Cell cycle and apoptosis genes in atherosclerosis

Lianne S.M. Boesten

2006

Cell cycle and apoptosis genes in atherosclerosis

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit van Leiden,
op gezag van de Rector Magnificus Dr.D.D. Breimer,
hoogleraar in de faculteit der Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens beluit van het College voor Promoties
te verdedigen op woensdag 1 maart 2006
klokke 14.15

door

Lianne Simone Mirjam Boesten

geboren te Tilburg in 1979

Promotiecommissie

Promotor:	Prof. Dr. L.M. Havekes
Co-promotores:	Dr. B.J.M. van Vlijmen Dr. M.P.J. de Winther, Universiteit Maastricht
Referent:	Prof. Dr. M.H. Hofker, Universiteit Maastricht
Overige leden:	Prof.Dr.A. v.d. Laarse Prof.Dr.A.C. Gittenberger-de Groot Dr.T. Kooistra,TNO-Kwaliteit van Leven, Leiden

ISBN: 90-8559-104-X

The study described in this thesis was supported by a grant of the Netherlands Heart Foundation (NHF-2000.051). The studies were performed at the Gaubius Laboratories of TNO Quality of Life and the Leiden University Medical Center, Leiden,The Netherlands.

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

The printing of this thesis was financially supported by the Dutch Atherosclerosis Society (DAS), TNO-Quality of Life, Leiden, The Netherlands and AstraZeneca, Sweden.

Cover: Paper spiral, photograph from Mauro Bighin, Padova, Italy

Print: Optima Grafische Communicatie B.V., Rotterdam

Let, als je naar een doel reist, goed op de weg.
De weg leert je de beste manier om er te komen
en verrijkt ons terwijl we hem bewandelen.

Paulo Coelho

Aan mijn ouders

Voor Michel

TABLE OF CONTENTS

Chapter 1

General Introduction

Cardiovascular diseases (CVD) have long been the leading cause of mortality and disability in developed countries, and it is rapidly becoming the number one killer in developing countries.[1] Worldwide 16.7 million people die from CVD each year. The primary cause of CVD is atherosclerosis, which is a multi-factorial disorder occurring in the large and medium-sized arteries of the body. Atherosclerosis is a complex disease that starts in childhood and progresses throughout life. Major risk factors for developing atherosclerosis are high blood pressure, high blood cholesterol, smoking, diabetes, and inherited genetic disposition. Although scientific advances in basic, clinical, and population research have been phenomenal, the complications of atherosclerosis such as myocardial infarction, stroke and peripheral vascular disease still makes it a prevailing disease in the western society. In the beginning 90s promising lipid lowering therapies predicted a strong reduction in cardiovascular deaths for the upcoming years, however in westernized societies it still accounts for 40% of the total number of annual deaths, indicating that treatment of atherosclerosis goes beyond lipid lowering solely. In addition to lipid accumulation, continuous cell proliferation (cell cycle) and cell death (apoptosis) processes are thought to play a central role in the development of atherosclerotic lesions. This chapter describes the general aspects of the development of atherosclerosis, discusses the role of cell cycle and apoptosis genes in atherosclerosis development in greater detail, and concludes with the outline of the thesis.

THE PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis comes from the Greek words athero (meaning gruel or paste) and sclerosis (hardness). Although the knowledge on atherosclerosis has expanded extremely the past couple of decades, the exact mechanism of initiating events is still unclear. Generally atherosclerosis can be considered as a form of chronic inflammation resulting from interaction between modified lipoproteins, monocyte-derived macrophages, T-cells, and the normal cellular elements of the arterial wall. This inflammatory process ultimately leads to the development of complex atherosclerotic lesions.[2]

Elevated plasma cholesterol levels are unique in being sufficient to drive the development of atherosclerosis even in the absence of other known risk factors.[3] Cholesterol together with triglycerides are the most important lipids in the circulation and are indispensable to various cellular processes. Cholesterol is necessary for the synthesis of cellular membranes, steroid-hormones and bile, whereas triglycerides function as a major energy source for the body. Cholesterol and triglycerides are lipophylic and are therefore transported in water-soluble lipoprotein particles. These lipoproteins are divided into different groups according to their density and size: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Chylomicrons are responsible for transport of dietary lipids, on the other hand, VLDL, LDL and HDL function to transport endogenous lipids. Increased LDL and VLDL cholesterol levels are associated with increased risk of cardiovascular disease.[4,5] These increased levels of plasma LDL and VLDL result in retention of the lipoproteins in the vascular wall, where they get modified (i.e. oxidation, proteolysis and aggregation).[4] Retention of the modified

lipoproteins leads to activation of the endothelium and expression of adhesion molecules. Following, monocytes from the circulation are recruited and adhere to the injured vessel wall where they subendothelially differentiate into macrophages due to the presence of various growth factors and cytokines (including interleukin-1 (Il-1), tumor necrosis factor-α (TNFα), and interferon-γ (IFNγ)). Subsequently, the macrophages become lipid-laden foam cells due to the continuous uptake of modified lipoproteins. The presence of foam cells in the vascular wall is called the fatty streak, the first occurrence of atherosclerosis.[6]

Although the recruitment of monocytes to the vascular wall and their differentiation into macrophages may initially serve as a protecting factor, progressive accumulation of these macrophages and subsequent production of cytokines, chemokines, and metalloproteinases and the continuous presence of modified lipoproteins results in the formation of more complicated atherosclerotic lesions.[3,7] Transition of the fatty streak towards the advanced atherosclerotic lesion is characterized by migration of smooth muscle cells (SMCs) from the medial layer of the artery into the intimal or subendothelial layers. SMCs can proliferate and take up modified lipoproteins contributing to foam cell formation. More importantly, SMCs synthesize extracellular matrix proteins that lead to the formation of a fibrous cap. The lesion continues to grow by the entrance of new mononuclear cells from the blood, which enter at the shoulders of the lesion. This is accompanied by cell proliferation, extracellular matrix production and the accumulation of extracellular lipid.[4] Gradually, the lesion develops towards an atheromatous or fibrofatty plaque[6] in which the fibrous cap overlies a pool of smooth muscle cells, lipid-laden macrophages, T-lymphocytes, necrotic debris and cholesterol crystals (Figure 1). Although advanced atherosclerotic lesions can grow sufficiently large to block blood flow, the most important clinical complication is the formation of a so-called vulnerable atherosclerotic lesion with an occluding thrombus, resulting in acute ischemia. The clinical outcome of acute ischemia is dependent on the site of the thrombus in the body and can be for example gangrene of the limbs, myocardial infarction or stroke.[4]

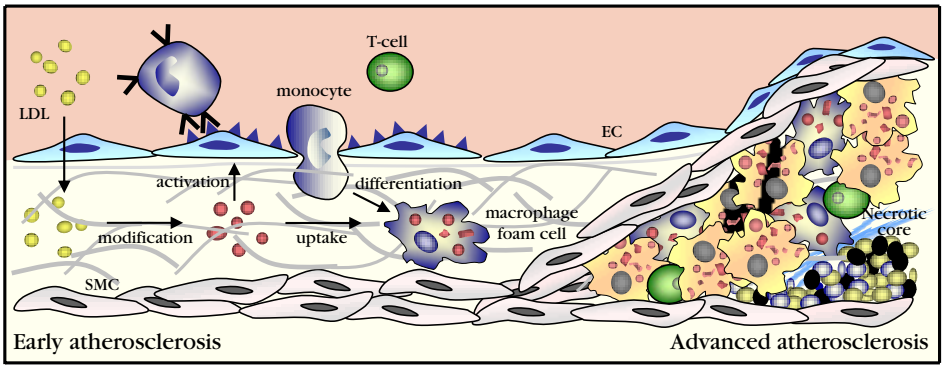


Figure 1. Atherosclerotic lesion formation from early to advanced atherosclerosis. Indicated are adhesion, migration, uptake of modified LDL and differentiation of monocytes to macrophage foam cells. Smooth muscle cells migrate and proliferate to form a fibrous cap, overlying a pool of lipid-laden macrophages, T-cells, necrosis and cholesterol crystals. SMC, smooth muscle cell; EC, endothelial cell; LDL, low density lipoprotein.

THE VULNERABLE ATHEROSCLEROTIC LESION

Every year over 19 million people worldwide are diagnosed with acute coronary syndromes (including: unstable angina, acute myocardial infarction, or sudden coronary death).[1] Most acute coronary syndromes are the consequence of the formation of an occluding thrombus at the site of the atherosclerotic lesion, which can arise from three different atherosclerotic lesion morphologies (rupture, erosion, and calcified nodules). Atherosclerotic lesion rupture is the most common type of complication, accounting for 60-75% of all cases. Pathological analysis of these ruptured lesions shows lesions with a necrotic core and an overlying thin disrupted fibrous cap heavily infiltrated by macrophages and T-lymphocytes. Activation of the clotting cascade results from contact of platelets with thrombogenetic agents of the core of the lesion leading to the formation of a thrombus. Second, erosions account for 25-40% of all coronary thrombi. Erosions are characterized by a luminal thrombus on top of a proteoglycan-rich lesion containing mostly SMCs with few inflammatory cells. Loss or dysfunction of the luminal endothelial cells is the primary cause of the formation of a thrombus. If present at all, there is no contact of the necrotic core with the overlying thrombus since the overlying fibrous cap is still intact. Last, the calcified nodule accounts only for 2-7% of all coronary thrombi. These lesions are characterized by the presence of calcified plates along with bony nodules that protrude into the vessel lumen, which contains disrupted endothelium. [8-11]

The cellular composition of an atherosclerotic lesion is an important determinant of its stability. In general, lipid-poor lesions with a prominent presence of fibroblasts, SMCs and collagen are relatively stable and resistant to rupture. On the other hand, lesions rich in cholesterol-loaded macrophages and extracellular lipid deposits, covered by a thin SMC-rich cap, are relatively soft and considered to be vulnerable to rupture.[12]

On cellular level, macrophages play a prominent role in creating a vulnerable lesion. They release chemokines (i.e. MCP-1, MCP-4, and RANTES) that attract additional macrophages, T-cells and mast cells into the site. Together they produce a pool of enzymes, including the family of matrix metalloproteinases (MMPs), which contribute to the degradation of the cap matrix and increase plaque vulnerability through secretion of collagenases, gelatinases, and stromolysin.[13,14] Eventually, foam cells contribute to the ongoing growth of the necrotic core, which is considered as a lesion destabilizing factor.[8]

Vulnerability to lesion rupture is not only characterized by the morphology of the lesion. In addition to lesion vulnerability, the vulnerable patient is also characterized by vulnerable blood (i.e. increased levels of C-reactive protein (CRP) and interleukin-6 (Il-6) and increased blood thrombogenicity) and a vulnerable myocardium (i.e. ECG abnormalities). Therefore, improved identification and treatment of vulnerable patients is a goal of great importance since it would result in major decreases in cardiovascular disease, morbidity and mortality.[9]

CELL PROLIFERATION AND CELL DEATH

Many physiological processes, including proper tissue development and homeostasis, require a delicate balance between cell gain (proliferation/cell cycle) and cell loss (apoptosis). All somatic cells proliferate via a mitotic process determined by progression through the cell cycle. Apoptosis (programmed cell death) occurs in a wide variety of physiological settings. Cell proliferation and apoptosis are coupled by cell-cycle regulators and apoptotic stimuli that affect both processes.[15] Normal cellular growth can be divided into five distinct phases (the cell cycle). The cell cycle is a conserved mechanism by which eukaryotic cells replicate themselves. Quiescent cells are found in the G_0 phase of the cell cycle and remain in a state in which messenger RNA (mRNA) and protein syntheses are minimal. A cell may stay in this state for years, but can re-enter the cycle at the first gap (G_1) phase when stimulated by growth factors. During G_1 the cell synthesizes series of mRNAs and proteins that are necessary for the next phase, the DNA synthesis (S) phase. Following the S-phase the cell enters a second gap (G_2) phase. During this phase the cell synthesizes additional mRNAs and proteins in preparation for cell division or mitosis (the M-phase), in which the cell divides into two daughter cells.[16] A number of checkpoints (restriction points) exist within the cell cycle to ensure that DNA synthesis and cell division proceed correctly. The two checkpoints occur at the G_1-S and the G_2-M transition. The checkpoints are also activated by DNA damage resulting in growth arrest and subsequent repair of the DNA damage. After damage repair, progression through the cell cycle resumes. If the damage cannot be repaired, the cell is eliminated through programmed cell death or apoptosis.[17] Thus, normal cellular proliferation is under tight regulations that control whether conditions are satisfactory for a particular cell to complete a round of division.

Apoptosis is a highly conserved mechanism by which eukaryotic cells commit suicide. It enables an organism to eliminate unwanted and defective cells through an orderly process of cellular disintegration avoiding undesirable inflammatory responses.[18] Apoptosis can be triggered by a wide variety of stimuli including DNA damage, oxidative stress, death receptor ligands, growth factor withdrawal, viral or bacterial infection, oncogenes, and irradiation.[19] Although the events inducing apoptosis may vary from cell to cell, there are basic features of a cell undergoing apoptosis: (1) cell shrinkage, (2) chromatin condensation, (3) DNA degradation, (4) protein fragmentation, (5) disassembly of organelles, and finally, (6) the collapse of cells into small apoptotic bodies that retain membrane integrity, which are removed by phagocytes. Apoptotic elimination of cells occurs during normal development and turnover, as well as in a variety of pathological conditions. Besides apoptosis, which is an active process, cells can also die as a part of a passive, degenerative, uncontrolled way of cell death, termed necrosis.

Necrosis represents a passive consequence of gross injury to the cell. It is morphologically different from apoptosis, and its physiological consequences are also very different from those of apoptosis.[20] Necrosis is characterised by cell swelling, loss of cytoplasmic membrane integrity, and mitochondrial damage. This leads to rapid depletion of energy levels, a breakdown of homeostatic control, cell membrane lysis, and release of intracellular contents, eventually resulting in an inflam-

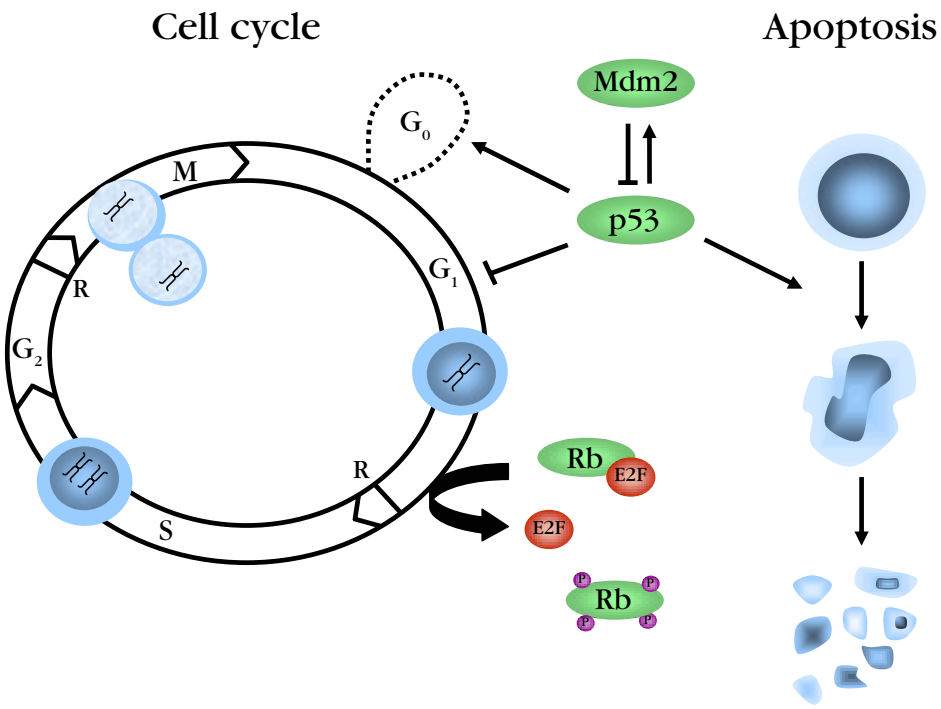


Figure 2. Coupling of cell cycle and apoptosis. Coupling cell cycle (left panel) and apoptosis (right panel) guarantees safe development and maintains homeostasis in organisms. Genes involved in both pathways are Rb and p53 (together with its inhibitor Mdm2). R, restriction point; P, phosphorylation

matory response, with damage to the surrounding cells. Necrosis must clearly be distinguished from apoptosis where cell death results from energy dependent, metabolically active, endogenous cellular processes and where the dying cells do not elicit an inflammatory reaction. The factors contributing to necrosis are mostly extrinsic in nature and therefore necrosis is mostly occurring under pathological conditions.[21] In contrast to apoptosis which occurs both under pathological and physiological circumstances.

CELL CYCLE AND APOPTOSIS GENES IN ATHEROSCLEROSIS

Cell proliferation and apoptosis are important processes in regulating macrophage and SMC numbers in the atherosclerotic lesion and thereby directly influence lesion stability.[22] Proliferating cells are present at all stages of atherosclerotic lesion development.[23-27] Although the ultimate signals that stimulate cell proliferation in the atherosclerotic lesions may be quite diverse, it is clear that cell proliferation is a crucial component of the atherogenic process.[28] Proliferation of SMCs contributes to atherosclerotic lesion stability. SMCs synthesize extracellular matrix proteins (i.e. collagen) that lead to the formation of a stable lesion covered by a fibrous cap. On the other hand, excessive SMC proliferation in restenosis is a direct complication of

surgical procedures such as balloon angioplasty or stent implantation used for the treatment of occluding atherosclerotic lesions.[29] Although peripheral macrophages are often considered mature (non-proliferating) cells, studies using combinations of different proliferation markers and macrophage-specific antibodies showed the presence proliferating macrophages in the lesion.[28,30,31] Macrophage proliferation is often considered detrimental to the atherosclerotic lesion, because an increase in the number of macrophages might result in an enhanced production of growth factors, cytokines, chemokines and metalloproteinases. This array of proteins stimulates the formation of an advanced atherosclerotic lesion.

Apoptosis (programmed cell death) is increasingly observed as atherosclerotic lesions develop, although the exact mechanism and consequences of apoptosis in the development and progression of atherosclerosis are still controversial.[32] Factors inducing apoptosis comprise high concentrations of oxidized LDL, oxysterols, Tumor Necrosis Factor-α (TNFα), Fas ligand, nitric oxide, growth factor withdrawal, hypoxia/ATP depletion and intracellular accumulation of unesterified (free) cholesterol (activating the Unfolded Protein Response (UPR) pathway).[33-35] Apoptotic cells within lesions are typically SMCs, macrophages and T-cells.[36] Increased SMC apoptosis has been detected in unstable compared with stable atherosclerotic lesions.[32] The loss of SMCs via apoptosis can be detrimental for plaque stability since most of the interstitial collagen fibers, which are important for the tensile strength of the fibrous cap, are produced by SMCs. In addition, apoptosis of SMCs is the basis for the generation of microparticels within the circulation, which act as potent procoagulant substrates both locally and systemically. The specific effect of macrophages apoptosis is even more controversial. Macrophages and macrophage apoptosis co-localize with sites of rupture, suggesting a direct causal role in rupture. Any reduction in macrophage numbers via apoptosis could improve plaque stability, due to less metalloproteinase activity and the decreased breakdown in collagen.[37] However, a decrease in macrophages would also reduce the scavenging of apoptotic SMCs and macrophages, allowing the cells to undergo secondary necrosis thereby increasing the inflammatory status and thrombogenicity of the lesion.[32] Hence, defining the exact role of proliferation and apoptosis in atherosclerosis will extend the knowledge on atherosclerotic lesion development and stability in general.

Cell proliferation and apoptosis are coupled by cell-cycle regulators and apoptotic stimuli that affect both processes.[15] Among these common cell-cycle regulators are Rb and p53 (and its inhibitor Mdm2).[17] The importance of these genes in maintaining homeostasis in embryonic and adult tissue becomes evident when concerning their roles in cancer development. Despite the more than 100 proto-oncogenes that have been identified, the pathways dominated by the two tumor suppressor genes Rb and p53 are the most frequently disrupted in cancer cells.[38] The unique role of these cell cycle and apoptosis genes in cancer puts a special interest for a role of these genes in atherosclerosis. Not the least because recently a series of shared molecular pathways have emerged that have in common a significant role in the pathogenesis and progression of both cancer and atherosclerosis.[39-42] Moreover, the proposed important role for both proliferation and apoptosis in determining atherosclerotic lesion composition and stability also opened the new era on the research of these processes in atherosclerosis via key genes such as p53, Rb, Mdm2, TNFα, and FasL.

Recent studies in mice demonstrated that genes involved in regulating cell cycle and apoptosis play an important role in the progression of atherosclerotic lesions coinciding with changes in the cellular composition.[43-53] P53, a tumour suppressor protein, plays a pivotal role in the cellular response to a range of environmental and intracellular stress signals (i.e. agents which cause DNA strand breaks, ultraviolet radiation, hyper-proliferation and hypoxia).[54] Mutations in p53 occur in about half of the human cancers, resulting in loss of apoptotic function. P53 belongs to a small family of related proteins that includes two other members p63 en p73.[55] P53 is a potent transcription factor, predominantly acting in the G_1 phase of cell cycle progression, regulating multiple downstream genes implicated in cell cycle control, apoptosis, differentiation, and senescence. Depending on many different factors that are both intrinsic and extrinsic to the cell, p53 activation results in activation of one of the abovementioned pathways.[56,57] In most cases, induction of p53 leads to an irreversible inhibition of cell growth, most decisively by activating apoptosis. The extent of DNA damage and p53 protein levels, however, are factors that contribute to making a choice between life and death. Mice homozygously knock out for p53 appear normal in embryogenesis and shortly after birth but are prone to the spontaneous development of a variety of neoplasms by 6 months of age. Hence, the p53 gene is dispensable for embryonic development but is required for the protection against formation of tumours.[58]

Recent studies demonstrated that p53 plays an important role in the progression of atherosclerotic lesions in mice. P53 is upregulated after various conditions of cellular stress found in atherosclerotic lesions, including DNA damage, hypoxia, oxidative stress and stress caused by oxidized lipoproteins.[59] Deletion of the tumour suppressor gene p53 strongly exacerbated atherosclerosis in different atherosclerosis-susceptible mouse models. Whole body p53 inactivation in apolipoprotein E-deficient (apoE$^{-/-}$) mice accelerated atherosclerosis by increased cellular proliferation.[47] In addition, hematopoietic inactivation of p53 via bone marrow transplantation in both APOE*3-Leiden[51] and LDL receptor deficient (LDLR$^{-/-}$)[48] mice confirmed the anti-atherogenic properties of the tumour suppressor gene. In addition to hematopoietic-derived p53, SMCs from human atherosclerotic lesions displayed increased sensitivity to p53-mediated apoptosis compared with normal SMCs.[60] Moreover, carotid artery lesions in apoE$^{-/-}$ mice treated locally with an adenovirus containing the p53 gene, displayed a phenotype that has been associated with increased vulnerability to plaque rupture.[52] Thus, abovementioned studies indicate an important role for p53 in atherosclerosis development.

P53 transcriptionally activates many target genes, one of which is its own inhibitor the murine double minute 2 (Mdm2) gene. Mdm2 was originally identified as an oncoprotein that binds to p53 and inhibits p53-mediated-transactivation. The human homologue of the Mdm2 gene is often found overexpressed in human cancers, particularly in breast tumours and carcinomas[61-64] and soft tissue sarcomas.[65-73] Mdm2 is an E3 ubiquitin ligase that mediates, together with enzymes E1 and E2, the ubiquitylation and proteasome-dependent degradation of p53.[74] Because Mdm2 inhibits p53 activity, it forms a negative feedback loop that tightly regulates p53 function. In turn, decreased p53 activity results in decreased Mdm2 to constitutive levels.[75] Mdm2 can also ubiquitinate itself and induce its own degradation.[76,77] *In*

vivo experiments demonstrated the importance of the Mdm2/p53 interaction.[78-81] Mice lacking Mdm2 are early embryonic lethal and die before implantation. This phenotype is completely rescued by concomitant deletion of p53, suggesting that p53 overexpression resulted in the embryonic lethal phenotype.

Early studies showed co-expression of p53 and Mdm2 in human carotid artery atherosclerotic lesions.[82] It was speculated that the destiny of individual p53 and Mdm2-co-expressing cells, either to undergo p53-dependent apoptosis or to re-enter the cycle of cell proliferation, may depend on the relative ratios of the two proteins. It was only recently that gene expression analysis showed that pro-apoptotic genes (p53, amongst others) are significantly more expressed in lesions causing acute coronary syndromes, whereas anti-apoptotic genes (Mdm2, amongst others) are more transcribed in stable angina atherosclerotic lesions.[83] Homozygous deletion of Mdm2 is an ideal method to specifically overexpress p53.[78-81] However, to date the embryonic lethality following homozygous allelic Mdm2 deletion hampered studies on the role of Mdm2 (and thereby p53 overexpression) in atherosclerosis.

Another important cell cycle regulatory gene, next to p53 and Mdm2, is Retinoblastoma (Rb). It is the first tumour suppressor gene identified molecularly and plays an important role in inhibiting cell proliferation. In addition, Rb can also act as an anti-apoptotic factor. The gene has been named after its disease Retinoblastoma, a rare childhood cancer of the retina which is caused by Rb inactivation. Rb is a nuclear phosphoprotein that arrests cells during the G_1-phase of the cell cycle by forming complexes with the members of the E2F transcription factor family. The E2F family of transcription factors has binding sites in the promoters of many of the genes that are involved in cell cycle progression.[84,85] In the cell, Rb is regulated via phosphorylation by cell cycle dependent kinases (CDKs) and cyclins, which, in turn, are inhibited by the cell cycle-inhibitor, p21. The identification of p21 as a p53 target gene implicated p53 in the upstream control and regulation of Rb.[86,87] Mutation or inactivation of both p53 and Rb have also been found in a variety of human tumours.[38,88-90] Mice homozygously knock out for Rb die at mid-gestation (E12-15) with defects in the haematopoietic system and impaired development of the central and peripheral nervous system resulting from massive cell death.[91-93]

Excessive proliferation of SMCs plays an important role in the pathobiology of different vascular occlusive diseases (i.e. atherosclerosis, (in-stent) restenosis, transplant vasculopathy). Therefore, earlier studies on the role of Rb in vascular diseases merely focussed on the role of Rb in SMC proliferation. Human plaque-derived SMCs show reduced proliferation and earlier senescence due to an increased ratio of the active form of Rb.[94] In addition, localized infection of the arterial wall with an adenovirus encoding a constitutively active non-phosphorylatable form of Rb significantly reduced medial vascular smooth muscle cell proliferation and restenosis in two animal models of balloon angioplasty.[43] Moreover, a phosphorylation-competent full-length and a truncated form of Rb inhibited vascular smooth muscle cell proliferation and neointima formation.[50] Although Rb is a key regulator of cell cycle progression in the G_1-phase (thereby directly affecting proliferation), more recent studies suggest that Rb activation is also seen in other stages of the cell cycle and in response to stresses, including hypoxia and DNA damage.[95,96]

Abovementioned studies indicate that cell cycle and apoptosis are important

processes in regulating macrophage and SMC numbers in the atherosclerotic lesion and might thereby directly influence lesion composition and stability.

APOPTOSIS AND INFLAMMATORY GENES IN ATHEROSCLEROSIS

The primary choice of cell death in atherosclerotic lesions is apoptosis. However, the harsh micro-environment, which is predominantly present in the growing complex atherosclerotic lesion, hampers the normal clearance of apoptotic bodies. Following, cumulative apoptotic bodies are ineffectively phagocytosed as a result of the presence of oxidized lipoproteins and lipids in the lesions or by the cholesterol-loaded state of the macrophage. It is a generally held concept that this situation precedes necrotic cell death as a result of the increased harmful content of the atherosclerotic lesion. Necrosis itself is more detrimental than apoptosis since necrosis by definition leads to an increased inflammatory status of the atherosclerotic lesion.[97]

Inflammation plays a key role in atherosclerosis. Immune cells dominate the early atherosclerotic lesions, their effector molecules accelerate progression of the lesions, and activation of inflammation can elicit acute coronary syndromes.[98] Besides macrophages also T- and B-lymphocytes have been reported to contribute to lesion development.[99-103] In addition, activated immune cells in the atherosclerotic lesion produce various inflammatory cytokines (interferon-γ (IFN-γ), interleukin-1 (Il-1), and Tumor Necrosis Factor-α (TNFα)), which induce the production of substantial amounts of interleukin-6 (Il-6). Il-6, in turn, stimulates the production of large amounts of acute-phase reactants, including C-reactive protein (CRP), serum amyloid A (SAA) and fibrinogen.[98] Thus, the local inflammatory process in the atherosclerotic artery leads to increased systemic blood levels of inflammatory cytokines and other acute-phase reactants. Therefore, measurements of these systemic cytokines and acute-phase reactants are particularly useful for clinical diagnosis. One of the key regulators of inflammation is the transcription factor nuclear factor κB (NF-κB). It controls transcription of many atherosclerosis-related genes, such as cytokines, chemokines, adhesion molecules, acute phase proteins, regulators of apoptosis, and cell proliferation. NF-κB plays an important role in directing both pro- and anti-inflammatory genes and also acts as a regulator of cell survival and proliferation in the atherosclerotic lesion.[104]

Different receptor-ligand couples play an important role in modulating both apoptotic and inflammatory processes. Tumor Necrosis Factor-α (TNFα) and one of its receptors TNFReceptor-1 (TNFR1) belong to the tumour necrosis factor receptor gene superfamily. This family comprise the so called "death receptors" from which the receptor-ligand couples TNFR1-TNFα and Fas-FasL are best characterized.[105] Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific death ligands (i.e. TNFα and FasL). These receptors can activate death caspases causing apoptosis of the cell. In addition to its role in apoptosis, TNFα is a pro-inflammatory cytokine that mediates key roles in acute and chronic inflammation, and infection.[106] Whereas binding of TNFα to TNFR1 (p55) activates responses associated with induction of adhesion molecule expression,[107] apoptosis,[108] and resistance to bacterial infection,[109,110] binding to TNFReceptor-2 (TNFR2, p75) activates induction of T cell proliferation,[111] induction of TNFα-mediated skin

tissue necrosis,[112] and modulation of TNFα-mediated pulmonary inflammation.[113] TNFα deficient mice develop normally, suggesting that TNFα does not have an irreplaceable role in prenatal tissue and organogenesis. However, TNFα deficient mice completely lack splenic primary B-follicles and cannot form organized follicular dendritic cell (FDC) networks and germinal centers.[114,115]

Although TNFα and its receptors are thought to be considerably important in a number of biological activities relevant to atherosclerosis, its function in atherogenesis remains unclear. Human association studies on TNFα polymorphisms are controversial varying from no,[116-118] weak[119] or strong[120] associations between different TNFα polymorphisms and coronary heart disease. Moreover, studies on the role of TNFα in atherosclerosis using several transgenic or knock out mouse models also yielded controversial results. TNFα ligand deficiency on a wild type C57BL/6 background showed variable effects varying from either no effect on early lesion development[121] to a reduction in atherosclerosis.[122] In addition, on the same background, TNFR1 deficiency did affect atherosclerosis formation, resulting in enhanced (early) lesion formation.[123] Overall, the abovementioned human and mouse studies demonstrate strong divergent results on the role of TNFα in atherosclerosis.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that, upon ligand activation, form heterodimers with the nuclear receptor RXR and bind to specific DNA sequences thereby transcriptionally regulating gene expression. PPARα and γ are the two main categories of these receptors, which are both characterized by their ability to influence cell apoptosis, inflammation, proliferation, differentiation, and as well lipid metabolism and glucose homeostasis.[124] PPARs are activated by ligands of physiological and pharmacological origin. PPARα is activated by polyunsaturated fatty acids and oxidized derivatives and by drugs of the fibrate family (i.e. fenofibrate and gemfibrozil).[125,126] Fibrates are clinically used to treat patients with lipid disorders and have been shown to reduce cardiovascular mortality. Ligands of PPARγ include naturally occurring fatty acid derivatives, prostaglandin derivatives and antidiabetic thiazolidinediones (glitazones), such as troglitazone, rosiglitazone, and pioglitazone.[127-129] PPARα and -γ agonists have shown positive effects on lipid metabolism in animal models and in clinical practice,[130-135] moreover several PPARγ agonists improve insulin resistance in type 2 diabetes.[136,137] In atherosclerosis PPARα and PPARγ activation results in reduction of atherogenic triglycerides and systemic plasma inflammatory proteins and raise HDL levels.[138] At a cellular level, PPARα/γ agonists act on most cell types involved in atherosclerosis reducing their involvement in the tissue response associated with lesion development.

MOUSE MODELS TO STUDY ATHEROSCLEROSIS

The mouse was generally regarded as a species that was resistant to the development of atherosclerosis. Therefore, early development of murine models for studying peripheral arterial disease focused on the identification of strains that were susceptible to atherosclerotic like lesion formation on high fat/high cholesterol diets. However, even the most sensitive strains (e.g. C57BL/6) required a diet high in cholesterol and the bile salt sodium cholate to develop atherosclerotic lesions. In the early 1990's the development of apolipoprotein E-deficient (apoE$^{-/-}$) mice revolu-

tionized the use of murine models in the study of cardiovascular disease.[139-141] These animals show strong elevated plasma cholesterol levels and vascular lesions similar in appearance to those observed in humans. The atherosclerotic lesions develop in the aortic root, the coronary arteries, and in the entire aorta at branch points of the major arteries in a time dependent manner.[139,142] ApoE-deficient mice on a chow diet spontaneously develop atherosclerosis, but feeding the mice a high fat diet, induces a strong acceleration of this process. Currently, the ApoE-deficient mouse is the most widely used experimental mouse model for studying atherosclerosis.

The LDL-receptor-deficient ($LDLR^{-/-}$) mouse, with its elevation in LDL levels, is also a useful model for studying atherosclerosis. In humans mutations in the gene for the LDL-receptor cause familial hypercholesterolemia, a major risk factor for developing atherosclerosis.[143] Homozygous LDLR-deficient mice show delayed clearance of VLDL and LDL from plasma.[144] In contrast to apoE-deficient mice, these mice do not manifest severe hypercholesterolemia on a chow diet and hence do not develop atherosclerosis. Upon feeding a high fat diet, plasma cholesterol levels increase strongly resulting in the formation of atherosclerotic lesions.[145,146]

In addition to the mouse models mentioned above, the APOE*3-Leiden mouse model is also frequently used for atherosclerosis and lipoprotein research. APOE*3-Leiden is a dominant negative mutant form of apolipoprotein E consisting of a tandem duplication of codons 120-126 in the apoE gene.[147-149] The introduction of this human mutation in a mouse resulted in a slight increase in cholesterol and triglyceride levels on a chow diet, whereas on a high fat diet cholesterol and triglyceride levels rose considerably.[150,151] In APOE*3-Leiden mice the degree of hypercholesterolemia can easily be adjusted to any desired level by varying in dietary contents. Moreover, APOE*3-Leiden mice have successfully been used in research on lipid-lowering and anti-atherosclerotic drugs and dietary supplements.[152-156]

Since the introduction of the abovementioned mouse models, the use of mice in atherosclerosis research has boomed over the last decade, driven by the development of knockout mice and transgenic animals. With these approaches, researchers have the tools to study the *in vivo* function of a specific gene product on an atherosclerotic background, while avoiding the difficulties associated with the use of antibodies or receptor agonists/antagonists (i.e. non-specificity, immunoreactivity, dosing and tachyphylaxis). Although, targeting specific genes of interest in atherosclerosis-susceptible mice elucidated the role of many genes in atherosclerosis, those genes that induce embryonic lethality associated with germline null alleles hampered the research on their role in atherogenesis. Site-specific recombinase (SSR) technology gives the opportunity to study the role of genes beyond the first required function of a gene, bypassing embryonic lethality associated with germline null alleles. SSR technology is a relatively new approach to induce gene deletion in a cell type of interest. The SSR Cre (an enzyme that causes recombination of the bacteriophage P1 genome) is able to recombine specific sequences of DNA with high fidelity without the need for cofactors. Therefore Cre has been used effectively to create gene deletions, insertions, inversions and exchanges in exogenous systems such as flies,[157-159] mammalian cell culture[160,161] and mice.[162-164] Cre recombines DNA at defined target sites, termed loxP sites, in actively dividing and post-mitotic cells, as well as in most tissue types. The activity of Cre involves DNA strand cleavage,

exchange and ligation.[165] The loxP sites consist of a 13 basepair (bp) palindromic sequence, or inverted repeats, separated by an 8 bp asymmetric core, or spacer sequence. Strand cleavage, exchange, and ligation occur within the spacers. Moreover, the development of ligand-regulated forms of Cre has added a temporal control to SSR activity to enable the induction of gene changes in late embryogenesis or in adult tissue. A successful strategy for inducing temporal SSR activity has involved fusing a mutant estrogen receptor (ER) ligand binding domain (LBD) to the C-terminus of Cre.[166-170] Currently, different ERs are available which do not bind endogenous ß-estradiol but are only responsive to the synthetic estrogen antagonist 4-hydroxytamoxifen (4-OHT). Two mouse lines are required for conditional gene deletion. A conventional transgenic mouse line with Cre expression in a specific tissue or cell type ("Cre-expressing mouse"), and a mouse strain that embodies a target gene (endogenous gene or transgene) flanked by two loxP sites ("floxed mouse"). Recombination (excision and consequently inactivation of the target gene) occurs only in those cells expressing Cre recombinase, leaving the target gene active in all other cells and tissues which do not express Cre. Cell proliferation and apoptosis are central themes in the cancer research field. As a result many "floxed-mice" are available aiming at different genes related to cell proliferation and apoptosis.[171-173] However, whereas Cre-loxP models are commonly used in cancer research,[172-176] the use of this system is still relatively new in the atherosclerosis research field.[177-180]

OUTLINE OF THIS THESIS

The general aim of the research presented in this thesis is to evaluate the role of different cell cycle and apoptosis genes in atherosclerosis. The knowledge on the role of cell cycle and apoptosis genes in atherosclerosis has become increasingly important over the last few years. To analyze these processes in greater detail in atherosclerosis development several key genes are studied (p53, Rb and Mdm2). To this end, we generated and analysed several mouse models. Because germline null alleles of the cell cycle genes of our interest (e.g. p53, Rb and Mdm2) lead to either formation of tumours after the age of 6 months (p53) or to embryonic lethality (Rb and Mdm2) we chose to use site-specific recombinase (SSR) technology, as described above. In addition, SSR technology gives the opportunity to study these genes in one single cell type of interest. To obtain cell type specificity, aiming at the two central cell types in atherosclerotic lesions, we used the lysozyme myeloid-Cre (LysMCre)[181] and the smooth muscle cell-Cre (SM-CreER^{T2}(ki))[182] mouse model for targeting macrophages and SMCs, respectively.

The tumour suppressor gene p53 has been shown to inhibit cell proliferation and stimulate apoptosis in many cell types. In **chapter 2** we address the role of macrophage p53 in atherosclerosis. A macrophage specific knock out for p53 demonstrated that p53 is major regulator of foam cell death in atherosclerotic lesions. To further extend the studies on cell cycle genes we targeted macrophage Retinoblastoma using a similar approach. Rb plays a pivotal role in regulating cell proliferation and apoptosis. **Chapter 3** describes that macrophage Rb plays a crucial role in atherosclerotic lesion development. Although SSR technology is a rather novel approach to study genes of interest, some genes turn out to be so vitally important

in maintaining homeostasis in adult tissues that SSR is not the solution to study the gene of interest in atherosclerosis. **Chapter 4** describes the mechanisms behind lethality induced via conditional deletion of Mdm2 in SMCs. Although lethality in this mouse model hampered studies on the role of SMC-Mdm2 in atherosclerosis, the mouse model showed that Mdm2 prevents accumulation of active p53 in quiescent SMCs and thereby the induction of p53-mediated necrotic cell death *in vivo*. Atherosclerosis, being a disease of the large and medium sized arteries also gives the advantage of proper accessibility for treatment. New technologies focusing on conditional, temporal and spatial gene deletion in atherosclerosis resulted, in addition to cell-type and time specificity, also in place specificity, which is described in **chapter 5**. TNFα is a protein often primarily described as a pro-inflammatory cytokine. On the contrary in **chapter 6** we demonstrate, using conventional whole body deletion of TNFα on an APOE*3-Leiden background, that TNFα is also a strong regulator of cell death in atherosclerotic lesions. Finally, **chapter 7** describes the effects of pharmacological regulation of PPARα and γ on atherosclerosis development in APOE*3-Leiden mice. These receptors influence cell inflammation, proliferation, differentiation, apoptosis and lipid and glucose homeostasis. **Chapter 8** discusses the results of these studies and the use of the SSR technology in the atherosclerosis research field, concluding with the implications for future research.

REFERENCES

1. Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, Stefanadis C, Moreno P, Pasterkamp G, Fayad Z, Stone PH, Waxman S, Raggi P, Madjid M, Zarrabi A, Burke A, Yuan C, Fitzgerald PJ, Siscovick DS, de Korte CL, Aikawa M, Juhani Airaksinen KE, Assmann G, Becker CR, Chesebro JH, Farb A, Galis ZS, Jackson C, Jang IK, Koenig W, Lodder RA, March K, Demirovic J, Navab M, Priori SG, Rekhter MD, Bahr R, Grundy SM, Mehran R, Colombo A, Boerwinkle E, Ballantyne C, Insull W, Jr., Schwartz RS, Vogel R, Serruys PW, Hansson GK, Faxon DP, Kaul S, Drexler H, Greenland P, Muller JE, Virmani R, Ridker PM, Zipes DP, Shah PK, Willerson JT. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. Circulation 2003;108:1664-1672.
2. Navab M, Berliner JA, Watson AD, Hama SY, Territo MC, Lusis AJ, Shih DM, Van Lenten BJ, Frank JS, Demer LL, Edwards PA, Fogelman AM. The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. Arterioscler Thromb Vasc Biol 1996;16:831-842.
3. Glass CK, Witztum JL. Atherosclerosis. the road ahead. Cell 2001;104:503-516.
4. Lusis AJ. Atherosclerosis. Nature 2000;407:233-241.
5. Nabel EG. Cardiovascular disease. N Engl J Med 2003;349:60-72.
6. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1995;92:1355-1374.
7. Ross R. Atherosclerosis is an inflammatory disease. Am Heart J 1999;138:S419-S420.
8. Dickson BC, Gotlieb AI. Towards understanding acute destabilization of vulnerable atherosclerotic plaques. Cardiovasc Pathol 2003;12:237-248.
9. Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, Stefanadis C, Moreno P, Pasterkamp G, Fayad Z, Stone PH, Waxman S, Raggi P, Madjid M, Zarrabi A, Burke A, Yuan C, Fitzgerald PJ, Siscovick DS, de Korte CL, Aikawa M, Airaksinen KE, Assmann G, Becker CR, Chesebro JH, Farb A, Galis ZS, Jackson C, Jang IK, Koenig W,

Lodder RA, March K, Demirovic J, Navab M, Priori SG, Rekhter MD, Bahr R, Grundy SM, Mehran R, Colombo A, Boerwinkle E, Ballantyne C, Insull W, Jr., Schwartz RS, Vogel R, Serruys PW, Hansson GK, Faxon DP, Kaul S, Drexler H, Greenland P, Muller JE, Virmani R, Ridker PM, Zipes DP, Shah PK, Willerson JT. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part II. Circulation 2003;108:1772-1778.
10. Schaar JA, Muller JE, Falk E, Virmani R, Fuster V, Serruys PW, Colombo A, Stefanadis C, Ward CS, Moreno PR, Maseri A, van der Steen AF. Terminology for high-risk and vulnerable coronary artery plaques. Report of a meeting on the vulnerable plaque, June 17 and 18, 2003, Santorini, Greece. Eur Heart J 2004;25:1077-1082.
11. Kolodgie FD, Virmani R, Burke AP, Farb A, Weber DK, Kutys R, Finn AV, Gold HK. Pathologic assessment of the vulnerable human coronary plaque 1. Heart 2004;90:1385-1391.
12. Fuster V, Badimon J, Chesebro JH, Fallon JT. Plaque rupture, thrombosis, and therapeutic implications. Haemostasis 1996;26 Suppl 4:269-284.
13. Dollery CM, McEwan JR, Henney AM. Matrix metalloproteinases and cardiovascular disease. Circ Res 1995;77:863-868.
14. Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res 2002;90:251-262.
15. Alenzi FQ. Links between apoptosis, proliferation and the cell cycle. Br J Biomed Sci 2004;61:99-102.
16. Li JM, Brooks G. Cell cycle regulatory molecules (cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors) and the cardiovascular system; potential targets for therapy? Eur Heart J 1999;20:406-420.
17. Pucci B, Kasten M, Giordano A. Cell cycle and apoptosis. Neoplasia 2000;2:291-299.
18. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell 1997;88:347-354.
19. Wyllie AH. Apoptosis: an overview. Br Med Bull 1997;53:451-465.
20. Kanduc D, Mittelman A, Serpico R, Sinigaglia E, Sinha AA, Natale C, Santacroce R, Di Corcia MG, Lucchese A, Dini L, Pani P, Santacroce S, Simone S, Bucci R, Farber E. Cell death: apoptosis versus necrosis (review). Int J Oncol 2002;21:165-170.
21. Bhatia M. Apoptosis versus necrosis in acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 2004;286:G189-G196.
22. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature JID - 0410462 1993;362:801-809.
23. Cavallero C, Turolla E, Ricevuti G. Cell proliferation in the atherosclerotic plaques of cholesterol-fed rabbits. 1. Colchicine and (3H)thymidine studies. Atherosclerosis 1971;13:9-20.
24. Stary HC, McMillan GC. Kinetics of cellular proliferation in experimental atherosclerosis. Radioautography with grain counts in cholesterol-fed rabbits. Arch Pathol 1970;89:173-183.
25. Walker LN, Reidy MA, Bowyer DE. Morphology and cell kinetics of fatty streak lesion formation in the hypercholesterolemic rabbit. Am J Pathol 1986;125:450-459.
26. SPRARAGEN SC, BOND VP, DAHL LK. Role of hyperplasia in vascular lesions of cholesterol-fed rabbits studied with thymidine-H3 autoradiography. Circ Res 1962;11:329-336.
27. McMillan GC, Stary HC. Preliminary experience with mitotic activity of cellular elements in the atherosclerotic plaques of cholesterol-fed rabbits studied by labeling with tritiated thymidine. Ann N Y Acad Sci 1968;149:699-709.
28. Rosenfeld ME, Ross R. Macrophage and smooth muscle cell proliferation in atherosclerotic lesions of WHHL and comparably hypercholesterolemic fat-fed rabbits. Arteriosclerosis 1990;10:680-687.
29. van der Hoeven BL, Pires NM, Warda HM, Oemrawsingh PV, van Vlijmen BJ, Quax PH, Schalij MJ, van der Wall EE, Jukema JW. Drug-eluting stents: results, promises and problems. Int J Cardiol 2005;99:9-17.
30. Sakai M, Kobori S, Miyazaki A, Horiuchi S. Macrophage proliferation in atherosclerosis.

