A in macrophages can indeed enhance foam cell formation *in vitro*. How this would turn out to effect atherosclerosis was assessed in the two atherosclerotic mouse models.

In chapter 6 the MSR1 transgenic mice were crossed onto an *ldlr* deficient background. After feeding these mice a high fat diet for 10 weeks they had severe hypercholesterolemia. No differences, however were found between the mice with or without the MSR1 in plasma cholesterol or triglyceride levels or lipoprotein profile. Measuring lesion area revealed that mice overexpressing MSR1 had smaller lesions as compared to mice without the MSR1 transgene. In addition, when cholesterol exposure was taken into account, the difference became even more significant. In this model the SR-A actually protected against atherosclerosis. Next, we assessed the effect of MSR1 overexpression in a completely different model. Therefore we performed a bone marrow transplantation to APOE3Leiden transgenic mice. Bone marrow from wildtype and MSR1 transgenic mice was isolated and transplanted to lethally irradiated APOE3Leiden

mice. This resulted in repopulation of the APOE3Leiden bone marrow with either wildtype or MSR1 transgenic bone marrow. Using this method we could easily assess the effects of MSR1 expression in bone marrow derived cells (macrophages) on atherosclerosis in APOE3Leiden mice. The mice were fed a high fat diet for 8 weeks. This resulted in milder hypercholesterolemia as compared to the *ldlr* deficient mice. Again, no effect was observed on lipid parameters. However, in the mice that had received the bone marrow with MSR1 expression, lesions appeared to be smaller. This difference did reach statistical significance when the individual differences in cholesterol exposure were taken into account. Again, in this completely different mouse model as compared to the *ldlr* knockout mouse, the SR-A protected against atherosclerosis. These observations completely uncouple the in vitro foam cell formation from the actual in vivo atherogenesis, as these two parameters actually opposed in our experiments. To explain the anti-atherogenic effects of the SR-A, additional SR-A functions may be important. We tested one of them, by examining in vitro macrophage activation in response to the bacterial surface molecule lipopolysaccharide (LPS). MSR1 macrophages showed a decreased response to LPS, as measured by nitric oxide production. Thus, the activation of MSR1 macrophages was changed as compared to wildtype macrophages, and this may contribute to the change in macrophage behaviour in atherosclerotic lesions, leading to the observed anti-atherogenic effects of the SR-A.

The SR-A occurs mainly in two isoforms, type I and type II, generated by alternative splicing of the same gene. They differ in their C-terminal domain. Type I contains a cystein-rich domain, which is lacking in the type II SR-A. Despite their structural difference no significant differences in binding properties of several SR-A ligands have been found between type I and type II. However, specific regulation of type I and II has been described. Generally spoken, type I appears to be upregulated upon macrophage differentiation, although most macrophages co-express type I and type II SR-A. The *in vivo* function of the cystein-domain in type I SR-A has not been elucidated yet.

To be able investigate the function of type I SR-A versus type II SR-A we generated type I specific knockouts, which is described in chapter 7. To accomplish this a replacement targeting vector was generated. Using this vector exon 10 of the murine SR-A gene, which is SR-AI specific, was replaced by a neomycin resistance cassette via homologous recombination in embryonic stem cells. This approach yielded heterozygous ES-cells, which were used to generate chimeric mice. Male chimeric mice were again bred and heterozygous offspring was intercrossed to yield homozygous SR-AI knockout mice. PCR analysis of cDNA generated from RNA isolated from macrophages from SR-AI wildtype ($SR\text{-}AI^{+/+}$) , heterozygous ($SR\text{-}AI^{+/-}$) and homozygous

(SR-AI$^{-/-}$) knockout mice was performed. This showed that in the SR-AI$^{-/-}$ mice no transcript for type I SR-A was present. However, some RNA was detected that was generated from splicing from exon 8 to exon 11. This resulted in a type III mRNA, which has been described recently as a dominant negative isoform of SR-A. When coexpressed with SR-AI or SR-AII, SR-AIII blocks SR-A function as measured by acetylated LDL uptake. However, the expression of type III SR-A in our SR-AI$^{-/-}$ mice was rather low. *In vitro* studies showed that SR-AI$^{+/+}$ and SR-AI$^{-/-}$ peritoneal macrophages exerted equal degradation and association of both oxidized and acetylated LDL. Absence of a difference in metabolism of these two forms of modified LDL confirm that SR-AI is not necessary in these processes in peritoneal macrophages. This mouse model gives the unique possibility to study the role of SR-AI and SR-AII in atherogenesis and other SR-A related processes, *in vivo*.

