Modulation of genes involved in inflammation and cell death in atherosclerosis-susceptible mice

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Chapter 1

General Introduction

Contents

Atherosclerosis

The main cause of cardiovascular disease (CVD) is atherosclerosis. Several risk factors associated with increased atherosclerosis have been identified, such as diabetes, lipid abnormalities, hypertension, cigarette smoking and physical inactivity. The cardiovascular burden has indeed been recognised as the tip of an iceberg, in which the immersed part is the preceding clustering of metabolic abnormalities. Atherosclerosis is a chronic inflammatory disease process affecting the vasculature, present at all ages, but develops in time[1]. It is a primary disease of the large and medium sized arteries and characterised by the focal accumulation of cells, fibrous tissue, lipids, debris and inflammatory blood constituents in the vessel wall, which result in narrowing of the lumen[2;3]. As age progresses, atherosclerosis can become clinically evident from the development of major complications, including pulmonary, myocardial or cerebral infarction and gangrene of the extremities. These complications account for up to 50% of all mortality in the USA, Europe and much of Asia[4;5].

Pathogenesis of atherosclerosis

Biomarkers

As previously mentioned several risk factors contribute to the susceptibility to atherosclerosis. One of the main risk factors of atherosclerosis is elevated plasma cholesterol and/or triglyceride levels. However, both cholesterol and triglycerides are important for many different cellular processes. Whereas cholesterol is important in the synthesis of membranes, steroid hormones and bile, triglycerides are the major energy source of the body. To distribute the lipids to the cells, they are transported in the circulation by lipoproteins. Lipoproteins consist of a hydrophobic core of neutral lipids (cholesteryl esters and triglycerides) surrounded by a monolayered shell of phospholipids, unesterified cholesterol and specific apolipoproteins. Through apolipoproteins lipoproteins can bind to cell surface receptors and facilitate lipoprotein metabolism. There are 5 major classes of lipoproteins, characterised by their size, density, electrophoretic mobility, lipid content and apolipoprotein composition: Chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high density lipoprotein (HDL)[6].

Atherosclerosis is associated with high plasma LDL[7] and low HDL levels. These are the classical markers in the risk assessment and prediction for atherosclerotic complications. It is suggested that although the inflammatory process in the arterial wall may be triggered in part by hyperlipidemia, there are other independent factors that drive the inflammatory process and contribute to the progression of the disease and its complications. In several patient studies elevated concentrations of plasma proteins C-reactive protein (CRP)[7],

soluble CD40 Ligand[8;9], soluble Tumor Necrosis Factor α (TNFα)[10], soluble TNF-Receptor 2[11], fibrinogen[12], serum amyloid A (SAA)[13] and von Willebrand Factor (vWF)[14] were proven to be independent risk markers in the prediction for cardiovascular events.

Atherosclerotic Plaque Formation

In hypercholesterolemic patients LDL can infiltrate the arterial intima. The area of deposition of LDL is determined by local hemodynamic flow patterns or "shear stress" of the vessel wall. More specifically, places with low average shear stress and high oscillatory shear stress show deposition[15]. Most commonly high density atherosclerotic lesions are observed in the aortic arch and bifurcations of the aortic arch, the femoral and carotid artery and the coronary artery[16]. The retention of LDL and its modification through oxidation or enzymatic attack causes focal activation of the endothelium and an inflammatory response in the arterial wall (Figure 1)[17;18]. This is the so-called response-to-injury theory[19]. Endothelial cells can be exposed to many forms of injury, including infectious, immunological, chemical, radiation and mechanical that has an impact on their cellular structure and function. As a result, markers such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and selectines are expressed, that cause homing of inflammatory cells to the site of retained and modified lipids[20-22]. Monocytes start rolling on the endothelium leading to their attachment and infiltration into the intima[1;23]. In the inflamed intima these monocytes are stimulated by macrophage colony-stimulating factor to differentiate into macrophages, which scavenge the modified LDL, accumulate the lipid and become foam cells. This stage is already called a fatty streak[24]. Depending on the balance of proatherogenic and antiatherogenic factors, fatty streaks may progress to advanced lesions[25] and others may regress. As a result of proatherogenic micro-environmental stimuli, macrophages cause inflammation and tissue damage by releasing cytokines, chemokines, proteases, oxygen and nitrogen radicals and other inflammatory molecules[26-28]. These molecules stimulate migration of fibroproliferative vascular smooth muscle cells (SMCs), derived from the underlying media or circulating progenitor cells, to the endothelium to form a protective fibrous cap[1]. Further progression of the plaque includes the accumulation of foam cells and the formation of a lipid core. Although not required for atherogenesis, other immunocellular components present in the advanced atherosclerotic plaque, despite their relative low numbers, are able to modulate the progression of the disease. Regulatory T and B-cells, mast cells, natural killer cells, neutrophils and dendritic cells play a role in the modulation of the response of SMCs and macrophage foam cells to the retained and modified lipids and drive the chronic inflammation that characterises the atherosclerotic disease[29]. Macrophage death by apoptosis or necrosis contributes to the formation of a necrotic core.

Macrophage death can be a consequence of cholesterol-toxicity, oxidative stress, inflammatory cytokines and growth factor depletion and results in extracellular lipid accumulation in the form of free cholesterol crystals[30]. Late events in plaque formation include cholesterol cleft formation and calcification. The progression to an advanced atherosclerotic plaque results in a complicated micro-environment involving an array of cell types and interactions (Figure 1)[31].

