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Dissecting Class A Scavenger Receptor Mediated Cell Adhesion and Lipoprotein Internalization

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Abstract

Macrophage Class A scavenger receptors (SR-A) are trimeric transmembrane glycoproteins that can bind a variety of ligands including modified lipoproteins and bacterial products. Through its ability to internalize these ligands, SR-A is thought to be involved in many physiological and pathophysiological processes such as host defense and atherosclerosis. In vitro, SR-A also mediates cell adhesion to modified extracellular matrix proteins. However, the physiological role of SR-A mediated cell adhesion is unknown.

The goal of this project was to dissect SR-A mediated cell adhesion and ligand internalization through structure-function studies and to develop a mutant of SR-A that specifically lacks the adhesive properties.

It appeared that SR-A mediated adhesion and ligand internalization depend on the same extracellular domain. Therefore, I investigated the role of cytoplasmic domains for SR-A mediated adhesion. Different cytoplasmic mutated SR-A constructs were stably expressed in human embryonic kidney (HEK 293) cells. I found that a mutated SR-A deficient in all but the six amino acids proximal to the membrane of the cytoplasmic tail (SR-$A_{\Delta 1-49}$) was able to mediate cell adhesion, but not receptor internalization. Substitution of the SR-A cytoplasmic tail with that of the transferrin receptor resulted in retention of this chimeric receptor in the endoplasmic reticulum demonstrating that the SR-A membrane proximal amino acids are also critical for transport of the receptor from the endoplasmic reticulum to the Golgi apparatus.

In summary, my results demonstrate that SR-A-mediated adhesion and internalization require distinct cytoplasmic domains and I developed a mutant SR-A (SR-$A_{\Delta 1-49}$) that specifically meditates cell adhesion. This mutant will make it possible to specifically address the physiological role of SR-A mediated adhesion.

Abstrakt

Die Makrophagen Scavenger-Rezeptoren der Klasse A (SR-A) sind trimere integrale Transmembranproteine, die eine Vielfalt von Liganden (z.B. modifizierte Lipoproteine und bakterielle Produkte) binden. Durch Internalisation dieser Liganden spielt SR-A eine wichtige Rolle in verschiedenen physiologischen und pathophysiologischen Vorgängen, unter anderem im Abwehrsystem und bei der Pathogenese der Arteriosklerose. In vitro kann SR-A auch die Adhäsion von Makrophagen zu modifizierten extrazellulären Matrixproteinen regulieren, die physiologische Bedeutung dieser Adhäsionseigenschaften ist allerdings noch unklar.

Das Ziel dieser Arbeit war es, die Adhäsionseigenschaften von den Internalisationeigenschaften des Rezeptors zu trennen und einen mutierten SR-A zu entwickeln, dem die Adhäsionseingenschaften fehlen.

Da Zelladhäsion und Internalisation von Liganden anscheinend von einer identischen extrazellulären Region des Rezeptors reguliert werden, habe ich die zytoplasmatische Region des Rezeptors erforscht. Veschiedene mutierte SR-A Konstrukte wurden in HEK-293 (human embryonic kidney) Zellen exprimiert. Mit diesem Ansatz konnte gezeigt werden, dass ein mutierter SR-A ($SR\text{-}A_{\Delta 1\text{-}49}$) mit einer verkürzten zytoplasmatischen Region von nur sechs Aminosäuren Zelladhäsion regulieren kann, aber nicht in der Lage ist, die Internalisation des Rezeptors zu regulieren. Studien mit einem chimären Rezeptor, in den der zytoplasmatische Teil von SR-A durch den des Transferrin Rezeptors ersetzt wurde, haben gezeigt, dass diese zytoplasmatische SR-A Region auch für den Transport vom Endoplasmatischen Reticulum zum Golgi Apparat nötig ist.

Es konnte gezeigt werden, dass Zelladhäsion und Internalisation von unterschiedlichen zytoplasmatischen Regionen reguliert werden. Es wurde ein mutierter SR-A entwickelt, der spezifisch die Zelladhäsion reguliert und anhand dieses Rezeptors kann die physiologische Bedeutung der Adhäsionseigenschaften untersucht werden.

Abbreviations

aa	amino acid(s)
AcLDL	acetylated low-density lipoprotein
ApoE	apolipoprotein E
bp	base pairs
BSA	bovine serum albumin
C-	carboxy-
d	density [g/ml]
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
E. coli	Escherichia coli
EndoH	endoglycosidase H
ER	endoplasmic reticulum
FBS	fetal bovine serum
HEK	human embryonic kidney
kDa	kilodalton
LDL	low-density lipoprotein
LDL-R	low-density lipoprotein receptor
N-	amino-
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SR-A	Class A scavenger receptor
TfR	transferrin receptor
TfR/SR-A	chimeric transferrin receptor class A scavenger receptor
u	unit
v	volume
w	weight

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I Introduction

1. Scavenger Receptors

Scavenger receptors are defined by their ability to recognize modified low-density lipoprotein (LDL). Although scavenger receptors are defined by this common function, scavenger receptors have heterogeneous molecular structures. Based on their structure, scavenger receptors are classified in an alphabetic scheme from A - G. The first class includes class A scavenger receptors (SR-A) and the structurally similar receptor MARCO (Figure 1). SR-A was the first member of the scavenger receptor family to be identified. In contrast to the receptor for native LDL, SR-A is not downregulated by intracellular cholesterol accumulation. Therefore, SR-A has been implicated in intracellular lipid accumulation during atherosclerosis. Meanwhile, a broad spectrum of ligands has been identified, and SR-A is thought to be involved in many physiological and pathophysiological processes.

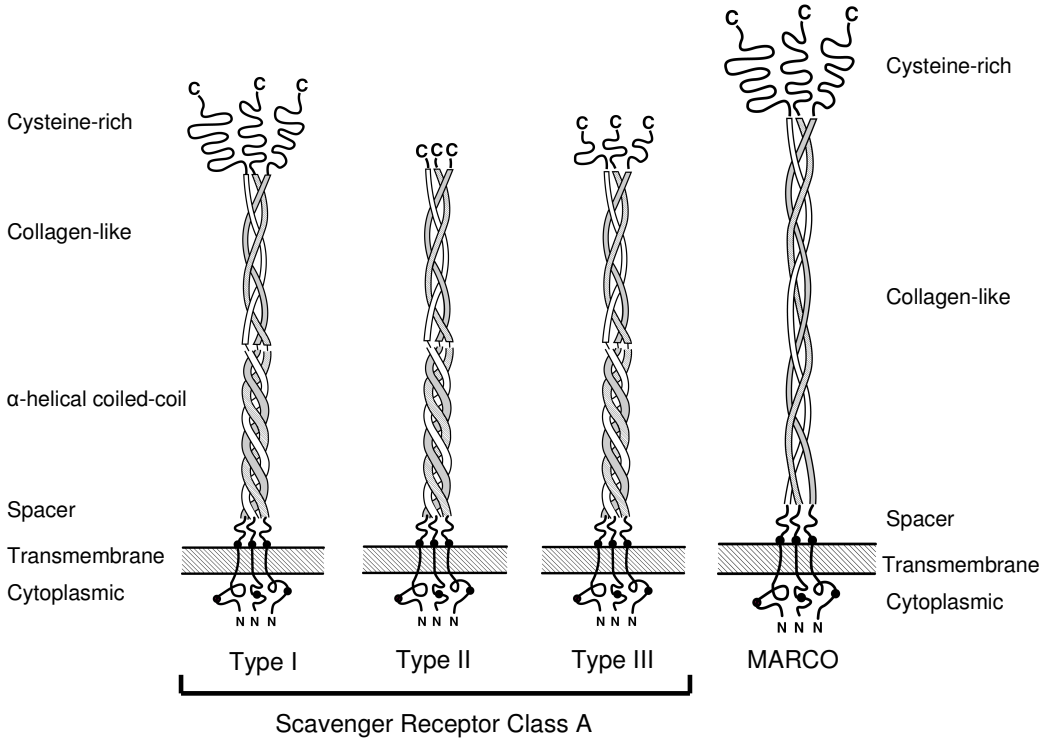
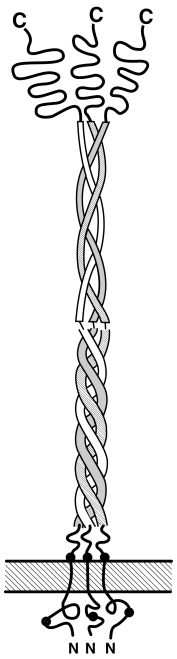


Figure 1: Proposed structures of the members of class A of scavenger receptors.

2. SR-A Structure

SR-A is a trimeric transmembrane glycoprotein consisting of six distinct domains: an N-terminal cytoplasmic, a transmembrane, and an extracellular part composed of short spacer, α-helical coiled-coil, collagen-like, and a varying C-terminal domain (Figure 1) (Kodama et al. 1990). Three different isoforms of SR-A exist, and these isoforms vary only in their C-terminal domain (Freeman et al. 1990; Naito et al. 1992; Ashkenas et al. 1993; Gough et al. 1998). The amino acids comprising the different domains of SR-A type I are shown in Figure 2. Each trimeric receptor consists of a Cys^{83} disulfide-linked dimer and a noncovalently associated monomer (Penman et al. 1991).



Cysteine-rich	349 - 458	(110 aa)
Collagen-like	277-348	(72 aa)
α-helical coiled-coil	114-276	(163 aa)
Spacer	82-113	(32 aa)
Transmembrane	56-81	(26 aa)
Cytoplasmic	1-55	(55 aa)

Figure 2: Structure of murine SR-A type I. The domains are indicated with the corresponding amino acid residues. The number of amino acids comprising the domains is shown in brackets.

SR-A cDNAs have been cloned from bovine liver (Rohrer et al. 1990; Kodama et al. 1990), murine macrophage cell line P338D1 (Freeman et al. 1990), human monocytic THP-1 cells (Matsumoto et al. 1990) and rabbit smooth muscle cells (Bickel et al. 1992; Doi et al. 1993). With a homology between 60 - 80 %, SR-A is highly conserved between the species (Ashkenas et al. 1993).

Originally the structure of SR-A was proposed to be a straight stalk. However, electron microscopy studies showed that the α-helical coiled coil and collagen-like domains are connected by an extremely flexible hinge allowing a variable angle from 0 to 180 degrees between these two domains. Under physiological conditions the predominant angle was 0 degree resulting in juxtaposition of the two domains (Figure 3) (Resnick et al. 1996).

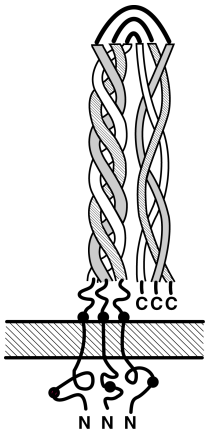


Figure 3: Proposed conformation of SR-A at physiological pH.

2.1. Cytoplasmic domain

(50 aa human, bovine and rabbit; 55 aa mouse) The cytoplasmic tail has been shown to play an important role in regulating SR-A function. A VXFD motif has been described to be essential for clathrin coated-pit-mediated endocytosis and also for the trafficking of the receptors to the cell surface (Morimoto et al. 1999). Furthermore, receptor phosphorylation has been shown to regulate cell surface expression as well as ligand internalization. The cytoplasmic tail contains 3 conserved phosphorylation sites that are conserved among the species: Ser^{21}, Thr^{30} and Ser^{49}. The mouse receptor has an additional site at

Ser^{36}. Fong et al. described serine, but no threonine phosphorylation during internalization of AcLDL (Fong et al. 1999). These investigators performed site-directed mutatgenesis replacing Ser^{21} and Ser^{49} with alanine to eliminate the phosphorylation sites. Elimination of Ser^{21} increased cell surface expression and uptake of AcLDL. Elimination of Ser^{49} did not affect cell surface expression, but slowed receptor internalization (Fong et al. 1999). In contrast, other investigators showed that mimicking phosphorylation by substitution of Ser^{49} with aspartate resulted into strongly reduced cell surface expression (Heider et al. 2001). Although the studies on the role of phosphorylation appear to be inconsistent, they support an important role for the cytoplasmic tail in receptor regulation.

2.2. Transmembrane domain

(26 aa) This transmembrane domain is a stretch of hydrophobic amino acids that anchors the receptor in the membrane.

2.3. Spacer domain

(32 aa) The spacer domain contains the cysteine residues (cys^{83}) involved in formation of the dimer, which noncovalently associates with a monomer to form the trimeric receptor. However, the disulfide-linked dimer formation was not required for trimer formation and SR-A function (Penman et al. 1991).

2.4. α-helical coiled-coil domain

(163 aa) This domain consists of 23 seven amino acid “heptads” repeats $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f)_n$. The first (a) and fourth (d) amino acids of these repeats have hydrophobic aliphatic side chains (leucine, isoleucine and valine), which form an interhelical hydrophobic core holding together the trimeric helix (Doi et al. 1993). Doi et al. have demonstrated that the α-helical coiled-coil is essential

for trimer formation expressing truncated forms of the scavenger receptor and analyzing timer formation (Doi et al. 1993).

A discontinuity of the heptad repeats has been identified at Asn^{203} and it has been proposed that the α-helical coiled-coil is divided into two functional subdomains (Suzuki et al. 1997). The N-terminal half of the domain is essential for stable trimer formation and a specific seven-residue sequence appears to be required (Suzuki et al. 1997; Frank et al. 2000). The C-terminal end determines pH dependent conformational changes (Suzuki et al. 1997). The conformation of this domain is a α-helical coiled-coil structure at pH 5, whereas the structure is random at pH 7 (Suzuki et al. 1997). Specifically, a buried glutamate (Glu^{242}) in this domain is thought to induce this pH-dependent conformational change (Suzuki et al. 1999). Furthermore, the pH dependent ligand dissociation depends on residue His^{260} within c-terminal end of the α-helical coiled-coil (Doi et al. 1994). Substitution of His^{260} sustained ligand binding, but abolished the ability to release the ligands in the lysosome, suggesting that this residue allosterically controls the pH dependent ligand release from the collagen-like domain (Doi et al. 1994).

A polymorphism of the murine sequence has been described at residue Leu^{168} and led to the identification of the binding site of the most commonly used antibody against murine SR-A, 2F8 (Fortin et al. 2000; Daugherty et al. 2000).

2.5. Collagen-like domain

(69 aa human; 72aa bovine, rabbit, mouse) The collagen-like domain has 23 (human) or 24 (bovine, rabbit, mouse) Gly-x-y tripeptide repeats that assemble into a collagenous triple helix. The amino acids in position y are mostly prolines or lysines that can be hydroxylated. The C-terminal 22 amino acids are highly conserved and contain a lysine cluster that forms a positively charged groove, which specifically interacts with the negatively charged ligands (Doi et al. 1993).

Truncated mutants lacking the conserved lysine cluster are unable to bind modified lipoproteins (Acton et al. 1993; Doi et al. 1993). Point mutations studies that substituted the conserved lysines against alanine, demonstrated that substitution of lys^{337} abolishes acLDL degradation and an additional substitution (either lys^{334} or lys^{340}) also abolishes oxLDL degradation (Doi et al. 1993).

Studies using synthetic SR-A peptides that contain the ligand binding domain (Gly^{323} to Lys^{340}) have shown that the trimeric structure is necessary for ligand binding. While the cross-linked trimeric form of the peptide can bind AcLDL, the single stranded peptide cannot (Tanaka et al. 1993; Tanaka et al. 1996). Using the same model peptide, it was demonstrated that not only the ligand binding, but also the ligand specificity is mediated by the lysine cluster, as the trimeric peptide has a ligand binding specificity similar to that of the natural receptor (Yamamoto et al. 1997).

2.6. Cysteine-rich domain

The carboxy-terminal domain varies between the three isoforms of SR-A. The carboxy-terminal domain of SR-A type I consists of 110 amino acids containing 6 cysteine residues (Kodama et al. 1990) and is referred to as cysteine-rich domain. The function of the cysteine-rich domain has not been defined yet. However, the identification of this domain lead to the identification of family of proteins characterized by scavenger receptor cysteine-rich (SRCR) domains (Freeman et al. 1990).

3. SR-A isoforms

The SR-A gene consists of 11 exons (Emi et al. 1993), and three SR-A isoforms are generated through alternative splicing of the mRNA (Freeman et al. 1990). Exons 2 - 8 are common to all isoforms. In addition, SR-A type I contains exons 10 and 11, SR-A type II contains exon 9, and SR-A type III contains exon 11 (Freeman et al. 1990; Gough et al. 1998).

The three isoforms vary only in their C-terminal domains. SR-A type I has a C-terminal cysteine-rich domain of 110 amino acids containing 6 cysteine residues (Kodama et al. 1990). This domain is truncated to six amino acids in the SR-A type II isoform (Rohrer et al. 1990) and to 49 amino acids containing 4 cysteines in the type III isoform (Gough et al. 1998).

Despite the structural differences in the cysteine-rich domain, SR-A type I and II have the same ligand specificity and affinity (Rohrer et al. 1990; Freeman et al. 1991) and the importance of the different isoforms remains unclear. SR-A type II has been reported to be the predominant isoform expressed in cultured murine macrophages, including P388D1 and RAW264 cells (Ashkenas et al. 1993). In contrast to SR-A I and II, SR-A type III does not mediate ligand internalization. Studies in CHO cells expressing SR-A type III have shown that the intracellular processing of this isoform is different from that of SR-A type I and II and therefore SR-A III does not localize to the cell surface. Furthermore, when coexpressed with type I or type II, SR-A type III functions as a dominant negative isoform (Gough et al. 1998). Therefore, SR-A III might function as a negative regulator of SR-A type I and II.

4. Intracellular processing and trafficking of SR-A

During synthesis, the monomeric protein chain is inserted into the membrane of the ER, whereby the extracellular domains are translocated into the ER lumen. In the ER lumen, the protein is N-linked glycosylated (Asn-X-Ser and Asn-X-Thr consensus sequences; 2 in spacer domain and 5 in α-helical coil). Furthermore, hydroxylation of prolines and/or lysines of the Gly-x-y repeats in the collagen-like domain occurs. The modified monomers assemble into precusor trimers consisting of a Cys^{83} disulfide-linked dimer and a non-covalently associated monomer. These precusor trimers are then transported from the ER to the cell surface through the Golgi apparatus, where further processing of the N-linked oligosaccharides converts the precusor into the endoglycosidase H-resistant mature form (Penman et al. 1991). An overview of the processing of SR-A is shown in Figure 4. The predicted molecular weight of the native monomer based on the amino acid sequence is 50 kDa (458 aa mouse) for type I and 38 kDa (354 aa mouse) for type II (Rohrer et al. 1990; Kodama et al. 1990). The addition of N-linked oligosaccharides results in apparent molecular weights of 64/55 kDa for the precursor monomers and 79/70 kDa for the mature monomers of type I/ type II, respectively (Table 1) (Penman et al. 1991). SR-A type III is processed differently from type I and II resulting in retention in the ER (Gough et al. 1998).

SR-A	kDa		
	native	precursor	mature
Type I	50	64	79
Type II	38	55	70

Table 1: Apparent molecular weights (in kDa) of SR-A type I and II at the different stages of intracellular processing.

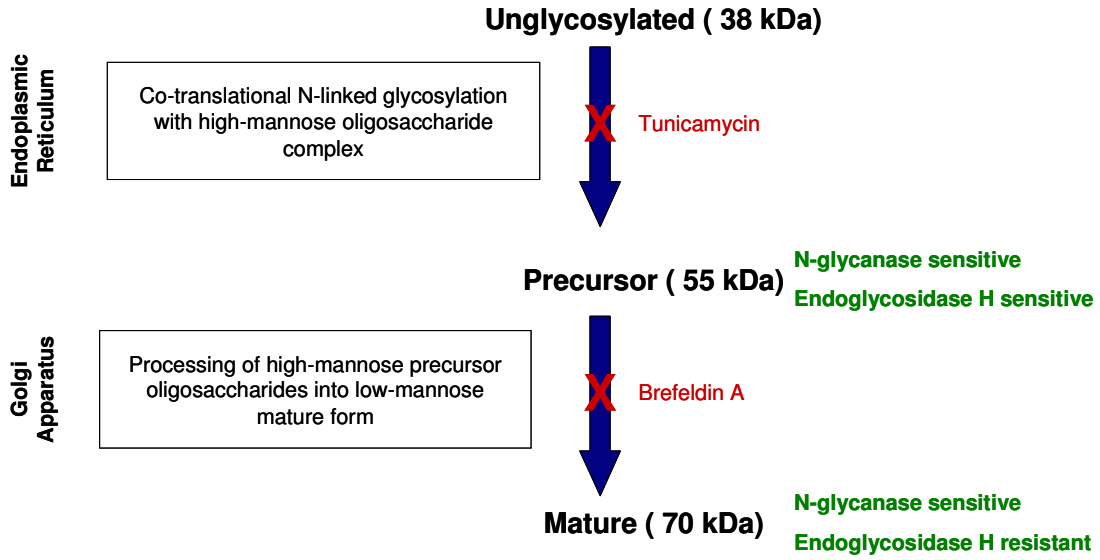


Figure 4: Processing of SR-A. After translocation of the SR-A carboxyterminal extracellular domains into the endoplasmic reticulum (ER), the monomeric SR-A is N-linked glycosylated and the glycosylated monomers assemble into trimers. These 'precursor' trimers are transported through the Golgi apparatus where further processing of the N-linked oligosaccharides converts the EndoH-sensitive precursor into the EndoH-resistant mature form expressed on the cell surface. The apparent molecular weight of the monomeric forms of SR-A type II is indicated. To study the processing of SR-A the inhibitors tunicamycin and brefeldin A can be used. Tunicamycin inhibits glycosylation of the native protein in the endoplasmic reticulum and brefeldin A inhibits maturation of the precursor in the Golgi apparatus.

