Modulation of Atherothrombotic Factors: Novel Strategies for Plaque Stabilization

Bot, I.

1 General Introduction

Contents

1. Atherosclerosis

Atherosclerosis is a multi-factorial disease of luminal narrowing of the larger arteries, of which the clinical manifestations (e.g. stroke and myocardial infarction) are the leading cause of death in the world. In general, atherosclerosis is a progressive disease, already initiating during childhood[1,2]. Early lesions progress during life, without clinical symptoms as arteries are capable of remodelling to compensate for luminal loss[3]. Depending on the composition of the atherosclerotic lesion and the affected artery, acute complications such as cerebral ischemia (stroke), angina pectoris, peripheral arterial occlusive disease and myocardial infarction may occur, which generally are the result of rupture of an advanced atherosclerotic plaque[4-6]. Upon rupture of an atherosclerotic plaque, the highly thrombogenic content of the plaque will be exposed to the circulation, initiating the blood coagulation cascade and thrombus formation[7,8]. The ensuing total arterial occlusion can lead to death.

Arteries that are particularly prone to atherosclerotic plaque formation are the coronary arteries, the carotid arteries at the bifurcation site and all main branching points of the aorta[9,10]. The high vulnerability of these arteries is attributable to hemodynamic flow factors, such as low shear stress, oscillatory flow and turbulent flow[11]. However, apart from a genetic predisposition to atherogenesis, various behavioral factors affect disease progression, such as smoking[12], high fat diet[13], stress and physical inactivity. Also, diabetes[14], hypertension[12], hyperhomocysteinemia[15] and obesity are related to an increased disease manifestation. Surgical intervention by e.g. bypass surgery, percutaneous transluminal coronary angioplasty (PTCA), stenting or atherectomy is frequently required to restore an impeded blood flow, however the success rate of these interventions is often impaired by recurrence of a lesion (so-called re-stenosis)[16].

2. Atherosclerotic Plaque Development

2.1. Lesion Initiation

Atherosclerosis is a process occurring in the medium and large sized arteries[17,18]. A normal artery consists of an endothelial layer covering the media of smooth muscle cells that is flanked by the internal and external elastic lamina. Outside the external elastic lamina, the artery is surrounded by adventitial tissue. Atherosclerosis is thought to start with endothelial malfunction at predisposed sites (e.g. arterial branches or bifurcations), caused by turbulent or oscillatory shear stress or atherogenic lipoproteins (VLDL, LDL): The so-called ″response to injury″ theory[19,20]. The activated endothelial cells respond by expressing adhesion molecules like E- and P-selectin which mediate the “rolling” of monocytes on top of the endothelium. Vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion

molecule-1 (ICAM-1) and some of the CC-Chemokine Receptors (CCRs) enable the subsequent adherence of circulating leukocytes to the endothelium. These leukocytes, expressing among others P-selectin glycoprotein ligand-1 (PSGL-1) and CC-Chemokine Receptor 2 (CCR2), migrate through the endothelial layer into the subendothelial space (Figure 1).

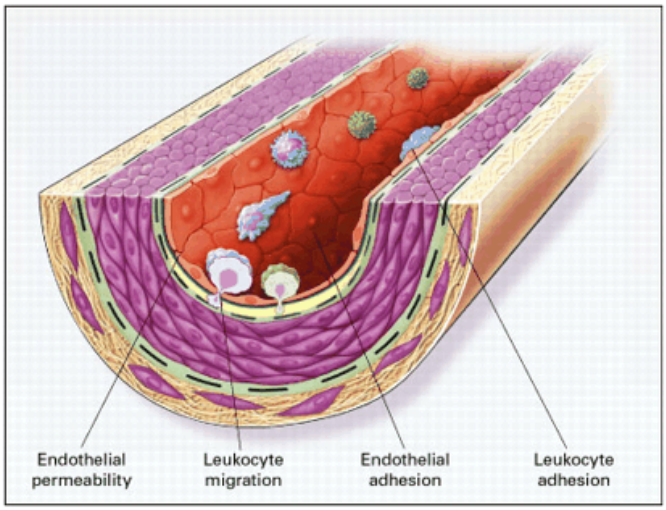

Figure 1. Initiation of atherosclerosis (adapted from R. Ross. *New Engl J Med*. 1999)[18].

The migrated leukocytes will differentiate into tissue macrophages in the presence of different cytokines such as Macrophage Colony Stimulating Factor (M-CSF), Tumor Necrosis Factor α (TNFα), Interferon γ (IFNγ), pro-inflammatory interleukins (e.g. Interleukins-1 and -2; IL-1, -2) and growth factors (like Transforming Growth Factor β (TGFβ), Platelet Derived Growth Factor (PDGF) and Insulin-like Growth Factor-1 (IGF-1))[18,21]. These macrophages progress into “foam cells” by ingesting cholesterol and modified lipoprotein particles, which have accumulated below the endothelial layer[22]. The initial stage of lesion progression is classified as a type I lesion according to the classification criteria of the American Heart Association (AHA) system introduced in 1995[23,24], which has been frequently updated afterwards[25]. These type I lesions progress into type II fatty streaks[26], which are still non-symptomatic, but are further enriched in lipid laden macrophages and contain T-lymphocytes. Also, medial vascular smooth muscle cells (vSMCs) start to migrate under the influence of PDGF, Fibroblast Growth Factor (FGF) and TGFβ, which are secreted by inflammatory cells and subsequently, the lesion progresses towards a type III lesion.

2.2. Lesion Progression

A type II fatty streak may progress into a type III intermediate lesion which contains small lipid deposits that are present extracellularly under a layer of migrated vSMCs. Type III plaques are recognized as true atherosclerotic or pre-atheroma plaques[23,24] and can be regarded as an intermediate stage between the fatty streak and an advanced atherosclerotic lesion. In type IV lesions the intimal lipid deposits have evolved into large cell-free lipid pools containing a substantial amount of cholesterol crystals, due to either apoptosis/necrosis of intimal lipid-laden macrophages or to retention of infiltrated lipoprotein particles. The type IV atheroma is the first stage of an advanced lesion possessing a lesion core and small intimal capillaries that originate from the *vasa vasorum*, which is described as the network of capillaries in the adventitia. These plaques are prone to become clinically symptomatic. During further progression, more fibroblasts and vSMCs migrate from the media into the intimal rim, tend to accumulate subendothelially and together with extracellular matrix material like collagen and proteoglycans, produce a fibrous cap covering the lipid core (Figure 2). The type V lesion is known as the fibro-atheroma[27] and most plaque ruptures take place in this lesion type, as these lesions are biomechanically vulnerable and are freely exposed to blood flow forces[4]. In fact, type V lesions are subdivided into 3 stages, of which the first (type Va) is described above, type Vb that is calcified and type Vc lesions, which are relatively lipid poor. Type IV and V lesion are, in practice, often difficult to discern, and nowadays frequently termed as “thick” and “thin” fibrous cap atheroma, respectively[4].

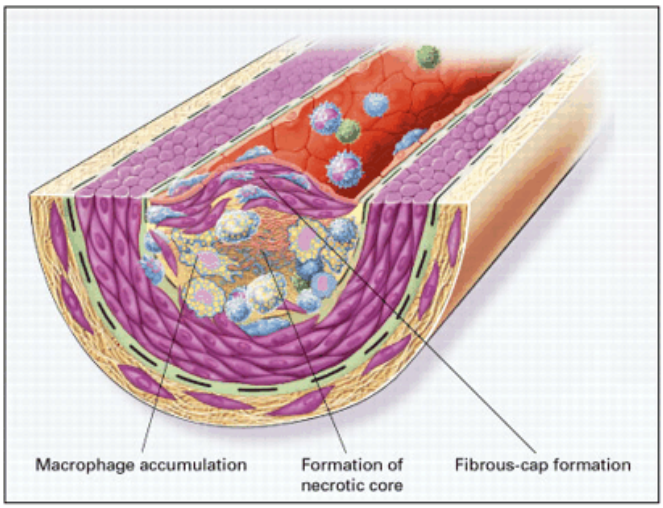

Figure 2. Progression of atherosclerosis (adapted from R. Ross. *New Engl J Med*. 1999)[18].