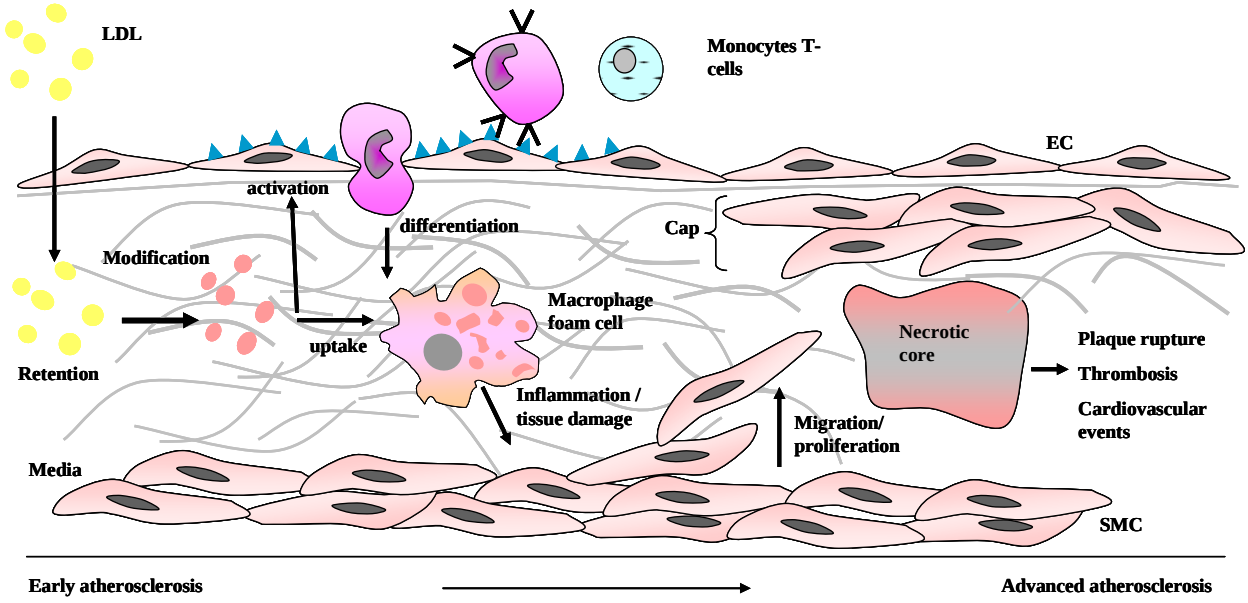

Figure 1. Schematic overview of atherogenesis from early to advanced atherosclerotic lesion formation. Adapted from Staels[32] and Hansson[23].

Atherosclerotic Plaque Vulnerability and Rupture

The cellular composition of atherosclerotic lesions is an important determinant for lesion stability. A stable lesion is rich in SMCs and has a thick fibrous cap. The macrophage-rich core is small. Instable lesions have a thin, collagen-poor cap and a large core enriched in inflammatory infiltrates with macrophages and lymphocytes, extra-cellular lipid and debris[33]. Several cellular processes can influence the plaque composition. Depending on the balance of ongoing processes advanced lesions have several potential fates, influenced by intrinsic and extrinsic mediators. Intrinsic mediators that can influence plaque composition and thereby stability and vulnerability are influx and efflux of cells and cholesterol, inflammation, migration, proliferation and cell death (via apoptosis or necrosis). Extrinsic mediators of plaque vulnerability are circumferential stress, hemodynamic shear stress, vasospasm, plaque fatigue and thrombosis or thrombolysis[34-37]. A positive balance ensures the progression of the plaque to a more stable, fibrotic or fibrocalcific phenotype. These plaques may or may not cause stenosis and stable angina.

A negative balance can lead to an unstable plaque phenotype. The morphologic outcome of unstable plaques is multidimensional, but these plaques are most prone to rupture.
Generally plaque rupture occurs at the shoulder area of the plaque. Here the concentration of macrophages is highest and the fibrous cap is weakest[38]. Macrophages weaken the fibrous cap in several ways: they secrete matrix metalloproteinases[39] (MMPs, like collagenases, elastases and stromelysins; MMP-1, 3, 9)[40;41] and cysteine proteases[42] (Cathepsins K and S)[43] that degrade the SMC-produced extra cellular matrix, they produce interferon γ (which can also be derived of T-lymphocytes), that retards SMC proliferation and inhibits the production of collagen by SMCs[44], and finally macrophages can induce SMC death. Once ruptured, contact of the necrotic core and/ or the exposed collagen and matrix promotes thrombosis by tissue factor expression[45]. This results in activation of the coagulation cascade, accumulation of platelets at the site of rupture, with the potential to form an occlusive thrombus, which is of greatest clinical significance. However, plaque rupture is not always a fatal event and does not always coincide with an occlusive thrombus. Ruptured plaques can be stenotic or not, with or without expansive remodeling. Non-ruptured plaques can show signs of erosion, calcified nodules, all with or without critical stenosis. Plaque rupture is even a proposed mechanism of plaque growth. This wide variety of culprit plaque phenotypes has lead to a listing of major criteria for the detection of a vulnerable plaque. Major criteria include: active inflammation, a thin cap with a large lipid core, endothelial denudation with superficial platelet aggregation, fissured or injured plaque and severe stenosis. Minor criteria are: superficial calcium nodules, intra plaque hemorrhage, endothelial dysfunction and expansive remodeling. Together with iron deposition and buried caps the last criteria are features that are associated with a vulnerable plaque but do not necesarily lead to rupture[35;46].