SR-A localized to the cell surface can mediate internalization of ligands or function as an adhesion molecule. SR-A internalizes ligands via clathrin-coated pit mediated endocytosis (Fukuda et al. 1986). The ligands dissociate from the receptor through allosteric changes in the acidic endosomes. The released ligands are transported to the lysosome where they are digested; while the receptors recycle back to the cell surface (Mori et al. 1994). Although it has been proposed that SR-A mediated adhesion results from the attempt to internalize an immobilized ligand, the mechanism by which SR-A mediates adhesion remains undefined.

5. SR-A expression

5.1. Tissue distribution

SR-A was first identified as receptor for acetylated low-density lipoprotein (AcLDL) on macrophages (Goldstein et al. 1979). While monocytes do not express SR-A, several cells of macrophage-lineage have been shown to express SR-A including various tissue macrophages in lung, spleen, lymph nodes and the specialized liver Kupffer cells and brain microglia cells (Naito et al. 1991; Hughes et al. 1995).

Although SR-A is mainly expressed on cells of macrophage-lineage, scavenger receptor expression is not limited to those cells. SR-A expression has been also detected in liver sinusoidal endothelial cells (Naito et al. 1991), aortic endothelial cells (Daugherty et al. 1997), smooth muscle cells and fibroblasts (Pitas 1990; Bickel et al. 1992; Mietus-Snyder et al. 2000).

5.2. Regulation of SR-A

Unlike the receptor for native LDL, SR-A is not downregulated by intracellular cholesterol accumulation. In contrast, it has been described that SR-A can be upregulated by modified lipoproteins such as AcLDL and oxLDL (Yoshida et al. 1998; Han et al. 1998).

Several regulators of SR-A expression have been described in cultured cells (Table 2). These regulators include several cytokines that are important mediators of inflammatory processes such as interferon-γ and tumor necrosis factor-α. However, in many cases contradictory effects have been reported. For example interferon-γ has been reported to upregulate as well as to

downregugate SR-A expression. in vivo, the regulation of SR-A is difficult to study as the cells are exposed to a complex environment of potential regulators.

Upregulation	**Downregulation**
monocyte/macrophage	
Lymphokines (Kotake et al. 1992)	Lymphokines (Fogelman et al. 1982)
IFN-γ (Lopes-Virella et al. 1987; Grewal et al. 1997; Cornicelli et al. 2000)	IFN-γ (Geng et al. 1992)
IL-4 (Cornicelli et al. 2000)	IL-6 (Liao et al. 1999)
IL-1β (Grewal et al. 1997)	TGF-β (Bottalico et al. 1991; Nishimura et al. 1998)
Phorbol esters (Via et al. 1989)	Phorbol esters (Leake et al. 1989; Fong et al. 1994)
Platelet derived products (Phillips et al. 1985)	LPS (Van Lenten et al. 1985)
Dexamethasone (Hirsch et al. 1986)	dexamethasone (Moulton et al. 1992)
M-CSF (de Villiers et al. 1994)	TNF-α (van Lenten et al. 1992; Hsu et al. 2000)
GM-CSF (Cornicelli et al. 2000)	GM-CSF (van der Kooij et al. 1996)
Serum (Fogelman et al. 1981; Pitas 1990)	Retionic acids (Horvai et al. 1995)
cytomegalovirus infection (Guetta et al. 1997)	PPAR agonists (Ricote et al. 1998)
oxLDL (Han et al. 1998)	Protein kinase C inhibitors (Akeson et al. 1991)
AcLDL (Han et al. 1998)	α-tocopherol (Teupser et al. 1999)
eicosapentaenoic acid (Saito et al. 1991)	essential fatty acid deficiency (Gavino et al. 1992)
Smooth muscle cell	
protein kinase c stimulation (Mietus-Snyder et al. 1997)	TGF-β (Gong et al. 1995)
serum (Pitas 1990)	
TNF-α (Li et al. 1995)	
IFN-γ (Li et al. 1995)	
PDGF (Inaba et al. 1992; Gong et al. 1995)	

Table 2: Regulators of SR-A in cultured macrophages and smooth muscle cells.

6. SR-A Ligands

SR-A binds a wide array of ligands (Table 3). In addition to modified lipoproteins, ligands include proteins, polynucleotides, polysaccharides and lipids. A common feature of SR-A ligands is that they are polyanionic; however, not all polyanions are ligands for SR-A. For example, among anionic polynucleotides SR-A binds polyinosine and polyguanosine, but not polycytidine or polyadenosine. Another example, among anionic polysaccharides, SR-A binds dextran sulfate and fucoidan, but not chondroitin sulfate or heparin. The fact that SR-A can distinguish between structurally similar polyanions demonstrates that SR-A selectively recognizes ligands.

Because of the ability to bind a wide array of ligands, SR-A is thought to be involved in many physiological and pathophysiological processes. Through its ability to bind modified forms of LDL (Goldstein et al. 1979), SR-A has been implicated in atherosclerosis. Furthermore, SR-A has been described to bind bacterial wall components such as lipopolysaccharide (LPS) (Hampton et al. 1991) and lipotechoic acid (Dunne et al. 1994), therefore SR-A is thought to play a role in host defense. By recognizing certain phospholipids such as phosphotidylserine (Platt et al. 1998) SR-A is also thought to be involved in clearance of apoptotic cells. Recognition of beta-amyloid fibrils (El Khoury et al. 1996) and secreted forms of amyloid beta precursor by SR-A (Santiago-Garcia et al. 2001) has implicated SR-A in Alzheimer's disease. As SR-A also binds advanced glycation end products (AGE) (Araki et al. 1995), SR-A might play a role in diabetes. Furthermore, SR-A recognizes several extracelluar matrix components that are associated with sites of tissue injury and SR-A mediated adhesion to such substrates might enhance macrophage retention. Finally, several polysaccharides (fucoidan, carrageenan) and polynucleotides (polyinosine and polyguanosine) have been shown to antagonize AcLDL binding and a non-peptide synthetic antagonist has been described (Lysko et al. 1999).

Groups	Examples of ligands	Possible physiological role
Modified proteins and lipoproteins	Acetylated LDL MDA-LDL Copper oxidized LDL MDA-albumin	Atherosclerosis
Bacterial products	Lipopolysaccharides Lipoteichoic acid	Host defense
Phospholipids	Phosphatidylserine	Phagocytosis of apoptotic cells
	beta-amyloid fibrils amyloid beta precursor	Alzheimer's Disease
Advanced glycation end products (AGE)	AGE-albumin	Diabetes
	Crocodolite asbestos	Pulmonary disease
Polysaccharides	Fucoidan Carageenan Dextran sulphate	Antagonst
Polynucleotides	Polyinosine Polyguanosine	Antagonist
synthetic antagonists	(E)-methyl 4-chloro-alpha-[4-(4-chlorophenyl)-1, 5-dihydro-3-hydroxy-5-oxo-1-(2-thiazolyl)-2H-pyrrol-2-ylidene]benzene acetate	Antagonist

Table 3: Ligands for SR-A and their possible physiological roles.

7. SR-A and disease

7.1. Atherosclerosis

The atherosclerotic disease process is characterized by lipid accumulation and leukocyte infiltration into the arterial wall, resulting in a chronic inflammatory, fibro-proliferative response. Clinical complications related to atherosclerosis include embolisms, stroke, and heart attack, which are the major causes of disability and death in the Western world (Ross 1993; Ross 1999).

SR-A has been implicated in atherosclerosis as receptor for modified lipoproteins on macrophages (Goldstein et al. 1979). Unlike the receptor for native LDL, SR-A is not downregulated by intracellular cholesterol accumulation. Therefore, uptake of modified LDL via SR-A might lead to unregulated cholesterol accumulation, resulting in the formation of lipid-laden macrophages referred to as 'foam cells'. Foam cell formation is one of the earliest hallmarks of atherosclerosis and these macrophage-derived foam cells are the major cell type in atherosclerotic lesions. Consequently, SR-A was thought to promote the development of atherosclerosis via foam cell formation. Supporting a role for SR-A in atherogenesis, immunohistological studies have demonstrated the presence of SR-A in atheroslerotic lesions (Matsumoto et al. 1990; Yla-Herttuala et al. 1991; Naito et al. 1992; Geng et al. 1995; Gough et al. 1999; Nakagawa-Toyama et al. 2001).

As SR-A was thought to mediate foam cell formation, it is generally considered to be proatherogenic. With the development of genetically modified mouse models it became possible to address the role of SR-A in atherogenesis in vivo. Since then, the effects of SR-A deficiency as well as SR-A overexpression on atherosclerosis have been studied in several mouse models of atherosclerosis (Table 4).

Mouse Strain/ Background	SR-A expression	Effect
C57Bl/6 No genetic manipulation	SR-A -/-	70% reduction in lesions (Kamada et al. 2001)
	SR-A -/-	no difference in serum cholesterol concentration; 86% in females and 81.5% in males reduction of lesions (Babaev et al. 2000)
	SR-A -/- after BMT	no difference in plasma cholesterol concentrations; 60% reduction in lesions (Babaev et al. 2000)
ApoE -/-	SR-A -/-	46% increase in plasma cholesterol concentrations; 58% decrease in lesion size (Suzuki et al. 1997)
	YAC SR-A transgenic after BMT	20% reduction of serum cholesterol (VLDL+LDL); no effect on lesion size (Van Eck et al. 2000)
LDL receptor -/-	SR-A -/-	18 % and 10 % decrease after 4 wks and 12 wks in plasma cholesterol concentrations; 28% and 23% reduction in lesions (Sakaguchi et al. 1998)
	SR-A -/- after BMT	no effect on plasma cholesterol concentrations; 60% (root) and 65% (en face) reduction in lesions (Babaev et al. 2000)
	SR-A transgenic after BMT	24% reduction of serum cholesterol (VLDL+LDL) 74% (root) and 40% (arch) decrease in lesions (Whitman et al. 2002)
	YAC SR-A transgenic after BMT	Increase of total serum choesterol (15-25%); no difference in lesion size (Herijgers et al. 2000)
apoE3Leiden transgenic	SR-A -/-	No effect on serum cholesterol in females, but 50% decrease in males. No statistical difference in lesion size (tendencies for larger lesions, especially female) but changes in cellular characteristics (more complex) (de Winther et al. 1999)
	YAC SR-A transgenic after BMT	35% reduction in lesion size, Abstract in Circulation 1999, not published.

Table 4: In vivo studies addressing the role of SR-A deficiency or overexpression in atherosclerosis in mouse models. The effects on plasma/serum cholesterol and atherosclerotic lesion development. YAC: yeast artificial chromosome, BMT: bone marrow transplantation.

Total SR-A deficiency in C57BL/6 mice, a mouse strain susceptible to diet-induced atherosclerosis, dramatically decreased lesions development by more than 70 % in two independent studies (Babaev et al. 2000; Kamada et al. 2001). Total SR-A deficiency was also studied in two C57BL/6 mouse strains that are genetically engineered to enhance atherosclerotic lesion development: Apolipoprotein E (apoE) and low-density lipoprotein receptor (LDL-R) deficient mice. In apoE deficient mice, SR-A deficiency decreased lesion development by 58 % (Suzuki et al. 1997). In LDL-R deficient mice, SR-A deficiency decreased lesion development by 23 % (Sakaguchi et al. 1998).

As SR-A is expressed on macrophages, endothelial cells and smooth muscle cells, bone-marrow transplantation studies were performed to specifically address the role of SR-A expressed on macrophages, which are derived from bone-marrow cells. Transplantation of SR-A deficient bone marrow into SR-A wild-type mice on a LDL-R deficient background reduced lesion development by 60% (Babaev et al. 2000).

In contrast to the studies on SR-A deficient mice described above which all support a pro-atherogenic role of SR-A, studies using SR-A transgenic mice support an anti-atherogenic role of SR-A. SR-A overexpression in macrophages after bone marrow transfer dramatically decreased lesion development in LDL-R deficient mice (Whitman et al. 2002). Overall, although the data are conflicting, the data from these in vivo studies demonstrates the importance of SR-A in atherosclerosis.

7.2. Host defense

SR-A is expressed mainly on macrophages, which are important cells during the innate immune response. Furthermore, SR-A recognizes bacterial cell wall products from gram-positive (lipoteichoic acid) (Dunne et al. 1994) and gram-negative (lipopolysaccharide; LPS) (Hampton et al. 1991) bacteria. Taken

together, expression on macrophages and recognition of bacterial products suggested a role for SR-A in host defense against bacterial infection. In vivo studies demonstrated that SR-A deficient mice are more susceptible to infection with gram-positive and gram-negative bacteria than wild-type mice.

SR-A deficient mice are more susceptible to injection of gram-positive pathogens such as *Listeria monocytogenes* (Suzuki et al. 1997; Ishiguro et al. 2001) and *Staphylococcus aureus* (Thomas et al. 2000). SR-A is thought to be protective from lethal gram-positive infection by recognition of lipoteichoic acid and consequent phagocytosis of the pathogens (Thomas et al. 2000).

SR-A deficient mice were also more susceptible to endotoxic shock induced by LPS, the bacterial wall product of gram-negative bacteria. Therefore, SR-A was suggested to protect against lethal endotoxic shock by scavenging LPS (Haworth et al. 1997; Kobayashi et al. 2000). It has also been shown that SR-A can not only bind, but also ingest gram-negative bacteria directly (E. coli) (Peiser et al. 2000).

Furthermore, SR-A has been shown to enhance host defense against herpes simplex virus type I (Suzuki et al. 1997) and repeated infestation with H. longicornis (Kabamoto et al. 2002). Finally, there is also evidence that SR-A is involved in adaptive immune response, as a role for SR-A in antigen presentation has been suggested (Nicoletti et al. 1999).

7.3. Alzheimer's Disease

Alzheimer's disease involves inflammatory processes and a pathological hallmark is the senile plaque. The senile plaque is characterized by accumulation of beta-amyloid fibrils, which exert cytotoxic effects on neurons, associated with microglia cells and astrocytes. In the normal brain, SR-A is only expressed on the perivascular cells surrounding arterioles (MATO cells) (Mato et al. 1996; Honda et al. 1998). However, in Alzheimer's disease SR-A is strongly expressed on microglia cells associated with senile plaques (Christie et al. 1996; Honda et al. 1998). SR-A mediates adhesion of microglia

cells to beta-amyloid fibrils resulting in the release of reactive oxygen species (El Khoury et al. 1996; El Khoury et al. 1998) and tumor necrosis factor (Meda et al. 1995). SR-A also has been shown to endocytose beta-amyloid fibrils (Paresce et al. 1996). Therefore, SR-A might play a role during Alzheimer's disease by modulating the inflammatory response and by scavenging beta-amyloid fibrils.

8. SR-A mediated adhesion

SR-A mediated adhesion has been discovered during the development of the rat monoclonal antibody against murine SR-A, designated 2F8 (Fraser et al. 1993). Unlike other cell lines, macrophages are able to attach to tissue culture plastic independent of divalent cations, which are required for integrin-mediated adhesion. 2F8 was able to completely inhibit divalent-cation independent adhesion of macrophages to tissue culture plastic. This adhesion was dependent on the presence of fetal calf serum, suggesting that SR-A mediates adhesion to a component of fetal calf serum that is immobilized on the tissue culture plastic (Fraser et al. 1993). The same investigators also demonstrated that divalent-cation independent adhesion of macrophages to frozen tissue sections could be completely inhibited by 2F8 (Hughes et al. 1995). In contrast to adhesion to tissue culture plastic, SR-A mediated adhesion to tissue sections did not require exogenous fetal bovine serum indicating that an endogenous adhesion substrate for SR-A is present within the tissues.

Several substrates have been identified that might function as substrate for SR-A mediated adhesion in tissues. SR-A mediated adhesion to modified extracellular matrix components prominent in the vessel wall has been described. These extracellular matrix proteins include collagen type IV, a component of the basement membrane, (el Khoury et al. 1994) and collagen type I (Gowen et al. 2000) and type III (Gowen et al. 2001), which are the most abundant components of the extracellular matrix in the vessel wall. Interestingly, SR-A mediates adhesion specifically to modified forms of the collagens. Such modifications include denaturation and glycation, which are

associated with tissue injury. Furthermore, SR-A mediated adhesion to certain proteoglycans that are increased in atherosclerotic lesions has been described (Santiago-Garcia et al. 2003). SR-A mediated adhesion of microglia cells to beta-amyloid fibrils might occur within the plaques of Alzheimer's disease. Thus, SR-A mediated adhesion might play an important physiological role by increasing macrophage retentention specifically at sites of tissue injury.

SR-A mediated adhesion of macrophages may attenuate disease progresssion by increasing the retention of macrophages at specific sites. In addition to scavenging modified lipoproteins or beta-amloid fibrils, retained macrophages may influence the disease progression through release of inflammatory mediators, growth factors and matrix degrading enzymes. Despite the in vitro evidence supporting a role for SR-A mediated adhesion, a role of SR-A mediated adhesion has never been demonstrated in vivo.

II Specific Aims

SR-A has been implicated in several disease processes through its ability to recognize and internalize a wide variety of ligands. However, SR-A also mediates cell adhesion. SR-A mediated adhesion may attenuate disease processes by increasing the retention of macrophages at sites of injury. However, the role of SR-A mediated adhesion in vivo is unclear. Due to a lack of understanding of the structural requirements and the cellular mechanisms involved in SR-A mediated adhesion, it has not been possible to specifically address this process in vivo. The goal of my thesis project was to dissect SR-A mediated adhesion and ligand internalization and to develop a mutant of SR-A that lacks the adhesive properties. Expression of such a mutant in SR-A deficient mice will then allow to define the role of SR-A mediated adhesion in vivo.

Specific Aim 1: To characterize the functional consequences of macrophage specific SR-A overexpression in vitro.

To address this aim, I studied lipoprotein internalization and the adhesive properties of peritoneal macrophages isolated from mice that overexpress SR-A under the control of a macrophage specific promoter (lyso-bSR-A mice) or from littermate control mice.

Specific Aim 2: To identify the extracellular motif of SR-A that mediates adhesion.

To address this aim, a SR-A specific adhesion assay that studies adhesion of the mouse macrophage cell line RAW.264 to collagenase-digested collagen type I was optimized. To identify the region of SR-A that contains the adhesion motif, fusion proteins homologous to different regions were used to compete for macrophage adhesion.

Specific Aim 3: To identify a distinct cytoplasmic domain required for SR-A mediated adhesion.

To address this aim, different cytoplasmic mutated SR-A constructs were expressed in HEK-293 cells and lipoprotein internalization and the adhesive properties of the cells were studied.