Ruptured lesions with an intramural or luminal thrombus or lesions containing hemorrhage are classified type VI atherosclerotic lesions. Type VI lesions without noticeable cap breaks are referred to as eroded[28,29]. To distinguish these differences, three different terms describe these subclasses: The “fibrous cap atheroma with erosion”, which has a thick fibrous cap and a luminal thrombus but without lumen-plaque core communication. Next is the “thin fibrous cap with plaque rupture”, where a luminal thrombus is in direct contact with the lipid core of the lesion. The third subtype describes the “calcified nodule with erosion”, with an eruptive nodular calcification with overlying luminal thrombus.

3. The Unstable Plaque

Type IV, V and VI plaques are considered “unstable” and give rise to the majority of clinical manifestations as stroke and myocardial infarction. Several factors may contribute to a reduced mechanical stability of atherosclerotic lesions, including matrix degradation, fibrous cap degradation and lipid core enlargement. In general, the size of the necrotic core and the strength of the overlying fibrous cap are in balance. When this balance is disturbed, the fibrous cap may rupture and the highly thrombogenic content of the lipid core may be extruded and comes in direct contact with the circulation leading to activation of the coagulation system, resulting in thrombus formation and possibly acute coronary syndromes or stroke. In particular, protein degradation by matrix metallo-proteinases (MMPs)[33,31] and cathepsins, vascular wall cell apoptosis[32,33] and platelets adherence[34] have been proven to induce or accelerate plaque destabilization. Also, increased levels of various pro-inflammatory interleukins and chemokines have been associated with plaque instability[35,36]. In the following chapter we will describe the individual identified players in plaque destabilization in more detail.

3.1. Matrix Remodelling

Matrix Metalloproteinases

Intraplaque expression of several members of the matrix metalloproteinase family (MMPs) is deemed to be correlated with reduced plaque stability. MMPs are mainly secreted by lesional macrophages and T-lymphocytes to degrade extracellular matrix components and to facilitate migration of these cells through the matrix. The MMPs are inhibited by Tissue Inhibitors of Metalloproteinases (TIMPs)[30] and the net activity depends on the balance between MMP expression and the presence of the inhibitor. The gelatinases (MMP-2 and MMP-9) are specialized in the degradation of collagen isoforms and elastin, whereas collagenases (e.g. MMP-1, -7) mainly digest fibrillar collagens. Stromelysin (MMP-3) is involved in the breakdown of proteoglycans, fibronectin and elastin, while it is also

capable of activation of other proteinases. Recently, Gelatinase-B (MMP-9) was shown to play a role in early plaque development[37], although especially in advanced atherosclerosis MMP-9 was found to be one of the most important MMPs for plaque instability. MMP-9 has, in a number of studies, been shown to be expressed in ruptured human lesions[38]. Especially in the shoulder region, which is the more vulnerable plaque region prone to rupture and in the core, an increased activity of MMP-9 was observed. Also, MMP-1 (interstitial collagenase)[38,39], MMP-3[40,41] and MMP-8 (neutrophil elastase)[42] have been associated with plaque instability. Lee *et al.* have shown that MMP-1 protein expression is especially upregulated in regions with high circumferential stress, hereby suggesting a role for MMP-1 in atherosclerotic plaque destabilization in advanced lesions[38]. Interestingly, ApoE$^{-/-}$ mice expressing human MMP-1 display reduced atherosclerotic lesion development, establishing that MMP-1 is important during lesion development with respect to matrix remodelling[43]. It is conceivable that inhibition of MMPs or correcting the MMP:TIMP balance may be useful in treating the symptoms of atherosclerosis[44].

Cathepsins

Cathepsins are cysteine proteases that are synthesized and targeted to acidic compartments, the lysosomes and endosomes, where they are activated to degrade their substrates, such as elastin and collagen. These compartments provide the cathepsins with the optimal pH for their activity. Cathepsins have been shown to be present in atherosclerotic plaques[46]. During the initial stage of atherosclerosis, macrophages express and secrete substantial amounts of cathepsins K, L and S, which can act pericellularly to degrade intimal matrix components, allowing the macrophages to migrate into the subendothelial space. Also at later stages of atherosclerosis progression, macrophages appear to produce the bulk of cysteine proteases in the atheroma, while human atherosclerotic lesions were shown to express relatively low levels of cystatin C, an endogenous inhibitor of these cathepsins[46]. Increased expression of cathepsin B in atheromatous plaques was shown to colocalize with macrophages[47] and in sections of human atherosclerotic lesions cathepsins S, K and L were visualized[48,49], all of which are able to degrade elastin and collagen and thus to destabilize the atherosclerotic plaque. Interestingly, inflammatory cytokines were found to increase cathepsin S secretion from macrophages. Cathepsin S/LDL receptor deficient double knockout (CatS$^{-/-}$/LDLr$^{-/-}$) mice were reported to have a decreased plaque formation as well as stage of plaque development. Also, the CatS$^{-/-}$/LDLr$^{-/-}$ mice demonstrated less elastin breaks[47]. Mice deficient in cystatin C and ApoE demonstrated to have disrupted arterial medial elastic laminae, thus increasing the risk of aneurysm formation[50]. However, the direct involvement of cysteine proteases in plaque rupture remains to be demonstrated.

3.2 Cellular Homeostasis: Apoptosis

Cellular homeostasis can be regarded as a balance between cell death and mitosis. Cells death may occur after exposure to heat, irradiation (UV, X-ray), oxidative stress or after infection with a pathogen. One of the cellular death mechanisms is apoptosis or programmed cell death, which is a process that all organisms display to dispose of cells in an efficient, highly selective manner[51]. In apoptosis, cells undergo a series of characteristic events, including cell shrinkage, DNA fragmentation and blebbing of the cell membrane (Table 1)[33]. The end-products of cellular apoptosis are apoptotic bodies or remnants, which may be phagocytosed or undergo secondary necrosis. Apoptosis does not elicit an inflammatory response or even may quench an ongoing response, although massive apoptosis may act pro-inflammatory. Apoptosis is cleary distinct from necrosis, a more conventional death mechanism, which results in enzymatic digestion and disruption of membranes of a cell that is accompanied by an inflammatory response.

Apoptosis is triggered via two mechanisms, the intracellular and the extracellular pathway. The former involves activation of membrane bound death receptors of the tumor necrosis receptor family (TNF-R) such as Fas (CD95) or the death receptors 3-6[52]. After binding of their trimerized ligands, the receptors aggregate and specific adapter proteins, e.g. Fas-associated death domain (FADD), are recruited. The receptor complexes will activate the caspase cascade, which in turn results in the activation of the terminal effector caspases 3, 6 and 7 that cleave the intracellular substrates necessary for cell survival, resulting in apoptosis[53]. The second apoptosis pathway proceeds via mitochondrial death signaling[54]. In this case, caspase 8 will cleave the pro-apoptotic protein Bid, which in turn binds and inactivates the anti-apoptotic Bcl-2, resulting in the release of cytochrome c and other mitochondrial proteins that activate the caspase cascade. Growth factor withdrawal and p53 activation induce apoptosis via this pathway.

Table 1. Characteristic features of apoptosis versus necrosis, adapted from Bennett, *Heart*, 2002[33].