Curr Opin Lipidol 2000;11:503-509.
31. Gordon D, Reidy MA, Benditt EP, Schwartz SM. Cell proliferation in human coronary arteries. Proc Natl Acad Sci U S A 1990;87:4600-4604.
32. Stoneman VE, Bennett MR. Role of apoptosis in atherosclerosis and its therapeutic implications. Clin Sci (Lond) 2004;107:343-354.
33. Feng B, Yao PM, Li Y, Devlin CM, Zhang D, Harding HP, Sweeney M, Rong JX, Kuriakose G, Fisher EA, Marks AR, Ron D, Tabas I. The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. Nat Cell Biol 2003;5:781-792.
34. Nhan TQ, Liles WC, Schwartz SM. Role of caspases in death and survival of the plaque macrophage. Arterioscler Thromb Vasc Biol 2005;25:895-903.
35. Tabas I. Apoptosis and plaque destabilization in atherosclerosis: the role of macrophage apoptosis induced by cholesterol. Cell Death Differ 2004;11 Suppl 1:S12-S16.
36. Kolodgie FD, Narula J, Guillo P, Virmani R. Apoptosis in human atherosclerotic plaques. Apoptosis 1999;4:5-10.
37. Kockx MM, Herman AG. Apoptosis in atherosclerosis: beneficial or detrimental? Cardiovasc Res 2000;45:736-746.
38. Sherr CJ. Cancer cell cycles. Science 1996;274:1672-1677.
39. Morganti M, Carpi A, Nicolini A, Gorini I, Glaviano B, Fini M, Giavaresi G, Mittermayer C, Giardino R. Atherosclerosis and cancer: common pathways on the vascular endothelium. Biomed Pharmacother 2002;56:317-324.
40. Ross JS, Stagliano NE, Donovan MJ, Breitbart RE, Ginsburg GS. Atherosclerosis: a cancer of the blood vessels? Am J Clin Pathol 2001;116 Suppl:S97-107.
41. Ross JS, Stagliano NE, Donovan MJ, Breitbart RE, Ginsburg GS. Atherosclerosis and cancer: common molecular pathways of disease development and progression. Ann N Y Acad Sci 2001;947:271-292.
42. Schwartz SM, Majesky MW, Murry CE. The intima: development and monoclonal responses to injury. Atherosclerosis 1995;118 Suppl:S125-S140.
43. Chang MW, Barr E, Seltzer J, Jiang YQ, Nabel GJ, Nabel EG, Parmacek MS, Leiden JM. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. Science 1995;267:518-522.
44. Condorelli G, Aycock JK, Frati G, Napoli C. Mutated p21/WAF/CIP transgene overexpression reduces smooth muscle cell proliferation, macrophage deposition, oxidation-sensitive mechanisms, and restenosis in hypercholesterolemic apolipoprotein E knockout mice. FASEB J 2001;15:2162-2170.
45. Diez-Juan A, Andres V. The growth suppressor p27(Kip1) protects against diet-induced atherosclerosis. FASEB J 2001;15:1989-1995.
46. Diez-Juan A, Perez P, Aracil M, Sancho D, Bernad A, Sanchez-Madrid F, Andres V. Selective inactivation of p27(Kip1) in hematopoietic progenitor cells increases neointimal macrophage proliferation and accelerates atherosclerosis. Blood 2004;103:158-161.
47. Guevara NV, Kim HS, Antonova EI, Chan L. The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo. Nat Med 1999;5:335-339.
48. Merched AJ, Williams E, Chan L. Macrophage-specific p53 expression plays a crucial role in atherosclerosis development and plaque remodeling. Arterioscler Thromb Vasc Biol 2003;23:1608-1614.
49. Merched AJ, Chan L. Absence of p21Waf1/Cip1/Sdi1 modulates macrophage differentiation and inflammatory response and protects against atherosclerosis. Circulation 2004;110:3830-3841.
50. Smith RC, Wills KN, Antelman D, Perlman H, Truong LN, Krasinski K, Walsh K. Adenoviral constructs encoding phosphorylation-competent full-length and truncated forms of the human retinoblastoma protein inhibit myocyte proliferation and neointima formation. Circulation 1997;96:1899-1905.
51. van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De WB, van Berkel TJ, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circ Res 2001;88:780-786.

52. der Thüsen JH, van Vlijmen BJ, Hoeben RC, Kockx MM, Havekes LM, van Berkel TJ, Biessen EA. Induction of atherosclerotic plaque rupture in apolipoprotein E-/- mice after adenovirus-mediated transfer of p53. Circulation 2002;105:2064-2070.
53. Zadelaar AS, Thüsen JH, Boesten LS, Hoeben RC, Kockx MM, Versnel MA, van Berkel TJ, Havekes LM, EA LB, van Vlijmen BJ. Increased vulnerability of pre-existing atherosclerosis in ApoE-deficient mice following adenovirus-mediated Fas ligand gene transfer. Atherosclerosis 2005;183(2):244-50.
54. Meek DW. The p53 response to DNA damage. DNA Repair (Amst) 2004;3:1049-1056.
55. Melino G, De L, V, Vousden KH. p73: Friend or foe in tumorigenesis. Nat Rev Cancer 2002;2:605-615.
56. Klein C, Vassilev LT. Targeting the p53-MDM2 interaction to treat cancer. Br J Cancer 2004;91:1415-1419.
57. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer 2002;2:594-604.
58. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CAJ, Butel JS, Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 1992;356:215-221.
59. Ko LJ, Prives C. p53: puzzle and paradigm. Genes Dev 1996;10:1054-1072.
60. Bennett MR, Littlewood TD, Schwartz SM, Weissberg PL. Increased sensitivity of human vascular smooth muscle cells from atherosclerotic plaques to p53-mediated apoptosis. Circ Res 1997;81:591-599.
61. Gudas JM, Nguyen H, Klein RC, Katayose D, Seth P, Cowan KH. Differential expression of multiple MDM2 messenger RNAs and proteins in normal and tumorigenic breast epithelial cells. Clin Cancer Res 1995;1:71-80.
62. Marchetti A, Buttitta F, Girlando S, Dalla PP, Pellegrini S, Fina P, Doglioni C, Bevilacqua G, Barbareschi M. mdm2 gene alterations and mdm2 protein expression in breast carcinomas. J Pathol 1995;175:31-38.
63. McCann AH, Kirley A, Carney DN, Corbally N, Magee HM, Keating G, Dervan PA. Amplification of the MDM2 gene in human breast cancer and its association with MDM2 and p53 protein status. Br J Cancer 1995;71:981-985.
64. Sheikh MS, Shao ZM, Hussain A, Fontana JA. The p53-binding protein MDM2 gene is differentially expressed in human breast carcinoma. Cancer Res 1993;53:3226-3228.
65. Cordon-Cardo C, Latres E, Drobnjak M, Oliva MR, Pollack D, Woodruff JM, Marechal V, Chen J, Brennan MF, Levine AJ. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. Cancer Res 1994;54:794-799.
66. Corvi R, Savelyeva L, Breit S, Wenzel A, Handgretinger R, Barak J, Oren M, Amler L, Schwab M. Non-syntenic amplification of MDM2 and MYCN in human neuroblastoma. Oncogene 1995;10:1081-1086.
67. Florenes VA, Maelandsmo GM, Forus A, Andreassen A, Myklebost O, Fodstad O. MDM2 gene amplification and transcript levels in human sarcomas: relationship to TP53 gene status. J Natl Cancer Inst 1994;86:1297-1302.
68. Habuchi T, Kinoshita H, Yamada H, Kakehi Y, Ogawa O, Wu WJ, Takahashi R, Sugiyama T, Yoshida O. Oncogene amplification in urothelial cancers with p53 gene mutation or MDM2 amplification. J Natl Cancer Inst 1994;86:1331-1335.
69. Ladanyi M, Cha C, Lewis R, Jhanwar SC, Huvos AG, Healey JH. MDM2 gene amplification in metastatic osteosarcoma. Cancer Res 1993;53:16-18.
70. Ladanyi M, Lewis R, Jhanwar SC, Gerald W, Huvos AG, Healey JH. MDM2 and CDK4 gene amplification in Ewing's sarcoma. J Pathol 1995;175:211-217.
71. Landers JE, Haines DS, Strauss JF, III, George DL. Enhanced translation: a novel mechanism of mdm2 oncogene overexpression identified in human tumor cells. Oncogene 1994;9:2745-2750.
72. Leach FS, Tokino T, Meltzer P, Burrell M, Oliner JD, Smith S, Hill DE, Sidransky D, Kinzler KW, Vogelstein B. p53 Mutation and MDM2 amplification in human soft tissue sarcomas. Cancer Res 1993;53:2231-2234.
73. Lianes P, Orlow I, Zhang ZF, Oliva MR, Sarkis AS, Reuter VE, Cordon-Cardo C. Altered

patterns of MDM2 and TP53 expression in human bladder cancer. J Natl Cancer Inst 1994;86:1325-1330.
74. Michael D, Oren M. The p53-Mdm2 module and the ubiquitin system. Semin Cancer Biol 2003;13:49-58.
75. Iwakuma T, Lozano G. MDM2, an introduction. Mol Cancer Res 2003;1:993-1000.
76. Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J Biol Chem 2000;275:8945-8951.
77. Honda R, Yasuda H. Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. Oncogene 2000;19:1473-1476.
78. Jones SN, Roe AE, Donehower LA, Bradley A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature 1995;378:206-208.
79. Montes de Oca LR, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 1995;378:203-206.
80. Mendrysa SM, McElwee MK, Michalowski J, O'Leary KA, Young KM, Perry ME. mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. Mol Cell Biol 2003;23:462-472.
81. Moll UM, Petrenko O. The MDM2-p53 interaction. Mol Cancer Res 2003;1:1001-1008.
82. Ihling C, Haendeler J, Menzel G, Hess RD, Fraedrich G, Schaefer HE, Zeiher AM. Co-expression of p53 and MDM2 in human atherosclerosis: implications for the regulation of cellularity of atherosclerotic lesions. J Pathol 1998;185:303-312.
83. Rossi ML, Marziliano N, Merlini PA, Bramucci E, Canosi U, Belli G, Parenti DZ, Mannucci PM, Ardissino D. Different quantitative apoptotic traits in coronary atherosclerotic plaques from patients with stable angina pectoris and acute coronary syndromes. Circulation 2004;110:1767-1773.
84. Dyson N. The regulation of E2F by pRB-family proteins. Genes Dev 1998;12:2245-2262.
85. Nevins JR. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ 1998;9:585-593.
86. el Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817-825.
87. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993;75:805-816.
88. Levine AJ, Momand J. Tumor suppressor genes: the p53 and retinoblastoma sensitivity genes and gene products. Biochim Biophys Acta 1990;1032:119-136.
89. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature 1991;351:453-456.
90. Weinberg RA. The retinoblastoma protein and cell cycle control. Cell 1995;81:323-330.
91. Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH, Bradley A. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 1992;359:288-294.
92. Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA. Effects of an Rb mutation in the mouse. Nature 1992;359:295-300.
93. Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van d, V, Hooper ML, Berns A, te RH. Requirement for a functional Rb-1 gene in murine development. Nature 1992;359:328-330.
94. Bennett MR, Macdonald K, Chan SW, Boyle JJ, Weissberg PL. Cooperative interactions between RB and p53 regulate cell proliferation, cell senescence, and apoptosis in human vascular smooth muscle cells from atherosclerotic plaques. Circ Res 1998;82:704-712.
95. Amellem O, Sandvik JA, Stokke T, Pettersen EO. The retinoblastoma protein-associated cell cycle arrest in S-phase under moderate hypoxia is disrupted in cells expressing HPV18 E7 oncoprotein. Br J Cancer 1998;77:862-872.

96. Ludlow JW, Nelson DA. Control and activity of type-1 serine/threonine protein phosphatase during the cell cycle. Semin Cancer Biol 1995;6:195-202.
97. Tabas I. p53 and atherosclerosis. Circ Res 2001;88:747-749.
98. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685-1695.
99. Watanabe T, Shimokama T, Haraoka S, Kishikawa H. T lymphocytes in atherosclerotic lesions. Ann N Y Acad Sci 1995;748:40-55.
100. Benagiano M, Azzurri A, Ciervo A, Amedei A, Tamburini C, Ferrari M, Telford JL, Baldari CT, Romagnani S, Cassone A, D'Elios MM, Del Prete G. T helper type 1 lymphocytes drive inflammation in human atherosclerotic lesions. Proc Natl Acad Sci U S A 2003;100:6658-6663.
101. de Boer OJ, van der Wal AC, Verhagen CE, Becker AE. Cytokine secretion profiles of cloned T cells from human aortic atherosclerotic plaques. J Pathol 1999;188:174-179.
102. Major AS, Fazio S, Linton MF. B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. Arterioscler Thromb Vasc Biol 2002;22:1892-1898.
103. Hansson GK. The B cell: a good guy in vascular disease? Arterioscler Thromb Vasc Biol 2002;22:523-524.
104. de Winther MP, Kanters E, Kraal G, Hofker MH. Nuclear factor kappaB signaling in atherogenesis. Arterioscler Thromb Vasc Biol 2005;25:904-914.
105. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305-1308.
106. Palladino MA, Bahjat FR, Theodorakis EA, Moldawer LL. Anti-TNF-alpha therapies: the next generation. Nat Rev Drug Discov 2003;2:736-746.
107. Mackay F, Loetscher H, Stueber D, Gehr G, Lesslauer W. Tumor necrosis factor alpha (TNF-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. J Exp Med 1993;177:1277-1286.
108. Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. Cell 1993;74:845-853.
109. Pfeffer K, Matsuyama T, Kundig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Kronke M, Mak TW. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell 1993;73:457-467.
110. Rothe J, Lesslauer W, Lotscher H, Lang Y, Koebel P, Kontgen F, Althage A, Zinkernagel R, Steinmetz M, Bluethmann H. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature 1993;364:798-802.
111. Tartaglia LA, Goeddel DV, Reynolds C, Figari IS, Weber RF, Fendly BM, Palladino MA, Jr. Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. J Immunol 1993;151:4637-4641.
112. Erickson SL, de Sauvage FJ, Kikly K, Carver-Moore K, Pitts-Meek S, Gillett N, Sheehan KC, Schreiber RD, Goeddel DV, Moore MW. Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. Nature 1994;372:560-563.
113. Peschon JJ, Torrance DS, Stocking KL, Glaccum MB, Otten C, Willis CR, Charrier K, Morrissey PJ, Ware CB, Mohler KM. TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. J Immunol 1998;160:943-952.
114. Marino MW, Dunn A, Grail D, Inglese M, Noguchi Y, Richards E, Jungbluth A, Wada H, Moore M, Williamson B, Basu S, Old LJ. Characterization of tumor necrosis factor-deficient mice. Proc Natl Acad Sci U S A 1997;94:8093-8098.
115. Pasparakis M, Alexopoulou L, Episkopou V, Kollias G. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J Exp Med 1996;184:1397-1411.
116. Allen RA, Lee EM, Roberts DH, Park BK, Pirmohamed M. Polymorphisms in the TNF-

alpha and TNF-receptor genes in patients with coronary artery disease. Eur J Clin Invest 2001;31:843-851.
117. Herrmann SM, Ricard S, Nicaud V, Mallet C, Arveiler D, Evans A, Ruidavets JB, Luc G, Bara L, Parra HJ, Poirier O, Cambien F. Polymorphisms of the tumour necrosis factor-alpha gene, coronary heart disease and obesity. Eur J Clin Invest 1998;28:59-66.
118. Koch W, Kastrati A, Bottiger C, Mehilli J, von Beckerath N, Schomig A. Interleukin-10 and tumor necrosis factor gene polymorphisms and risk of coronary artery disease and myocardial infarction. Atherosclerosis 2001;159:137-144.
119. Keso T, Perola M, Laippala P, Ilveskoski E, Kunnas TA, Mikkelsson J, Penttila A, Hurme M, Karhunen PJ. Polymorphisms within the tumor necrosis factor locus and prevalence of coronary artery disease in middle-aged men. Atherosclerosis 2001;154:691-697.
120. Vendrell J, Fernandez-Real JM, Gutierrez C, Zamora A, Simon I, Bardaji A, Ricart W, Richart C. A polymorphism in the promoter of the tumor necrosis factor-alpha gene (-308) is associated with coronary heart disease in type 2 diabetic patients. Atherosclerosis 2003;167:257-264.
121. Schreyer SA, Vick CM, LeBoeuf RC. Loss of lymphotoxin-alpha, but not tumor necrosis factor-alpha reduces atherosclerosis in mice. J Biol Chem 2002;277(14):12364-8.
122. Canault M, Peiretti F, Mueller C, Kopp F, Morange P, Rihs S, Portugal H, Juhan-Vague I, Nalbone G. Exclusive expression of transmembrane TNF-alpha in mice reduces the inflammatory response in early lipid lesions of aortic sinus. Atherosclerosis 2004;172:211-218.
123. Schreyer SA, Peschon JJ, LeBoeuf RC. Accelerated atherosclerosis in mice lacking tumor necrosis factor receptor p55. J Biol Chem 1996;271:26174-26178.
124. Puddu P, Puddu GM, Muscari A. Peroxisome proliferator-activated receptors: are they involved in atherosclerosis progression? Int J Cardiol 2003;90:133-140.
125. Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A 1997;94:4312-4317.
126. Staels B, Dallongeville J, Auwerx J, Schoonjans K, Leitersdorf E, Fruchart JC. Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation 1998;98:2088-2093.
127. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. Cell 1995;83:813-819.
128. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). J Biol Chem 1995;270:12953-12956.
129. Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell 1998;93:229-240.
130. Tailleux A, Torpier G, Mezdour H, Fruchart JC, Staels B, Fievet C. Murine models to investigate pharmacological compounds acting as ligands of PPARs in dyslipidemia and atherosclerosis. Trends Pharmacol Sci 2003;24:530-534.
131. Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. JAMA 1986;256:2835-2838.
132. Rubins HB, Robins SJ, Collins D, Fye CL, Anderson JW, Elam MB, Faas FH, Linares E, Schaefer EJ, Schectman G, Wilt TJ, Wittes J. Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group. N Engl J Med 1999;341:410-418.
133. Frick MH, Elo O, Haapa K, Heinonen OP, Heinsalmi P, Helo P, Huttunen JK, Kaitaniemi P, Koskinen P, Manninen V, . Helsinki Heart Study: primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia. Safety of treatment, changes in risk factors, and incidence of coronary heart disease. N Engl J Med 1987;317:1237-1245.
134. Elkeles RS, Diamond JR, Poulter C, Dhanjil S, Nicolaides AN, Mahmood S, Richmond

W, Mather H, Sharp P, Feher MD. Cardiovascular outcomes in type 2 diabetes. A double-blind placebo-controlled study of bezafibrate: the St. Mary's, Ealing, Northwick Park Diabetes Cardiovascular Disease Prevention (SENDCAP) Study. Diabetes Care 1998;21:641-648.
135. Effect of fenofibrate on progression of coronary-artery disease in type 2 diabetes: the Diabetes Atherosclerosis Intervention Study, a randomised study. Lancet 2001;357:905-910.
136. Rosenblatt S, Miskin B, Glazer NB, Prince MJ, Robertson KE. The impact of pioglitazone on glycemic control and atherogenic dyslipidemia in patients with type 2 diabetes mellitus. Coron Artery Dis 2001;12:413-423.
137. Winkler K, Konrad T, Fullert S, Friedrich I, Destani R, Baumstark MW, Krebs K, Wieland H, Marz W. Pioglitazone reduces atherogenic dense LDL particles in nondiabetic patients with arterial hypertension: a double-blind, placebo-controlled study. Diabetes Care 2003;26:2588-2594.
138. Duez H, Fruchart JC, Staels B. PPARS in inflammation, atherosclerosis and thrombosis. J Cardiovasc Risk 2001;8:187-194.
139. Reddick RL, Zhang SH, Maeda N. Atherosclerosis in mice lacking apo E. Evaluation of lesional development and progression. Arterioscler Thromb 1994;14:141-147.
140. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 1992;71:343-353.
141. van Ree JH, van den Broek WJ, Dahlmans VE, Groot PH, Vidgeon-Hart M, Frants RR, Wieringa B, Havekes LM, Hofker MH. Diet-induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. Atherosclerosis 1994;111:25-37.
142. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler Thromb 1994;14:133-140.
143. Brown MS, Goldstein JL. Expression of the familial hypercholesterolemia gene in heterozygotes: mechanism for a dominant disorder in man. Science 1974;185:61-63.
144. Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE, Herz J. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest 1993;92:883-893.
145. Ishibashi S, Goldstein JL, Brown MS, Herz J, Burns DK. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. J Clin Invest 1994;93:1885-1893.
146. Ishibashi S, Herz J, Maeda N, Goldstein JL, Brown MS. The two-receptor model of lipoprotein clearance: tests of the hypothesis in "knockout" mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. Proc Natl Acad Sci U S A 1994;91:4431-4435.
147. Havekes L, de Wit E, Leuven JG, Klasen E, Utermann G, Weber W, Beisiegel U. Apolipoprotein E3-Leiden. A new variant of human apolipoprotein E associated with familial type III hyperlipoproteinemia. Hum Genet 1986;73:157-163.
148. van den Maagdenberg AM, de Knijff P, Stalenhoef AF, Gevers Leuven JA, Havekes LM, Frants RR. Apolipoprotein E*3-Leiden allele results from a partial gene duplication in exon 4. Biochem Biophys Res Commun 1989;165:851-857.
149. Wardell MR, Weisgraber KH, Havekes LM, Rall SC, Jr. Apolipoprotein E3-Leiden contains a seven-amino acid insertion that is a tandem repeat of residues 121-127. J Biol Chem 1989;264:21205-21210.
150. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van Vlijmen B, van der BH, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. J Biol Chem 1993;268:10540-10545.
151. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der BH, HogenEsch H, Frants RR, Hofker MH, Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. J Clin Invest 1994;93:1403-1410.

152. van Vlijmen BJ, Mensink RP, 't Hof HB, Offermans RF, Hofker MH, Havekes LM. Effects of dietary fish oil on serum lipids and VLDL kinetics in hyperlipidemic apolipoprotein E*3-Leiden transgenic mice. J Lipid Res 1998;39:1181-1188.
153. van Vlijmen BJ, Pearce NJ, Bergo M, Staels B, Yates JW, Gribble AD, Bond BC, Hofker MH, Havekes LM, Groot PH. Apolipoprotein E*3-Leiden transgenic mice as a test model for hypolipidaemic drugs. Arzneimittelforschung 1998;48:396-402.
154. Post SM, de Roos B, Vermeulen M, Afman L, Jong MC, Dahlmans VE, Havekes LM, Stellaard F, Katan MB, Princen HM. Cafestol increases serum cholesterol levels in apolipoprotein E*3-Leiden transgenic mice by suppression of bile acid synthesis. Arterioscler Thromb Vasc Biol 2000;20:1551-1556.
155. Volger OL, Mensink RP, Plat J, Hornstra G, Havekes LM, Princen HM. Dietary vegetable oil and wood derived plant stanol esters reduce atherosclerotic lesion size and severity in apoE*3-Leiden transgenic mice. Atherosclerosis 2001;157:375-381.
156. Volger OL, van der BH, de Wit EC, van Duyvenvoorde W, Hornstra G, Plat J, Havekes LM, Mensink RP, Princen HM. Dietary plant stanol esters reduce VLDL cholesterol secretion and bile saturation in apolipoprotein E*3-Leiden transgenic mice. Arterioscler Thromb Vasc Biol 2001;21:1046-1052.
157. Golic KG, Lindquist S. The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. Cell 1989;59:499-509.
158. Dang DT, Perrimon N. Use of a yeast site-specific recombinase to generate embryonic mosaics in Drosophila. Dev Genet 1992;13:367-375.
159. Xu T, Rubin GM. Analysis of genetic mosaics in developing and adult Drosophila tissues. Development 1993;117:1223-1237.
160. Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci U S A 1988;85:5166-5170.
161. O'Gorman S, Fox DT, Wahl GM. Recombinase-mediated gene activation and site-specific integration in mammalian cells. Science 1991;251:1351-1355.
162. Lakso M, Sauer B, Mosinger B, Jr., Lee EJ, Manning RW, Yu SH, Mulder KL, Westphal H. Targeted oncogene activation by site-specific recombination in transgenic mice. Proc Natl Acad Sci U S A 1992;89:6232-6236.
163. Orban PC, Chui D, Marth JD. Tissue- and site-specific DNA recombination in transgenic mice. Proc Natl Acad Sci U S A 1992;89:6861-6865.
164. Dymecki SM. Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. Proc Natl Acad Sci U S A 1996;93:6191-6196.
165. Sadowski PD. The Flp recombinase of the 2-microns plasmid of Saccharomyces cerevisiae. Prog Nucleic Acid Res Mol Biol 1995;51:53-91.
166. Metzger D, Clifford J, Chiba H, Chambon P. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. Proc Natl Acad Sci U S A 1995;92:6991-6995.
167. Feil R, Brocard J, Mascrez B, LeMeur M, Metzger D, Chambon P. Ligand-activated site-specific recombination in mice. Proc Natl Acad Sci U S A 1996;93:10887-10890.
168. Kellendonk C, Tronche F, Monaghan AP, Angrand PO, Stewart F, Schutz G. Regulation of Cre recombinase activity by the synthetic steroid RU 486. Nucleic Acids Res 1996;24:1404-1411.
169. Brocard J, Warot X, Wendling O, Messaddeq N, Vonesch JL, Chambon P, Metzger D. Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. Proc Natl Acad Sci U S A 1997;94:14559-14563.
170. Schwenk F, Kuhn R, Angrand PO, Rajewsky K, Stewart AF. Temporally and spatially regulated somatic mutagenesis in mice. Nucleic Acids Res 1998;26:1427-1432.
171. Grier JD, Yan W, Lozano G. Conditional allele of mdm2 which encodes a p53 inhibitor. Genesis 2002;32:145-147.
172. Jonkers J, Meuwissen R, van Der GH, Peterse H, van d, V, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet 2001;29:418-425.
173. Marino S, Vooijs M, van Der GH, Jonkers J, Berns A. Induction of medulloblastomas in

p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev 2000;14:994-1004.
174. Colnot S, Decaens T, Niwa-Kawakita M, Godard C, Hamard G, Kahn A, Giovannini M, Perret C. Liver-targeted disruption of Apc in mice activates beta-catenin signaling and leads to hepatocellular carcinomas. Proc Natl Acad Sci U S A 2004;101:17216-17221.
175. Lin SC, Lee KF, Nikitin AY, Hilsenbeck SG, Cardiff RD, Li A, Kang KW, Frank SA, Lee WH, Lee EY. Somatic mutation of p53 leads to estrogen receptor alpha-positive and -negative mouse mammary tumors with high frequency of metastasis. Cancer Res 2004;64:3525-3532.
176. Vooijs M, van d, V, te RH, Berns A. Flp-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse. Oncogene 1998;17:1-12.
177. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 2003;112:1176-1185.
178. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. Science 2003;300:329-332.
179. Feil S, Hofmann F, Feil R. SM22alpha modulates vascular smooth muscle cell phenotype during atherogenesis. Circ Res 2004;94:863-865.
180. Espirito Santo SM, Pires NM, Boesten LS, Gerritsen G, Bovenschen N, van Dijk KW, Jukema JW, Princen HM, Bensadoun A, Li WP, Herz J, Havekes LM, van Vlijmen BJ. Hepatic low-density lipoprotein receptor-related protein deficiency in mice increases atherosclerosis independent of plasma cholesterol. Blood 2004;103:3777-3782.
181. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 1999;8:265-277.
182. Kuhbandner S, Brummer S, Metzger D, Chambon P, Hofmann F, Feil R. Temporally controlled somatic mutagenesis in smooth muscle. Genesis 2000;28:15-22.

Chapter 2

Macrophage p53 and atherosclerosis

Macrophage p53 controls foam cell death in atherosclerotic lesions of apolipoprotein E deficient mice

Lianne S.M. Boesten[1,5];A. Susanne M. Zadelaar [2,5];Anita van Nieuwkoop[1,5]; Lihui Hu[1]; Amina F.A.S.Teunisse[3];Aart G. Jochemsen[3]; Bastiaan Evers[6]; Marion J.J. Gijbels[7,8]; Bart J.M. van Vlijmen[4]; Louis M. Havekes[1,2,5]; Menno P.J. de Winther[7]

[1]Dept. of General Internal Medicine, [2]Dept. of Cardiology, [3]Dept. of Molecular and Cell Biology, [4]Hemostasis and Thrombosis Research Center, Dept. of Hematology, Leiden University Medical Center, Leiden,The Netherlands, [5]TNO-Quality of Life/ Gaubius Laboratory, Leiden The Netherlands, [6]The Netherlands Cancer Institute, Amsterdam,The Netherlands, [7]Department of Molecular Genetics, Cardiovascular Research Institute Maastricht, [8]Department of Pathology, Maastricht University, Maastricht,The Netherlands

ABSTRACT

The cellular composition of an atherosclerotic lesion is determined by many factors including cell infiltration, proliferation and cell death, either by apoptosis or necrosis. The tumor suppressor gene p53 has been shown to regulate both cell proliferation and cell death in many cell types. To study the role of macrophage p53 in the development of atherosclerosis, we generated apoE-deficient mice with a macrophage-restricted deletion of p53 (LysMCre^{+} p53$^{loxP/loxP}$ apoE$^{-/-}$ mice) and control littermates (p53$^{loxP/loxP}$ apoE$^{-/-}$ mice) and analyzed early and advanced atherosclerosis development. Absence of macrophage p53 did not affect lesion area in both early and advanced atherosclerosis, neither in the aortic root nor in the aortic arch and thoracic aorta. In early atherosclerosis, absence of macrophage p53, resulted in reduced apoptosis (-59%), however without changes in lesion composition. In contrast, in advanced atherosclerosis, reduced apoptosis (-37%) upon absence of macrophage p53, coincided with increased necrotic death (+96%), increased foam cell content (+24%), and reduced lipid core formation (-41%). Proliferation was not affected by the absence of macrophage p53 in both early and advanced atherosclerosis. Hence, our data point towards an important role for macrophage p53 in induction of foam cell apoptosis and prevention of lesional necrosis.

Atherosclerosis is an inflammatory disease of the large vessels in which macrophages play a central role.[1,2] Accumulation of macrophage foam cells in the vessel wall results in the formation of fatty streaks. These lesions may be reversible and may not cause clinical symptoms. However, macrophage accumulation within the arterial intima sets the stage for progression of the atheroma and evolution into more complicated lesions that can cause clinical symptoms by eventual rupture or erosion of the plaque.[3] Changes in the cellular composition of an atherosclerotic lesion are important in modulating the risk of acute coronary syndromes. Cell proliferation and cell death are crucial processes in regulating cell numbers in the atherosclerotic lesion and may thereby directly influence lesion composition and stability.[4]

The p53 tumor suppressor protein is an essential gene in cell proliferation and cell death and plays a pivotal role in the cellular response to a range of environmental and intracellular stress signals.[5] Mutations in p53 occur in about half of the human cancers, resulting in loss of apoptotic function. P53 is a potent transcription factor, predominantly acting in the G1 phase of cell cycle progression, regulating multiple downstream genes implicated in cell cycle control, apoptosis, differentiation, and senescence.[6,7] In atherosclerosis, p53 was immunohistochemically visualized in human carotid atheromatous lesions in virtually all cell types (macrophages, smooth muscle cells, endothelial cells).[8] Recent mouse studies demonstrated that p53 plays an important role in the progression of atherosclerotic lesions. Whole body p53 inactivation in apolipoprotein E-deficient (apoE$^{-/-}$) mice accelerated atherosclerosis primarily by increased cellular proliferation.[9] In addition, using bone marrow transplantation in both apoE*3-Leiden[10] and LDL receptor deficient (LDLR$^{-/-}$)[11] mice it was shown that p53 of hematopoietic origin is, at least in part, responsible for the inhibition of atherogenesis in these models. Hence, these studies show an important role for both macrophage- and lymphocyte-derived p53 (or in addition: their interplay) in the development of atherosclerosis.

To distinguish the effects of p53 deficiency specifically in macrophages from processes affected by lymphocyte-p53 deficiency, we employed a conditional deletion approach using the Cre-loxP system. Macrophage specific p53 deletion was accomplished by combining mice carrying a p53 allele that was flanked by loxP sites[12] with LysMCre mice.[13] Expression of Cre in the myeloid lineage in the LysMCre mice will result in cell specific deletion of p53 and thereby give macrophages that lack p53. Using this approach, we found that absence of macrophage p53 did not affect lesion area in both early and advanced atherosclerosis, neither in the aortic root nor in the aortic arch and thoracic aorta. In early atherosclerosis, absence of macrophage p53 resulted in reduced apoptosis, however without changes in lesion composition. In contrast, in advanced atherosclerosis, reduced apoptosis due to absence of macrophage p53, coincided with increased necrotic death, increased foam cell content and reduced lipid core formation. These studies indicate that macrophage p53 is primarily involved in determining atherosclerotic lesion composition by controlling the balance of lesional apoptosis and necrosis.

METHODS

Mice and diet

The experimental animals were obtained by combining mice carrying the floxed p53 gene[12] with LysMCre mice[13] (a generous gift from Dr. B.E. Clausen, AMC, The Netherlands and Dr. I. Forster, University of Cologne, Germany), and apoE-deficient mice[14] resulting in mice that are homozygously floxed for p53 and deficient for apoE and that either express Cre in their macrophages (LysMCre^{+} p53$^{loxP/loxP}$ apo E$^{-/-}$ further referred to as p53^{del}) or do not express Cre and remain wildtype for p53 (p53$^{loxP/loxP}$ apoE$^{-/-}$ further referred to as p53^{fl}). Mice were genotyped by polymerase chain reaction (PCR) for LysMCre,[13] p53$^{loxP/loxP}$,[12] and apoE[14] status. For experiments, 8 week old male p53^{del} and littermate control p53^{fl} mice were used. Mice were fed a semi-synthetic cholesterol-rich diet composed essentially according to Nishina et al.[15] supplemented with cocoa butter (15%, by weight) and cholesterol (0.25%, by weight), without cholate (Hope Farms, Woerden, The Netherlands). The animals were fed the cholesterol-rich diet for either 7 weeks (early atherosclerosis development, n=18 and n=21 for p53^{del} and p53^{fl} mice, respectively) or 11 weeks (advanced atherosclerosis development, n=17 and n=15 for p53^{del} and p53^{fl} mice, respectively). Mice were given food and water ad libitum. All animal work was approved by institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government.

Quantification of macrophage p53 by Western blotting

Peritoneal macrophages were obtained from p53^{del} and p53^{fl} mice four days after intraperitoneal injection of 1 ml thioglycollate broth (3% wt/vol.) by flushing the peritoneum with 10 ml ice-cold PBS. Macrophages were washed with RPMI 1640 containing 10% foetal calf serum and the cells of each mouse were subsequently divided over two 6-cm culture plates. After 2 hours the duplicate dishes were either mock-treated or incubated with a combination of etoposide (20 μM, Sigma Aldrich) and proteasome inhibitor MG132 (20 μM, Sigma Aldrich) for 2 hours. Subsequently, non-adherent cells were removed by washing twice with ice-cold PBS, and cells were harvested in Giordano buffer ((50 mM Tris HCl, pH 7.4; 250 mM NaCl; 0.1% Triton X-100; 5 mM EDTA), supplemented with protease inhibitors) and analyzed by Western blot as described previously.[16] Lysates from mouse embryo fibroblast derived from wild type mice or homozygous p53/mdm2-deficient double knock out (DKO) mice were used as positive and negative controls, respectively. Blots were incubated with anti-p53 (Ab-7; Merck Biochemicals) and after stripping with anti-αTubulin (CloneDM1A; Sigma-Aldrich) as loading control. Protein bands were detected by enhanced chemiluminescence with the use of the SuperSignal West-Dura kit (Pierce), visualized by autoradiography or by imaging with the ChemiGenius XE3 (Syngene, Cambridge, UK). Quantification of the p53 and tubulin protein levels was performed with use of the Syngene GeneTools software.

Blood sampling and analysis

Blood samples were collected, after 4 hours fasting, in EDTA-coated vials (Sarstedt,

Nümbrecht, Germany) by bleeding from the tail vein. Plasma cholesterol and triglyceride levels were measured enzymatically using commercially available kits (Roche Diagnostics GmbH, Mannheim, Germany). Total blood leukocyte ($CD45^+$), T-cell ($CD3^+$), B-cell ($CD19^+$) and myeloid ($CD11b^+$) numbers were determined by FACS analysis (FACSCalibur, BD Biosciences, California, USA) following standard protocol (TruCOUNT, BD Biosciences, California, USA), as described before.[17]

Atherosclerosis analysis

After either 7 or 11 weeks on a cholesterol-rich diet mice were sacrificed. Hearts were overnight fixed in formalin (pH 7.4) and embedded in paraffin. The aorta was snap-frozen and stored at -80°C. To quantify cross-sectional lesion area in the aortic root formalin-fixed hearts were processed as described before.[10,18] Sections of the aortic root were routinely stained with hematoxylin-phloxine-saffran (HPS) for morphometric analysis. Areas were determined using Leica Qwin image software (EIS, Asbury, NJ).

For *en face* analysis of lesion area in the aortic arch and thoracic aorta, the aorta was cleaned in situ from periadventitial tissue, dissected from the aortic arch down to the iliac bifurcation, opened longitudinally, pinned on a silicone basement and stained with Oil-Red-O. The percentage of surface area covered by atherosclerotic lesions (Oil-Red-O positive area) was quantified starting from the top of the aortic arch 1 cm down towards the thoracic aorta by computer-assisted analysis[19] (n=9 vs. n=10 for early atherosclerosis and n=9 vs. n=9 for advanced atherosclerosis for $p53^{del}$ and $p53^{fl}$ mice, respectively). All analyses were performed double blindly without prior knowledge of the genotype.

Lesion composition analysis

For compositional analysis of the lesions lipid core, necrosis and macrophage content were determined. Lipid core area was defined by the presence of cholesterol clefts, extracellular lipids and the complete absence of nuclei. In addition, necrosis was defined by the presence of pyknosis, karyorrhexis, or complete absence of nuclei.[20] Lipid core area and necrosis area were measured using morphometric analysis, as described above. Serial sections were stained for macrophages using a rabbit antibody to mouse macrophages (AIA-312040, 1/1500, Accurate Chemical and Scientific). AIA-312040-positive areas were quantified as described before.[17]

To label DNA-synthesizing cells the mice received 5'-Bromo-2'-Deoxyuridine (BrdU, Sigma; 60 mg/kg, intraperitoneally) for 3 consecutive days prior to sacrification. Sections were stained for proliferation using a monoclonal mouse anti-BrdU antibody (DAKO A/S Denmark) and for apoptosis using the TUNEL technique according to manufacturer's protocol (In situ cell detection kit POD, Roche Diagnostics GmbH, Mannheim, Germany). Numbers of BrdU- or TUNEL-positive nuclei were expressed per total atherosclerotic lesion area, corrected for lipid core area, as the lipid core is an acellular area of the atherosclerotic lesion without nuclei and therefore by definition does not contain any (BrdU- or TUNEL- positive) nuclei.

Statistical analysis

Statistical analyses were performed using Graphpad Prism 4.03. All data groups were

first tested for normality. If the data were distributed normally, groups were compared using Welch's corrected t-test. If data were not distributed normally, groups were compared using Mann-Whitney rank sum test. Data are expressed as mean±SD. P-value < 0.05 was regarded as significant.

RESULTS

General characteristics of apoE-deficient $p53^{del}$ mice

Littermate male $apoE^{-/-}$ mice either lacking p53 in their macrophages ($p53^{del}$) or wildtype for p53 ($p53^{fl}$) were fed a cholesterol-rich diet for 7 weeks (early atherosclerosis) or 11 weeks (advanced atherosclerosis). During the study, the mice appeared healthy and displayed no signs of abnormalities. As shown in Table 1, both after 7 and 11 weeks of a cholesterol-rich diet challenge, mean body weight was not different between $p53^{del}$ and $p53^{fl}$ mice. Plasma cholesterol, triglyceride and hematocrite levels (Table 1) and lipoprotein profiles (data not shown) did not differ between $p53^{del}$ and $p53^{fl}$ mice. Moreover, absence of macrophage p53 did not affect circulating T-cell, B-cell or myeloid cell concentrations (Table 1) as analyzed after 11 weeks feeding a cholesterol-rich diet.

Table 1. General characteristics of male $p53^{del}$ and $p53^{fl}$ mice after feeding a cholesterol-rich diet for 7 weeks (early atherosclerosis development) or 11 weeks (advanced atherosclerosis development)

		$p53^{fl}$	$p53^{del}$	$p53^{fl}$	$p53^{del}$
Atherosclerosis development		Early		Advanced	
Weight (g)		29.5±2.8	29.8±3.0	29.4±3.6	28.3±2.2
Plasma lipid levels (mmol/L)	Cholesterol	31.7±8.0	28.4±7.4	47.0±16.3	39.9±16.4
	Triglycerides	1.3±0.6	1.2±0.6	1.1±0.4	1.1±0.6
Hematocrite		0.47±0.02	0.47±0.02	0.47±0.02	0.47±0.04
Blood leukocytes (10^6 cells/mL)	T-cells ($CD3^+$)	n.d.	n.d.	1.8±0.4	1.7±0.5
	B-cells ($CD19^+$)	n.d.	n.d.	4.9±1.1	3.7±1.4
	Myeloid cells ($CD11b^+$)	n.d.	n.d.	3.6±0.5	3.7±1.3

n.d. = not determined

To confirm deletion of p53 in macrophages, we analyzed p53 protein levels in thioglycollate-elicited macrophages using Western blot analysis. Yields of thioglycollate-elicited peritoneal macrophages from $p53^{del}$ and $p53^{fl}$ mice were similar (data not shown). Western blot analysis showed that p53 was virtually absent in thioglycollate-elicited macrophages from $p53^{del}$ animals, while it could easily be detected in $p53^{fl}$ macrophages (Figure 1A). To further increase p53 signals in the macrophages, we treated the cells with the DNA damaging agent etoposide in the presence of the proteasome inhibitor MG132. Such treatments are known to increase the levels of the very unstable p53 protein.[21] As can be seen in figure 1A, this treatment strongly increased p53 levels in $p53^{fl}$ macrophages and resulted in the presence of

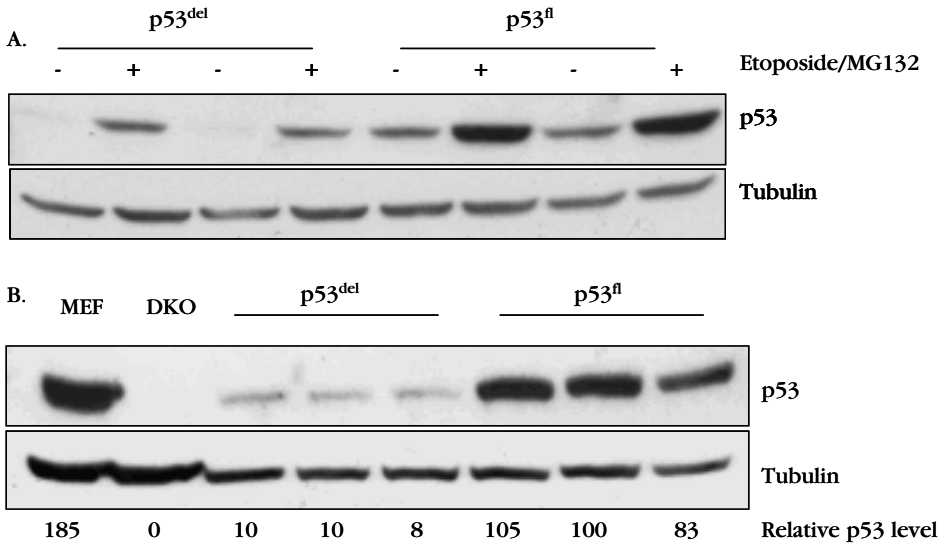


Figure 1. (A.) Protein extracts from $p53^{del}$ and $p53^{fl}$ macrophages, either mock-treated or treated with etoposide and proteasome inhibitor MG132, were analyzed for p53 and α-tubulin expression. (B.) p53 protein expression levels in lysates from representative $p53^{del}$ and $p53^{fl}$ macrophages treated with etoposide and MG132. P53 protein levels in $p53^{del}$ macrophages were correlated to the p53 expression levels in $p53^{fl}$ macrophages, corrected for the loading control α-tubulin. MEF: lysate from wild type mouse embryo fibroblasts (positive control); DKO: lysate from homozygous p53/mdm2 double knock out mouse embryo fibroblasts (negative control)

a p53 band in the $p53^{del}$ cells, indicating that some remaining p53 was left. Semi-quantitative PCR confirmed that some wild type allele was left in the $p53^{del}$ macrophages (data not shown). The relative difference in p53 levels between $p53^{del}$ and $p53^{fl}$ macrophages was quantified using Western blots of etoposide/MG132-treated cells and showed a reduction of approximately 90% in p53 protein levels in $p53^{del}$ macrophages as compared to $p53^{fl}$ mice (Figure 1B). Moreover, titration on Western blot using lysates from $p53^{fl}$ macrophages to obtain p53 protein levels similar to that of $p53^{del}$ macrophages also required over 10-fold dilution confirming the >90% reduction in p53 protein levels (data not shown). These data show that cell specific deletion of p53 results in a strong (>90%) reduction in p53 protein levels in macrophages.

Analysis of atherosclerotic lesion area

Mice fed the cholesterol-rich diet for 7 weeks (early atherosclerosis) or 11 weeks (advanced atherosclerosis) were sacrificed for collection of heart, aorta, and other organs. Morphometric analysis of the total atherosclerotic lesion area in the aortic root showed no difference between $p53^{del}$ and $p53^{fl}$ in early lesion size (P=0.40, Figure 2A and B) and in advanced lesion size (P=0.12, Figure 2A and B). In addition, *en face* analysis of Oil-Red-O stained aortas did also not reveal a difference in relative lesion area between $p53^{del}$ and $p53^{fl}$ mice on early (P=0.84, Figure 3 A and B) and advanced atherosclerosis development (P=0.84, Figure 3A and B). Hence,

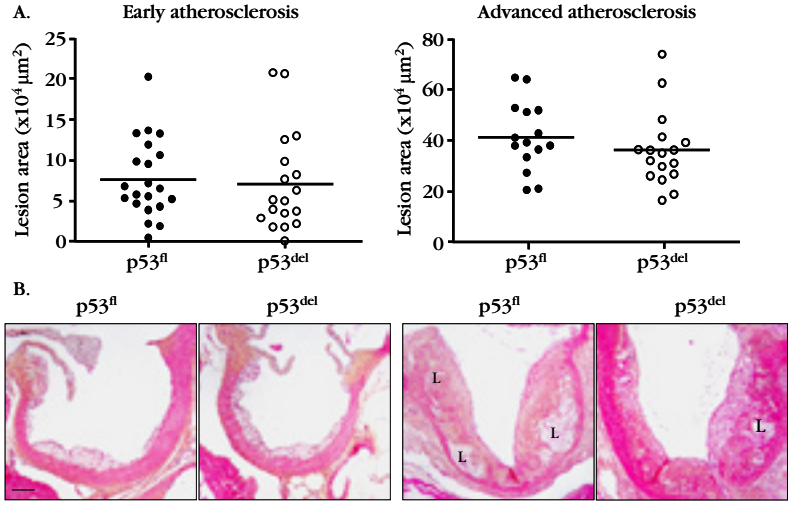


Figure 2. Aortic root atherosclerosis in $p53^{del}$ and $p53^{fl}$ mice. (A.) Early and advanced aortic root atherosclerosis in $p53^{fl}$ (closed circles) and $p53^{del}$ (open circles) mice. Symbols indicate individual mice. Line represents mean area for each group. (B.) Representative photomicrographs of atherosclerotic lesions in $p53^{fl}$ and $p53^{del}$ mice. Serial sections were stained with HPS. L: lipid core, magnification 50x, scale bar 100 µm.

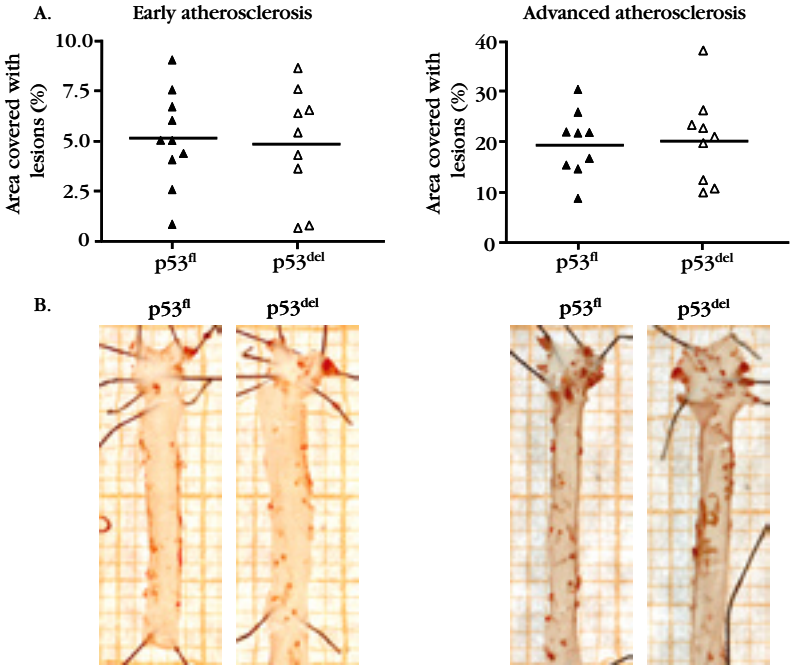


Figure 3. Aortic arch and thoracic aorta atherosclerosis in $p53^{del}$ and $p53^{fl}$ mice. (A.) Early and advanced atherosclerosis in $p53^{fl}$ (closed triangles) and $p53^{del}$ (open triangles) mice. Symbols indicate individual mice. Line represents mean area for each group. (B.) Representative Oil-Red-O stained aortas of $p53^{fl}$ and $p53^{del}$ mice in early and advanced atherosclerosis development.

absence of macrophage p53 did not affect total atherosclerotic lesion area, both in early and advanced atherosclerotic lesions at two different regions in the aortas of apoE-deficient mice.

