Therapeutic and imaging potential of peptide agents in cardiocascular disease

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1. Inflammation and vasculopathies

1.1 Atherosclerosis

Cardiovascular disease is the major cause of death in the USA and most European countries. Only in the USA, almost 1 million people die from cardiovascular disease every year. With 36.3 percent of all deaths, cardiovascular disease related mortality exceeds that of any other cause of death including cancer. In Asia, cardiovascular mortality is rapidly burgeoning due to the changed diet and life style and fast economic development and now represents 34% of all casualties (American Heart Association 2007).

It is widely established that cardiovascular disorders are the principal clinical manifestation of atherosclerosis[1]. Atherosclerosis is a disease of the inner layer of medium sized muscular arteries (e.g. coronary and carotid arteries) and large elastic arteries such as aorta and iliac vessels[2,3]. While silent during the initial stages of disease, atherosclerosis can cause severe clinical complications such as occlusive thrombosis after rupture of an atherosclerotic lesion. In the heart, plaque rupture may lead to angina pectoris, myocardial infarction and eventually heart failure. In the brain, atherosclerosis manifests itself in stroke, and in peripheral vessels in hypertension, aneurysm, peripheral ischemia and renal impairment[4,5].

Many risk factors have been shown to predispose to atherosclerosis, including age, gender, diabetes mellitus, hypercholesterolemia, smoking, elevated homocysteine, high blood pressure, obesity, sedentary lifestyle and infectious microorganism such as herpesvirus or *Chlamydia pneumoniae*[6-9]. The principal risk factors such as hyperlipidemia (e.g. hydroxymythelglutaryl coenzyme A (HMG-CoA) inhibitors, fibrates), hypertension (e.g. angiotensin-converting enzyme inhibitors, angiotensin-receptor blockers, calcium channel antagonists, β2-blockers) and hypercoagulance (e.g. cumarin, aspirin, Cox-2 inhibitors) are targeted in cardiovascular therapy and these treatments form the major approach in the prevention and therapy of atherosclerosis. For those patients with symptomatic coronary artery disease, bypass graft surgery, percutaneous transluminal coronary angioplasty (PTCA) and stents are established and effective (surgical) interventions in the treatment of this disease. Unfortunately, 10-20 percent of patients receiving surgical or noninvasive treatment are facing the daunting problem of restenosis[10].

1.2 Pathology of atherosclerosis

Our understanding of the pathophysiology of atherosclerosis has substantially evolved over the last three decades. In 1970s, the discovery that a low density lipoprotein (LDL) receptor mutation is underlying familial hypercholesterolemia has linked lipids and lipoproteins to atherosclerosis and nowadays LDL is viewed as a major culprit. Further research has led to Russell Ross to launch his ‘response to injury’ hypothesis[11]. In 1980s, the frequent incidence of restenosis has drawn research focus to growth factors and SMC proliferation. From 1990s onwards, the

identification of inflammatory cells and molecules in the atherosclerotic lesion has led to the notion that multiple molecular and cellular immune processes are involved in atherogenesis[12,13], and that atherosclerosis can be regarded as a chronic inflammatory disease.

Inflammation in the initiation stage of atherosclerosis

Endothelial dysfunction is one of the earliest events in atherosclerosis. Two key features of dysfunction are loss of endothelium-dependent vasodilation and an increased expression of endothelial adhesion molecules. Recruitment of monocytes and lymphocytes to the intima is triggered by the progressive accumulation of oxidized LDL (oxLDL) in the subendothelial tissue and the subsequent induction of proinflammatory mediator production by endothelial cells. Circulating leukocytes will bind weakly to selectins like P-and E-selectins on the activated endothelium through sialylated ligands[14]. Increased expression of vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecules-1 (ICAM-1) will then enable the firm arrest of circulating leukocytes to the endothelium via ligand very late antigen 4 (VLA-4) and CD11 integrins[15]. Once adherent to the endothelium, leukocytes penetrate the endothelium by transjunctional diapedesis. The recruitment process is directed by various chemoattractant chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR2[16,17]. Once infiltrated into the arterial wall, monocytes differentiate into resident macrophages under the agency of macrophage colony stimulating factor (M-CSF)[18]. Monocyte derived macrophages will acquire an increased expression of scavenger receptors such as SR-AI or CD36, which will mediate the internalization of modified LDL[19]. Monocyte derived macrophages also have increased expression of toll like receptors (TLRs)[20], which are activated by lipopolysaccharides (LPS), heat-shock protein (HSP 60)[21] and oxLDL[22]. The formation of lipid-laden macrophages, the so-called foam cells, as a result of the continuous accumulation of lipid droplets in the cytoplasm of macrophages is viewed as a hallmark in atherosclerosis.