Apoptosis	**Necrosis**
Condensation/clumping of nuclear chromatin	Nuclear chromatin non-specifically degraded
Loss of cell–cell contact, cell shrinkage, and fragmentation, with formation of membrane bound processes and vesicles containing fragments of nuclear material or organelles	Cell volume increases
Adjacent cells phagocytose the end product, the apoptotic body	
Minimal disruption of cell membranes or release of lysosomal enzymes, with consequently little inflammatory reaction	Cell membrane integrity lost early, release of lysosomal enzymes and subsequent inflammation
Organelle structure and function maintained until late into the process	Organelle structure and function lost early

In atherogenesis, apoptosis is an important process[3,55,56], especially in later stages of plaque progression (type IV-VI). Plaque rupture is associated with thinning of the vSMC rich fibrous cap and indeed, apoptotic vSMCs have been detected in the shoulder region of atherosclerotic lesions[57]. Also, apoptosis of SMCs was found to be increased in unstable plaques compared to stable lesions. In addition, apoptosis of medial SMCs might induce aneurysm formation. Endothelial cell apoptosis is one of the underlying pathways to induce plaque erosion, which is often the cause of CVD-related death among younger women[58].

The role of macrophage apoptosis in advanced atherosclerotic lesions remains somewhat controversial. On the one hand macrophage apoptosis might be beneficial to plaque stability as it will be accompanied by a reduced secretion of matrix degrading enzymes and it may reduce plaque growth in initial lesions. On the other hand, apoptosis of macrophages leads to an increased size of the plaque core if the apoptotic remnants cannot be cleared, which may result in a disbalance between lipid core size and fibrous cap strength in more advanced lesions[59]. Also, the apoptotic bodies left in the atheroma contain large amounts of activated Tissue Factor (TF), hereby enhancing the prothrombotic potential after rupture of the plaque[60].

In conclusion, apoptosis occurs in all cells of the atherosclerotic lesion and contributes to plaque growth, lipid core size and especially plaque rupture followed by its thrombotic complications. Although the exact contribution of apoptosis of each cell type to plaque destabilization remains somewhat unclear, the main view focuses on anti-apoptotic therapy for plaque stabilization.

3.3. Plaque Inflammation: Key Factors

Intimal Inflammation

The intima of an atherosclerotic plaque contains different cell types such as endothelial cells, vascular smooth muscle cells, macrophages and T-lymphocytes, which express inflammatory mediators in response to injury. Cytokines are small cell-regulatory proteins that are key players in the initiation and control of the inflammatory process. Cytokines are known to be involved in the process of atherosclerotic lesion formation and can roughly be divided into six families: interleukins, the tumor necrosis factor-1 family, interferons, colony stimulating factors, chemokines and growth factors, although considerable overlap between the different families exists. As multiple cytokines act in concert to mediate the inflammatory process, the balance between the anti- and pro-inflammatory cytokines and growth factors will determine the net outcome of the effect of these mediators.

Anti-inflammatory interleukins, such as IL-10, can reduce adhesion molecule expression and inhibit proteolytic enzymes and coagulation factor (TF) expression[61]. Various pro-inflammatory cytokines have been conclusively shown to contribute to the development of atherosclerosis, e.g. TNFα, interleukin-12 (IL-12)[62], IL-18[63] and IL-1[64.65]. IL-8, also known as

CXC-chemokine Ligand 8 (CXCL8) and hence member of both the interleukin and the chemokine subfamily, activates monocytes and directs migration of monocytes across the endothelium[66]. Interleukins in particular involved in plaque destabilization may be IL-1 and IL-18[63,67]. Overexpression of IL-18 in carotid artery plaques results in an increased unstable phenotype[68]. The cytokine IFNγ, secreted by T-lymphocytes in the human plaque, inhibits the production of collagen overlying the lipid core. Recently, it was reported that patients with acute coronary syndromes had increased blood levels of TNFα, IL-6 and sCD40L[69]. In concert, Waehre *et al.*[70] documented that in patients with unstable angina, the TNFα/IL-10 balance was highly enhanced, while treatment with IL-10 inhibited the release of TNFα, IL-8 and TF.

For therapeutic approaches, inhibition of the pro-inflammatory interleukins or TNFα could result in reduced atherosclerotic lesion progression. For example, Interleukin-1 Converting Enzyme (ICE)[71,72] is involved in 2 processes important during the development of an atherosclerotic plaque, notably apoptosis and inflammation. ICE converts pro-interleukin 1β (pro-IL-1β) into its active form IL-1β and similarly activates pro-interleukin 18 (pro-IL-18) into IL-18[73]. IL-1β is a highly pro-inflammatory cytokine as described above and also IL-18 has been associated with plaque destabilization[68]. Furthermore, ICE is involved in the induction of apoptosis, as ICE is also known as caspase-1, one of the initiators of the apoptosis cascade[74]. Inhibition of ICE by the cowpox virus CrmA protects cells infected with the cowpox virus from clearance by preventing IL-1β release[75,76]. Also, smooth muscle cells produce the serine protease inhibitor PI-9, decreased levels of which have been found in unstable plaques, which resulted in increased IL-1β expression[77,78].

Chemokines represent a family of structurally related chemotactic cytokines, which are classified in subgroups (CC, CXC, C, CXXXC) according to their N-terminal sequence[79]. Recent evidence suggests that Monocyte Chemoattractant Protein-1 (MCP-1)[80], which is the natural ligand for the chemokine receptor CC-chemokine receptor 2 (CCR2), contributes to thrombin generation and thrombus formation by inducing TF production[81]. The CC-chemokines Thymus (TARC, CCL17)[82], Pulmonary and Activation-Regulated Chemokine (PARC, CCL18)[83] and Macrophage Derived Chemokine (MDC, CCL19) have been identified in macrophage-rich areas of atherosclerotic lesions[84]. Also, CXC chemokines like IL-8 (CXCL8)[85], Monokine Induced by IFNγ (MIG, CXCL9)[86], Stromal cell Derived Factor-1α (SDF-1α, CXCL12)[87] and the transmembrane chemokines as CXCL16[88] and fractalkine[82,89] have been detected in atherosclerotic plaques. As macrophages play an important role in the initiation and progression of atherosclerotic lesion development, one can expect that these chemokines, either independently or in concert, are instrumental in atherogenesis. In more unstable plaques, chemokines may be important as well, as SDF-1α[87,90], MDC[91] and fractalkine[92] have been shown to induce platelet activation, which can result in platelet aggregation and adhesion in the

presence of adenosine 5'-diphosphate (ADP) or thrombin. In addition, chemokine-mediated platelet activation leads to degranulation and deposition of platelet chemokines as Platelet Factor 4 (PF4), macrophage inflammatory protein 1 (MIP-1) or RANTES (regulated on activation, normal T cell expressed and secreted)[93], which in turn can further enhance the attraction of monocytes to the (unstable) plaque[94]. Thus via the activation of chemokines, activated platelets will be engaged in the local inflammatory response at the site of activation and may therefore contribute to the development of atherosclerosis[95]. Indeed, one of the major platelet-activating chemokines, SDF-1α, was identified within unstable atherosclerotic plaques[90,96] and it is conceivable that it could play a role in the formation of a platelet-rich thrombus after plaque disruption.

Regulatory Pathways in Inflammation

Cytokines (e.g. IL-2, TNFα, CD40L and IFNγ) are expressed after activation and nuclear translocation of various transcription factors such as nuclear factor κB (NFκB)[97], Nuclear Factor of Activated T-cells (NFAT)[98], Myocyte Enhancer Factor-2 (MEF-2)[99] or activated protein-1 (AP-1). Plausibly, inhibition of transcription factor activation might lead to reduced expression of these pro-inflammatory cytokines and could attenuate atherogenesis. Immunosuppressive drugs Cyclosporin A (CsA) or FK506 (tacrolimus) inhibit signaling pathways via inhibition of NFAT and NFκB activation[100,101]. CsA and FK506 interact with specific immunophillins cyclophilin A or FK506 Binding Protein 12 (FKBP12), respectively, to inhibit calcineurin signaling in a Ca^{2+}/calmodulin–dependent fashion and thus cytokine gene expression[102]. This calcineurin-signaling pathway was first described in T-cells, but appeared also functionally active in all vascular cell types, which makes inhibition of this pathway also in atherosclerosis a therapeutic option to reduce the ongoing inflammation.

