Cell turnover and immune cell activation: key factors in the control of plaque progression and phenotype in atherosclerosis?

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Cell Turnover and Immune Cell Activation: Key Factors in the Control of Plaque Progression and Phenotype in Atherosclerosis?

Cell Turnover and Immune Cell Activation: Key Factors in the Control of Plaque Progression and Phenotype in Atherosclerosis?

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Universiteit Maastricht,
op gezag van Rector Magnificus,
Prof. dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 15 februari 2001 om 14.00 uur

door

Esther Lutgens
4 November 1975, geboren te Heerlen.

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Ανδρα μοι εννεπε, Μουσα, πολυτροπον ος μαλα πολλα
πλαγχθη, επει Τροιης ιερον πτολιεθρον επερσε
πολλων δ' ανθρωπων ιδεν ασθεα και νοον εγνω,
πολλα δ' ο γ εν πονται παθεν αλγεα ον καταθυμον,
αρνυμενος ην τε ψυχην και νοστον εταιρων.

(Homerus, Odyssee, Aanhef).

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List of abbreviations

AA	abdominal aorta
ACE	angiotensin converting enzyme
AEC	amino-3-9-ethylcarbazole
AHA	American Heart Association
Ang	angiotensin II
ANP	atrial natriuretic peptide
ApoE	apolipoprotein E
ASMA	α -smooth muscle actin
Bak	bcl-2 associated K protein
Bax	bcl-2 associated X protein
Bcl-2	B-cell leukemia 2
BCT	brachiocephalic trunk
bFGF	basic fibroblast growth factor
BKLF	basic Kruppel like factor
BrdU	5'-bromo-2'-deoxyuridine
CA	carotid artery
CAK	cdk activating kinase
CCR2	CC-chemokine receptor 2
Cdk	cyclin dependent kinase
CTLA4	cytotoxic-T-lymphocyte-associated protein 4
DAB	3,3'-diaminobenzidine
DNA	deoxyribo nucleic acid
ECM	extracellular matrix
EEL	external elastic lamina
EGF	epidermal growth factor
EGR-1	early growth response gene 1
EL	elastic lamina
ERK	extracellular regulated kinase
ES-cells	embryonic stem cells
fc	fibrous cap
fcR	foam cell rich
Gas6	growth arrest specific gene 6
GLA	γ -carboxyglutamic acid
gm-CSF	granulocyte-macrophage colony stimulating factor
GRO- α	growth related oncogen
HE	haematoxinilin and eosin
HUVEC	human umbilical vein endothelial cells
HFC diet	high-fat/ high-cholesterol diet
HSP	heat shock protein
ICAM-1	intracellular adhesion molecule 1
ICE	interleukin converting enzyme
IDL	intermediate density lipoprotein

IEL	internal elastic lamina
IFN γ	interferon gamma
IGF	insulin like growth factor
IGFBP	IGF-binding protein
IL	interleukin
IP	intra-peritoneal
IRF-1	interferon regulatory factor-1
JNK	c-jun N-terminal kinase
kD	kiloDalton
KIF3b	kinesin superfamily protein 3b
lc	lipid core
LDL	low density lipoprotein
LDL-R	LDL-receptor
LKLF	lung Kruppel-like factor
MAPK	mitogen activated protein kinase
MCP-1	macrophage chemoattractant protein 1
MHR23b	mouse homologue of RAD23b
MIP	macrophage inflammatory protein
MLC	myosin light chain
MMP	matrixmetalloproteinase
NA	not available
NC	not classified
ND	non-diseased
NF-E2	nuclear factor E2
NF κ B	nuclear factor kappa B
NO	nitric oxide
OxLDL	oxidized LDL
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PT(C)A	percutaneous transluminal (coronary) angioplasty
RAG-1	recombinase activated gene 1
RANTES	regulated on activation normal T-lymphocyte expressed and secreted
Rb	retinoblastoma
RNA	ribo nucleic acid
SLE	systemic lupus erythematosus
SMC	smooth muscle cell
SM-MHC	smooth muscle- myosin heavy chain
SR	scavenger receptor
sh	shoulder
TA	thoracic aorta
TBS	tris buffered saline
TGF β	transforming growth factor beta