Cell death and inflammation in the atherosclerotic vessel wall

Progression of a lesion depends on the balance of proatherogenic and antiatherogenic factors. These factors determine lesion composition and thereby plaque stability and vulnerability to rupture. Since inflammation and cell death are thought to be important processes in the onset, progression and transition towards advanced and complex atherosclerotic lesions they are described more extensively below.

Cell Death

Cell death is a major mechanism to remove unwanted, aged, or damaged cells. Opposite to, but together with proliferation cell death ensures tissue homeostasis. Proliferation and cell death are linked in the cell cycle. Normally cells are quiescent, residing in the gap 0 phase (G0). Upon stimulation by for instance growth factors they enter the cell cycle at

gap phase 1 (G1). Thereafter, they go into the S phase followed by gap phase 2 (G2) and the M phase to close the cycle again at G1. In G1 the cell prepares for DNA synthesis in the S-phase, recruiting all necessary elements. After DNA synthesis, the G2 phase arranges everything for the cell to go into mitosis in the M phase. The G phases are the checkpoints of the cell cycle, in which is decided whether a cell is allowed to go into the next phase. If DNA is damaged or a phase was improperly finished, repair mechanisms are activated or in case of too much damage programmed cell death is induced[47;48].

Cells may die in various ways. Cell death can occur in a disorganized, energy independent manner associated with swelling and a final burst, spilling the cells contents, a mode known as necrosis. Apoptosis on the other hand, also known as programmed cell death, involves a highly ordered sequence of events. It is energy dependent, associated with cell shrinkage and involves features as nuclear condensation and fragmentation, membrane blebbing and formation of apoptotic bodies. Unlike necrotic cells, apoptotic cells and bodies usually retain an intact cellular membrane and are promptly removed by adjacent cells or tissue macrophages, avoiding damage to neighbouring tissue and inflammation. Apoptotic cell death applies to embryonic development, morphogenesis, adult tissue turnover and several pathological processes[49;50].

Atherosclerosis is such a pathological process, in which apoptosis occurs. It was found that apoptosis was increased in plaque compared with normal vessel and also the frequency of apoptosis was higher in ruptured than in stable plaques[51-53]. Apoptosis can occur in different stages of atherosclerosis. It can be involved in the iniation of atherosclerosis. Although endothelial cells are known to be resistant to apoptosis in some situations[54;55], apoptosis of the endothelium does occur. In early phases of atherosclerosis apoptotic cell death of recruited macrophages and lipid laden foam cells can even cause regression of the plaque. However, foam cell macrophages can be protected from apoptosis induced by ox-LDL via a scavenger receptor A related mechanism[50;56]. Alternatively, macrophages contribute to progression of atherosclerosis via apoptosis by the secretion of inflammatory cytokines such as TNFα, IFNγ and IL-1β, that are known for their apoptosis inducing and “priming” capacities with SMCs[57]. In advanced atherosclerosis apoptosis is involved by enlargement of the necrotic core and weakening of the fibrous cap by SMC loss.

Inflammation

Inflammation is the normal response of the body to protect tissues from infection, injury or disease. The inflammatory response, recruiting leukocytes to the site of inflammation, usually promotes healing. However, when uncontrolled, acute inflammation can become chronic. Atherosclerosis is a chronic disease, characterised by inflammation.

Inflammation plays a role in every stage of atherosclerotic lesion formation. At initiation the endothelium is activated, causing upregulation of inflammatory mediators such as adhesion molecules and chemoattractants[58-60]. SMC induce the expression of macrophage chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) that induce massive macrophage immigration[61]. The influx of inflammatory cells such as monocytes and T-lymphocytes increases with plaque progression, secreting inflammatory cytokines, chemokines and proteases[1;62;63]. It is assumed that proteolytic activity is driven by inflammatory activity, especially in the vulnerable shoulder areas of the plaque[64]. Macrophage foam cells enrich the highly inflammatory necrotic core. Macrophage-induced cell death can contribute to inflammation and necrotic core formation. Cell death in the form of necrosis is the major trigger for inflammation. The cell membrane bursts, spilling the highly inflammatory contents of the cell. The inflammatory reaction from necrotic cells may drive the atherosclerotic process leading to advanced lesion formation and increased vulnerability to rupture. On the other hand, cell death in the form of apoptosis is supposed to be a clean mechanism of cell removal. However, when apoptotic bodies are not readily cleared and reside in the tissue, they do harm neighbouring cells by exposure of membrane phosphatidylserines and loss of anticoagulant components. Apoptotic vascular SMCs even acquire a thrombin-generating capacity. Furthermore, apoptotic cells increase tissue factor on their cell surface[65-67]. In these ways apoptotic cells promote a procoagulant and proinflammatory environment. This highly inflammatory environment puts all lesional cells in a hyper-reactive highly-sensitized state, making the plaque more prone to rupture.

Genes involved in cell death and inflammation in atherosclerosis

Inflammation and cell death are important processes in the development and the transition towards advanced and complex atherosclerotic lesions. These processes take place during atherogenesis at different time points and as a result multiple genes are involved at different stages. This is supported by the presence of gene products involved in these processes in the advanced atherosclerotic plaque cells, including retinoblastoma (Rb), bax, c-myc, p21, p27, p53, Fas, Tumor Necrosis Factor alpha (TNFα) and Nuclear Factor-kappa B (NF-κB)[68;69]. In this thesis we investigated several key players in the abovementioned processes and they will be dicussed in their function and atherosclerotic environment in more detail below (Figure 2).