Adventitial Inflammation

The adventitia, the perivasclar tissue, is becoming increasingly important in atherosclerosis research. The adventitia consists of extracellular matrix material, a capillary blood vessel network (*vasa vasorum*), fibroblasts, progenitor cells and also macrophages. During progression of atherosclerotic lesion development, also inflammation of the adventitia appears to be increased[103,104]. As compared to that of non-ruptured lesions, the adventitia of ruptured lesions was shown to consist of significantly more inflammatory cells, like monocytes, T-lymphocytes and mast cells (Figure 3). Moreover, medial fibrosis and the number of elastic lamina breaks were increased in ruptured lesions. In culprit lesions, significantly more CD4- and CD8-positive lymphocytes were observed at the adventitial rim, accompanied by an increased amount of capillaries, compared to stable atherosclerotic lesions.

Mast cells were found in the shoulder region of atherosclerotic lesions where these cells are associated with plaque rupture[105,106]. Bone marrow

derived mast cells migrate into almost all vascularized tissues, where they complete their maturation and reside in a quiescent state close to epithelia, blood vessels and nerves[107]. Interestingly, activated mast cells were found to be abundantly present also in the adventitia of atherosclerotic lesions and their number correlated with the stage of atherosclerotic plaque development and the incidence of plaque rupture[108,109].

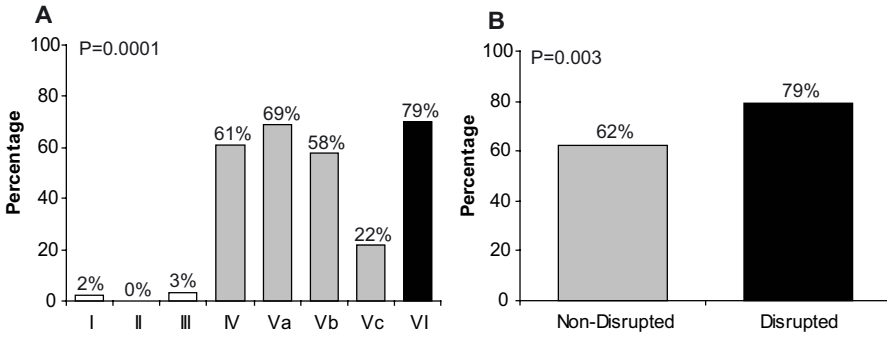

Figure 3. Adventitial inflammation in early and advanced atherosclerotic plaques. (A) Bar graph showing incidence of adventitial inflammation in early plaques and advanced plaques. (B) Bar graph showing adventitial inflammation in advanced, nondisrupted (left), and disrupted plaques (right) (adapted from Moreno *et al*., *Circulation*, 2002)[103].

As mast cells contain among others a range of mast cell proteases (chymase, tryptase), histamine, heparin and TNFα[110], activation of this inflammatory cell type may strongly impact on atherosclerotic lesion development and plaque morphology. The mast cell proteases chymase and tryptase are capable of activating MMP-1 and -3[111,112], causing degradation of the extracellular matrix (ECM) components (e.g. collagen), necessary for the stability of the plaque. Activated mast cells also secrete MMP-9[113]. Furthermore, chymase induces SMC apoptosis by degrading fibronectin, a matrix component necessary for SMC adhesion and survival[114,115]. By secreting chymase and TNFα, activated mast cells are able to promote endothelial cell apoptosis[116]. Furthermore, chymases convert Angiotensin I to Angiotensin II similar to angiotensin coverting enzyme (ACE), activate TGFβ-1 and IL-1β and modulate lipid metabolism by degrading LDL, thus facilitating foam cell formation[117]. In conclusion, mast cells and derived granulae constituents can have profound effects on plaque morphology and stability, although it is not quite clear how and when mast cell are activated in atherosclerotic lesions and in its adventitia.

Interestingly, also hypertension seems to be associated with increased adventitial inflammation, as Angiotensin II has been found to stimulate among others the adventitia to generate reactive oxygen species (ROS), which in turn lead to endothelial dysfunction and inflammation[118]. Activation of ROS induces the upregulation of endothelin-1, adhesion molecules,

nuclear factor-kappa B (NFκB) and other inflammatory mediators, which all contribute to the progression of vascular disease and atherogenesis.

The *vasa vasorum*, the network of adventitial capillaries (Figure 4), is increasingly recognized as an important factor in atherosclerotic lesion development, as it is a major source of intimal neovessels[119-121], although luminal infiltration of neovessels may occur as well. The exact mechanism of neovessel formation from the *vasa vasorum* into the plaque is only poorly understood[122]. Possibly, intimal hypoxia and ischemia may induce the expression of Hypoxia-Inducible Factor (HIF-1)[123], which in turn upregulates the expression of Vascular Endothelial Growth Factor (VEGF) and other angiogenic factors by the endothelial cells of the *vasa vasorum*. Additionally, activated macrophages, particularly in the inner core of the atheroma, stimulate the angiogenic system by inducing endothelial cell secretion of FGF and VEGF[122], which further induce endothelial cell proliferation. *In vivo* models have revealed that oxidative stress induced endothelial dysfunction could promote enhanced adventitial inflammation and revascularization of the *vasa vasorum*. The *vasa vasorum* also provides another means for inflammatory cells and plasma constituents into the plaques, which is critical in plaque progression. In *post mortem* studies, hyperplasia of the *vasa vasorum* and consequential macrophage infiltration were found to be associated with plaque rupture. Currently, the high density of *vasa vasorum* is considered as one of the determinants of a "vulnerable plaque"[120].

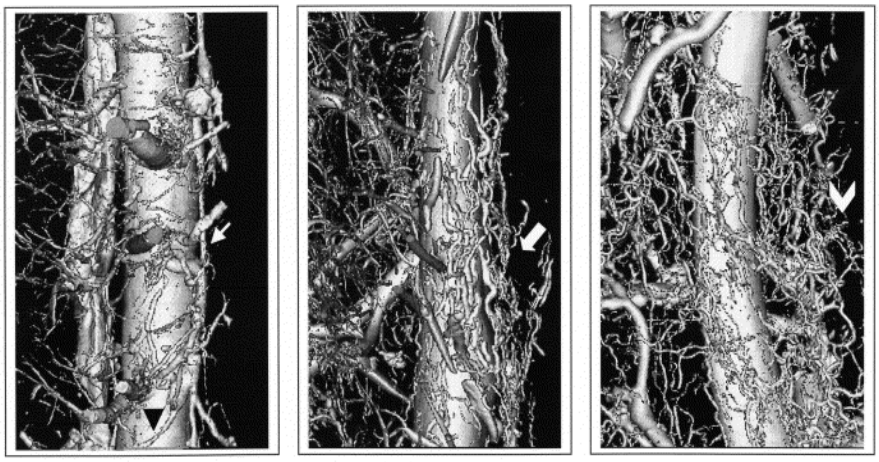

Figure 4. Microscopic computed tomography images of a coronary artery from a normal pig (left) and a pig in which atherosclerosis was promoted by feeding a hypercholesterolemic diet for 4 (middle) and 12 weeks (right) (adapted from Herrmann *et al*., *Cardiovasc. Res*., 2001)[124].