In chapter 8 the role of apolipoprotein E (apoE) mutants in atherogenesis and organ specific pathology was examined in mice deficient for both the low density lipoprotein receptor (*ldlr*) and *apoe*, carrying either the APOE2(158) transgene (APOE2), the APOE3Leiden transgene or no transgene. The apoE variants were crossed back on an *apoe-ldlr* double knockout background to investigate the human apoE specific effects in the presence of similar lipid levels. These mice have similarly elevated serum cholesterol levels (30-40 mM) on a chow diet. At twelve weeks of age all mice had severe atherosclerosis but no differences were found between the three groups. In all three groups complete necroscopy was performed at the age of 8-10 months. Microscopical evaluation revealed that the mice differed in other pathology, including xanthomas, glomerulosclerosis, myocardial degeneration, lesions in the brain and pericentral macrovesicular steatosis in the liver. The xanthomas were equally present in both transgenic groups but were more severe in the *apoe*$^{-/-}$-*ldlr*$^{-/-}$ group. The other phenomena were most severe in the *apoe*$^{-/-}$-*ldlr*$^{-/-}$ and APOE3Leiden-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ groups and less in the APOE2-*apoe*$^{-/-}$-*ldlr*$^{-/-}$ mice. We conclude that, in the *apoE.ldlr* double knockout mice, APOE3Leiden expression can only reduce some aspects of the phenotype, while APOE2 expression is more capable of rescuing the hyperlipidemia associated pathology in these mice.

SAMENVATTING IN HET NEDERLANDS

In dit proefschrift wordt het onderzoek naar de rol van de scavenger receptor klasse A (SR-A) in de ontwikkeling van atherosclerose beschreven. Atherosclerose is de belangrijkste oorzaak van sterfte door hart- en vaatziekten. Het wordt gekenmerkt door de stapeling van met vet geladen macrofagen in de vaatwand, geinitieerd door en samengaand met een sterke onstekingsreaktie. Deze zogenaamde schuimcellen ontstaan door de ongeremde opname van gemodificeerde lipoproteinen uit de vaatwand, via scavenger receptoren. De SR-A was de eerst bekende scavenger receptor die in vitro het ontstaan van vet-geladen cellen kon mediëren, die gelijkenis vertonen met de schuimcellen die in atherosclerotische lesies aanwezig zijn. Expressie van SR-A in getransfecteerde CHO-cellen resulteert in de vorming, na incubatie met acetyl LDL, schuimcellen. Daarnaast komt de SR-A hoog tot expressie in de vaatwand en is het SR-A ligand, geoxideerd LDL, aangetoond in lesies. Gebaseerd op deze feiten werd de SR-A een belangrijke rol in atherosclerose toegedicht.

Om de exacte rol van de SR-A te onderzoeken werd het onderzoek, beschreven in dit proefschrift, uitgevoerd.

Uitgaande van de resultaten van Kodama's groep, verkregen met SR-A deficiente muizen in combinatie met apoE knockout of LDL-receptor knockout muizen, werd verwacht dat afwezigheid van de SR-A in APOE3Leiden muizen zou resulteren in minder atherosclerose. In hoofdstuk 3 werden zowel mannetjes als vrouwtjes APOE3Leiden muizen bekeken, met en zonder SR-A. Na 10 weken hoog vet dieet, werd het ontstane atherosclerotische lesie oppervlak bepaald en werden de lesies morfologisch gekarakteriseerd. Tot onze verassing leidde afwezigheid van de SR-A niet tot kleinere lesies. Er was zelf een trend naar grotere lesies. De lesies werden gedetaileerder bestudeert en categoriseerd volgens hun ernst. Drie categoriën werden gemaakt. Type I lesies, de 'fatty streaks', bevatten alleen schuimcellen; in type II lesies, de 'mild plaques', zijn ook fibrotische cellen aanwezig en type III lesies, de 'severe plaques', bevatten veel fibrose, schade aan de media en necrose. Er bleek dat, naast de vergroting in lesie oppervlak, de lesies in de SR-A deficiente muizen ernstiger waren, in vergelijking met de muizen die wildtype waren voor het SR-A allel. Deze resultaten laten duidelijk zien dat in APOE3Leiden muizen afwezigheid van de SR-A, atherosclerose niet vermindert maar zelfs verergert. In onze experimenten bleek de SR-A dus een anti-atherogene factor te zijn.