Tumor Necrosis Factor Alpha

As a member of the tumor necrosis factor (TNF) superfamily, tumor necrosis factor alpha (TNFα) is an inducer of apoptosis, a cytokine and central mediator of inflammatory reactions[70]. Next to macrophages of the atherosclerotic plaque, also visceral adipocytes can be a source of inflammatory cytokines, secreting TNFα and interleukin-6 (Il-6)[71]. In turn, these can stimulate hepatic production of CRP, which is a strong predictive marker for cardiovascular events and may directly influence the progression of vascular disease[72]. TNFα also reduces lipoprotein lipase activity, thereby inducing an atherogenic lipoprotein pattern[73]. Binding of TNFα to its receptors TNFR1 (p55) or TNFR2 (p75) induces a broad range of responses, including inflammation, differentiation, proliferation and cell death[74]. Secreted by SMCs, macrophages and T-lymphocytes, TNFα is generally considered highly atherogenic[57;75], although evidence of human and murine studies on early lesion development is equivocal on the role of TNFα in atherosclerosis[76-80].

Fas and Fas Ligand Death Receptor Couple

Fas (45kd) is one of the major apoptosis receptors, belonging to the tumor necrosis factor receptor (TNF-R) superfamily. When it binds to its couple Fas Ligand (FasL) (40kd), a cascade of events leads to the rapid induction of programmed cell death. This includes the clustering of receptors at the cell surface, the formation of a death inducing signaling complex (DISC) and the activation of several proteolytic caspases[81]. It is a very potent pathway and known to be involved in tissue homeostasis, the down-regulation of immune reactions and T-cell-mediated toxicity[82]. Beside this, FasL is known to have a gatekeeper function in immune privileged tissues[83]. This includes the vessel wall where endothelial cells express FasL to keep out inflammatory cells. All cell types in the atherosclerotic plaque express Fas[84;85]. SMCs, however, do not go into apoptosis until after "priming" by cytokines, as TNFα, Il-1 or IFNγ[86]. Interestingly, some cell types in the plaque can also express Fas Ligand, and may themselves become triggers for apoptosis[87;88].

Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) negatively interfere with inflammatory gene expression. Amongst other actions, they have been reported to lower CRP[89]. PPAR agonists mimic the structure and biological functions of free fatty acids and as a consequence bind to specific transcription factors, the PPARs, which are member of the nuclear hormone receptors. They are activated upon ligand binding in the cytoplasm and migrate to the nucleus to heterodimerize with retinoic acid X receptor (RXR). The heterodimer can bind specific PPAR response elements in the regulatory regions of several target genes. As a result, the expression of those genes may be activated or

repressed. There are 3 PPAR subtypes: PPARα, PPARγ and PPARβ/δ. PPARα is mostly expressed in liver and mainly involved in lipid metabolism. PPARγ is preferentially expressed in adipose tissue and involved in adipogenic differentiation and glucose homeostasis. Not so much is known about PPARβ/δ[90]. However, all PPARs are known to be expressed in cells of the atherosclerotic vessel wall[91]. PPAR agonists have shown to exert more effects that go beyond effects than can be attributed to lipid metabolism and glucohomeostasis. As yet they revolve around putative effects on endothelial function, inflammation, cell cycle, thrombosis, plaque stability, and immune regulation.

P53

P53 is a tumor suppressor gene and guardian of the cell cycle. The function of p53 is to keep the cell from progressing through the cell cycle if there is DNA damage. Either repair mechanisms are switched on or in case of too much damage cell death is induced. Defects in the cell cycle either by mutations in p53 or other key regulators may result in unlimited proliferation and tumorigenesis. P53 is a cytosolic protein, but can also act as a nuclear transcription factor, inducing the expression of genes involved in a range of processes. Downstream targets of p53 regulate processes like proliferation, cell death, differentiation and senescence[92;93]. The negative regulator of p53 is Mdm2. It is an ubiquitination enzyme, responsible for the breakdown of p53. P53 activity is regulated by phosphorylation, thereby p53 is released of Mdm2[94;95]. Histological evidence was found for the presence of p53 and Mdm2 in the plaque[96]. Knocking out p53 on a whole body level in apoE$^{-/-}$ mice aggravated atherosclerosis through an increase in p53-controlled proliferation[97]. ApoE3*Leiden mice show accelerated atherosclerosis after bone marrow transplantation with p53$^{-/-}$ bone marrow, as a result of enhanced macrophage accumulation[98]. In contrast, ectopic overexpression of p53 in the cap of plaques of apoE$^{-/-}$ mice rendered the plaque highly prone to rupture by inhibition of proliferation and increasing apoptosis of SMCs[99]. These studies show an important role for p53 in proliferation and cell death of the plaque. Depending on the affected cell type and location p53 can have detrimental effects on the stability of the plaque.

Nuclear Factor-kappa B

Nuclear factor-kappa B (NF-κB) is a central regulator in inflammation. It is involved in inflammation, proliferation and differentiation and can both protect against and contribute to apoptosis[100]. NF-κB can influence or be influenced by an array of genes, including the ones described above[101;102]. Furthermore, NF-κB is present in all atherosclerotic plaque cell types and can be involved in all stages of atherosclerotic lesion formation. It is suggested that shear stress may prime the vascular wall by inducing steady state levels of

NFκB. Later, on additional threat (oxidized LDL), NFκB is activated to mediate expression of adhesion molecules (ICAM, VCAM) and chemoattractants (MCP1). Activation of infiltrating monocytes by NFκB leads to the expression of inflammatory cytokines (TNFα) that can promote SMC proliferation[103;104]. NFκB can induce apoptosis via regulation of death receptors and ligands (FasL, TNF-Related Apoptosis Inducing Ligand (TRAIL), TNFα), upregulation of p53 expression, but also prevent apoptosis via cellular inhibitors of apoptosis (FLIP)[102;105].