Angiogenesis and ensuing adventitial *vasa vasorum* neovascularization of the intima may predispose to intraplaque hemorrhage (IPH), which has been associated with plaque instability[125]. Kolodgie *et al*.[126] has recently provided compelling evidence that intraplaque hemorrhage often colocalizes with leaky microvessels and may significantly contribute to the expansion of

the lipid core. When erythrocytes accumulate in the lesion, the free cholesterol from the erythrocyte membranes is deposited in the core, unbalancing the equilibrium between lipid core size and cap thickness. Excess cholesterol that is taken up by macrophages induces apoptosis of these cells[127]. Moreover, IPH will increase macrophage infiltration, platelet deposition and foam cell formation, all factors that destabilize plaques. Macrophage apoptosis will be accompanied by enhanced TF activity in the plaque which in turn increases VEGF expression and angiogenesis, thus creating a self-perpetuating circuit. In patients with peripheral artery disease, both VEGF and TF levels were significantly increased and the expression of both factors appeared to be interrelated, suggesting a direct link between thrombosis and angiogenesis[121]. Focal inhibition of angiogenesis could results in reduced *vasa vasorum* development and decreased plaque formation[128].

Platelets in Atherosclerosis

Platelets are blood cells that originate from megakaryocytes in the bone marrow. They serve as cells that circulate to discriminate between intact and injured endothelium. Activated platelets, together with the activated coagulation cascade, are key mediators in thrombus formation. Multiple receptor-ligand interactions, including that of von Willebrand factor (vWF) binding with platelet GPIbα and GPIIβ/IIIα, P-selectin and sulfatides, collagen binding with collagen receptors, as well as platelet receptors stimulation (e.g. via ADP receptors) appear to orchestrate in the process of arterial thrombus formation[129].

Apart from mediating thrombosis, platelets are also suggested to play a role in the initiation and progression of atherosclerotic lesions. During atherogenesis, platelets attach directly or after tethering to monocytes to the disrupted endothelium without eliciting a direct thrombotic response. Inflamed endothelial cells display an enhanced secretion of vWF, which results in increased platelet adherence to the endothelium[130].

Not only platelet agonists, such as ADP and thrombin, are able to activate platelets, but also, as described above, various chemokines and cytokines (e.g. PF-4[91] and recently SDF-1α[90]). SDF-1α will do so in a CXCR4 dependent fashion, resulting in platelet aggregation and adhesion. The macrophage chemokine TARC[86], shown to be present in atherosclerotic lesions, has recently been demonstrated to enhance platelet activation via platelet CCR4. Activated platelets are a rich source of pro-inflammatory cytokines (e.g. CD40 ligand, IL-1β) and chemokines (PF-4, RANTES)[131]. Platelet surface exposed P-selectin may bind to PSGL-1 on monocytes and endothelial cells, facilitating leukocyte attachment to the endothelium[132], their subsequent migration through the endothelial lining after which they will be activated resulting in increased cytokine production. Platelets may, via CD40L, stimulate endothelial cells and vSMCs to express MCP-1 and IL-8, which are crucial for leukocyte recruitment[133].

Platelets also express cyclooxygenase-1 and -2 (COX-1, -2). Interestingly, inhibition of COX-2, the isoform involved in prostacyclin synthesis, leads to reduced atherosclerotic lesion development[134], although contradictory results have been reported[135]. Likewise, inhibition of COX-1, the key enzyme in thromboxane synthesis, retarded atherogenesis[135]. In summary, platelets are essential for thrombus formation and are involved in the inflammatory process, either directly via the production of cytokines or indirect via adhesion to leukocytes.

3.4. Lipid Accumulation

During atherogenesis, lipids accumulate in the core of the lesion. These lipids enter the plaque via influx of modified low density lipoprotein (LDL) particles, which are phagocytosed by macrophages, rendering them foam cells and increasing the plaque lipid core. Therefore, modified LDL (e.g. minimally modified, mildly oxidized or oxidized LDL) is widely recognized as a key factor in the pathogenesis of atherosclerosis and its thrombotic complications[136], as it activates endothelial cells, vSMCs and platelets, which are all involved in the progression of atherosclerosis. LDL particles contain different atherogenic lipids, such as oxPAPC[137], lysophosphatidylcholine (lysoPC), phosphatidic acid (PA), lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P)[138,139]. Lysophosphatidic acid (LPA) showed to be an important mediator for the pro-atherogenic actions of LDL[138]. LPA is formed during mild oxidation of LDL and is the main active compound in mildly oxidized LDL and minimally modified LDL. LPA can also be enzymatically produced by different cell types from PC and PA. LPA was originally known to be a key precursor in *de novo* lipid synthesis, but it has emerged as an intercellular phospholipid messenger with a wide variety of biological activities. Amongst others, LPA was found to induce actin cytoskeletal reorganization and cell shape changes thereby inducing smooth muscle contraction[140-142] and platelet aggregation[143]. In addition LPA promotes macrophage survival[140,144], stimulates growth of fibroblasts, vSMCs[145,146], endothelial cells and induces vSMC TF expression[147]. In the early phase of atherosclerosis LPA induces barrier dysfunction through stimulation of endothelial cell stress-fiber and gap formation and by increasing the expression of monocyte adhesion molecules[138], including E-selectin and VCAM-1, thus stimulated monocyte binding.

Not only is LPA an important constituent of mildly oxidized LDL, it also accumulates in the lipid-rich core of atherosclerotic plaques. LPA is the primary platelet-activating lipid of the plaque, which may after plaque rupture contribute to the increased risk of intra-arterial thrombus formation in late stages of atherosclerosis[148-149]. Two sources account for the accumulation of LPA in the lipid core of atheromas. Mildly oxidized LDL contains LPA and part of the LPA in the plaque is probably directly deposited through LDL that enters the arterial wall and undergoes oxidation. Additionally, LPA is likely to be synthesized *de novo* from its precursors by plaque macrophages and

smooth muscle cells[150]. LPA augments signal transduction via three LPA receptors, known as Endothelial Differentiation Gene-2, -4 and -7 and recently, PPARγ was recognized as an intracellular LPA receptor[151]. It is conceivable that modulation of the LPA content of the plaque will significantly affect plaque thombogenicity.

4. Atherothrombosis

Atherothrombosis, defined as the process of which atherosclerotic lesions develop a thrombus, is characterized by a ruptured atherosclerotic lesion containing a superimposed thrombus, which is the major cause of the acute coronary syndromes (e.g., MI, stroke, transient ischemic attack (TIA) or peripheral artery diseases) and death[152]. Atherosclerotic plaques contain a wide variety of thrombogenic and procoagulant factors such as TF, which enhance the risk of occluding thrombi after rupture. Apart from the aforementioned lipid factors, protein factors like fibrinogen, fibrin degradation products (FDP) and TF[153,154] are main compounds of the lipid core. The coagulation and fibrinolytic systems are complex and tightly regulated[155,156], making the identification of individual factors and its contribution to the risk of atherothrombosis in atherosclerotic lesions very difficult.

Nevertheless, a key role has been attributed to TF, which together with factor VIIa, induces the extrinsic coagulation cascade (Figure 5) resulting in thrombus formation. TF is normally inactive, however is released by endothelial cells after injury to induce wound healing. In atherosclerosis, plaque macrophages were seen to contain large amounts of active TF. T-lymphocytes induce TF production in macrophages via CD40/CD40 ligand (CD40L) tethering[157]. Oxidized LDL (oxLDL) has, in several papers, been shown to enhance TF expression in monocytes, macrophages, endothelial cells and vSMCs[158]. In particular, macrophage apoptosis will result in the release of residual TF-rich apoptotic bodies or microparticles, which are deposited in the plaque. These plaque microparticles can also serve as a major source of the levels of circulating TF that were found to be elevated in patients with acute coronary syndromes[60,159,160]. Interestingly, monocyte TF expression is also upregulated after association with platelets. The original hypothesis that TF is not able to activate platelets by itself, has recently been revised and the current notion is that TF can generate very small amounts of thrombin at the site of injury that, in turn, will activate platelets and subsequently trigger the coagulation cascade. To control the procoagulant effects of TF, Tissue Factor Pathway Inhibitors (TFPI-1 and to a lesser extent TFPI-2) are locally produced in the plaque and inhibition of TF by TFPI has been associated with a reduction of plaque thrombogenicity[161].