Cell proliferation and cell death

To investigate whether macrophage p53 deficiency affects cell turnover in the lesions both cell proliferation and cell death were followed during atherosclerosis development. For analysis of cell proliferation mice were injected daily with BrdU for 3 days before the end of the experiment. In both the early and advanced atherosclerosis group the incidence of $BrdU^+$ cells did not differ between $p53^{del}$ and $p53^{fl}$ mice (Figure 4A), indicating that macrophage p53 deficiency did not affect lesional proliferation in both early and advanced atherosclerosis.

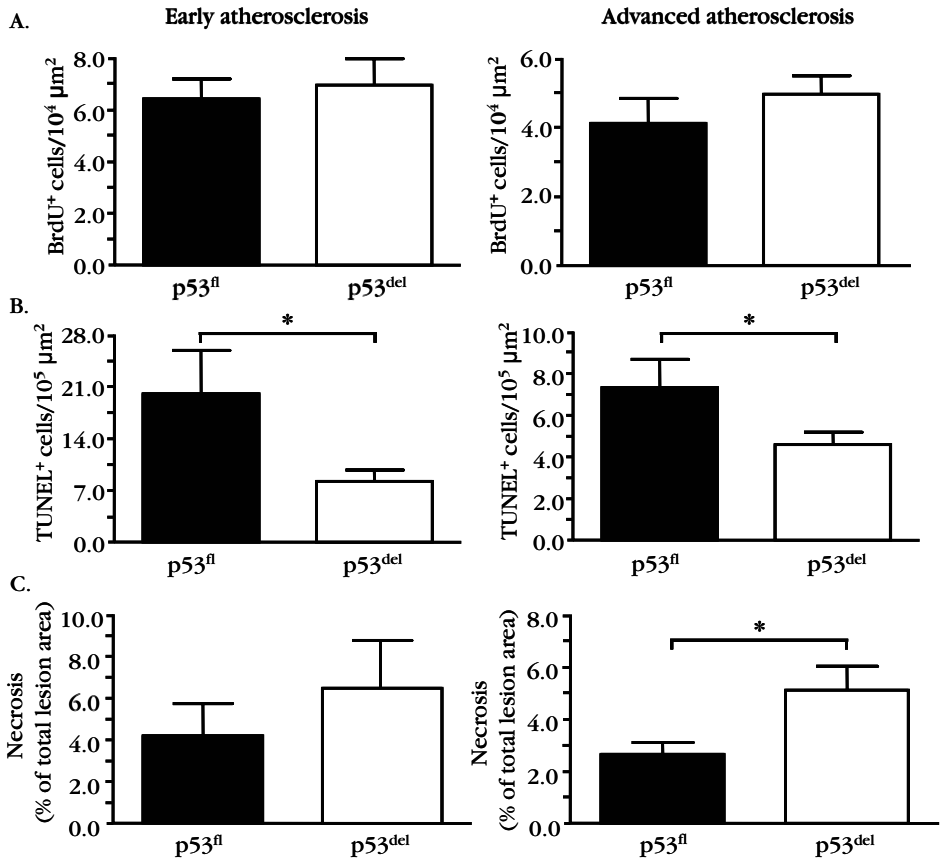


Figure 4. Proliferation, apoptosis and necrosis in $p53^{fl}$ and $p53^{del}$ mice. (A.) Proliferation was detected by BrdU-staining. Bars represent the number of $BrdU^+$ nuclei per lesion area. (B.) Apoptosis was detected by TUNEL staining. Bars represent the number of $TUNEL^+$ nuclei per lesion area. (C.) Necrosis, defined by the presence of pyknosis, karyorrhexis, or complete absence of nuclei, was analyzed on HPS sections. Bars represent necrotic area per lesion area. Black bars, $p53^{fl}$ mice; white bars $p53^{del}$ mice. Error bars indicate SEM. *$P<0.05$.

To investigate cell death in the atherosclerotic lesions apoptosis and necrosis were quantified. Apoptotic cells in the atherosclerotic lesions were identified as TUNEL-positive cells. Strikingly, the incidence of TUNEL-positive nuclei was strongly reduced in $p53^{del}$ mice in both early and advanced atherosclerosis development (-59% and -37%, respectively, $P<0.05$, Figure 4B). In addition to apoptosis, necrosis in the lesions was quantified. Necrosis in the advanced atherosclerosis group was 2-fold increased in the $p53^{del}$ group, as compared to the controls ($P<0.05$, Figure 4C). A similar trend was observed in early atherosclerosis although not significant (Figure 4C). These analyses show that macrophage p53 deletion prevents lesional macrophage apoptosis both in early and advanced atherosclerosis. In addition, prevention of macrophage apoptosis in advanced atherosclerosis switches the cell death pathway towards necrotic cell death.

Macrophages and lipid core

To analyze whether the changes in lesional cell death in $p53^{del}$ mice coincided with changes in atherosclerotic lesion composition we performed a more detailed phenotypic analysis of the lesions. Macrophage area was detected by immunostaining and lipid core area was defined by the presence of cholesterol clefts, extracellular lipids and complete absence of nuclei. In the early atherosclerosis group both macrophage area and lipid core content were not affected by macrophage restricted-p53 deletion (Table 2). However, quantification of macrophage area in the advanced atherosclerosis group revealed a 24% increase in $p53^{del}$ mice as compared to the $p53^{fl}$ control mice ($P<0.05$, Table 2). Interestingly, these changes coincided with a strong 41% reduction in the lipid core content of the lesions in $p53^{del}$ mice as compared to $p53^{fl}$ control mice ($P<0.05$, Table 2, Figure 2B, right panel). Hence, the decrease in lesional apoptosis in $p53^{del}$ mice coincides with a change in cellular lesion composition towards atherosclerotic lesions with an increased macrophage area and a decreased lipid core.

Table 2. Characteristics of early (7 weeks cholesterol-rich diet) and advanced (11 weeks cholesterol-rich diet) atherosclerotic lesions in $p53^{del}$ and $p53^{fl}$ mice

	$p53^{fl}$	$p53^{del}$	$p53^{fl}$	$p53^{del}$
Atherosclerosis development	Early		Advanced	
Lesion macrophage area (% of total lesion area)	66.4±10.5	63.6±11.5	42.2±8.2	52.5±9.8*
Lipid core (% of total lesion area)	3.1±3.6	2.5±3.3	11.1±4.2	6.5±3.7*

* $P<0.05$

DISCUSSION

In the present study, we investigated the role of macrophage p53 in the pathogenesis of atherosclerosis. Absence of macrophage p53 did not affect lesion area of early and advanced atherosclerosis analyzed both at the level of the aortic root and at the level of the aortic arch and thoracic aorta in ApoE-deficient mice. However, detailed analysis of atherosclerosis in the aortic root revealed that apoptosis of foam

cells was strongly reduced in $p53^{del}$ mice at both time points, while proliferation was unaffected. Moreover, the reduction in lesional apoptosis in $p53^{del}$ mice coincided with increased necrosis in the advanced atherosclerotic lesions. The changes in lesional cell death were accompanied by an increase in relative macrophage area and a decrease in relative lipid core area in the advanced atherosclerosis group. Hence, these studies demonstrate that macrophage p53 is a major mediator of foam cell apoptosis and inhibition of this pathway results in a shift of cell death towards necrotic death of lesional macrophages, thereby affecting lesion composition.

Previous bone marrow transplantation (BMT) studies demonstrated that p53 of hematopoietic origin clearly has atheroprotective properties.[10,11] Bone marrow harbors not only progenitors for the myeloid lineage including macrophages, but also the progenitors from which ultimately T-cells and B-cells originate. In atherosclerosis, T-cell derived cytokines as well as B-cell mediated antibody production clearly contribute to atherosclerosis progression.[1] Moreover, p53 is shown to be important in T- and B-cell turnover.[22,23] Hence, the atheroprotective effect of bone marrow derived p53 could be attributed in part to T and B-cell-specific p53. In this light it is of interest that hematopoietic p53 deficiency (i.e. combined lymphocyte and macrophage p53 deficiency) results in strong effects on the size of the lesions, while this study demonstrates that macrophage-restricted p53 deficiency results to more subtle effects confined to lesional macrophage turnover, eventually affecting lesion composition. Combining these data it can be suggested that T/B-cell p53 is involved in modulating size i.e. growth of the lesions. Future studies, using conditional approaches for lymphocyte-specific deletion of p53 may shine more light onto the role of lymphocyte-derived p53 in atherogenesis.

Additionally, the differences in findings on quantitative lesion area may also partly be attributed to the differences in choice of atherosclerotic background. Whereas the LDL-receptor deficient mouse model[11] and the APOE*3-Leiden mouse model[10] show mild atherosclerosis development, the $apoE^{-/-}$ mouse is a model of accelerated atherosclerosis development leading to complex atherosclerotic lesions.[24] Moreover, absence of ApoE inhibits cholesterol efflux from macrophages and thereby more progressively stimulates the formation of foam cells, our cells of interest.[25] Although the clearance of apoptotic cell remnants is attenuated in $apoE^{-/-}$ mice[26] the foam cell rich lesions enabled us to establish the role of macrophage p53 on macrophage turnover in the atherosclerotic lesions.

Based on the current study we hypothesize that in early lesions foam cells preferentially die quickly via a relatively clean apoptotic death which requires p53. However, eventually, the composition of the lesions may prevent complete proper phagocytosis of the apoptotic cells, resulting in secondary necrosis[27] and the formation of a lipid core. In contrast, upon impaired functioning of the p53 pathway in macrophages, lesional foam cells are no longer directed to die via the apoptotic pathway. This is in line with recent *in vitro* data of Mercer et al. showing that $p53^{-/-}$ peritoneal macrophages exhibit reduced apoptosis.[28] As a consequence of the inability to die via apoptosis, death of foam cells may be delayed, but eventually it results in increased death via necrosis, due to extensive lipid accumulation. Previously, it was also shown that inhibition of p53 in mouse embryonic fibroblasts results in a shift of NO-induced cell death towards relatively more necrosis and less apoptosis.[29]

The alternative necrotic death pathway of foam cells may be more slowly and is generally considered to be more detrimental since necrosis leads to the release of pro-inflammatory and pro-thrombotic substances. Hence, macrophage necrosis is detrimental to atherosclerotic lesion development, whereas macrophage apoptosis can be considered more beneficial in lesion development and plaque stability.Therefore, our data point towards an important role for macrophage p53 in controlling foam cell death by induction of apoptosis and prevention of lesional necrosis.

In conclusion, we demonstrate that macrophage p53 guarantees safe foam cell death via apoptosis and thereby prevents lesional necrosis, which directly affects lesion composition. Lesion composition rather than lesion size determines its vulnerability and thereby its clinical relevance.This implies that local targeting of processes that regulate p53 mediated apoptosis may be a powerful target for therapeutic intervention in coronary artery disease. Recently, the use of drug-eluting stents has emerged as a highly promising local approach to reduce in-stent restenosis.[30] The different drugs (i.e. rapamycin, flavopiridol) used in these drug-eluting stents target different apoptosis and proliferative genes, including p53.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J. Jonkers for his contribution to the experiments leading to the manuscript.This work was supported by the Netherlands Heart Foundation (NHS 2000.051) and the Netherlands Organization for Scientific Research (NWO 902-26-242). B.J.M. van Vlijmen is a fellow of the Royal Netherlands Academy of Arts and Sciences, and M.P.J. de Winther is supported by NWO (906.02.075 and 917.66.329).

REFERENCES

1. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685-1695.
2. Lusis AJ. Atherosclerosis. Nature 2000;407:233-241.
3. Libby P. Changing concepts of atherogenesis. J Intern Med 2000;247:349-358.
4. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature JID - 0410462 1993;362:801-809.
5. Meek DW.The p53 response to DNA damage. DNA Repair (Amst) 2004;3:1049-1056.
6. Klein C, Vassilev LT. Targeting the p53-MDM2 interaction to treat cancer. Br J Cancer 2004;91:1415-1419.
7. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer 2002;2:594-604.
8. Ihling C, Menzel G, Wellens E, Monting JS, Schaefer HE, Zeiher AM. Topographical association between the cyclin-dependent kinases inhibitor P21, p53 accumulation, and cellular proliferation in human atherosclerotic tissue. Arterioscler Thromb Vasc Biol 1997;17:2218-2224.
9. Guevara NV, Kim HS, Antonova EI, Chan L.The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo. Nat Med 1999;5:335-339.
10. van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De WB, van Berkel TJ, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circ Res 2001;88:780-786.

11. Merched AJ, Williams E, Chan L. Macrophage-specific p53 expression plays a crucial role in atherosclerosis development and plaque remodeling. Arterioscler Thromb Vasc Biol 2003;23:1608-1614.
12. Jonkers J, Meuwissen R, van Der GH, Peterse H, van d, V, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet 2001;29:418-425.
13. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 1999;8:265-277.
14. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 1992;258:468-471.
15. Nishina PM, Verstuyft J, Paigen B. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. J Lipid Res 1990;31:859-869.
16. Meulmeester E, Maurice MM, Boutell C, Teunisse AF, Ovaa H, Abraham TE, Dirks RW, Jochemsen AG. Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. Mol Cell 2005;18:565-576.
17. Boesten LS, Zadelaar AS, van Nieuwkoop A, Gijbels MJ, de Winther MP, Havekes LM, van Vlijmen BJ. Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-leiden transgenic mice. Cardiovasc Res 2005;66:179-185.
18. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 1987;68:231-240.
19. Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. J Lipid Res 1995;36:2320-2328.
20. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 2003;112:1176-1185.
21. Damalas A, Ben Ze'Ev A, Simcha I, Shtutman M, Leal JF, Zhurinsky J, Geiger B, Oren M. Excess beta-catenin promotes accumulation of transcriptionally active p53. EMBO J 1999;18:3054-3063.
22. Moller MB, Gerdes AM, Skjodt K, Mortensen LS, Pedersen NT. Disrupted p53 function as predictor of treatment failure and poor prognosis in B- and T-cell non-Hodgkin's lymphoma. Clin Cancer Res 1999;5:1085-1091.
23. Ward JM, Tadesse-Heath L, Perkins SN, Chattopadhyay SK, Hursting SD, Morse HC, III. Splenic marginal zone B-cell and thymic T-cell lymphomas in p53-deficient mice. Lab Invest 1999;79:3-14.
24. Rosenfeld ME, Polinsky P, Virmani R, Kauser K, Rubanyi G, Schwartz SM. Advanced atherosclerotic lesions in the innominate artery of the ApoE knockout mouse. Arterioscler Thromb Vasc Biol 2000;20:2587-2592.
25. Meir KS, Leitersdorf E. Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol 2004;24:1006-1014.
26. Grainger DJ, Reckless J, McKilligin E. Apolipoprotein E modulates clearance of apoptotic bodies in vitro and in vivo, resulting in a systemic proinflammatory state in apolipoprotein E-deficient mice. J Immunol 2004;173:6366-6375.
27. Tabas I. p53 and atherosclerosis. Circ Res 2001;88:747-749.
28. Mercer J, Figg N, Stoneman V, Braganza D, Bennett MR. Endogenous p53 protects vascular smooth muscle cells from apoptosis and reduces atherosclerosis in ApoE knockout mice. Circ Res 2005;96:667-674.
29. McLaughlin LM, Demple B. Nitric oxide-induced apoptosis in lymphoblastoid and fibroblast cells dependent on the phosphorylation and activation of p53. Cancer Res 2005;65:6097-6104.
30. van der Hoeven BL, Pires NM, Warda HM, Oemrawsingh PV, van Vlijmen BJ, Quax PH, Schalij MJ, van der Wall EE, Jukema JW. Drug-eluting stents: results, promises and problems. Int J Cardiol 2005;99:9-17.

Chapter 3

Macrophage Rb and atherosclerosis

Macrophage Retinoblastoma deficiency leads to enhanced atherosclerosis development in ApoE-deficient mice

Lianne S.M. Boesten[1,4]; A. Susanne M. Zadelaar[2,4]; Anita van Nieuwkoop[1,4]; Lihui Hu[1]; Jos Jonkers[5]; Bob van de Water[6]; Marion J.J. Gijbels[7,8]; Ingeborg van der Made[7]; Menno P.J. de Winther[7]; Louis M. Havekes[1,2,4]; Bart J.M. van Vlijmen[3]

[1]Dept. of General Internal Medicine, [2]Dept. of Cardiology, [3] Hemostasis and Thrombosis Research Center, Dept. of Hematology, Leiden University Medical Center, Leiden, [4]TNO-Quality of Life/Gaubius Laboratory, Leiden, [5] The Netherlands Cancer Institute, Amsterdam, [6]Div. of Toxicology, Leiden Amsterdam Center for Drug Research [7]Department of Molecular Genetics, Cardiovascular Research Institute Maastricht, [8]Department of Pathology, Maastricht University, Maastricht, The Netherlands

ABSTRACT

The cellular composition of an atherosclerotic lesion is determined by cell infiltration, proliferation and apoptosis. The tumor suppressor gene retinoblastoma (Rb) has been shown to regulate both cell proliferation and cell death in many cell types.

To study the role of macrophage Rb in the development of atherosclerosis, we used apoE-deficient mice with a macrophage-restricted deletion of Rb (Rb^{del} mice) and control littermates (Rb^{fl} mice). After 12 weeks feeding a cholesterol-rich diet, the Rb^{del} mice showed a 51% increase in atherosclerotic lesion area with a 39% increase in the relative number of advanced lesions. Atherosclerotic lesions showed a 13% decrease in relative macrophage area and a 46% increase in relative smooth muscle cell area, reflecting the more advanced state of the lesions. The increase in atherosclerosis was independent of *in vitro* macrophage modified lipoprotein uptake or cytokine production. Whereas macrophage-restricted Rb deletion did not affect lesional macrophage apoptosis, a clear 2.6-fold increase in lesional macrophage proliferation was observed.

These studies demonstrate that macrophage Rb is a suppressing factor in the progression of atherosclerosis by reducing macrophage proliferation.

Cardiovascular disease (CVD) has long been the leading cause of mortality and disability in developed countries, and it is rapidly becoming the number one killer in developing countries.[1] The primary cause of CVD is atherosclerosis, which is a multi-factorial complex disease that starts in childhood and progresses throughout life. Atherosclerosis is initiated by subendothelial accumulation of cholesterol-engorged macrophages. Gradually, these lesions develop towards more advanced lesions characterized by increased deposition of extracellular lipid cores, fibrous material, and necrosis covered by a smooth muscle cell (SMC)-rich cap.[2,3] The cellular composition of an atherosclerotic lesion is an important determinant of its stability. In general, lesions rich in cholesterol-loaded macrophages and extracellular lipid deposits are prone to rupture. On the other hand, lipid-poor lesions with a prominent presence of fibroblasts, SMCs and collagen are relatively stable and resistant to rupture.[4] Cell proliferation and cell death are important processes in regulating macrophage and SMC numbers in the atherosclerotic lesion and may thereby directly influence lesion stability.[5]

Indeed, recent mouse studies demonstrated that genes involved in regulating cell proliferation and cell death play an important role in progression of the atherosclerotic lesion coinciding with changes in the cellular composition. Deletion of the tumor suppressor gene p53, an essential molecule in both cell proliferation and apoptosis, strongly exacerbated atherosclerosis in apoE-deficient (apoE$^{-/-}$),[6] LDL receptor deficient (LDLR$^{-/-}$)[7] and APOE*3-Leiden[8] mice. Recently Merched et al. showed that the p53 downstream target p21, an inhibitor of cell cycle progression via inactivation of cyclin-CDK complexes during the G1 phase of the cell cycle, has strong pro-atherogenic functions.[9] In addition, mouse studies showed that (hematopoietic) inactivation of p27, another cyclin-CDK regulating cell cycle inhibitor, exacerbated atherosclerosis on an apoE$^{-/-}$ background.[10,11] Taken together, these studies indicate an important role for p53, p21, and p27 in controlling atherogenesis.

Retinoblastoma (Rb), the first tumor suppressor gene identified molecularly, also plays a pivotal role in regulating cell proliferation and apoptosis. Rb is a nuclear phosphoprotein that arrests cells during the G1-phase of the cell cycle by forming complexes with the members of the E2F transcription factor family. The E2F family of transcription factors has binding sites in the promoters of many of the genes that are involved in cell-cycle progression.[12] In addition, loss of Rb function can trigger a p53-dependent apoptotic pathway, which may serve as an intrinsic protective mechanism to eliminate cells in which the Rb pathway is deregulated.[13] This is supported by the finding that Rb-deficient mice die in mid-gestation with widespread apoptosis.[14-16]

Although Rb is known to be a major factor in cell cycle progression and cell death, to date the exact role of Rb in atherosclerosis has not been elucidated. To investigate the role of macrophage-Rb in the development of atherosclerosis we crossed mice with a macrophage specific Rb deficiency[17,18] onto an apoE$^{-/-}$ background. Effects of macrophage Rb deletion on atherosclerosis development were evaluated using morphometric analysis of atherosclerotic lesion area and classification of lesion severity. In addition, detailed immunohistochemical analyses were performed to analyze lesion composition and the contribution of cell proliferation and cell death to atherosclerosis development upon macrophage-restricted Rb deletion.

METHODS

Mice and diet

The experimental animals were obtained by combining mice carrying the floxed Rb gene[18] with LysMCre mice,[17] and apoE-deficient mice[19] resulting in mice homozygously deficient for macrophage-Rb and apoE (LysMCre^{+} Rb$^{loxP/loxP}$apoE$^{-/-}$ or Rb^{del}) and control (LysMcre-negative) littermates that only lack apoE (Rb$^{loxP/loxP}$apoE$^{-/-}$ or Rb^{fl}). Mice were genotyped by polymerase chain reaction (PCR) for LysMCre,[17] Rb$^{loxP/loxP18}$ and apoE[19] status. For experiments, 8 weeks old male Rb^{del} (n=17) and littermate control Rb^{fl} (n=13) were used. Mice were fed a semi-synthetic cholesterol-rich diet composed essentially according to Nishina et al.[20] supplemented with cocoa butter (15%, by weight) and cholesterol (0.25%, by weight), without cholate (Hope Farms, Woerden, The Netherlands). Mice were given food and water ad libitum. All animal work was approved by institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government.

Quantification of macrophage Rb deletion by Southern blotting

Southern blotting for the quantification of the deletion of Rb was performed as described before.[21] Mice were intraperitoneally injected with 1 ml thioglycollate broth (3% wt/vol.). After 4 days, DNA was isolated from PBS flushed peritoneal macrophages and digested with Pst1. Southern blots were hybridized with a 450-bp Pst1-PvuII probe, detecting a 5.0 kb wild type allele and floxed allele and a 4.5 kb deleted allele.

Blood sampling and analysis

Blood samples were collected in EDTA-coated vials (Sarstedt, Nümbrecht, Germany) by bleeding from the tail vein. Plasma cholesterol and triglyceride levels were measured enzymatically using commercially available kits (Roche Diagnostics GmbH, Mannheim, Germany). Total blood leukocyte (CD45^{+}), T-cell (CD3^{+}), B-cell (CD19^{+}) and monocyte/granulocyte (CD11b^{+}) numbers were determined by FACS analysis (FACSCalibur, BD Biosciences, California, USA) following standard protocol (TruCOUNT, BD Biosciences, California, USA), as described before.[22] Qualitative analysis of peripheral blood was performed on May-Grunwald-Giemsa (MGG) stained blood smears, according to standard procedures. The acute phase inflammatory markers Serum Amyloid A (SAA, BioSource International, Inc, Camarillo, CA) and fibrinogen[23] were analyzed by ELISA on plasma samples according to standard protocols.

Atherosclerosis analysis

After 12 weeks on a cholesterol-rich diet, mice were sacrificed. Heart and aorta were perfused with PBS, formalin fixed (pH 7.4) overnight and embedded in paraffin. From the entire aortic root area of the heart, four 5 μm cross-sections with an interval of 40 μm were used for quantification of atherosclerotic lesion area.[24] Sections of the aortic root area were routinely stained with hematoxylin-phloxine-saffran (HPS) for morphometric analysis, and characterization of the lesion and with Sirius red for collagen. Areas were determined using Leica Qwin image software (EIS, Asbury, NJ).

Lipid core area was defined by the presence of cholesterol clefts and extracellular lipids. In addition, necrosis was defined by the presence of pyknosis, karyorrhexis, or complete absence of nuclei.[25] Lipid core area and necrosis area were measured using morphometric analysis, as described above.

Atherosclerotic lesions were classified on severity (i.e. early lesions or advanced lesions) as described before.[22] The number observed in each category is expressed as a percentage of the total number of lesions observed within one group of mice (Rb^{del} or control Rb^{fl} group). All analyses were performed double blindly without prior knowledge of the genotype.

Immunohistochemistry

Serial sections were stained for macrophages and SMCs using a rabbit antibody to mouse macrophages (AIA-312040, 1/1500, Accurate Chemical and Scientific) and a monoclonal alpha-smooth muscle cell actin antibody (1/1600, DAKO A/S, Denmark), respectively. AIA-312040-positive and alpha-smooth muscle cell actin-positive areas were quantified using threshold values that discriminated between antibody-positive and antibody negative lesion areas, as described before.[8] In addition, nuclear counting in AIA-312040-positive and alpha-smooth muscle cell actin-postive areas was performed for quantification of macrophage and SMC numbers. Analysis on individual lesions was performed on lesions ranging from 0-50x10^3 μm^2 (n=16 individual lesions for both Rb^{del} and Rb^{fl} mice) and 50-150x10^3 μm^2 (n=24 and n=16 individual lesions for Rb^{del} and Rb^{fl} mice, respectively). To label proliferating cells, sections were stained using a monoclonal rat anti-mouse Ki-67 antibody (DAKO A/S Denmark) and for apoptosis using the Terminal Deoxynucleotidyl Transferase End-Labeling (TUNEL) technique (In situ cell detection kit POD, Roche Diagnostics GmbH, Mannheim, Germany).[8,26] Macrophages and SMCs positive for either Ki-67 or TUNEL were expressed as a percentage of the total number of macrophages and SMCs present.[22]

To analyze monocyte differentiation, spleen cryo-sections of Rb^{del} and Rb^{fl} mice were stained for macrophage markers using the antibodies: FA-11 (a kind gift from S. Gordon, Oxford University, UK), Mac1 (a kind gift from G. Kraal, VUMC, The Netherlands), ERTR9 (a kind gift from G. Kraal, VUMC, The Netherlands), and F4/80 (a kind gift from W. Buurman, UM, The Netherlands).

Uptake of modified lipoproteins and cytokine measurements in *in vitro* cultured bone marrow derived macrophages

Bone marrow-derived macrophages (BMM) were obtained according to standard procedures. Culturing, analyses and modified lipoprotein uptake experiments were performed as described by Kanters et al.[25] TNFα and interleukin-10 production were quantified at 0, 3, 6, and 24 hours after LPS (O111:B4, Sigma-Aldrich) stimulation (100 ng/ml) by ELISA (Biosource, Etten-Leur, The Netherlands).[25]

Statistical analysis

Data were analyzed using the non-parametric Mann-Whitney rank sum test (Graphpad Software, San Diego California USA). Data are expressed as mean±SD. Frequency data for lesion classification were compared by means of the Fisher's exact test.

Correlation analysis was performed using the Spearman's rank order correlation. *P*-value < 0.05 was regarded as significant.

RESULTS

General characteristics of apoE-deficient Rb^{del} mice

Male Rb^{del} (n = 17) and control Rb^{fl} (n = 13) littermates were fed a cholesterol-rich diet from 8 weeks of age on. During the study, the mice appeared healthy and displayed no signs of abnormalities. As shown in Table 1, after 12 weeks of a cholesterol-rich diet challenge, mean body weight was not different between Rb^{del} and Rb^{fl} mice. Plasma cholesterol and triglyceride levels (Table 1) and lipoprotein profiles (data not shown) were not different between Rb^{del} and Rb^{fl} mice. Moreover, absence of macrophage Rb did not affect hematocrite, $CD3^+$, $CD19^+$, and $CD11b^+$ leukocyte concentrations (Table 1). Subdivision of the $CD11b^+$ population did not reveal an effect of LysMCre-induced Rb deletion on either circulating $CD11b^+$ monocyte or circulating $CD11b^+$ granulocyte numbers. Additional, detailed pathological analysis of May-Grunwald Giemsa (MGG) stained blood smears also did not yield any abnormalities in the peripheral blood of Rb^{del} mice.

The degree of Rb deletion is dependent on the effectiveness of the Cre-recombinase in deleting the loxP-flanked Rb allele. Yields of thioglycollate-elicited peritoneal macrophages isolated from Rb^{fl} and Rb^{del} mice, for quantification of Rb deletion by Southern blot analysis, were similar (data not shown). Southern blot analysis revealed that in Cre-recombinase expressing mice the deletion of the floxed allele was almost complete in the heterozygous state ($LysMCre^+Rb^{loxP/WT}$;Figure 1A, lane 2) and complete in the homozygous state ($LysMCre^+Rb^{loxP/loxP}$; Figure 1A, lane 3) confirming effective deletion of Rb in macrophages.

Table 1. General characteristics of male Rb^{del} and Rb^{fl} mice after 12 weeks of feeding a cholesterol-rich diet.

		Rb^{fl}	Rb^{del}
Weight (g)		26.0±0.9	26.0±3.0
Plasma lipid levels (mmol/L)	Cholesterol	31.7±9.5	28.6±8.3
	Triglycerides	1.8±1.0	1.8±1.3
Hematocrite		0.49±0.02	0.49±0.03
Blood leukocytes (10^6 cells/mL)	$CD3^+$ cells	3.5±1.3	3.1±0.9
	$CD19^+$ cells	8.1±2.4	7.2±2.8
	$CD11b^+$ cells	4.0±0.6	4.4±1.2
	$CD11b^+$ monocytes	1.0±0.3	0.8±0.2
	$CD11b^+$ granulocytes	3.2±0.6	3.6±1.1
Inflammation parameters (μg/ml)	SAA	41.7±10.1	140.4±186.7
	Fibrinogen	2.5±0.5	2.2±0.2

Analysis of atherosclerotic lesion area

Comparable body weight, blood composition, plasma lipid levels, and complete deletion of the Rb-floxed allele in $LysMCre^+Rb^{loxP/loxP}$ mice, allowed us to selectively

dissect the effect of macrophage Rb on atherosclerosis development in these mice. Mice fed the cholesterol-rich diet for 12 weeks were sacrificed for collection of heart, aorta, and other organs. Morphometric analysis of total atherosclerotic lesion area revealed a significant 51% increase in the Rb^{del} mice, as compared with control Rb^{fl} mice (26.8±13.2 vs. 17.7±9.4 x10^4 μm^2, P=0.04, Figure 1B). In addition, lesion classification (early vs. advanced) showed that Rb^{del} mice had a significantly lower incidence of early lesions (21.6% vs. 43.5%, P=0.04) and a significantly increased incidence of advanced lesions (78.4% vs. 56.4%, P=0.04) as compared to Rb^{fl} control mice, indicating an enhanced progression of atherosclerosis in Rb^{del} mice (Figure 1C).

To evaluate whether this enhanced atherosclerosis in Rb^{del} mice is due to an enhanced uptake of modified LDL and/or differences in inducble cytokine production in macrophages, we performed *in vitro* analysis using bone marrow derived macrophages (BMM). BMM from Rb^{del} and Rb^{fl} mice did not differ in endocytosis of either oxidized LDL or acetylated LDL in two different doses (Figure 2A). LPS stimulation of BMM resulted in an increase in both TNFα and Il-10 production in Rb^{del} and Rb^{fl} BMM (Figure 2B and C). However, Rb^{del} BMM did not differ from Rb^{fl} control BMM in LPS-induced TNFα and Il-10 production. These data show that Rb^{del} mice have normal modified LDL uptake and are not affected in the production of either the pro-inflammatory cytokine TNFα or the anti-inflammatory cytokine Il-10.

In addition, plasma levels of the acute phase inflammatory marker Serum Amyloid A (SAA) and fibrinogen were not significantly affected (Table 1), although

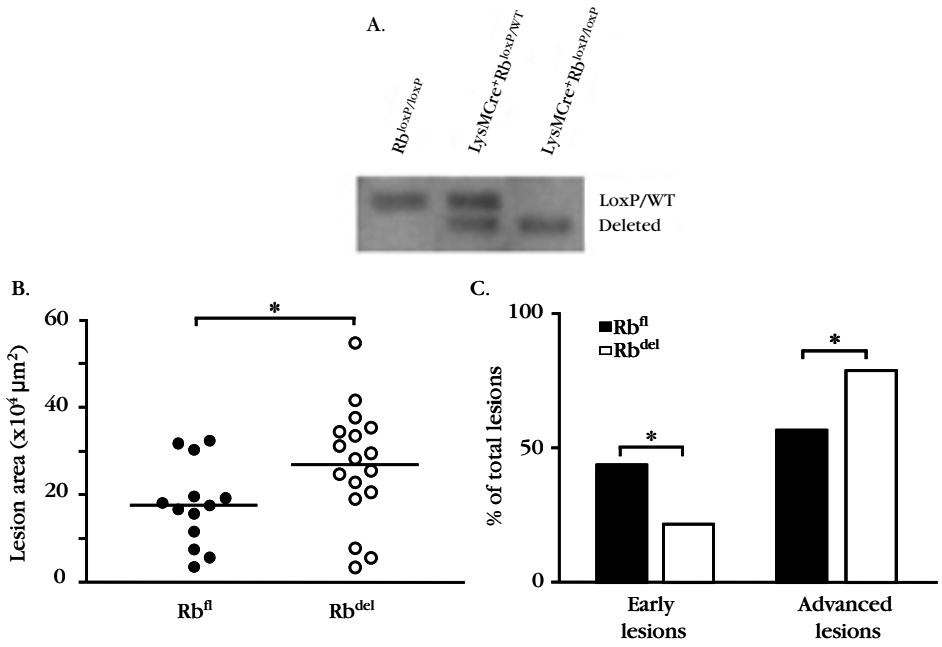


Figure 1. (A.) Southern blot analysis of thioglycollate-elicited peritoneal macrophages. (B.) Aortic atherosclerotic lesion area in Rb^{fl} (closed circles, n = 13) and Rb^{del} (open circles, n = 17) mice. Line represents mean area for each group. (C.) Lesion classification of Rb^{fl} (black bars) and Rb^{del} (white bars) mice. *P<0.05.

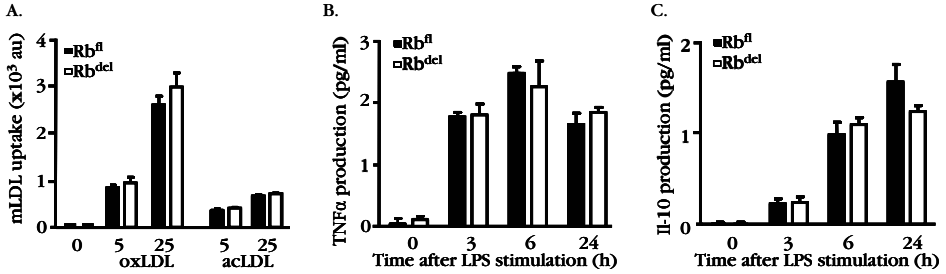


Figure 2. (A.) Uptake of oxidized LDL and acetylated LDL in different doses (μg/mL) by BMM from Rb^{fl} (black bars) and Rb^{del} (white bars) mice. (B, C) Rb^{fl} and Rb^{del} macrophages were stimulated with LPS. TNFα (B.) and Il-10 (C.) production were measured in the supernatant by ELISA.

SAA showed a tendency towards an increase. To evaluate a possible relationship between inflammation and atherosclerotic lesion area a Spearman's rank order correlation analysis was performed. No correlations were found for either Rb^{del} or Rb^{fl} mice between SAA levels and atherosclerotic lesion areas (Correlation coefficient of 0.06 for Rb^{del} (P = 0.83) and 0.33 for Rb^{fl} (P = 0.35) mice). In line with this, no correlations were found either between fibrinogen levels and atherosclerotic lesion areas (Correlation coefficient of -0.04 for Rb^{del} (P = 0.89) and -0.30 for Rb^{fl} (P = 0.32) mice). These data indicate that inflammation, as measured by plasma SAA and fibrinogen levels, does not contribute to the observed difference in atherosclerotic area between Rb^{del} and Rb^{fl} mice.

Moreover, Rb plays a critical role in monocytic differentiation.[27] To evaluate whether the enhanced atherosclerosis in Rb^{del} mice is due to a defect in monocyte differentiation into macrophages, we immunohistochemically stained spleen sections of Rb^{del} and Rb^{fl} mice. No differences were found on the differentiation markers FA-11, Mac1, ERTR9, and F4/80 between Rb^{del} and Rb^{fl} mice (data not shown) indicating that macrophage specific deletion of Rb does not affect the differentiation of monocytes into macrophages.

Histopathological analysis of atherosclerotic lesions

Histopathological analysis of atherosclerotic lesions in the aortic valve area revealed the presence of foam cell rich fatty streaks and fibrous plaques with a lipid core and a cap covering necrotic material, cholesterol clefts and extracellular lipids in both Rb^{del} and Rb^{fl} mice. Figure 3 shows representative photomicrographs of atherosclerotic lesions in Rb^{fl} (A-C) and Rb^{del} (D-F) mice stained with HPS for morphometric analysis (A and D), for macrophages (B and E) and for smooth muscle cells (C and F). Quantification of the lesion area positive for the anti-mouse macrophage polyclonal antibody AIA-312040 in Rb^{del} mice showed a significant 13% decrease in lesion macrophage area as compared to the Rb^{fl} control mice (P=0.03, Table 2). However, analysis of macrophage area on individual lesions within the same size range (0-50x10^3 μm^2 and 50-150x10^3 μm^2, Table 2) showed no difference in macrophage area between Rb^{del} and Rb^{fl} mice. These data indicate that the decrease in relative macrophage area in the lesions of Rb^{del} mice is the result of an enhanced

Table 2. Characteristics of atherosclerotic lesions in Rb^{del} and Rb^{fl} mice.

	Rb^{fl}	Rb^{del}
Lesion macrophage area (% of total lesion area)	62.5±13.5	54.2±12.7*
Individual lesion size		
0-50x10^3 µm^2	74.7±20.0	75.2±18.4
50-150x10^3 µm^2	48.6±18.7	46.3±14.6
Macrophage density (per 10^3 µm^2 macrophage area)	3.6±1.0	3.1±1.2
Lesion SMC area (% of total lesion area)	2.8±1.6	4.1±1.8*
Individual lesion size		
0-50x10^3 µm^2	2.8±1.9	4.6±2.6
50-150x10^3 µm^2	3.5±2.5	4.7±2.6
SMC density (per 10^3 µm^2 SMC area)	0.9±0.5	1.2±0.9
Collagen area (% of total lesion area)	18.5±10.0	24.9±12.3
Lipid core area (% of total lesion area)	5.3±4.5	6.8±3.2
Necrosis area (% of total lesion area)	2.7±3.2	4.3±4.0

* P<0.05

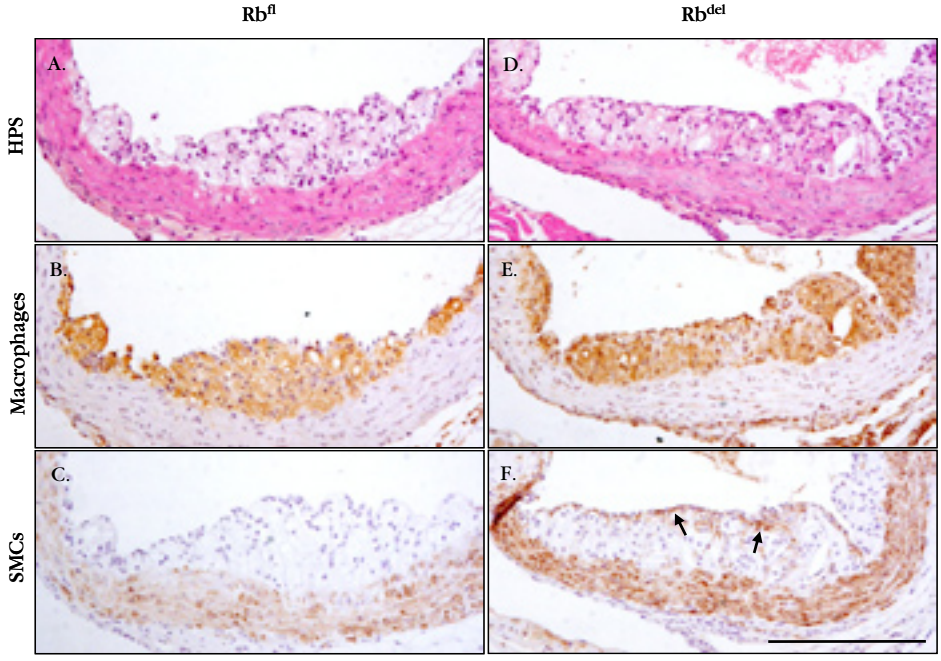


Figure 3. Representative lesions of Rb^{fl} (A-C.) and Rb^{del} (D-F) mice. Sections were stained with HPS (A and D.), macrophage-specific antibody (B and E.) or alpha-smooth muscle cell actin antibody (C and F). Arrows indicate the presence of a SMC rich cap (F). Magnification 100x, scale bar 100 µm.

progression of atherosclerosis, a primary characteristic of advanced lesion formation,[28] rather than an additional effect of macrophage specific Rb deletion on lesion composition. In the sections stained with the AIA-312040 antibody, it was demonstrated that monocyte adherence to the lesions, as a parameter for endothelial cell activation, was not affected (4.4±2.5 vs. 4.4±2.3 monocytes/lesion for Rb^{del} and Rb^{fl} mice, respectively). Analysis of the SMC area showed a significant 46% increase in lesions in the Rb^{del} mice (P=0.05, Table 2). Analysis of SMC area on individual lesions within the same size range (0-50x10^3 μm^2 and 50-150x10^3 μm^2, Table 2) showed no difference between Rb^{del} and Rb^{fl} mice indicating that the increase in SMC area was also a result of the enhanced progression of atherosclerotic lesions,[28] rather than an additional macrophage-specific Rb effect on lesion composition. The increase in SMC area in the Rb^{del} mice coincided with a non-significant 35% increase in collagen area (P=0.26, Table 2). Both parameters indicate the presence of a thicker fibrous cap in Rb^{del} mice. Nuclear counting revealed no effect of macrophage Rb deficiency on macrophage and SMC numbers (macrophage and SMC density, Table 2) in the atherosclerotic lesions. The lipid core defined by the presence of cholesterol clefts and extracellular lipids was not shown to be affected by macrophage Rb deficiency (P=0.23, Table 2). To complete lesion composition analysis the necrotic core was analyzed. Rb^{del} mice showed a (non-significant) doubling of the necrotic core as compared to Rb^{fl} mice (P=0.13, Table 2).

Cell death and cell proliferation

To investigate whether macrophage-specific deletion of Rb affects cell death in the atherosclerotic lesions, TUNEL-positivity was determined. Lesions of Rb^{del} mice showed an incidence of 1.3±1.0% TUNEL-positive macrophages which did not differ significantly from the incidence of 1.1±1.0% TUNEL-positive macrophages for Rb^{fl} mice (Figure 4A). In addition, the incidence of TUNEL-positive SMCs in Rb^{del} mice (0.5±0.5%) did not differ significantly from the Rb^{fl} control group (0.5±0.5%, Figure 4A), indicating that macrophage Rb deficiency did not affect lesional apoptosis.

To study the effect of macrophage-specific Rb deletion on lesional proliferation nuclear protein Ki-67-positivity was determined for both lesional macrophages and SMCs. As shown in Figure 4B, lesions of Rb^{del} mice showed a significant 2.6-fold increase in the incidence of Ki-67-positive macrophages as compared to lesions of Rb^{fl} mice (3.3±2.4% vs. 1.3±1.2% Ki-67-positive macrophages for Rb^{del} and Rb^{fl} mice, respectively, P=0.02, Figure 4B and C). The incidence of Ki-67-positive SMC nuclei was not affected by the macrophage Rb genotype (0.7±0.6% vs. 0.5±0.4% Ki-67-positive SMCs for Rb^{del} and Rb^{fl} mice, respectively, Figure 4B). Hence, the increased atherosclerosis in Rb^{del} mice coincides with increased proliferation of macrophages in the lesions of these mice.

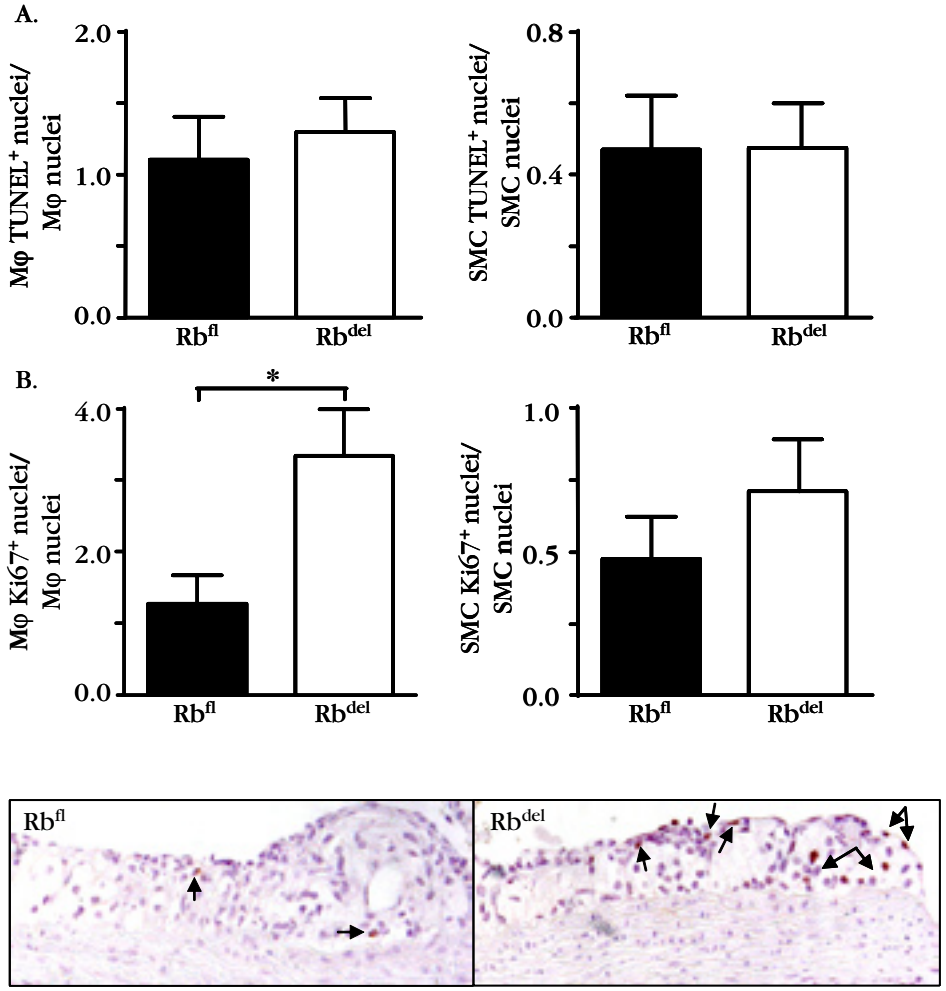


Figure 4. (A.) Apoptosis and (B.) proliferation in macrophages (left panel) and SMCs (right panel) in the atherosclerotic lesions of Rb^{fl} (black bars) and Rb^{del} (white bars) mice. Error bars indicate SEM. *P<0.05. (C.) Representative Ki-67 stained lesions of Rb^{fl} (left panel) and Rb^{del} (right panel) mice. Arrows indicate Ki-67 positive cells. Magnification 150x.