T-lymphocytes play an important role in the initiation of atherosclerosis as well. They infiltrate into the intima by binding to VCAM-1. The chemokine receptor CXCR3 expressed by T-cells can bind chemokines present in the plaque including inducible protein-10 (IP-10), monokine induced by IFN-γ (Mig) and I-TAC, which further facilitates T lymphocytes infiltration. Once recruited, T lymphocytes will be activated by macrophages or DC, which process and present antigens derived from oxLDL, HSP60 and microorganisms and help to elicit an immune response. The ensuing T-helper 1 (Th1) cell response results in the production of IL-12, IL-15, IL-18 etc. As a consequence, Th1 cells will produce inflammatory cytokines including IL-1, IFNγ, TNFα, and express CD40L. The Th1 response will in turn activate EC and macrophages to produce proteases, to increase ICAM-1 expression, halt SMC proliferation, and facilitate coagulant production and thrombus formation. The Th2 cells response, characterized by increased IL-4 and IL-10 production, can attenuate the plaque inflammation by production of IL-10 and TGF-β.

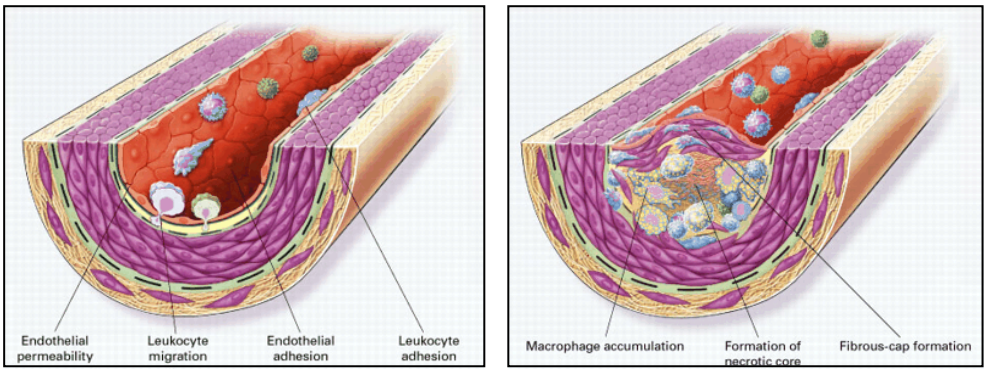

Figure 1 Initiation (left) and progression (right) of atherosclerosis (adapted from R. Ross. Atherosclerosis - An inflammatory disease. New Engl J Med. 1999;340:115-126)[18].

Inflammation in the progression of atherosclerosis

The transition from a fatty streak into a more advanced lesion is characterized by SMC proliferation and migration from media to the intima, where they synthesize extracellular matrix and contribute importantly to fibrous cap formation. During this fibroatheromatous stage, growth factors such as PDGF production by infiltrated macrophage will stimulate SMCs proliferation and matrix synthesis, a process that is inhibited by IFN-γ derived from activated T-cells. Arterial remodeling and progressive narrowing of the lumen is a predominant feature at this stage of disease development.

Inflammation in the advanced lesions and thrombosis complication

The most important complication of atherosclerosis is thrombosis. Thrombus formation usually results from physical disruption of vulnerable plaques consisting of a thin fibrous cap surrounding a lipid-rich necrotic core and a large number of inflammatory cells. This so-called advanced or complicated lesion displays a massive accumulation of lipid and cell debris. In contrast, stable plaques are characterized by a small lipid pool, thick fibrous cap and the absence of outward remodeling[23]. Three fundamentally different types of plaque disruption can be discerned: superficial EC erosion, microvasculature and fibrous cap fracture. The majority of cases of coronary thrombosis result from rupture of the protective fibrous cap, which prevents direct contact between blood and plaque entrapped procoagulant factors (tissue factor, platelet activating factor and lysophosphatidic acid). Interstitial collagens confer most of the tensile strength to the fibrous cap, but can be disintegrated during inflammatory processes. For example, IFN-γ can inhibit collagen production by vSMC. Proteolytic collagenases (MMP-1, -8, -13) and gelatinases (MMP-2, -9)[24] will be upregulated by IL-1β, TNF-α and CD40L and degrade the extracellular matrix[25]. The eroded fibrous cap is weakened and fragile to hemodynamic forces and stretch. In addition, vulnerable plaques usually contain a large number of T-lymphocytes and macrophages but few vSMCs owing to oxidative stress and inflammation induced apoptosis. During plaque rupture,

platelets will be activated by thrombin to form a thrombus, which either will lead to an acute myocardial infarction or to wound healing.

1.3 Restenosis

Restenosis literally means the ***re***occurrence of ***stenosis***. It generally refers to the re-narrowing of an artery or blood vessel after vascular intervention. Restenosis occurs within 3 months in 30–40% of patients receiving angioplasty. Although the incidence was reduced to 10-20% with the advent of the stent, a wire mesh tube to keep the artery opened after angioplasty, restenosis still sharply reduces the therapeutic efficacy of angioplastic interventions[26-29].