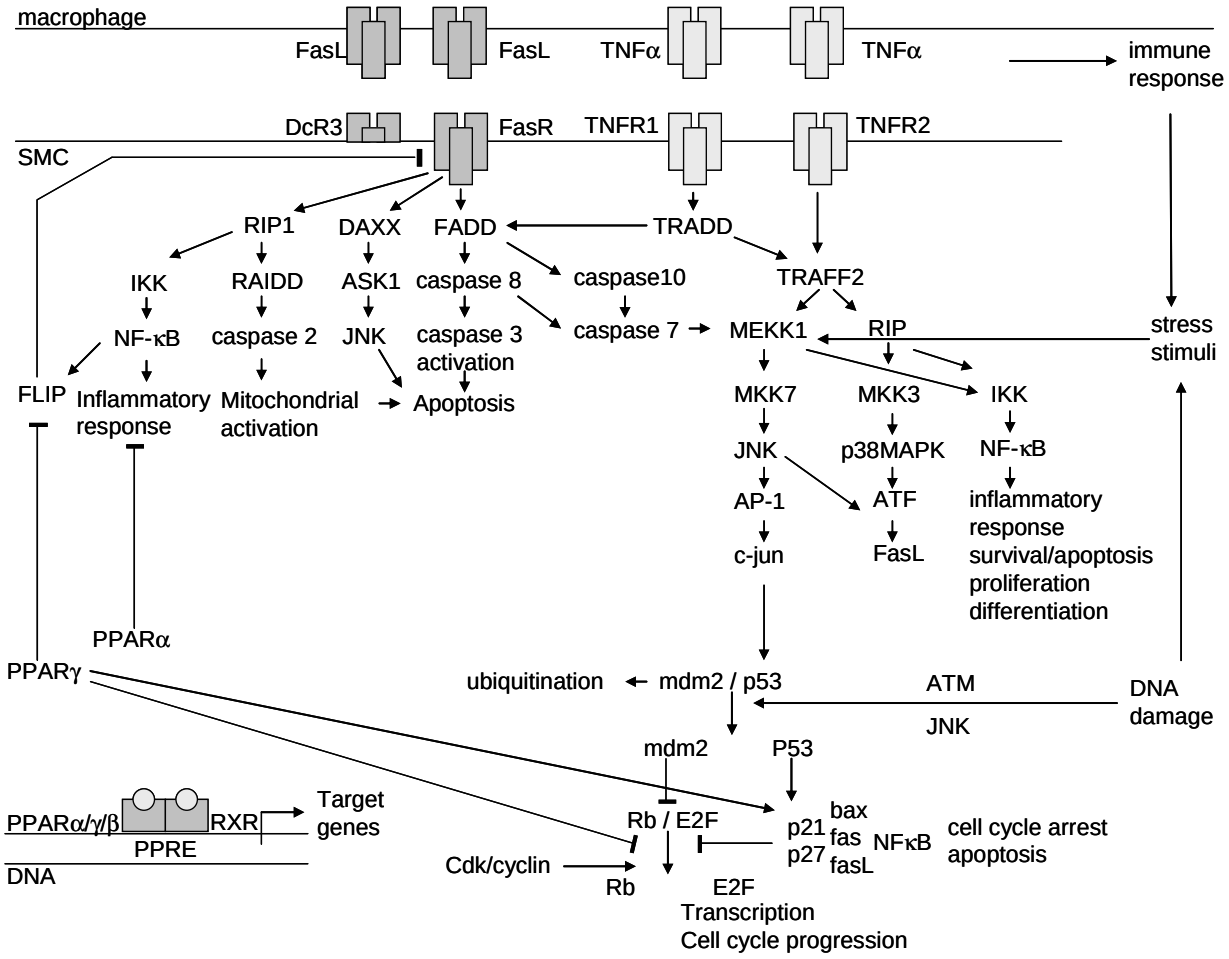

Figure 2. Schematic overview of genes involved in cell death and inflammation in atherosclerosis

Treatment of atherosclerosis

Surgical Intervention

At the end stage of atherosclerosis, after occlusion of the vessel, tissue can be rescued from ischemia and death by arterial bypassing or percutaneous transluminal coronary angioplasty (PTCA). Although the initial success of these interventions is high, the major drawbacks are the occurrence of restenosis and thrombosis. Restenosis is characterised by excessive proliferation of SMCs after intervention. Often the consequence of these

processes is renewed symptoms and the need for repeated intervention in up to 50% of patients[106;107]. Nowadays PTCA can be combined with stent placement. Bare metal stent placement can reduce restenosis, however, is accompanied by in-stent restenosis in 20-30% of patients within 6-9 months[108;109]. Stent placement is also still accompanied by the risk of thrombosis and is therefore combined with aspirin and anti-platelet treatment. This led to the development of state-of-the-art stents that have a polymer coating, releasing agents against restenosis. Ongoing clinical trials use drug eluting stents that can release anti-proliferating agents, such as rapamycin or paclitaxel. These stents narrowed the window of reblockage and retreatment to 1-3% at one year[110;111]. Investigation of other drug eluting stents containing anti-inflammatory agents (dexamethasone, aspirin) or coated stents with anti-coagulants (heparin) is still preliminary[112]. The long-term effects of these coated stents on the underlying atherosclerotic plaque are not known yet[113].