Om de mogelijkheden om de rol van de SR-A in atherosclerose te bestuderen uit te breiden, werd een muismodel gemaakt dat de SR-A overexpresseert. Om dit te

bereiken wilden we een natuurlijk overexpressie model maken, gebruik makend van het complete humane SR-A gen (MSR1) in zijn natuurlijk genomische omgeving. Dit beperkte ons tot het gebruik van grote DNA vectoren omdat de MSR1 regio 80 kb groot is. Screening van literatuur en genomische banken resulteerde in de isolatie van 2 grote (>1 Mb) gist artificiële chromosomen (YAC's) die MSR1 bevatten. Op YAC 766_a_12 lag MSR1 naast één van de vector armen. Omdat een YAC van 1Mb bij oocyte injectie moeilijk heel te houden is en omdat de kans op additionele genen in het 1Mb gebied groot is, werd deze YAC verkleind. Hiervoor werd een set nieuwe YAC fragmentatie vectoren gemaakt (hoofdstuk 4). Deze vectoren kunnen worden gebruikt om YAC kleiner te maken door nieuwe vector armen, met nieuwe selektie markers, te laten recombineren met door het genoom verspreide alu-repeats. Op deze manier werd de YAC verkleind naar de gewenste grootte.

In hoofdstuk 5 werd, gebruik makend van deze vectoren, een nieuwe YAC van 180 kb (yHSR1) gemaakt, met MSR1 in het midden. Transgene muizen lieten een natuurlijke expressie patroon zien, d.w.z. expressie werd gedetecteerd in geïsoleerde macrofagen en in verschillende weefsel macrofagen. Hiernaast, leek de regulatie van MSR1 in macrofagen uit MSR1 muizen ook normaal. De overexpressie verhoogde het metabolisme van gemodificeerd LDL door geisoleerde macrofagen. Afbraak van acetyl LDL was ongeveer 2-3 verhoogd in MSR1 macrofagen in vergelijking met wildtype macrofagen. Na overnacht incubatie met acetyl LDL, onstonden er in MSR1 macrofagen meer schuimcellen, gemeten na oil red O kleuring; en stapelde er meer cholesterol ester in macrofagen. Voorgaande SR-A overexpressie experimenten maakten gebruik van cDNA constructen in CHO cellen. Wij laten nu zien dat overexpressie van de SR-A, met zijn eigen promoter en enhancer elementen, inderdaad schuimcel vorming kan mediëren. Wat voor effect dit op atherosclerose zou hebben werd in twee atherosclerose muis modellen bestudeerd.

In hoofdstuk 6 werden de MSR1 muizen gekruist op een ldlr deficiente achtergrond. Na 10 weken hoog vet dieet hadden deze muizen zware hypercholesterolemie. Er werden geen verschillen gevonden tussen muizen met of zonder MSR1 in plasma cholesterol of triglyceride waardes of in het lipoproteine profiel. Lesie oppervlakte metingen lieten zien dat muizen die MSR1 overexpresseerden, kleinere lesies hadden dan muizen zonder het MSR1 transgen. Dit verschil werd nog significanter als rekening werd gehouden met de individuele cholesterol belastingen van de muizen. In dit model beschermde de SR-A dus tegen atherosclerose. Vervolgens, werd het effect van MSR1 overexpressie bekeken in een totaal ander muismodel. Hiervoor werd een beenmerg transplantatie naar APOE3Leiden muizen uitgevoerd. Beenmerg uit wildtype en MSR1 transgene muizen werd getransplanteerd naar letaal bestraalde APOE3Leiden muizen. Dit resulteerde in een repopulatie van het