TIMP	tissue inhibitor of matrix metalloproteinases
TNF	tumor necrosis factor
TNF-R	tumor necrosis factor receptor
TUNEL	terminal deoxynucleotidyl transferase nick end labeling
UBF	upstream binding factor
VCAM	vascular cell adhesion molecule
VLDL	very low density lipoprotein
VSMC	vascular smooth muscle cell

Chapter 1

General Introduction

General

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General

1. Epidemiology

Atherosclerosis is the leading cause of morbidity, disability and mortality in the western world. The name, derived from Greek, refers to thickening of the arterial intima (*sclerosis* 'hardening'), and accumulation of lipid (*athere* 'gruel'), that characterize the typical atherosclerotic lesion. Atherosclerosis is a systemic disease of the large arteries that affects all persons to a greater or lesser extent, and progresses with age. The atherogenic process silently starts in early childhood¹⁻³, and becomes clinically evident in later life, mostly from the fifth/sixth decade⁴.

Depending on the arterial bed affected, and the characteristics of the individual lesions, atherosclerosis leads to distinct clinical manifestations, which may be quite heterogeneous. Atherosclerosis of the coronary arteries may cause angina pectoris and myocardial infarction. Atherosclerosis of the arteries supplying the central nervous system can lead to transient cerebral ischemia and stroke. In the peripheral circulation, atherosclerosis jeopardizes limb viability and may cause intermittent claudication and gangrene. Involvement of the splanchnic circulation can result in mesenteric ischemia and bowel infarction, and involvement of the renal circulation may cause hypertension and renal infarction. The kind of complication, i.e. acute or chronic, is dependent on the composition of the atherosclerotic lesion, and the remodeling response of the artery (see below). Acute complications are usually the result of rupture of an advanced atherosclerotic plaque. This 'rupture' exposes the thrombogenic *athere* to the blood, which results in thrombus formation and acute occlusion of the vessel.⁵⁻⁷ On the other hand, chronic, slowly developing complications are the result of a growing advanced stable plaque that slowly encroaches on the lumen.⁸

Despite our familiarity with the clinical symptoms of the disease, its pathogenesis remains poorly understood. However, it is known that atherosclerosis is a multifactorial disease, and that both genetic and environmental factors, such as different types of hyperlipidemias⁹, hypertension⁹, hyperhomocysteinemia¹⁰, diabetes¹¹, high fat diet¹², stress, sedentary lifestyle¹³, and smoking⁹, accelerate the progression of atherosclerosis into a clinically symptomatic stage.¹⁴⁻¹⁶

New pharmacological intervention with HMG Co-A reductase inhibitors has reduced the incidence of symptomatic atherosclerotic disease by 25%-42%¹⁹⁻²¹. Blood pressure lowering and the cessation of smoking also contribute significantly to a reduction of atherosclerosis related mortality and morbidity.^{14,17,18,293} Other, preventive measures include treatment with ACE-inhibitors¹⁹⁻²¹, the supplementation of anti-oxidants, for example vitamin E²²⁻²⁴ as well as diet and life style changes that need to be initiated early in life to achieve the most effect.^{13,14} Atherosclerosis nevertheless remains the primary cause of mortality in the industrialized world and is rapidly acquiring the same importance in the developing world. Moreover, the epidemiology of atherosclerosis is changing with an increase in acute, thrombotic manifestations of the disease, caused by plaque rupture. These thrombotic

manifestations result in more mortality and morbidity than the non-thrombotic disease states caused by atherosclerosis.

The aim of the current thesis is to further elucidate the pathogenesis of atherosclerosis. It concentrates on the role of cell turnover and inflammation in lesion initiation, progression and phenotype. Experiments were performed on human atherosclerotic tissue obtained during autopsy or vascular surgery, or in mouse models of atherosclerosis. Interventions in these mouse models comprise genetic disruption studies and antibody studies.

In this general introduction, the pathogenesis of atherosclerosis, including the 'response to injury' hypothesis, histological classification, and vascular remodeling will be discussed, followed by an overview of the most commonly used mouse models of atherosclerosis. Subsequently, background information will be provided on the process of cell turnover in general, and specifically in vascular disease, followed by a description of inflammation with a focus on the CD40-CD40L system, and its role in vascular disease. Finally, the hypothesis of the thesis will be outlined.