Pharmacological Intervention

Up to now there is no cure for atherosclerosis or its sequelae. Dietary intervention like energy restriction and low-fat diets have shown to be effective in weight reduction, and cholesterol reduction. Often this type of life style modification is hard to comply with and then pharmacologic therapy should be considered. Preventing treatments are aimed at the primary risk factors. Treatment is mainly aimed at improving hypertension and lowering cholesterol. Hypertension can be improved by anti-hypertensive agents such as angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, calcium antagonists or anti-coagulatory drugs[114-117].

There are many ways to lower cholesterol. Bile acid sequestrants (cholestyramines) induce an increase in hepatic bile acid production from cholesterol. Thereby intrahepatic cholesterol is decreased, resulting in increased clearance of LDL and VLDL from the plasma[118]. Ezetimibe is an example of a cholesterol absorption inhibitor at the level of the small intestine. State of the art compounds that lower plasma cholesterol are statines and fibrates.

Statins inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyses the rate-limiting step in cholesterol biosynthesis. Hereby, sterol regulatory element binding proteins (SREBPs) are activated and consequently the LDL-receptor is upregulated in the liver, resulting in enhanced LDL clearance from the plasma[119]. Prava- and lovastatin are produced from fungal metabolites, whereas other statins such as simva-, fluva-, atorva-, and rosuvastatin are (semi-)synthetic. Prava-, and rosuvastatin are hydrophilic compounds; the other statins are highly lipophilic[120]. Fibrates mimic the structure and biological functions of free fatty acids. As a result they bind to specific transcription factors, the PPARs. Fibrates bind PPARα, which is highly expressed in liver

and muscle[121]. Thereby, fibrates improve the atherogenic lipoprotein profile by decreasing triglyceride levels, raising HDL cholesterol and favourably modifying LDL particle size and density[122]. Additional to their cholesterol lowering capacities, both statins and fibrates are thought to exert "pleiotropic" effects. These are suggested to be associated with their anti-inflammatory actions. Anti-inflammatory actions of both agents include reducing adhesion of leukocytes, proliferation of macrophages, secretion of MMPs, tissue factor procoagulant gene expression and lowering CRP[123-126]. Furthermore, PPARα agonism interferes with the activation of NFκB, resulting from competition for co-activators, and thereby exerts this anti-inflammatory action[127]. Lipid modifying interventions can reduce inflammation as measured by CRP levels[128]. However, currently no direct treatment for the underlying chronic inflammation of atherosclerosis exists.

Mouse models to study atherosclerosis

Mouse Models

Clinical investigations, population studies and cell culture experiments have provided important clues to the pathogenesis of vascular disease. Experiments, in which interactions between cells and tissues on one side and haemodynamic and immunologic influences on the other side are expected, need to be performed in for instance mouse models *in vivo*. In contrast to humans, mice have a well-defined genetic background and environmental factors are easily controlled. They are easy to breed and can be modified genetically. However, atherosclerosis does not develop in mice under normal conditions. For example even the wild-type C57Bl/6 mice, that are most prone to the development of atherosclerosis, need to be challenged with a severe atherogenic diet containing cholate to develop only fatty streaks after several months[129]. Genetically modified mice in which specific genes are knocked out or overexpressed have been generated, which are more suitable to study hyperlipidemia and atherosclerosis[130-132].

Atherosclerotic Mouse Models

When lipoprotein metabolism is affected by targeted deletion of the apoE gene (apoE$^{-/-}$ mice) or deletion of the LDL receptor (LDLR$^{-/-}$ mice) mice develop hypercholesterolemia and apoE$^{-/-}$ mice even develop spontaneous atherosclerosis[133-135]. The LDLR$^{-/-}$ and the apoE$^{-/-}$ are the most commonly used mice in vascular research. However, the LDLR$^{-/-}$ mouse is a more moderate model than the apoE$^{-/-}$ when cholesterol levels (12 vs 15-20mM) and severity of hyperlipidemia and atherosclerosis are considered[136]. Another drawback of the apoE$^{-/-}$ mouse is that it lacks apoE, which has been shown to be involved in atherogenesis by mediating cholesterol efflux from foam cells in the atherosclerotic vessel wall[137].

Humanized Atherosclerotic Mouse Models

An important difference between mice and men is the lipoprotein distribution. In mice, the main lipoprotein class in plasma is the anti-atherogenic HDL, whereas in humans the predominant lipoproteins are VLDL and LDL. Therefore, a mouse model with a more human-like lipoprotein profile was developed: the apoE*3-Leiden mouse. Unlike the previous mentioned mice, lipoprotein metabolism is not blocked, but impaired by the E*3Leiden mutation. The apoE*3Leiden mutation is a mutant form of apoE, characterised by a 7-amino acid tandem repeat of residues 120-126 and yields a mature protein of 306 amino acid residues[138;139]. Single allelic mutation yields elevated plasma triglycerides and cholesterol (10-12mM) mainly confined to the VLDL and LDL lipoprotein fractions. Thereby, the lipoprotein profiles show close resemblance to that of humans[140;141]. In apoE*3Leiden mice plasma cholesterol levels can easily be titrated by adjusting the dietary cholesterol intake. These mice show a clear releationship between aortic lesion size and plasma cholesterol exposure[142]. ApoE*3Leiden mice have shown to be responsive to anti-hyperlipidemic treatment using several (pharmacological) compounds, such as statins and fibrates[143], but also fish oil[144] and stanol esters[145;146]. Therefore this established model for hyperlipidemia and atherosclerosis is also a suitable model for the investigation of anti-atherosclerotic and pleiotropic properties of hypolipidemic drugs[147].