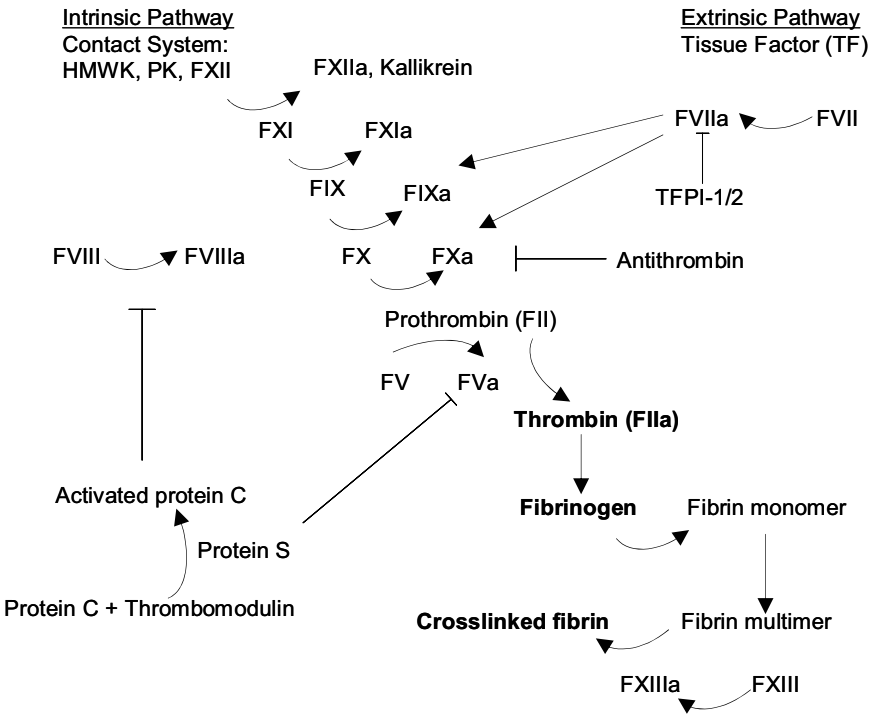

Figure 5. The coagulation cascade. Legend: HWMK = High molecular weight kininogen, PK = Prekallikrein, TFPI = Tissue factor pathway inhibitor. Black arrow = conversion/activation of factor. ⊤ = action of inhibitors. Curved arrows = reactions catalysed by activated factor.

Fibrinolysis is essential in the degradation of a blood clot and for restoring the blood flow[162]. The plasmin/plasminogen system, also known as the blood fibrinolytic system, comprises an inactive pro-enzyme plasminogen, which is converted by Plasminogen Activators to the active plasmin, which degrades fibrin into soluble FDPs. Two different Plasminogen Activators[163] have been identified: tissue-type Plasminogen Activator (tPA), which is primarily involved in the dissolution of fibrin in the circulation and urokinase type Plasminogen Activator (uPA), which binds to a specific receptor (uPAR) to activate cell-bound plasminogen. Next to its role in fibrinolysis, uPA is involved in pericellular matrix degradation via activation of growth factors and proteinases. The fibrinolytic activity is controlled by two dedicated Plasminogen Activator Inhibitors (i.e. PAI-1 and PAI-2). The PAIs belong to a major subgroup of protease inhibitors, the so-called serpins, which are single-chain proteins that act as irreversible covalent 'suicide' protease inhibitors. Elevated levels of PAI-1 were reported to be associated with atherosclerosis and an increased thrombotic tendency[164,165], while PAI-1 deficiency is accompanied by increased fibrinolysis and bleeding disorders[166,167]. Animal studies showed that PAI-1 did not affect *de novo*

atherogenesis in hypercholesterolemic mice[168], whereas other studies have demonstrated PAI-1 deficiency to be atheroprotective in early atherosclerosis[169] or to accelerate atherosclerotic plaque progression[170]. Conversely, mice deficient in plasminogen activators or plasminogen were generally more susceptible to inflammation or injury triggered thrombosis. Overexpression of uPA promoted neointima and aneurysm formation[171], which is probably due to increased plasmin levels and increased remodelling of the extracellular matrix in the vascular wall. Furthermore, tPA or uPA may contribute to the initiation of atherosclerosis by inducing P-selectin and platelet activating factor (PAF)[172] as well as to plaque rupture, either directly or indirectly, by activation of MMPs[173]. Plasmin, at least *in vitro*, is known to directly activate pro-MMPs-1 and -9[174] into their mature forms, resulting in increased matrix degradation.

Other plasma components have also been associated with an enhanced thrombotic risk. Lp(a) is a lipoprotein that structurally resembles LDL and consists of LDL covalently linked to apo(a). Lp(a) has pro-inflammatory properties as it increases expression of ICAM-1, VCAM-1 and E-selectin by endothelial cells[165]. This apo(a) is very homologous to plasminogen and may act prothrombotic by competing with plasminogen for fibrin binding sites and inhibit TFPI expression, conferring a prothrombotic status[176,177]. Increased plasma homocysteine concentration has been marked as prothrombogenic by interfering with the binding of tPA to its receptor and increasing PAI-1 expression by vSMCs and endothelial cells[178,179].

5. Research Tools for Therapy Development

Preclinical research on atherosclerosis largely depends on representative *in vitro* and *in vivo* models of atherosclerosis. *In vitro* models are supportive in that they allow studying single cell type responses to different atherogenic stimuli. However, it does not incorporate interaction between multiple cell types and only poorly mimics the complexity of the human atheroma. This pitfall is obviously circumvented when using an animal model of atherosclerosis. These models have been highly valuable in atherosclerosis research, as it gives information on the underlying mechanisms of in the development of atherosclerosis and is useful for the preclinical screening of therapeutic strategies. Results obtained in different animal models can not always be extrapolated to the human situation, but many *in vivo* studies have provided evidence for anti-atherogenic therapies. Numerous species have up to now been used to elucidate the mechanisms of atherosclerotic lesion development, such as non-human primates[26,27], swine[180], rabbits[181,182], rats and mice.

5.1 Mouse Models

Since the last two decades, the mouse has emerged as the model of choice in atherosclerosis research. The advantages of using mice are clear: they are small, relatively cheap and there are currently several transgenic and knockout mice available to study the role of single genes. For studies on atherosclerotic lesion development, conventional wild-type mice are not ideal, with their high resistance to atherogenic stimuli. The C57Bl/6 mouse may develop fatty streak like lesions in the aorta when fed a rather unphysiological high-cholesterol, cholate containing diet[183]. Transgene and knockout technology has enabled the generation of mouse strains that are prone to lesion development, the most important being the ApoE deficient ($ApoE^{-/-}$) [184,185], the ApoE*3-Leiden transgenic[186] and the LDL receptor deficient ($LDLr^{-/-}$) mouse[187]. While the latter two develop atherosclerosis when fed a high cholesterol diet, the $ApoE^{-/-}$ mouse develop plaques even when put on a chow diet. The $ApoE^{-/-}$ mice already suffer from hypercholesterolemia on chow diet, due to the lack of apolipoprotein E, which is required to clear lipoprotein particles from the plasma. These mice spontaneously develop large complex atherosclerotic plaques[188]. These lesions are characterized by foam cell formation, a smooth muscle cell cap, lipid accumulation, a high collagen content and the presence of a necrotic core. The LDL receptor deficient mice have elevated levels of total cholesterol, similar to humans with familial hypercholesterolemia having defective LDL receptors and develop macrophage-rich lesions upon feeding of a high cholesterol diet. Compared to $ApoE^{-/-}$ mice, $LDLr^{-/-}$ mice develop atherosclerotic lesions more slowly and lesions are less severe. In addition to these two knockout models, an ApoE*3-Leiden transgenic mouse has been developed as a model for familial dysbetalipoproteinemia. These mice express a dominant dysfunctional lipoprotein E*3-Leiden and these mice exhibit high levels of cholesterol, mainly in very low density lipoprotein (VLDL) and LDL, and high triglyceride levels, which results in initial and advanced atherosclerotic lesions in the sinus valves and the carotid arteries upon cholesterol feeding[186].