III Materials and Methods

1. Materials

1.1. Buffers and solution

Ampicillin stock (100 mg/ml)
1 g ampicillin
Dissolve 10 ml dH_2O
Sterile filter and aliquot in 1 ml tubes
Store at -20°C
Do not freeze-thaw more than 5 times

Luria broth (LB)

10 g	peptone
5 g	yeast extract
5 g	NaCl

Dissolve in 1 L of dH_2O
Autoclave
Store at 4°C

Lysis buffer

25 mM	(N-morpholino)ethanesulfonic acid (MES)
150 mM	NaCl
60 mM	octylglucopyranoside
1 % (v/v)	Triton-X 100
	pH 6.4
	store at 4°C

Protease inhibitor cocktail (1x)

500 mM	AEBSF
150 nM	Aprotinin
1 mM	E-64
0.5 mM	EDTA
1 mM	Leupeptin
	Store as 100 x stock solution at -20°C

5 x PBS

6.25g	K_2HPO_4
1.93 g	KH_2PO_4
48.84 g	NaCl
	Dissolve in 1l dH2O
	Store at 4°C

1 x PBS

Dilute 5 x PBS 1:5 (pH 7.4)

1.2. List of Manufacturers

Accurate, Westbury, NY

Amersham, Piscataway, NJ

Beckman, Fullerton, CA

Becton Dickinson, Franklin Lakes, NJ

Bio-Rad, Hercules, CA

Calbiochem, San Diego, CA

Cellgro, Herndon, VA

Fisher Scientifc, Pittsburgh, PA

GibcoBRL Life Technologies, Grand Island, NY

ICN, Costa Mesa, CA

Invitrogen, Carlsbad, CA

Jackson, West Grove, PA

Pierce, Rockford, IL

Promega, Madison, WI

Millipore, Bedford MA

Mirus, Madison, WI

Molecular Probes, Eugene, OR

Nalge Nunc International; Naperville, IL

Novagen, Madison, WI

PerSeptive Biosystems, Foster City, CA

Roche, Nutley, NJ

Santa Cruz, Santa Cruz, CA

Sigma, St. Louis, MO

Serotec, Raleigh, NC

Stratagene, La Jolla, CA

2. Molecular biology

2.1. Primer sequences

The nucleotide sequences of the primers used to amplify murine SR-A (mouse strain BALB/C, gene bank accession number L04275) and murine TfR (gene bank accession number X57349) are shown in table 5. Numbers after gene (SR-A or TfR) indicate the first (sense) or last (antisense) nucleotide of the gene bank nucleotide sequences amplified by the primer. If the primers were designed to contain restriction sites, the restriction enzyme is stated and the restriction sites in the primer sequences are indicated in bold. All primers were obtained from the Macromolecular Structure Analysis Facility at the University of Kentucky in lysophilized form. Primers were resuspended in dH_2O to a final concentration of 100 pmol/µl and stored at –20°C.

Primer	Restriction site	Nucleotide Sequence
SR-A 6 sense	Hind III	5' CGC GGG **AAG CTT** GCA ATG ACA AAA GAG ATG 3'
SR-A 153 sense	Hind III	5' CGC GGG **AAG CTT** GCA ATG GCT AAG TTG AAG TCC TTC AAG 3'
SR-A 171 sense	blunt	5' GCT GCC CTC ATT GCT CTC TAC CTC CTT GTG 3'
SR-A 171 sense	Hind III	5' CGC GGG **AAG CTT** GCA ATG GCT GCC CTC ATT GCT CTC 3'
SR-A 348 sense	Sal I	5' CGC GGG **GTC GAC** TTT ACA ATT ATC ATG GCA 3'
SR-A 834 sense	Sal I	5' CGC GGG **GTC GAC** GGG CCT CCT GGA CCC CAA 3'
SR-A 833 antisense	Not I	5' CGC GGG **GCG GCC GC**T TGA ATG AAG GTG ATC TT 3'
SR-A 1049 antisense	Not I	5' CGC GGG C**GC GGC CGC** TAC ATC CCC CTT CTC 3'
SR-A 1067 antisense	Xho I	5' GGG **CTC GAG** TTA TAC TGA TCT TGA TCC GCC 3'
TfR 1 sense	Hind III	5' CGC GGG **AAG CTT** GCA ATG ATG GAT CAA GCC 3'
TfR 171 antisense	blunt	5' TCT GAC ACT AGC CTT CAT GTT ATT GTC GGC 3'

Table 5: Nucleotide sequences and generated restriction sites of primers.

2.2. RNA Isolation

Total RNA was isolated using the SV total RNA isolation system (Promega) according to the manufacturer's protocol. Briefly, by addition of the SV RNA lysis buffer (containing guanidine thiocyanate and β-mercaptoethanol) the cells were lysed, cellular proteins denatured, nucleoprotein complexes

disrupted and nucleases present in the cell lysates inactivated. Cell debris and denatured proteins were removed by centrifugation while the RNA remains in solution. RNA is precipitated out of solution by addition of ethanol and then bound to a silica surface of glass fibers in a spin basket by centrifugation. To digest contaminating genomic DNA, RNase-free DNAse I was added to the spin basket. After washing with the SV RNA wash solution, the RNA is eluted from the spin basket in nuclease-free H_2O. The RNA concentration was determined as described (2.5.) and the RNA was stored at – 70°C.

2.3. RT-PCR

The Access RT-PCR system (Promega) was used for all RT-PCR reactions. This is a one tube, two-enzyme system, which allows reverse transcription of mRNA into cDNA and amplification of the cDNA in one reaction mixture. AMV reverse transcriptase transcribes the mRNA into cDNA (RT) and the thermostable Tfl DNA polymerase amplifies the cDNA (PCR). The reaction mixtures were prepared as shown in table 6 using 0.5 µg of total RNA in a final reaction volume of 50 µl. The parameters of the thermocycle reaction are shown in table 7.

Reagent	**Volume**	**Final concentration**
RNA in H_2O	x µl	
5x reaction buffer	10 µl	1x
Sense primer (100 pmol/µl)	0.5 µl	2 pmol/µl
Antisense primer (100 pmol/µl)	0.5 µl	2 pmol/µl
dNTP mixture (10mM each)	1 µl	0.2 mM
25 mM $MgSO_4$	6 µl	3 mM
AMV reverse transcriptase (5 u/µl)	1 µl	0.1 u/µl
Tfl DNA polymerase (5u/µl)	1 µl	0.1 u/µl
dH_2O	x µl	
Final volume	50 µl	

Table 6: Reaction mixture for RT-PCR using the Access RT-PCR system (Promega).

Step	Process	Parameters	Cycles
1	RT	48°C for 45 min	1
2	Denaturation	94°C for 2 min	1
3	Denaturation Annealing Synthesis	94°C for 30 s 60°C for 60 s 68°C for 120 s	40
4		hold at 4°C	1

Table 7: Parameters of the thermocycle reaction used for RT-PCR using the Access RT-PCR system (Promega).

RT-PCR was used to amplify the cDNA encoding the mouse TfR from mouse liver RNA. RT-PCR was also used to assess mRNA expression of SR-A receptor constructs in HEK-293 cells with primers that amplify a fragment corresponding to the SR-A transmembrane and extracellular domains common to all SR-A constructs. The primers used for RT-PCR and are shown in table 8 (for primer sequences see 2.1. table 5). Reactions performed in the absence of RNA or reverse transcriptase were used as negative controls. RT-PCR products were analyzed by agarose gel electrophoresis (2.6.).

cDNA	Sense primer	Antisense primer	Product
TfR cyto	TfR 1 Hind III	TfR 171 blunt	171 bp (1-171)
SR-$A_{\Delta 1-55}$	SR-A 171 Hind III	SR-A 1067 Xho I	897 bp (171-1067)

Table 8: Primers used for RT-PCR and size of the cDNA product. Numbers in brackets indicate corresponding nucleotide of gene bank sequences (SR-A gene bank accession number L04275 and TfR gene bank accession number X57349).

2.4. PCR

A master mix was used for PCR reactions (Promega). The reaction mixtures were prepared as shown in Table 9. The parameters of the thermocycle reaction are shown in table 10.

Reagent	Volume	Final concentration
DNA in H_2O	1 µl	
Sense primer (100pmol/µl)	1 µl	2 pmol/µl
Antisense primers (100pmol/µl)	1 µl	2 pmol/µl
2 x Mastermix: *Taq* Polymerase (50 Units/mL) MgCl (3mM) dATP, dCTP, dGTP, dTTP (400 µM each)	25 µl	1x: 0.025 units/µl 1.5 mM 200 µM
dH_2O	21 µl	
Final volume	50 µl	

Table 9: Reaction mixture for PCR using a mastermix (Promega).

Step	Process	Parameters	Cycles
1	Denaturation	94°C for 5 min	1
2	Denaturation Annealing Synthesis	94°C for 60 s 60°C for 60 s 72°C for 90 s	35
3	Final extension	72°C for 5 min	1
4		hold at 4°C	1

Table 10: Parameters of the thermocycle reaction used for RT-PCR using the Access RT-PCR system (Promega).

PCR was used to generate cDNA inserts to create expression vectors for fusion protein isolation from E. coli (pET23b) and for SR-A construct expression in HEK-293 cells (pcDNA5/FRT/TO). The primers used are shown in table 11 (for primer sequences see 2.1. table 5). Reactions performed in

the absence of DNA were used as negative controls. PCR products were analyzed by agarose gel electrophoresis (2.6.).

cDNA	Sense primer	Antisense primer	Product
SR-A ahc+CL	SR-A 348 Sal I	SR-A 1049 Not I	702 bp (348-1049)
SR-A ahc	SR-A 348 Sal I	SR-A 833 Not I	486 bp (348-833)
SR-A CL	SR-A 834 Sal I	SR-A 1049 Not I	216 bp (834-1049)
SR-A	SR-A 6 Hind III	SR-A 1067 Xho I	1067 bp (6-1067)
SR-$A_{\Delta 1-55}$	SR-A 171 Hind III	SR-A 1067 Xho I	897 bp (171-1067)
SR-$A_{\Delta 1-49}$	SR-A 153 Hind III	SR-A 1067 Xho I	915 bp (153-1067)
TfR cyto	TfR 1 Hind III	TfR 171 blunt	171 bp (1-171)
SR-A TM+EC	SR-A 171 blunt	SR-A 1067 Xho I	897 bp (171-1067)

Table 11: Primers used to amplify SR-A and TfR cDNAs and size of the resulting product. Numbers in brackets indicate corresponding nucleotides of the SR-A (accession number L04275) and TfR (accession number X57349) gene bank sequences. ahc = α-helical coiled coil; CL=collagen-like; TM= tranmembrane; EC=extracellular domain of SR-A type II. SR-$A_{\Delta 1-55}$ and SR-$A_{\Delta 1-49}$ are the cDNAs encoding SR-A type II lacking the first 55 or 49 amino acids of the cytoplasmic tail.

2.5. DNA and RNA concentration

DNA and RNA concentration were determined by spectroscopy. Absorbances at 260 nm (A_{260}) and at 280 nm (A_{280}) were measured. To assess purity of the isolated RNA or DNA, the ratio A_{260}/A_{280} was calculated. Pure RNA exhibits a A_{260}/ A_{280} of 2.0. Pure DNA exhibits a A_{260}/A_{280} of 1.8. A lower ratio indicates contamination with protein. A higher ratio indicates contamination with reagents used for isolation.

An absorbance of 1.0 at A_{260} equals 50 µg/µl of DNA and 40 µg/µl of RNA. Therefore, the DNA and RNA concentration were calculated as follows:

DNA concentration [µg/µl] = A_{260} x 50 x dilution factor

RNA concentration [µg/µl] = A_{260} x 40 x dilution factor

2.6. Agarose gel electrophoresis

TBE buffer (10x)

890mM	Tris-borate
890mM	boric acid
20mM	EDTA
	pH 8.3
	store at room temperature

TBE buffer (1 x)

Dilute 10 x buffer 1: 10 in dH_20

To analyze the size of the PCR and RT-PCR products, 5 µl of the reaction volume were mixed with 1 µl of 6x loading dye (Promega) and then separated on a 1 % agarose gel containing 0.02 % ethidium bromide (Fisher) in 1x TBE buffer (Promega). To determine product size the pGEM molecular weight marker was used (Promega). Electrophoresis was performed at 100 V for about 30 min using a horizontal electrophoresis chamber (Bio-Rad) filled with 1 x TBE buffer. Ethidium bromide stained DNA was visualized using a Kodak Image Station 440.

2.7. Restriction enzyme digest

The cDNA inserts generated by RT-PCR or PCR were designed to create restriction sites that allow insertion into the multiple cloning site of the expression vector. The cDNA inserts and expression vector DNA (1 - 5 µg) were digested with the respective restriction enzymes (Promega). For cloning into the pET23b vector (used for generating fusion proteins) the restriction enzymes Sal I and Not I were used in buffer D. For cloning into the pcDNA5/FRT/TO vector (used for SR-A construct expression in HEK-293 cells) the restriction enzymes Hind III and Xho I were used in buffer C. Buffers were provided with the enzymes and in these buffers the activity of the enzymes is 75 – 100 %. To enhance enzyme activity, AcBSA was added at a final concentration of 100 µg/mL. Per µg of DNA 10 units of each restriction

enzyme (10 U/µl) were used. Enzymes are provided in 50 % glycerol and for optimal enzyme activity the final glycerol concentration was kept < 5 % in the final volume of 50 -100 µl. The digest was incubated at 37°C for 1 h.

2.8. DNA purification

To purify PCR products and digested vector DNA, the reaction mixtures were resolved by gel electrophoresis and the DNA was extracted from the gels using the rapid gel extraction kit (GibcoBRL) following the manufacturer's protocol. To purify digested PCR products, the rapid PCR purification kit (GibcoBRL) was used following the manufacturer's protocol.

2.9. DNA ligation

The DNA Quick ligation kit (Roche) was used to ligate cDNA inserts into the expression vectors. Briefly, the digested and purified vector DNAs (100ng) and insert DNAs (vector DNA and insert DNA at a molar ratio of 1:3) were mixed in a total volume of 20 µl containing 10 µl of 2x DNA ligation buffer. After addition of 1 µl T4 DNA ligase (5 U/µl), the mixture was incubated for 30 min at room temperature. To amplify the ligated vector DNA, E. coli JM109 were transformed using the ligation mixture.

2.10. Transformation of E.coli

LB Agar plates

6.4 g of powered LB agar

Dissolve in 200 ml dH_2O

Autoclave

Cool < 60°C and add 250 µl ampicillin stock (125 µg/ml)

Store at 4°C

E. coli competent cells (Promega) were thawed on ice and 20 ul of cells were transferred into a pre-chilled 1.5 mL polypropylene Eppendorf tube. Then, 3 µl of the ligation reaction or 1 - 5 ng of purified vector DNA was added and cells were kept on ice for 10 min. As positive control, transformation with 0.1ng/µl non-digested vector DNA was performed. As negative control, transformation without vector DNA was performed. The cells were heat-shocked at 42°C for 30 seconds and then kept on ice for 2 min Then, 80 µl of S.O.C. medium were added. Cells were incubated in an orbital shaker at 225 rpm and 37°C for one hour to allow expression of the ampicillin resistance gene from the vector. 20 and 50 µl of the reaction were spread out on LB agar plates containing ampicillin (125 µg/mL). The plates were incubated at 37°C overnight to allow growth of ampicillin resistant colonies. Colonies were screened by PCR for the presence of the inserts using the primers that were used to generate the inserts.

2.11. Storage of E. coli

80 % glycerol
8 ml glycerol
2 ml dH_2O
Autoclave
Store at room temperature

For storage of E.coli, glycerol stocks were prepared. E. coli containing the vectors were grown in 10 mL of LB the presence of selection antibiotics until the culture reached an optical density (OD) of OD_{600} = 0.6 – 0.8. Then, 0.9 ml of cell suspension were transferred to a cryovial and 0.1 ml of 80% glycerol were added. Glycerol stocks were stored at – 70°C.

2.12. Isolation of vector DNA

E. coli colonies selected on LB Agar plates were inoculated in 10 mL LB containing ampicillin (100 µg/ml) and grown overnight. Vector DNA was isolated from E.coli cultures using the Wizard Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer's protocol. The DNA concentration was determined and the DNA was stored at – 20°C.

2.13. Generation expression vectors

a) PET23b for fusion protein expression in E. coli

The PET23b vector of the pET system (Novagen) was used to generate fusion proteins homologous to the α-helical coiled-coil, to the collagen-like domain or to both of these domains of the murine scavenger receptor. The respective cDNA encoding the proteins were amplified by PCR with primers generating 5' Sal I and 3' Not I restriction sites. The primer sequences and product sizes are shown in table 5.

After the correct product size was confirmed by agarose gel electrophoresis, the cDNA products were purified, digested with Sal I and Not I, and cloned into the PET23b expression vector. E. coli JM109 were transformed and selected on LB agar plates. Colonies were screened for cDNA inserts by PCR using the primers that were used to generate the inserts. Positive colonies of each insert were grown in LB overnight and vector DNA was isolated. The sequence of the inserts was confirmed at the Macromolecular Structure Analysis Facility at the University of Kentucky.

b) pcDNA5/FRT/TO for SR-A construct expression vectors in HEK cells

The pcDNA5/FRT/TO expression vector of the Flp-In™ T-REx™ system (Invitrogen) was used to generate HEK cells that express the different SR-A constructs. The cDNAs encoding full-length SR-A, SR-A lacking all 55 amino acids of the N-terminal cytoplasmic tail (SR-$A_{\Delta 1-55}$), and SR-A lacking the first 49 amino acids of the cytoplasmic tail (SR-$A_{\Delta 1-49}$) were amplified by PCR from SR-A type II murine cDNA (gene bank accession no. L04275). The primers were designed to generate 5' Hind III and 3' Xho I restriction sites. The primer

sequences and products sizes are shown in Table 5. Deleting the cDNA encoding the amino-terminal amino acids eliminated the translation start codon. Therefore, the 5' PCR primers used to amplify SR-$A_{\Delta 1-55}$ and SR-$A_{\Delta 1-49}$ were designed to insert a methionine translation start codon and a spacer alanine codon. To replace the cytoplasmic tail of SR-A with that of the transferrin receptor (TfR/SR-A), the cDNA encoding the N-terminal 57 amino acids of the murine transferrin receptor (gene bank accession no. X57349) was amplified by RT-PCR from RNA isolated from mouse liver and inserted 5' to the cDNA encoding the SR-A transmembrane and extracellular domains.

The RT-PCR and PCR products were resolved by agarose gel electrophoresis. After the correct product size was confirmed, the cDNA products were purified, digested by the appropriate restriction enzymes, and cloned into pcDNA5/FRT/TO expression vector (Invitrogen). The sequence of the inserts was confirmed at the Macromolecular Structure Analysis Facility at the University of Kentucky. The amino acids sequences of the receptor constructs are shown in Table 14.

3. Generation of fusion proteins

IPTG (100 mM)
2.38 g IPTG
Dissolve in 100 ml dH_2O
Store at -20°C

Fusion proteins homologous to the α-helical coiled-coil, the collagen-like domain or both of these domains of the murine scavenger receptor were generated using the Novagen pET system. An overview of the system is shown in Figure 5. The cDNAs, which are the target genes, encoding the SR-A domains, were cloned into the PET23b expression vector and transformed into E. coli BL21(DE3) host cells as described in the molecular biology section. Expression of the target gene is under the control of the T7 RNA polymerase promoter. E.coli BL21 (DE3) host cells carry the T7 RNA polymerase gene (DE lysogen). The T7 RNA polymerase and the target gene

expression are under the control of the lac repressor, which is expressed constitutively from the E. coli genome. Therefore, expression of the target gene is induced by addition of the lactose analogue IPTG (0.4 mM) to the growth medium. Expression of the target gene results in His•Tag fusion proteins (6 consecutive histidine residues).

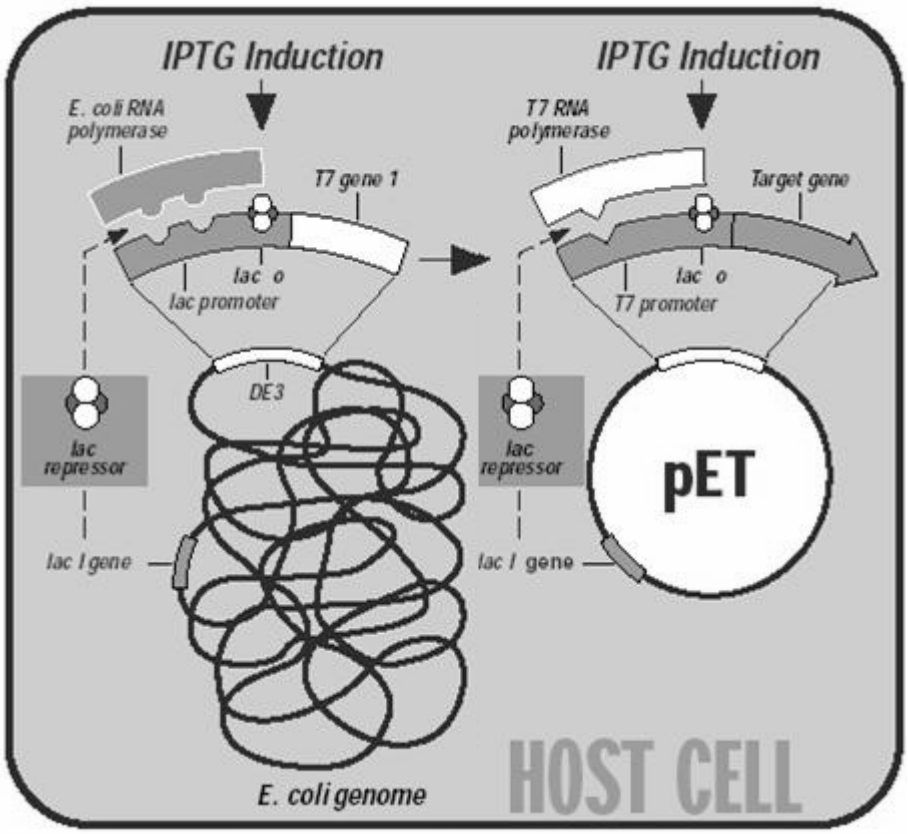


Figure 5: Overview of the pET expression system used to express fusion protein homologous to SR-A domains. Expression of the target gene in the E. coli host cells is induced by addition of IPTG.