Accelerated Atherosclerotic Mouse Models

Places where atherosclerosis is measured in mice can vary. Spontaneous atherosclerosis is mostly assessed in cross sections of the aortic valve area or by en face measurements of the aortic arch and descending aorta. These studies are time consuming. Recently the bracchiocephalic artery is also used, however it is more studied as a vessel, in which features of the vulnerable plaque are observed[148]. Since these two arteries are not very accessible for mechanical modulation to study the effect of local modulation of genes in atherosclerosis, several other models of accelerated atherosclerosis were developed. One example is the carotid artery using a perivascular silastic collar in combination with a high fat/ high cholesterol diet to induce plaque formation[149]. The other is in the femoral artery using a polyethylene cuff to induce neointima formation in combination with a chow diet or accelerated atherosclerosis with a high fat/ high cholesterol diet[150] (Figure 3).

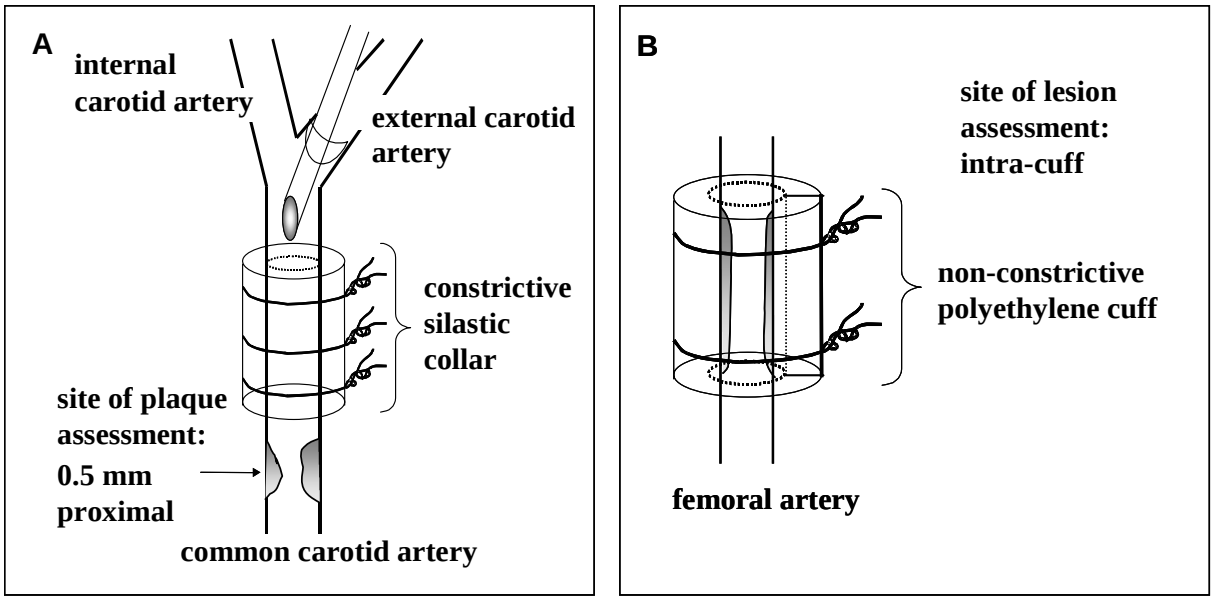

Figure 3. Examples of models of accelerated atherosclerotic lesion formation. A, carotid artery. B, Femoral artery. Adapted from von der Thüsen[149] and Quax.

Systems for modulation of inflammation and cell death

There are several ways to find out the effect of a particular gene. Most commonly the effects are explored via loss or gain of function studies i.e. via downregulation or overexpression of a gene. Standard gene knock-out and transgenic animal models have been highly informative. However, early embryonic lethality or complex phenotypes often obscure the roles of subject genes at later stages of development or in specific tissues.

Viruses

To overcome these problems adenoviruses can be used to temporarily overexpress a gene at the desired point in time. Opposed to retroviruses, they can transfect dividing as well as non-dividing cells. Gene expression from adenoviruses fades out after 2-3 weeks[151;152]. However, sometimes the long-term effects of a gene need to be studied and then lentiviruses may be a solution. Lentiviruses give stable expression of your gene of interest, since the information is integrated into the DNA[153]. A disadvantage of these procedures is that they are not local and not tissue specific.

Conditional Gene Targeting

To gain precise information about the role of a gene in a specific cell type at a critical stage of disease or development, a more sophisticated approach is required that allows for flexible control of gene expression. Conditional gene targeting may provide a means to circumvent certain limitations of conventional gene targeting. Examples are the site-specific recombinase (SSR) systems Cre-*loxP*, Flp-*FRT* and ΦC31-*att*[154]. Using

conditional targeting genes can be switched "on" and "off". In this thesis we used the Cre/loxP system to delete genes[155;156]. To add temporal control to the SSR activity we used a mutant estrogen receptor ligand binding domain fused to the Cre recombinase, which is responsive to the ligand tamoxifen or its metabolite 4-hydroxytamoxifen[157-160]. Cell-type specificity can be achieved by choosing a cell-type-specific promotor in front of the Cre recombinase. Upon systemical or local application of the ligand, this will bind the ligand binding domain (LBD) attached to the Cre recombinase, that is normally transcribed and residing in the cytoplasm, but is then targeted to the nucleus. Cre recombines DNA at specific target sites, termed *lox*P, integrated in the DNA around the gene of interest. By assuring the relative orientation of the *lox*P sites is directly repeated, the DNA will be excised. The order of events follow strand cleavage, excision and thereafter ligation[161;162]. The excision reaction is effectively irreversible, due to loss of the excised DNA, and therefore the gene is permanently "knocked-out".