As lesion development in these atherosclerosis-prone mice is rather slow, manipulation strategies have been elaborated to speed up atherogenesis. Atherosclerosis was considerably accelerated after cuff placement at the femoral artery[189] or after application of a perivascular silastic collar at the carotid arteries of hypercholesterolemic mice (Figure 6)[190]. Wire injury in the carotid artery of high fat diet fed mice results in a neointima like lesion, as also in vein graft atherosclerosis and artery ligation models. When studying atherothrombosis in mice, we have to deal with the attendant fact that in mice true and spontaneous plaque rupture and subsequent thrombus formation has hardly ever been observed. Recently, Johnson *et al*. have thoroughly investigated the brachiocephalic artery of $ApoE^{-/-}$ mice for indications of plaque rupture[191].

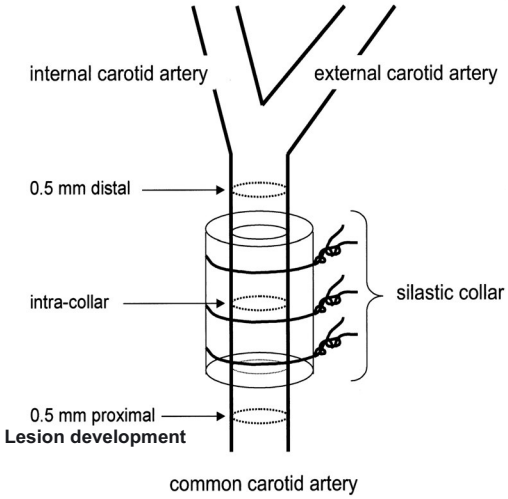

Figure 6. Schematic representation of the carotid collar model to induce atherosclerotic lesion development in hypercholesterolemic mice (adapted from von der Thüsen *et al*., *Circulation*, 2001)[190].

While they did observe mainly healed cap ruptures and intraplaque hemorrhage, no actual thrombotic occlusions were observed. Thus, the relevance of this model for plaque rupture research has been disputed. However, intraplaque hemorrhage is a phenomenon which is more often observed in mouse models as compared to plaque rupture[192,193] or thrombus formation and as described previously, intraplaque hemorrhage is deemed to be associated with plaque destabilization.

Manipulation of the atherosclerotic plaque to induce plaque rupture may increase the incidence of atherothrombosis. Indeed, von der Thüsen *et al*. have shown that adenoviral overexpression of the pro-apoptotic gene *p53* in collar-induced carotid artery lesions resulted in increased rate of plaque rupture in ApoE$^{-/-}$ mice[194]. Although this is a promising development in the investigation of the mechanism of plaque rupture, it has been criticized due to the low incidence of thrombotic events.

5.2 Gene Modulation Approaches

Next to transgenesis, gene modulation is a very important tool in gene function research. As described above, the use of knockout mice has provided loads of information on gene function, however local or cell-specific down-regulation of the target gene is sometimes preferred. Overexpression or downregulation of a target gene may reveal the actual function of a gene in disease mechanisms. Overexpression of a target gene can be reached either by transfection with naked DNA or by non-viral vector gene delivery,

which are generally rather inefficient, or by making use of viral vectors, which are more efficient in the delivery of target genes *in vitro* and *in vivo*. Adenoviruses[195], adeno-associated viruses[196], retroviruses[197] and lentiviruses[198] have most frequently been used for transient or long-term overexpression of target genes *in vivo*, while downregulation often is more informative in establishing gene function, however it is much more of a challenge.

Strategies for inhibition of gene expression have been known since over two decades. In 1978, an oligonucleotide sequence was utilized for sequence-specific interference with translation of the target gene[199]. So-called antisense oligodeoxynucleotides (as-ODNs) were used to manipulate gene expression, thereby identifying gene function[200,201]. It was readily acknowledged, that several backbone modifications had to be made to stabilize the ODNs without affecting its inhibitory activity and also efficient local intracellular delivery of the antisense molecules was shown crucial for effective downregulation. Unfortunately, the mode of action of as-ODNs appeared to vary depending upon the backbone of the ODN. For example, negatively charged ODNs, such as phosphodiesters and phosphorothioates, elicit RNAse H-mediated cleavage of the target mRNA.

A second technology to reduce gene expression is the application of ribozymes. Ribozymes are small RNA sequences with targeted endonuclease activity. They occur naturally, but can also be artificially engineered for specific genes[202,203]. In ribozymes, the catalytic domain is flanked at both sides with short sequences complementary to the target gene mRNA which are responsible for target-specific nuclease activity. Ribozyme sequences can be inserted in transcription vectors for prolonged activity, which is an advantage as compared to antisense sequences. A disadvantage of ribozymes is the limitation in the choice of target, as it was suggested that the cleavage site of the target gene requires a GUX triplet and successful application of ribozymes depends, similar as for as-ODNs technology, on the stability and efficient delivery of the ribozymes.

RNA Interference

RNA interference is a recently discovered, evolutionary conserved gene silencing mechanism in which small interfering RNA (siRNA) units repress the expression of genes carrying an identical sequence. SiRNAs, expressed in all eukaryotic cells, are thought to represent a cellular defence mechanism against bacterial or viral infection or genomic intruders like transposons[204,205]. The expression of many eukaryotic key genes in the cellular differentiation is regulated by small double-stranded RNAs. RNA interference (RNAi) was discovered by Lee *et al*.[206] and by Fire *et al.*[207] during research on the development of *Caenorhabditis Elegans*. Fire *et al*. found that sense RNA, used as a control during antisense oligonucleotide experiments, led to reduced gene expression. RNAi was first thought to be operational only in primitive organisms like *C. Elegans* and in *Drosophila*, but subsequent studies by Elbashir *et al*.[208] demonstrated that this mechanism

was also active in mammalian cells. Target gene expression was shown to be efficiently abolished by transfection of mammalian cells with 21-25 nucleotides long double-stranded RNA.
The advent of RNAi technology has instigated a true revolution in functional genomics and allowed the *in situ* knockdown of genes. Synthetic short double-strand DNA sequences of 21-25 nucleotides in length that were introduced into the host cell were reported to be efficient in inducing selective mRNA degradation and in suppressing gene expression[209]. Recently, Brummelkamp *et al.*[210] have generated a mammalian expression vector, encoding a short hairpin dsRNA (shRNA) transcript under the control of the RNA polymerase-III dependent H1 promoter for sustained silencing of a transgene.