DISCUSSION

In the present study, we investigated the role of macrophage Rb in the pathogenesis of atherosclerosis. ApoE-deficient mice lacking macrophage Rb displayed accelerated atherosclerosis. This was characterized by the presence of more advanced lesions that were rich in smooth muscle cells and collagen and poor in macrophages (Figure 5). *In vitro* analysis showed that the enhanced atherosclerosis in macrophage Rb deficient mice was independent of modified lipoprotein uptake or cytokine production. Whereas macrophage specific Rb deletion did not affect

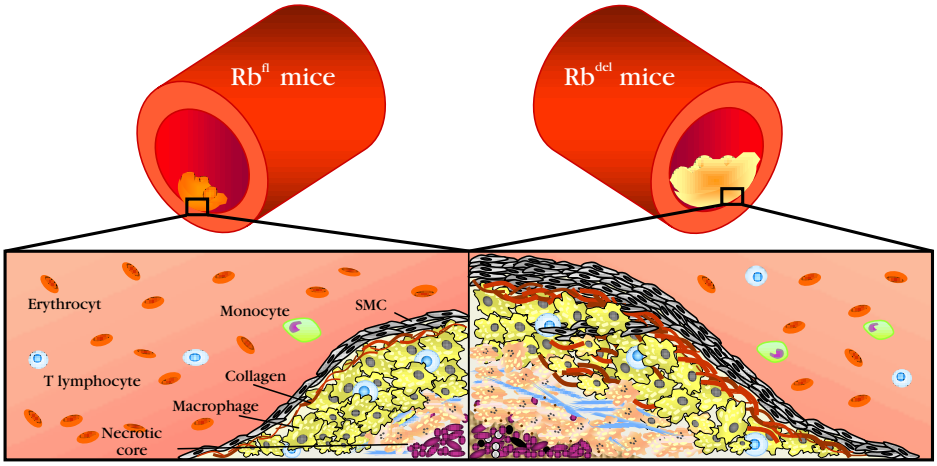


Figure 5. Schematic drawing illustrating lesion development upon macrophage-restricted Rb deletion. Macrophage-restricted Rb deletion leads to enhanced atherosclerosis development characterized by increased lesion area and the presence of more advanced lesions rich in smooth muscle cells and collagen and poor in macrophages.

the systemic inflammation markers SAA and fibrinogen, monocyte differentiation or macrophage apoptosis, lesional macrophage proliferation was strongly increased. These studies demonstrate that macrophage Rb is a suppressing factor in the progression of atherosclerosis.

Bennett et al. demonstrated that human plaque-derived VSMCs show reduced proliferation and earlier senescence due to an increased ratio of the active form of Rb.[29] In addition, localized infection of the arterial wall with an adenovirus encoding a constitutively active non-phosphorylatable form of Rb significantly reduced medial vascular smooth muscle cell proliferation and restenosis in two animal models of balloon angioplasty.[30] Moreover, a phosphorylation-competent full-length and a truncated form of Rb inhibited vascular smooth muscle cell proliferation and neointima formation.[31] These data, together with our data, indicate that Rb can be a strong modulator of vascular disease both at the level of SMCs and macrophages.

To define the molecular pathways underlying Rb function in vascular disease our findings may support an initial mechanistic explanation for a role of macrophage Rb in atherosclerosis. We showed that increased macrophage proliferation may underlie the formation of more advanced lesions in Rb^{del} mice. Surprisingly, increased macrophage proliferation did not coincide with increasing effects on macrophage area or number. Remarkably, macrophage area was even decreased upon Rb deletion. Detailed analysis of individual lesions showed that the decrease in macrophage area reflected the more advanced state of the lesions in Rb^{del} mice, rather than an additional effect of Rb deletion on lesion (macrophage) composition.[4] In general, macrophages produce growth factors, cytokines, chemokines and metallopoteinases which play an important role in the development and progression of atherosclerotic lesions. This diverse array of bio-active molecules activates the surrounding endothelium and SMCs. Following, the lesion becomes increasingly complex with the presence of SMCs, lipid-laden macrophages, T-lymphocytes, a necrotic core and

cholesterol crystals covered by a fibrous cap.[2,3,32] During atherosclerosis development, the macrophage population in Rb^{del} mice exhibited an increased proliferative state. We hypothesize that this increased proliferative state resulted in enhanced lesion pathology via increased production of growth factors, cytokines, chemokines and metalloproteinases. This array of proteins stimulated the formation of an advanced atherosclerotic lesion, characterized by a relative decrease in macrophage content and a relative increase in SMC content. Hence our data suggest that Rb protects against excessive macrophage proliferation and thereby against enhanced atherosclerosis progression.

Bergh et al. showed that Rb plays a critical role in monocytic and neutrophilic lineage commitment of normal human bone marrow progenitor cells.[27] In addition, macrophages play a central role in red blood cell development. Erythroblast islands, required for red blood cell development, are present in the liver of fetuses and in the bone marrow of adults. These erythroblast islands consist of a central macrophage that supplies nutrients to the surrounding erythroblasts and degrades the nuclei from the enucleated circulating red blood cells. Recently, it was shown that Rb-deficient murine fetuses have a severe defect in macrophage maturation and fail to form functional erythroblast islands resulting in lethal anemia characterized by the persistence of nucleated erythroid cells in the peripheral blood.[33] However, we did not observe differences in monocyte differentiation analyzed using different macrophage markers (e.g. FA-11, Mac1, ERTR9, and F4/80) or in macrophage maturation characterized by an aberrant blood composition (e.g. nucleated red blood cells) as analyzed by FACS, hematocrite levels and May-Grunwald Giemsa stained blood smears. Thus, differences in either monocyte differentiation or macrophage maturation during embryonic development or in adult life that might affect atherosclerosis development in Rb^{del} mice have not been found.

In addition to defining the molecular pathways underlying Rb function in atherosclerosis our findings may also have direct clinical significance. Unregulated cell proliferation has been implicated in the etiology of a variety of vascular proliferative diseases including atherosclerosis and (in-stent) restenosis after PTCA or placement of a stent.[34-37] Recently, the use of drug-eluting stents has emerged as a highly promising approach to reduce in-stent restenosis.[38] In addition to the vascular smooth muscle cells (VSMCs), macrophages also play a crucial role in the formation of neointima via the stimulation of VSMC migration and proliferation at the injury site.[39] The different drugs used in drug-eluting stents most often target proliferative state of the cells. Therefore, regulation of the Rb gene via drug-eluting stents might prove a promising approach since activation of Rb both at the level of VSMCs [30,31] and macrophages (present study) is shown to be beneficial for inhibition of vascular disease.

Our observation that Rb^{del} mice after 12 weeks feeding a high-fat diet have a tendency towards increased plasma SAA levels, may point towards a role for Rb in modulating the inflammatory status in apoE-deficient mice. This might not be a very plausible explanation, however, for the following reasons. Firstly, our *in vitro* experiments showed that Rb^{del} macrophages do not respond differently from Rb^{fl} macrophages regarding LPS-induced production of TNFα and Il-10. Secondly, SAA and fibrinogen levels on a standard chow diet (data not shown) and fibrinogen levels

after 12 weeks feeding a high-fat diet (Table 1) did not differ between Rb^{del} and Rb^{fl} mice. Finally, correlation analysis for SAA (and fibrinogen) and the total lesion areas in both Rb^{del} and Rb^{fl} mice negated that inflammation contributes to atherosclerosis development in these mice. The absence of a correlation between SAA and total area in this mouse model also excludes the possibility that the observed increased SAA level is the result of an increase in lesion area as recently described by O'Brien et al.[40] Thus, changes in inflammatory status due to macrophage specific Rb deletion as an explanation for the observed increase in atherosclerosis is highly unlikely.

In contrast to conditional $Rb^{loxP/loxP}$ mice, conventional homozygous Rb deficient mice die at mid-gestation, displaying impaired neurogenesis, fetal liver erythropoiesis and lens development, which was related to a defect in proliferation and end-stage differentiation in several lineages.[14-16] Pituitary-specific inactivation of the Rb gene resulted in pituitary tumors due to hyperproliferation and impairment of apoptosis.[21] In the present study, LysMCre-mediated deletion of the floxed-Rb allele did not result in any abnormality at the level of circulating $CD45^+/CD11b^+$ blood leukocytes or resident cells in liver, spleen or bone marrow (data not shown). Hence, the Rb gene product has a pivotal role in controlling cell division, cell death and cell differentiation of neuronal, erythropoetic, lens and pituitary cells, but not in controlling these cellular processes in the blood granulocyte and monocyte population. Moreover, the application of the site-specific recombinase technology (i.e. the LysMcre mouse model in combination with the $Rb^{loxP/loxP}$ mouse model) creates a first and unique opportunity to study the role of macrophage Rb in pathophysiology of disease in general.

In conclusion, we demonstrate that deletion of the tumor suppressor gene Rb specifically in macrophages enhances atherosclerosis development. Combined with our previous and comparable findings for p53 in macrophages,[8] we conclude that Rb and p53, in addition to their suppressing function in cancer, have a suppressing function for atherosclerosis development.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. T. Kooistra and Dr. K. Willems-van Dijk for critically reading the manuscript, B. Evers for Southern blot analysis and M. van Nimwegen for FACS analysis. This work was supported by the Netherlands Heart Foundation (NHS 2000.051) and the Netherlands Organization for Scientific Research (NWO 902-26-242). M.P.J. de Winther is a NWO-fellow (906-02-075), and B.J.M. van Vlijmen is a fellow of the Royal Netherlands Academy of Arts and Sciences.

REFERENCES

1. Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, Stefanadis C, Moreno P, Pasterkamp G, Fayad Z, Stone PH, Waxman S, Raggi P, Madjid M, Zarrabi A, Burke A, Yuan C, Fitzgerald PJ, Siscovick DS, de Korte CL, Aikawa M, Juhani Airaksinen KE, Assmann G, Becker CR, Chesebro JH, Farb A, Galis ZS, Jackson C, Jang IK, Koenig W, Lodder RA, March K, Demirovic J, Navab M, Priori SG, Rekhter MD, Bahr R, Grundy SM, Mehran R, Colombo A, Boerwinkle E, Ballantyne C, Insull W, Jr., Schwartz RS, Vogel R, Serruys PW, Hansson GK, Faxon DP, Kaul S, Drexler H, Greenland P, Muller JE, Virmani

R, Ridker PM, Zipes DP, Shah PK, Willerson JT. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. Circulation 2003;108:1664-1672.
2. Libby P. Inflammation in atherosclerosis. Nature 2002;420:868-874.
3. Lusis AJ. Atherosclerosis. Nature 2000;407:233-241.
4. Fuster V, Badimon J, Chesebro JH, Fallon JT. Plaque rupture, thrombosis, and therapeutic implications. Haemostasis 1996;26 Suppl 4:269-284.
5. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature JID - 0410462 1993;362:801-809.
6. Guevara NV, Kim HS, Antonova EI, Chan L. The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo. Nat Med 1999;5:335-339.
7. Merched AJ, Williams E, Chan L. Macrophage-specific p53 expression plays a crucial role in atherosclerosis development and plaque remodeling. Arterioscler Thromb Vasc Biol 2003;23:1608-1614.
8. van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De WB, van Berkel TJ, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circ Res 2001;88:780-786.
9. Merched AJ, Chan L. Absence of p21Waf1/Cip1/Sdi1 modulates macrophage differentiation and inflammatory response and protects against atherosclerosis. Circulation 2004;110:3830-3841.
10. Diez-Juan A, Andres V. The growth suppressor p27(Kip1) protects against diet-induced atherosclerosis. FASEB J 2001;15:1989-1995.
11. Diez-Juan A, Perez P, Aracil M, Sancho D, Bernad A, Sanchez-Madrid F, Andres V. Selective inactivation of p27(Kip1) in hematopoietic progenitor cells increases neointimal macrophage proliferation and accelerates atherosclerosis. Blood 2004;103:158-161.
12. Dyson N. The regulation of E2F by pRB-family proteins. Genes Dev 1998;12:2245-2262.
13. Harbour JW, Dean DC. Rb function in cell-cycle regulation and apoptosis. Nat Cell Biol 2000;2:E65-E67.
14. Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van d, V, Hooper ML, Berns A, te RH. Requirement for a functional Rb-1 gene in murine development. Nature 1992;359:328-330.
15. Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA. Effects of an Rb mutation in the mouse. Nature 1992;359:295-300.
16. Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH, Bradley A. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 1992;359:288-294.
17. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 1999;8:265-277.
18. Marino S, Vooijs M, van Der GH, Jonkers J, Berns A. Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev 2000;14:994-1004.
19. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 1992;258:468-471.
20. Nishina PM, Verstuyft J, Paigen B. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. J Lipid Res 1990;31:859-869.
21. Vooijs M, van d, V, te RH, Berns A. Flp-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse. Oncogene 1998;17:1-12.
22. Boesten LS, Zadelaar AS, van Nieuwkoop A, Gijbels MJ, de Winther MP, Havekes LM, van Vlijmen BJ. Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-leiden transgenic mice. Cardiovasc Res 2005;66:179-185.
23. Kockx M, Gervois PP, Poulain P, Derudas B, Peters JM, Gonzalez FJ, Princen HM, Kooistra T, Staels B. Fibrates suppress fibrinogen gene expression in rodents via activation of the peroxisome proliferator-activated receptor-alpha. Blood 1999;93:2991-2998.

24. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 1987;68:231-240.
25. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 2003;112:1176-1185.
26. Kockx MM, Muhring J, Knaapen MW, De Meyer GR. RNA synthesis and splicing interferes with DNA in situ end labeling techniques used to detect apoptosis. Am J Pathol 1998;152:885-888.
27. Bergh G, Ehinger M, Olsson I, Jacobsen SE, Gullberg U. Involvement of the retinoblastoma protein in monocytic and neutrophilic lineage commitment of human bone marrow progenitor cells. Blood 1999;94:1971-1978.
28. Dickson BC, Gotlieb AI. Towards understanding acute destabilization of vulnerable atherosclerotic plaques. Cardiovasc Pathol 2003;12:237-248.
29. Bennett MR, Macdonald K, Chan SW, Boyle JJ, Weissberg PL. Cooperative interactions between RB and p53 regulate cell proliferation, cell senescence, and apoptosis in human vascular smooth muscle cells from atherosclerotic plaques. Circ Res 1998;82:704-712.
30. Chang MW, Barr E, Seltzer J, Jiang YQ, Nabel GJ, Nabel EG, Parmacek MS, Leiden JM. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. Science 1995;267:518-522.
31. Smith RC, Wills KN, Antelman D, Perlman H, Truong LN, Krasinski K, Walsh K. Adenoviral constructs encoding phosphorylation-competent full-length and truncated forms of the human retinoblastoma protein inhibit myocyte proliferation and neointima formation. Circulation 1997;96:1899-1905.
32. Libby P, Geng YJ, Aikawa M, Schoenbeck U, Mach F, Clinton SK, Sukhova GK, Lee RT. Macrophages and atherosclerotic plaque stability. Curr Opin Lipidol 1996;7:330-335.
33. Iavarone A, King ER, Dai XM, Leone G, Stanley ER, Lasorella A. Retinoblastoma promotes definitive erythropoiesis by repressing Id2 in fetal liver macrophages. Nature 2004;432:1040-1045.
34. Forrester JS, Fishbein M, Helfant R, Fagin J. A paradigm for restenosis based on cell biology: clues for the development of new preventive therapies. J Am Coll Cardiol 1991;17:758-769.
35. Ip JH, Fuster V, Badimon L, Badimon J, Taubman MB, Chesebro JH. Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. J Am Coll Cardiol 1990;15:1667-1687.
36. Liu MW, Roubin GS, King SB, III. Restenosis after coronary angioplasty. Potential biologic determinants and role of intimal hyperplasia. Circulation 1989;79:1374-1387.
37. Schwartz RS, Holmes DR, Jr., Topol EJ. The restenosis paradigm revisited: an alternative proposal for cellular mechanisms. J Am Coll Cardiol 1992;20:1284-1293.
38. van der Hoeven BL, Pires NM, Warda HM, Oemrawsingh PV, van Vlijmen BJ, Quax PH, Schalij MJ, van der Wall EE, Jukema JW. Drug-eluting stents: results, promises and problems. Int J Cardiol 2005;99:9-17.
39. El Omar MM, Dangas G, Iakovou I, Mehran R. Update on In-stent Restenosis. Curr Interv Cardiol Rep 2001;3:296-305.
40. O'Brien KD, McDonald TO, Kunjathoor V, Eng K, Knopp EA, Lewis K, Lopez R, Kirk EA, Chait A, Wight TN, deBeer FC, LeBoeuf RC. Serum Amyloid A and Lipoprotein Retention in Murine Models of Atherosclerosis. Arterioscler Thromb Vasc Biol 2005;25:785-90.

Chapter 4

Mdm2 protects terminally differentiated SMCs

Mdm2 protects terminally differentiated smooth muscle cells from p53-mediated cell death with a necrotic morphotype

Lianne S.M. Boesten[1,3,*]; A. Susanne M. Zadelaar[2,3,*]; Sarah De Clercq[4,*]; Sarah Francoz[4]; Anita van Nieuwkoop[1,3]; Erik A.L. Biessen[5]; Aart G. Jochemsen[6]; Chris Zurcher[7]; Louis M. Havekes[1,3]; Jean-Christophe Marine[4,†]; Bart J.M. van Vlijmen[8]

[1]Dept. of General Internal Medicine, [2]Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands, [3]TNO-Gaubius Laboratory, Biomedical Research Unit, Leiden, The Netherlands, [4]Laboratory For Molecular Cancer Biology, Flanders Interuniversity Institute For Biotechnology (VIB), Ghent, Belgium, [5]Dept. of Biopharmaceutics, Leiden Amsterdam Center for Drug Research, Leiden, The Netherlands, [6]Dept. of Molecular and Cell Biology, Leiden University Medical Center, Leiden, The Netherlands, [7]Dept. of Veterinary Pathology, Utrecht University, Utrecht, The Netherlands, [8]Hemostasis and Thrombosis Research Center, Dept. of Hematology, Leiden University Medical Center, Leiden, The Netherlands.

*These authors contributed equally to this study.

ABSTRACT

p53 is a potent inhibitor of cell growth and an inducer of apoptosis. During embryonic development and in adult homeostatic tissues, Mdm2 inhibits the growth suppressive activities of p53. However, whether tight surveillance of p53 activity is required in quiescent cells is unknown. To test this, conditional inactivation of *mdm2* was carried out in smooth muscle cells (SMCs) *in vivo*. Upon SMC-specific *mdm2* inactivation, mice rapidly became ill and died. Necropsy showed small intestinal dilation, and histological analyses indicated a severe reduction in the number of intestinal SMCs. Increased p53 levels and activity was detected in the remaining SMCs, and the phenotype was completely rescued on a p53-null background. Surprisingly, SMCs did not exhibit signs of apoptotic cell death but had a necrotic morphotype. These results show that Mdm2 prevents accumulation of active p53 in quiescent SMCs and thereby the induction of p53-mediated necrotic cell death *in vivo*.

The p53 tumor suppressor protein plays a critical role in suppressing tumor formation by inducing two types of anti-proliferative responses: cell-cycle arrest and apoptotic cell death.[1] Cell cycle arrest is mediated by transcriptional induction of genes whose products inhibit cell cycle progression, such as $p21^{Waf1/Cip12}$ or Ptprv.[3] The apoptotic function of p53 depends on both transcription-dependent and independent mechanisms.[4]

The importance of p53 in tumor suppression is highlighted by the observation that virtually all human cancers display an impaired p53 response. This is achieved either through direct inactivating mutations within the *p53* gene, or through aberrant expression of proteins acting in the p53 pathway. For instance, Mdm2 is overexpressed in human tumors of diverse origin.[5,6] The *mdm2* gene was originally identified as an amplified and overexpressed gene in a spontaneously transformed mouse BALB/c cell line.[7] Its transformation potential was later demonstrated and explained by the ability of Mdm2 to inactivate p53.[8,9]

Lack of functional *mdm2* is lethal in early mouse embryos, which die before implantation. This dramatic phenotype is completely rescued by concomitant deletion of *p53*.[10,11] In addition, mice with a hypomorphic *mdm2* allele have lower body weight and higher rates of p53-dependent apoptosis in various tissues. Notably, this phenotype is only observed in a subset of actively dividing cells, such as lymphocytes, and in the crypts of the small intestine, which could indicate that, in adult mice, Mdm2 restraints p53 activity only in homeostatic tissues.[12] Together, clear genetic evidence highlights the importance of the p53/Mdm2 interaction. However, limitations of the existing mouse models, such as early embryonic lethality of the constitutive null mutation, preclude analysis of the function of Mdm2 in a spatial and temporal specific manner.

In addition, these models do not allow firm establishment of the role of Mdm2 in the regulation of p53 stability. It was indeed shown that, beside the ability of Mdm2 to bind p53 in its transactivation domain and to interfere with p53-transcriptional activity, Mdm2 acts as an E3 ubiquitin ligase responsible for the ubiquitination of p53 and itself.[13-16] It was later proposed that Mdm2 mediates monomeric p53 ubiquitination on multiple lysine residues, rather than polyubiquitination, as previously thought.[17] Because chains of multiple ubiquitin molecules are necessary for efficient protein degradation, the data suggested that the enzymatic activity of Mdm2 might not be sufficient for optimal degradation of p53, and that other proteins must aid in polyubiquitination and degradation of p53 *in vivo*. More recent data indicated that Mdm2 differentially catalyzes either monoubiquitination or polyubiquitination of p53 in a dosage-dependent manner.[18] The authors proposed that Mdm2-mediated polyubiquitination and nuclear degradation occurs only in specific contexts, such as when Mdm2 is malignantly overexpressed. On the other hand, Mdm2-mediated monoubiquitination and subsequent cytoplasmic translocation of p53 may represent an important means of p53 regulation in unstressed cells, in which Mdm2 is maintained at low/physiological levels. In addition, in mice with the hypomorphic *mdm2* allele, the level of p53 protein was not coordinately increased, suggesting that Mdm2 can inhibit p53 function in a manner independent of degradation.[12] Moreover, other cellular ubiquitin ligases, such as Pirh2, Cop-1, yin-yang and ARF-BP1, were reported to also promote p53 ubiquitination and degradation.[19-22] Thus,

while Mdm2 is a key regulator of p53 function *in vivo*, p53 degradation may be mediated through both Mdm2-dependent and Mdm2-independent pathways *in vivo*.

Here, we show that specific inactivation of *mdm2* in terminally differentiated smooth muscle cells (SMCs) results, concomitantly with severe cell loss, in increased p53 protein levels and transcriptional activity. Interestingly, SMCs undergoing cell death did not show evidence of caspase-3 activation and DNA fragmentation, but displayed hallmarks of necrosis. Together, Mdm2 is critical for the regulation of p53 steady state levels and activity in quiescent cells *in vivo*. Moreover, the data indicate that increased p53 activity *in vivo* can lead to cell death with a necrotic morphotype, and consequently can go undetected when using apoptosis-specific methodology.

MATERIALS AND METHODS

Transgenic Mice

To achieve SMC-specific *mdm2* deletion we combined mice that carry a tamoxifen-inducible Cre-recombinase under control of the SMC specific SM22 promoter (SM-CreER^{T2}(ki) mice)[23] and mice carrying the *mdm2* gene modified by flanking exons 5 and 6 with two loxP sites (*mdm2*$^{FM/FM}$)[24] to create SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice. To determine p53 dependent effects of *mdm2* deletion SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice were crossed with p53 knock out (p53$^{-/-}$) mice[25] resulting in SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$ mice. In addition, to facilitate the monitoring of Cre activity *in vivo*, we combined the SM-CreER^{T2}(ki) mice and the *Rosa*26 reporter mouse line[26] to generate SM-CreER^{T2}(ki);*Rosa*26 mice. The SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$, SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$, SM-CreER^{T2}(ki);R*osa*26 and their control littermates mice were born at the expected Mendelian frequency, developed normally and were genotyped by PCR, as described previously.[23,24,26]

Conditional deletion of *mdm2* and quantification of recombination

Mice, aged 8-10 weeks, were injected intra-peritoneally with 100 µl of 20mg/ml tamoxifen (TMX, Sigma) or vehicle (peanut oil) for 0, 2, 5, and 7 continuous days. Intra-peritoneal TMX injections did not result in liver toxicity as measured by serum alanine aminotransferase (ALAT) levels (40.6±12.7 for *mdm2*$^{FM/FM}$ mice vs. 36.3±16.0U/l for SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice; P=0.754, ALT, Roche). Recombination of the FM allele (226bp) was assessed by PCR.[24] Recombination in SM-CreER^{T2}(ki);*Rosa*26 mice was quantified by counting β-galactosidase positive (β-gal^{+}) cells and was expressed as a percentage of the total number of cells (Figure 1C).

Tissue preparation and histology

Mice were sacrificed and a complete gross necropsy was performed. Organs and selected tissues, including oesophagus, stomach, jejunum, proximal and distal ileum, colon, aorta, urinary bladder and liver were sampled for further investigation. Sampled organs were either directly snap frozen in sterile eppendorf tubes and stored at -80°C or fixed in phosphate buffered formalin pH 7.4 and embedded in

paraffin. Of the tissues sampled and fixed in formalin microscopical analysis was performed of 5 μm routinely stained hematoxylin-phloxin-saphron (HPS), hematoxylin-eosin (HE) or 4'-6-Diamidino-2-phenylindole (DAPI) sections. In addition, slides were stained with antibodies against SM-α-actin (clone 1A4, dilution 1:1500, DAKO), pro-caspase-3 (1:1000, Cell Signaling), cleaved form of caspase-3 (1:1000, Cell Signaling), p53 (CM5, 1:1000, Novocastra Lab Ltd.). DNA fragmentation (ISEL staining) was assessed with the FragEL kit (Oncogene Research Products) according to the manufacturer's directions and sections were counterstained with methyl green (Vector Laboratories).

LCM and Q-PCR

Laser Capture Microdissection (LCM) samples were prepared from frozen sections of three control and three SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice and pooled. Total RNA was extracted using the PicoPure RNA isolation kit and amplified using the RiboAmp RNA Amplification Kit according to manufacturer's instructions (Acturus Bioscience). 1 μg of total RNA from each pool was reverse-transcribed using a SuperScript kit (Invitrogen). These assays were performed following the manufacturer's specifications (PE Applied Biosystems). Primer pairs and TaqMan probes were designed by Applied Biosystems (Assays on demand).

Electron Microscopy

Tissue samples were immersed in a fixative solution of 2 % paraformaldehyde and 2.5 % glutaraldehyde and postfixed in 1% OsO4 with 1.5% K3Fe(CN)6 in 0.1 M NaCacodylate buffer, pH 7.2. Samples were dehydrated through a graded ethanol series, including a bulk staining with 2% uranyl acetate at the 50% ethanol step followed by embedding in Spurr's resin. Ultra thin sections, made on a Ultracut E microtome (Reichert-Jung), were post-stained in an ultrostainer (Leica,Herburgg, Switzerland) with uranyl acetate and lead citrate. Sections were viewed with a transmission electron microscope 1010 (JEOL,Tokyo, Japan).

Statistical Analysis

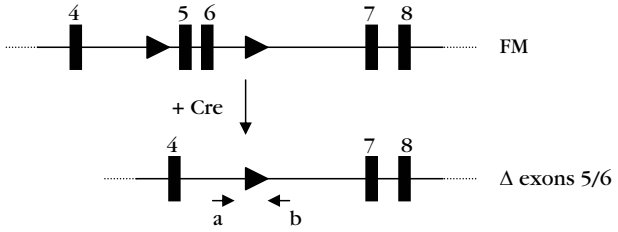
All data are represented as mean±SD. Data were analysed using the non-parametric Mann-Whitney rank sum test. P-values less than 0.05 were regarded as statistically significant.

RESULTS

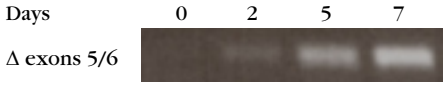
Strategy for conditional bi-allelic inactivation of *mdm2* in quiescent smooth muscle cells *in vivo*

To test whether Mdm2 is required for regulating p53 stability and activity in quiescent cells *in vivo*, we specifically inactivated *mdm2* in G_0 smooth muscle cells (SMCs). To this end, conditional inactivation of *mdm2* was carried out in mice harbouring *mdm2* floxed alleles and a tamoxifen (TMX)-inducible Cre-recombinase under control of the SM22 promoter (SM-CreER^{T2}(ki) mice).[23] The *mdm2* floxed allele (FM) had been previously described.[24] It carries a *lox*P recombination site in

A.



B.



C.

Oesophagus	Stomach	Proximal Ileum	Distal Ileum	Colon	Bladder	Aorta
7.5±2.5	61.5±8.8	55.3±4.2	39.8±3.1	47.4±5.5	92.5±4.2	6.7±3.1

D.

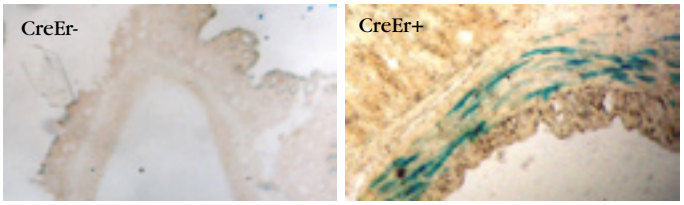


Figure 1. (A.) Schematic representation of part of the *mdm2* floxed allele (FM) and the Cre-mediated recombination event. The a and b arrows designated the position of the primers used to detect the Cre-mediated recombination by PCR. (B.) PCR analysis showing increased detection of the Cre-mediated recombination event, using primers a and b, in the intestine of CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice treated for 0, 2, 5 and 7 days with TMX. (C.) Percentage of recombination of SMC-rich SM-CreERT2(ki);*Rosa*26 organs/tissues after 7 days of intraperitonal TMX administration. No recombination was observed in TMX or vehicle-treated control *Rosa*26 mice. (D.) Detection of *lacZ* reporter gene expression in the stomach of SM-CreERT2(ki);*Rosa*26 mice and *Rosa*26;CreER-negative control mice after 7 days of TMX administration.

intron 4 and another in intron 6 (Figure 1A). Cre-mediated recombination therefore yields an *mdm2* allele lacking exons 5 and 6, which encode for most of the p53-binding domain. Mice homozygous for the FM allele (or *mdm2*$^{FM/FM}$) appear normal; however, ubiquitous deletion of exons 5 and 6 *in vivo* results in an embryonic lethality similar to the *mdm2* null allele.[24]

We first examined by PCR the extent of recombination at the *mdm2* locus in SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice (Figure 1B) and compared it with the Cre activity at the *Rosa26* locus in SM-CreER^{T2}(ki);*Rosa*26 mice at various sites containing SMCs (Figure 1C). SMCs were identified both morphologically and immunohistochemically by SM-α-actin staining (Figure 2). In order to induce the latent CreER fusion protein, mice were injected daily with TMX for seven days, and then analyzed. Upon

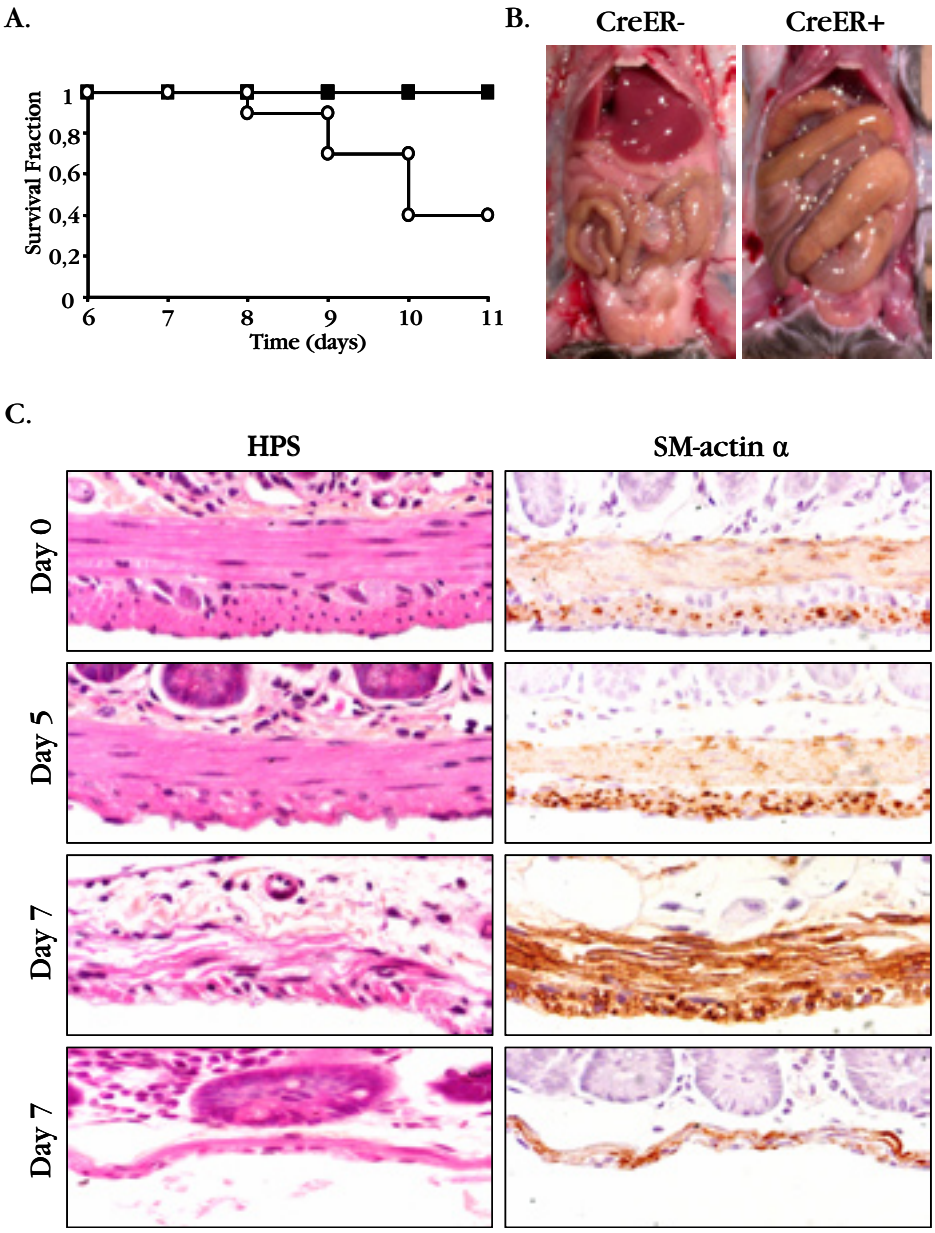


Figure 2. (A.) Kaplan-Meier curves of age-matched CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice (open circles, n=21) and control CreER-negative mice (filled squares, n=15). Mice were TMX-treated for 7 consecutive days and followed thereafter. (B.) Gross appearance of control (left panel) and CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice (right panel) after 7 days of intra-peritoneal tamoxifen administration. (C.) HPS (left panels) and IHC for SM-α-actin (right panels) staining of the small intestine of CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice 0, 5, 7 days following TMX treatment (magn. 200x).

TMX injection, we found that SMCs from the gastroinstestinal (GI) tract, particularly the stomach (Figure 1D) and proximal ileum, were stably marked (β-gal$^+$), whereas little reporter activity was found in cells of the cardiovascular system such as in the aorta. Since efficient recombination of the *mdm2* locus was observed in the SMCs of the small intestines (proximal ileum), we concentrated our studies at this site.

TMX-treated SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice exhibit severe lesions in the SMC-containing layers of the intestinal wall and eventually die

Following 7 days of TMX administrations, the body weight of SM-CreER^{T2}(ki); *mdm2*$^{FM/FM}$ mice decreased as compared to TMX-treated control Cre-ER-negative mice. Moreover, SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice were not responsive to stimuli and were hunchbacked with ruffled coat. Strikingly, illness proceeded to death from day 8 on (Figure 2A). In contrast, TMX-treated *mdm2*$^{FM/FM}$ CreER-negative mice appeared normal and did not differ from vehicle-treated SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ and vehicle treated *mdm2*$^{FM/FM}$ CreER-negative mice. SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice were sacrificed for gross necropsy and histopathological analysis.

The stomach and small intestine of TMX-treated SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice adhered to spleen and liver, appeared vulnerable and friable, were filled with soft materials, and were loose. The consistently abnormal findings included liver and spleen atrophy and dilation of the small intestinal, which varied between mice, but could be considerable (Figure 2B). This dilation was associated with a decreased length of the small intestine (from pylorus to ileo-cecal junction: 44.0±3.5 for control mice, 32.0±2.0 cm for SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice; P=0.007).

The lesions in the dilated small intestine of SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice treated with TMX were limited to the lamina interna and externa of the muscularis (M.) propria of the intestinal wall, consistent with a specific activity of the Cre in the SMCs. The architecture of the lamina M. interna was disturbed due to a decrease in cell layers and irregular alignment of the SMCs. The number of SMC layers was reduced from 6-8 to 3-5, with multifocal irregular increase of intercellular spaces, tapering of SMCs with wavy ends and, irregular, often shortened hyperchromatic nuclei (Figure 2C). In some extreme cases, the SMCs were simply either missing or unrecognizable.

Alterations of the SMC-containing intestinal wall and lethality upon SMC-specific *mdm2* inactivation are entirely p53-dependent

A large body of evidence suggests that Mdm2 can function both dependently and independently of p53. In agreement, Mdm2 binds several proteins involved in the regulation of cell cycle progression and survival other than p53, such as p19/ARF, p63 and p73, Rb, and E2F-1/DP-1.[27] In order to test whether the phenotype observed in the SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice is p53-dependent, they were crossed with p53-null mice (p53$^{-/-}$)[25] to create SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$ mice. Strikingly, as observed in control mice, TMX injection in SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$ mice did not cause death (Figure 3A). Gross necropsy did not reveal differences in liver and spleen weight and small intestinal length as compared to control mice. Histological examination did not reveal any obvious lesions, disorganization of intestinal cell alignment or loss of cell viability in SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$ (Figure 3B,

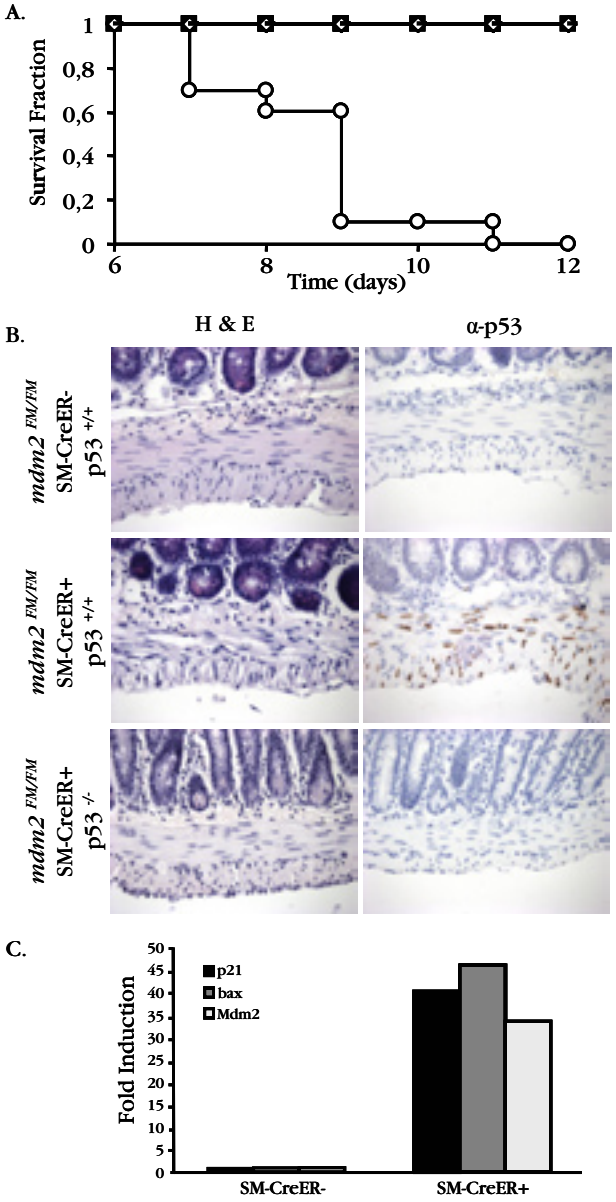


Figure 3. (A.) Kaplan-Meier curves of age-matched CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice (open circles, n=11) and control *mdm2*$^{FM/FM}$;CreER-negative mice (filled squares, n=10) and CreER^{T2}(ki);*mdm2*$^{FM/FM}$;*p53*$^{-/-}$ mice (open diamonds, n=6) following 7 days of intra-peritoneal TMX administration. (B.) H&E staining and p53 immunostaining of the small intestine of mice with the indicated genotypes (magn. 60x). (C.) Q-RT-PCR analysis shows induction of expression of p53 target genes in laser-capture microdissected SMCs of mice with the indicated genotypes. Microdissected samples from three different mice were pooled before the analysis. The data represent therefore the mean expression levels in these three mice treated independently.

left panels). We therefore concluded that loss of *mdm2* in the SMCs of the GI tract causes loss of cell viability and acute lethality in a manner that is completely dependent on the presence of functional p53.

SMC-specific *mdm2* inactivation leads to increased p53 stability and transcriptional activity

To determine whether specific deletion of *mdm2* in the SMCs allows the level of p53 protein to increase, we performed immunostaining for p53. In sections of the proximal ileum from *mdm2*$^{FM/FM}$;CreER-negative mice injected with TMX, no p53 staining could be detected (Figure 3B). In contrast, nuclei of the SMCs of SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice showed marked p53 immunoreactivity (Figure 3B, right panels). Importantly, no staining was observed in sections from SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$ mice, confirming the specificity of the p53 detection method (Figure 3B). These results suggest that p53 is maintained at low levels in a strict Mdm2-dependent manner in terminally differentiated SMCs. In addition, p53 was not only stabilized but it was also functionally active, as indicated by concomitant upregulation of several p53-target genes such as *p21*$^{Waf1/Cip1}$ and the pro-apoptotic gene *bax* in laser-capture microdissected-SMCs in the proximal ileum of SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice, as determined by Q-RT-PCR (Figure 3C). Of note, *mdm2* Δexons5/6 transcripts, were also found upregulated in the SMCs of SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice.

SMC-specific *mdm2* inactivation does not cause apoptotic cell death

Since one of the main p53 antiproliferative activities is induction of caspase-dependent apoptotic cell death, we hypothesized that the hypocellularity observed following *mdm2* inactivation was a consequence of increased p53-mediated apoptosis. To investigate this possibility, we first performed staining using an antibody specifically recognizing the activated form of the main effector caspase, caspase-3. No significant activation of caspase-3 could be observed both in control and SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$TMX-treated mice (Figure 4). Interestingly, the non-activated form of caspase-3 (pro-caspase-3) was not significantly detected in the SMCs of both control (CreER-negative) and SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$TMX-treated mice (Figure 4). This observation, thus, provides a simple explanation for absence of activated caspase-3 in the SMCs of TMX-treated mice. Absence of caspase-3 protein was also previously reported in rat and mouse adult skeletal muscles, despite the abundant presence of its mRNA.[28] Since other effector caspases might compensate for the lack of caspase-3 in these cells, we explored the presence of other apoptotic signatures. Apoptosis is morphologically defined by several hallmarks including nuclear shrinkage (pyknosis), chromatin condensation, DNA degradation and nuclear fragmentation (karyorrhexis) with formation of apoptotic bodies. Examination of H&E or DAPI staining did not reveal the presence of apoptotic bodies or pyknotic nuclei. Moreover, *in situ* end labeling (ISEL)[29] (Figure 4) and TUNEL assays (data not shown) did not detect DNA fragmentation. To verify that our detection methods could detect both pro-caspase-3 and apoptosis, sections of E15.5 embryos expressing p53 specifically in post-mitotic neurons deficient for mdm2 were analyzed (Francoz *et al.*, submitted 2005). Clear and specific staining for pro-caspase-3, the activated form

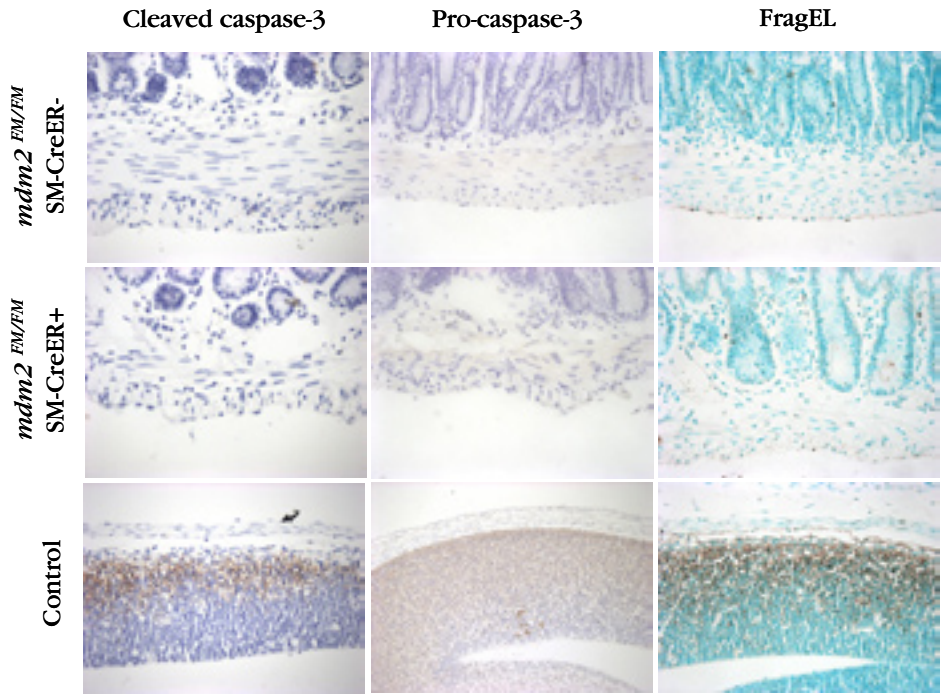


Figure 4. Immunostaining for the activated form of caspase-3 and pro-caspase-3 (non-cleaved caspase-3) and ISEL staining of the small intestine of mice with the indicated genotypes and in the lateral ventricle region of the cerebral cortex of E16.6 embryos expressing p53 specifically in post-mitotic neurons deficient for *mdm2* (control).

of caspase-3 and DNA fragmentation were detected in these embryos (Figure 4), confirming the sensitivity and the specificity of both methods. Together, the observed phenotype and multifocal irregular increase of intercellular spaces with tapering SMCs is not suggested to coincide with an increase in apoptotic cell death.