2. Pathogenesis

2.1 Introduction

During the last two centuries, clinicians and researchers have been fascinated by the process of atherosclerosis. The first investigations were performed by pathologists. In 1858, Virchow, the founder of cellular pathology, presented a lecture on the 'atheromatous affection of arteries'. He distinguished 3 processes. At first, the *simple fatty metamorphosis*, which is characterized by the transformation of the existing histological elements into a state of fatty degeneration. Second, the *stage of irritation*, comparable to the stage of swelling, and enlargement, seen in other states of inflammation. Lastly, the stage of *atheromatous degeneration*, with cholestearine plates, granule cells and fat granules, and hard lumps of half softened substance resulting in the *pultaceous* character of the atheromatous matter.²⁶ In 1908, sir William Osler, the pioneer of internal medicine stated that 'the stability of tubing of any sort depends on the structure and on the sort of material used: and so it is with the human being. With the poor variety of elastic and muscular fibers in the blood vessels, some are unable to resist the wear and tear of daily life... in time they become old, in threescore or fourscore years the limit of their endurance is reached and they wear out'.²⁷

Nowadays, we have a more profound knowledge of processes and features of the different stages of atherosclerosis, but many aspects are based on the observations of ancient research described above. This outline will focus on the pathogenesis of atherosclerosis. First, the 'response to injury' theory will be discussed, followed by a description of the latest histological classification, an overview of the site and age dependent progression of atherosclerosis and lastly, the aspects of remodeling of the entire vasculature during atherogenesis.

2.2 *Response to Injury*

One of the most important theories, that has contributed to a better (molecular) understanding of the pathogenesis of atherosclerosis is the 'response to injury' hypothesis. In 1973, the group of R. Ross postulated that atherosclerosis is the result of endothelial denudation.²⁸ They hypothesized that endothelial denudation caused migration of smooth muscle cells (SMCs) into the intima, followed by proliferation of these cells, increased lipid deposition and extracellular matrix synthesis.^{28,29} This hypothesis was derived from the observation that balloon-catheter induced arterial denudation in macaque monkeys resulted in the development of 'fibromusculoelastic lesions' (fig. 1.1).³⁰

Over the years, the hypothesis was adapted to newer insights. In 1993, endothelial dysfunction triggered by shear stress and (ox)LDL, rather than denudation was considered to be the trigger of atherogenesis.^{31,32} Furthermore, besides the SMC and endothelial cell, macrophages and T-lymphocytes were considered to play major roles in the disease process. Molecules considered to act in cell recruitment, migration, proliferation, apoptosis and the control of lipid and protein synthesis were growth factors (PDGF, bFGF, IGF, TGF β) and cytokines (IL1, TNF α , IL2, IFN γ) (fig. 1.1).

Nowadays, endothelial activation, and the subsequent chronic inflammatory response, comprising upregulation of the immune system, is considered to play the major role in the progression of atherosclerosis.³³ The most important players responsible for the initiation and progression of atherosclerosis are the macrophage and the T-lymphocyte. Endothelial activation induces upregulation of leukocyte adhesion molecules (like P- and E-selectin, ICAM-1 and VCAM), that mediate monocyte recruitment into the intima. Also, growth factors (PDGF, bFGF, IGF, TGF β), chemokines (MCP-1, IL8) and cytokines (IL1, TNF α , IL2, IFN γ , gm-CSF, M-CSF) mediate the differentiation of monocytes into macrophages, the transition into macrophage derived foam cells, and the activation of macrophages and T-lymphocytes. Furthermore, these molecules are responsible for recruitment and proliferation of cells into the intima, apoptosis of these cells and the synthesis and degradation of the extracellular matrix (fig. 1.1).