1.4 Pathology of in-stent restenosis

Gruntzig was the first to document that restenosis was directly caused by the angioplasty[30]. Our understanding to the molecular basis of restenosis is rapidly growing and inflammation appears to play a pivotal role at the site of stent placement. The mechanisms underlying in-stent restenosis are illustrated in Fig. 2.

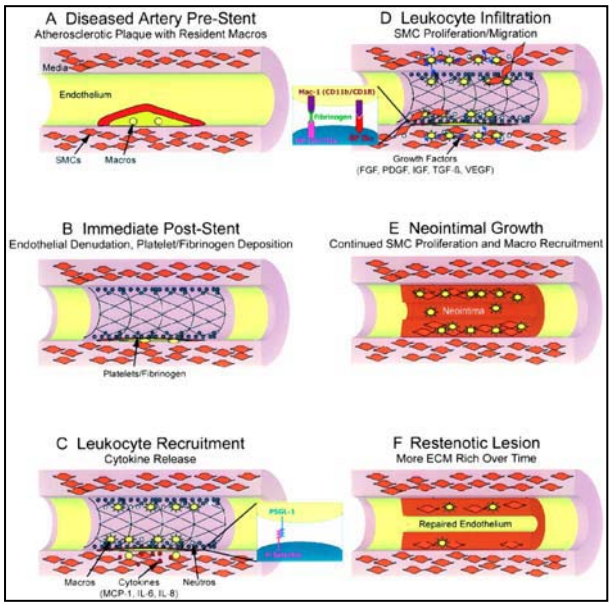

Figure 2 Scheme of restenosis cascade. (Adapted from Welt, F. G.P. *et al.* Arterioscler Thromb Vasc Biol 2002; 22:1769-1776)

The initial consequences immediately after stent deployment are deendothelialization, plaque crush, often with dissection into the tunica media and even adventitia. A layer of platelets and fibrin is then deposited at the injured site. Activated platelets on the injured surface express adhesion molecules such as selectins[31-33]. Circulating leukocytes attach to the activated platelets via platelet receptors such as PSGL-1 and begin a rolling process along the injured surface. Tight binding of leukocytes to the platelet-fibrin layer proceeds via leukocyte integrins and platelet receptors, LFA-1 (Lymphocyte Function-Associated Antigen-1, CD11a/CD18), Mac-1 (CD11b/CD18), and p150, 95 (CD11c/CD18)[34], which bind to ICAM-1[35], fibrinogen[36] or to heparin and heparan sulfate[37]. The migration of leukocytes across the platelet-fibrin layer and infiltration into the tissue is driven by chemokines released from vSMCs and macrophages. Chemokines, such as MCP-1 importantly participate in the recruitment of monocytes, and promote monocyte accumulation

and neointimal hyperplasia[38,39]. The CXC chemokine, IL-8, was seen to play a critical role in the recruitment of monocytes and neutrophils to the site of vascular injury[40]. Also IL-1β and IL-6 levels were significantly increased in the coronary sinus after stent implantation[41].

In addition, PDGF secreted from platelets, VEGF secreted from EC and other growth factors secreted by vSMC such as fibroblast growth factor (FGF) and insulin like growth factor (IGF), contribute to SMC proliferation and migration as well. The neointima consists of vSMCs, extracellular matrix, and macrophages recruited over several weeks. In the end, the injury will reendothelialize and heal.

Next to leukocytes and platelets, stem cells and bone marrow derived EC/SMC may contribute to neointima formation. It is generally believed that in atherosclerosis intimal vSMCs originate locally from vSMCs of the medial layer. In analogy it was assumed that neointimal cells in restenosis are derived from medial SMCs as well. However, part of the intimal vSMC-like cells appeared to be derived from blood cells rather than from medial vSMCs[42]. Pluripotent hematopoietic stem cells (HSCs) were found not only to give rise to hematopoietic blood cells, but also to epithelial cells[43], hepatocytes[44], cardiomyocytes[45], SMC[46,47] and EC[48,49], which indicate that bone marrow cells/stem cells have the potential to contribute to vascular remodeling. Evidence has been gain to support the hypothesis that circulating vascular progenitor cells may contribute to atherosclerosis[50,51]. It is shown that after cardiac transplantation as much as 10% of arteriole and 2.6% of vSMCs were of host origin[52,53], indicating effective migration of stem cells from the recipient to the grafted heart. Recent evidence points to a contribution of SMC progenitors and stromal cell-derived factor (SDF)-1α to neointima formation after arterial injury[54-56]. The CXC chemokine SDF-1, also named CXCL-12, is produced by multiple stromal cell types in the bone marrow, and by epithelial cells in many organs. CXCR4 (CD184), the seven transmembrane G-protein coupled receptor of SDF-1, is widely expressed by a variety of cell types including hematopoietic, endothelial, stromal and neuronal cells, and upregulated under conditions of severe vascular injury and hypoxia. The SDF-1α/CXCR4 axis is instrumental in vascular remodeling by recruiting a subset of SMC progenitors in response to medial apoptosis and stretch, and acts in concert with platelets, epitomizing its importance for tissue repair and identifying it as a prime chemokine target to limit lesion development. However, considering the relatively low percentage and largely unproven contribution of HSC/bone marrow–derived vascular cells in vascular remodeling, their importance in human restenosis disease needs further scrutiny.