Evidence for p53-dependent necrotic cell death following SMC-specific *mdm2* inactivation

Electron microscopy (EM) confirmed absence of pyknosis and karyorrhexis with formation of apoptotic bodies. However, EM revealed that many of the remaining SMCs had a morphotype identical to that seen in necrosis[30,31] including a "mottled" nucleus caused by clumped, but not marginalised, and only loosely packed chromatin (Figure 5). Most of the nuclei appeared largely intact, however, in some cases nuclear membrane detachment and rupture were apparent. The affected cells also showed dilated mitochondria and cytoplasmic vacuoles (not shown). External membrane rupture and swelling of cytoplasmic organelles was also evident (Figure 5). In agreement, clusters of roughly intact nuclei, which are no longer surrounded by cytoplasmic membranes, were also observed (Figure 5). Various intermediate aspects could be observed, which ranged from apparently intact cells that had a mottled nucleus, to cells that had a mottled nucleus and gross membrane alterations. Finally,

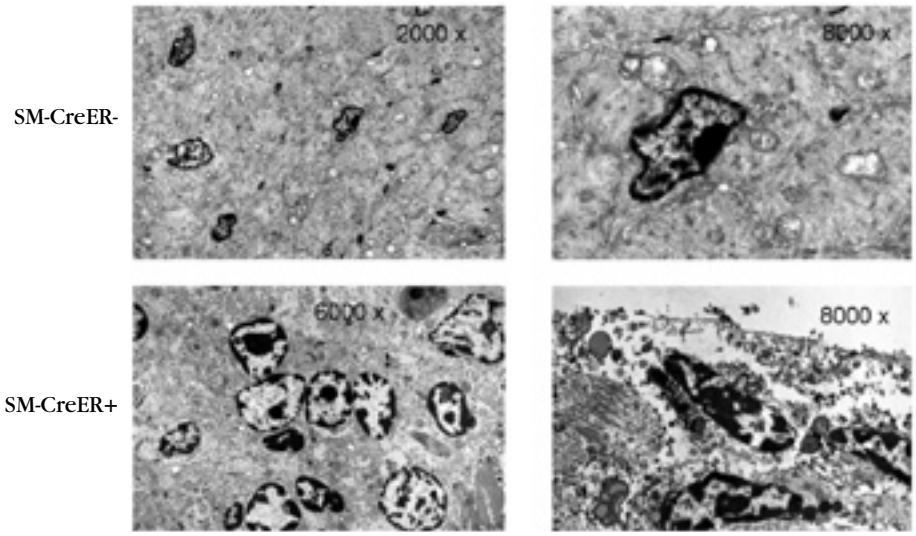


Figure 5. Electron microscopy of the small intestine of control (creER-negative; top panels) and CreER^{T2}(ki);*mdm2*$^{FM/FM}$ (low panels) TMX-treated mice. Nuclei in control tissues are sparse, equally distributed and surrounded by a plasma membrane. Nuclei in tissues from CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice are clustered and not surrounded by a plasma membrane. Evidence of plasma membrane ruptures is shown in the bottom right panel. Magnifications are indicated.

whereas apoptosis includes phagocytosis, even heavily altered cells appeared not to be within phagocytes. Surprisingly, absence of phagocytosis did not lead in this case to local signs of inflammation, but this absence might be related to local anatomical constraints.

DISCUSSION

It is well documented that the p53 protein is maintained at low levels in embryonic and adult tissues; however, it remained to be established whether p53 degradation occurs in a strict Mdm2-dependent manner. In addition, while downregulation of Mdm2 appears to be sufficient to activate a p53 response in homeostatic tissues,[12] its relevance in non-proliferating, terminally differentiated cells remained unexplored. Here, we provide the first genetic evidence that Mdm2 plays a key role in the regulation of p53 levels and activity in quiescent, terminally differentiated, SMCs.

Notably, we have recently confirmed this finding in other cellular contexts. Indeed, conditional inactivation of mdm2 was achieved in mouse embryonic fibroblasts (MEFs), in neuronal stem/progenitor cells and in post-mitotic neurons (Francoz et al., submitted 2005), in erythroid progenitor cells (Maetens et al., in preparation), in thymocytes and cardiac muscle cells (G. Lozano, Personal communication). In all these settings, an increase in p53 levels and activity was observed and this activation of function resulted in caspase 3-dependent cell death *in vivo*. Thus, even if other E3 protein ligases have been reported to induce p53 ubiquitination and

degradation,[19-22] none of these proteins can fully compensate for loss of Mdm2 function *in vivo*. Therefore, even if these data do not exclude the possibility that these proteins might aid in p53 degradation, they strongly suggest that p53 degradation *in vivo* occurs through Mdm2-dependent pathways.

Mdm2 ubiquitination activity and the physical interaction between Mdm2 and p53 have become the targets of adjuvant chemotherapies designed to sensitize human tumors to cancer therapies.[32-35] Indeed, there is evidence that cancer cells are more sensitive to activation of p53 apoptotic function than the resting host cells. However, the data presented herein suggest that Mdm2 is critical for maintaining p53 activity at low levels also in quiescent, terminally differentiated cells. Therefore, this study raises concerns about the benefit of such approaches for the patients. Indeed, our data would predict that Mdm2 inhibition of function *in vivo* would be detrimental not only to cancer cells but also to most of the resting host cells.

Interestingly, the data presented herein also provide the first compiling evidence that p53 is able to activate a caspase-3-independent cell death program with a necrotic morphotype *in vivo*. All studies converge to the crucial role of both the mitochondrial pathway (cytochrome c release,[36] ROS production and/or $\Delta\psi m$ drop)[37] and activation of caspase-9 and its downstream caspases[38] in p53-induced apoptosis. In agreement with this view, Apaf-1[39] and several genes encoding for the BH3-containing proapoptotic proteins such as Bax, Noxa and Puma are among the many reported p53-regulated genes and key mediators of p53-induced apoptosis *in vivo*.[40,41] Caspase-3, the main effector caspase acting dowstream of caspase-9, is also of fundamental importance for many forms of apoptosis[42] and required for p53-induced apoptosis. Since we show here that caspase-3 protein is either absent or expressed at very low levels (below detectable levels) in the SMCs, these data provide a simple explanation for our failure to detect apoptosis, despite the fact that p53 was stabilized and transcriptionally active in these cells. One possibility is that p53 once activated provokes, through some of its mediators, mitochondrial damages that are sufficient to induce cell death in a caspase-independent manner. We could indeed detect in *mdm2*-deficient SMCs high levels of expression of bax, a protein particularly important for the triggering of mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release. MOMP frequently marks the "point of no return" of the lethal process. It is clear today that artificial or genetic inhibition of caspases is often not sufficient to avoid physiological cell death, and in this case this often leads to a shift in the morphology of cell death, from classical apoptosis to "apoptosis-like cell death", autophagic cell death or necrosis.[43] For instance, the loss of interdigital cells in the mouse embryo, a prototype of mitochondrial apoptotic cell death, still occurs by necrosis in mice deficient for Apaf-1.[30] Similarly, because they do not express caspase-3, the *mdm2*-deficient SMCs could undergo necrotic cell death following activation of MOMP by p53. In order to test whether necrotic cell death observed in these cells occurs through a MOMP-dependent mechanism, one could check whether overexpression of Bcl-2, a BH3-containing protein that can inhibit mitochondrial channel opening, is able to rescue the phenotype. Alternatively, eventhough less likely, p53 might be able to activate directly a genetic program leading to necrotic cell death *in vivo* in a tissue-specific manner. Interestingly, p53 was recently shown to be able to activate a MOMP-independent (and Bcl-2

insensitive) cell death program in cultured cells.[44] The existence of such a pathway *in vivo* remains, however, to be demonstrated. A careful analysis of the transcription program activated by p53 in the *mdm2*-deficient SMCs might help to identify key mediator(s) of this putative pathway.

Regardless the molecular mechanism, this study opens new perspectives for cancer therapies. For instance, inactivation of Apaf-1, which is essential for p53-induced caspase-dependent apoptosis, may contribute to the low frequency of p53 mutations observed in therapy-resistant melanomas.[38] The ability of p53 to induce a caspase-independent and MOMP-independent type of cell death may be the basis for new therapies killing cells in which p53 is wild-type but have acquired defects in the signaling pathways that are downstream p53.

ACKNOWLEDGMENTS

We thank the technical staffs of TNO-Biomedical Research, Dieter Defever, Ines Bonk and Riet De Rycke for their excellent technical assistance. We thank Guillermina Lozano for the *mdm2* conditional knockout mice. This work was supported by Public Health Service grant 2000B051 from the Netherlands Heart Foundation and by grants 902-26-242 and 912-02-03 from NWO/ ZonMw. The research of B.J.M. van Vlijmen has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. S. Francoz was supported by grants from "Télévie (FNRS)". This work was supported in part by grants from Association for International Cancer Research (J-C Marine), Belgium Federation against Cancer (non profit organization; J-C Marine) and by EC FP6 funding (AG Jochemsen and J-C Marine). This publication reflects only author's views. The commission is not liable for any use that may be made of the information herein.

REFERENCES

1. Lane DP. Cancer. p53, guardian of the genome. Nature 1992;358:15-16.
2. el Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817-825.
3. Doumont G, Martoriati A, Beekman C, Bogaerts S, Mee PJ, Bureau F, Colombo E, Alcalay M, Bellefroid E, Marchesi F, Scanziani E, Pelicci PG, Marine JC. G1 checkpoint failure and increased tumor susceptibility in mice lacking the novel p53 target Ptprv. EMBO J 2005;24:3093-3103.
4. Chipuk JE, Green DR. p53's believe it or not: lessons on transcription-independent death. J Clin Immunol 2003;23:355-361.
5. Momand J, Jung D, Wilczynski S, Niland J. The MDM2 gene amplification database. Nucleic Acids Res 1998;26:3453-3459.
6. Riemenschneider MJ, Buschges R, Wolter M, Reifenberger J, Bostrom J, Kraus JA, Schlegel U, Reifenberger G. Amplification and overexpression of the MDM4 (MDMX) gene from 1q32 in a subset of malignant gliomas without TP53 mutation or MDM2 amplification. Cancer Res 1999;59:6091-6096.
7. Fakharzadeh SS, Trusko SP, George DL. Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. EMBO J 1991;10:1565-1569.
8. Cahilly-Snyder L, Yang-Feng T, Francke U, George DL. Molecular analysis and

chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. Somat Cell Mol Genet 1987;13:235-244.
9. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 1992;69:1237-1245.
10. Jones SN, Roe AE, Donehower LA, Bradley A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature 1995;378:206-208.
11. Montes de Oca LR, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 1995;378:203-206.
12. Mendrysa SM, McElwee MK, Michalowski J, O'Leary KA, Young KM, Perry ME. mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. Mol Cell Biol 2003;23:462-472.
13. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature 1997;387:296-299.
14. Honda R, Tanaka H, Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 1997;420:25-27.
15. Honda R, Yasuda H. Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. Oncogene 2000;19:1473-1476.
16. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. Nature 1997;387:299-303.
17. Lai Z, Ferry KV, Diamond MA, Wee KE, Kim YB, Ma J, Yang T, Benfield PA, Copeland RA, Auger KR. Human mdm2 mediates multiple mono-ubiquitination of p53 by a mechanism requiring enzyme isomerization. J Biol Chem 2001;276:31357-31367.
18. Li M, Brooks CL, Wu-Baer F, Chen D, Baer R, Gu W. Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science 2003;302:1972-1975.
19. Chen D, Kon N, Li M, Zhang W, Qin J, Gu W. ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. Cell 2005;121:1071-1083.
20. Dornan D, Wertz I, Shimizu H, Arnott D, Frantz GD, Dowd P, O'Rourke K, Koeppen H, Dixit VM. The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature 2004;429:86-92.
21. Leng RP, Lin Y, Ma W, Wu H, Lemmers B, Chung S, Parant JM, Lozano G, Hakem R, Benchimol S. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell 2003;112:779-791.
22. Sui G, Affar eB, Shi Y, Brignone C, Wall NR, Yin P, Donohoe M, Luke MP, Calvo D, Grossman SR, Shi Y. Yin Yang 1 is a negative regulator of p53. Cell 2004;117:859-872.
23. Kuhbandner S, Brummer S, Metzger D, Chambon P, Hofmann F, Feil R. Temporally controlled somatic mutagenesis in smooth muscle. Genesis 2000;28:15-22.
24. Grier JD, Yan W, Lozano G. Conditional allele of mdm2 which encodes a p53 inhibitor. Genesis 2002;32:145-147.
25. Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, Weinberg RA. Tumor spectrum analysis in p53-mutant mice. Curr Biol 1994;4:1-7.
26. Mao X, Fujiwara Y, Orkin SH. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. Proc Natl Acad Sci U S A 1999;96:5037-5042.
27. Iwakuma T, Lozano G. MDM2, an introduction. Mol Cancer Res 2003;1:993-1000.
28. Ruest LB, Khalyfa A, Wang E. Development-dependent disappearance of caspase-3 in skeletal muscle is post-transcriptionally regulated. J Cell Biochem 2002;86:21-28.
29. Gavrieli Y, Sherman Y, Ben Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493-501.
30. Chautan M, Chazal G, Cecconi F, Gruss P, Golstein P. Interdigital cell death can occur through a necrotic and caspase-independent pathway. Curr Biol 1999;9:967-970.
31. Searle J, Kerr JF, Bishop CJ. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol Annu 1982;17 Pt 2:229-259.
32. Issaeva N, Bozko P, Enge M, Protopopova M, Verhoef LG, Masucci M, Pramanik A, Selivanova G. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. Nat Med 2004;10:1321-1328.

33. Midgley CA, Desterro JM, Saville MK, Howard S, Sparks A, Hay RT, Lane DP. An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. Oncogene 2000;19:2312-2323.
34. Vassilev LT. Small-molecule antagonists of p53-MDM2 binding: research tools and potential therapeutics. Cell Cycle 2004;3:419-421.
35. Yang Y, Ludwig RL, Jensen JP, Pierre SA, Medaglia MV, Davydov IV, Safiran YJ, Oberoi P, Kenten JH, Phillips AC, Weissman AM, Vousden KH. Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. Cancer Cell 2005;7:547-559.
36. Gao CF, Ren S, Zhang L, Nakajima T, Ichinose S, Hara T, Koike K, Tsuchida N. Caspase-dependent cytosolic release of cytochrome c and membrane translocation of Bax in p53-induced apoptosis. Exp Cell Res 2001;265:145-151.
37. Li PF, Dietz R, von Harsdorf R. p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. EMBO J 1999;18:6027-6036.
38. Soengas MS, Alarcon RM, Yoshida H, Giaccia AJ, Hakem R, Mak TW, Lowe SW. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. Science 1999;284:156-159.
39. Moroni MC, Hickman ES, Lazzerini DE, Caprara G, Colli E, Cecconi F, Muller H, Helin K. Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol 2001;3:552-558.
40. Jeffers JR, Parganas E, Lee Y, Yang C, Wang J, Brennan J, MacLean KH, Han J, Chittenden T, Ihle JN, McKinnon PJ, Cleveland JL, Zambetti GP. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell 2003;4:321-328.
41. Michalak E, Villunger A, Erlacher M, Strasser A. Death squads enlisted by the tumour suppressor p53. Biochem Biophys Res Commun 2005;331:786-798.
42. Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev 1998;12:806-819.
43. Kroemer G, Martin SJ. Caspase-independent cell death. Nat Med 2005;11:725-730.
44. Godefroy N, Lemaire C, Renaud F, Rincheval V, Perez S, Parvu-Ferecatu I, Mignotte B, Vayssiere JL. p53 can promote mitochondria- and caspase-independent apoptosis. Cell Death Differ 2004;11:785-787.

Chapter 5

Conditional gene targeting in atherosclerosis

Local Cre-mediated gene recombination in vascular smooth muscle cells in mice

Lianne S.M. Boesten[2,3*];A. Susanne M. Zadelaar[1,3*]; Nuno M.M. Pires[1,3]; Anita van Nieuwkoop[2,3]; E.A.L. Biessen[4]; J. Wouter Jukema[1]; Louis M. Havekes[1,2,3]; Bart J.M. van Vlijmen[5]; Ko Willems van Dijk[6]

[1]Dept. of Cardiology, [2]Dept. of General Internal Medicine, Leiden University Medical Center, [3]Dept. of Biomedical Research, TNO-Quality of Life, [4]Dept. of Biopharmaceutics, Leiden Amsterdam Center for Drug Research, [5]Hemostasis and Thrombosis Research Center, Leiden University Medical Center, [6]Dept. of Human and Clinical Genetics, Sylvius Laboratory, Leiden, The Netherlands.

[*] These authors contributed equally to this study

ABSTRACT

Here we describe a means to conditionally modify genes at a predefined and localized region of the vasculature using a perivascular drug delivery device (PDD). A 4-hydroxytamoxifen (4-OHT)-eluting PDD was applied around the carotid or femoral artery of a mouse strain, carrying both the tamoxifen-inducible and smooth muscle cell (SMC)-specific Cre-recombinase (SM-Cre-ER^{T2}) transgene and a stop-floxed -galactosidase gene in the Rosa26 locus: the SM-CreER^{T2}(ki)/rosa26 mouse.

A dose and time curve of 0-10% (w/w) 4-OHT and 0-14 days application of the PDD in SM-CreER^{T2}(ki)/rosa26 mice showed optimal gene recombination at 1% (w/w) 4-OHT loading at 7 days post application (carotid artery 2.4±1.8%; femoral artery 4.0±3.8% of SMCs). The unique 4-OHT-eluting PDD allowed us to achieve SMC-specific recombination in the same order of magnitude as compared to systemic tamoxifen administration. In addition, recombination was completely confined to the PDD-treated vessel wall segment.

Thus, local application of a 4-OHT-eluting PDD results in vascular SMC-specific Cre-mediated recombination in SM-CreER^{T2}(ki)/rosa26 mice without affecting additional SMCs.

Pathological processes, such as atherosclerosis and post-angioplasty restenosis, occur in highly localized regions of the vasculature.[1] Studying these processes using genetic modification may thus require a restriction to the area that is conditionally gene targeted. Moreover, some conditional alterations to smooth muscle cells (SMCs) of the vasculature as a whole may not be compatible with life, but should be addressed in a limited area of a vessel. To temporally and conditionally modify genes in a predefined and localized region of a blood vessel, we used a perivascular drug delivery device (PDD). The perivascular drug-eluting cuff has been used to study the effect of pharmaceutical compounds on neointima formation or restenosis.[2] The PDD is very suitable for local drug delivery and can simultaneously induce neointima formation.[2]

Via the release of 4-hydroxytamoxifen (4-OHT) from the PDD we studied the temporal and conditional modification of genes in a predefined and localized region of a blood vessel. The PPD was applied in a mouse strain that carried both the mutant estrogen receptor ligand binding domain, responsive to tamoxifen and SMC-specific Cre-recombinase (SM-Cre-ER^{T2}) transgene[3] and a stop-floxed β-galactosidase gene in the Rosa26 locus:[4] the SM-CreER^{T2}(ki)/ rosa26 mouse.

The unique 4-OHT-eluting PDD allowed us to achieve SMC-specific recombination in the same order of magnitude as compared to systemic tamoxifen administration. In addition, recombination was completely confined to the SMCs of the PDD-treated vessel wall segment. These data indicate that the novel 4-OHT-eluting PDD is an efficient tool to specifically induce highly localized Cre-mediated recombination in the SM-CreER^{T2}(ki)/rosa26 mouse.

METHODS

Transgenic mice

Mice that carry a tamoxifen-inducible Cre-recombinase under control of the smooth muscle cell (SMC)-specific SM22 promoter (SM-CreER^{T2}(ki) mice)[3] were crossed with the rosa26 reporter mouse line[4] to generate SM-CreER^{T2}(ki)/rosa26 mice. SM-CreER^{T2}(ki)/rosa26 mice were genotyped for the SM-CreER^{T2}(ki) promoter[3] and the rosa26 transgene.[4] Homozygous SM-CreER^{T2}(ki)/rosa26 littermates 8-10 weeks of age were compared in experiments. All animal work was approved by the regulatory authority of the institutional experimental animal committee.

Perivascular delivery device

The Poly(ε-caprolactone)-based perivascular delivery devices (PDD) were manufactured as previously described.[5,6] In brief, 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich Chemicals BV, Zwijndrecht, The Netherlands) was first blended with PEG before this blend was mixed with molten PCL at 70 °C. The PCL:PEG ratio was 4:1 (w/w). Drug-loaded PDD were made from the blended molten 4-OHT–polymer mixture and designed to fit around the femoral and carotid arteries of mice (Figure 1). Drug-eluting PDD had the shape of a longitudinal cut cylinder with an internal diameter of 0.5 mm, an external diameter of 1 mm, a length of 2 mm, and a weight of approximately 5 mg. PDD were loaded with 1%, 2.5%, 5% and 10% (w/w) n=5 and *in vitro*

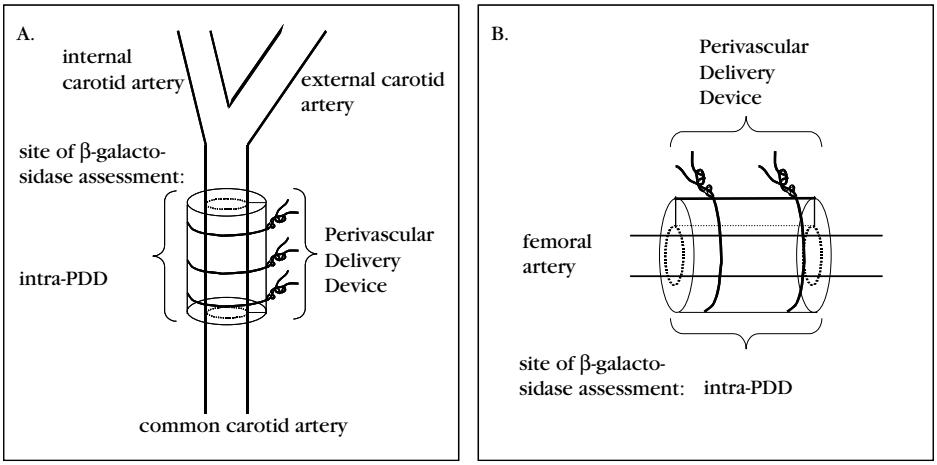


Figure 1. Local 4-hydroxytamoxifen (4-OHT) application using the perivascular delivery device (PDD) at the level of (A.) the carotid and (B.) femoral arteries.

release profiles were performed as previously described.[2,7] PDD of each composition were placed in 20 ml glass scintillation vials and cooled to 4°C. Five milliliters of iced-cold PBS pH 7.4 containing 0.2% bovine serum albumin (fraction V, Roche Diagnostics, Mannheim, Germany) were placed on top of the cuffs followed by 5 ml of n-octanol. The n-octanol formed an upper immiscible phase on top of the PBS. 4-OHT is far more soluble in n-octanol than in PBS (log Poctanol/water=3.2), which ensured rapid partition into the octanol phase. The vials were capped and incubated at 37°C. The concentration of 4-OHT in the octanol phase was analyzed by UV-VIS absorbance methods (Pharmacia LKB Ultrospec III, Peak Tek Inc., Glenside, USA) at a 4-OHT-specific wavelength (247 nm) using a double beam UV/VIS spectrophotometer (UVIKON 933, Kontron Instruments Ltd, Milan, Italy). This octanol phase was replaced back into the vial. A calibration graph of 4-OHT in n-octanol was established by measuring the absorbance of a 0-50 μg/ml range of standards in n-octanol.

Conditional gene targeting, histology and quantification of recombination

To achieve local recombination, SM-CreER^{T2}(ki)/rosa26 and control littermate rosa26 mice were anaesthetized and a PDD was placed around the carotid or femoral arteries as described,[2,8,9] containing vehicle, 0.1, 0.3, 1, 3 or 10% (w/w) 4-OHT for 7 (n=6 arteries/group) or 14 (n=8 arteries) continuous days. In order to achieve systemic recombination of the rosa26 transgene, SM-CreER^{T2}(ki)/rosa26 and control rosa26 mice (n=5/group) were injected intraperitoneally with 100 μl 20 mg tamoxifen (TMX, Sigma) for 7 continuous days. Next to carotid and femoral arteries, several SMC-rich organs were harvested from SM-CreER^{T2}(ki)/rosa26 and control rosa26 mice, including aorta, stomach, intestines and bladder to evaluate the site-specificity of 4-OHT induced recombination. β-Galactosidase activity was demonstrated as described[10] by staining of 20 μm cryosections. β-Galactosidase positive cells were counted and expressed as a percentage of the total number of morphologically identified SMCs. Antibodies against PECAM-1 (CD31, 1:200, Sigma, St. Louis, USA) were used to stain endothelial cells.

Statistical Analysis

All data are represented as mean±SD. Data were analysed using the non-parametric Mann-Whitney rank sum test. P-values less than 0.05 were regarded as statistically significant.

RESULTS

In vivo application of the perivascular delivery device

We developed a perivascular poly-(ε-caprolactone)-based delivery device (PDD) loaded with a tamoxifen derivative 4-hydroxytamoxifen (4-OHT) to restrict conditional recombination to a predefined and localized region of the vasculature in a susceptible mouse strain. PDDs loaded with a dose range of 4-OHT were generated to make a release profile *in vitro*. 4-OHT release from the PDDs was sustained and dose-dependent for at least 3 weeks.

To determine the optimal loading concentration of 4-OHT in the PDDs, leading to the highest levels of recombination *in vivo*, PDDs were placed around carotid and femoral arteries with a dose range from 0 to 10% (w/w) 4-OHT for 7 days. Arteries were examined for β-galactosidase-positive SMCs and morphology. At a loading of 0.1% 4-OHT recombination was hardly detectable, while at 0.3% 4-OHT 2.2±2.1% SMC-recombination for carotid and 1.5±1.5% for femoral arteries was found (Figure 2). At a loading concentration of 1%, 4-OHT recombination was increased to 4.0±3.8% for femoral and 2.4±1.8% for carotid arteries (Figure 2, Figure 3B and F, Table 1) and was not significantly different between both arteries (P=0.361). At a loading of 3 or 10% 4-OHT recombination was approximately 3-fold decreased as compared to 1% loaded PDDs (Figure 2, Figure 3C, G and D, H). Increasing the application time of the 1% 4-OHT-loaded PDDs around carotid and femoral arteries from 7 to 14 days did not affect the percentage of SM-recombination (data not shown). Importantly, no

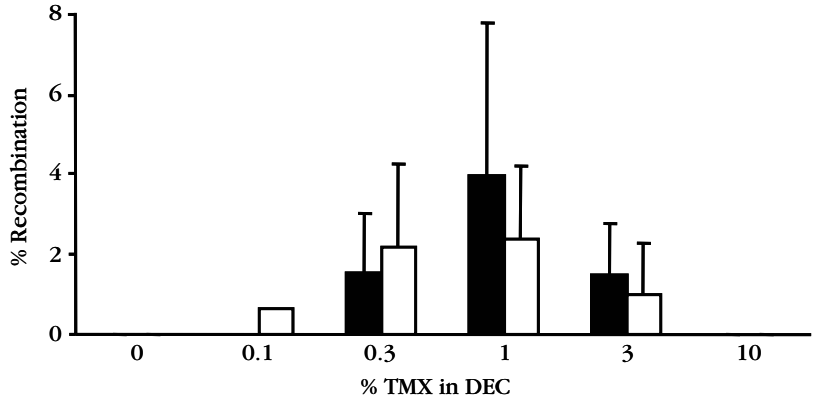


Figure 2. Percentage of medial gene recombination in femoral artery (black bars) and carotid artery (white bars) after incubation with PDDs containing 0, 0.1, 0.3, 1, 3, 10% (w/w) 4-OHT for 7 days. Success of recombination is shown as the number of β-galactosidase-positive SMCs as a percentage of the total number of SMCs.

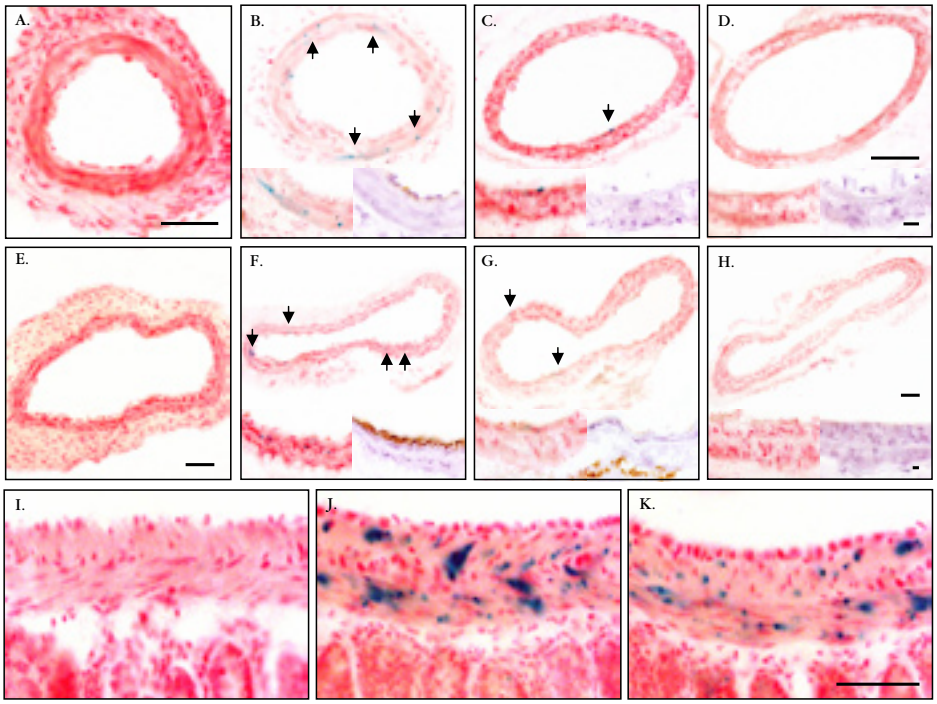


Figure 3. (A-H.) Microscopic images of β-galactosidase (top row, counterstained with nuclear fast red, magn. 200x and left insert, magn. 600x) and PECAM-1 staining (right insert, magn. 600x) of representative cross-sections of (A-D.) femoral and (E-H.) carotid arteries of SM-CreERT2(ki)/rosa26 mice treated with a (A, E.) 0, (B, F.) 1, (C, G.) 3 or (D, H.) 10% (w/w) 4-OHT-loaded PDD for 7 days. (I-K.) Microscopic images of β-galactosidase stained cross-sections of the intestines (I.) without 4-OHT, (J.) 1% 4-OHT-loaded PDD and (K.) systemic 4-OHT administration. Arrows indicate β-galactosidase-positive cells. Scale bar= 50μm.

β-galactosidase positive cells were detected in the aorta, stomach, intestines or the bladder (both 0.0±0.0%), indicating that recombination was restricted to the site of PDD application. No recombination was observed in SM-CreER^{T2}(ki)/rosa26 mice treated with empty PDDs, neither in control rosa26 mice receiving a 4-OHT loaded or empty PDDs. In conclusion, 1% (w/w) 4-OHT-loading for PDDs and application for one week yielded the highest percentage of SM-recombination.

Systemic application of tamoxifen via intraperitoneal (IP) injection for 7 days resulted in β-galactosidase positive staining in cryosections of several SMC-rich organs (aorta 6.7±3.1, femoralis 7.3±1.3, carotis 2.1±0.5 (Table 1) vs. stomach 61.5±5.8, intestines 47.6±9.4 (Figure 3I, J and K), bladder 92.5±4.2). In the femoral artery, systemic tamoxifen administration resulted in a 1.8-fold higher level of SMC recombination as compared to the 4-OHT-eluting PDD. In the carotid artery, the 4-OHT-eluting PDD allowed us to achieve similar levels of SMC-specific recombination as compared to systemic tamoxifen administration.

Table 1. Recombination in vascular SMCs of SM-CreER^{T2}(ki)/rosa26 mice after 7 days of local 4-OHT or systemic tamoxifen administration.

Organ	Administration	
	Local	Systemic
	0.05 mg/PDD	*2 mg/day*
	Recombination (%)	
Femoralis	4.0±3.8	7.3±1.3
Carotis	2.4±1.8	2.1±0.5
Aorta	n.d.	6.7±3.1

n.d. = not determined

Morphological analysis

After application of the 1% 4-OHT PDD to carotid and femoral arteries for 7 days no morphological abnormalities were observed. However, using 3% 4-OHT-loaded PDDs we observed medial thickening as a result of massive increase of medial SMCs and using 10% PDDs this coincided with occasional hemorrhage of the media, as derived from presence of red blood cells in the media (Figure 3 C, G and D, H). Furthermore, the CD31-positive endothelial lining was affected (Figure 3, right insets), as compared to 1% loaded PDDs. In conclusion, local application of PDDs loaded above 1% 4-OHT hampers SM-recombination of both the carotid and femoral vessel wall, as a result of toxic side-effects.

DISCUSSION

In the present study, we describe the means to conditionally modify genes at a predefined and localized region of a blood vessel using a perivascular drug delivery device (PDD). When applied to SM-CreER^{T2}(ki)/rosa26 mice, a dose and time curve of 4-OHT released from the PDD showed optimal gene recombination at 1% (w/w) 4-OHT loading at 7 days post application (carotid artery 2.4±1.8%; femoral artery 4.0±3.8% of SMCs). No gene recombination could be detected in vehicle treated SM-CreER^{T2}(ki)/rosa26, 4-OHT treated control rosa26 mice, gastrointestinal SMCs or other regions of the vasculature (0.0±0.0%). Thus, local application of a 4-OHT-eluting PDD results in highly localized SMC-specific Cre-mediated recombination in SM-CreER^{T2}(ki)/rosa26 mice at levels that are in the same order of magnitude to systemic tamoxifen administration, but without affecting additional SMCs.

The efficiency of systemic versus local application of 4-OHT in carotid and femoral arteries is similar at 2-7%. This efficiency could neither be increased by loading more 4-OHT in the PDD (Figure 2), nor by increasing the exposure time of the PDD (data not shown). In contrast, higher 4-OHT dosages in the PDD actually resulted in vascular toxicity (Figure 3). The dose-response curve of locally delivered 4-OHT to the vessel wall and the results of systemic 4-OHT administration seems to justify the notion that the efficiency of SMC recombination in carotid and femoral arteries of SM-CreER^{T2}(ki)/rosa26 mice is maximal at 2-7%.

In our experiments, we observed a more than 10-fold difference in recombination in vascular versus gastrointestinal SMCs. This difference in susceptibility to recombination has also been observed by Feil et al.[3] One explanation for this phe-

nomenon may be that the activity of the SM22 promoter fragment used in the SM-CreER^{T2}(ki) construct is decreased in vascular SMCs versus gastrointestinal SMCs. However, indirect analysis of SM22 promoter activity by measuring Cre mRNA levels using quantitative real-time PCR did reveal relatively high expression levels in both vascular and gastrointestinal SMCs in our mice (data not shown). Alternatively, the difference in recombination efficiency between vascular and gastrointestinal SMCs could be caused by differences in accessibility of the loxP sites for the Cre enzyme.[11] In the present study we did not further address this topic.

The present study indicates that, using the SM-CreER^{T2} mouse model, vascular recombination efficiency does not exceed 8% in femoral and 4% in carotid arteries. These levels are not sufficient to study genes that potentially show a mild phenotype upon activation or deletion. The SM-CreER^{T2}(ki) mice would be more suitable for loss-of-function or gain-of-function experiments of targets, that will have a dramatic impact upon subtle presence or absence. Examples of such targets are specific secretory proteins (cytokines, chemokines, enzymes) and oncogenes. In the case of secretory tissue inhibitor of metalloproteinase-3 (TIMP-3) recent data showed that a 8-10% adenoviral transduction efficiency resulted in potent effects on gelatinolytic activity, apoptosis and vascularization of melanomas.[12] Apoptosis and matrix breakdown are important processes implicated in local vascular diseases such as atherosclerosis and restenosis. In addition, studies aiming at the investigation of the vasculature using systemic TMX treatment could result in lethality as a result of whole body SMC targeting. The induced lethality can be circumvented by local TMX application using the PDD. Thus, the limited recombination levels achieved with the PDDs in the SM-CreER^{T2}(ki) model could still be sufficient when the right target genes are considered.

The application of a 4-OHT-eluting device to locally induce the ER^{T2}-driven Cre-recombinase gene is particularly useful in case the applied tissue-specific promoter does not display a sufficiently narrow expression pattern. In this respect, it is noteworthy to mention that the 4-OHT-eluting polymer PDD, when size adapted and placed at the gastrointestinal tract, can also be used to induce local gene recombination in SMCs of the stomach and intestine (data not shown). Thus, this technology enables physical limitation to the 4-OHT exposed area that can subsequently undergo Cre-mediated recombination.

ACKNOWLEDGEMENTS

This study was supported by the Netherlands Organization of Scientific Research (NWO/ZonMw grant no. 902-26-242) and the Netherlands Heart Foundation (grant no. 2000.051). The research of B.J.M.v.V. has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. J.W.J. is a clinical established investigator of the Netherlands Heart Foundation (2001D032).

REFERENCES

1. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685-1695.

2. Pires NM, van der Hoeven BL, de Vries MR, Havekes LM, van Vlijmen BJ, Hennink WE, Quax PH, Jukema JW. Local perivascular delivery of anti-restenotic agents from a drug-eluting poly(epsilon-caprolactone) stent cuff. Biomaterials 2005;26:5386-5394.
3. Kuhbandner S, Brummer S, Metzger D, Chambon P, Hofmann F, Feil R. Temporally controlled somatic mutagenesis in smooth muscle. Genesis 2000;28:15-22.
4. Mao X, Fujiwara Y, Orkin SH. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. Proc Natl Acad Sci U S A 1999;96:5037-5042.
5. Signore PE, Machan LS, Jackson JK, Burt H, Bromley P, Wilson JE, McManus BM. Complete inhibition of intimal hyperplasia by perivascular delivery of paclitaxel in balloon-injured rat carotid arteries. J Vasc Interv Radiol 2001;12:79-88.
6. Winternitz CI, Jackson JK, Oktaba AM, Burt HM. Development of a polymeric surgical paste formulation for taxol. Pharm Res 1996;13:368-375.
7. Jackson JK, Zhang X, Llewellen S, Hunter WL, Burt HM. The characterization of novel polymeric paste formulations for intratumoral delivery. Int J Pharm 2004;270:185-198.
8. Lardenoye JH, Delsing DJ, de Vries MR, Deckers MM, Princen HM, Havekes LM, van Hinsbergh VW, van Bockel JH, Quax PH. Accelerated atherosclerosis by placement of a perivascular cuff and a cholesterol-rich diet in ApoE*3Leiden transgenic mice. Circ Res 2000;87:248-253.
9. Moroi M, Zhang L, Yasuda T, Virmani R, Gold HK, Fishman MC, Huang PL. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. J Clin Invest 1998;101:1225-1232.
10. Chai Y, Jiang X, Ito Y, Bringas P, Jr., Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 2000;127:1671-1679.
11. Mao X, Fujiwara Y, Chapdelaine A, Yang H, Orkin SH. Activation of EGFP expression by Cre-mediated excision in a new ROSA26 reporter mouse strain. Blood 2001;97:324-326.
12. Ahonen M, Ala-Aho R, Baker AH, George SJ, Grenman R, Saarialho-Kere U, Kahari VM. Antitumor activity and bystander effect of adenovirally delivered tissue inhibitor of metalloproteinases-3. Mol Ther 2002;5:705-715.

Chapter 6

TNFα and atherosclerosis

Tumor Necrosis Factor-α promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice

Lianne S.M. Boesten[1,4]; A. Susanne M. Zadelaar[2,4]; Anita van Nieuwkoop[1,4]; Marion J.J. Gijbels[5,6]; Menno P.J. de Winther[5]; Louis M. Havekes[2,4]; Bart J.M. van Vlijmen[3]

[1]Department of General Internal Medicine, [2]Department of Cardiology, [3]Department of Hematology, Leiden University Medical Center, [4]TNO-PG/Gaubius Laboratory, Leiden, The Netherlands, [5]Department of Molecular Genetics, Cardiovascular Research Institute Maastricht, [6]Department of Pathology, Maastricht University, Maastricht, The Netherlands

ABSTRACT

Tumor Necrosis Factor-α (TNFα) is a pleiotropic cytokine exerting both inflammatory and cell death modulatory activity, and is thought to play a role in the pathogenesis of atherosclerosis. Studies in mice indicated that TNFα affects atherosclerosis minimally or not under conditions that allow fatty streak formation. Here, we examined the possible role of TNFα in advanced and complex atherosclerotic lesions.

To induce atherosclerosis, TNFα-deficient (*Tnf-/-*) APOE*3-Leiden and control APOE*3-Leiden only mice were fed a cholesterol-rich diet. Comparable levels of plasma cholesterol and triglycerides and the systemic inflammatory parameters, serum amyloid A and soluble intercellular adhesion molecule-1 were found in APOE*3-Leiden*Tnf-/-* and control mice. Although absence of TNFα did not affect the quantitative area of atherosclerosis, APOE*3-Leiden*Tnf-/-* mice had a higher relative number of early lesions (46.1% vs. 21.4%) and a lower relative number of advanced lesions (53.9% vs. 78.6%, P=0.04). In addition, the advanced lesions in APOE*3-Leiden*Tnf-/-* mice showed less necrosis (9.9±12.1% vs. 23.4±19.3% of total lesion area, P=0.04) and an increase in apoptosis (1.5±1.5% vs. 0.4±0.6% of total nuclei, P=0.03).

Our data indicate that TNFα stimulates the formation of lesions towards an advanced phenotype, with more lesion necrosis and a lower incidence of apoptosis.

Inflammatory processes are involved in all stages of atherosclerotic lesion development.[1,2] Cytokine-mediated pro-inflammatory responses are thought to positively contribute to the atherogenic process. Tumor Necrosis Factor-α (TNFα) is a central mediator of inflammatory reactions.[3] TNFα is a member of the TNF ligand family and binding to its receptors TNFR1 (p55) and TNFR2 (p75) leads to activation of downstream targets.[4] Whereas binding of TNFα to TNFR1 activates responses associated with induction of adhesion molecule expression,[5] apoptosis,[6] and resistance to bacterial infection,[7,8] binding to TNFR2 activates induction of T cell proliferation,[9] induction of TNFα-mediated skin tissue necrosis,[10] and modulation of TNFα-mediated pulmonary inflammation.[11]

Although TNFα and its receptors are thought to be considerably important in a number of biological activities relevant to atherosclerosis, its function in atherogenesis remains unclear. The TNFα gene has a number of polymorphisms, some of which affect transcription and secretion.[12] These polymorphisms have been associated with a number of infectious, inflammatory and immune diseases including atherosclerosis and coronary artery disease (CHD).[12] However, human association studies are controversial varying from no,[12-14] a weak[15] or a strong[16] association between TNFα polymorphisms and CHD. Moreover, studies on the role of TNFα in atherosclerosis using several transgenic or knock out mouse models also yielded controversial results. TNFα ligand deficiency on a wild type C57BL/6 background did not affect early lesion development.[17] However, on the same background, TNF receptor 1 deficiency did affect atherosclerosis formation, resulting in enhanced (early) lesion formation. This unexpected result was attributed to increased macrophage scavenger receptor activity and consequent increased uptake of atherogenic lipoproteins.[18] Very recently, as a part of mouse studies on a non-cleavable transmembrane form of TNFα, TNFα-deficiency on a C57BL/6 background was demonstrated to reduce atherosclerosis.[19] This effect however coincided with a significant decrease in plasma VLDL and increase in plasma HDL levels. Hence, a direct anti-atherosclerotic effect of TNFα deficiency could not be concluded from this experiment. Overall, the above recombinant mouse studies suggest that TNFα may have subtle pro-atherogenic properties, possibly related to an unexpected TNFα-mediated effect on atherogenic lipoproteins, at least in a setting of a C57BL/6 background.

Variable results were also obtained upon (pharmacological) modulation of TNFα expression. Specific immunization against TNFα was not effective in preventing the formation of advanced lesions in apoE-deficient mice.[20] Capturing TNFα using a TNFα receptor homologue had only a modest and gender-dependent inhibitory effect on the formation of early lesions in the apoE-deficient setting.[21] On the contrary thalidomide, a TNFα production inhibitor, was capable of inhibiting the early atherogenesis in apoE-deficient mice.[22]

The majority of these mouse studies focuses on the role of TNFα in early lesion development in relatively atherosclerosis-resistant C57BL/6 mice[17-19] and young apoE-deficient mice[20,22] that display only minimal advanced lesion formation. Inflammation and cell death are important processes in the development and transition towards advanced and complex atherosclerotic lesions and may be modulated by TNFα. Therefore, we examined the role TNFα under conditions of advanced lesion formation. To this end, we crossbred TNFα deficient (*Tnf-/-*) mice with APOE*3-

Leiden transgenic mice. APOE*3-Leiden is a mutant form of apoE, characterized by a 7-amino acid tandem repeat of residues 120-126 and yields a mature protein of 306 amino acid residues. Family studies have demonstrated that this mutation is associated with a dominantly inherited form of familial dysbetalipoproteinemia (FD).[23-25] APOE*3-Leiden transgenic mice have a lipoprotein profile that is very similar to the profile of patients with FD in which the elevated plasma cholesterol and triglyceride levels are mainly confined to the VLDL/LDL-sized lipoprotein fractions. Therefore, APOE*3-Leiden transgenic mice are an established model for hyperlipidemia and atherosclerosis.[26] We investigated the effect of TNFα on advanced lesion formation and on lesion composition. Our data indicate that TNFα, independent of the atherogenic lipoprotein profile, stimulates lesional necrosis, decreases the incidence of apoptosis and the progression of lesions towards a more advanced phenotype.

METHODS

Mice and diet

The experimental animals were obtained by crossbreeding *Tnf-/-* mice[27] with APOE*3-Leiden transgenic mice.[28] Offspring was analyzed for their TNFα status using polymerase chain reaction (PCR) analysis and presence of the human APOE*3-Leiden transgene by ELISA.[29]

For experiments, 8 weeks old female APOE*3-Leiden*Tnf-/-* and littermate control APOE*3-Leiden*Tnf+/+* mice were used. Mice (n=31) were fed a semi-synthetic cholesterol-rich diet composed essentially according to Nishina et al.[30] supplemented with cocoa butter (15%, by weight) and cholesterol (1%, by weight), without dietary cholate. The diet was purchased from Hope Farms, Woerden, The Netherlands. Mice were bred and housed under specific pathogen-free conditions and given food and water ad libitum. All animal work was approved by institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Blood sampling and analysis

Blood samples were collected in EDTA-coated vials (Sarstedt, Nümbrecht, Germany) by bleeding from the tail vein. Plasma cholesterol and triglyceride levels were measured enzymatically using commercially available kits (Roche Diagnostics GmbH, Mannheim, Germany).

Total blood leukocyte ($CD45^+$), T-cell ($CD3^+$), B-cell ($CD19^+$) and monocyte/granulocyte ($CD11b^+$) numbers were determined by FACS analysis (FACSCalibur, BD Biosciences, California, USA) of whole blood using a PerCP-CY5.5-conjugated rat anti-mouse CD45 monoclonal antibody, a fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD3 monoclonal antibody, a R-Phycoerythrin (R-PE) conjugated rat anti-mouse CD19 monoclonal antibody, and an allophycocyanin (APC)-conjugated rat anti-mouse CD11b monoclonal antibody, respectively following standard protocol (TruCOUNT, BD Biosciences, California, USA).

Serum Amyloid A (SAA, acute phase protein) ELISA (BioSource International, Inc, Camarillo, CA) and Mouse soluble Intercellular Adhesion Molecule-1 (sICAM-1, marker for endothelial cell activation) ELISA (Pierce Biotechnology, Inc, Rockford, IL) were performed on plasma samples according to standard protocols.[31]

Atherosclerosis analysis

After 20 weeks of a cholesterol-rich diet, mice were sacrificed, heart and aorta were isolated and embedded in tissue tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) and stored at -80˚C. From the entire aortic root area of the heart, four 7 μm cross-sections with an interval of 42 μm were used for quantification of atherosclerotic lesion area.[32] Sections were routinely stained with toluidin blue for morphometric analysis, characterization and categorization of the lesions, and with Sirius red for quantification of collagen area. Lesion area was determined using Leica Qwin image software (EIS, Asbury, NJ). All analyses were performed blindly without prior knowledge of the genotype.

Atherosclerotic lesions were categorized on severity. Two types of categories were discerned: (1) early lesions were fatty streaks containing only foam cells (type I-II lesions) and (2) advanced lesions showed foam cells in the media and presence of fibrosis, cholesterol clefts, mineralization and/or necrosis covered by a collagenous cap (type III-V lesions).[29] The number observed in each category is expressed as a percentage of the total number of lesions observed within one group of mice (APOE*3-Leiden*Tnf-/-* or APOE*3-Leiden*Tnf*+/+ control group).

Sections were stained for macrophages using rat FA11 antibody to mouse macrophages (a kind gift from S. Gordon, Oxford University, UK), for smooth muscle cells using a monoclonal α-smooth muscle cell actin antibody (Sigma-Aldrich, St. Louis, USA), for T-cells using KT3 rat antibody (a kind gift from G. Kraal, VUMC, The Netherlands), and areas were quantified as described previously.[33,34] For the detailed phenotypic sub-analysis of the advanced lesions (macrophage area, SMC area, collagen area and the number of T-cells) only those mice were included that displayed advanced atherosclerotic lesions (APOE*3-Leiden*Tnf*+/+ n=13, APOE*3-Leiden-*Tnf-/-* n=14)

Quantification of lesion necrosis and apoptosis

During pathological examination of the lesions, lesional necrosis was defined by the presence of pyknosis, karyorrhexis, or complete absence of nuclei, as described before.[33] Necrosis area was measured using the method for total lesion area measurements, as described above. Apoptosis was quantified using the TUNEL technique.[35] Only those TUNEL-positive nuclei were included that displayed morphological features of apoptosis including cell shrinkage, aggregation of chromatin into dense masses, and nuclear fragmentation. For the detailed phenotypic sub-analysis of the advanced lesions, on lesion necrosis and apoptosis, only those mice were included that displayed advanced atherosclerotic lesions (APOE*3-Leiden*Tnf*+/+ n=13, APOE*3-Leiden*Tnf-/-* n=14).

Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, California, USA). Data were tested for normality using the Kolmogorov-Smirnov test.

All data following normality were tested using Welch's corrected *t*-test. Data are expressed as mean±SD, unless stated otherwise. *P*-value < 0.05 was regarded as significant. Frequency data for lesion categorization were compared by means of the Fisher's exact test.

RESULTS

General

Female APOE*3-Leiden*Tnf-/-* (n=18) mice and APOE*3-Leiden*Tnf*+/+ (n=13) littermate controls were fed a cholesterol-rich diet. The mice appeared healthy and displayed no signs of abnormalities. After 20 weeks feeding the cholesterol-rich diet, APOE*3-Leiden*Tnf-/-* mice were significantly lower in body weight as compared to APOE*3-Leiden*Tnf*+/+ control mice (Table 1).

Following cholesterol-rich diet feeding, both APOE*3-Leiden*Tnf-/-* and control mice had comparable levels of plasma cholesterol and plasma triglyceride levels (Table 1). As determined by FPLC analysis on pooled plasma, lipoprotein distribution did not differ between APOE*3-Leiden*Tnf-/-* and APOE*3-Leiden*Tnf*+/+ control mice (Figure 1).

Analysis of $CD45^+$ blood leukocytes showed that absence of TNFα resulted in an approximate doubling of circulating $CD3^+$ (*P*=0.01) and $CD19^+$ (*P*=0.06) cells (T- and B-cells, respectively), leaving the $CD11b^+$ cell population (monocytes/granulocytes) unaffected (Table 1).

Plasma levels of serum amyloid A (SAA) and soluble intercellular adhesion molecule-1 (sICAM-1) were comparable in APOE*3-Leiden*Tnf-/-* and APOE*3-Leiden-*Tnf*+/+ control mice indicating no difference in inflammation (SAA) or endothelial cell activation (sICAM) between the two groups (Table 1).

Table 1. Characteristics of female APOE*3-Leiden*Tnf*+/+ and APOE*3-Leiden*Tnf-/-* mice after 20 weeks feeding a cholesterol-rich diet

Parameters	**E3L*Tnf*+/+**	**E3L*Tnf-/-***
Weight (g)	25.7±2.6	22.6±1.5*
Plasma cholesterol (mmol/l)	21.7 ± 3.8	19.4 ± 4.2
Plasma triglycerides (mmol/l)	2.0 ± 0.7	2.3 ± 1.5
$CD3^+$ (10^6 cells/ml)	1.8 ± 0.6	3.2 ± 1.0*
$CD19^+$ (10^6 cells/ml)	3.8 ± 2.2	6.1 ± 2.0
$CD11b^+$ (10^6 cells/ml)	2.6 ± 0.9	2.7 ± 1.5
SAA (μg/ml)	16.0±4.5	12.8±28.4
sICAM (μg/ml)	23.8±2.1	23.6±2.0

* *P*<0.05, as compared to APOE*3-Leiden*Tnf*+/+ control mice

Atherosclerosis analysis

Mice fed the cholesterol-rich diet for 20 weeks were sacrificed for collection of heart and aorta. Histopathological analysis of atherosclerotic lesions of the aortic valve area in both APOE*3-Leiden*Tnf-/-* and control mice revealed that the majority

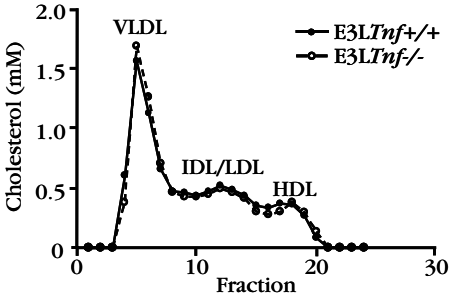


Figure 1. Plasma lipoprotein profile after size fractionation of pooled plasma samples of APOE*3-Leiden*Tnf*+/+ (closed symbols) and APOE*3-Leiden*Tnf-/-* (open symbols) after 20 weeks of feeding the cholesterol-rich diet.

of the lesions were fibrous plaques with a lipid core and a cap covering necrotic material (pyknosis, karyorrhexis, or complete absence of nuclei), cholesterol clefts and extra-cellular lipids. As determined by computer-assisted morphometry, mean total lesion area did not differ significantly between APOE*3-Leiden*Tnf*+/+ and APOE*3-Leiden*Tnf-/-* mice (5.8±2.8 and 5.1±4.5x104 μm^2, respectively, P=0.64, Figure 2A). While a difference in total lesion area was absent, lesion categorization (early vs. advanced) revealed a difference between APOE*3-Leiden*Tnf-/-* and control mice (Figure 2B). APOE*3-Leiden*Tnf-/-* mice had a significant higher incidence of early lesions (46.1% vs. 21.4%) and a significant decreased incidence of advanced lesions (53.9% vs. 78.6%) as compared to APOE*3-Leiden*Tnf*+/+ controls (P=0.04).

A more detailed phenotypic sub-analysis analysis of the advanced lesions only, did not yield any effect of TNFα status on lesion macrophage area, lesion smooth muscle cell area or collagen area. In addition, the number of lesional T cells did not differ between the groups (Table 2). However, pathological analysis revealed that advanced lesions in APOE*3-Leiden*Tnf-/-* mice had smaller areas of pyknosis, karyor-

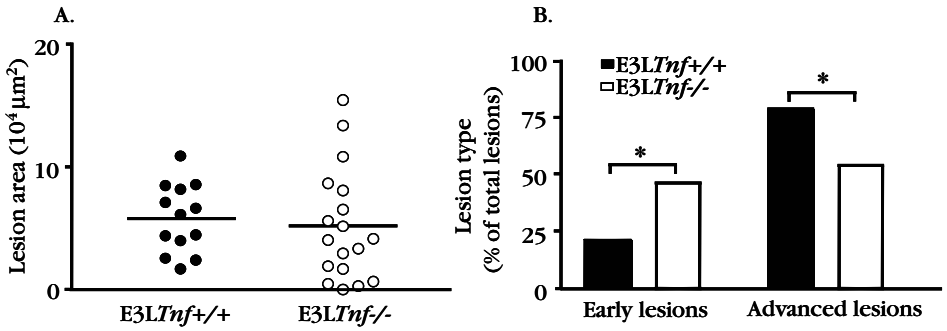


Figure 2. (A.) After 20 weeks of feeding the cholesterol-rich diet, the extent of atherosclerosis in APOE*3-Leiden*Tnf*+/+ (closed symbols, n=13) and APOE*3-Leiden*Tnf-/-* (open symbols, n=18) mice was quantified at the level of the aortic valves. Each data point represents the lesion area per mouse. Line represents mean area for each group. (B.) Lesion categorization of APOE*3-Leiden*Tnf*+/+ (black bars) and APOE*3-Leiden*Tnf-/-* (white bars) mice. Lesions were categorized according to severity and are shown as percentage of the total number of lesions present. Frequency data were compared by means of the Fisher's exact test, P=0.04.

Table 2. Characteristics of advanced atherosclerotic lesions in APOE*3-Leiden*Tnf*+/+ and APOE*3-Leiden *Tnf*-/- mice

Lesion composition	E3L*Tnf*+/+	E3L*Tnf*-/-
Lesion macrophage area (% of total lesion area)	39.0±14.0	38.7±18.3
Lesion SMC area (% of total lesion area)	9.0±5.6	10.9±7.1
Collagen area (% of total lesion area)	73.5±21.9	78.9±13.9
T-cells (number of cells/total lesion area)	1.8±1.1	1.3±1.4

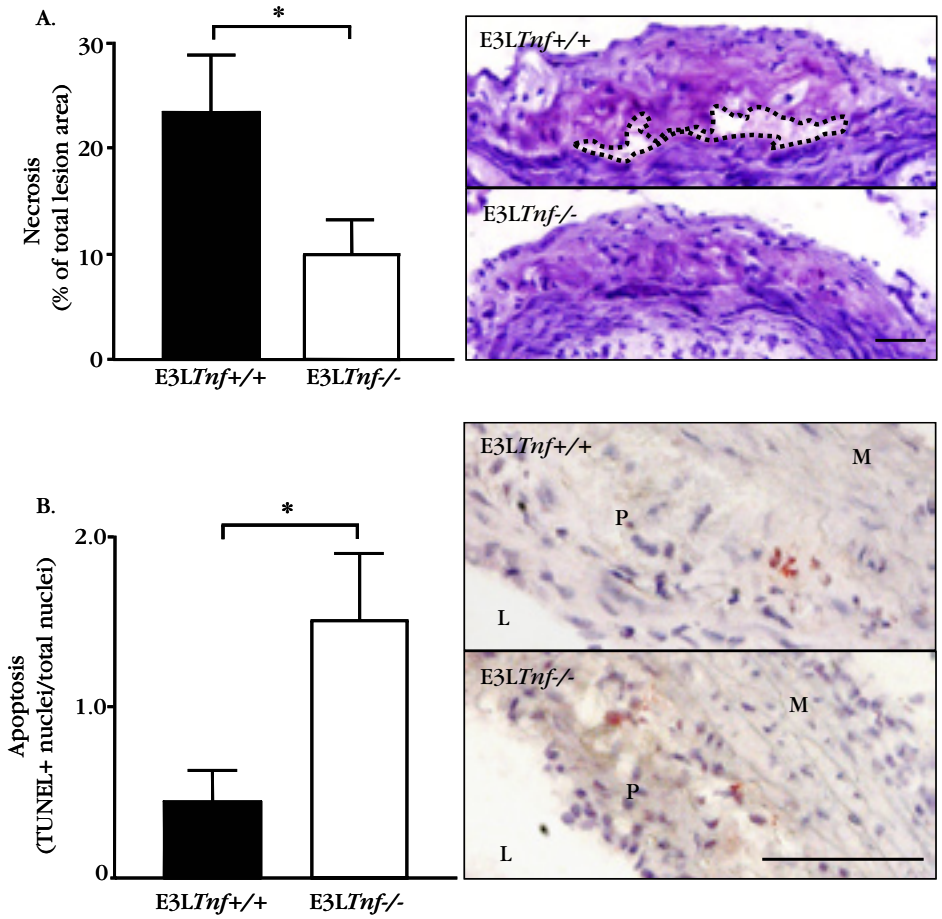


Figure 3. (A.) Necrosis (P=0.04) and (B.) Apoptosis (P=0.03) in advanced lesions of APOE*3-Leiden*Tnf*+/+ (black bar, n=13) and APOE*3-Leiden*Tnf*-/- (white bar, n=14). Error bars indicate SEM. Inserts: representative pictures of advanced lesions in APOE*3-Leiden*Tnf*+/+ and APOE*3-Leiden*Tnf*-/- mice. (A.) The black dotted line indicates the necrotic area (magn. 40x, scale bar 100 μm). (B.) The arrows indicate TUNEL-positive nuclei. L: lumen, P: plaque, M: media, (magn 100x, scale bar 100 μm).

rhexis, or complete absence of nuclei as compared to advanced lesions in control mice, suggesting that the TNFα-status affects lesional necrosis. The early atherosclerotic lesions did not contain any necrotic area in both groups. Computer-assisted morphometric analysis demonstrated that advanced lesions in APOE*3-Leiden*Tnf-/-* mice had significant smaller areas covered by necrosis as compared to advanced lesions in controls (9.9±12.1% vs. 23.4±19.3% of total lesion area, P=0.04, Figure 3A).

TUNEL-positive nuclei (apoptotic cells) were only found in advanced lesions of both APOE*3-Leiden*Tnf-/-* and control mice. The location of the TUNEL-positive nuclei (i.e. core or cap) did not differ between APOE*3-Leiden*Tnf-/-* and control mice (data not shown). In addition, the majority of the TUNEL-positive nuclei was located in the core of the advanced lesions, in both APOE*3-Leiden*Tnf-/-* and control mice. Remarkably, the incidence of TUNEL-positive nuclei in advanced lesions of APOE*3-Leiden-*Tnf-/-* mice was 3.4-fold higher as observed for lesions in control mice (1.5±1.5% vs. 0.4±0.6% of total number of cells, P=0.03, Figure 3B). Hence, in APOE*3-Leiden*Tnf-/-* mice a decrease in lesional necrosis coincides with increased apoptosis.

DISCUSSION

In the present study we examined the role of TNFα in the formation of advanced lesions. Using atherosclerosis-susceptible APOE*3-Leiden mice crossbred with TNFα-deficient mice we found that TNFα modulates lesional cell death by increasing necrosis and decreasing the incidence of apoptosis. Moreover, TNFα progresses the lesions towards a more advanced phenotype.

Very recently, Branén et al. also showed that TNFα stimulates atherosclerosis development using apoE/*Tnf* knock out mice.[36] ApoE deficiency affects local cholesterol homeostasis in macrophages and inflammatory reactions within the atherosclerotic vessel.[37] In the current study the role of TNFα was assessed specifically in advanced atherosclerotic lesion development on an APOE*3-Leiden background (with endogenous apoE still present) preventing the possible local effects due to the absence of apoE. Importantly, besides the differences in study-design, both studies demonstrate that TNFα can be considered a pro-atherogenic cytokine in atherosclerosis development.

Inflammation and cell death are important processes in the development and transition towards advanced and complex atherosclerotic lesions.[38-40] Our observation that TNFα promotes advanced lesion formation, and on top of that increases the extent of necrosis in these lesions, is in line with the role of TNFα function in modulating inflammatory processes and cell death.[3,4,6-8,10,11]

Studies on human endarterectomy specimens showed that unstable atherosclerotic lesions are characterized by a necrotic core consisting of dead macrophages, macrophage debris and extracellular lipid covered by a fibrocellular cap.[41] Therefore, necrosis is often considered to be a negative risk factor for plaque stability. The observation that TNFα increases the ratio of necrosis versus apoptosis suggests that TNFα modulates the lesion towards a more instable phenotype. Moreover, *in vitro* studies have shown that TNFα stimulates both macrophages and smooth muscle cells to synthesize matrix proteases[42,43] and in such a way can contribute to plaque instability by degrading the fibrotic cap. Hence, both through increased necrosis

and increased matrix protease activation TNFα may contribute to the formation of instable plaques that are prone to rupture.

The reduction in lesional necrosis upon TNFα deletion coincided with an increase in lesional apoptosis. TNFα is known to exert its effector actions, partially, through the activation of the pro-inflammatory transcription factor, NF-κB.[4] Absence of TNFα may promote apoptotic cell death via a reduction in NF-κB activation. Reduced NF-κB activation does not only lead to a beneficial reduced transcription of pro-inflammatory cytokines and chemokines, but also to a reduction in transcription of anti-apoptotic factors (i.e. Bcl-xL, Bcl-2, IAPs).[44] Apoptosis is often considered to be beneficial for atherosclerosis, since in contrast to necrosis, it may prevent the release of matrix-degrading enzymes and pro-inflammatory substances from the dying foam cells. Our data suggest that apoptosis forms part of a beneficial process since it coincides with a less advanced lesion phenotype.

Uysal et al. demonstrated that the total weight gain in obese *Tnf+/+* mice on a high fat diet was larger than that of obese *Tnf-/-* mice, which was related to a decrease in fat-pad weight.[45] Also our APOE*3-Leiden mice, fed a cholesterol-rich diet, that additionally contained 15% fat, displayed reduced body weight upon TNFα deletion (Table 1). Hence, our data support a role for TNFα in body weight development. Although body weight is not shown to be a predictive parameter for atherosclerosis development in mice,[46,47] it remains open to question whether TNFα-mediated reduction in body weight contributes to the observed TNFα-mediated reduction in advanced lesion formation.

Upon characterization of the TNFα deficient mice Marino et al. reported that these mice have no significant abnormalities in the distribution of lymphocytes, granulocytes or monocyte population in thymus, spleen or peripheral blood.[48] Remarkably, we found an increase in circulating T-cells in TNFα deficient mice, that was independent of the diet and of the APOE*3-Leiden transgene (data not shown). This suggests that TNFα might play a role in determining circulating T-cells in mice already under basal conditions. In this light it is worthwhile to mention that the higher levels of circulating T-cells did not translate to a higher number of T-cells in the advanced atherosclerotic plaque.

In conclusion, we have demonstrated that TNFα enhances progression of lesions towards a more advanced phenotype. One may speculate that drugs that inhibit TNFα expression or capture TNFα biological action may inhibit advanced lesion formation. Future studies are required to demonstrate whether, next to a reduction in early lesions,[21,22] TNFα can be used as a target to prevent the formation of advanced lesions.

ACKNOWLEDGEMENTS

This work was supported by the Netherlands Heart Foundation (NHS 2000.051), the Netherlands Organization for Scientific Research (NWO 902-26-242), M.P.J. de Winther is a NWO-fellow (906-02-075), and B.J.M. van Vlijmen is a fellow of the Royal Netherlands Academy of Arts and Sciences. The authors wish to thank Ingeborg van der Made for her technical assistance.

REFERENCES

1. Ross R. Atherosclerosis is an inflammatory disease. Am Heart J 1999;138:S419-S420.
2. Wick G, Schett G, Amberger A, Kleindienst R, Xu Q. Is atherosclerosis an immunologically mediated disease? Immunol Today 1995;16:27-33.
3. Camussi G, Albano E, Tetta C, Bussolino F. The molecular action of tumor necrosis factor-alpha. Eur J Biochem 1991;202:3-14.
4. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305-1308.
5. Mackay F, Loetscher H, Stueber D, Gehr G, Lesslauer W. Tumor necrosis factor alpha (TNF-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. J Exp Med 1993;177:1277-1286.
6. Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. Cell 1993;74:845-853.
7. Pfeffer K, Matsuyama T, Kundig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Kronke M, Mak TW. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell 1993;73:457-467.
8. Rothe J, Lesslauer W, Lotscher H, Lang Y, Koebel P, Kontgen F, Althage A, Zinkernagel R, Steinmetz M, Bluethmann H. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature 1993;364:798-802.
9. Tartaglia LA, Goeddel DV, Reynolds C, Figari IS, Weber RF, Fendly BM, Palladino MA, Jr. Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. J Immunol 1993;151:4637-4641.
10. Erickson SL, de Sauvage FJ, Kikly K, Carver-Moore K, Pitts-Meek S, Gillett N, Sheehan KC, Schreiber RD, Goeddel DV, Moore MW. Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. Nature 1994;372:560-563.
11. Peschon JJ, Torrance DS, Stocking KL, Glaccum MB, Otten C, Willis CR, Charrier K, Morrissey PJ, Ware CB, Mohler KM. TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. J Immunol 1998;160:943-952.
12. Allen RA, Lee EM, Roberts DH, Park BK, Pirmohamed M. Polymorphisms in the TNF-alpha and TNF-receptor genes in patients with coronary artery disease. Eur J Clin Invest 2001;31:843-851.
13. Koch W, Kastrati A, Bottiger C, Mehilli J, von Beckerath N, Schomig A. Interleukin-10 and tumor necrosis factor gene polymorphisms and risk of coronary artery disease and myocardial infarction. Atherosclerosis 2001;159:137-144.
14. Herrmann SM, Ricard S, Nicaud V, Mallet C, Arveiler D, Evans A, Ruidavets JB, Luc G, Bara L, Parra HJ, Poirier O, Cambien F. Polymorphisms of the tumour necrosis factor-alpha gene, coronary heart disease and obesity. Eur J Clin Invest 1998;28:59-66.
15. Keso T, Perola M, Laippala P, Ilveskoski E, Kunnas TA, Mikkelsson J, Penttila A, Hurme M, Karhunen PJ. Polymorphisms within the tumor necrosis factor locus and prevalence of coronary artery disease in middle-aged men. Atherosclerosis 2001;154:691-697.
16. Vendrell J, Fernandez-Real JM, Gutierrez C, Zamora A, Simon I, Bardaji A, Ricart W, Richart C. A polymorphism in the promoter of the tumor necrosis factor-alpha gene (-308) is associated with coronary heart disease in type 2 diabetic patients. Atherosclerosis 2003;167:257-264.
17. Schreyer SA, Vick CM, LeBoeuf RC. Loss of lymphotoxin-alpha, but not tumor necrosis factor-alpha reduces atherosclerosis in mice. J Biol Chem 2002;277(14):12364-8.
18. Schreyer SA, Peschon JJ, LeBoeuf RC. Accelerated atherosclerosis in mice lacking tumor necrosis factor receptor p55. J Biol Chem 1996;271:26174-26178.
19. Canault M, Peiretti F, Mueller C, Kopp F, Morange P, Rihs S, Portugal H, Juhan-Vague I, Nalbone G. Exclusive expression of transmembrane TNF-alpha in mice reduces the inflammatory response in early lipid lesions of aortic sinus. Atherosclerosis

2004;172:211-218.
20. Hansen PR, Chew M, Zhou J, Daugherty A, Heegaard N, Jensen P, Mouritsen S, Falk E. Freunds adjuvant alone is antiatherogenic in apoE-deficient mice and specific immunization against TNFalpha confers no additional benefit. Atherosclerosis 2001;158:87-94.
21. Elhage R, Maret A, Pieraggi MT, Thiers JC, Arnal JF, Bayard F. Differential effects of interleukin-1 receptor antagonist and tumor necrosis factor binding protein on fatty-streak formation in apolipoprotein E-deficient mice. Circulation 1998;97:242-244.
22. Chew M, Zhou J, Daugherty A, Eriksson T, Ellermann-Eriksen S, Hansen PR, Falk E. Thalidomide inhibits early atherogenesis in apoE-deficient mice. APMIS Suppl 2003;113-116.
23. Havekes L, de Wit E, Leuven JG, Klasen E, Utermann G, Weber W, Beisiegel U. Apolipoprotein E3-Leiden. A new variant of human apolipoprotein E associated with familial type III hyperlipoproteinemia. Hum Genet 1986;73:157-163.
24. van den Maagdenberg AM, de Knijff P, Stalenhoef AF, Gevers Leuven JA, Havekes LM, Frants RR. Apolipoprotein E*3-Leiden allele results from a partial gene duplication in exon 4. Biochem Biophys Res Commun 1989;165:851-857.
25. Wardell MR, Weisgraber KH, Havekes LM, Rall SC, Jr. Apolipoprotein E3-Leiden contains a seven-amino acid insertion that is a tandem repeat of residues 121-127. J Biol Chem 1989;264:21205-21210.
26. Groot PH, van Vlijmen BJ, Benson GM, Hofker MH, Schiffelers R, Vidgeon-Hart M, Havekes LM. Quantitative assessment of aortic atherosclerosis in APOE*3 Leiden transgenic mice and its relationship to serum cholesterol exposure. Arterioscler Thromb Vasc Biol 1996;16:926-933.
27. Pasparakis M, Alexopoulou L, Episkopou V, Kollias G. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J Exp Med 1996;184:1397-1411.
28. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van Vlijmen B, van der BH, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. J Biol Chem 1993;268:10540-10545.
29. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der BH, HogenEsch H, Frants RR, Hofker MH, Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. J Clin Invest 1994;93:1403-1410.
30. Nishina PM, Verstuyft J, Paigen B. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. J Lipid Res 1990;31:859-869.
31. Horie Y, Chervenak RP, Wolf R, Gerritsen ME, Anderson DC, Komatsu S, Granger DN. Lymphocytes mediate TNF-alpha-induced endothelial cell adhesion molecule expression: studies on SCID and RAG-1 mutant mice. J Immunol 1997;159:5053-5062.
32. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 1987;68:231-240.
33. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 2003;112:1176-1185.
34. van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De WB, van Berkel TJ, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circ Res 2001;88:780-786.
35. Kockx MM, Muhring J, Knaapen MW, De Meyer GR. RNA synthesis and splicing interferes with DNA in situ end labeling techniques used to detect apoptosis. Am J Pathol 1998;152:885-888.
36. Branen L, Hovgaard L, Nitulescu M, Bengtsson E, Nilsson J, Jovinge S. Inhibition of

Tumor Necrosis Factor-{alpha} Reduces Atherosclerosis in Apolipoprotein E Knockout Mice. Arterioscler Thromb Vasc Biol 2004;24(11):2137-42.
37. Curtiss LK, Boisvert WA. Apolipoprotein E and atherosclerosis. Curr Opin Lipidol 2000;11:243-251.
38. Geng YJ, Libby P. Progression of atheroma: a struggle between death and procreation. Arterioscler Thromb Vasc Biol 2002;22:1370-1380.
39. Libby P. Inflammation in atherosclerosis. Nature 2002;420:868-874.
40. Libby P. Vascular biology of atherosclerosis: overview and state of the art. Am J Cardiol 2003;91:3A-6A.
41. Tabas I. Apoptosis and plaque destabilization in atherosclerosis: the role of macrophage apoptosis induced by cholesterol. Cell Death Differ 2004;11 Suppl 1:S12-S16.
42. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Unemori EN, Lark MW, Amento E, Libby P. Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. Circ Res 1994;75:181-189.
43. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Libby P. Enhanced expression of vascular matrix metalloproteinases induced in vitro by cytokines and in regions of human atherosclerotic lesions. Ann N Y Acad Sci 1995;748:501-507.
44. Karin M, Ben Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 2000;18:621-663.
45. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature 1997;389:610-614.
46. Lyngdorf LG, Gregersen S, Daugherty A, Falk E. Paradoxical reduction of atherosclerosis in apoE-deficient mice with obesity-related type 2 diabetes. Cardiovasc Res 2003;59:854-862.
47. Schreyer SA, Lystig TC, Vick CM, LeBoeuf RC. Mice deficient in apolipoprotein E but not LDL receptors are resistant to accelerated atherosclerosis associated with obesity. Atherosclerosis 2003;171:49-55.
48. Marino MW, Dunn A, Grail D, Inglese M, Noguchi Y, Richards E, Jungbluth A, Wada H, Moore M, Williamson B, Basu S, Old LJ. Characterization of tumor necrosis factor-deficient mice. Proc Natl Acad Sci U S A 1997;94:8093-8098.

Chapter 7

PPARα/γ agonism and atherosclerosis

The dual PPARα/γ agonist tesaglitazar reduces atherosclerosis development beyond its plasma cholesterol-lowering effects in APOE*3-Leiden transgenic mice

A Susanne M Zadelaar[1,2]; Lianne SM Boesten[2,3]; J Wouter Jukema[1]; Bart JM van Vlijmen[4]; Teake Kooistra[2]; Jef J Emeis[2]; Erik Lundholm[5]; German Camejo[5]; Louis M Havekes[1,2,3]

[1]Department of Cardiology, Leiden University Medical Center; [2]TNO-Gaubius Laboratory, Biomedical Research Unit; [3]Department of General Internal Medicine, Leiden University Medical Center; [4]Hemostasis and Thrombosis Research Center, Leiden University Medical Center, Leiden, The Netherlands; [5]Astra Zeneca, Mölndal, Sweden.

ABSTRACT

We investigated whether the dual PPARα/γ agonist tesaglitazar has anti-atherogenic effects in APOE*3-Leiden mice with normal and reduced insulin sensitivity.

APOE*3-Leiden transgenic mice were fed either a low-fat (LF) diet or a high-fat (HF), insulin-resistance-inducing diet. In both LF and HF-fed mice, one group received a high-cholesterol supplement (1% wt/wt; HC group). A second group received the same HC supplement along with tesaglitazar 0.5 μmol/kg diet (T group). A third (control) group received a low cholesterol supplement (0.1% wt/wt; LC group), which resulted in plasma cholesterol levels similar to those of the T group. In both HF- and LF-fed mice, tesaglitazar decreased plasma cholesterol by 20% compared with the respective HC groups; cholesterol levels were similar in the T and LC groups. In LF-fed mice, tesaglitazar reduced atherosclerosis in the aortic root up to 65%, whereas the cholesterol-matched LC group had a reduction of 38%. In HF-fed mice, tesaglitazar produced a 92% reduction in atherosclerosis, while a 56% reduction was seen in the cholesterol-matched LC group. Furthermore, tesaglitazar treatment significantly reduced lesion number beyond that expected from cholesterol lowering, and induced a shift to less severe lesions. Concomitantly, tesaglitazar reduced macrophage-rich and collagen areas in both HF- and LF-fed mice. In addition, tesaglitazar treatment reduced inflammatory markers, including plasma serum amyloid A levels, the number of adhering monocytes, and nuclear factor κB activity in the vessel wall.

Tesaglitazar has anti-atherosclerotic effects that go beyond plasma cholesterol lowering. These effects were more pronounced in HF-fed mice. Tesaglitazar may exert these actions via anti-inflammatory effects.

Agonists of the peroxisome proliferator-activated receptor (PPAR) α have positive effects on lipid metabolism both in animal models and in clinical practice.[1-3] Agonists of PPAR γ– the thiazolidinediones rosiglitazone and pioglitazone – improve insulin resistance in type 2 diabetes, and pioglitazone improves the dyslipidemia associated with insulin resistance.[4-6] In addition to these effects, both PPARα and γ agonists have anti-inflammatory properties,[7,8] and, therefore, have the potential to provide additional cardiovascular benefit.[9]

PPARα and -γ agonists appear to act at two different levels to counteract atherosclerosis. Systemically, they ameliorate the atherogenic lipid profile by reducing plasma free fatty acids and triglycerides, and increasing high-density lipoprotein (HDL) cholesterol levels.[10] At the cellular level, PPAR agonists act on most cell types involved in atherosclerosis, including endothelial cells, smooth muscle cells (SMCs), macrophages and lymphocytes, reducing their involvement in the tissue response associated with plaque development.These agonists dampen the systemic response to inflammation by reducing levels of plasma proteins such as C-reactive protein (CRP), tumor necrosis factor (TNF) α and interferon (IFN) γ;[11] inhibiting interleukin (IL) 2 and TNFα secretion by monocytes;[12] and reducing IL-1-induced secretion of IL-6 via nuclear factor (NF) κB signaling pathways in SMCs.[13,14]

PPAR agonists have a number of other actions that positively modulate vascular effects. In the endothelium, for example, they inhibit production of the vasoconstrictor endothelin-1[15,16] and inhibit cytokine-induced expression of the adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion molecule-1.[17] In monocyte/macrophages, chemotaxis by monocyte chemotactic peptide-1 and proteolytic enzyme activity by matrix metalloproteinase-9 are inhibited,[18-20] and the proliferation and migration of SMCs are inhibited.[21] Both PPARα and -γ stimulate ATP-binding cassette transporter A1 expression, thereby promoting cholesterol efflux from macrophages[22] and possibly cholesterol excretion into the gut.

In the clinical setting, PPARα agonists reduce cardiovascular disease (CVD) risk, especially in subjects with insulin resistance.[23] PPARγ agonists have been to shown reduce the progression of intima-media thickening in patients with coronary artery disease,[24] and recent evidence suggests that pioglitazone reduces the incidence of myocardial infarction and stroke in patients with type 2 diabetes and pre-existing CVD.[25] Dual PPARα/γ agonists, which are at an earlier stage of clinical development, have been shown to improve both glucose and lipid abnormalities in patients with insulin resistance and type 2 diabetes.[26,27]

Tesaglitazar is a dual PPARα/γ agonist that has demonstrated positive effects on plasma glucose and lipid abnormalities in animal models of type 2 diabetes and metabolic syndrome.[28] Based on their effects in animal models, it has been proposed that dual PPARα/γ agonists may have additional benefits, beyond their cholesterol-lowering effect, in reducing components of insulin resistance that contribute to atherosclerosis and cardiovascular disease.[28,29] In this study, we examined whether tesaglitazar can confer additional cardiovascular benefit using APOE*3-Leiden transgenic mice, an established model of human hyperlipidemia and atherosclerosis.

When fed a high-cholesterol diet, APOE*3-Leiden transgenic mice develop a human-like lipoprotein profile, which includes elevated plasma levels of very-low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and low-densi-

ty lipoprotein (LDL) and leads to the development of atherosclerosis. In addition, when fed a high-calorie, high-fat, high-cholesterol diet, these mice develop insulin resistance. Depending on their plasma cholesterol levels, APOE*3-Leiden mice develop atherosclerotic lesions that have comparable morphological, histological and immunohistochemical characteristics to human lesions.[30-32] Since plasma cholesterol levels in APOE*3-Leiden transgenic mice can be titrated to any level by adjusting dietary cholesterol intake, we were able to study the effects of tesaglitazar on atherogenesis, independent of its total plasma cholesterol lowering effect. In addition, we were able to examine these effects under both normal and mild insulin-resistant conditions.

METHODS

Animals

Female heterozygous APOE*3-Leiden transgenic mice (3–4 months of age), characterized by an ELISA for human apoE,[31] were used. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO). Animals were provided by TNO-Biomedical Research.

Diets

During a run-in period of 3 weeks, animals received either a high-fat/high-cholesterol (HF/HC) diet, containing 23% (wt/wt) bovine lard, or a low-fat/high-cholesterol (LF/HC) Western-type diet, containing 15% (wt/wt) cocoa butter.[31]

After the run-in period, the HF/HC mice were matched for age and cholesterol level into 3 groups of 17 mice each (Table 1). The mice maintained the HF diet in addition to one of the following three treatments. The high-cholesterol (HF/HC) group received a diet containing 1% (wt/wt) cholesterol. The tesaglitazar-treated group (HF/T) received the same diet as the HC group, but the diet was supplemented with tesaglitazar (0.5 μmol/kg diet), equaling 20 μg/kg body weight per day. Tesaglitazar [(S)-2-Ethoxy-3-[4-[2-(4-methylsulphonyloxyphenyl) ethoxy]phenyl propanoic acid] was provided by AstraZeneca R&D, Mölndal, Sweden. The low-cholesterol (HF/LC) group received a diet containing 0.1% (wt/wt) cholesterol to titrate the plasma cholesterol level to that of the T group, as deduced from previous experiments in our lab. The LC group served as the cholesterol-matched control. The three groups of HF-fed mice were treated for 28 weeks.

Table 1. Diets used during the study

Diet	**Treatment**			**Duration**
	High cholesterol (1% wt/wt)	High cholesterol and tesaglitazar (1% wt/wt; 0.5 μmol/kg)	Matched low cholesterol (0.1% wt/wt)	
High Fat	HF/HC	HF/T	HF/LC	28 weeks
Low Fat	LF/HC	LF/T	LF/LC	16 weeks

Animals that received the LF/HC diet during the run-in period were similarly randomized into three groups containing 17 mice. In addition to their LF diet, the three groups received treatment as described above, except that treatment lasted 16 weeks. Thus, the three treatment groups were LF/HC, LF/T and LF/LC. All animals had free access to food and water. Body weight and food intake were monitored throughout the study.

Analysis of plasma

After a 4-hour fast, commercially available kits were used to measure total plasma cholesterol (No. 1489437; Roche Diagnostics) and triglyceride levels (No. 337-B; Sigma Diagnostics). Cholesterol exposure was calculated as the area under the curve of cholesterol levels versus time in weeks. Lipoprotein distribution was determined by fast performance liquid chromatographic (FPLC) size fractionation (Pharmacia).[31]

Glucose and insulin levels were determined following sacrifice at week 28 for HF-fed animals and week 16 for LF-fed animals. Plasma glucose was measured using commercial reagents (No. 2319 and 2320; Instruchemie) and plasma insulin was measured using a mouse specific ELISA (10-1150-01, Alpco). Homeostasis model assessment-insulin resistance (HOMA-IR), a surrogate measure of insulin resistance, was calculated as the product of fasting insulin (μU/mL) and glucose (mmol/L) concentrations divided by 22.5.[33] Plasma fibrinogen (home-made mouse kit)[34] and serum amyloid A (SAA; Biosource) were measured using specific ELISAs.

Analysis of atherosclerosis

After 28 weeks (for HF-fed mice) and 16 weeks (for LF-fed mice), animals were sacrificed and the hearts were harvested, fixed and embedded in paraffin.[31] Serial 5-μm cross-sections of the entire aortic valve area were prepared and stained with hematoxylin-phloxin-saffron (HPS) for histological analysis, and with Sirius Red to quantify the collagen area. Atherosclerotic lesions were categorized into types I–V, as described previously.[31] Cross-sectional lesion areas were quantified using Leica Qwin morphometric software.[35] Four sections of each specimen were analyzed at 40-μm intervals to determine the average lesion number, type, and area.[36] In addition, descending aortas were isolated and snap frozen until further analysis. During later analysis, vessels were cleaned of adherent fat, and then stained for lipids using Oil red O for "en face" morphometry of the atherosclerotic lesion area (Leica Qwin morphometric software). All analyses were performed blind, without prior knowledge of feeding regime or treatment.

The number of monocytes adhering to atherosclerotic plaques may give an indication of endothelial activation, and thereby of the inflammatory status of the plaque. Macrophages were detected using AIA31240 antiserum (1:3000, Accurate Chemical and Scientific). The inflammatory status of plaques was further examined by estimating the local presence of NFκB (a major regulatory component of inflammatory reactions) in the plaque. NFκB was detected using mouse anti-human p65-NFκB (F-6, 1:100, Santa Cruz Biotechnology). The level of NFκB-positive staining was scored in the cytoplasm and nucleus for both macrophages and endothelial cells 0-2 (0=no positivity, 1=1 to 5 positive cells, 2=above 5 positive cells).

Statistical analysis

Non-parametric Mann-Whitney U-tests were used to analyze treatment differences, unless stated otherwise. Probability values of $P<0.05$ (two-sided) were considered significant. Frequency data for lesion categorization were compared using the Fisher's exact test. All data are presented as mean ± SD.

RESULTS

Plasma lipids and lipoprotein profiles

In both the HF and LF-fed mice, body weight (Figure 1A) and food intake (data not shown) did not differ between the three treatment groups during the study periods. In the HF-fed mice, plasma cholesterol levels were 22% lower in the tesaglitazar-treated group than in the HF/HC group (Figure 1B). A similar pattern was seen in the LF-fed mice, with plasma cholesterol levels 21% lower in the tesaglitazar-treated group than in the LF/HC group (Figure 1B). As required by the experimental design (Table 1), the total plasma cholesterol levels were similar in the HF/LC and HF/T groups and in the LF/LC and LF/T groups.

Lipoprotein profiles of the mice showed that tesaglitazar decreased VLDL cholesterol levels in both HF- and LF-fed mice (data not shown). Additionally, following tesaglitazar treatment, a lipoprotein fraction appeared with a size between LDL and HDL lipoproteins (Figure 1C). Western blot analysis revealed that this lipoprotein fraction was poor in apoAI and apoB, but rich in apoE (data not shown).

As derived from the area under the curve of Figure 1B, the HF/HC and LF/HC groups had significantly increased exposure to cholesterol compared with the respective tesaglitazar-treated and LC groups (Figure 1D). There was no significant difference in cholesterol exposure between tesaglitazar-treated and LC control groups. Triglyceride levels were significantly lower in tesaglitazar-treated groups compared with HC groups (Figure 1E) with both HF and LF diets ($P<0.05$).

Plasma tesaglitazar levels reached 38.6±11.4 nmol/L for the HF groups and 41.4±11.7 nmol/L for the LF groups (n.s.).

Insulin sensitivity

Changes in glucose and insulin levels during the study are shown in Table 2. In HF-fed mice, the HOMA-IR index indicated insulin resistance in HF/HC mice at 28 weeks. HOMA-IR was significantly lower in both the HF/T and HF/LC groups compared with the HF/HC group. In LF-fed mice, only tesaglitazar treatment significantly reduced HOMA-IR compared with both LF/HC and LF/LC mice ($P<0.05$).

Atherosclerosis

Tesaglitazar reduced atherosclerosis in treated mice compared with the respective HC groups and cholesterol-matched LC groups. In HF-fed mice, "en face" preparations of the descending aorta showed that lesion area was reduced by 34% in the tesaglitazar-treated group compared with the HF/HC group, and by 21% compared with the cholesterol-matched HF/LC control group (Figure 2A). These changes did not reach statistical significance. In the LF-fed mice, lesion area was significantly

Table 2. HOMA-IR calculations as a measure for insulin resistance in high-fat- and low-fat-fed mice.

Diet	Treatment	Weeks	Glucose (mmol/L)	Insulin (µg/L)	HOMA-IR
High fat					
	HC		6.8±0.8	1.3±0.8	11.1±6.8
	T	28	5.7±0.5*	0.6±0.4*	4.3±3.2*
	LC		5.5±0.6*	0.5±0.4*	3.4±2.7*
Low fat					
	HC		5.6±0.6	0.7±0.4	5.0±3.4
	T	16	5.1±0.4*	0.5±0.4	2.9±2.4*
	LC		5.5±0.5	0.5±0.5	3.7±3.2

HOMA-IR = Insulin (µU/mL) x (Glucose (mmol/L)/22.5)
* Significantly different from HC, P<0.05
HC: high cholesterol; T: tesaglitazar; LC: low cholesterol

reduced by 28% (P<0.05) in the tesaglitazar-treated group compared with the LF/HC group, and by 16% (n.s.) compared with the cholesterol-matched LF/LC group (Figure 2B).

Consistent with the descending aorta data, cross-sections of the aortic valve area showed that tesaglitazar reduced atherosclerosis (Figure 2B). In HF-fed mice, treatment with tesaglitazar significantly reduced total lesion area by 92% compared with the HF/HC group, and by 83% compared to the cholesterol-matched HF/LC control group (P<0.05). In the LF-fed mice, tesaglitazar treatment resulted in a significant 65% reduction in total lesion area compared with the LF/HC group and a non-significant 43% reduction in total lesion area compared with the cholesterol-matched LF/LC control group.

In the same cross-sections, the average number of lesions per animal did not differ significantly between the HC and LC control groups in HF-fed mice (Figure 2C). However, treatment with tesaglitazar significantly reduced the average number of lesions by 73% compared with the HF/HC group and by 67% compared with the cholesterol-matched HF/LC group (P<0.05). Treatment with tesaglitazar did not affect the average number of lesions in LF-fed mice (Figure 2C). When lesions were categorized as either mild or severe in HF-fed mice, there was a significant shift (P<0.05) from severe to mild lesions in tesaglitazar-treated animals (Figure 2D). Although there was a similar trend seen in LF-fed mice (Figure 2D), there was no difference in mild and severe lesion categorization between the LF/T and LF/LC groups.

To further characterize the atherosclerotic lesions, we measured macrophage and collagen areas in cross-sections serial to those used for morphometry (Figure 3). In HF-fed mice, the macrophage-positive area was larger in the HC group compared with the tesaglitazar and LC groups (Figure 3A,C). Moreover, the macrophage-positive area was smaller in the tesaglitazar group than in the cholesterol-matched LC group. The collagen-positive areas followed a similar trend (Figure 3A,C). Since the total cross-sectional lesion area was larger in LF-fed mice than in HF-fed mice, macrophage and collagen areas were also larger and the absolute areas followed the lesion area trend (data not shown). When expressed as a percentage of the total

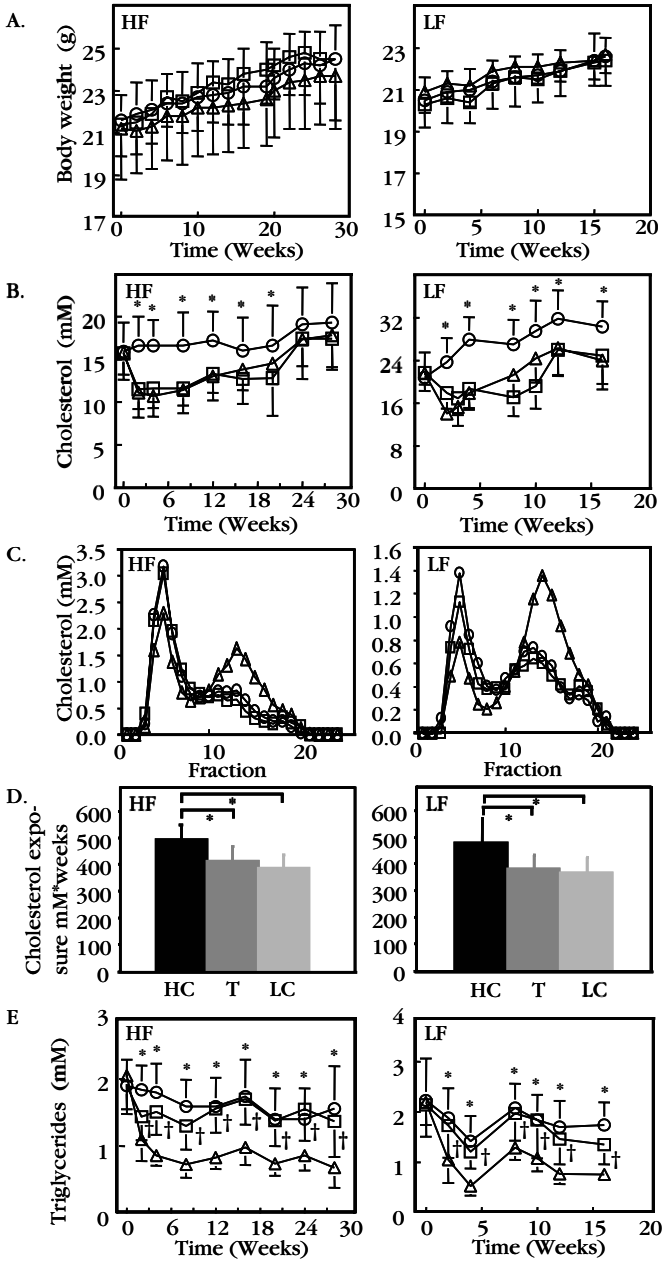


Figure 1. Effect of tesaglitazar on plasma lipids in APOE3*Leiden mice with (left panels) and without (right panels) insulin resistance. (A.) body weight over time (B.) plasma cholesterol over time (C.) lipoprotein profiles (D.) total cholesterol exposure (E.) plasma triglycerides over time. To increase plasma cholesterol levels at 20 weeks, the HF/LC dietary cholesterol was increased from 0.1% to 0.5% cholesterol (wt/wt). Plasma cholesterol levels in the HF/LC group returned to levels comparable to the T-group by 24 weeks. Circles = HF/HC or LF/HC; triangles = HF/T or LF/T; squares = HF/LC or LF/LC; *P<0.05

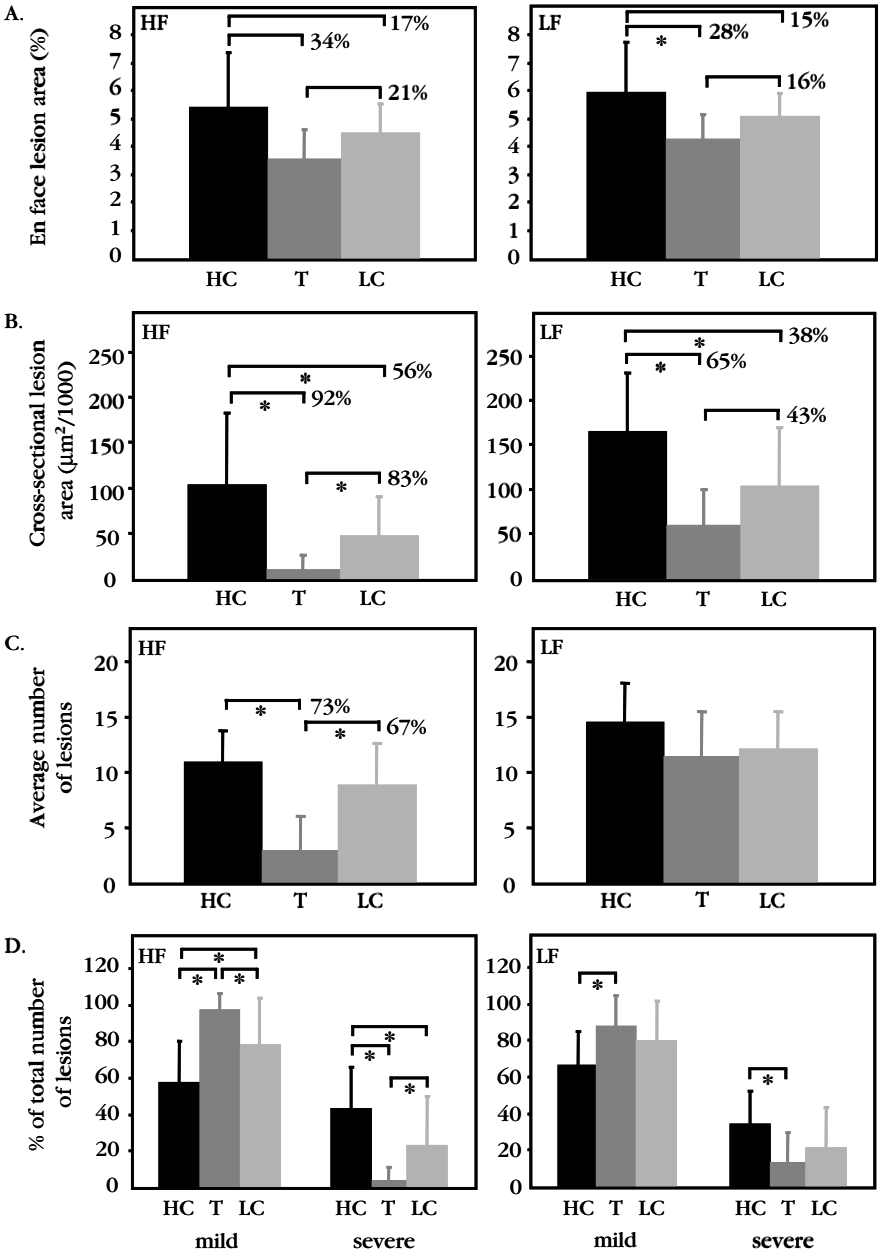


Figure 2. Effect of tesaglitazar on atherosclerosis in the aorta of APOE3*Leiden mice with (left) and without (right) insulin resistance. Shown are (A.) the aortic "en face" atherosclerotic lesion area (B.) the cross-sectional lesion area in the aortic valve area (C.) total number of lesions and (D.) lesion severity. *P<0.05

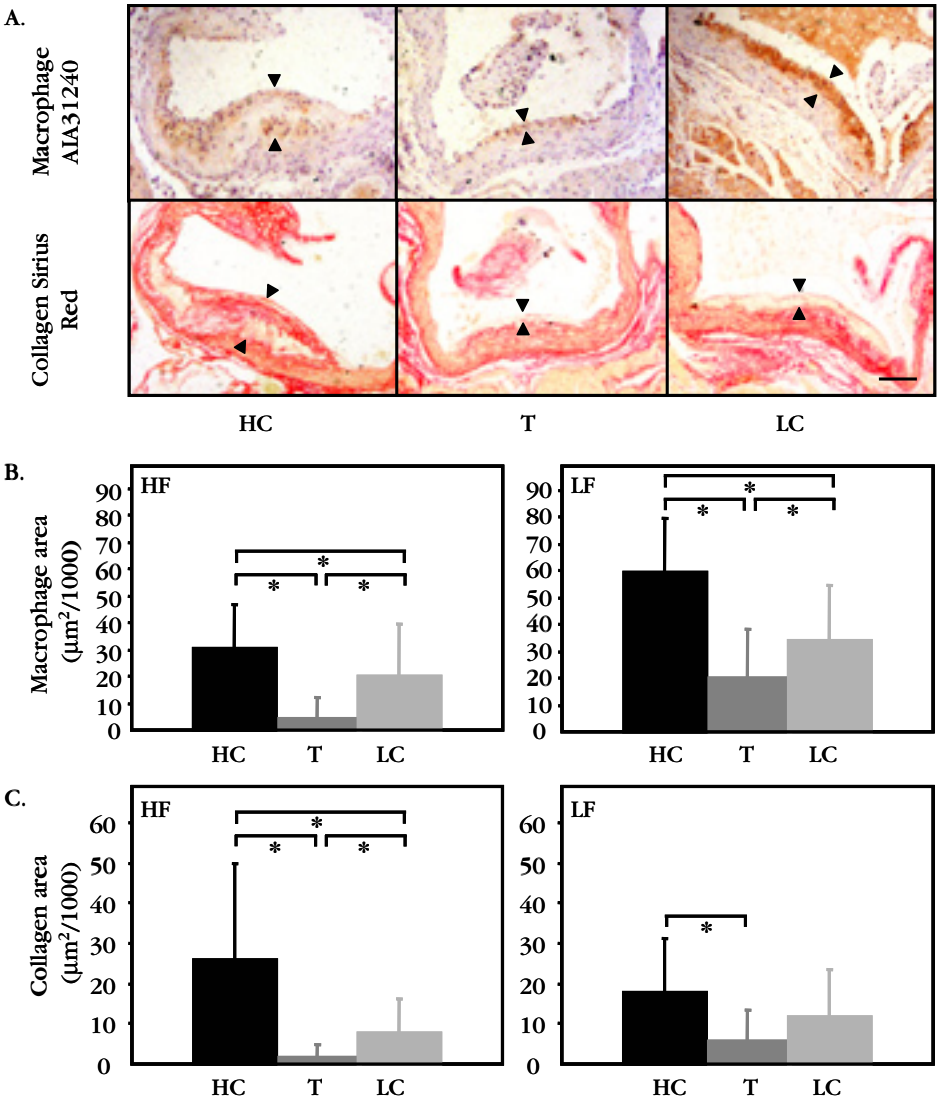


Figure 3. Cross-sectional lesion characteristics. (A.) Representative microscopic images of macrophage and collagen staining of HF-fed mice. (B.) macrophage area (C.) collagen area. *P<0.05

lesion area, collagen decreased and macrophages increased in accordance with the shift to less severe lesions.