Cells transformed with the expression vectors were grown in 200 ml LB until the culture reached an optical density (OD) of OD_{600} = 0.5 – 1.0. Fusion protein expression was induced for 3 h by addition of IPTG (0.4 mM) to the broth. To isolate the expressed fusion protein, the cells were separated from the broth by centrifugation at 1,000 g for 10 min. To confirm fusion protein expression, cell lysates were separated by SDS-PAGE and fusion proteins were detected by Western blotting with a His•Tag antibody (Santa Cruz).

After expression was confirmed, the fusion proteins were isolated from the cell lysates by immobilized metal affinity chromatography using His•bind quick columns (Novagen). The His•Tag sequence of the fusion proteins binds to divalent cations (Ni^{2+}) immobilized on His•Bind resins of the columns. After unbound proteins are washed away, the fusion proteins are recovered by elution with imidazole and dialyzed against PBS.

4. Protein chemistry

4.1. Preparation of cell lysates

Cell lysates of HEK-293 cells were prepared by incubating cells in lysis buffer for 30 min on ice in the presence of protease inhibitors (Calbiochem). After lysis, insoluble material was removed by centrifugation at 13,000 g for 10 min and the supernatant was transferred to a fresh tube.

4.2. Protein concentration

Protein concentrations of the cell lysates were determined based on the Lowry assay (Lowry et al. 1951) using a colorimetric assay according to the manufacturer's instructions (D_C Protein Assay, Bio-Rad). Absorption of samples was measured at 655 nm using a microplate reader (Bio-Rad). Bovine serum albumin at concentrations between 0.1 mg/ml and 1.6 mg/ml was used to generate a standard curve. All samples and standards were assayed in duplicate.

4.3. Immunoprecipitation

In some experiments, SR-A receptors were immunoprecipitated from cell lysates or culture media using the rat SR-A monoclonal antibody, 2F8 (3 µg/ml; Serotec) and anti rat IgG-coated magnetic beads (PerSeptive Biosystems). Briefly, cell lysates were adjusted to 200 µl in lysis buffer containing protease inhibitors (Calbiochem). 2F8 was added one hour before addition of 50 µl anti rat IgG-coated magnetic beads. Samples were incubated overnight rotating at 4°C to allow formation of the antigen-antibody-complexes bound to the beads. The beads were pelleted by centrifugation at 2,000 rpm for 5 min at 4°C. After washing twice in 1 ml lysis buffer containing protease inhibitors, 25 µl of reducing sample buffer were added to the bead pellet. Samples were boiled at 100°C for 10 min and resolved by SDS-PAGE.

4.4. Biotinylation of cell surface proteins

Cells were plated in 6-well plates (0.5 x 10^6 cells/well) in DMEM/FBS and induced. Induced cells were trypsinized, transferred into 1.5 mL eppendorf tubes, washed twice in 1 ml ice-cold PBS. To prevent internalization, cells were kept on ice. Cell surface proteins were biotinylated in suspension (1 mg/ml EZ-Link sulfo-NHS-LC-Biotin in PBS, Pierce) at 4°C for 30 min according to the manufacturer's instructions. To remove non-bound biotin, cells were washed with three times with ice-cold PBS. Subsequently, cell lysates were prepared and adjusted to 200 µl in lysis buffer containing protease inhibitors. Biotinylated surface proteins were precipitated from lysates using streptavidin-coated magnetic beads (PerSeptive Biosystems). A volume of 50 µl of beads was added to the cell lysates and incubated rotating at 4°C overnight. Cell surface proteins (bead pellet) and intracellular proteins (supernatant) of the precipitation were resolved under reducing conditions by SDS-PAGE and SR-A was detected by Western blotting.

4.5. N–glycanase (PNGase F) and endoglycosidase H digestion

To cleave all N-linked oligosaccharides, cell lysate protein (10 µg) was digested with N-glycanase (PNGase F; Sigma) in buffer (50 µl; 50 mM $NaHPO_4$, 0.1% SDS, 0.05 M β-mercaptoethanol, pH 7.5) according to the manufacturer's protocol. Briefly, proteins were denatured by heating to 100°C for 5 min. After cooling, Triton X-100 (0.75% v/v) and N-glycanase were added and the reaction was incubated for 3 h at 37°C.

To cleave precursor N-linked oligosaccharides, cell lysate protein (10 µg) was digested with endoglycosidase H (EndoH, Sigma) in buffer (50 µl; 50 mM $NaHPO_4$, 0.1% SDS, 0.05 M β-mercaptoethanol, pH 5.5) according to the manufacturer's protocol, which was identical to that used for N-glycanase digestion with the exception of the addition of Triton X-100.

4.6. SDS-PAGE

10 % SDS
10 g sodium docecyl sulfate (SDS)
Dissolve in 100 ml dH_2O
Store at room temperature

1.5 M Tris
7.38 g Tris HCl
30.78 g Tris base
Dissolve in 200 ml dH_2O
pH 8.8
Store at 4°C

0.5 M Tris
12.0 g Tris base
Dissolve in 200 ml dH_2O
pH 6.8
Store at 4°C

20 % APS (wt/v)
0.2 g ammonium persulfate
Dissolve in 1.0 ml dH_2O
Store at 4°C

Running buffer (10 x)
30 g Tris
144 g Glycine
10 g SDS
Dissolve in 1 l dH_2O
Store at room temperature

Running buffer (1 x)
dilute 50 mL of 10 x running buffer to 500 mL with dH_2O

Sample buffer, reducing (2 x)
6.0 ml dH_2O
0.5 ml 0.5 M Tris, pH 6.8
1 ml glycerol
1 ml SDS (10 % w/v)
0.75 ml bromophenol blue
0.2 ml β-mercaptoethanol
Store at 4°C
For non-reducing buffer omit β-mercaptoethanol

Proteins were resolved by discontinuous SDS-polyacrylamide gel elecrophoresis (SDS-PAGE) using the MINI-PROTEAN II System (BioRad). By the addition of SDS, an anionic detergent, proteins are denatured and charged negatively. Thus, proteins are separated independent of charge based on their molecular weight. Proteins were resolved under reducing or non-reducing conditions as indicated. The resolving gel was overlayed with a stacking gel. The preparation of the gels is shown in table 12. The gel electrophoresis was run at 50 V until the samples reached the stacking gel. Then, the voltage was increased to 130 V and electrophoresis was continued until the loading dye reached the bottom of the resolving gel. Prestained markers were used to determine the molecular weight (BioRad).

Separating gels (20 ml)						
Percent	**Acrylamide/bis**	**1.5 M Tris**	**H_2O**	**10 % SDS**	**20 % APS**	**TEMED**
7.5	2.5	2.5	4.9	100 µl	30 µl	60 µl
15	5		2.4			
Stacking gels (5 ml)						
Percent	**Acrylamide/bis**	**0.5 M Tris**	**H_2O**	**10 % SDS**	**20 % APS**	**TEMED**
4	0.65	1.25	3.05	50 µl	30 µl	60 µl

Table 12: Preparation of separating and stacking gels for SDS-PAGE. All volumes are ml unless stated otherwise. The final volumes are sufficient for two minigels. APS and TEMED are added last for polymerization of the gels.

4.7. Western blotting

Primary antibodies			
Antibody	**Antigen**	**Raised in**	**Dilution**
2F8	SR-A	Rat	3 µg/ml
SR-A antiserum	SR-A	Guinea pig	1:100
His•Tag	His•Tag	Rabbit	1:1,000
Secondary antibodies			
Antibody	**Antigen**	**Raised in**	**Dilution**
Anti rat	Rat IgG	Goat	1:10,000
Anti guinea pig	Guinea pig IgG	Goat	1:10,000
Anti rabbit	Rabbit IgG	Goat	1:10,000

Table 13: Antibodies used for Western blotting. All antibodies were diluted in TNT.

Transfer buffer

3 g	Tris base
14.4 g	glycine
	Dissolve in 800 ml dH_2O
	Add 200 ml methanol
	Store at 4°C

TNT

10 mM Tris	10 ml 500 mM pH 8.0
140 mM NaCl	15 ml 5M
0.1 % Tween 20 (v/v)	500 µl
	Dilute to 500 ml dH_2O
	Store at room temperature

Blocking solution

1.25 g	dry milk
25 ml	TNT

Proteins resolved by SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) by electrophoresis at 100 V (300 – 400 mA) for 90 min on ice. To enhance protein binding to the membranes, the membranes were dried after the transfer.

Antigens bound to the membrane were detected by Western blotting as follows. All steps were performed at room temperature using an orbital shaker. Dried PVDF membranes were rewetted in ethanol and soaked in dH_2O for 5 min. Then, the membranes were blocked with dry milk (5% wt/v) in TNT for 30 min. Membranes were incubated with primary antibodies for 20 min. SR-A receptor proteins resolved under reducing conditions were detected using a guinea pig antiserum developed to a fusion protein of the extracellular SR-A domain as described previously (Daugherty et al. 2000). To detect oligomeric forms of the receptors, cell lysates were resolved under non-reducing conditions and receptor proteins detected with 2F8 (3 µg/mL, Serotec), which also recognizes an extracellular epitope of SR-A (Daugherty et al. 2000). His•Tag labeled fusion proteins were resolved under reducing

conditions and detected with a rabbit anti His•Tag antibody. After washing 5 times for 2 min in TNT. Primary antibody binding was detected by incubation with horseradish peroxidase labeled secondary antibodies (Jackson; 1:10,000 dilution). After washing 5 times for 2 min in TNT, horseradish peroxidase activity was visualized by enhanced chemiluminescense (ECL; Pierce) using a Kodak Image Station 440.

4.8. Immunofluorescence

Cells were plated on 2-chamber (50,000 cells/chamber) LAB-TEK glass slides (Nalge Nunc International) and receptor proteins were detected as previously described (Post et al. 2002). Briefly, cells were induced where indicated, washed twice with phenol-red free DMEM (37°C), fixed in paraformaldehyde (4 % w/v), and permeabilized with Triton-X (0.1 % v/v in PBS). Nonspecific binding sites were blocked by incubation with BSA (1% w/v in PBS) for 30 min. To detect expressed receptors, cells were incubated with 2F8 (3 ug/mL in PBS; Serotec) for 15 min. After washing twice with PBS, primary antibody binding was detected by incubation with an Alexa-Fluor488 labeled goat anti-rat antibody (2 µg/mL in PBS; Molecular Probes) for 20 min. Cell nuclei were stained with DAPI (300 nM in PBS; Molecular Probes) for 5 min. After washing twice in PBS, cells were mounted in Mowiol embedding medium (37°C) containing n-propyl gallate (1% w/v), cover slipped and dried horizontally overnight at 4°C. Images were captured digitally by fluorescence microscopy (Excitation filter 465 – 495) with constant exposure times.

5. Lipoprotein isolation and modification

EDTA/saline solution

1 mM	EDTA
0.15 M	NaCl
	pH 7.4
	Store at room temperature

LDL (d = 1.019 – 1.063 g/ml) was isolated by sequential ultracentrifugation of EDTA-anticoagulated plasma obtained from healthy normolipidemic volunteers (Havel et al. 1955).

5.1. VLDL and IDL

The first step is isolation of the VLDL and IDL fraction (d < 1.019 g/ml). The density of the plasma (d = 1.006) is adjusted to 1.019 by addition of KBr (17.14 mg/ml). After adjusting the density, the plasma is transferred into ultracentrifuge tubes (Beckman) and spun at 40,000 rpm at 10 °C for 18 h in a Beckman ultracentrifuge. A phase separation will occur. The white top phase (d < 1.019) contains the VLDL and IDL particles. This phase is removed and discarded. The lower yellow phase (d > 1.019) contains LDL, HDL and proteins.

5.2. LDL

The lower phase (d > 1.019) is transferred into a measuring cylinder and the density is adjusted to 1.063 by addition of KBr (58.78 mg/ml). After adjusting the density, the plasma is transferred into ultracentrifuge tubes (Beckman) and spun at 50,000 rpm at 10 °C for 18 h. Again, a phase separation will occur. The top yellow phase (d = 1.019 – 1.063) contains the LDL particles. To further purify the LDL containing phase, the top yellow phase is transferred into a fresh centrifuge tube and mixed with EDTA/saline solution adjusted to d = 1.063 (80.77 mg/ml KBr) and centrifuged as above. The purified top phase is then transferred into dialysis tubing (Spectrum) and dialyzed against EDTA/saline (d = 1.006) with at

least three changes of the EDTA/saline at 4 °C. After dialysis LDL is filter sterilized (0.22 µm pore size) and the protein concentration is determined by Lowry assay after delipidation of the sample.

5.3. Preparation of acetylated LDL

Saturated sodim acetate:
0.574 g sodium acetate
Dissolve in 1 ml dH_2O

Acetylated LDL (AcLDL) was prepared by chemical modification of LDL with acetic anhydride (Fisher) as described by Basu et al.. (Basu et al. 1976). LDL (1 ml), isolated as described above (5.2), was placed into a glass tube (16x100) and an equal volume of saturated sodium acetate solution was added. During constant stirring on ice, acetic anhydride (2.5 µl) was added at 0, 15, 30, 45 min of incubation and incubation was continued for another 30 min. Acetylation was confirmed by agarose gel electrophoresis and comparison to unmodified LDL. As described for LDL, acetylated LDL was dialyzed against EDTA/saline, sterile filtered and the protein concentration was determined.

6. Cell culture

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL) containing glutamine (1%), penicillin (10 U/ml), streptomycin (10 µg/ml) and heat-inactivated fetal bovine serum (10% FBS; GibcoBRL) (DMEM/FBS) unless stated otherwise. Cells, culture reagents, and materials were handled under sterile conditions. Cells were cultured in a humidified incubator with 5 % CO_2/95 % air at 37°C.

6.1. Mouse peritoneal macrophages

Mouse peritoneal macrophages were harvested by peritoneal lavage. Mice were anesthetized and sacrificed by cervical dislocation. The outer skin was cut and 5 ml of ice-cold sterile saline were injected into the peritoneal cavity through the intact abdominal musculature with a 5 ml syringe and an 18-gauge needle. The saline was slowly withdrawn back into the syringe and transferred into a conical tube on ice. Macrophages were spun down by centrifugation at 300 g for 15 min at 4°C, washed and plated for experiments in tissue culture dishes or onto glass slides in DMEM/FBS.

6.2. RAW.264

The mouse macrophage cell line RAW.264 was cultured in about 13 ml of DMEM/FBS in T-75 tissue culture flasks. When the cells reached about 80 - 90 % confluency, cells were scraped and resuspended in 10 mL and 1 ml of the cells suspension (1:10 split) was transferred into a fresh T-75 flask containing 12 ml media.

6.3. HEK-293 expressing SR-A constructs

a) HEK-293 host cells

To generate cells that express the different SR-A constructs, we used the Flp-In™ T-REx™ system (Invitrogen). An overview of the system is shown if Figure 5. This system allows tetracycline-inducible expression after integration of the cDNA into a specific genomic site. An important advantage of this system is that similar levels of expression can be obtained upon induction of cells transfected with the different SR-A constructs. Flp-In™ T-REx™ HEK-293 host cells were used, as HEK-293 cells do not endogenously express SR-A and have been used previously to study SR-A function (Post et al. 2002).

HEK-293 host cells were cultured in T-75 tissue culture flasks in 13 ml DMEM/FBS containing the selection antibiotics blasticidin (15 µg/ml) and zeocin (100 µg/ml). When the cells reached a confluency of about 80 -90 %, cells were detached by incubation in 5 mL Trypsin/EDTA for 5 - 10 minutes. Trypsin activity was stopped by addition of 5 mL DMEM/FBS. The cell suspension was transferred into a 15 ml centrifuge tube and the cells were spun down by centrifugation at 300 g for 5 min. The cells were resuspended in 10 ml DMEM/FBS containing selection antibiotics and 1 ml of the cells suspension (1:10 split) was transferred into a fresh T-75 flask with 12 ml DMEM/FBS containing selection antibiotics. For transfection cells were plated (day 1 of transfection procedure) into 6-well plates in 2 ml (5×10^5/well).

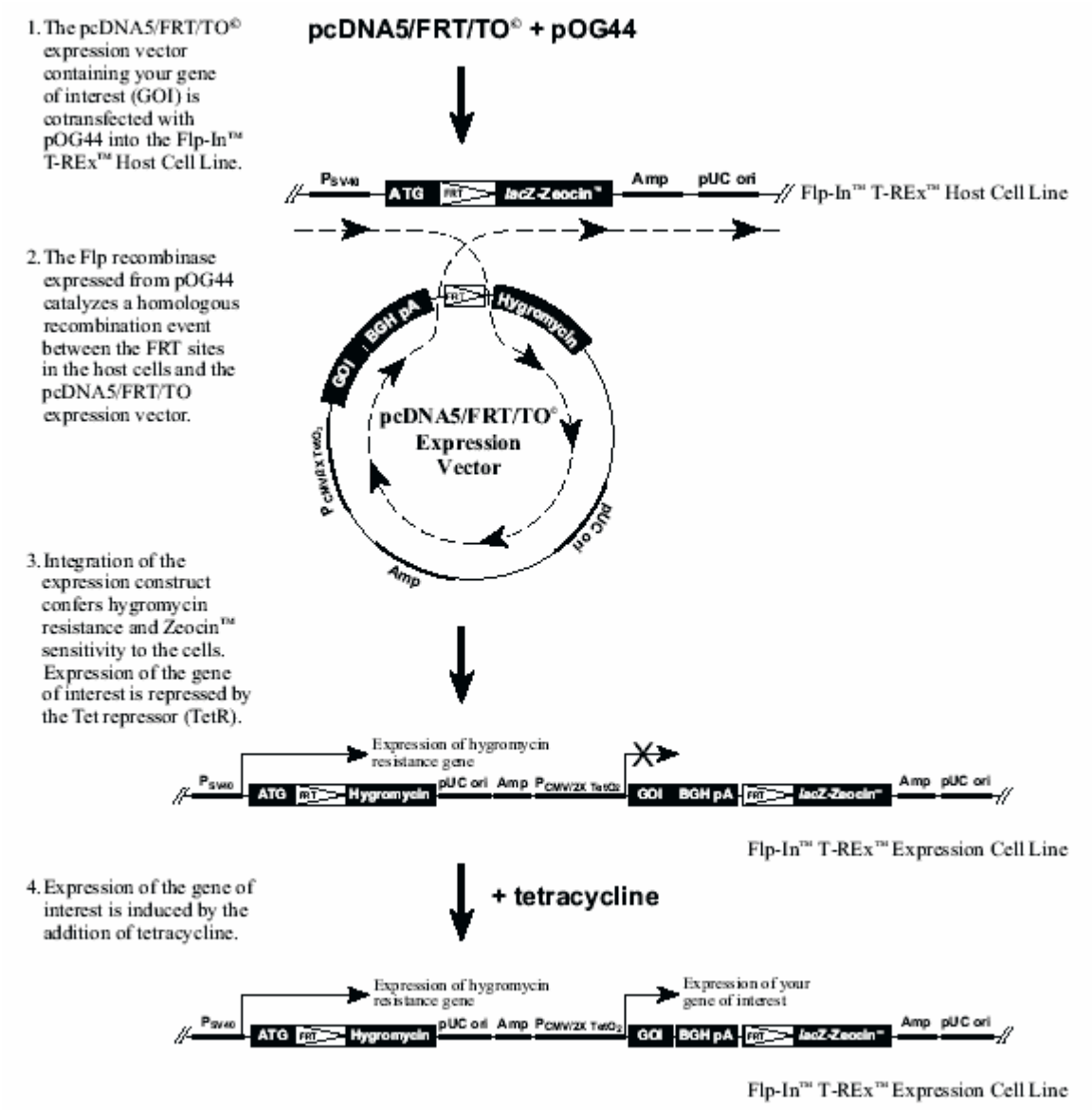


Figure 6: Overview of the Flp-In™ T-REx™ expression system used to generate HEK-293 cells expressing the different SR-A constructs.

b) Transfection and selection of HEK-293 cells

Approximately 24 hours after plating (day 2), HEK-293 host cells were co-transfected with the vectors pcDNA5/FRT/TO (0.2 µg) containing the SR-A constructs and pOG44 (1.8 µg), encoding Flp recombinase, which mediates insertion of pcDNA5/FRT/TO into the genomic integration site, using the TransIT-293 transfection reagent according to the manufacturer's protocol (Mirus). Briefly, 6 µl of the TransIT-293 reagent were added to 244 µl DMEM, mixed by vortexing and incubated for 5 min. In a separate tube, the vector

DNAs were mixed in 250 µl DMEM. The two mixtures were combined, mixed by gentle pipetting and incubated at room temperature for 20 min to allow the TransIT-293/DNA complex to form. This mixture was added dropwise to the HEK-293 host cells. The cells were incubated with the TransIT-293/DNA complex for 24 h. On day 3, the transfection media was replaced with DMEM/FBS containing blasticidin (15 µg/ml). After transfection, Flp recombinase is expressed and mediates integration of the pcDNA5/FRT/TO into the genome. Integration of pcDNA5/FRT/TO eliminates zeocin resistance and confers hygromycin B resistance. Thus, transfected cells were selected for stable integration of the SR-A constructs into the genome with hygromycin B (200 µg/ml) starting on day 4. The selection media was changed every 2 - 3 day and resistant colonies appeared about 2 weeks after tranfection. Tranfected cells were cultures are described for host cells in DMEM/FBS containing the selection antibiotics blasticidin (15 µg/ml) and hygromycin B (100 µg/ml).

c) Induction of construct expression of HEK-293

Receptor construct expression was induced by adding tetracycline to the culture media for 16 h unless stated otherwise. A tetracycline stock solution (1mg/ml) was prepared in ethanol and stored protected from light at - 20°C. Concentration response studies showed that a concentration of 0.5 µg/ml tetracycline induced maximal protein expression (data not shown).

d) Storage of cells

When cells were passage into a new T-75 flask as described above, 0.7 ml of the 10 ml cell suspension was transferred into 1.5 ml cryovials containing 0.2 ml FBS and 0.1 ml DMSO. The cells were stored at - 70°C for 24 h and then transferred into liquid nitrogen.