APOE3Leiden beenmerg met wildtype of MSR1 beenmerg. Op deze manier kon makkelijk het effect bestudeerd worden van MSR1 overexpressie in beenmerg afgeleide cellen (macrofagen) op atherosclerose in APOE3Leiden muizen. De muizen kregen 8 weken een hoog vet dieet. Dit resulteerde in een hyperlipidemie die milder was dan bij de ldlr deficiënte muizen. Opnieuw werden er geen verschillen in lipide parameters waargenomen. Echter, de muizen die MSR1 beenmerg hadden gekregen, leken kleinere atherosclerotische lesies te hebben. Dit verschil werd significant als rekening werd gehouden met de individuele verschillen in cholesterol belasting. Opnieuw, ook in dit compleet van de ldlr deficiente muis verschillende model, beschermde de SR-A tegen atherosclerose. Deze observaties koppelen de in vitro schuimcel vorming los van de in vivo atherogenese, omdat deze twee parameters tegenovergestelde resultaten lieten zien in onze proeven. Om dit anti-atherogene effect van de SR-A te verklaren, kunnen andere SR-A functies belangrijk zijn. We testten er één van, door de activering van macrofagen te meten, in reactie op een behandeling met het bacteriële oppervlakte molecuul lipopolysaccharide (LPS). MSR1 macrofagen lieten een verlaagde reactie op LPS zien, gemeten aan de productie van stikstof monoxide. Dus, de activering van MSR1 macrofagen was veranderd in vergelijking met wildtype macrofagen en dit zou bij kunnen dragen aan een veranderd gedrag van de macrofaag in de atherosclerotische lesie, wat kan resulteren in het anti-atherogene effect van de SR-A.

De SR-A komt voornamelijk voor in twee isoformen, type I en type II, geproduceerd door alternatieve splicing van één gen. Ze verschillen in hun C-terminale domein. Type I bevat een cystein-rijk domein, dat afwezig is in type II SR-A. Ondanks het structurele verschil zijn er geen verschillen aangetoond in bindingseigenschappen voor verschillende SR-A liganden. Er is wel specifieke regulatie van type I en type II beschreven. In het algemeen, lijkt type I opgereguleerd te worden tijdens macrofaag differentiatie, alhoewel de meeste macrofagen wel beide typen co-expresseren. De in vivo functie van het cysteine-rijke domein van de type I SR-A is nog niet bekend.

Om de specifiek functie van type I versus type II te onderzoeken, hebben we type I specifieke knockouts gemaakt (hoofdstuk 7). Om dit te bereiken werd een replacement targeting vector gemaakt. Met deze vector, werd exon 10, die specifiek is voor type I SR-A, vervangen door een neomycine resistentie gen via homologe recombinatie in embryonale stamcellen. Dit leverde heterozygote ES-cellen op die gebruikt werden om chimere muizen mee te maken die uiteindelijk homozygote SR-AI knockout muizen op leverden. cDNA uit macrofagen van SR-AI wildtype (SR-AI$^{+/+}$), heterozygote (SR-AI$^{+/-}$) en homozygote (SR-AI$^{-/-}$) knockout werd met PCR gekarakteriseerd. Dit liet zien dat in SR-AI$^{-/-}$ muizen geen type I transcript aanwezig is. Er werd echter wel een RNA product gedetecteerd dat afkomstig was van splicing tussen exon 8 en exon 10. Dit leverde type

III mRNA op, wat in de literatuur beschreven is als een dominant negatieve isoform van de SR-A. Bij co-expressie met type I of type II SR-A, blokkeert type III, SR-A functie, gemeten aan de afbraak van acetyl LDL. De expressie van type III in onze SR-AI$^{-/-}$ muizen was echter erg laag. In vitro studies toonden aan dat SR-AI$^{+/+}$ en SR-AI$^{-/-}$ macrofagen een gelijke degradatie en associatie van zowel acetyl LDL als oxLDL laten zien. Afwezigheid van een verschil in metabolisme van deze twee vormen van gemodificeerd LDL toont aan dat de SR-AI voor deze processen niet belangrijk is in peritoneaal macrofagen. Dit muismodel geeft de unieke mogelijkheid om de rol van type I en type II SR-A in atherogenese en andere SR-A gerelateerde processen in vivo te bestuderen.