Inflammatory markers

SAA levels were significantly reduced (P<0.05) in tesaglitazar-treated groups compared with HC groups in both HF-fed (-50.5%) and LF-fed mice (-20.9%) (Figure 4A). In HF-fed mice, tesaglitazar treatment reduced SAA levels further than LC treatment

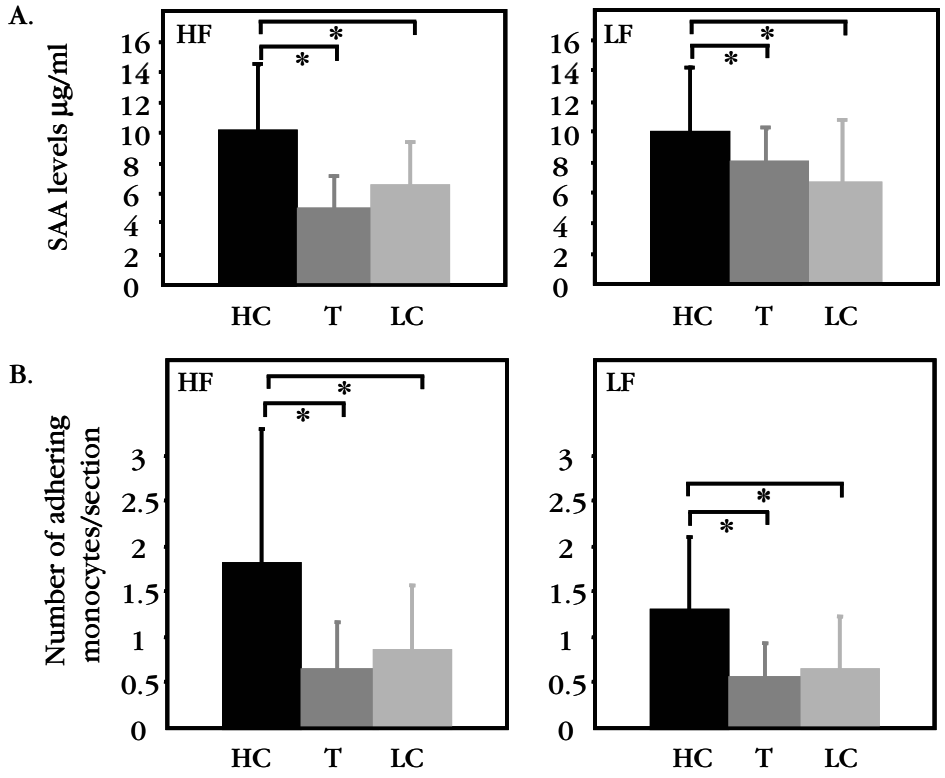


Figure 4. Effect of tesaglitazar on inflammatory parameters in APOE3*Leiden mice with (left) or without (right) insulin resistance. (A.) plasma SAA level (B.) monocyte adherence. P<0.05

(-23%). Fibrinogen levels were unaffected (data not shown). In both HF-fed and LF-fed mice there were fewer adhering monocytes in tesaglitazar-treated groups compared with HC groups (Figure 4B).There were no differences between the tesaglitazar-treated groups and the cholesterol matched LC control groups.

P65-NFκB staining was found in the cytosol and nuclei of both endothelial cells and macrophages (Figure 5A). SMCs remained unstained. Positively stained endothelial cells were observed on plaques.When observed on normal vessel walls, the positively stained cells were in close proximity to the shoulder regions of plaques. In both HF-fed and LF-fed mice, P65-NFκB expression (Figure 5B,C) followed the same pattern as total lesion numbers (Figure 2C).

DISCUSSION

This study showed that tesaglitazar has atherosclerosis reducing capacities in APOE*3-Leiden transgenic mice that cannot be attributed solely to its reduction of plasma total cholesterol.This anti-atherosclerotic effect was more notable when the animals were placed on a diet that generated insulin resistance, obesity and moderate hypertriglyceridemia, conditions that contribute to metabolic syndrome in

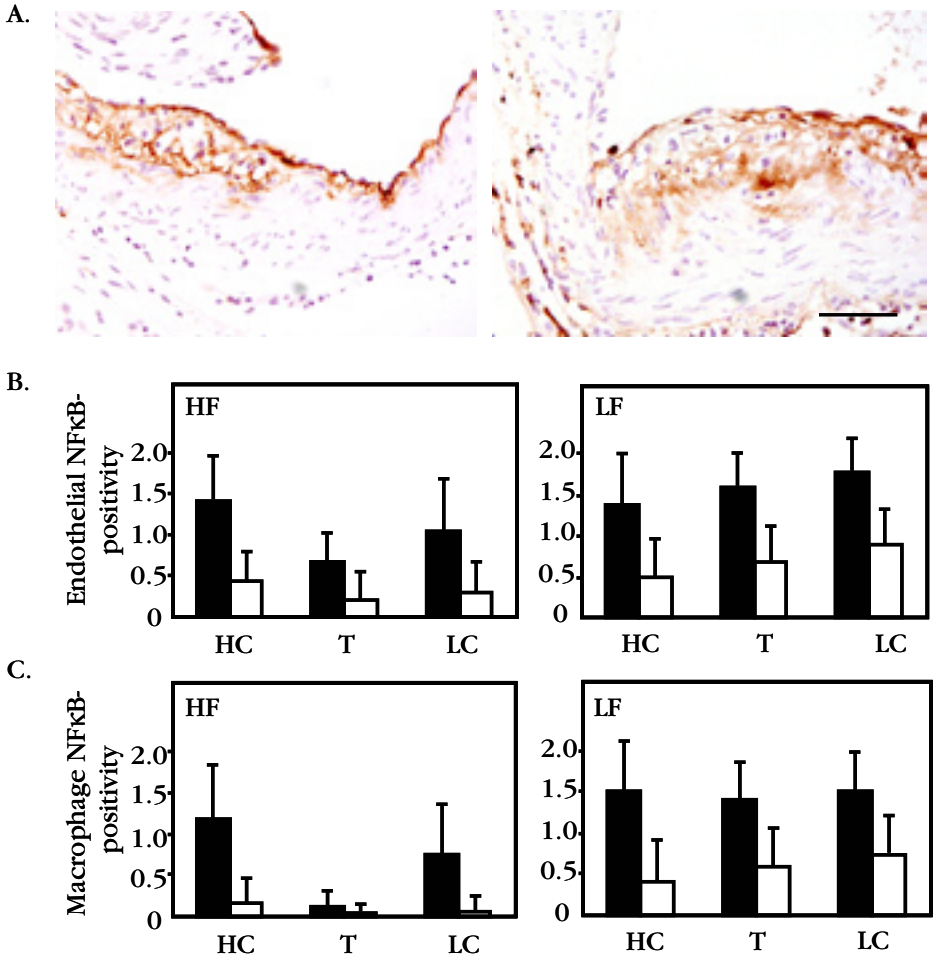


Figure 5. Effect of tesaglitazar on the inflammatory marker NFκB in atherosclerotic plaques of APOE3*Leiden mice with (left) or without (right) insulin resistance. (A.) Representative microscopic pictures of p65-NFκB-positive staining of atherosclerotic plaques (scale bar=100 μm). (B.) Scoring of endothelial NFκB positivity in the cytosol (black bars) and nucleus (white bars). (C.) Scoring of macrophage NFκB-positivity in the cytosol (black bars) and nucleus (white bars). *P<0.05

humans. The mechanism by which tesaglitazar reduced atherogenesis in these mice involved direct actions on the pro-inflammatory tissue response of vascular cells.

The hyperlipidemic APOE*3-Leiden mice used here have a lipoprotein profile that is more similar to the human profile than those of either apoE$^{-/-}$ or LDLr$^{-/-}$ mice. In agreement with previous studies with APOE*3-Leiden mice,[30,37] we were able to titrate plasma cholesterol levels by adjusting dietary cholesterol intake. Previous studies have also shown that these mice respond to hypolipidemic drugs; treatment with statins reduces plasma cholesterol[38,39] and treatment with a PPARα agonist reduces both plasma cholesterol and triglyceride levels (unpublished data). In addi-

tion, we showed in an earlier dose-finding study that APOE*3-Leiden mice respond to the dual PPARα/γ agonist tesaglitazar. In the present study, we aimed for mild cholesterol lowering with tesaglitazar, in order to investigate direct anti-atherosclerotic effects on the vascular wall. At a dose of tesaglitazar 0.5 μmol/kg diet (or 20 μg/kg body weight/day), a mild decrease in plasma cholesterol of approximately 20% was achieved.

The lipoprotein profiles of the mice suggested that treatment with tesaglitazar resulted in the formation of an additional particle, sized between the LDL and HDL fractions. Western blotting characterized the particle as poor in apoAI and apoB, but rich in apoE (data not shown). Similar lipoprotein profiles have been observed following treatment of APOE*3-Leiden mice with the PPARα agonist fenofibrate (unpublished data). Since cholesteryl ester transfer protein is not expressed in mice, these particles could represent large apoE-rich HDL.[40] Furthermore, the appearance of these large apoE-rich particles during tesaglitazar treatment was associated with a decrease in atherosclerosis, suggesting that they may have favorable anti-atherosclerotic properties. However, it remains unclear whether the accumulation of these particles is clinically relevant, or a mouse-specific effect.

To examine the pleiotropic effects of tesaglitazar that might contribute to a reduction in atherosclerosis beyond that provided by lipoprotein changes, we analyzed the levels of anti-inflammatory markers SAA and fibrinogen in plasma, and examined adhering monocytes and vascular NFκB expression in atherosclerotic plaques. We found a decrease in plasma SAA levels for tesaglitazar-treated mice, but no change in plasma fibrinogen levels. In tesaglitazar-treated mice, fewer monocytes adhered to the endothelium over plaques, coinciding with decreased NFκB expression at the same location. Previous studies have shown evidence for anti-inflammatory activities of PPAR α and -γ agonists, due to upregulation of IκB, leading to decreased NFκB/C-EBPβ complexes and suppression of C-reactive protein synthesis.[41] Our model provides further evidence of the anti-inflammatory effects of tesaglitazar, including reduced total lesion area as a result of decreased relative macrophage and collagen areas. Both effects contributed to the observed decrease in plaque severity in drug-treated animals. These anti-inflammatory effects were more pronounced in the tesaglitazar groups than in the LC groups, and were thus not due to cholesterol lowering per se.

Although the anti-inflammatory effect of tesaglitazar was observed in both HF- and LF-fed mice, the effect of tesaglitazar was greater under HF-fed conditions. This might be ascribed to differences in the level of insulin resistance between the two groups. However, we cannot exclude the possibility that the difference might be due to the relative length of the treatment periods (28 vs. 16 weeks), or to a difference in plasma cholesterol levels.

In summary, the dual PPARα/γagonist tesaglitazar showed significant anti-atherogenic effects in this mouse model, especially in animals with moderate insulin resistance. These positive results did not solely result from tesaglitazar-induced reductions in total cholesterol levels. In addition to the beneficial effects on lipid and glucose abnormalities previously shown in animal models of type-2 diabetes and the metabolic syndrome, tesaglitazar also demonstrated anti-inflammatory and anti-atherosclerotic effects in the vascular wall. The results from this study show that

the beneficial effects of tesaglitazar on atherosclerosis involve a number of different pathways.

ACKNOWLEDGEMENTS

This study was supported by the Netherlands Organization of Scientific Research (NWO/ZonMw grant no. 902-26-242), the Netherlands Heart Foundation (grant no. 2000.051) and AstraZeneca, Mölndal, Sweden. J.W.J. is a clinical established investigator of the Netherlands Heart Foundation (2001D032). We thank the technicians of TNO-Biomedical Research and AstraZeneca-Sweden for their excellent technical assistance.

REFERENCES

1. Frick MH, Elo O, Haapa K, Heinonen OP, Heinsalmi P, Helo P, Huttunen JK, Kaitaniemi P, Koskinen P, Manninen V, . Helsinki Heart Study: primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia. Safety of treatment, changes in risk factors, and incidence of coronary heart disease. N Engl J Med 1987;317:1237-1245.
2. Rubins HB, Robins SJ, Collins D, Fye CL, Anderson JW, Elam MB, Faas FH, Linares E, Schaefer EJ, Schectman G, Wilt TJ, Wittes J. Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group. N Engl J Med 1999;341:410-418.
3. Tailleux A, Torpier G, Mezdour H, Fruchart JC, Staels B, Fievet C. Murine models to investigate pharmacological compounds acting as ligands of PPARs in dyslipidemia and atherosclerosis. Trends Pharmacol Sci 2003;24:530-534.
4. Lebovitz HE, Dole JF, Patwardhan R, Rappaport EB, Freed MI. Rosiglitazone monotherapy is effective in patients with type 2 diabetes. J Clin Endocrinol Metab 2001;86:280-288.
5. Rosenblatt S, Miskin B, Glazer NB, Prince MJ, Robertson KE. The impact of pioglitazone on glycemic control and atherogenic dyslipidemia in patients with type 2 diabetes mellitus. Coron Artery Dis 2001;12:413-423.
6. Winkler K, Konrad T, Fullert S, Friedrich I, Destani R, Baumstark MW, Krebs K, Wieland H, Marz W. Pioglitazone reduces atherogenic dense LDL particles in nondiabetic patients with arterial hypertension: a double-blind, placebo-controlled study. Diabetes Care 2003;26:2588-2594.
7. Xu J, Storer PD, Chavis JA, Racke MK, Drew PD. Agonists for the peroxisome proliferator-activated receptor-alpha and the retinoid X receptor inhibit inflammatory responses of microglia. J Neurosci Res 2005;81:403-411.
8. Zingarelli B, Cook JA. Peroxisome proliferator-activated receptor-gamma is a new therapeutic target in sepsis and inflammation. Shock 2005;23:393-399.
9. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685-1695.
10. Duez H, Fruchart JC, Staels B. PPARS in inflammation, atherosclerosis and thrombosis. J Cardiovasc Risk 2001;8:187-194.
11. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 1998;391:82-86.
12. Marx N, Duez H, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors and atherogenesis: regulators of gene expression in vascular cells. Circ Res 2004;94:1168-1178.
13. Delerive P, De Bosscher K, Besnard S, Vanden Berghe W, Peters JM, Gonzalez FJ, Fruchart JC, Tedgui A, Haegeman G, Staels B. Peroxisome proliferator-activated receptor alpha

negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappaB and AP-1. J Biol Chem 1999;274:32048-32054.
14. Poynter ME, Daynes RA. Peroxisome proliferator-activated receptor alpha activation modulates cellular redox status, represses nuclear factor-kappaB signaling, and reduces inflammatory cytokine production in aging. J Biol Chem 1998;273:32833-32841.
15. Delerive P, Martin-Nizard F, Chinetti G, Trottein F, Fruchart JC, Najib J, Duriez P, Staels B. Peroxisome proliferator-activated receptor activators inhibit thrombin-induced endothelin-1 production in human vascular endothelial cells by inhibiting the activator protein-1 signaling pathway. Circ Res 1999;85:394-402.
16. Martin-Nizard F, Furman C, Delerive P, Kandoussi A, Fruchart JC, Staels B, Duriez P. Peroxisome proliferator-activated receptor activators inhibit oxidized low-density lipoprotein-induced endothelin-1 secretion in endothelial cells. J Cardiovasc Pharmacol 2002;40:822-831.
17. Pasceri V, Wu HD, Willerson JT, Yeh ET. Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor-gamma activators. Circulation 2000;101:235-238.
18. Jackson SM, Parhami F, Xi XP, Berliner JA, Hsueh WA, Law RE, Demer LL. Peroxisome proliferator-activated receptor activators target human endothelial cells to inhibit leukocyte-endothelial cell interaction. Arterioscler Thromb Vasc Biol 1999;19:2094-2104.
19. Lee H, Shi W, Tontonoz P, Wang S, Subbanagounder G, Hedrick CC, Hama S, Borromeo C, Evans RM, Berliner JA, Nagy L. Role for peroxisome proliferator-activated receptor alpha in oxidized phospholipid-induced synthesis of monocyte chemotactic protein-1 and interleukin-8 by endothelial cells. Circ Res 2000;87:516-521.
20. Shu H, Wong B, Zhou G, Li Y, Berger J, Woods JW, Wright SD, Cai TQ. Activation of PPARalpha or gamma reduces secretion of matrix metalloproteinase 9 but not interleukin 8 from human monocytic THP-1 cells. Biochem Biophys Res Commun 2000;267:345-349.
21. Goetze S, Xi XP, Kawano H, Gotlibowski T, Fleck E, Hsueh WA, Law RE. PPAR gamma-ligands inhibit migration mediated by multiple chemoattractants in vascular smooth muscle cells. J Cardiovasc Pharmacol 1999;33:798-806.
22. Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, Teissier E, Minnich A, Jaye M, Duverger N, Brewer HB, Fruchart JC, Clavey V, Staels B. PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. Nat Med 2001;7:53-58.
23. Fazio S, Linton MF. The role of fibrates in managing hyperlipidemia: mechanisms of action and clinical efficacy. Curr Atheroscler Rep 2004;6:148-157.
24. Sidhu JS, Kaposzta Z, Markus HS, Kaski JC. Effect of rosiglitazone on common carotid intima-media thickness progression in coronary artery disease patients without diabetes mellitus. Arterioscler Thromb Vasc Biol 2004;24:930-934.
25. Dormandy JA, Charbonnel B, Eckland DJ, Erdmann E, Massi-Benedetti M, Moules IK, Skene AM, Tan MH, Lefebvre PJ, Murray GD, Standl E, Wilcox RG, Wilhelmsen L, Betteridge J, Birkeland K, Golay A, Heine RJ, Koranyi L, Laakso M, Mokan M, Norkus A, Pirags V, Podar T, Scheen A, Scherbaum W, Schernthaner G, Schmitz O, Skrha J, Smith U, Taton J. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. Lancet 2005;366:1279-1289.
26. Fagerberg B, Edwards S, Halmos T, Lopatynski J, Schuster H, Stender S, Stoa-Birketvedt G, Tonstad S, Halldorsdottir S, Gause-Nilsson I. Tesaglitazar, a novel dual peroxisome proliferator-activated receptor alpha/gamma agonist, dose-dependently improves the metabolic abnormalities associated with insulin resistance in a non-diabetic population. Diabetologia 2005;48:1716-1725.
27. Saad MF, Greco S, Osei K, Lewin AJ, Edwards C, Nunez M, Reinhardt RR. Ragaglitazar improves glycemic control and lipid profile in type 2 diabetic subjects: a 12-week, double-blind, placebo-controlled dose-ranging study with an open pioglitazone arm.

Diabetes Care 2004;27:1324-1329.
28. Ljung B, Bamberg K, Dahllof B, Kjellstedt A, Oakes ND, Ostling J, Svensson L, Camejo G. AZ 242, a novel PPARalpha/gamma agonist with beneficial effects on insulin resistance and carbohydrate and lipid metabolism in ob/ob mice and obese Zucker rats. J Lipid Res 2002;43:1855-1863.
29. Zuckerman SH, Kauffman RF, Evans GF. Peroxisome proliferator-activated receptor alpha,gamma coagonist LY465608 inhibits macrophage activation and atherosclerosis in apolipoprotein E knockout mice. Lipids 2002;37:487-494.
30. Groot PH, van Vlijmen BJ, Benson GM, Hofker MH, Schiffelers R, Vidgeon-Hart M, Havekes LM. Quantitative assessment of aortic atherosclerosis in APOE*3 Leiden transgenic mice and its relationship to serum cholesterol exposure. Arterioscler Thromb Vasc Biol 1996;16:926-933.
31. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der BH, HogenEsch H, Frants RR, Hofker MH, Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. J Clin Invest 1994;93:1403-1410.
32. van Vlijmen BJ, Pearce NJ, Bergo M, Staels B, Yates JW, Gribble AD, Bond BC, Hofker MH, Havekes LM, Groot PH. Apolipoprotein E*3-Leiden transgenic mice as a test model for hypolipidaemic drugs. Arzneimittelforschung 1998;48:396-402.
33. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-419.
34. Kockx M, Gervois PP, Poulain P, Derudas B, Peters JM, Gonzalez FJ, Princen HM, Kooistra T, Staels B. Fibrates suppress fibrinogen gene expression in rodents via activation of the peroxisome proliferator-activated receptor-alpha. Blood 1999;93:2991-2998.
35. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 1987;68:231-240.
36. Volger OL, Mensink RP, Plat J, Hornstra G, Havekes LM, Princen HM. Dietary vegetable oil and wood derived plant stanol esters reduce atherosclerotic lesion size and severity in apoE*3-Leiden transgenic mice. Atherosclerosis 2001;157:375-381.
37. Kleemann R, Princen HM, Emeis JJ, Jukema JW, Fontijn RD, Horrevoets AJ, Kooistra T, Havekes LM. Rosuvastatin reduces atherosclerosis development beyond and independent of its plasma cholesterol-lowering effect in APOE*3-Leiden transgenic mice: evidence for antiinflammatory effects of rosuvastatin. Circulation 2003;108:1368-1374.
38. Delsing DJ, Post SM, Groenendijk M, Solaas K, van der BH, van Duyvenvoorde W, de Wit EC, Bloks VW, Kuipers F, Havekes LM, Princen HM. Rosuvastatin reduces plasma lipids by inhibiting VLDL production and enhancing hepatobiliary lipid excretion in ApoE*3-leiden mice. J Cardiovasc Pharmacol 2005;45:53-60.
39. Verschuren L, Kleemann R, Offerman EH, Szalai AJ, Emeis SJ, Princen HM, Kooistra T. Effect of low dose atorvastatin versus diet-induced cholesterol lowering on atherosclerotic lesion progression and inflammation in apolipoprotein E*3-Leiden transgenic mice. Arterioscler Thromb Vasc Biol 2005;25:161-167.
40. Pitas RE, Innerarity TL, Arnold KS, Mahley RW. Rate and equilibrium constants for binding of apo-E HDLc (a cholesterol-induced lipoprotein) and low density lipoproteins to human fibroblasts: evidence for multiple receptor binding of apo-E HDLc. Proc Natl Acad Sci U S A 1979;76:2311-2315.
41. Kleemann R, Verschuren L, de Rooij BJ, Lindeman J, de Maat MM, Szalai AJ, Princen HM, Kooistra T. Evidence for anti-inflammatory activity of statins and PPARalpha activators in human C-reactive protein transgenic mice in vivo and in cultured human hepatocytes in vitro. Blood 2004;103:4188-4194.

Chapter 8

General Discussion

Cardiovascular diseases (CVD) are the leading cause of mortality and disability in the industrialized world, and it is rapidly becoming the number one killer in developing countries.[1] Atherosclerosis is the primary cause of cardiovascular disease, a multi-factorial disorder occurring in the large and medium-sized arteries of the body. Although in the beginning 90s promising lipid lowering therapies predicted a strong reduction in cardiovascular deaths for the upcoming years, in westernized societies it still accounts for 40% of the total number of annual deaths, indicating that treatment of atherosclerosis goes beyond lipid lowering solely. In addition to lipid accumulation, continuous cell proliferation (cell cycle) and cell death (apoptosis) processes are thought to play a central role in the development of atherosclerotic lesions. Proliferation and apoptosis are important processes in regulating macrophage and smooth muscle cell (SMC) numbers in the atherosclerotic lesion and may thereby directly influence lesion composition and stability. The research described in this thesis was designed to identify the role of cell cycle and apoptosis genes in atherosclerosis. The major conclusions concerning the studies on cell cycle and apoptosis genes in atherosclerosis and the importance of site-specific recombinase (SSR) technology in atherosclerosis research are discussed in this chapter, concluding with the potential implications for future research.

CELL CYCLE AND APOPTOSIS GENES IN ATHEROSCLEROSIS

Many physiological processes, including proper tissue development and homeostasis, require a delicate balance between cell proliferation and apoptosis. Cell proliferation and apoptosis are linked by cell-cycle regulators and apoptotic stimuli that affect both processes.[2] Among these common cell-cycle regulators are Rb, p53, and its inhibitor Mdm2. The importance of these genes in maintaining homeostasis becomes evident if one considers that despite the identification of more than 100 proto-oncogenes, the pathways dominated by the two tumor suppressor genes Rb and p53 are the most frequently disrupted in cancer.[3] The unique role of these cell cycle and apoptosis genes in cancer puts a special interest for a role of these genes in atherosclerosis. Not the least because recently a series of shared molecular pathways have emerged that have in common a significant role in the pathogenesis and progression of both cancer and atherosclerosis.[4,5]

Initial *in vitro* studies demonstrated a central role for p53 in human plaque vascular smooth muscle cells (VSMCs). These plaque-derived VSMCs showed an increased sensitivity to p53-mediated apoptosis.[6] Moreover, plaque VSMCs displayed slower rates of cell proliferation and earlier senescence due to a higher ratio of active Rb.[7] Furthermore, p53 was shown to be able to mediate apoptosis of these cells through Fas transport from cytoplasmatic stores.[8] These studies were initial indications that p53 (and downstream targets) play a potentially important role in determining atherosclerotic lesion composition and stability, and thereby opened a new era of research on these processes in atherosclerosis via key genes such as p53, Rb, and Mdm2.

Following, to unravel molecular mechanisms underlying the role of p53 in atherosclerosis murine studies were performed. These studies started using conventional knock out mice crossbred to different atherosclerosis-susceptible back-

grounds (i.e. apoE$^{-/-}$, LDLR$^{-/-}$ and APOE*3-Leiden mice) and gradually became more cell-type specific using bone marrow transplantations. Initial studies started with whole body deficiency of p53[9], followed by hematopoietic inactivation of p53 via bone marrow transplantations.[10,11] Whole body and hematopoietic p53 deficiency indicated strong anti-atherogenic properties of the tumour suppressor gene. In **chapter 2** we completed the research on the role of p53 in the development of atherosclerosis by studying macrophage p53 using LysMCre^{+} p53$^{loxP/loxP}$ apoE$^{-/-}$ mice. Whole body inactivation of p53 can induce severe side-effects interfering with the atherosclerotic process.[12] In addition, hematopoietic inactivation using bone marrow transplantation studies[10,11] targets a diverse spectrum of bone marrow derived cells. Cells originating from bone marrow are macrophages, T- and B-cells, SMCs,[13] and endothelial cells.[14,15] With the use of the LysMCre^{+} p53$^{loxP/loxP}$ apoE$^{-/-}$ mouse model we scaled our research down to one single cell-type (macrophages) and in addition prevented development of severe side-effects as a result of whole body p53 deletion. This mouse model allowed us to define that macrophage p53 plays a minimal role in atherosclerotic lesion size but has a unique role in inducing foam cell apoptosis, preventing lesional necrosis and thereby affects lesion composition and progression.

Both p53 and Rb are potent inhibitors of cell cycle progression. In contrast to the comprehensive studies on the role of p53 in the development of atherosclerosis, we were the first group showing a role for (macrophage) Rb in the development of atherosclerosis (**chapter 3**). Under identical experimental conditions macrophage Rb deficiency showed to have more pronounced effects on atherosclerotic lesion size than macrophage p53 deficiency. This difference might be ascribed to the extent of successful gene deletion. However, a more plausible explanation could be that each individual gene plays its role in different processes. Rb is important in controlling the progression of the cell cycle from G1-phase to S-phase, principally by binding to and inactivating the E2F transcription factors, and in addition acts as an anti-apoptotic factor.[16] Deletion of macrophage Rb showed that this gene has strong anti-atherogenic properties. On the other hand, p53, next to regulating proliferation and apoptosis, also is a potent transcription factor inducing the expression of many downstream target genes. These target genes can be divided in the following sub-categories: (1) genes involved in cell cycle control, (2) genes involved in apoptosis, (3) genes involved in DNA repair, (4) genes involved in angiogenesis, (5) genes involved in cellular stress response. Although currently around 60 target genes have been identified, it is predicted that the human genome contains 200-300 p53 target genes.[17] Thus, p53 targets many genes amongst which several might have either pro- or anti-atherosclerotic effects, giving a possible explanation for the less pronounced effects on atherosclerotic lesion size. Hence, as a result of the multi-targeted nature of p53, definition of macrophage p53 as an anti-atherosclerotic gene is more complex.

How do the different studies on cell cycle and apoptosis genes as described in this thesis and by others expand our knowledge on the role of these two processes in atherosclerosis? From literature it is know that SMC apoptosis can selectively weaken the fibrous cap, thereby accelerating the process towards plaque rupture.[18,19] Human plaque-derived smooth muscle cells displayed increased rates of

spontaneous apoptosis and high susceptibility to p53-mediated apoptosis.[6,20] In addition, adenovirus-induced p53[21] or FasL[22] overexpression in murine carotid artery lesions resulted in increased apoptosis and in a phenotype that has been associated with increased vulnerability to plaque rupture, whereas lesion size was unaffected in both studies. In **chapter 2** we showed that macrophage p53 deletion induced a decrease in macrophage apoptosis resulting in differences in lesion composition and again leaving atherosclerotic lesion size unaffected. Concerning earlier studies on proliferation in atherosclerosis it was shown that human plaque-derived SMCs show reduced proliferation and earlier senescence due to an increased ratio of the active form of Rb.[7] In addition, localized infection of the arterial wall with an adenovirus encoding a constitutively active non-phosphorylatable form of Rb[23] or a phosphorylation-competent full-length and a truncated form of Rb significantly[24] inhibited vascular smooth muscle cell proliferation and neointima formation in different animal models of balloon angioplasty. Moreover, mouse studies showed that (hematopoietic) inactivation of p27, a cyclin-CDK regulating cell cycle inhibitor, resulted in an increase in lesional proliferation, thereby exacerbating atherosclerosis in apoE$^{-/-}$ mice.[25,26] Finally, in **chapter 3** we demonstrated that increased macrophage proliferation (via macrophage Rb deletion) also enhanced atherosclerotic lesion size. Summarizing, we can conclude that lesional apoptosis has primarily qualitative effects on atherosclerotic lesion development (affecting lesion composition and stability), whereas lesional proliferation has primarily quantitative effects on atherosclerotic lesion development (affecting lesion size).

Analysis of proliferation and cell death (either apoptosis or necrosis) form the common denominator in the different chapters of this thesis (**chapters 2, 3, 4, and 6**). Both in **chapter 2 and 6** we showed that a reduction in apoptosis, either via deletion of macrophage p53 (**chapter 2**) or via active TNFα (**chapter 6**), resulted in enhanced death via necrosis, thereby stimulating the formation of advanced atherosclerotic lesions. Moreover, our earlier studies on the role of hematopoietic p53 in the development also showed this similar trend (B.J.M. van Vlijmen and L.S.M. Boesten et al.).[10] Apoptosis often precedes necrosis in the formation of an advanced atherosclerotic lesion and thereby results in the release of cellular contents from dying cells.This may lead one to suggest that apoptosis itself strongly contributes to lesion pathology, by leading to the release of harmful molecules and finally the formation of a necrotic core.[27,28] However, our current data challenge this traditional point of view that apoptosis is harmful and suggest that apoptosis itself is a direct protective factor in the development of atherosclerosis.

Our studies led to the following hypothesis behind cell death in atherosclerotic lesions:

In early lesions foam cells preferentially die quickly via a relatively clean apoptotic death followed by phagocytosis and disposal of apoptotic bodies.This process limits the number of cells in early lesions.[27] However, it is the harsh microenvironment in the growing lesion that hampers the normal clearance of apoptotic bodies. Following, these accumulating apoptotic bodies are ineffectively phagocytosed, partly as a result of cytoplasmic overload of macrophages and competition among oxidized red blood cells, oxidized LDL and apoptotic bodies for the same receptor(s) on the macrophages,[29] thereby promoting the inflammatory status of the lesion. Eventu-

ally, it is this increase in inflammatory status that promotes (secondary) necrosis[27] and thereby the formation of an advanced atherosclerotic lesion. Necrosis of foam cells may be more slowly but is more detrimental since necrosis itself leads to the release of pro-inflammatory and pro-thrombotic substances. The increase in the inflammatory status of the lesion goes beyond processes involved in the apoptotic machinery. Thus, foam cell apoptosis is in principal a beneficial process, leading to a reduction in the production of cytokines, chemokines, and metalloproteinases, thereby reducing lesion pathology. However, it is the complicated atherosclerotic environment that restricts proper execution of apoptosis.

In addition to its role in regulating apoptosis and necrosis in atherosclerosis (as described above) TNFα is more often described as a strong pro-inflammatory cytokine in different diseases.[30,31] However, to date, studies on the role of TNFα in atherosclerosis yielded controversial results. TNFα deficiency on a wild type C57BL/6 background did not affect early lesion development.[32] On the contrary, another research group demonstrated that TNFα-deficiency, also on a C57BL/6 background, reduced atherosclerosis.[33] A direct anti-atherosclerotic effect of TNFα deficiency could not be concluded from these experiments because they also showed an unexpected TNFα-mediated effect on atherogenic lipoproteins. In addition, **chapter 6** describes a subtle role for TNFα in the development of advanced atherosclerosis. In accordance with the data on early atherosclerosis of Schreyer et al.[32] we do not demonstrate an effect of TNFα deficiency on the size of atherosclerotic lesions. TNFα deficiency solely results in less advanced lesions as a result of a shift in cell death towards apoptosis at the expense of necrosis. None of the three abovementioned studies showed an effect of TNFα deficiency on either systemic or local inflammatory parameters. This brings to doubt the generally held concept that TNFα has strong pro-inflammatory properties in atherosclerosis development. Therefore, we conclude that the pro-inflammatory properties of TNFα play a minor role in the development of atherosclerosis. Hence, the primary effects of TNFα on atherosclerosis development are at the level of cell death regulation.

TNFα and one of its receptors TNFR1 belong to the tumour necrosis factor receptor gene superfamily. This family comprises the so called "death receptors" from which the receptor-ligand couples Fas-FasL and TNFR1-TNFα are best characterized.[34] Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific death ligands (i.e. TNFα and FasL). These receptors can activate death caspases within seconds of ligand binding, causing apoptosis of the cell within hours. Different vascular studies aiming at the role of Fas-FasL in atherosclerosis demonstrated that this couple inhibited the infiltration of inflammatory cells, thereby inhibiting the progression of the disease.[35-38] However, our study on the role of FasL in pre-existing lesions in apoE$^{-/-}$ deficient mice, showed that FasL expression increased apoptosis in the SMC-rich caps of the lesions, thereby remodelling the lesions towards a more vulnerable phenotype (A.S.M. Zadelaar and L.S.M. Boesten et al.).[22] Taken together, these two studies indicate that the actions at the level of atherosclerosis development of these two ligands of the death receptor family (TNFα and FasL), are merely attributable to their activity at the level of cell death (both apoptosis and necrosis), and not at the level of inflammation.

MOUSE MODELS TO STUDY CELL CYCLE AND APOPTOSIS GENES IN ATHEROSCLEROSIS

Throughout this thesis we made use of state-of-the-art SSR mouse models to study the role of cell cycle and apoptosis genes in atherosclerosis. In this part of the discussion the advantages and disadvantages of the different mouse model technologies applied in atherosclerosis research will be discussed.

Conventional whole body knock out models can be designed to introduce the desired genetic changes into the germ line, thereby affecting all tissues during the entire lifespan of the resulting mouse. However, several limitations of the conventional germ line gene-targeting approach hamper analysis of target genes. As the mutation will be already present in the first developing cell, an embryonic lethal phenotype might be provoked (i.e. Rb and Mdm2-germline null alleles), precluding any further functional analysis during embryogenesis and/or adulthood. In addition, pleiotropic effects as a compensatory reaction to the introduced germ line mutation are often observed. Moreover, when the gene of interest has a wide expression pattern, its inactivation might induce a highly complicated accumulative phenotype involving multiple tissues. Hypomorphic mutations, which is a mutation that reduces, but does not completely eliminate, the function of a gene, could partly be a solution to these problems. Mice carrying for example one hypomorphic Mdm2 allele (*mdm2*^{puro}) and a known *mdm2* null allele showed that Mdm2 is critical for regulating p53 under homeostatic conditions.[39] However, it still might be important to completely delete a gene at a specific developmental time point or during a particular stage in disease.[40] Thus, although conventional whole body knock out mice boomed our knowledge on multiple genes under physiological and pathological conditions, the abovementioned limitations activated researchers to search for alternative approaches to study genes. Concerning the atherosclerosis research field several different approaches have been applied the last couple of years.

Adenovirus vectors can be used to efficiently overexpress a gene of interest *in vivo*.[41-43] Replicative deficient adenoviruses, in which the E1 genes have been replaced with an appropriate transgene and transcriptional regulatory element(s), can be used to efficiently infect most replicating and nonreplicating cell types *in vivo*, lacking the ability to regenerate infectious progeny after an initial injection into mice. Compared with the production of knock out/transgenic animals, adenoviruses are more convenient and less expensive to prepare. Moreover, they can be used alone and in combinations to rapidly produce large numbers of animals expressing one or more transgenes. Finally, they ultimately may have potential applications for human gene therapy. Adenovirus vectors injected intravenously home to the liver and a single intravenous injection of mice results in the selective transduction of 10% to 100% of the hepatocytes in these animals. This also directly shows the disadvantage of using adenoviruses. *In vivo* atherosclerosis research using adenoviruses gives the opportunity to study genes associated with the liver (mainly lipoprotein-related genes), which has been successfully performed for several genes including: apolipoprotein A-I (APOA-I),[44] secreted macrophage scavenger receptor-AI (SR-AI),[45] and plasma phospholipids transfer protein (PLTP).[46] However, when one wants to study a gene in a specific cell type in the vessel wall or at the site of

the lesion, intravenously injected adenoviruses can't be used. Von der Thusen et al.[21] and Zadelaar et al.[22] anticipated on this limitation by locally incubating a collar-induced carotid artery lesion using an adenovirus encoding p53 or FasL, respectively. Using this approach, solely the endothelial and smooth muscle cells are affected (leaving macrophages and other lesional cells unaffected). Again, this can either be an advantage or a disadvantage, depending on the type of cell one wants to target. Thus adenoviruses are perfect tools to systemically target liver associated genes or locally target EC and SMC associated genes in carotid artery lesions.

Bone marrow transplantation (BMT) studies opened the field for cell-type specific research in atherosclerosis research.[47] In combination with the different mouse models for atherosclerosis (i.e. apoE$^{-/-}$, LDLR$^{-/-}$ and APOE*3-Leiden mice) this approach enabled researchers to study macrophage-specific genes. Although BMT studies extended our knowledge on the role of multiple genes in atherosclerosis, years of research also revealed the disadvantages of using this technique in atherosclerosis research. Graft-versus-host disease is the most common technical problem associated with BMT. In addition, the radiation used for the recipient mice to remove its bone marrow often makes the mice seriously ill requiring a long recovering period, with often many premature deaths. However, a careful titration of the applied radiation for each single mouse strain prevents illness in the recipient mice. Although often claimed as a cell-type specific approach, BMT targets many cell types. Next to macrophages, also smooth muscle cells, endothelial cells, T-cells and B-cells develop from bone marrow progenitor cells.[13-15,46] Thus, earlier published studies using BMT analyzed the role of a gene of interest in all the abovementioned cell types. Therefore, concerning BMT studies in atherosclerosis, the term "macrophage-derived" is currently being replaced by "hematopoietic-derived".

Alternative mouse models for atherosclerosis research also include conditional "gain of function" and knock-in mouse models. Next to inactivation of a gene ("loss of function") also activation of a gene ("gain of function") can be achieved. Transcriptional transactivation, used to activate transgenes in gain of function experiments, is more widely used than DNA recombination, because the latter is irreversible.[48] Tetracycline-dependent regulatory systems are most often used for transcription transactivation systems ("Tet-on" and "Tet-off"). These systems use a chimeric transactivator to control transcription of the gene of interest from a silent promoter. Depending on the system used target genes are expressed in presence or absence of the inducer doxycyclin with impressive induction levels, reaching in some tissues five orders of magnitude.[40,49] Alternatively, knock-in experiments are used to place a transgene (either cDNA or a reporter construct) under the transcriptional control of an endogenous gene. The most widely used knock-in strategy is the replacement of a gene by a reporter gene (e.g. LacZ or GFP) to monitor its expression patters during development, in adult mice or during a disease (atherosclerosis), both in a spatial and temporal matter.[49] The APOE2 knock-in mouse model is a clear example of the use of knock-in techniques in lipid research. In the APOE2 knock-in mouse, the endogenous mouse ApoE gene has been replaced by the human Apolipoprotein E2 (APOE2) gene, a relatively common recessive allele, which is the main cause of type III hyperlipidemia in humans.[50]

Mouse models expressing conditionally regulated genes (pioneering work from

the laboratory of Klaus Rajewsky[51]) initiated a new era for all scientific areas. Using the site-specific recombinase (SSR) technology, those genes that induce embryonic lethality associated with germline null alleles, could now be studied in a wide variety of diseases. In the following decade, from the introduction of the SSR technique, there has been a tremendous expansion in the number of Cre-expressing and floxed mice. Currently an excellent list of all Cre-expressing and floxed mice has been established and is available on: http://www.mshri.on.ca/nagy. In addition, with the introduction of the spatiotemporally controlled genes (deletion of a gene at a time point of interest) shifted the research question from: "What is the role of this gene in this disease?" towards "At what particular stage does this gene play a role in this disease?". Although SSR techniques were first applied in developmental and cancer research, the use of these systems is now more often adapted in research on many different diseases (i.e. atherosclerosis[52-55], diabetes[56], and multiple sclerosis[57]).

However, any new technique also brings its limitations. When considering the first practical limitations, one encounters that Cre recombinase can cause chromosomal rearrangements/aberrations and is speculated to be involved in causing cell cycle arrest.[58-61] Applying the SSR technique in the atherosclerosis research field particularly brings extensive breeding work before atherosclerosis experiments can be performed. In addition to combining the Cre-expressing mouse with the floxed-mouse, an atherosclerosis-susceptible background (i.e. apoE$^{-/-}$, LDLR$^{-/-}$ or APOE*3-Leiden mice) also needs to be introduced. At least two years of breeding and genotypic analysis are required to achieve a triple homozygous mouse line. Thereafter, atherosclerosis experiments can be initiated. Concerning these time-consuming breedings, a time reducing approach has been described by Kanters et al., who first combined the Cre-expressing and floxed mouse lines and subsequently performed a BMT onto an atherosclerotic-susceptible background. [55]

Another potential drawback is that the targeting constructs for both Cre-expression and the floxed-gene might be present on the same chromosome. As a consequence, homozygous floxed-embryos are not formed or die in utero in a very early stage (these topics have not been addressed in the current thesis). We experienced this unfortunate practical problem while breeding the LysMCre mouse strain[62] (lysozyme M gene: chromosome 10, genome coordinates: 116966783-116971716) with Mmd2$^{loxP/loxP}$ mouse strain[63] (Mdm2 gene: chromosome 10, genome coordinates: 117379898-117401709). With both targeting constructs present on the same chromosome, only 410 kb apart, homozygous LysMCre^{+} Mdm2$^{loxP/loxP}$ ApoE$^{-/-}$ mice were not formed, resulting in the birth of heterozygous LysMCre^{+} Mdm2$^{loxP/+}$ ApoE$^{-/-}$ mice only. Although atherosclerosis experiments were performed using these heterozygous LysMCre^{+} Mdm2$^{loxP/+}$ ApoE$^{-/-}$ mice, as described in **chapter 2 and 3** for p53 and Rb respectively, heterozygous deletion of macrophage Mdm2 did not affect atherosclerosis development. Additional Western blot analysis on macrophages from LysMCre^{+} Mdm2$^{loxP/+}$ mice did not show a (partial) upregulation of p53, indicating that one functional Mdm2 allele is sufficient to keep p53 levels in constraint. These coincidental practical shortcomings hampered studies on Mdm2 deletion (and thereby p53 overexpression) in lesional macrophages. Hence, *in vivo* modulation of macrophage Mdm2 is not conceivable with the current tools available (LysMCre mice and Mdm2$^{loxP/loxP}$ mice).