In general, ISR is characterized by a larger lumen size, less (or no) negative remodeling and enhanced chronic inflammation in comparison with balloon angioplasty induced restenosis[57,58]. Thus there is a demand for more selective agents possessing both anti-inflammatory and anti-proliferative properties. Until recently, the only effective treatment for ISR was brachytherapy, a form of radiotherapy where a radioactive source is placed inside the treatment area, which

is however not universally accepted, due to thrombotic side effects and the radioactivity. Drug eluting stents (DES) containing the immunosuppressive agent Sirolimus (rapamycin) or the cytostatic agent paclitaxel have shown encouraging reductions in ISR. Sirolimus or tacrolimus (FK506), normally used as immunosuppressants but recently discovered to also inhibit the proliferation of vSMC, proved to be quite effective in preventing restenosis. However, they are toxic to non-immune cells in adjacent vascular tissue. Therefore more selective and less toxic agents are desired.

2. Targeting atherosclerosis by cell surface receptors

2.1 Scavenger receptor AI, culprit in macrophage cholesterol accumulation and atherosclerosis

The modified lipoprotein receptor was first described by Brown and Goldstein in 1979[59]. Because of its ability to bind a broad range of negatively charged polyanions, such as modified LDL, the receptor was referred to as *S*cavenger *R*eceptor (SR). Since then, SR has received great interest in the study of atherosclerosis and host defense. In recent years, several new members of the scavenger receptor family have been identified on the basis of their ability to recognize modified lipoproteins[60,61]. The members are classified as follows: scavenger receptor (SR) class A consists of SR-AI, SR-AII, SR-AIII, and the macrophage receptor with collagenous structure (MARCO); class B consists of SR-BI and CD36; and class C contains only the Drosophila SR-C. Classes D, E, F, G, H, I and J were more recently described and show no structural similarity with class A, B, or C receptors (see Fig. 1 for classes and structures). Not all of the scavenger receptors are implicated in atherosclerosis. SR-AI, CD36 (SR class B), CD68 (SR class D), lectin-like oxidized LDL receptor (LOX-1, SR class E), scavenger receptors expressed by endothelial cells (SREC, SR class F), and scavenger receptors for phosphatidyl serine and oxidized lipoprotein (SR-PSOX, SR class G) were all shown to be involved in foam cell formation, an early event in atherogenesis. A common feature among scavenger receptors is their capacity to internalize modified LDL. Currently, the main focus on macrophage scavenger receptors was given to SR-A (SRAI/AII) and SR-B (CD36 and SR-BI). SR-AI/II and CD36 were believed to play a proatherogenic role, whereas SR-BI was indicated as atheroprotective, at least for the development of advanced lesions.

SR-A structure and its ligand

SR-A was first purified and cloned from bovine liver membrane in 1988. It is a trimeric transmembrane glycoprotein consisting of six distinct domains[62-64]. Three isoforms exists of SR-A: SR-AI, SR-AII and SRA-III, which differ in the cysteine-rich domain and/or collagen-like domain responsible for ligand binding[65-68]. As shown in Fig. 3, SR-AII lacks the 110- amino acid C-terminal cysteine-rich domain of SR-AI. However, isoform I and II are remarkably similar in ligand binding

profile. In 1998, SR-AIII was identified as an alternative splicing product[69]. It has the cysteine-rich C-terminal domain but lacks the C-terminal collageneous domain, which had been attributed to ligand binding.

SR-A has very broad ligand specificity but most of its ligands are polyanionic molecules. In addition, the spatial orientation of the negative charge was also shown to be important in ligand binding[70]. SR-A not only recognizes chemically modified lipoproteins (e.g. oxLDL and AcLDL) but also polyribonucleotides (e.g. polyinosinic (poly I) and polyguanylic acid (poly G)); polysaccharides (e.g. dextran sulfate and fucoidin) and anionic macromolecules such as polyvinyl sulfate and lipopolysaccharide (LPS); Gram-positive bacteria, lipoteichoic acid and Lipid IV_A[71-79]. Although SR-A is characterized as a modified lipoprotein receptor, it displays a clearcut preference for oxLDL over acLDL[80].