7. Cholesterol ester synthesis in peritoneal macrophages

Isolated peritoneal macrophages were incubated with the stated concentrations of AcLDL protein/ml and ^{3}H-oleate (0.1 mM; Amersham) complexed with fatty acid-free BSA in a molar ratio of 5:1 for 5 h at 37°C in DMEM. The media was removed, and the cells were washed twice with ice-cold Tris buffer (pH 7.4). Cellular lipids were extracted by two 30-min incubations with 1ml hexane–isopropanol (3:2, v/v) containing carrier lipid (triolein and cholesterol palmitate). The lipid extracts were dried under nitrogen, redissolved in chloroform-methanol (2:1, v/v), and separated by thin-layer chromatography with petroleum ether:diethyl ether:acetic acid 84:15:1 (v/v/v). Esterified cholesterol was identified by exposure to iodide vapor, scraped into EcoLite scintillation fluid (ICN). Cholesteryl ^{3}H-oleate was quantified by liquid scintillation counting using a Beckman LS 3801 counter. Cell proteins were solubilized in 0.5 ml 0.1 N NaOH for 16 h at room temperature and protein content was determined using the Bio-Rad D_c protein assay (Bio-Rad). The amount of cholesteryl ^{3}H-oleate was normalized to cell protein.

8. Spreading of peritoneal macrophages

Peritoneal macrophages isolated from lyso-bSR-A transgenic or non-transgenic littermate mice were resuspended in DMEM/FBS and plated on 8-well LabTek glass slides (10^5 cells/well) for up to 24 hrs and fixed with 4 % paraformaldehyde. After non-specific binding sites were blocked with non-immune goat serum (15 µl/ml), cells were immunostained with a rabbit antiserum to mouse macrophages (1:1,000 dilution; Accurate). Primary antibody binding was detected with a biotinylated goat anti rabbit secondary antibody (1:200 dilution; Vector). Antibody binding was visualized using the ABC Kit (Vector) and the red chromogen ACE. The area of cells was quantified using Image Pro software.

9. RAW.264 macrophage adhesion assay

We modified an assay described by Gowen and colleagues to investigate SR-A mediated macrophage adhesion (Gowen et al. 2000). Collagen type I coated 96-well plates (Becton Dickinson) were digested with collagenase (2.5 µg/mL PBS, Sigma) for 30 min at 37°C and then washed three times with PBS (200 µl). RAW 264 macrophages were washed and resuspended to a final density of 1.5 x 10^6 cells/ml in Hanks buffered saline solution (HBSS) devoid of divalent cations (Cellgro). Incubation in the absence of divalent cations will eliminate integrin-mediated adhesion, as integrin-mediated adhesion is dependent on divalent cations. To each well 100 µl of cell suspension (1.5 x 10^5 cells/well) were added and incubated for 60 min at 37°C to allow cells to adhere. Non-adherent cells were removed by washing three times with PBS (200 µl). Adherent cells were fixed in 4 % paraformaldehyde (200 µl/well) for 15 min and stained with hematoxylin (50 µl/well) for 5 min. Fixed and stained cells were washed with PBS (200 µl) twice and kept in PBS (100 µl) for analysis. Adhesion was quantified by counting cells per field at 10x magnification using a light microscope and ImagePro Software.

To inhibit SR-A mediated adhesion, the cells were incubated with the rat monoclonal antibody against SR-A, 2F8 (Serotec), or antagonists of the SR-A specific ligand acLDL, polyinosine and fucoidan, at the stated concentrations for 15 min at room temperature before the cells were added to the wells. As negative controls, cells were incubated with an isotype-matched antibody (rat IgG2b, Serotec) or structurally similar compounds of the antagonists polycytidine and chondroitin respectively at the same concentrations and conditions. To investigate whether a fusion protein homologous to the extracellular domain can compete for SR-A mediated cell adhesion, the fusion protein was added to the modified collagen coated wells at the stated concentrations in 50 µl PBS and incubated for 30 min before addition of the cells.

10. Tunicamycin and brefeldin A treatment of HEK-293 cells

To inhibit N-linked glycosylation in the endoplasmic reticulum (ER), HEK-293 cells were cultured in presence of tunicamycin (5 µg/ml, Sigma). To inhibit maturation of N-linked oligosaccharides in the Golgi apparatus, HEK-293 cells were cultured in presence of brefeldin A (5 µg/ml, Sigma). These inhibitors were added 60 min before inducing cells with tetracycline and were present throughout induction. Cell lysates were prepared 6 h after addition of tetracycline.

11. AcLDL internalization of HEK-293 cells

Cells were plated into 6-well plates (0.5 x 10^6 cells/well) in DMEM/FBS and induced. Following incubation of induced cells in serum-free DMEM for 2 h, fluorescently labeled AcLDL (2.5 µg/ml, Alexa-Fluor488 AcLDL, Molecular Probes) was added to the media and incubation was continued for another 2 h. Cells were trypsinized with 0.5 ml T/E, washed twice with 2 ml ice cold PBS and resuspended in 200 µl PBS for analysis. AcLDL internalization was assessed by quantifying cell-associated fluorescence (Flow Cytometry Core Facility, University of Kentucky). Fluorescence was gated for individual live cells and the fluorescence associated with approximately 20,000 cells determined for each sample. To assess non-specific AcLDL cell-association, polyinosine (10 µg/ml) was added 5 min before addition of fluorescently-labeled AcLDL.

12. Cell adhesion assay of HEK-293 cells

HEK-293 cells were plated in 96-well plates (2 x 10^4 cells/well) in DMEM/FBS and induced where indicated. Adhered cells were incubated in EDTA-solution (0.2 g/ml, 37°C) for 10 min to eliminate divalent-cation dependent adhesion. After washing with PBS (37°C), cells remaining adhered were quantified using the CyQUANT assay (Molecular Probes) according to the manufacturer's instructions. To normalize cell adhesion to the total number of cells plated, an untreated plate was analyzed.

IV Results

1. Macrophages from SR-A transgenic mice

To better understand the role of SR-A expressed on macrophages in vivo, transgenic mice that express bovine SR-A (bSR-A) under the control of the macrophage specific lysozyme promoter (lyso-bSR-A) were generated (Daugherty et al. 2001). To assess the functional consequences of lyso-bSR-A expression, SR-A mediated ligand internalization and cell adhesion of peritoneal macrophages from lyso-bSR-A transgenic mice were compared to those of peritoneal macrophages from nontransgenic littermate mice.

To assess SR-A mediated internalization, I determined cholesterol ester synthesis in response to AcLDL. Cultured peritoneal macrophages were incubated with ^{3}H-oleate in the presence or absence of AcLDL (5 µg/ml) for 5 h. Then, lipids were extracted from the cells and separated by thin layer chromatography. Cholesterol ester synthesis was determined by quantifying the radioactivity of ^{3}H-oleate incorporated into cholesterol ester. AcLDL increased cholesterol ester synthesis in macrophages isolated from nontransgenic mice by 5-fold (figure 7). This increase was augmented by 2-fold in macrophages isolated from transgenic mice. This finding demonstrates that lyso-bSR-A expression enhanced SR-A mediated ligand internalization.

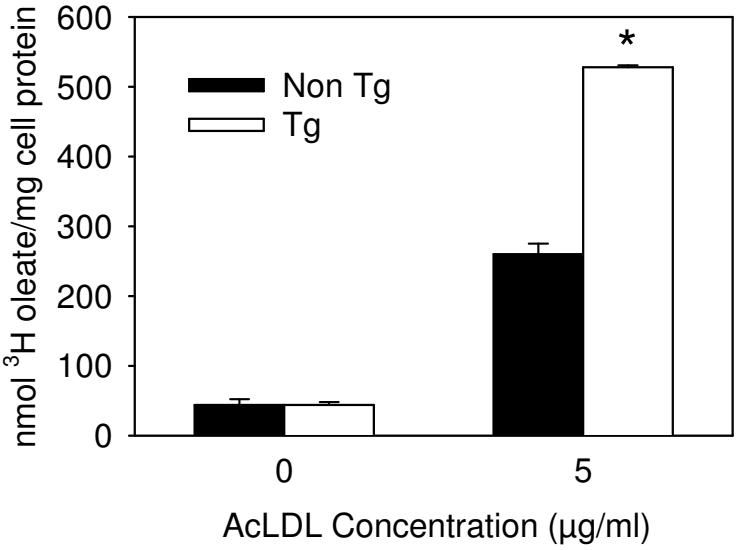


Figure 7: Peritoneal macrophages from non-transgenic (black bars) and lyso-bSR-A transgenic (white bars) littermates were incubated with AcLDL and ^{3}H-oleate at 37°C for 5 hours. Cholesterol esters were extracted and radioactivity content was quantified. Histobars represent the mean of six observations from two experiments, and bars represent the SEM. * P < 0.001

To assess the SR-A mediated cell adhesion, I anaylzed cell spreading of peritoneal macrophages in culture. Peritoneal macrophages isolated from SR-A deficient mice adhered weakly and did not spread when cultured on glass for 24 h, whereas those from the wild-type mice adhered tightly and displayed a spread morphology (Suzuki et al. 1997). To investigate whether macrophages isolated from lyso-bSR-A mice display enhanced cell spreading, I cultured peritoneal macrophages from SR-A transgenic mice on glass slides. After 3, 6 and 24 h cells were fixed and immunostained with a macrophage antiserum. By visual inspection macrophages from transgenic mice appeared more spread than those from nontransgenic mice at all time points (Figure 8A and B). Quantification of the area of the cells confirmed this observation (Figure 8C). After 24 h, there were a significantly increased number of macrophages with an area greater than 400 µm^2 ($P < 0.001$). The increased

cell spreading of macrophages from lyso-bSR-A transgenic mice demonstrates that lyso-bSR-A expression enhanced SR-A mediated adhesion.

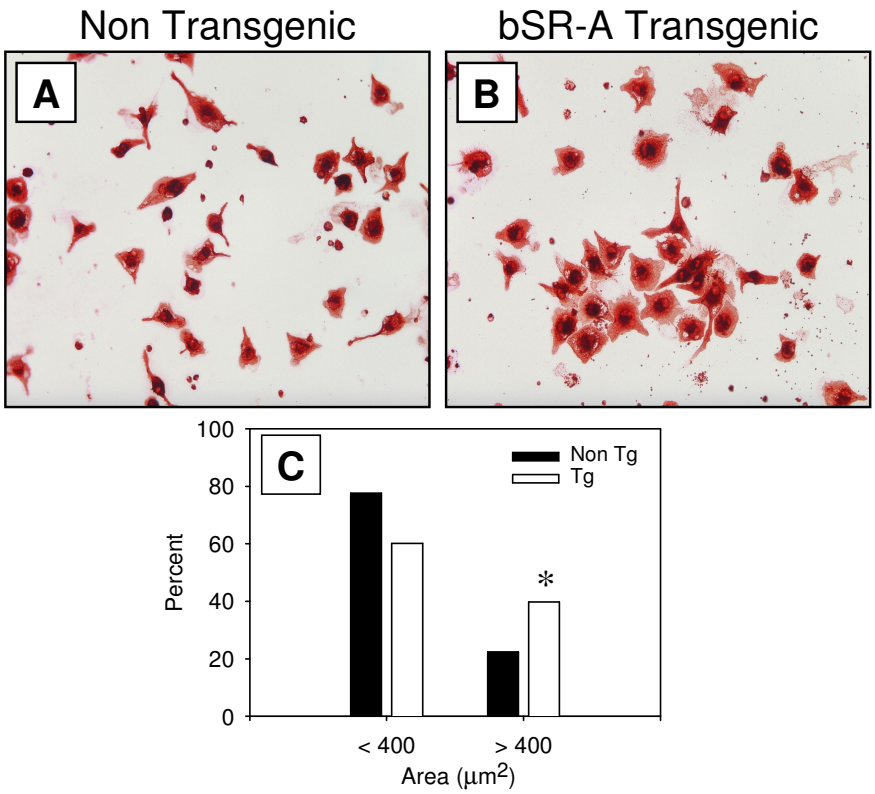


Figure 8: Peritoneal macrophages from non-transgenic (A) (black bars) and lyso-bSR-A transgenic (B) (white bars) littermates were cultured for 24 h on glass slides, immunostained with a macrophage antiserum and surface area was quantified (C). * P < 0.001

Taken together, our in vitro results demonstrate that lyso-bSR-A transgenic mice have increased macrophage SR-A activity. SR-A overexpression enhanced ligand internalization and cell spreading of peritoneal macrophages and both processes might occur in lyso-bSR-A mice in vivo.

Subsequent in vivo studies were performed to address the role of enhanced macrophage SR-A activity of lyso-bSR-A transgenic mice in a model of

inflammation (Daugherty et al. 2001). These studies found that lyso-bSR-A expression significantly increased the development of carrageenan-induced granulomas. Importantly, this increase was due to increased number of macrophages and not due to enhanced lipid deposition. This finding demonstrated that SR-A expression is associated with increased macrophage retention/recruitment during inflammatory processes in vivo and supports an important physiological role of SR-A mediated adhesion.

2. The extracellular domain of SR-A that mediates adhesion

SR-A mediated adhesion may play an important physiological role. Using SR-A transgenic mice, I have shown that increased SR-A expression enhanced macrophage spreading in vitro. This finding suggested that SR-A can increase macrophage adhesion. Subsequent studies in our laboratory using these transgenic mice have shown increased macrophage retention/recruitment in a model of inflammation in vivo (Daugherty et al. 2001). Furthermore, several extracellular matrix components that are associated with sites of tissue injury including modified types of collagen and certain proteoglycans have been identified as adhesion substrates for SR-A (el Khoury et al. 1994; Gowen et al. 2000; Santiago-Garcia et al. 2003). Therefore, SR-A mediated adhesion may play an important role in retention of macrophage at sites of tissue injury.

To be able to address the role of SR-A mediated adhesion specifically, I performed studies in order to identify the extracellular motif that mediates adhesion. I optimized an SR-A dependent adhesion assay described previously by Gowen et al.. (Gowen et al. 2001). RAW.264 macrophages adhere weakly to fibrillar (native) collagen type I. However, these cells displayed enhanced adhesion to monomeric, heat-denatured and collagenase-treated collagen type I collagen. The adhesion was independent of divalent-cations and could be blocked by 2F8 and the SR-A ligand dextran sulfate. Initial studies have shown that SR-A mediated adhesion to serum-coated surface can be completely blocked by 2F8. I studied adhesion of RAW264 macrophages to collagenase-treated collagen type I in serum-free and divalent cation-free HBSS.

RAW264 macrophages were incubated in 96-well tissue culture plates coated with collagenase-treated collagen type I. After one hour, non-adherent cells were removed and adherent cells were quantified as described in Figure 9.

I found that 2F8 specifically inhibited macrophage adhesion while an isotype matched control antibody had no effect (Figure 9).

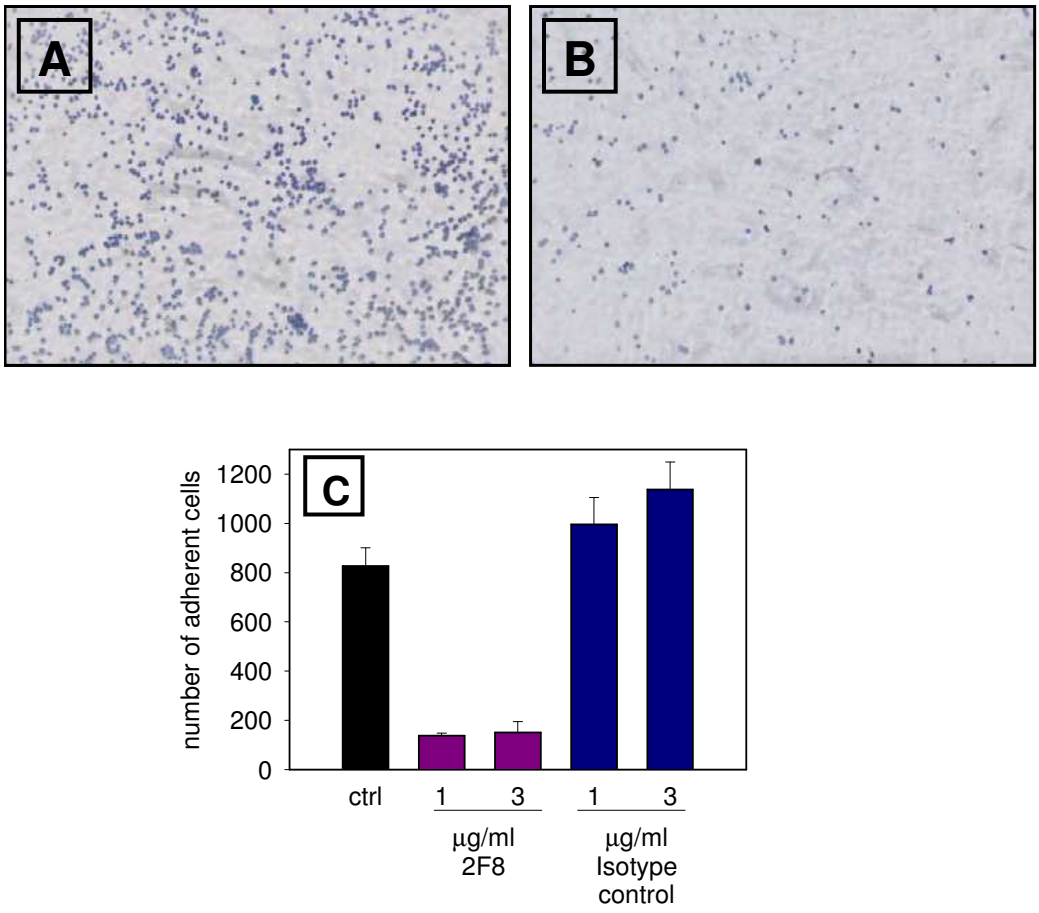


Figure 9: RAW264 macrophage were preincubated with 2F8, a monoclonal antibody against SR-A, or an isotype matched control antibody (rat IgG2b) for 15 min. Cells (1.5 x 10^5 cells/well) were plated in 96-well plates coated with collagenase-treated collagen type I. After incubation for one hour, non-adherent cells were removed. Adherent cells were fixed in paraformaldehyde and stained with hematoxylin. Light microscopic pictures of adherent cells were captured (10x magnification) with a digital camera and the number of adherent cells was determined for one field (10x) per well using ImagePro Software. Representative images of untreated control (A) or 2F8 treated (B) adherent macrophages. Quantification of adherent cells (C). Data is shown as mean ± SEM of six replicates.