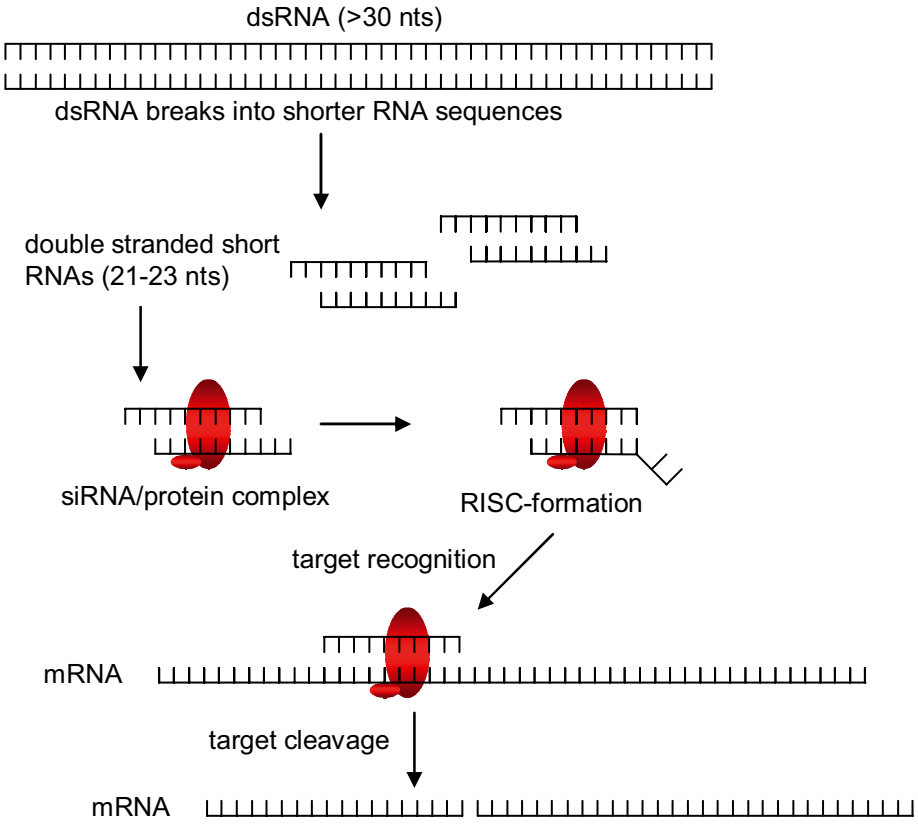

Figure 7. Proposed mechanism for RNA interference.

Gene silencing can be initiated by introduction of a synthetic siRNA or by transfection with a RNA polymerase III promoter containing vector that codes for a shRNA precursor with sequence homology to the target gene sequence. The precursors (either dsRNA or the shRNAs) are cleaved into

21-25 nucleotide long fragment siRNAs by a cytoplasmic RNase III-like enzyme called Dicer. The synthetically introduced siRNAs do not require processing by the Dicer complex, although it has been shown that siRNAs, which are not cleaved by Dicer, silence gene expression less efficiently. After cleavage by Dicer, one strand (the guide strand) will remain bound to the Dicer protein and will attract the Argonaute protein 2 to form the RNA-induced Silencing Complex (RISC, Figure 7). This complex binds the target mRNA sequence and will degrade the target gene mRNA. The RISC-entrapped guide strand is presumably protected to degradation and can therefore cleave many copies of target mRNA[206].

Delivery of siRNA into mammalian cells by means of transfection will result in transient expression of the siRNA, which can be desirable for treatment of acute diseases, e.g. viral infection. However, for more long-term silencing, viral expression vectors for shRNA are the more obvious choice. Currently, various expression vectors are available for the delivery of shRNAs and reports have described adeno- or adeno-associated virus (AAV) and retrovirus mediated shRNA delivery[211]. However, by far the most widely applied vector for shRNA transfer to mammalian cells is the lentiviral vector[212]. Now the safety of the 3rd generation vectors, which are self-inactivating and unable to replicate after infection, is warranted[213], the potential of lentivirus can be optimally exploited. Unlike conventional retroviruses, lentivirus is able to infect non-replicating, quiescent cells[214] without inducing differentiation of the host cells, which is especially important when inserting a shRNA into stem cells.

6. Study Aims

Plaque rupture and the subsequent thrombotic complications such as occlusion of an artery is the actual cause of death of atherosclerosis. We propose that atherosclerotic plaque stabilization and reduction of the thrombotic potential of plaque constituents could be very valuable in the treatment of atherothrombosis and in reducing the mortality rate of atherosclerotic complications. In this thesis, it was aimed to increase plaque stability and to reduce plaque thrombogenicity by means of matrix stabilization and by inhibition of apoptosis and inflammation. As a second objective, the identification a number of important new targets for future therapeutic intervention was intended.

7. Thesis Outline

Prevention of atherothrombotic complications as myocardial infarction or stroke due to occlusive thrombus formation after plaque rupture is likely to result in a decreased cardiovascular death rate in the Western Society. As treatment with anticoagulant drugs includes a risk of bleeding disorders,

improving plaque stability and reducing the thrombogenic potential of an atherosclerotic lesion could offer a suitable therapeutic alternative. In this thesis, it was aimed to shift atherosclerotic lesion development to a more stable phenotype, focussing on vascular wall constituents and factors involved in atherosclerotic lesion development and atherothrombosis.

In the first part of the thesis, the focus was on matrix stabilization and cellular lipid homeostasis. In Chapters 2 and 3, the potential of application of three viral proteinase inhibitors, Serp-1, Cytokine Response Modifier A (CrmA) and Serp-2 for stabilizing atherosclerotic plaques was studied. Serp-1 is a serine protease inhibitor similar to PAI-1 that may act by inhibiting the uPA/uPAR pathway. In this study, a continuous infusion was applied from the very start of lesion development in ApoE$^{-/-}$ mice. Moreover, the effect of Serp-1 infusion on morphology and stability of advanced plaques has been determined. In a subsequent study, the therapeutic effect of CrmA and Serp-2 was addressed, which are both viral so-called cross-class protease inhibitors that interact with cystein and serine proteases. Both inhibitors were known to inhibit Interleukin-1 Converting Enzyme (ICE), which is a key caspase in the control of inflammation and apoptosis. We evaluated the morphology of collar-induced atherosclerotic lesions in ApoE$^{-/-}$ mice after daily injections of CrmA and Serp-2, where special attention was given to effects on apoptosis.

In Chapter 4 the LPA homeostasis of carotid artery plaques of LDLr$^{-/-}$ mice was evaluated. As described in section 3.4., LPA is one of the primary platelet activating lipids and thus, at least in part, responsible for the thrombogenic activity of the lipid core. In humans, the abundant presence of LPA in carotid artery lesions has already been firmly established, however the actual mechanism of intimal LPA accumulation is largely unknown. The LPA content of advanced mouse lesions appeared to be very similar to that of human carotid artery specimens and these findings prompted us to further analyse LPA metabolism in mouse plaques. In this study we have determined the expression levels of key enzymes and proteins involved in LPA homeostasis.

The second part of this thesis will focus more on modulation of inflammation pathways and plaque stability. Immunosuppression may considerably influence atherosclerotic lesion development. Leukocytes and cytokines produced by these cells have been convincingly shown as key mediators in this process. In Chapter 5 we have applied immunosuppressive therapy by means of FK506 treatment in ApoE$^{-/-}$ mice equipped with perivascular carotid artery collars at different stages of lesion development.

In Chapter 6 we have studied an inflammatory cell type, the mast cell, which, in human samples of plaque rupture, has been shown to be abundantly present in the adventitia of affected arteries. A relationship between the mast cell content of the adventitia and the severity of disease was discovered, while it remained uncertain whether these mast cells causally contributed to or had been recruited in response to plaque rupture. In this study, we have attracted mast cells to the adventitia of carotid artery

lesions in ApoE$^{-/-}$ mice by an adapted sensitization/challenge protocol and evaluated its effect on plaque morphology.

Bone marrow transplantation has also proven its usefulness in atherosclerosis research[215-217]. Leukocytes play a significant role in the development of atherosclerotic lesions at all stages. By replacement of the bone marrow derived leukocytes from the recipient with knockout or transgenic donor cells, the contribution of a specific leukocyte gene to plaque development can be determined. For numerous leukocyte genes, a role in atherosclerosis has been successfully established by bone marrow transplantation. However, the generation of knockout mice is very laborious and even impossible when deletion of the target gene leads to embryonic lethality. Also, graft-versus-host responses seriously limit use of this powerful technique. In Chapter 7 a new experimental methodology is described to facilitate leukocyte gene function research by means of bone marrow transplantation. We show in this study that it is possible to generate knockdowns by transducing bone marrow cells with lentivirus containing a shRNA and subsequent transplantation into irradiated recipient mice. At 7 weeks after transplantation, the shRNA sequence was still found to be expressed in the hematopoietic cell lineage of the recipients, resulting in effective knockdown of the target gene.

Finally, Chapter 8 will provide a discussion of the most relevant findings of this thesis and offer an overview of future perspectives of these studies and their therapeutic implications.

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