Next to targeting macrophages (**chapters 2 and 3**) we also aimed at targeting the other central cell type in atherosclerotic lesions: SMCs. To this end we crossbred $p53^{loxP/loxP}$,[64] $Rb^{loxP/loxP}$,[65] $Mdm2^{loxP/loxP}$,[63] mice, and the rosa26[66] reporter mouse line with the SM-CreER^{T2}(ki)[67] mouse line. Although all four mouse lines were viable, we were unable to reproduce the data of Kuhbandner et al.[67] using the SM-CreER^{T2}(ki)/rosa26 mouse model. The data described in **chapters 4 and 5** define the difficulties which hampered studies on SMC-p53, -Rb and -Mdm2 in atherosclerosis. Opposite to the published study of Kuhbandner et al.[67] we demonstrated only a 2-8% gene deletion efficiency in the vasculature of SM-CreER^{T2}(ki)/rosa26 mice both after systemic and local application of tamoxifen and 4-hydroxytamoxifen, respectively (**chapter 5**). This is much less efficient than the reported 60% gene deletion in the vasculature by Kuhbandner et al. In addition, the data described in **chapter 4** show a phenotype after inducible SMC-specific Mdm2 deletion only in the gastro-intestinal (GI) tract, inducing lethality in adult mice, leaving the vasculature unaffected. Although not described in this thesis we performed multiple optimization procedures to increase the number of cells displaying gene deletion (β-Gal positive cells) in the vasculature of SM-CreER^{T2}(ki)/rosa26 mice, however all attempts proved to be unsuccessful. Combination of these data forced us to decide to no longer use the SM-CreER^{T2}(ki) mouse model for research on gene deletion (p53, Rb and Mdm2) in the vasculature.

Various factors might have contributed to the failure of this model in atherosclerosis research. Overestimation of the degree of gene deletion in the vasculature, as a consequence of the choice of the reporter mouse model by Kuhbandner et al,[67] could be an underlying cause. In addition, SM22 promoter activity might have been affected or even reduced at our sampling time points. Although studies using a non-inducible SM22-Cre transgenic mouse line argue against this point of view[52,68] showing successful gene deletion from birth on using the SM22 promotor. Finally, the mixed background or differences in accessibility of the loxP sites between the vasculature and the gastro-intestinal tract (GI-tract) for the Cre enzyme might have attributed to the low efficiency of gene deletion in the vasculature using the SM-CreER^{T2}(ki) mouse model. Which of these options, or a combination of them, is the cause of the limitation of the SM-CreER^{T2}(ki) mouse model concerning studies in the vasculature, remains subject to speculation. In this light, it is worthwhile to mention that 5 years after the publication of Kuhbandner et al. no data have been published using the SM-CreER^{T2}(ki) mouse model, although various research groups attempted to introduce this mouse model in their research lines. In this thesis we show that the SM-CreER^{T2}(ki) mouse model is not suitable for research concerning the vasculature but can be efficiently applied for research focussing at gene deletion in the GI-tract.

FUTURE PERSPECTIVES

The findings on cell cycle and apoptosis genes described in this thesis may provide a possible starting point for pharmacological intervention or further specialized application of Cre-loxP models in atherosclerosis, as discussed below.

Stimulation of p53 and/or Rb may prove beneficial in inhibition of atheroscle-

rosis development. However, one should consider that both proteins are of vital importance in all cells present in the body. Therefore, systemic pharmacological modulation of these genes is not applicable for the treatment of atherosclerosis. In contrast, local pharmacological modulation of these genes might prove efficacious in the treatment of atherosclerosis. Local pharmacological treatment is currently often used in treatment of occluding atherosclerotic lesion and (in-stent) restenosis after PTCA or placement of a stent.[69-72] To treat these unregulated proliferative diseases, the use of drug-eluting stents has emerged as a highly promising local approach.[73] The different drugs (i.e. rapamycin, paclitaxel) used in these drug-eluting stents successfully target cell cycle genes (p53 and Rb, amongst others). This local approach gives an unique opportunity to locally target p53 and Rb, since activation of these genes both at the level of SMCs[23,24,74,75] and macrophages (this thesis) is shown to be beneficial for inhibition of vascular disease. In addition, one could envision that stents coated with a combination of pharmacological compounds targeting cell cycle, apoptosis and inflammatory genes may prove increasingly efficacious in the treatment of cardiovascular disease.

The Cre-loxP system induced major advancements in many scientific areas. Future research will focus on the refinement of these techniques. The need for transgenic mouse lines that tissue-specifically and inducibly express Cre-recombinase in the appropriate cell type will increase. Designing a truly macrophage-specific Cre-expressing mouse line will further improve state of the art atherosclerosis research, as the currently available LysMcre mouse line targets both macrophages and granulocytes.[62] In addition, both research on atherosclerosis and restenosis will thrive on the development of a well-functioning inducible SMC-specific Cre-expressing mouse line. Following, multiple genes involved in lesion stability and rupture may be analysed by the combination of this inducible SMC-specific mouse model and local application of a 4-hydoxytamoxifen loaded perivascular delivery device.[76] With the introduction of Cre-loxP models in atherosclerosis the opportunities to determine the contribution of each single gene in the disease process are within reach. Understanding the contribution of each gene/pathway in this disease may yield novel (pharmacological) ways to interfere in atherosclerosis development.

Taking the complex pathogenesis of cardiovascular diseases into account, targeting a single gene or process, although it might be an attractive candidate, may prove to be inadequate therapeutically. Although current (lipid-lowering) treatments for atherosclerosis show considerable progress, combinatorial therapies will prove most efficacious. Despite the considerable difficulties involved, the use of combinatorial therapy aiming at (1) lifestyle interventions (i.e. food and physical habits), (2) lipid therapies (i.e. statins, fibrates), (3) inflammation (i.e. regulation by PPARs) and (4) targeting cell cycle and apoptosis genes on lesional or cellular level, might prove the most effective way to reduce the burden of atherosclerosis.

REFERENCES

1. Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, Stefanadis C, Moreno P, Pasterkamp G, Fayad Z, Stone PH, Waxman S, Raggi P, Madjid M, Zarrabi A, Burke A, Yuan C, Fitzgerald PJ, Siscovick DS, de Korte CL, Aikawa M, Juhani Airaksinen

KE, Assmann G, Becker CR, Chesebro JH, Farb A, Galis ZS, Jackson C, Jang IK, Koenig W, Lodder RA, March K, Demirovic J, Navab M, Priori SG, Rekhter MD, Bahr R, Grundy SM, Mehran R, Colombo A, Boerwinkle E, Ballantyne C, Insull W, Jr., Schwartz RS, Vogel R, Serruys PW, Hansson GK, Faxon DP, Kaul S, Drexler H, Greenland P, Muller JE, Virmani R, Ridker PM, Zipes DP, Shah PK, Willerson JT. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. *Circulation* 2003;108:1664-1672.

2. Alenzi FQ. Links between apoptosis, proliferation and the cell cycle. *Br J Biomed Sci* 2004;61:99-102.
3. Sherr CJ. Cancer cell cycles. Science 1996;274:1672-1677.
4. Morganti M, Carpi A, Nicolini A, Gorini I, Glaviano B, Fini M, Giavaresi G, Mittermayer C, Giardino R. Atherosclerosis and cancer: common pathways on the vascular endothelium. Biomed Pharmacother 2002;56:317-324.
5. Ross JS, Stagliano NE, Donovan MJ, Breitbart RE, Ginsburg GS. Atherosclerosis: a cancer of the blood vessels? Am J Clin Pathol 2001;116 Suppl:S97-107.
6. Bennett MR, Littlewood TD, Schwartz SM, Weissberg PL. Increased sensitivity of human vascular smooth muscle cells from atherosclerotic plaques to p53-mediated apoptosis. Circ Res 1997;81:591-599.
7. Bennett MR, Macdonald K, Chan SW, Boyle JJ, Weissberg PL. Cooperative interactions between RB and p53 regulate cell proliferation, cell senescence, and apoptosis in human vascular smooth muscle cells from atherosclerotic plaques. Circ Res 1998;82:704-712.
8. Bennett M, Macdonald K, Chan SW, Luzio JP, Simari R, Weissberg P. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science 1998;282:290-293.
9. Guevara NV, Kim HS, Antonova EI, Chan L. The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo. Nat Med 1999;5:335-339.
10. van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De WB, van Berkel TJ, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circ Res 2001;88:780-786.
11. Merched AJ, Williams E, Chan L. Macrophage-specific p53 expression plays a crucial role in atherosclerosis development and plaque remodeling. Arterioscler Thromb Vasc Biol 2003;23:1608-1614.
12. Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, Weinberg RA. Tumor spectrum analysis in p53-mutant mice. Curr Biol 1994;4:1-7.
13. Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat Med 2002;8:403-409.
14. Asahara T, Murohara T, Sullivan A, Silver M, van der ZR, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964-967.
15. Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MA, Storb RF, Hammond WP. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92:362-367.
16. Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR. The E2F transcription factor is a cellular target for the RB protein. Cell 1991;65:1053-1061.
17. Tokino T, Nakamura Y. The role of p53-target genes in human cancer. Crit Rev Oncol Hematol 2000;33:1-6.
18. Davies MJ, Thomas AC. Plaque fissuring–the cause of acute myocardial infarction, sudden ischaemic death, and crescendo angina. Br Heart J 1985;53:363-373.
19. Lee RT, Libby P. The unstable atheroma. Arterioscler Thromb Vasc Biol 1997;17:1859-1867.
20. Bennett MR, Evan GI, Schwartz SM. Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. J Clin Invest 1995;95:2266-2274.

21. der Thusen JH, van Vlijmen BJ, Hoeben RC, Kockx MM, Havekes LM, van Berkel TJ, Biessen EA. Induction of atherosclerotic plaque rupture in apolipoprotein E-/- mice after adenovirus-mediated transfer of p53. Circulation 2002;105:2064-2070.
22. Zadelaar AS, Thusen JH, LS MB, Hoeben RC, Kockx MM, Versnel MA, van Berkel TJ, Havekes LM, EA LB, van Vlijmen BJ. Increased vulnerability of pre-existing atherosclerosis in ApoE-deficient mice following adenovirus-mediated Fas ligand gene transfer. Atherosclerosis 2005.
23. Chang MW, Barr E, Seltzer J, Jiang YQ, Nabel GJ, Nabel EG, Parmacek MS, Leiden JM. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. Science 1995;267:518-522.
24. Smith RC, Wills KN, Antelman D, Perlman H, Truong LN, Krasinski K, Walsh K. Adenoviral constructs encoding phosphorylation-competent full-length and truncated forms of the human retinoblastoma protein inhibit myocyte proliferation and neointima formation. Circulation 1997;96:1899-1905.
25. Diez-Juan A, Andres V. The growth suppressor p27(Kip1) protects against diet-induced atherosclerosis. FASEB J 2001;15:1989-1995.
26. Diez-Juan A, Perez P, Aracil M, Sancho D, Bernad A, Sanchez-Madrid F, Andres V. Selective inactivation of p27(Kip1) in hematopoietic progenitor cells increases neointimal macrophage proliferation and accelerates atherosclerosis. Blood 2004;103:158-161.
27. Tabas I. p53 and atherosclerosis. Circ Res 2001;88:747-749.
28. Geng YJ. Molecular signal transduction in vascular cell apoptosis. Cell Res 2001;11:253-264.
29. Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. Arterioscler Thromb Vasc Biol 2005;25:1256-1261.
30. Palladino MA, Bahjat FR, Theodorakis EA, Moldawer LL. Anti-TNF-alpha therapies: the next generation. Nat Rev Drug Discov 2003;2:736-746.
31. Kwon B, Kim BS, Cho HR, Park JE, Kwon BS. Involvement of tumor necrosis factor receptor superfamily(TNFRSF) members in the pathogenesis of inflammatory diseases. Exp Mol Med 2003;35:8-16.
32. Schreyer SA, Vick CM, LeBoeuf RC. Loss of lymphotoxin-alpha, but not tumor necrosis factor-alpha reduces atherosclerosis in mice. J Biol Chem 2002.;277(14):12364-8
33. Canault M, Peiretti F, Mueller C, Kopp F, Morange P, Rihs S, Portugal H, Juhan-Vague I, Nalbone G. Exclusive expression of transmembrane TNF-alpha in mice reduces the inflammatory response in early lipid lesions of aortic sinus. Atherosclerosis 2004;172:211-218.
34. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305-1308.
35. Luo Z, Sata M, Nguyen T, Kaplan JM, Akita GY, Walsh K. Adenovirus-mediated delivery of fas ligand inhibits intimal hyperplasia after balloon injury in immunologically primed animals. Circulation 1999;99:1776-1779.
36. Schneider DB, Vassalli G, Wen S, Driscoll RM, Sassani AB, DeYoung MB, Linnemann R, Virmani R, Dichek DA. Expression of Fas ligand in arteries of hypercholesterolemic rabbits accelerates atherosclerotic lesion formation. Arterioscler Thromb Vasc Biol 2000;20:298-308.
37. Yang J, Sato K, Aprahamian T, Brown NJ, Hutcheson J, Bialik A, Perlman H, Walsh K. Endothelial overexpression of Fas ligand decreases atherosclerosis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2004;24:1466-1473.
38. Wang T, Dong C, Stevenson SC, Herderick EE, Marshall-Neff J, Vasudevan SS, Moldovan NI, Michler RE, Movva NR, Goldschmidt-Clermont PJ. Overexpression of soluble fas attenuates transplant arteriosclerosis in rat aortic allografts. Circulation 2002;106:1536-1542.
39. Mendrysa SM, McElwee MK, Michalowski J, O'Leary KA, Young KM, Perry ME. mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. Mol Cell Biol 2003;23:462-472.

40. Bockamp E, Maringer M, Spangenberg C, Fees S, Fraser S, Eshkind L, Oesch F, Zabel B. Of mice and models: improved animal models for biomedical research. Physiol Genomics 2002;11:115-132.
41. Brody SL, Crystal RG. Adenovirus-mediated in vivo gene transfer. Ann N Y Acad Sci 1994;716:90-101.
42. Kozarsky KF, Wilson JM. Gene therapy: adenovirus vectors. Curr Opin Genet Dev 1993;3:499-503.
43. Kremer EJ, Perricaudet M. Adenovirus and adeno-associated virus mediated gene transfer. Br Med Bull 1995;51:31-44.
44. Pastore L, Belalcazar LM, Oka K, Cela R, Lee B, Chan L, Beaudet AL. Helper-dependent adenoviral vector-mediated long-term expression of human apolipoprotein A-I reduces atherosclerosis in apo E-deficient mice. Gene 2004;327:153-160.
45. Jalkanen J, Leppanen P, Narvanen O, Greaves DR, Yla-Herttuala S. Adenovirus-mediated gene transfer of a secreted decoy human macrophage scavenger receptor (SR-AI) in LDL receptor knock-out mice. Atherosclerosis 2003;169:95-103.
46. Yang XP, Yan D, Qiao C, Liu RJ, Chen JG, Li J, Schneider M, Lagrost L, Xiao X, Jiang XC. Increased atherosclerotic lesions in apoE mice with plasma phospholipid transfer protein overexpression. Arterioscler Thromb Vasc Biol 2003;23:1601-1607.
47. Linton MF, Atkinson JB, Fazio S. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. Science 1995;267:1034-1037.
48. Lewandoski M. Conditional control of gene expression in the mouse. Nat Rev Genet 2001;2:743-755.
49. van der WL, Adams DJ, Bradley A. Tools for targeted manipulation of the mouse genome. Physiol Genomics 2002;11:133-164.
50. Huang Y, Schwendner SW, Rall SC, Jr., Mahley RW. Hypolipidemic and hyperlipidemic phenotypes in transgenic mice expressing human apolipoprotein E2. J Biol Chem 1996;271:29146-29151.
51. Gu H, Zou YR, Rajewsky K. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell 1993;73:1155-1164.
52. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. Science 2003;300:329-332.
53. Espirito Santo SM, Pires NM, Boesten LS, Gerritsen G, Bovenschen N, van Dijk KW, Jukema JW, Princen HM, Bensadoun A, Li WP, Herz J, Havekes LM, van Vlijmen BJ. Hepatic low-density lipoprotein receptor-related protein deficiency in mice increases atherosclerosis independent of plasma cholesterol. Blood 2004;103:3777-3782.
54. Feil S, Hofmann F, Feil R. SM22alpha modulates vascular smooth muscle cell phenotype during atherogenesis. Circ Res 2004;94:863-865.
55. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 2003;112:1176-1185.
56. Lu Y, Herrera PL, Guo Y, Sun D, Tang Z, LeRoith D, Liu JL. Pancreatic-specific inactivation of IGF-I gene causes enlarged pancreatic islets and significant resistance to diabetes. Diabetes 2004;53:3131-3141.
57. Hisahara S, Araki T, Sugiyama F, Yagami K, Suzuki M, Abe K, Yamamura K, Miyazaki J, Momoi T, Saruta T, Bernard CC, Okano H, Miura M. Targeted expression of baculovirus p35 caspase inhibitor in oligodendrocytes protects mice against autoimmune-mediated demyelination. EMBO J 2000;19:341-348.
58. Loonstra A, Vooijs M, Beverloo HB, Allak BA, van Drunen E, Kanaar R, Berns A, Jonkers J. Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. Proc Natl Acad Sci U S A 2001;98:9209-9214.
59. Schmidt EE, Taylor DS, Prigge JR, Barnett S, Capecchi MR. Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. Proc Natl Acad Sci U S A 2000;97:13702-13707.

60. Silver DP, Livingston DM. Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. Mol Cell 2001;8:233-243.
61. Adams DJ, van der WL. Are we creating problems? Negative effects of Cre recombinase. Genesis 2001;29:115.
62. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 1999;8:265-277.
63. Grier JD, Yan W, Lozano G. Conditional allele of mdm2 which encodes a p53 inhibitor. Genesis 2002;32:145-147.
64. Jonkers J, Meuwissen R, van Der GH, Peterse H, van d, V, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet 2001;29:418-425.
65. Marino S, Vooijs M, van Der GH, Jonkers J, Berns A. Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev 2000;14:994-1004.
66. Mao X, Fujiwara Y, Orkin SH. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. Proc Natl Acad Sci U S A 1999;96:5037-5042.
67. Kuhbandner S, Brummer S, Metzger D, Chambon P, Hofmann F, Feil R. Temporally controlled somatic mutagenesis in smooth muscle. Genesis 2000;28:15-22.
68. Holtwick R, Gotthardt M, Skryabin B, Steinmetz M, Potthast R, Zetsche B, Hammer RE, Herz J, Kuhn M. Smooth muscle-selective deletion of guanylyl cyclase-A prevents the acute but not chronic effects of ANP on blood pressure. Proc Natl Acad Sci U S A 2002;99:7142-7147.
69. Forrester JS, Fishbein M, Helfant R, Fagin J. A paradigm for restenosis based on cell biology: clues for the development of new preventive therapies. J Am Coll Cardiol 1991;17:758-769.
70. Ip JH, Fuster V, Badimon L, Badimon J, Taubman MB, Chesebro JH. Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. J Am Coll Cardiol 1990;15:1667-1687.
71. Liu MW, Roubin GS, King SB, III. Restenosis after coronary angioplasty. Potential biologic determinants and role of intimal hyperplasia. Circulation 1989;79:1374-1387.
72. Schwartz RS, Holmes DR, Jr., Topol EJ. The restenosis paradigm revisited: an alternative proposal for cellular mechanisms. J Am Coll Cardiol 1992;20:1284-1293.
73. van der Hoeven BL, Pires NM, Warda HM, Oemrawsingh PV, van Vlijmen BJ, Quax PH, Schalij MJ, van der Wall EE, Jukema JW. Drug-eluting stents: results, promises and problems. Int J Cardiol 2005;99:9-17.
74. Scott S, O'Sullivan M, Hafizi S, Shapiro LM, Bennett MR. Human vascular smooth muscle cells from restenosis or in-stent stenosis sites demonstrate enhanced responses to p53: implications for brachytherapy and drug treatment for restenosis. Circ Res 2002;90:398-404.
75. Yonemitsu Y, Kaneda Y, Tanaka S, Nakashima Y, Komori K, Sugimachi K, Sueishi K. Transfer of wild-type p53 gene effectively inhibits vascular smooth muscle cell proliferation in vitro and in vivo. Circ Res 1998;82:147-156.
76. Pires NM, van der Hoeven BL, de Vries MR, Havekes LM, van Vlijmen BJ, Hennink WE, Quax PH, Jukema JW. Local perivascular delivery of anti-restenotic agents from a drug-eluting poly(epsilon-caprolactone) stent cuff. Biomaterials 2005;26:5386-5394.

Summary

The work described in this thesis was aimed at identifying the role of cell cycle and apoptosis genes in atherosclerosis. Atherosclerosis is the primary cause of cardiovascular disease, a multi-factorial disorder occurring in the large and medium-sized arteries of the body. Although in the beginning 90s promising lipid lowering therapies predicted a strong reduction in cardiovascular deaths for the upcoming years, in westernized societies it is still the underlying cause of about 40% of all deaths, indicating that treatment of atherosclerosis goes beyond lipid lowering solely. In addition to lipids, continuous cell growth (cell cycle), cell death (i.e. apoptosis and necrosis) and inflammatory processes play a central role in the development and maintenance of atherosclerotic lesions. To investigate in detail the role of several cell cycle and apoptosis genes in atherosclerosis we generated and characterized several mouse models as described in this thesis.

Based on the findings that germline null alleles of the cell cycle genes of our interest (i.e. p53, Rb and Mdm2) lead to either the formation of tumors after the age of 6 months (p53) or embryonic lethality (Rb and Mdm2) we chose to use site-specific recombinase (SSR) technology. To obtain cell type specificity we used either the LysMcre or the SM-CreER^{T2}(ki) mouse model for targeting macrophages or SMCs, respectively.

The tumor suppressor gene p53 has been shown to inhibit cell proliferation and stimulate apoptosis in many cell types. To study the role of macrophage p53 in the development of atherosclerosis, we generated apoE-deficient mice with a macrophage-restricted deletion of p53 and control littermates and analyzed early and advanced atherosclerosis development (**chapter 2**). Absence of macrophage p53 did not affect lesion area in both early and advanced atherosclerosis, neither in the aortic root nor in the aortic arch and thoracic aorta. In early atherosclerosis, absence of macrophage p53 resulted in reduced apoptosis, though without changes in lesion composition. In contrast, in advanced atherosclerosis, reduced apoptosis upon absence of macrophage p53 coincided with increased necrotic death, increased foam cell content, and reduced lipid core formation. Proliferation was not affected by the absence of macrophage p53 in both early and advanced atherosclerosis. Hence, these studies demonstrate that macrophage p53 is a major mediator of foam cell apoptosis and inhibition of this pathway results in a shift of cell death towards necrotic death of lesional macrophages, thereby affecting lesion composition.

To expand the knowledge on the role of cell cycle genes in vascular disease *in vivo* we investigated the role of macrophage Retinoblastoma (Rb) in atherosclerosis development. The tumor suppressor gene Rb has been shown to regulate both cell proliferation and cell death in many cell types. In **chapter 3** we describe the role of macrophage Rb in atherosclerosis development in apoE-deficient mice. To this end, we fed a cholesterol-rich diet for 12 weeks to apoE-deficient mice with a macrophage-restricted deletion of Rb and control littermates. Macrophage-restricted Rb deletion resulted in a strong increase in atherosclerotic lesion area. In addition, the increase in atherosclerosis was characterized by the presence of more advanced lesions that were rich in smooth muscle cells and poor in macrophages. Additional analyses showed that the increase in atherosclerosis was independent of *in vitro* macrophage modified lipoprotein uptake or cytokine production. Immunohistochemical analysis showed that macrophage-restricted Rb deletion did not affect

lesional macrophage apoptosis, but lesional macrophage proliferation was strongly increased. These studies clearly demonstrate that macrophage Rb is a suppressing factor in the progression of atherosclerosis via reduction of macrophage proliferation.

The Mdm2 oncoprotein inhibits p53 activity during embryonic development and in adult homeostatic tissues. Overexpression of p53 can be achieved by specific inactivation of its inhibitor Mdm2 (**chapter 4**). To this end, conditional allelic inactivation of Mdm2 was carried out in mice harboring Mdm2 floxed alleles and a tamoxifen-inducible Cre-recombinase under control of the SM22 promoter (SM-CreER^{T2}(ki) mice), resulting in mice that inducibly lack Mdm2 in their smooth muscle cells. This mouse model would allow us to study p53 overexpression in the SMC-rich cap of atherosclerotic lesions. However, upon SMC-specific Mdm2 deletion mice became rapidly ill and died, hampering studies on the role of SMC-p53 in atherosclerosis. Unexpectedly, the mouse model showed that Mdm2 prevents accumulation of active p53 in quiescent SMCs and thereby the induction of p53-mediated necrotic cell death *in vivo*.

Chapter 5 describes a means to conditionally and locally modify genes of the vasculature using a perivascular drug delivery device (PDD). A 4-hydroxytamoxifen (4-OHT)-eluting PDD was applied around the carotid or femoral artery of a mouse strain, carrying both the tamoxifen-inducible and smooth muscle cell (SMC)-specific Cre-recombinase (SM-Cre-ER^{T2}(ki)) transgene and a stop-floxed β-galactosidase gene in the Rosa26 locus. A dose and time curve of 0-10% (w/w) 4-OHT and 0-14 days application of the PDD showed optimal gene recombination at 1% (w/w) 4-OHT loading at 7 days post application. Recombination was similar to the level achieved by systemic tamoxifen administration and was completely confined to the PDD-treated vessel wall segment. Thus, local application of a 4-OHT-eluting PDD results in vascular SMC-specific Cre-mediated recombination without affecting additional SMCs.

In addition to cell proliferation and cell death, inflammation plays a key role in the development of atherosclerosis. Immune cells are of paramount importance in early atherosclerosis development and their effector molecules accelerate progression of atherosclerosis. Tumor necrosis factor-α (TNFα) is a pleiotropic cytokine exerting both cell death and inflammatory activity. Although TNFα and its receptors are thought to be considerably important in a number of biological activities relevant to atherosclerosis, its complete function in atherogenesis remains unclear. Earlier studies in mice indicated that TNFα affects atherosclerosis minimally or not in early atherosclerosis development. To study the role of TNFα in advanced and complex atherosclerotic lesions we crossbred TNFα-deficient mice onto an APOE*3-Leiden background (**chapter 6**). To induce atherosclerosis development the mice were fed a cholesterol-rich diet. Mice deficient for TNFα and their control littermates, showed comparable levels of plasma cholesterol and triglycerides and the systemic inflammatory parameters, serum amyloid A (SAA) and soluble intercellular adhesion molecule-1 (sICAM). Although absence of TNFα did not affect the quantitative area of atherosclerosis, mice deficient for TNFα had a higher relative number of early lesions and a lower relative number of advanced lesions. In addition, the advanced lesions in TNFα deficient mice showed a decrease in necrosis and an increase in apoptosis. Hence, TNFα stimulates the formation of lesions towards an advanced

phenotype, with more lesion necrosis and a lower incidence of apoptosis.

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors present in several organs and cell types. PPAR alpha and gamma are the two main categories of these receptors, which are both characterized by their ability to influence cell proliferation, differentiation, apoptosis, and inflammation as well as lipid metabolism and glucose homeostasis via transcriptional activation or repression of target genes or via DNA-binding-independent pathways. In atherosclerosis PPAR-α and PPAR-γ activation results in reduction of atherogenic triglycerides and systemic plasma inflammatory proteins, raise HDL levels and improve insulin resistance. At a cellular level, PPARα/γ agonists act on most cell types involved in atherosclerosis reducing their involvement in the tissue response associated with lesion development. In **chapter 7** the combined PPARα/γ agonist tesaglitazar was investigated on its anti-atherogenic effects in APOE*3Leiden mice with normal and reduced insulin sensitivity. APOE*3-Leiden transgenic mice were fed either a low-fat (LF) or high-fat (HF) insulin-resistance-inducing diet. In both LF and HF-fed mice, one group received a high-cholesterol supplement. A second group received the same HC diet, additionally supplemented with tesaglitazar. A third control group received a low cholesterol supplement, resulting in plasma cholesterol levels similar to those of the tesaglitazar-group. In this study we showed that tesaglitazar has anti-atherosclerotic effects, analyzed both by cross-sectioning at the level of the aortic root and by *en face* analysis of the aortic arch. These anti-atherosclerotic effects go beyond plasma total cholesterol lowering, and were more pronounced in animals on high-fat diet. In addition, tesaglitazar treatment reduced inflammatory parameters as plasma SAA levels, the number of adhering monocytes, and NFκB activity in the vessel wall. The mechanism by which tesaglitazar exerts its anti-atherosclerotic actions beyond plasma cholesterol lowering could therefore be associated with its anti-inflammatory effects.

Samenvatting

In dit proefschrift wordt het onderzoek naar de rol van celcyclus en apoptose genen in atherosclerose beschreven. Atherosclerose is de belangrijkste oorzaak van cardiovasculaire ziekten, een multi-factoriële aandoening die voornamelijk de grote en middelgrote vaten van het lichaam aantast. Alhoewel in het begin van de jaren 90 veelbelovende lipide verlagende therapieën een sterke reductie in cardiovasculaire mortaliteit voorspelden, is atherosclerose nog steeds de onderliggende oorzaak in 40% van alle sterfgevallen in de westerse samenleving. Dit geeft aan dat de behandeling van atherosclerose meer vereist dan enkel het verlagen van plasmalipiden. Naast plasma lipiden spelen continue celgroei (celcyclus), celdood (apoptose en necrose) en ontstekingsprocessen een belangrijke rol in de ontwikkeling van atherosclerotische lesies. Om de rol van verschillende celcyclus en apoptose genen in de ontwikkeling van atherosclerose te bestuderen, hebben we verschillende muismodellen gemaakt en gekarakteriseerd.

Uitgaande van de bevindingen dat het volledig uitschakelen van de genen van onze interesse (nl. p53, Rb en Mdm2) leidt tot òf de vorming van tumoren wanneer de muizen 6 maanden en ouder zijn (bij p53) òf embryonale letaliteit (bij Rb en Mdm2) hebben we gebruik gemaakt van "site-specific recombinase (SSR)" technologie. Met deze techniek bereikten we celspecificiteit door het gebruik van het LysMCre muismodel (voor macrofagen) of het SM-CreER^{T2}(ki) muismodel (voor gladde spiercellen), twee celtypes die van groot belang zijn in atherosclerotische lesies.

Het tumor suppressor gen p53 remt celproliferatie en stimuleert apoptose in veel verschillende celtypes. Om de rol van macrofaag p53 in de ontwikkeling van atherosclerose te bestuderen, werden apoE-deficiënte muizen met een macrofaag-specifieke p53 deletie en controle muizen gemaakt (**hoofdstuk 2**). Macrofaag p53 werd in milde en ernstige atherosclerose bestudeerd. Macrofaag specifieke p53 deletie had geen effect op het oppervlak van de milde en ernstige atherosclerotische lesies, niet in het hartkleppengebied en ook niet in de aortaboog en thoracale aorta. In de milde atherosclerotische lesies leidde afwezigheid van macrofaag p53 tot een afname in apoptose. Dit had geen effect op de atherosclerotische lesie samenstelling. Echter, in de ernstige atherosclerotische lesies, leidde de afname van apoptose in p53-deficiënte muizen tot een toename in necrotische celdood, een toename van het schuimcel oppervlak, en een afname in de lipide afzettingen. Macrofaag-specifieke deletie van p53 had geen invloed op celproliferatie in zowel de milde als de ernstige atherosclerotische lesies. Deze studie laat zien dat macrofaag p53 een sterke regulator is van schuimcelapoptose en remming van deze route leidt tot een verschuiving van apoptotische celdood naar necrotische celdood van macrofagen, direct resulterend in veranderingen in lesie samenstelling.

Om de kennis van de rol van celcyclus genen in vasculaire ziekten verder te verbreden, hebben we de rol van macrofaag Retinoblastoma (Rb) in atherosclerose ontwikkeling onderzocht. Het tumor suppressor gen Rb reguleert zowel cel proliferatie als celdood in veel verschillende celtypes. In **hoofdstuk 3** beschrijven we de rol van macrofaag Rb in de ontwikkeling van atherosclerose zoals bestudeerd in apoE-defciënte muizen. Gedurende 12 weken werden macrofaag-specifieke Rb deficiënte en controle muizen een cholesterol-rijk dieet gevoerd. Macrofaag-specifieke Rb deletie resulteerde in een sterke toename van het atherosclerotisch lesie

oppervlak. Bovendien werd de toename in atherosclerose gekarakteriseerd door de aanwezigheid van meer ernstige lesies met veel gladde spiercellen en weinig macrofagen. *In vitro* analyses lieten zien dat de toename in atherosclerose onafhankelijk was van opname van gemodificeerde lipoproteïnen of productie van cytokines door macrofagen. Macrofaag proliferatie was sterk verhoogd in de atherosclerotische lesies van macrofaag-specifieke Rb deficiënte muizen. Daartegenover, had macrofaag-specifieke Rb deletie geen effect op apoptose in de lesies. Deze studie laat duidelijk zien dat macrofaag Rb een remmende factor is in de ontwikkeling van atherosclerose via de reductie van macrofaag proliferatie.

Oncoproteine Mdm2 remt p53 activiteit tijdens embryonale ontwikkeling en in volwassen homeostatische weefsels. Overexpressie van p53 kan worden geïnduceerd door specifieke inactivatie van zijn remmer Mdm2 (**hoofdstuk 4**). Om dit te bereiken werden muizen waarvan het Mdm2 gen "gefloxed" is, gecombineerd met muizen die het tamoxifen-inducеerbare Cre-recombinase onder de controle van de SM22 promotor hebben (SM-CreER^{T2}(ki) muizen). Dit resulteerde in muizen die induceerbaar deficiënt waren voor Mdm2 in gladde spiercellen. Dit model had als doel om p53 overexpressie te bestuderen in de gladde spiercel-rijke cap van atherosclerotische lesies. Maar, na deletie van Mdm2 specifiek in gladde spiercellen, werden de muizen snel ziek en gingen dood, waardoor studies naar de rol van gladde spiercel p53 in atherosclerose niet uitgevoerd konden worden. Extra analyses aan het muismodel leverde onverwachts het bewijs dat Mdm2 ophoping van actief p53 in rustende gladde spiercellen voorkomt en daarmee de inductie van p53-gemedieerde necrotische celdood *in vivo*.

Hoofdstuk 5 beschrijft een methode om conditioneel en lokaal genen te modificeren in de vaatwand met een "perivasculaire drug delivery device (PDD)". Een 4-hydroxytamoxifen (4-OHT) PDD werd rond de carotis en femoralis van een muisstam geplaatst, die zowel het tamoxifen-inducеerbare en gladde spiercel-specifieke Cre-recombinase (SM-CreER^{T2}(ki)) transgen als het "stop-floxed -galactosidase" gen in de Rosa26 locus heeft. Een dosiscurve van 0-10% (w/w) 4-OHT en tijdscurve van 0-14 dagen applicatie van de PDD resulteerde in optimale gen recombinatie bij 1% (w/w) 4-OHT, 7 dagen post applicatie. De hiermee verkregen recombinatie was gelijk aan de niveaus die bereikt werden met systemische toediening van tamoxifen en beperkte zich volledig tot het PDD-behandelde gedeelte van de vaatwand. Dus, lokale toediening van een 4-OHT PDD resulteerde in vasculaire gladde spiercel specifieke Cre-gemedieerde gen recombinatie zonder andere gladde spiercellen te beïnvloeden.

Naast celgroei en celdood speelt ontsteking een belangrijke rol in de ontwikkeling van atherosclerose. Immuuncellen zijn van groot belang in vroege atherosclerose ontwikkeling en hun "effector" moleculen versnellen de progressie van atherosclerose. Tumor necrosis factor-α (TNFα) is een cytokine met zowel celdood als ontstekings modulerende activiteit. Hoewel TNFα en zijn receptoren van belang zijn in een aantal biologische activiteiten die zeer relevant zijn voor atherosclerose, is de volledige rol van TNFα in de ontwikkeling van atherosclerose nog onduidelijk. Eerdere muizen studies lieten zien dat TNFα vroege atherosclerose ontwikkeling niet tot nauwelijks beïnvloedt. Om de rol van TNFα in ernstige en complexe atherosclerotische lesies te onderzoeken hebben we TNFα-deficiënte muizen gekruist

met APOE*3-Leiden muizen (**hoofdstuk 6**). Vervolgens werden de muizen gevoerd met een cholesterol-rijk dieet om atherosclerose te induceren. TNFα-deficiënte en controle muizen vertoonden gelijke niveaus van plasma cholesterol en triglyceriden en de systemische ontstekingsparameters "serum amyloid A" en "soluble intercellular adhesion molecule-1". Hoewel TNFα deficiëntie geen invloed had op het atherosclerose lesie oppervlak, hadden TNFα-deficiënte muizen een relatief hoger aantal vroege en relatief lager aantal ernstige lesies. Bovendien vertoonden de ernstige lesies in TNFα-deficiënte muizen een afname in necrose en een toename in apoptose. We concluderen dat TNFα de vorming van ernstigere lesies met meer necrose en een lagere incidentie van apoptose stimuleert.

"Peroxisome proliferator activated receptoren" (PPAR) zijn nucleaire receptoren die aanwezig zijn in verschillende organen en celtypes. PPAR alpha (α) en gamma (γ) zijn de belangrijkste subtypes van deze receptoren en beïnvloeden cel proliferatie, differentiatie, apoptose en ontsteking, maar ook lipide metabolisme en glucose homeostase via transcriptionele activatie of repressie van genen of via DNA-bindings-onafhankelijke routes. In atherosclerose leidt PPARα en PPARγ activatie tot een reductie van atherogene triglyceriden en systemische ontstekingseiwitten, een toename in HDL niveaus en een verbetering van insuline resistentie. Op cellulair niveau werken PPARα/γ agonisten op de meeste celtypen die van belang zijn in atherosclerose, waardoor de betrokkenheid van deze celtypen in de ontwikkeling van de atherosclerotische lesie wordt verminderd. In **hoofdstuk 7** onderzochten we de anti-atherogene effecten van de gecombineerde PPARα/γ agonist tesaglitazar in APOE*3-Leiden muizen, met een normale en gereduceerde insuline gevoeligheid. De APOE*3-Leiden muizen werden in twee verschillende dieet groepen ingedeeld. De eerste groep kreeg een laag-vet dieet en de tweede groep een hoog-vet insuline-resistentie-inducerend dieet. In zowel de laag-vet als de hoog-vet muizen kreeg één groep een hoog-cholesterol supplement. Een tweede groep kreeg ook dit hoog-cholesterol supplement, maar nu met tesaglitazar als toevoeging. Een derde groep kreeg een laag-cholesterol supplement, wat resulteerde in plasma cholesterol niveaus die gelijk waren aan de plasma cholesterol niveaus in de tesaglitazar behandelde groep. Analyses van atherosclerose, in zowel het hartkleppengebied als in de aortaboog (*en face* analyse), lieten zien dat tesaglitazar anti-atherosclerotische effecten heeft. Deze anti-atherosclerotische effecten werden niet enkel geïnduceerd door plasma cholesterol verlaging en bleken sterker in muizen op een hoog-vet dieet. Tesaglitazar behandeling resulteerde ook in een afname van ontstekings parameters zoals "plasma serum amyloid A" (SAA), het aantal aanhechtende monocyten en "Nuclear Factor Kappa-B" (NFκB) activiteit in de vaatwand. Het zijn waarschijnlijk deze anti-inflammatoire eigenschappen van tesaglitazar die zorgen voor een additionele reductie in atherosclerose.

Curriculum Vitae
List of publications

Lianne Simone Mirjam is geboren op 17 september 1979 te Tilburg. In 1997 behaalde zij haar VWO diploma aan het Interconfessioneel Hofstad College te Den Haag. In september van datzelfde jaar begon zij aan de studie Bio-Farmaceutische Wetenschappen aan de Universiteit Leiden. De propadeuse werd behaald in 1998, waarna vervolgens het doctoraal examen in juni 2001 werd behaald (cum laude). In het kader van het doctoraal examen heeft zij bij de vakgroep Biofarmacie van het Leiden/Amsterdam Center for Drug Research (Prof. Dr. Th.J.C. van Berkel) en TNO-Preventie en Gezondheid, Gaubius Laboratorium (Prof. Dr. Ir. L.M. Havekes) haar afstudeervak gevolgd.

Van juni 2001 tot mei 2005 was zij werkzaam als assistent in opleiding (a.i.o.) in dienst van de Nederlandse Hartstichting (NHS, project 2000.051) bij Algemene Interne Geneeskunde van het Leids Universitair Medisch Centrum en TNO-Kwaliteit van Leven, afdeling Vascular and Metabolic Diseases. Gedurende deze periode werd onder begeleiding van Dr. B.J.M. van Vlijmen, Dr. M.P.J. de Winther en Prof. Dr. Ir. L.M. Havekes het in dit proefschrift beschreven onderzoek verricht.

Vanaf november 2005 is zij in opleiding tot Klinisch Chemicus in het Leids Universitair Medisch Centrum en het Diaconessenhuis te Leiden.

FULL PAPERS

Boesten L.S.M., Zadelaar A.S.M., van Nieuwkoop A., Gijbels M.J.J., de Winther M.P.J., Havekes L.M., van Vlijmen B.J.M. Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice. *Cardiovascular Research (2005) 66(1):179-85.*

Boesten L.S.M.*, Zadelaar A.S.M.*, Pires N.M.M., van Nieuwkoop A., Biessen E.A.L., Jukema J.W., Havekes L.M., van Vlijmen B.J.M., Willems-van Dijk K. Local cre-mediated gene recombination in vascular smooth muscle cells in mice. *Transgenic Research (2005). In press.*

Boesten L.S.M., Zadelaar A.S.M., van Nieuwkoop A., Hu L., Jonkers J., Gijbels M.J.J., van der Made I., de Winther M.P.J., Havekes L.M., van Vlijmen B.J.M. Macrophage retinoblastoma deficiency leads to enhanced atherosclerosis development in apoE-deficient mice. *The FASEB Journal (2006). In press.*

Boesten L.S.M., Zadelaar A.S.M., van Nieuwkoop A., Hu L., Teunisse A.F.A.S., Jochemsen A.G., Evers B., Gijbels M.J.J., van Vlijmen B.J.M. Havekes L.M., de Winther M.P.J. Macrophage p53 controls foam cell death in atherosclerotic lesions of apolipoprotein E deficient mice. *Submitted for publication.*

Boesten L.S.M.*, Zadelaar A.S.M.*, Francoz S., De Clercq S., van Nieuwkoop A., Biessen E.A.L., Jochemsen A.G., Zürcher C., Havekes L.M., Marine J.C., van Vlijmen B.J.M. Mdm2 protects terminally differentiated smooth muscle cells from p53-mediated caspase-independent cell death. *Submitted for publication.*

Zadelaar A.S.M., **Boesten L.S.M.**, Jukema J.W., van Vlijmen B.J.M., Kooistra T., Emeis J.J., Lundholm E., Camejo G., Havekes L.M. The PPARα/γ dual agonist tesaglitazar reduces atherosclerosis development beyond its plasma cholesterol-lowering effects in APOE*3-Leiden transgenic mice. *Submitted for publication.*

van Vlijmen B.J.M., Gerritsen G., Franken A.L., **Boesten L.S.M.**, Kockx M.M., Gijbels M.J., Vierboom M.P., van Eck M., van De Water B., van Berkel TJ, Havekes L.M. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. *Circulation Research (2001) 88:780-6.*

Espirito Santo S.M., Pires N.M., **Boesten L.S.M.**, Gerritsen G., Bovenschen N., van Dijk K.W., Jukema J.W., Princen H.M., Bensadoun A., Li W.P., Herz J., Havekes L.M., van Vlijmen B.J.M. Hepatic low-density lipoprotein receptor-related protein deficiency in mice increases atherosclerosis independent of plasma cholesterol. *Blood (2004) 103:3777-82.*

Pires N.M.M., Schepers A., van der Hoeven B.L., de Vries M.R., **Boesten L.S.M.**, Jukema J.W., Quax P.H.A. Histopathologic alterations following local delivery of dexamethasone to inhibit restenosis in murine arteries. *Cardiovascular Research (2005) 68(3):415-24*

Zadelaar A.S.M., von der Thüsen J.H., **Boesten L.S.M.**, Hoeben R.C., Kockx M.M., Versnel M.A., van Berkel T.J.C., Havekes L.M., Biessen E.A.L., van Vlijmen B.J.M. Inreased vulnerability of pre-existing atherosclerotic plaques in apolipoprotein E-/- mice following adenovirus mediated transfer of Fas ligand. *Atherosclerosis (2005) 183(2):244-250*

Monraats P.S.*, Pires N.M.M.*, Schepers A., Agema W.R.P., **Boesten L.S.M.**, de Vries M.R., Zwinderman A.H., de Maat M.P.M., Doevendans P.A.F.M., de Winter R.J., Tio R.A., Waltenberger J., 't Hart L.M., Frants R.R., Quax P.H.A., van Vlijmen B.J.M., Havekes L.M., van der Laarse A., van der Wall E.E., Jukema J.W. Tumor necrosis factor-α plays an important role in restenosis development. *FASEBJ (2005) 19(14):1998-2004.*

Hu L., **Boesten L.S.M.**, Bovenschen N., Huisman M.V., Berbée J.F.P., Havekes L.M., van Vlijmen B.J.M., Tamsma J.T. Macrophage low-density lipoprotein receptor-related Protein deficiency enhances atherosclerosis in mice. *Submitted for publication.*

***** These authors contributed equally to the study.

ABSTRACTS

Boesten L.S.M., Zadelaar A.S.M., van Nieuwkoop A., Gijbels M.J.J., de Winther M.P.J., Havekes L.M., van Vlijmen B.J.M. Tumor necrosis factor-α promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice. *Vasculaire Geneeskunde 2004;2:10.*

Zadelaar A.S.M., **Boesten L.S.M.**, von der Thüsen J.H., Hoeben R.C., Kockx M.M., Havekes L.M., Biessen E.A.L., van Vlijmen B.J.M. Induction of atherosclerotic plaque rupture in apolipoprotein $E^{-/-}$ mice after local adenovirus mediated transfer of Fas ligand. *Vasculaire Geneeskunde 2003.*

Zadelaar A.S.M., **Boesten L.S.M.**, von der Thüsen J.H., Hoeben R.C., Kockx M.M., Havekes L.M., Biessen E.A.L., van Vlijmen B.J.M. Induction of atherosclerotic plaque rupture in apolipoprotein $E^{-/-}$ mice after local adenovirus mediated transfer of Fas ligand. *Circulation Supplement 2003;108(17):IV-223.*

Zadelaar A.S.M., **Boesten L.S.M.**, van Nieuwkoop A., Jukema J.W., Kooistra T., Lundholm E., Camejo G., Havekes L.M. Tesaglitazar, a dual PPARα/γ agonist, reduces atherosclerosis in APOE*3-Leiden transgenic mice. *Diabetes Supplements 2005.*