To verify further that the assay is specific for SR-A, the cells were incubated with polyinosine and fucoidan, antagonists of AcLDL binding, before addition to the collagen I coated wells. Polyinosine and fucoidan inhibited macrophage adhesion, whereas the structural similar compounds polycytidine and chondroitin, respectively, had no effect (Figure 10).

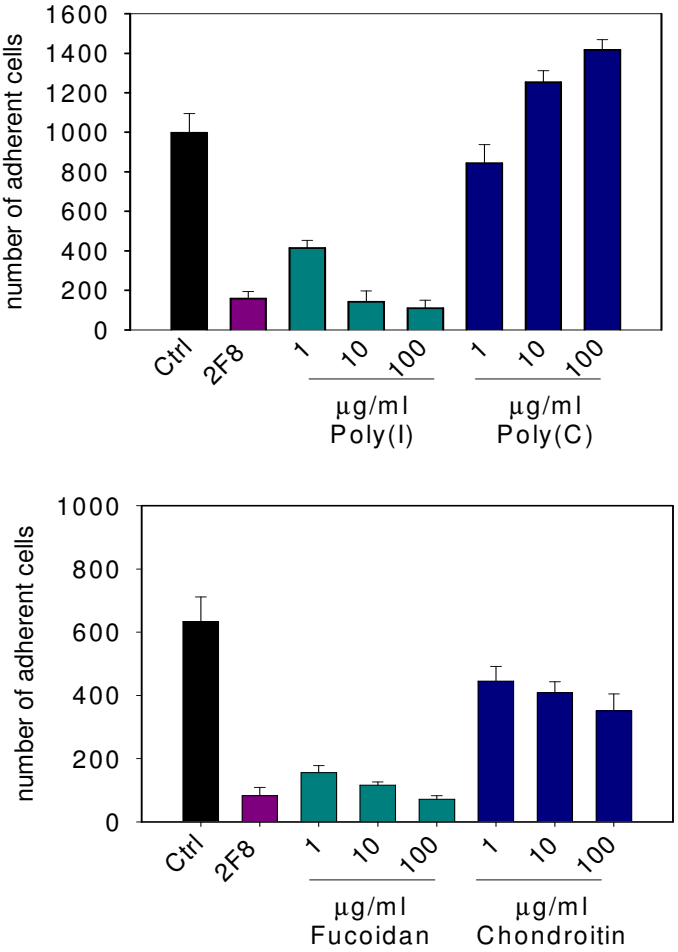


Figure 10: AcLDL antagonists (Poly(I) and Fucoidan) or structurally similar compounds (Poly(C) and Chondroitin), that do not antagonize AcLDL binding were preincubated for 15 min with RAW.264 macrophages before the adhesion assay was performed as described in Figure 9. As positive control cells were preincubated with 2F8 (3 µg/µl). A. polyinosine (poly(I)) but not polycytidine (poly(C)) inhibited adhesion. B. fucoidan but not chondroitin inhibited adhesion. Data is shown as mean ± SEM of six replicates.

To define the region of SR-A that mediated adhesion, I proposed to use fusion proteins homologous to SR-A to compete for SR-A mediated cell adhesion using this assay. I demonstrated that a fusion protein homologous to the extracellular α-helical coiled-coil and collagen-like domains could compete for macrophage adhesion (Figure 11).

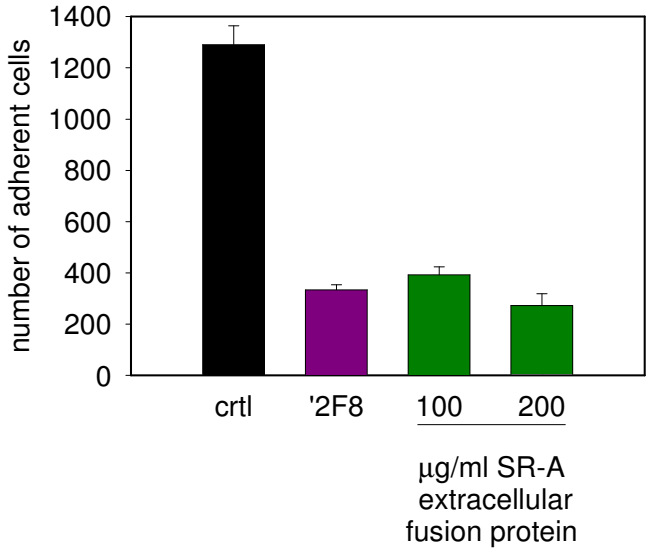


Figure 11: A fusion protein homologous to the extracellular domain of SR-A was incubated with the collagenase-treated collagen type I for 30 min and 2F8 (3 µg/µl) was incubated with the RAW264 macrophages for 15 min before the adhesion assay was performed as described in Figure 9. Data is shown as mean ± SEM of three replicates.

Overall, I confirmed that the adhesion assay is specific for SR-A mediated adhesion. Furthermore, I showed that a fusion protein containing the adhesion motif can compete for SR-A mediated adhesion demonstrating the feasibility of my approach to define the region containing the adhesion motif. However, during the course of the studies other investigators demonstrated that SR-A mediated adhesion requires the same domain that mediates binding of soluble ligands during internalization (Gowen et al. 2001). Thus, it appeared unfeasible to disrupt the adhesive properties without also disrupting ligand internalization.

3. Cytoplasmic domains mediating for SR-A functions

Previous studies have indicated that SR-A mediated adhesion and internalization have common extracellular requirements (Gowen et al. 2001). Consequently it has been postulated that SR-A mediated adhesion results from an attempt to internalize an immobilized ligand. In contrast, a recent study showed that clathrin does not co-localize with SR-A in the filopodia-like projections associated with increased adhesion of SR-A expressing cells (Post et al. 2002). This finding suggests that SR-A mediated adhesion is a process that is independent of clathrin-coated pit mediated endocytosis.

3.1. Expression and post-transcriptional processing

To investigate the role of the cytoplasmic tail in SR-A-mediated adhesion and to determine whether SR-A-mediated adhesion and internalization require distinct cytoplasmic domains, I expressed full-length SR-A or different SR-A constructs (Table 14) under the control of a tetracycline-inducible promoter in HEK 293 cells.

Receptor construct	Amino acid sequence of amino-terminal cytoplasmic tail
	1 10 20 30 40 50 55
SR-A (full-length)	MTKEMTENQRLCPHEREDADCSSESVKFDARSMTASLPHSTKNGPSVQEKLKSFK
SR-A$_{\Delta 1-49}$	MAKLKSFK
SR-A$_{\Delta 1-55}$	MA
	1 10 20 30 40 50 57
TfR/SR-A	MMDQARSAFSNLFGGEPLSYTRFSLARQVDGDNSHVEMKLAADEEENADNNMKASVR

Table 14: Amino acid sequences of the amino-terminal cytoplasmic tail of SR-A receptor constructs. Full-length murine SR-A has a 55 amino acid amino-terminal cytoplasmic tail. SR-A$_{\Delta 1-49}$ lacks the first 49 amino acids and SR-A$_{\Delta 1-55}$ lacks all 55 amino acids of the cytoplasmic tail. TfR/SR-A is a chimeric receptor, in which all 55 amino acids of the SR-A cytoplasmic tail are replaced by the first 57 amino acids of the murine transferring recptor (TfR). The transmembrane and extracellular domains are identical for all receptor constructs and correspond to amino acids 56 to 354 of SR-A type II.

I found that deleting the entire 55 amino acid cytoplasmic tail (SR-A$_{\Delta 1-55}$) greatly diminished SR-A protein abundance compared to that of SR-A full-length expressing cells (Figure 12A). SR-A protein was not detectable in the culture media indicating that the decreased cellular SR-A$_{\Delta 1-55}$ protein abundance was not due to secretion into the media (data not shown). In contrast to complete deletion of the cytoplasmic tail, retaining the membrane-proximal six amino acids by deleting amino acids 1 to 49 (SR-A$_{\Delta 1-49}$) restored protein abundance of SR-A (Figure 12A). SR-A mRNA abundance was similar for all SR-A constructs (Figure 12B). Thus, my results demonstrate that the six membrane-proximal amino acids are required for post-transcriptional processing of SR-A.

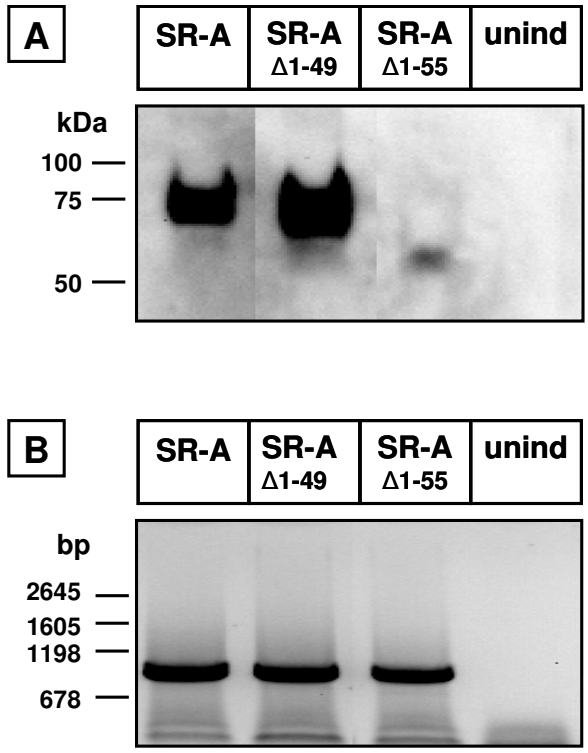


Figure 12: Expression of SR-A constructs in HEK cells. *A.* SR-A protein was immunoprecipitated from cell lysates (100 µg) prepared from uninduced (unind) cells or cells induced to express the indicated SR-A construct. Precipitated proteins were resolved by SDS-PAGE under reducing conditions and SR-A was detected by Western blotting. SR-A protein expression was not detectable in lysates prepared from uninduced cells. The blot shown is representative of results obtained in two separate experiments. *B.* RT-PCR was performed using primers that amplify a 897 bp fragment spanning the transmembrane and extracellular part of SR-A from total RNA (500 ng) isolated from induced cells. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. SR-A mRNA expression was not detectable in uninduced cells. No products were detected in control reactions performed without RT or without RNA. Data shown are representative of two separate experiments.

3.2. Cell surface localization and receptor internalization

To study the role of the membrane-proximal amino acids in SR-A trafficking, I analyzed cell surface localization of SR-$A_{\Delta 1-49}$. Cell surface localization of SR-$A_{\Delta 1-49}$ was greater than that of full-length SR-A (Figure 13).

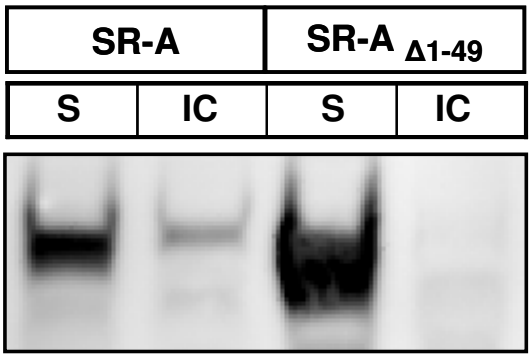


Figure 13: Cell surface localization of full-length and SR-$A_{\Delta 1-49}$ in HEK cells. Cells surface proteins of induced cells were biotinylated and cell lysates were prepared. Biotinylated proteins were precipitated from lysates (50 µg) using streptavidin-coated beads. Total cell surface protein (S, pellet) and 1/10 of the intracellular protein (IC, supernatant) were resolved by SDS-PAGE under reducing conditions and SR-A was detected by Western blotting. The blot shown is representative of three separate experiments.

To address the role of the membrane-proximal amino acids for SR-A internalization, I examined internalization of the SR-A ligand AcLDL by quantifying cell-associated fluorescence after incubation with fluorescently labeled AcLDL for 2 h (Figure 14). As shown previously, HEK-293 cells expressing full-length SR-A cells efficiently internalized AcLDL (Post et al. 2002). However, cell-associated fluorescence detected in cells expressing SR-$A_{\Delta 1-49}$ was only 14 % of that detected in cells expressing full-length SR-A (Figure 14B). The small amount of fluorescence associated with SR-$A_{\Delta 1-49}$ expressing cells most likely reflects AcLDL binding to cell surface receptors. The increased cell-associated fluorescence observed in SR-A and SR-$A_{\Delta 1-49}$ expressing cells was completely blocked by the SR-A antagonist polyinosine demonstrating specificity. Thus, although SR-$A_{\Delta 1-49}$ is able to bind ligand, SR-$A_{\Delta 1-49}$ does not mediate ligand internalization.

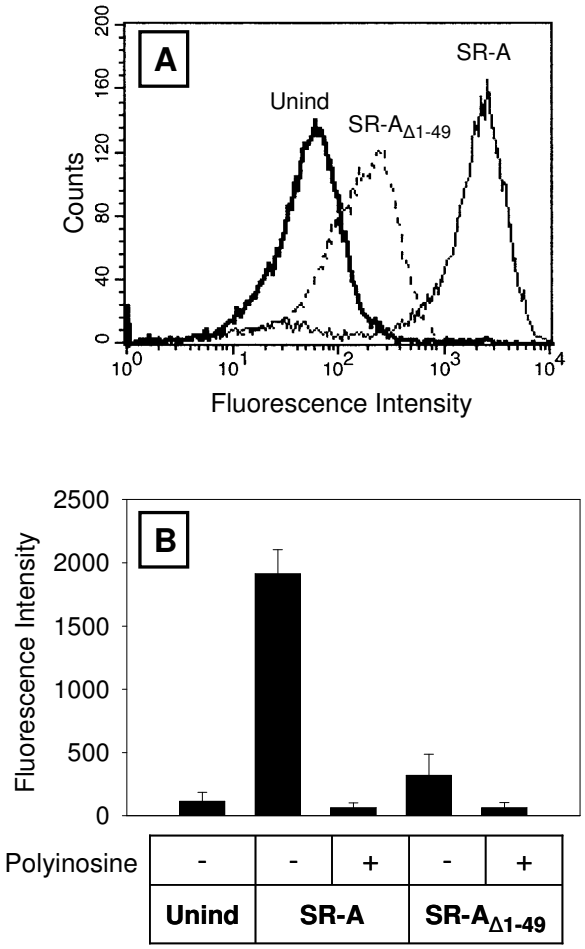


Figure 14: AcLDL internalization of SR-A and SR-A$_{\Delta 1-49}$ expressing HEK cells. Cells were induced as indicated. Following incubation in serum-free DMEM for 2 h, fluorescently labeled AcLDL (2.5 µg/ml, Alexa-Fluor488 AcLDL) was added and cells were incubated for another 2 h. AcLDL uptake was assessed by quantifying cell-associated fluorescence of uninduced, SR-A expressing, and SR-A$_{\Delta 1-49}$ expressing cells A. Histograms of cell-associated fluorescence. Data is representative of two separate experiments. B. Quantification of two separate experiments. To determine non-specific AcLDL association, polyinosine (10 µg/ml) was added before addition of AcLDL. Data are the mean fluorescence intensities ± SD of two separate experiments.

3.3. Intracellular trafficking from the endoplasmic reticulum to the Golgi apparatus

Previous studies have associated SR-A cell surface localization and receptor internalization with a common cytoplasmic motif (Morimoto et al. 1999). However, the finding that SR-$A_{\Delta 1-49}$ localized to the cell surface, but did not internalize ligand, suggests that SR-A trafficking to the cell surface and internalization have distinct cytoplasmic requirements. To address the specific role of the membrane-proximal amino acids in SR-A trafficking and whether an internalization motif is sufficient to mediate cell surface localization, I generated a chimeric receptor in which the entire cytoplasmic tail of SR-A was replaced by 57 amino acids of the TfR cytoplasmic tail (TfR/SR-A; Table 14) omitting a similar membrane-proximal domain. Like SR-A, the TfR is a type II transmembrane receptor that mediates internalization via clathrin-coated pits. However, whereas the cytoplasmic tail of SR-A is still poorly characterized, the cytoplasmic tail of TfR has been studied extensively and contains a well-characterized internalization motif (YTRF; 15).

To study the trafficking and intracellular processing of the chimeric TfR/SR-A, I assessed the ability of TfR/SR-A to form oligomers by resolving cell lysates from induced cells under non-reducing conditions. TfR/SR-A was expressed and assembled into oligomers similar to SR-A. Monomers of TfR/SR-A and SR-A were detected at a molecular weight higher than predicted from the amino acid sequences (about 38 kDa) suggesting that both receptors were post-translationally modified (Figure 15A). However, the apparent molecular weight of TfR/SR-A was lower than SR-A's indicating that the post-translational processing was different.

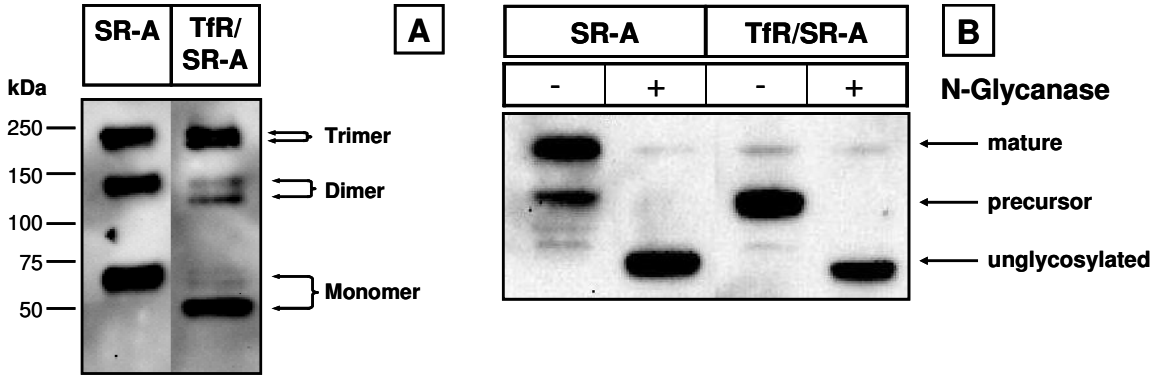


Figure 15: Expression and processing of SR-A and TfR/SR-A in HEK cells. A. Cell lysates were prepared from induced cells and resolved by SDS-PAGE under non-reducing conditions. SR-A and TfR/SR-A were detected by Western blotting. Arrows indicate the oligomeric forms of the receptors. B. Cells were induced for 6 h and cell lysates were prepared. As indicated, lysates were treated with N-glycanase to cleave all N-linked oligosaccarides and were then resolved under reducing conditions by SDS-PAGE. SR-A and TfR/SR-A were detected by Western blotting. Each blot is representative of results obtained in at least two separate experiments.

To examine the post-translational processing of the SR-A receptor constructs, I analyzed N-linked oligosaccharides of the receptors. An overview of the post-translational processing of SR-A's N-linked oligosaccharides is shown in Figure 4. To demonstrate N-linked gycosylation, I digested cell lysates prepared from induced cells with N-glycanase (Figure 15B), which cleaves all N-linked oligosaccharides. The deglycosylated monomeric proteins were detectable at 38 kDa for both SR-A and the chimeric TfR/SR-A indicating that the differences in apparent molecular weight resulted from differences in N-linked oligosaccharides. To test whether the N-linked oligosaccharides of the receptors differ in Golgi processing, lysates prepared from induced cells were digested with EndoH (Figure 16A). EndoH cleaves the high-mannose precursor N-linked oligosaccharides attached in the ER, but not the low-mannose mature oligosaccharides that are formed after processing in the Golgi. In contrast to SR-A (Figure 16A) and SR-$A_{\Delta 1\text{-}49}$ (data not shown), TfR/SR-A (Figure 16A) was EndoH-sensitive indicating that TfR/SR-A was not processed into the EndoH-resistant mature form by the Golgi apparatus.