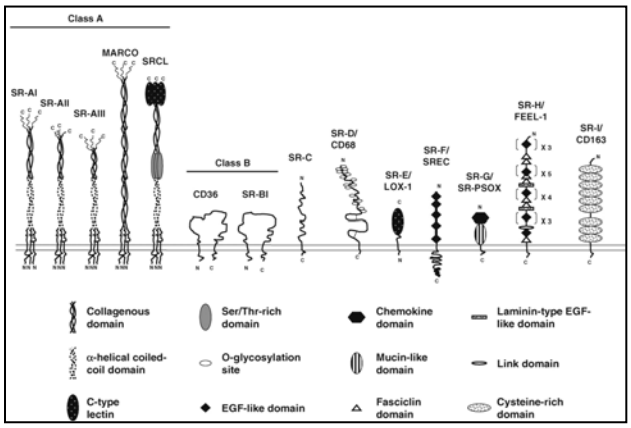

Figure 3 Classification and structure of scavenger receptors. (Adapted from Krieger M. The other side of scavenger receptors: pattern recognition for host defense. *Curr Opin Lipidol* 1997;8:275-280)[81].

SRA Expression

SR-AI/II is mainly expressed in resident macrophages of various organs including liver Kupffer cells[82-84] but also by ECs[85-87] and vSMCs[88]. Freshly isolated monocytes have a low expression level of SR-AI/II but it increases rapidly after differentiation into macrophages[89]. Interestingly, SR-AI was found to be considerably overexpressed in atherosclerotic lesions *in vivo*[90-96]. SR-A were found to mediate the accumulation of modified LDL by macrophages and other plaque cells leading to the formation of lipid laden foam cells, as was observed after overexpression of SR-A by Chinese hamster ovary cells and macrophages[97]. SR-A expression is modulated by a large number of cytokines and growth factors. PDGF[98], M-CSF[99] and phorbol esters have been shown to induce the monocyte differentiation into macrophages and increase SR-A expression. TNFα[100], TGFβ[101], IFNγ, LPS[102] and granulocyte macrophage colony-stimulating factor (GM-CSF)[103] were reported to decrease SR-A expression. SR-A ligands such as minimally modified LDL (MM-LDL) have been shown to modulate SR-A expression at an mRNA and protein level[104]. Even pathogens such as human cytomegalovirus do affect SR-A expression[105], while Calmette-Guerin bacillus was seen to increase SR-A expression in Kupffer cells[106].

The role of SR-A in atherosclerosis: friend or foe?

Theoretically, modified LDL is toxic to the endothelium and by removing these atherogenic particles from the blood circulation, SR-A should have a protective role against atherosclerosis. However, inadequate handling of these internalized deleterious materials will lead to lipid-rich foam cell formation and atherogenesis. Indeed evidence is overwhelming that SR-A, together with CD36, is the major receptor responsible for foam cells formation. The importance of SR-A in atherosclerosis was first shown in 1997 by Suzuki *et al*[107], demonstrating that lesion formation was 60% lower in SR-A-deficient ApoE$^{-/-}$ mice as compared to SR-A containing ApoE$^{-/-}$ mice. Subsequent study by Sakaguchi *et al* revealed a minor reduction of lesion formation at two time points in SR-A-deficient Ldlr$^{-/-}$ mice. The lesions were composed mainly of macrophage-derived foam cells and these foam cells in the SR-A$^{-/-}$ Ldlr$^{-/-}$ mice did express other scavenger receptors like MARCO, CD36 and CD68[108]. Following study by Babaev *et al* generated C57Bl/6 mice or Ldlr$^{-/-}$mice deficient in macrophage SR-A. A similar reduction (60%) in both cases in lesion area as compared with SR-A expression mice was found, suggested that SR-AI/II significantly contributes to atherosclerotic lesion formation and atherogenosis[109]. While these studies supported a proatherogenic role for SR-A *in vivo,* bone marrow transplantation from SR-A overexpressing mice to Ldlr$^{-/-}$ and ApoE$^{-/-}$ recipients did not reveal any differences in lesion development[110,111]. Basal macrophage SR-A expression should be sufficient to remove modified lipoproteins from the arterial wall. Nevertheless, contradictory in vivo results fueled doubts on the alleged role of SR-A as pro-atherogenic mediator. In 1999, de Winther *et al*[112] observed more severe lesions in SR-A-deficient mice backcrossed to apoE3 Leiden mice on cholate containing high fat diet as compared to control mice. In 2005, Moore *et al*[113] reported that ApoE$^{-/-}$ mice lacking SR-A or CD36 showed a significant reduction in macrophage lipid accumulation, but associated with an increased aortic sinus lesion size. Their data suggest that alternative lipid uptake mechanisms should occur when SR-A or CD36 are absent. A perturbing fact was that plasma cholesterol levels were 40% higher in their SR-A/ApoE double knockout mice which might lead to atherosclerosis in the long run. Van Eck *et al* recently showed that SR-BI was proatherogenic after 4 weeks on western type diet but antiatherogenic after 9 and 12 weeks in Ldlr knockout mice. A similar biphasic activity pattern may apply to SR-A. Therefore, a critical reassessment of the role of SR-A in atherosclerosis is still necessary. Different stages and especially advanced lesion formation should be examined as well to fully appreciate the role of the SR-A in atherogenesis.