2.3 *Progression of atherosclerosis according to the AHA classification of 1995*

Since the 1950s, several histological classifications have been developed. The most recent, published in 1995 and updated in 2000, was developed by the Council on Arteriosclerosis, Thrombosis and Vascular Biology of the American Heart Association.^{1,4,34-36} It is based upon the principles of the 'response to injury' theory outlined above. This classification divides the pathogenesis of atherosclerosis into six different stages, based on histological characteristics (fig. 1.2 and 1.3). Type I-III lesions comprise initial atherosclerotic lesions, that are clinically silent, while type IV and V lesions are advanced stable or instable atherosclerotic lesions, that both can progress to the clinically most dangerous type VI (thrombotic) lesions.^{4,33,34}

Atherogenesis starts with monocyte adhesion to the endothelium, diapedesis of monocytes into the intima (that differentiate into tissue macrophages) and accumulation of lipids into these macrophages (foam cells). This stage is called the

Evolution of the ‘Response to Injury’ hypothesis

Fig. 1.1 Evolution of the ‘response to injury’ hypothesis in the last three decades, based upon reviews by R. Ross.^{30,33}

Fig. 1.2
Progression of atherosclerosis according to the AHA classification of 1995

fatty streak, or, according to the AHA, a type I-II lesion. Macroscopically, these lesions look like small lipid yellow dots.^{4,34,37,38} They have been reported to occur as early as in infancy and childhood.¹⁻³ When monocyte recruitment continues, the fatty streak lesion progresses into a type III lesion, a plaque containing also extracellular lipids (pre-atheroma).³⁴ Further progression results in the development of advanced atherosclerotic lesions. The first advanced stage of atherosclerosis, prone to become clinically symptomatic is the atheroma (type IV lesion). The type IV lesion contains a necrotic core with extracellular lipids and cholesterol-ester crystals.⁴ During the progression of atherosclerosis, (myo)fibroblasts and SMCs migrate into the intima and form, together with extracellular matrix elements (collagen), a fibrous cap that overlies the atherosclerotic plaque. This lesion type is called the fibro-atheroma (type V lesion). Type V lesions are subdivided into Va (large lipid core), Vb (=VII) (calcification) and type Vc (=VIII)(fibrous).⁴ Type IV and V lesions can either have a stable plaque morphology, characterized by a thick fibrous cap, high SMC/collagen content, a low amount of T-lymphocytes, and a small lipid core, or an unstable plaque morphology, which is characterized by a thin fibrous cap, a large lipid core, a low SMC/collagen content, a low amount of T-lymphocytes, and a thin media.^{7,33,39} The thin fibrous cap of unstable type IV and type V lesions is prone to rupture, thereby exposing the thrombogenic material of the lipid core. The resulting lesion type is the clinically most symptomatic type VI lesion

Fig. 1.3
Figure describing the most important features and cell types of the six different stages of atherosclerosis according to the AHA classification of 1995.

(complicated lesion) (fig. 1.2 and 1.3).^{4,7} Type VI lesions are ruptured atherosclerotic plaques, characterized by fissures, hematoma and thrombus formation, causing occlusion of the vessel lumen. This causes acute ischemia often resulting in necrosis and loss of function of the affected organ. Type VI lesions are therefore associated with most of the morbidity and mortality caused by atherosclerosis.^{4,33}

Recently, Virmani et al. proposed some modifications to the AHA classification of 1995, since autopsy studies revealed a number of lesion types that are difficult to define by the present classification, especially the advanced atherosclerotic

lesions.³⁵ For example, the distinction between type IV and V lesions is often difficult, when a thin fibrous cap is present. Instead of type IV, or instable type V, the modification proposes to call it a 'thin fibrous cap atheroma'. Type VI lesions comprise ruptured plaques, with thrombus formation. However, plaques showing thrombus are often not ruptured, but only show plaque erosion. Therefore, the modification proposes three different terms for the former type VI lesion. First, it distinguishes the 'fibrous cap atheroma with erosion', which contains a thick fibrous cap with a luminal thrombus, but shows no communication of the thrombus with the necrotic core. Second, the 'thin fibrous cap atheroma with plaque rupture', in which the fibrous cap is ruptured and the luminal thrombus communicates with the underlying lipid core, and third, the 'calcific nodule with erosion' with an eruptive nodular calcification with overlying luminal thrombus.³⁵ These modifications should not be used as an alternative classification, but as a way to describe features of atherosclerotic lesions of a specific AHA type in more detail.