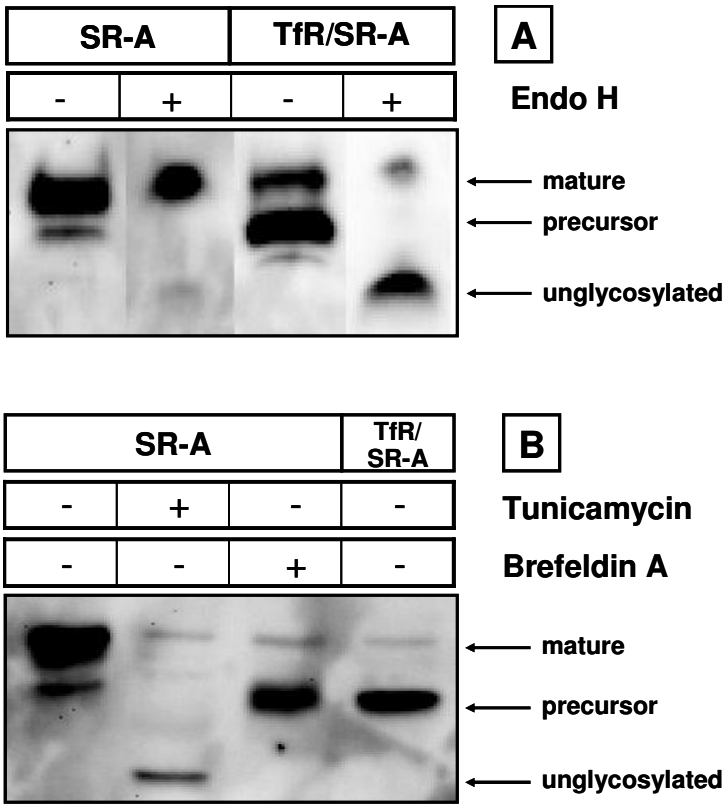


Figure 16: A. HEK cells were induced for 6 h and cell lysates prepared. As indicated, lysates were treated with EndoH to cleave only precursor N-linked oligosaccharides and were then resolved under reducing conditions by SDS-Page. SR-A and TfR/SR-A were detected by Western blotting. B. Cells were treated with tunicamycin (5 µg/ml) to inhibit formation of N-linked precursor oligosaccharides or with brefeldin A (5 µg/ml) to prevent maturation of the N-linked precursor oligosaccarides 60 min before and during induction (6 h). Then, cell lysates were prepared and analyzed as in A. Each blot is representative of results obtained in at least two separate experiments.

The EndoH sensitivity of TfR/SR-A suggests that the oligosaccharides of TfR/SR-A are analogous to those of the endoH-sensitive SR-A precursor. To confirm this, cells were treated before induction either with brefeldin A, which

disrupts the Golgi apparatus thereby preventing conversion of the precursor into the mature form, or with tunicamycin, an inhibitor of N-linked glycosylation (Figure 16B). I found that the apparent molecular weight of the SR-A precursor detected in brefeldin A treated cells was the same as that of TfR/SR-A in untreated cells. Tunicamycin treatment confirmed the results obtained after N-glycanase treatment showing that both receptors were N-linked glycosylated. Together, the data demonstrate that TfR/SR-A is translated and subsequently glycosylated in the ER, but the glycosylated precursor is not further processed in the Golgi apparatus. Because TfR/SR-A assembles into trimers, it seems unlikely that TfR/SR-A is retained in the ER due to misfolding of the protein. As expected, TfR/SR-A was not detected on the cell surface using the biotinylation protocol described above (data not shown).

Overall, our results from experiments studying the trafficking and intracellular processing of the different SR-A constructs demonstrate that an internalization motif is not sufficient to mediate trafficking to cell surface. Furthermore, the membrane-proximal amino acids of SR-A are sufficient for cell surface localization and are specifically required for trafficking of SR-A from the ER to the Golgi apparatus.

3.4. Cell spreading and adhesion

To investigate the cell morphology of cells expressing the SR-A constructs, we examined SR-A localization by fluorescence microscopy (Figure 17). Consistent with previous findings (Post et al. 2002), SR-A expressing HEK cells exhibited a cell morphology characterized by filopodia-like projections and increased cell spreading (Figure 17, panels A and B). As expected from the lack of surface expression, cells expressing TfR/SR-A (panels C and D) exhibited an intracellular immunostaining pattern and no increased cell spreading. Interestingly, SR-$A_{\Delta 1\text{-}49}$ expressing cells (panels E and F) exhibited filopodia-like projections and increased cell spreading that was similar to that observed for cells expressing full-length SR-A.

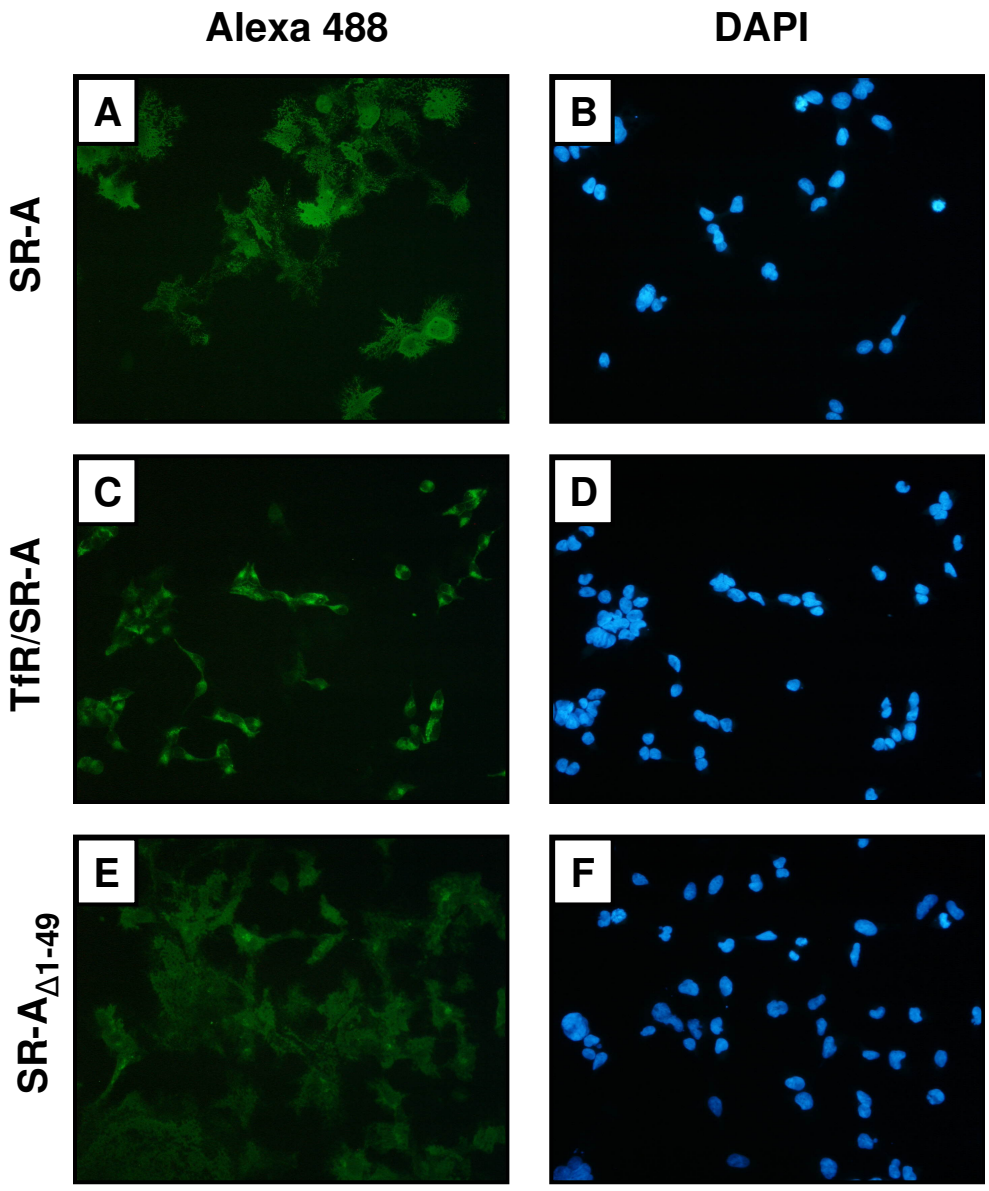


Figure 17: Cellular morphology of SR-A, TfR/SR-A and SR-A$_{\Delta 1-49}$ expressing HEK cells. Cells were plated on glass slides and SR-A (A and B), TfR/SR-A (C and D), and SR-A$_{\Delta 1-49}$ (E and F) expression was induced. After the induced cells were fixed and permeabilized, SR-A construct expression was detected using 2F8 and a secondary Alexa-Fluor488 labeled anti-rat antibody (A, C, E). No fluorescence was detectable in uninduced cells (not shown). The nuclei were stained with DAPI (B, D, F).

As the presence of the filopodia-like projections and increased cell spreading are associated with increased cell adhesion of SR-A expressing cells, we analyzed cell adhesion of cells expressing the different SR-A constructs. Consistent with increased spreading of SR-A and SR-$A_{\Delta 1\text{-}49}$ expressing cells, both receptors were able to increase cell adhesion to the same extent (Figure 18), demonstrating that the membrane-proximal amino acids are sufficient for SR-A-mediated adhesion. As expected from the lack of surface expression, TfR/SR-A (Figure 18) did not increase cell adhesion.

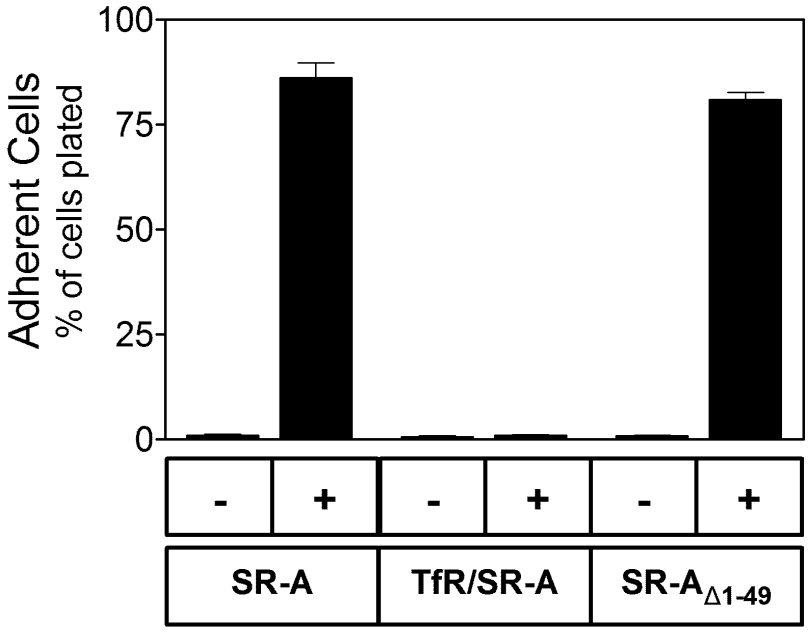


Figure 18: Divalent-cation independent cell adhesion of HEK cells. Cells were plated in 96-well plates (20,000 cells/well) and induced as indicated. Then, cells were incubated in EDTA solution (0.2 g/ml) for 10 min to eliminate divalent-cation dependent adhesion. Cell that remained adhered were quantified by CyQUANT assay. Adhesion is expressed as percent of total cells plated. Data shown is representative of two separate experiments.

V Discussion

1. SR-A transgenic mice

I have characterized functional changes in peritoneal macrophages isolated from lyso-bSR-A transgenic mice, which express SR-A in a macrophage-specific manner. Expression of bSR-A mRNA had been confirmed by RT-PCR. Because there is no antibody available that specifically recognizes bSR-A, the protein expression of the bSR-A transgene cannot be determined specifically. However, I did confirm bSR-A protein expression by characterizing functional changes. I found that bSR-A expression enhanced ligand internalization and cell spreading of peritoneal macrophages from lyso-bSR-A transgenic mice. Therefore, my results demonstrate that lyso-bSR-A transgenic mice express the transgene and exhibit enhanced macrophage SR-A activity in vitro.

Mouse models are powerful tools to study the role of specific genes in vivo. Mice are genetically well characterized and genetic manipulations allow deletion or overexpression of genes. The effect of deleting or overexpressing a gene can then be studied in different disease models. Initial studies in SR-A deficient mice have demonstrated the importance of SR-A in host defense and atherosclerosis (Suzuki et al. 1997). Whereas at this time it is well established that SR-A has a protective role in host defense, the role of SR-A in atherogenesis is still controversial.

Although mice had been considered to be resistant against atherosclerosis, in 1985 it was described that several inbred mouse strain develop diet-induced atherosclerosis, when fed a non-physiological diet containing high concentrations of saturated fat (21%), cholesterol (1.25%) and cholate (0.5%) (Paigen et al. 1985). The most susceptible mouse strain was the C57BL/6, and since then the C57BL/6 became the most widely used inbred strain to study atherosclerosis.

Atherosclerotic lesions of C57BL/6 mice are comparable to early stage lesions in humans; however, the lesions are small and restricted to the aortic root making analysis difficult. To enhance lesion development in C57BL/6 mice, genetic manipulations that interfere with the lipoprotein metabolism have been introduced. Deletion of apoE (Zhang et al. 1992; Plump et al. 1992), which is necessary for the clearance of cholesterol-rich lipoproteins, and deletion of the LDL receptor (LDL-R) (Ishibashi et al. 1994), which mediates cellular uptake of lipoproteins, cause hypercholesterolemia and enhance atherosclerotic lesion development. The lesions of apoE and LDL-R deficient C57BL/6 mice are more advanced than those in wild-type C57BL/6 mice and resemble several stages of human lesions. Furthermore, lesion development is not restricted to the aortic root; lesions develop throughout the aortic tree facilitating analysis of lesion development. Therefore, apoE and LDL-R deficient C57BL/6 mice are the most commonly used mouse models of atherosclerosis.

SR-A deficiency and overexpression have been studied in several mouse models of atherosclerosis including wild-type and apoE or LDL-R deficient C57BL/6 (Table 4). However, the results are inconsistent: initial studies demonstrated a proatherogenic role of SR-A, but consequent studies also have demonstrated no effect or antiatherogenic effects. One reason for the inconsistent results might be that SR-A is expressed on several cell types and the contribution of SR-A on the various cell types might vary in different models.

The use of a cell-specific promoter in lyso-bSR-A transgenic mice made it possible to address specifically macrophage SR-A overexpression. Studies in our laboratory using the lyso-bSR-A transgenic mice demonstated an antiatherogenic effect of macrophage-specific overexpression of SR-A after bone marrow transfer into LDL-R mice (Whitman et al. 2002). However, another group of investigators addressed macrophage specific deficiency of SR-A and demonstrated a proatherogenic effect. (Babaev et al. 2000). Thus, even the results from studies addressing the role of macrophage specific SR-A expression are inconsistent.

Another reason for the inconsistent results of studies addressing the role of SR-A in atherosclerosis might be that the contribution of the different functions mediated by SR-A such as lipoprotein internalization and adhesion might vary. However, to date it has not been possible to specifically address the relative contributions of SR-A mediated lipoprotein internalization and adhesion.

2. SR-A mediated lipoprotein internalization

I have shown that overexpression of SR-A enhanced internalization of AcLDL in peritoneal macrophages isolated from lyso-bSR-A transgenic mice. It is well established that SR-A mediated internalization of modified lipoproteins can result in cholesterol ester accumulation and thus foam cell formation in vitro (Brown et al. 1979).

The role of SR-A mediated internalization during foam cell formation in vitro has been confirmed in SR-A deficient mice. In vitro studies in peritoneal macrophages isolated from SR-A deficient mice showed that SR-A deficiency decreases AcLDL degradation by 80 % and OxLDL degradation by 30 % (Suzuki et al. 1997). In vivo, SR-A deficiency decreased atherosclerotic lesion development. However, SR-A deficient mice develop atherosclerotic lesions rich in foam cells (Suzuki et al. 1997). Taken together, these findings support the notion that SR-A is involved in foam cells formation during atherosclerosis, but demonstrate that other scavenger receptors are also involved.

Although SR-A was the first scavenger receptor that was identified and consequently thought to mediate foam cell formation during atherogenesis, by now several other scavenger receptors, defined by their ability to recognize modified lipoproteins, have been described. Several of the other scavenger receptors have been detected in addition to SR-A in atherosclerotic lesions and thus suggested to be involved in uptake of modified lipoproteins during

atherogenesis. These receptors include: CD36 (Endemann et al. 1993), macrosialin/CD68 (Ramprasad et al. 1996), lectin-like oxidized LDL receptor (LOX-1) (Sawamura et al. 1997) scavenger receptor for phosphotidylserine and oxidized lipoproteins (SR-PSOX) (Shimaoka et al. 2000; Minami et al. 2001). However, the contribution of these receptors to foam cell formation and atherosclerotic lesion development remains controversial. The redundancy of the system makes it difficult to address the role of individual receptors. Mice genetically deficient in individual receptors are a powerful tool to elucidate the role of individual receptors, but a potential problem is that compensatory mechanisms might develop and confound the results.

One of the scavenger receptors considered to potentially play a role during atherosclerois is macrosialin, the mouse homolog of human CD68. Macrosialin had been suggested as receptor for oxLDL on the basis of ligand blotting studies (Ramprasad et al. 1996), and macrosialin is expressed in atherosclerotic lesions (de Villiers et al. 1998). However, it has been recently shown that oxLDL does not bind to macrosialin expressed on the cell surface of transfected COS-7 cells demonstrating that macrosialin does not play a role in metablolism of oxLDL (De Beer et al. 2003).

Another scavenger receptor that has been detected in atherosclerotic lesions is CD36. The important role of CD36 in atherogenesis has been confirmed in CD36 deficient mice on an ApoE deficient background (Febbraio et al. 2000). In vitro studies in peritoneal macrophages isolated from these mice showed that CD36 deficiency decreased OxLDL degradation by more than 60 %. In vivo, CD36 deficiency decreased lesion development by 77 % (in the aortic tree) when fed a Western diet and by 45% (in aortic sinus) when fed a normal diet. Similar to the finding in SR-A deficient mice, CD36 deficiency did not prevent foam cell formation. Studies in mice deficient in SR-A and CD36 will be necessary to define whether other scavenger receptors in addition to SR-A and CD36 play a role during atherogensis.

Recently, mice that are deficient in SR-A and CD36 were described (Kunjathoor et al. 2002). Althought atherosclerotic lesion development in these mice has not been characterized, in vitro studies addressed the role of these receptors during foam cell formation. Peritoneal macrophages isolated from these double deficient mice degraded 75 -90 % less modified lipoproteins (including AcLDL, mildly and extensively oxLDL and myeloperoxidase modified LDL). Peritoneal macrophages isolated from the double deficient mice did not accumulate cholesterol esters in response to modified lipoproteins demonstrating that SR-A and CD36 are the main receptors mediating macrophage foam cell formation in vitro. As macrophages from these SR-A and CD36 mice deficient mice are protected from foam cell formation in vitro, it will be interesting to see whether these mice are protected from atherosclerosic lesion development.

Although SR-A promotes cholesterol accumulation in vitro, it remains controversial whether SR-A promotes foam cell formation in vivo. The accumulation of cholesterol esters induced by SR-A mediated lipoprotein internalization is reversible (Brown et al. 1979). In vivo, cholesterol acceptors are present and they might allow cholesterol efflux from the macrophages thus preventing foam cell formation. Therefore, internalization of modified lipoproteins via SR-A does not necessary lead to foam cell formation.

Furthermore, it is unclear to which extent SR-A is involved in internalization of modified lipoproteins in vivo. Although SR-A deficiency decreases degradation of modified lipoproteins by macrophages in vitro, it has been shown that modified lipoproteins can be rapidly metabolized in SR-A deficient mice (Ling et al. 1997; Van Berkel et al. 1998). There is evidence supporting that SR-A might predominantly function as adhesion molecule in vivo. Studies using the lyso-bSR-A mice found that overexpression of SR-A enhanced granuloma formation by increasing macrophage retention, while it did not increase intracellular lipid accumulation (Daugherty et al. 2001). It also has been shown that SR-A mediated internalization of AcLDL is abolished if macrophages

adhere via SR-A (el Khoury et al. 1994). Therefore, it is possible that in vivo SR-A functions as adhesion molecule to increase macrophage retention while other scavenger receptors mediate uptake of modified lipoprotein.

3. SR-A mediated adhesion

I found that overexpression of SR-A in lyso-bSR-A transgenic mice enhanced macrophage spreading in addition to increased internalization of lipoproteins. The finding that macrophages from SR-A transgenic mice exhibit increased spreading in culture demonstrates the adhesive properties of SR-A. Our finding is consistent with the observation that SR-A deficient macrophages exhibit slowed spreading (Suzuki et al. 1997). Furthermore, HEK cells, which do not express SR-A and are usually weakly adherent, exhibit a more adherent phenotype upon transfection with SR-A (Robbins et al. 1998; Post et al. 2002). Although the adhesive properties of SR-A are established in vitro, the role of SR-A mediated adhesion in vivo is not known.