Modulation of SR-A function

In the last two decades, our group and others have gathered increasing knowledge of scavenger receptors biology. SR-A is deemed to play an important role in foam cell formation and atherogenesis, and intervention in its function may inhibit foam cell formation and possibly lesion development. However, scavenger receptor family acts together in a dynamic network. When a single scavenger encoded-gene/

receptor is knocked down or knocked out, other partners such as SR-BI or CD36 will compensate to replace its function. Therefore, blocking its function by selective SR-AI antagonists might not produce significant anti-atherosclerotic activity. Our current understanding is that SR-AI and CD36 are the major scavenger receptors responsible for modified LDL uptake, and SR-A/CD36/ ApoE or SR-A/CD36/Ldlr triple knock out mice should allow us to validate the actual contribution of SR-A. These experiments are undergoing in our lab.
In addition, the abundant expression of SR-AI on macrophage enriched sites of inflammation and atherosclerotic lesions and the fact that this receptor mediates the efficient endocytosis of bound substrates indicates that SR-AI may not only be an interesting target for therapeutic intervention in atherosclerosis and inflammation but also for drug delivery and targeted imaging approaches. Given the complex chemical nature of the macromolecular substrates for SR-AI it is not surprising that ligand design for this receptor has been rather unsuccessful and has only resulted in a single report on a synthetic, rather unselective, SR-A antagonist[114]. In chapter 3, we describe a novel unbiased approach for the design of SR-AI antagonists involving the use of phage display on a synthetic receptor containing the ligand-binding pocket of SR-AI.

2.2 CD40/CD40L receptor/ligand dyad

The interaction of CD40/CD40L plays an important role in both humoral and cell-mediated immune responses. Genetic mutations in either CD40 or CD40L are often accompanied by severe immunodeficiency such as X-linked hyper-IgM syndrome (for a review, see[115-119]). Interruption of CD40L-CD40 signaling by administration of an anti-CD40L antibody prevents autoimmune diseases such as collagen-induced arthritis, lupus nephritis, acute or chronic graft-versus-host disease, multiple sclerosis, thyroiditis and atherosclerosis[25,116,118]. Initially, the expression of CD40 and CD40L were considered to be mostly in B lymphocytes and $CD4^+$ T-lymphocytes, respectively. However, a lot of immune and nonimmune cells were shown to express both CD40 and CD40L. For example, $CD4^+$ T-lymphocytes, B lymphocytes, EC, SMC monocytes, dendritic cells, macrophages and epithelial cells express both CD40 and CD40L. In addition, $CD34^+$ progenitor cells and fibroblasts were seen to express CD40. Mast cells, NK cells, platelets contain CD40L which is instrumental in proper cell function. The CD40/CD40L regulated humoral and cellular immune responses interweave intimately and are at the very heart of many inflammatory processes including atherosclerosis.

CD40 and CD40L signaling

CD40 belongs to the TNF receptor (TNFR) superfamily, while CD40L is a member of the TNF gene superfamily[120]. CD40 is a 48 kDa type I transmembrane protein that comprises 277 amino acids[119]. The CD40L gene encodes a 261-amino acid protein and transcription yields a 39 kDa type II transmembrane protein[119]. Until recently, most information on CD40 signaling was obtained from studies in B

lymphocytes, but these findings also apply to atheroma-associated cells. Activation of CD40 signaling requires multimerization of the receptor[121]. CD40 presents as a monomeric or preformed trimer on the cell surface to facilitate the binding of trimeric CD40L and trigger downstream signaling pathways[122]. CD40 signaling requires the association of one or two of its cytoplasmic domains with specific binding partners termed TNFR-associated factors (TRAFs)[115]. The TRAF family consists of six known members, of which TRAF1, TRAF2, TRAF3, and TRAF6 directly, and TRAF5 indirectly associate with CD40. TRAF2, TRAF5, and TRAF6 activate stress-activated protein kinases (SAPK) and NF-κB. TRAF3 activate NF-κB[123]. In addition, TRAF6 activates the ERK1/2 and p38 mitogen-activated protein kinase (MAPK), while TRAF2 and TRAF6 can activate the c-Jun N-terminal kinase (JNK) signaling pathway[124-128]. In general, CD40 signaling will activate three MAPK signaling pathways (e.g. ERK, p38 and JNK) as well as that of the transcription factors NF-κB, AP-1, and NF-AT.