In hoofdstuk 8 werd de rol van apolipoprotein E (apoE) in atherogenese en orgaan specifieke pathologie bestudeerd in muizen die deficient zijn voor zowel muize *apoe* als de *ldlr*, met ofwel een APOE2(158) transgen, een APOE3Leiden transgen of geen transgen. De apoE varianten werden op een *apoe-ldlr* dubbel knockout achtergrond gekruist om de specifieke effecten van de humane apoE varianten te bestuderen onder gelijke hyperlipidemische omstandigheden. Deze muizen hebben gelijke cholesterolniveau's van rond de 30-40 mM op een chow dieet. Op een leeftijd van 12 weken hadden alle muizen zware atherosclerose maar er werd geen verschil gevonden tussen de drie groepen. Op de drie groepen werd volledige sectie uitgevoerd op de leeftijd van 8-10 maanden. Microscopische observatie liet zien dat de muizen wel verschilden in de andere pathologische verschijnselen, waaronder xanthoma vorming, glomerulosclerose, hartspier afbraak, lesies in de hersenen en macrovesiculaire steatose in de lever. De xanthoma's waren in beide transgene groepen even ernstig, maar ernstiger in de *apoe-ldlr* groep, zonder transgen. De andere verschijnselen waren het ernstigst in de *apoe-ldlr* en APOE3Leiden-*apoe-ldlr* groepen en minder sterk in de APOE2-apoe-ldlr muizen. Wij concluderen dat in *apoe-ldlr* muizen, APOE3Leiden slechts in mindere mate het fenotype kan verbeteren, terwijl APOE2 beter in staat is om deze met hyperlipidemie geassocieerde pathologie te verminderen.

LIST OF PUBLICATIONS

Full papers

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7. Willems van Dijk K, Van Vlijmen BJM, De Winther MPJ, Van 't Hof B, Van der Zee A, Van der Boom H, Havekes LM, Hofker MH. Hyperlipidemia of APOE2(ARG158-CYS) and APOE3-Leiden transgenic mice is predominantly modulated by low density lipoprotein receptor expression.

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Published Abstracts:

1. M.P.J. de Winther, M.J.J. Gijbels, K. Willems van Dijk, P.J.J. van Gorp, T. Kodama, R.R. Frants, L.M. Havekes, M.H. Hofker (1997), Atherosclerosis in scavenger receptor deficient APOE3Leiden transgenic mice, *Atherosclerosis* 134: 33.

2. M.P.J. de Winther, M.J.J. Gijbels, K. Willems van Dijk, P.J.J. van Gorp, T. Kodama, R.R. Frants, L.M. Havekes, M.H. Hofker (1997), Enhanced atherosclerosis in macrophage scavenger receptor deficient APOE3Leiden transgenic mice, *Circulation* (suppl.) vol.96 no.8: I-490.

3. M.P.J. de Winther, K. Willems van Dijk, B.J.M. van Vlijmen, M.J.J. Gijbels, E.R. Wijers, M. Breuer, R.R. Frants, L.M. Havekes, M.H. Hofker (1998), Macrophage specific overexpression of the human macrophage scavenger receptor in transgenic mice, using a 180kb yeast artificial chromosome, leads to enhanced foam cell formation of isolated peritoneal macrophages, *Circulation* (suppl.) vol.98 no.17: I-464

Curriculum Vitae

Menno Paulus Johannes de Winther werd geboren op 29 augustus 1970 in Nijmegen. In 1988 behaalde hij zijn VWO diploma aan het Marnix College te Ede. In datzelfde jaar begon hij de studie Biologie aan de Universiteit Utrecht. Hiervoor werd in 1990 de propedeuse behaald. Het doctoraal examen werd in 1994 afgelegd, met als specialisaties celbiologie en chemische biologie. In het kader van het doctoraal examen werden twee onderzoeksstages gedaan. De eerste vond plaats bij het RITOX (Research Institute for Toxicology) in Utrecht onder begeleiding van dr. C. Toxopeus en dr. B.J.J. Blaauboer, met als titel 'Disturbance of energy- and calcium homeostasis in relation to cell death'. Hierna werd een stage afgelegd bij de projectgroep stofwisselingsfysiologie van de vakgroep experimentele dierkunde van de Universiteit Utrecht. Deze was getiteld 'Expression and Regulation of Apolipophorin-III in *Locusta migratoria*' en werd begeleid door dr. P.M.M. Weers en Prof.dr. D.J. van der Horst.

Eind 1994 werd hij bij NWO aangesteld als onderzoeker in opleiding op een samenwerkingsproject tussen de vakgroep Anthropogenetica van het Leids Universitair Medisch Centrum en TNO-Preventie en Gezondheid in het Gaubius Laboratorium in Leiden. Gedurende deze periode werd het onderzoek beschreven in dit proefschrift uitgevoerd onder leiding van dr. M.H. Hofker en Prof.dr. L.M. Havekes.

Vanaf september 1999 werkt hij voor een periode van een half jaar aan de Sir William Dunn School of Pathology te Oxford in de groep van Prof.dr. S. Gordon. Hier zal hij werken aan de rol van de scavenger receptor in hersenvliesontsteking veroorzaakt door *N. meningitidis.*