Pharmacological or Dietary Supplements

Another way to target genes is via pharmacological treatment or dietary supplements. Pharmacological agents that have a well-known anti-atherosclerotic effect are lipid lowering agents such as statins and fibrates[163-166]. Anti-cancer therapeutics such as rapamycin and paclitaxel, are anti-proliferative agents that inhibit restenosis[167]. Examples of dietary supplements can be the stanol esters and vitamin E[168;169]. However, again this is not local and not cell-type specific, when pathological processes such as atherosclerosis occur in highly localized regions of the vasculature. To limit side-effects a restriction to the area that is gene targeted is required.

Local Gene Targeting

Several ways to target more local have been invented, although not yet directly applicable in the clinic, they rather facilitate studies in laboratory animals. Adenoviruses can be specifically targeted to an atherosclerotic plaque developed in the carotid artery by temporal ligation and local incubation[170]. To gain tissue specificity, recent developments show that adenoviruses can be modified and targeted to certain tissues by adapting the fibre knobs[171-173]. Certain areas can be incubated with pluronic or agar gels releasing compounds locally[174]. Drug releasing polymers may allow the same (chitosan)[175;176].

Outline of this thesis

In this thesis we focus on atherosclerosis as the main cause of cardiovascular disease. Since inflammation and cell death are important processes in the onset and progression of atherosclerosis, we investigate the role of several genes involved in inflammation and cell

death in the vessel wall and their effect on atherosclerosis. We use several ways to modulate gene expression in various atherosclerosis-susceptible mice.

Since inflammation and cell death are two important processes in the transition towards advanced and complex atherosclerotic lesions, we investigate the involvement of two genes of the tumor necrosis factor (TNF) superfamily. The first gene of our focus is TNFα, an inducer of apoptosis and a central inflammatory cytokine. Although TNFα and its receptors are thought to be of considerable importance in a number of biological activities relevant to atherosclerosis, its function in atherogenesis remains unclear. Opposed to previous studies on the role of TNFα in early lesion development, **chapter 2** discusses the role of TNFα under conditions of advanced lesion formation. To this end TNFα is deleted on whole body level in ApoE*3Leiden mice and we evaluate the effect on advanced lesion formation and lesion composition.

Chapter 3 describes the experiment performed to study Fas Ligand, which is another member of the TNF superfamily, in atherosclerosis. Fas Ligand is the major trigger for apoptotic cell death. In the advanced atherosclerotic plaque, cells are in a hyper-reactive highly-sensitized state. The presence of FasL and its receptor Fas in human atherosclerotic plaques, as well as the fact that macrophages can induce apoptosis in SMCs by Fas/FasL interactions *in vitro*, have fuelled speculation about the role of the Fas/FasL pathway of apoptosis in lesion remodelling and plaque vulnerability. In the present study, we investigate whether local overexpression of FasL in caps of pre-existing atherosclerotic lesions of apoE$^{-/-}$ mice can induce lesion remodelling and rupture-related events. We assess the effects on plaque morphology and composition in a time-course of FasL expression.

Since SMCs are the only cells producing the structurally important collagen, weakening them under the influence of inflammatory cytokines and rendering them more susceptible to cell death may lead to plaque destabilization and remodelling towards a more vulnerable phenotype. The guardian of cell cycle, the tumor suppressor gene p53, is tightly negatively regulated by mdm2. Thus far, this potent inducer of proliferation and cell death is known to be upregulated only under stress conditions and in homeostatic tissues. Therefore, **chapter 4** first focuses on whether tight surveillance of p53 activity is also required in terminally differentiated non-stressed smooth muscle cells. To improve our targeting we aim at using a temporal and conditional model. Via tamoxifen administration and the SM22CreER^{T2}(ki) model we induce the systemic and SMC specific deletion of Mdm2, consequently upregulating p53.

Often pathological processes such as atherosclerosis and restenosis occur in highly localized regions of the vasculature. To avoid systemical side-effects a restriction to the area that is conditionally gene targeted is required. To this end, **chapter 5** discusses the

use of a polymer drug eluting device (PDD) for 4-hydroxytamoxifen (4-OHT) administration in combination with the conditional SM22CreER^{T2}(ki) model. To characterise the model we use the reporter ROSA26 mouse line. To optimize gene recombination we use a dose and time curve. Furthermore, we determine the localization and specificity of the induced recombination. Finally, we compare the vascular SMC recombination levels achieved with the unique 4-OHT-eluting PDD to the recombination levels with systemic tamoxifen administration.

In **chapter 6** we take a different approach and use a pharmacological compound to stimulate the PPARs and modulate their target genes. Tesaglitazar is a potent dual PPARα/γ agonist which has shown positive effects on the plasma glucose and lipid derangements in animal models of type 2 diabetes and the metabolic syndrome. Based on their effects in animal models it has been proposed, that combined PPARα/γ agonists may have additional benefits in reducing components of insulin resistance contributing to atherosclerosis and thereby to cardiovascular disease. We therefore investigate the anti-atherosclerotic effect of tesaglitazar both under normal and mild insulin-resistant conditions in the atherosclerosis-susceptible apoE*3Leiden mouse, beyond its total plasma cholesterol lowering effect. We determine the atheroprotective effects of tesaglitazar from plaque composition and several inflammation parameters.

The results obtained in these studies and future perspectives are discussed in **chapter 7**.

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