The proinflammatory and proatherogenic role of CD40/CD40L

CD40 signaling profoundly contributes to atherogenesis at all stages of disease development. Until now, the actual stimuli for CD40/CD40L expression in EC, SMC, macrophage and lymphocytes in the initial plaque are still unclear. Amongst others oxLDL and antigen presentation were seen to stimulate CD40L expression[129,130]. The most important stimulus for CD40/CD40L expression during the initial stage of atherosclerosis is believed to be INF-γ[119]. CD40/CD40L interactions can mediate several of the processes considered crucial in plaque formation. During leukocyte rolling, adhesion and migration, CD40 ligation on ECs and SMCs induces the expression of leukocyte adhesion molecules such as VCAM-1, ICAM-1, LFA-1, P-selectin and E-selectin[25,131-134]. Ligation of CD40 on ECs, SMCs, macrophages, and T lymphocytes will also trigger the expression and release of chemokines within the human atheroma, such as IL-8, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), SDF-1, and MCP-1[135]. Next to chemokines, CD40 ligation also induces chemokine receptor expression such as CCR1, CCR5 and CCR7[25,119]. In the atheroma, CD40 activation promotes a Th1 type immune response[130], inducing IL-1, IL-12, IL-15, IL-18 expression and suppression IL-4 and IL-10R expression[136-138]. During lesion progression, CD40 mediates SMC migration and angiogenesis via induction of VEGF and COX-2 expression[139]. In the more advanced atheroma, CD40 ligation leads to vulnerable plaque formation by promoting extracellular matrix degradation. In vascular EC, SMC and macrophages, CD40 can activate all thirteen of the MMP family members (MMP-1 to MMP-13)[25,119]. CD40 ligation on EC, SMC and macrophages strongly induces the TF expression and reduces the expression of thrombomodulin[140-144], which results in a net accumulation of procoagulant materials in vulnerable lesions. Since activated platelets also express CD40L[145], positive feedback can amplify the local inflammatory responses and lead to severe

thrombus. Finally, a wealth of evidence exists that CD40 signaling acts through a positive feedback loop. For example, CD40L stimulates IL-15 expression, which will synergize with IL-12 to promote INFγ synthesis. As mentioned above, INFγ in turn can potently induce CD40/CD40L expression. Another positive feedback loop proceeds through CD-40 induced IL-15 as this cytokine can increase CD40L expression[146]. In addition, CD40L can induce CD40 and its own expression[147].

Interruption of CD40/CD40L ligation as a therapy to atherosclerosis

Given the prominent role of CD40 signaling to promote atherosclerosis, interruption of CD40/CD40L ligation might be beneficial to address the disease. Actually, interruption of CD40 signaling was shown to have a profound effect on atherogenesis, both in the initial and in the advanced stage. Treatment of mice with antibody against mouse CD40L reduced the size of aortic lesions by 59% and their lipid content by 79% in Ldlr-deficient mice that had been fed a high-cholesterol diet for 12 weeks, resulting in significantly fewer macrophages/T lymphocytes and a decreased expression of VCAM-1 and MMPs in comparison with the control[148]. Lutgens *et al* demonstrated that CD40L and ApoE double knockout mice had reduced lesion formation with a lipid-poor, collagen-rich, more stable phenotype and with a reduced T-lymphocyte/macrophage content[149].

In addition, CD40/CD40L not only is pivotal in the initiation and progression of atherosclerosis, but also affects plaque stability. Schönbeck *et al* showed that anti-CD40L antibody treatment of Ldlr-deficient mice during the second half of a 26-week regimen of high-cholesterol diet did not regress, but did significantly reduce the progression of established atherosclerotic. Except for halting lesion progression, anti-CD40L treatment favored a more stable phenotype[150]. Similarly, in $apoE^{-/-}$ mice treated with an anti-CD40L antibody for 12 weeks lesion size was unaffected but displayed a more favorable lipid-poor collagen-rich stable plaque phenotype. In both mouse models, a pronounced increase in collagen content, vascular smooth muscle cell/myofibroblast content, and fibrous cap thickness were observed, whereas T-lymphocyte, lipid core, inflammatory cytokine and macrophage content were reduced[151]. In conclusion, interruption of CD40/CD40L will prevent the initiation and progression of atherosclerosis and even may ameliorate plaque stability.

Although a few studies illustrated the potential of CD40/CD40L blockade by humanized monoclonal antibodies (humanized 5C8), to prevent allograft rejection[152,153], severe side effects like thrombo-embolic complications have been reported as well[154,155] which will hamper clinical application. Obviously, blockage of CD40/CD40L ligation will sharply influence the immune system and lead to some undesirable side effects. Nevertheless, Ketorolac/heparin administration was found to be effective in preventing the thromboembolism associated with anti-CD154 mAb treatment[155], suggesting that the anti-CD40L antibody may have induced platelet activation and aggregation which can be overcome by targeted

medication during therapy. Therefore, more selective and less toxic small molecule inhibitors as alternatives are urgently desired.