SR-A-mediated adhesion may have important physiological roles by increasing the retention of macrophages. Macrophages are important mediators of inflammatory processes by releasing inflammatory mediators, growth factors and matrix degrading enzymes. SR-A is expressed strongly on macrophages at sites of tissue injury associated with inflammatory processes such as atherosclerotic lesions and senile plaques of Alzheimer's disease. While in resident macrophages adhesion is mainly mediated by integrins (Albelda et al. 1990), upon activation of macrophages SR-A mediated adhesion is upregulated (van Velzen et al. 1999). In vivo studies using the lyso-bSR-A transgenic mice demonstrated a role of SR-A-mediated adhesion in a model of inflammation (Daugherty et al. 2001). The macrophage-specific overexpression of SR-A in lyso-bSR-A mice enhanced granuloma formation after subcutaneous injection of carrageenan. This enhanced granuloma

formation was associated with an increase in macrophage numbers suggesting that SR-A enhanced macrophage recruitment and/or retention. Thus, SR-A mediated adhesion might be a mechanism for increasing macrophage retention specifically at sites of tissue inflammation.

Further support for the hypothesis that SR-A may be involved in macrophage retention at sites of tissue inflammation is provided by the identification of adhesion substrates that are associated with sites of inflammation. SR-A mediates adhesion specifically to modified extracellular matrix proteins such as collagen type I, III, and IV (el Khoury et al. 1994; Gowen et al. 2000). These modifications of the extracellular matrix included denaturation and glycation and may occur during certain pathophysiological conditions such as hyperglycemia or inflammation. Recently, SR-A also has been shown to mediate adhesion to proteoglycans of the extracellular matrix that are present in atherosclerotic lesions (Santiago-Garcia et al. 2003). Furthermore, SR-A mediates adhesion of microglial cells to beta-amyloid fibrils which accumulate in plaques of Alzheimer's disease (El Khoury et al. 1996). Thus, SR-A might increase macrophage retention at sites of inflammation through adhesion to modified extracellular matrix proteins.

4. Extracellular requirements for SR-A mediated adhesion

Despite the evidence supporting an important role of SR-A mediated adhesion, the relative contribution of SR-A mediated adhesion in vivo is unknown, as it has not been possible to block SR-A mediated adhesion specifically. To be able to address the role of SR-A mediated adhesion specifically, my aim was to identify the motif in the extracellular domains of SR-A that mediates adhesion. If a specific adhesion motif is defined, this motif can be mutated by site-directed mutagenesis to disrupt the adhesion properties of SR-A specifically. Expression of a SR-A mutant specifically lacking the adhesion properties in SR-A deficient mice will allow defining the role of SR-A's adhesion properties in vivo.

To identify the adhesion motif, I modified a SR-A dependent adhesion assay described previously by Gowen et al. (Gowen et al. 2000). Gowen et al. demonstrated that adhesion of RAW.264 macrophages to collagenase-digested collagen type I is mediated by SR-A. I chose this assay as collagenase-digested collagen type I might be a substrate for SR-A mediated adhesion during atherosclerosis in vivo. Collagen type I is a major extracellular matrix protein within the arterial wall and its synthesis is increased in atherosclerotic lesions (Rekhter et al. 1993). Moreover, during atherogenesis several matrix-degrading enzymes that have collagenase activity are present in the lesion (Galis et al. 1994).

It has been shown that SR-A mediated adhesion can be blocked by the monoclonal SR-A antibody, 2F8, and by antagonists of AcLDL binding (Fraser et al. 1993; el Khoury et al. 1994; Gowen et al. 2000). Therefore, the macrophages were preincubated with 2F8 or the antagonists polyinosine and fucoidan to confirm the specificity of the assay. Unlike integrin-mediated adhesion, SR-A mediated adhesion does not depend on divalent cations; therefore, the adhesion assay was performed in the absence of divalent cations to exclude integrin-mediated adhesion. I found that 2F8 blocked SR-A mediated macrophage adhesion, whereas an isotype matched control antibody had no effect (Figure 9). Furthermore, I found that polyinosine and fucoidan, also inhibited macrophage adhesion, whereas the structural similar compounds polycytidine and chondroitin, respectively, had no effect (Figure 10). Thus, these results demonstrate that under the conditions of the assay macrophage adhesion is mainly mediated by SR-A.

Although studies using monoclonal antibodies and SR-A ligands are suitable to address whether adhesion is mediated by SR-A, due to the conformation of SR-A such studies cannot be used to define the location of the adhesion motif. The conformation of SR-A was shown to resemble a hairpin, in which

the α-helical coiled coil and collagen-like domains are juxtapositional (Figure 3) (Resnick et al. 1996). Therefore, binding of antibodies or ligands to one domain can mask binding sites in the other domain through steric hindrance. For example, although the binding epitope for 2F8 is located in the N-terminal region of the α-helical coiled coil (Daugherty et al. 2000), 2F8 is able to antagonize AcLDL binding, which occurs at the C-terminal end of the collagen-like domain. Similarly, the adhesion motif might be masked through steric hindrance. Consequently, another approach is necessary to define the adhesion motif.

To define the adhesion motif, I proposed to compete for SR-A mediated macrophage adhesion with peptides homologous to SR-A. Peptides containing the adhesion motif will compete for SR-A binding sites of the adhesion matrix and therefore block SR-A mediated macrophage adhesion. It has been previously demonstrated that preincubation of modified collagen type IV with a peptide homologous to part of the collagen-like domain of SR-A could decrease SR-A mediated adhesion (el Khoury et al. 1994). Unlike observed for inhibition of AcLDL binding with peptides (Yamamoto et al. 1997), which required trimeric peptides, monomeric peptides were sufficient to inhibit adhesion. Therefore, I proposed to use fusion proteins to compete for SR-A mediated adhesion. To confirm that these monomeric fusion proteins are able to inhibit macrophage adhesion, I preincubated the digested collagen type I with a fusion protein homologous to the α-helical coiled coil and the collagen-like domains. These domains are common to SR-A type I and II, which are both able to mediate adhesion and thus, the adhesion motif must be located within these domains. I found that that this fusion protein could block SR-A mediated macrophage adhesion (Figure 11). Therefore, I verified that fusion proteins containing the adhesion motif can compete for SR-A binding. By analyzing the inhibitory properties of fusion proteins that are sequentially truncated, the region that contains the adhesion motif could have been narrowed down. Finally, site directed mutants of this region could have been used to define the adhesion motif.

However, during the course of these studies, structure-function studies by other investigators have also addressed the localization of the adhesion motif. While transient expression of full-length SR-A type II of COS cells conferred adhesion to collagen type I, expression of a truncated receptor lacking the distal portion of the collagenous domain did not (Gowen et al. 2001). Furthermore, a peptide homologous to 10 amino acids of the collagen-like domain containing the residues critical for ligand binding could block macrophage adhesion (Gowen et al. 2001). Taken together, the data strongly support that SR-A-mediated adhesion depends on the same region in the collagen-like domain of SR-A that mediates binding of soluble ligands during internalization. Thus, it does not appear to be feasible to generate a SR-A receptor mutant specifically lacking the adhesion properties, without disrupting binding of soluble ligands.

Due to the inability to separate SR-A-mediated adhesion from ligand internalization using 2F8 and SR-A ligands as described above (Fraser et al. 1993; el Khoury et al. 1994; Gowen et al. 2000; Gowen et al. 2001), it has been hypothesized that SR-A-mediated adhesion results from the attempt to internalize an immobilized ligand (Fong et al. 1999). The finding that SR-A mediated adhesion and internalization of soluble ligands depend on the same region of the extracellular domain supports this hypothesis. In contrast to data supporting a common mechanism for adhesion and internalization, recent studies showed that clathrin does not co-localize with SR-A in the filopodia-like projections associated with the enhanced adhesion of SR-A expressing cells (Post et al. 2002). This observation provided indirect evidence that SR-A-mediated adhesion is a process that is distinct from clathrin-coated pit internalization.

5. The role of cytoplasmic domains in SR-A function

As it appeared to be unfeasible to generate a mutant of SR-A specifically lacking the adhesion properties by modifying the extracellular domains, I wanted to investigate the role of the cytoplasmic tail in SR-A-mediated

adhesion. In particular, I wanted to address whether SR-A-mediated adhesion and internalization require distinct cytoplasmic domains. To address this question, I expressed different SR-A constructs under the control of an inducible promoter in stably transfected HEK-293 cells.

HEK-293 cells do not express endogenous SR-A and are a weakly adherent cell line. It has been shown that HEK-293 exhibit a more adherent phenotype upon transfection with SR-A (Robbins et al. 1998). Therefore, use of this cell line provides a good model to address the adhesive properties of expressed SR-A receptor constructs. Furthermore, transfected HEK-293 cells have been used to study SR-A mediated internalization of modified lipoproteins (Lysko et al. 1999; Post et al. 2002). It was also shown that SR-A mediated internalization is similarly regulated in HEK-293 cells expressing SR-A and mouse peritoneal macrophages (Post et al. 2002). Therefore, this cell line also provides a good model to address SR-A mediated internalization. To generate HEK-293 cells that express the different SR-A constructs, we used the Flp-In^{TM} T-REx^{TM} system (Invitrogen). This system allows tetracycline-inducible expression after integration of the cDNA into a single specific genomic site. An important advantage of this system is that similar levels of expression can be obtained upon induction of cells transfected with different cDNAs. Taken together, HEK-293 cells expressing the different SR-A constructs using the Flp-In^{TM} T-REx^{TM} system provide a good model to compare the adhesive and internalization properties of the different SR-A constructs.

I found that complete truncation of the cytoplasmic tail (SR-A$_{\Delta 1\text{-}55}$) dramatically decreased the protein abundance of SR-A compared to that of cells expressing full-length SR-A. This finding demonstrates that the cytoplasmic tail is critical for the processing of SR-A. As we have shown that the mRNA abundance of the receptor constructs is similar upon induction, the decreased protein abundance was not due to differences in mRNA transcription, but due to differences in post-transcriptional processing.

An initial step in the post-transcriptional processing of SR-A is insertion of the nascent protein into the membrane of the ER. The process whereby transmembrane proteins are inserted into the ER membrane and adopt a type I (carboxy-terminal cytoplasmic tail) or type II (amino-terminal cytoplasmic tail) topology is not fully understood (Wilkinson et al. 1997). It is generally thought that type I and II transmembrane proteins are targeted to the ER and then inserted and anchored in the membrane via a process involving signal-anchor sequences located in the hydrophobic amino acids of the transmembrane domains. However, I found that the transmembrane domain of SR-A (SR-$A_{\Delta 1-55}$) was not sufficient for the post-transcriptional processing of SR-A.

In contrast to the dramatically decreased protein abundance of SR-A after complete truncation of the cytoplasmic tail (SR-$A_{\Delta 1-55}$), retaining the membrane-proximal amino acids of the cytoplasmic tail (SR-$A_{\Delta 1-49}$) restored SR-A protein abundance. Three of the six retained SR-A membrane-proximal amino acids (KLKSFK) are positively charged. Charged membrane-proximal amino acids have been shown to affect the topology of transmembrane proteins (Spiess 1995). Thus, the membrane-proximal charged residues might be critical during ER membrane insertion of SR-A.

Binding of a number of cytosolic proteins is involved in processing of newly synthesized proteins and membrane translocation. As the cytoplasmic tail is the N-terminal end of SR-A, this part is synthesized first and it might be critical for binding of proteins during early processing. One group of cytosolic proteins that many newly synthesized proteins interact transiently with is chaperones (Beckmann et al. 1990). For example, binding of the chaperone heat shock protein (Hsp) 70 is required during apolipoprotein B synthesis and ER translocation (Zhou et al. 1995). Binding of Hsp70 and Hsp90 has also beenshown to mediate translocation across the mitochondrial membrane (Ellis 2003). It has been shown that the Hsp70 and Hsp90 directly bind to the cytoplasmic tail of SR-A (Nakamura et al. 2002). Thus, the membrane-

proximal amino acids might provide a recognition site for proteins critical during membrane insertion.

To address the specific requirement for the membrane-proximal amino acids, I replaced the cytoplasmic tail of SR-A with the first 57 amino acids of the transferrin receptor (TfR) omitting the TfR charged membrane-proximal amino acids (KPKR). I chose the TfR for a number of reasons. Like SR-A, TfR is a type II transmembrane protein that mediates clathrin-coated pit endocytosis. In contrast to SR-A, endocytosis is the only function mediated by TfR. The cytoplasmic motif (YTRF) of the TfR required for internalization is well-defined and mutation of this motif dramatically reduces TfR internalization (Jing et al. 1990). If the chimeric TfR/SR-A had been expressed on the cell surface, TfR/SR-A could have been used to specifically address the role of internalization of SR-A ligands. TfR/SR-A should be able to mediate ligand internalization via the YTRF internalization motif of the TfR. Moreover, after mutation of the internalization motif TfR/SR-A could have been used to address the role of SR-A cell surface localization. Comparison of the adhesive properties of cells expressing the internalization deficient TfR/SR-A and SR-$A_{\Delta 1\text{-}49}$ could have addressed whether cell surface localization alone is sufficient for SR-A mediated adhesion or whether the membrane-proximal amino acids of SR-A are specifically required for SR-A mediated adhesion.

However, although TfR/SR-A expression was detectable in whole cell lysates, TfR/SR-A was not detectable on the cell surface. We analyzed the intracellular processing and found, that the chimeric TfR/SR-A receptor was glycosylated in the ER, but not processed in the Golgi apparatus indicating that the receptor was not transported from the ER to the Golgi. The glycosylated TfR/SR-A monomer is similar to the glycosylated full-length SR-A monomer formed in the ER. Because TfR/SR-A assembles into trimers, it

seems unlikely that TfR/SR-A is retained in the ER due to misfolding. I conclude that the membrane-proximal amino acids are specifically required for trafficking from ER to the Golgi.

The mechanism by which proteins traffic from the ER to Golgi remains controversial (Klumperman 2000). The *bulk-flow model* proposes that proteins are transported from the ER to the Golgi by default. In contrast, the *selective export model* proposes that proteins are selectively exported based on the presence of ER-export signals (Gorelick et al. 2001). Several cytosolic proteins are involved in trafficking of newly synthesized transmembrane proteins. Thus, the membrane-proximal amino acids might provide a critical recognition site for proteins involved in selective ER export.

In addition to being required for trafficking from the ER to the Golgi, the membrane-proximal amino acids were sufficient for cell surface localization of the receptor. Although SR-$A_{\Delta 1\text{-}49}$ was expressed primarily on the cell surface, SR-$A_{\Delta 1\text{-}49}$ failed to internalize ligand. The lack of SR-$A_{\Delta 1\text{-}49}$ internalization may cause the increased cell surface localization of SR-$A_{\Delta 1\text{-}49}$ (Figure 13). A well-defined internalization motif is not present in the cytoplasmic tail of SR-A. However, Morimoto et al. have suggested that a single motif, VXFD, is required for both SR-A internalization and cell surface localization (Morimoto et al. 1999). In contrast, my results show that the membrane-proximal amino acids are sufficient for cell surface localization, but that receptor internalization depends on a distinct motif, possibly the VXFD motif.

Although $SRA_{\Delta 1\text{-}49}$ did not mediate ligand internalization, the receptor mediated cell adhesion. The mechanism by which SR-A mediates cell adhesion remains unclear. It has been shown, that SR-A-mediated adhesion involves changes in the actin cytoskeleton and the formation of focal adhesions. The ability of $SRA_{\Delta 1\text{-}49}$ to enhance cell adhesion suggests that the membrane-proximal amino acids of SR-A are sufficient to mediate these processes. Likewise, the cytoplasmic membrane-proximal amino acids of the

adhesion molecule L1 have been shown to be sufficient for association of this receptor with the cytoskeleton (Dahlin-Huppe et al. 1997). Thus, the membrane-proximal amino acids of SR-A might be a binding site for cytosolic proteins involved in formation of focal adhesions. However, cell surface localization might be sufficient to mediate cell adhesion. For example the uPA receptor, a GPI anchored protein, has been shown to mediate cell adhesion through association with integrins (Wei et al. 1996). Similarly, SR-A might associate though its extracellular domains with another receptor to mediate adhesion.

VI Conclusions and Future Directions

SR-A overexpression enhances lipoprotein internalization and adhesive properties of macrophages in vitro. Both functions might play an important physiological role, but their relative contribution in vivo is unknown. It appeared that these two functions depend on the same extracellular region of SR-A. Thus, to dissect SR-A mediated cell adhesion and lipoprotein internalization, I investigated the role of cytoplasmic domains for SR-A function. I found that the cytoplasmic tail plays a critical role in SR-A processing and trafficking. Furthermore, I identified a mutant of SR-A that specifically mediates cell adhesion, but not ligand internalization. This finding demonstrates that SR-A mediated adhesion and internalization require distinct cytoplasmic domains and therefore must be distinct cellular processes.

The development of the $SRA_{\Delta 1\text{-}49}$ mutant that specifically mediates cell adhesion will make it possible to study SR-A-mediated adhesion. Cell culture studies can elucidate the mechanism by which the adhesion is regulated. Expression of $SRA_{\Delta 1\text{-}49}$ in a SR-A deficient mouse model can address the contribution of SR-A mediated adhesion in various physiological and pathophysiologial processes such as atherosclerosis.

VII Summary

Class A scavenger receptors (SR-A) are trimeric transmembrane glycoproteins that are mainly expressed in macrophages. SR-A can bind a variety of ligands including modified lipoproteins and bacterial products. Through its ability to internalize these ligands, SR-A is thought to be involved in many physiological and pathophysiological processes such as host defense and atherosclerosis. In vitro, SR-A also mediates cell adhesion to modified extracellular matrix proteins. Thus, SR-A mediated adhesion may play an important physiological role by increasing the retention of macrophages. However, to date it has not been possible to define specifically the physiological role of SR-A mediated cell adhesion.

The goal of my thesis project was to dissect SR-A mediated cell adhesion and ligand internalization and to develop a mutant of SR-A that specifically lacks the adhesive properties.

In initial studies I characterized peritoneal macrophages from transgenic mice that overexpress SR-A in macrophages. SR-A overexpression augmented cholesterol ester deposition of peritoneal macrophages in response to AcLDL indicating increased internalization of AcLDL. Furthermore, these cells exhibited enhanced cell spreading. This finding suggested that overexpression of SR-A can increase macrophage adhesion.

To develop a mutant of SR-A lacking the adhesion properties, I performed studies to define the adhesion motif in the extracellular domain of SR-A in order to determine if the extracellular motif involved in adhesion can be distinguished from the motif involved in ligand binding during internalization. I studied SR-A mediated adhesion of RAW.264 macrophages to denatured collagen type I. SR-A mediated adhesion was inhibited by the rat SR-A monoclonal antibody 2F8 and by the SR-A antagonists polyinosine and fucoidan. Furthermore, a fusion protein homologous to the extracellular domain of SR-A competed for cell adhesion. My approach was to define the adhesion motif by testing the ability of shorter fusion proteins to compete for cell adhesion. However, using this approach it was not

possible to define an extracellular adhesion motif that is distinct from the lipoprotein binding domain, as it appeared that ligand binding during receptor internalization and adhesion depend on the same extracellular domain.

Because the cytoplasmic tail is required for receptor internalization, I investigated the role of cytoplasmic domains for SR-A mediated functions. Different cytoplasmic mutated SR-A constructs were stably expressed in human embryonic kidney (HEK 293) cells. Complete deletion of the cytoplasmic tail ($SRA_{\Delta 1-55}$) greatly diminished SR-A protein expression. However, a mutated SR-A deficient in all but the six amino acids proximal to the membrane of the cytoplasmic tail ($SRA_{\Delta 1-49}$) was expressed and localized to the cell surface. Substitution of the SR-A cytoplasmic tail with that of the transferrin receptor (TfR/SR-A) resulted in retention of this chimeric receptor in the endoplasmic reticulum demonstrating that the SR-A membrane proximal amino acids are critical for trafficking of the receptor from the endoplasmic reticulum to the Golgi apparatus. Furthermore, these membrane proximal amino acids were sufficient for SR-A mediated cell adhesion, but not for receptor internalization.

My results demonstrate that the cytoplasmic tail plays a critical role for SR-A processing and trafficking. Furthermore, SR-A-mediated adhesion and internalization require distinct cytoplasmic domains and therefore must be distinct cellular processes. The identification of a SR-A mutant that specifically meditates cell adhesion, such as $SRA_{\Delta 1-49}$, will make it possible to specifically address the physiological role of SR-A mediated adhesion.

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