3. Targeting restenosis by transcriptional inhibition

The expression of a series of proinflammatory cytokines, such as IL-2, IL-6, TNFα, IFNγ and CD40L requires coordinate activation and translocation of transcription factors, including NFκB, NFAT and Myocyte Enhancer Factor-2 (MEF-2). The presence of the aforementioned cytokines provides a permissive milieu for vSMC migration, dedifferentiation and proliferation. The dedifferentiated vSMC will acquire an embryonic non-contractile and synthetic state phenotype. These events are deemed as important steps in the development of restenosis after vascular angioplasty/stenting and are at least in part regulated by calcineurin/NFAT signaling. Transcriptional inhibition of calcineurin/NFAT signaling may enable to simultaneously reduce the expression of pro-inflammatory cytokines and growth factors and may diminish restenosis. In addition, since restenosis has been identified as an inflammatory disorder, traditional immunosuppressants may provide a new therapeutic option to dampen the underlying inflammation. In Chapter 2, we give a detailed discussion on selective inhibition of NFAT as a potential therapy to cardiovascular disorders.

4. Phage display in drug discovery and targeted imaging

Phage display is a molecular technology that allows the presentation of peptide and protein libraries on the surface of filamentous phage. It was first described by Smith *et al* in 1985[156]. Phage display is a powerful tool to identify the individual partners of protein-protein interactions and one of the most established techniques to generate lead molecules in drug discovery (for review, see[156-158]). Depending on the position of the DNA insert, peptides are displayed at the N-terminus of pIII (3-5 copies) or pVIII (~2000 copies) coat protein. Expressed peptides can be linear or constrained according to the phage vectors adopted. The pIII system is fit to express very long sequenced peptides ranging from 6-40 amino acids, whereas the pVIII is particularly useful for the construction of cyclic peptide libraries. The in vitro selections are performed by an iterative process called biopanning. As shown in Fig. 4, a library of recombinant bacteriophage displaying different peptide sequences can be fractionated by affinity selection in six steps.

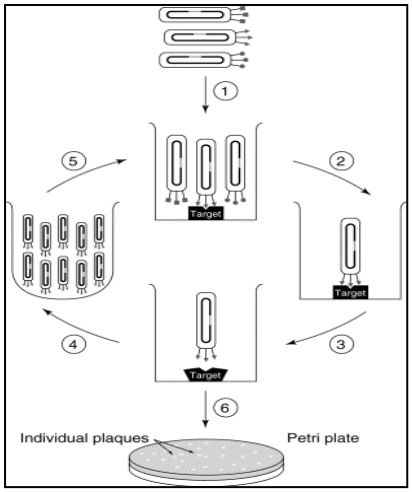

Figure 4 Biopanning selection of peptide phage display. Adapted from Kay *et al*. Drug discovery today, 1998, 3(8), 370-378

The library will first be incubated with the immobilized targets. After incubation, non-binding phage or nonspecific binding phage are removed by repeated washes. The bounded phage is recovered by acid elution followed by amplification in E. coli. The enriched library is selected in a new screening cycle. After a few rounds of selection, the phage pool will be enriched in conserved sequences. Individual phage particle will be isolated and sequenced. The peptide encoded by the DNA insert in the phagemid clone will be sequenced. Finally, the biological functionality of expressed recombinants or synthetic peptides will be elucidated.
Peptide phage display has been successfully applied in new drug discovery and targeted imaging for atherosclerosis. *In vivo* phage display had led to plaque targeting peptides for use in imaging strategies[159,160]. Peptides can also constitute promising leads for anti-atherogenic or anti-atherothrombotic therapy. Peptides with high specificity for E-selectin, ICAM-1[161], LOX-1[162] and MCP-1[163,164] have already been discovered by this technique. Recently, we have used phage display to identify a selective human P-selectin peptide antagonist[165,166], which may hold promise in the treatment of atherosclerosis and restenosis. Phage display derived cyclic peptides specific for MMP-2 and MMP-9 were shown to potently inhibit the migration and proliferation of human EC and tumor cells[167], and may possibly also be able to prevent erosion of the fibrous cap. Further, selected peptides can be used to facilitate drug delivery, improve gene administration[168] and enhance the selectivity of contrast agents for vascular imaging[160].

5. Thesis outline:
Chapter 2 reviews the regulatory pathways in calcineurin/NFAT signaling and its importance to restenosis and other cardiovascular disorders. Furthermore, we describe the potential of a selective peptide inhibitor, VIVIT, which may be superior in selective targeting of NFAT than CsA in anti-immune and anti-proliferative therapy. Chapter 3 describes a study on the development of a selective peptide antagonist for SR-AI by phage display and elaborates on its potential as an imaging agent for the atherosclerotic plaque. Chapter 4 reports on the development of a selective CD40 targeting peptide by phage display. In chapter 5 we present synthetic VIVIT peptides as promising therapeutics in the treatment of restenosis and we have characterized the effects of VIVIT based NFAT inhibition on key cells in restenosis. In chapter 6 we communicate on the rational design and characterization of a selective bipartite peptide inhibitor of NFAT, which is more potent than VIVIT and less toxic than CsA, for use in anti-inflammatory therapy.

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