2.4 *Age and site specific progression*

In humans, the first atherosclerotic plaques develop at sites with adaptive intimal thickening. These sites include arterial branches, curvatures and bifurcations, sites at which alterations of blood flow occur, such as decreased shear stress and increased turbulence.⁴⁰ Also, in animal models of atherosclerosis, plaques develop at these specific sites.⁴¹⁻⁴³ The ApoE^{-/-} mouse (the most frequently used hyperlipidemic mouse model) shows a cranio-caudal atherosclerotic lesion progression, starting at the aortic arch and its main branchpoints and progressing to the femoral arteries.⁴¹ The lesions typically develop in low shear stress areas of the vasculature, i.e. the low flow side of branch points and the lesser curvature of the aortic arch.⁴¹ In humans, a detailed report regarding the progression of atherosclerosis throughout the arterial tree is still lacking. However, the first significant atherosclerotic plaques seem to develop in the coronary and carotid arteries, and also the abdominal aorta is prone to develop lesions, most probably because of its many branchpoints and its different composition compared to the thoracic aorta. The thoracic aorta diameter is larger than that of the abdominal aorta, and has a larger number of transmedial lamellar units.⁴⁴ The thoracic aorta also contains relatively more elastin and less collagen than the abdominal aorta, which is therefore stiffer and less compliant.⁴⁵

Fatty streak lesions (AHA type I and II) in the coronary arteries are already observed in 45% of children in the first 7 months of life.^{3,46} At the age of 1, these lesions disappear, to reappear more substantially in 53% of the individuals at puberty.^{2,46} Advanced atherosclerotic plaques (type III-VI), although not clinically symptomatic, are present from the third decade of life (34%). These plaques increase in number and can progress into clinically significant lesions during the following decades of life.^{2,46}

2.5 Remodeling

For a long time, the arterial system was considered to be a rigid tube that exhibited luminal narrowing in response to a growing plaque mass. However, in 1987, Glagov, and many others thereafter, reported that coronary arteries show compensatory enlargement with maintenance of the vessel lumen in response to plaque progression. It was stated that the capacity of the vessel wall to show compensatory enlargement was limited, and that when the limit of 40% stenosis has been reached, luminal narrowing would develop.⁴⁷ Now we know that compensatory enlargement, also termed outward remodeling, as well as constrictive remodeling can occur.⁴⁸⁻⁵⁰ The mode of remodeling (compensatory vs constrictive) varies strongly over the length of the same artery, between the different arteries, and between different individuals.^{48,51,52} Compensatory enlargement prevents, and constrictive remodeling accelerates narrowing of the lumen, inducing clinically significant stenoses.^{53,54} Interestingly, arterial cross-sections with a large atherosclerotic plaque and massive compensatory enlargement showed histological features of plaque instability, like more inflammation, large necrotic cores and thin fibrous caps,^{55,56} suggesting that a large lumen at angiography not always reflects a non- or less-diseased vessel.

Mechanisms of arterial remodeling in primary atherosclerosis are largely unknown, and most of the postulated mechanisms are derived from observations in balloon injured animals. In primary atherosclerosis, plaque growth narrows the lumen and shear stress changes. The endothelium reacts by producing vasodilators (for example NO), and compensatory enlargement develops.⁵⁷ When this mechanism does not suffice, inadequate compensatory enlargement will ensue.⁵⁸⁻⁶¹ In addition, it is reported that decreased blood flow causes arterial wall shrinkage (constrictive remodeling), but precise mechanisms are largely unknown.^{62,63}

On the other hand, compensatory enlargement is thought to result from involution of the support structure of the arterial wall.^{64,65} During atherogenesis, collagen, as well as elastin is degraded. This often results in an atrophic, elastolytic media and decreased collagen content in the adventitia. Matrix degrading substances, responsible for media degradation are MMP-3, -9, -12 and -13.⁶⁶ As a response, the artery is able to enlarge, often resulting in aneurysm formation.^{64,66,67}

Although compensatory and constrictive remodeling has been extensively reported in different animal models after balloon angioplasty, like the baboon^{68,69}, the rat, the rabbit⁷⁰ and the pig⁷¹, an animal model of *de novo* atherosclerosis that shows constrictive remodeling together with significant stenosis is still lacking. So far, the majority of animal models of primary atherosclerosis, only show compensatory enlargement, but fail to show significant stenosis or constrictive remodeling.^{63,70,72} ApoE-/- mice show compensatory remodeling, and even aneurysm formation in the thoracic, abdominal and common carotid artery.^{66,73} Interestingly, luminal narrowing, although not significant, was observed in the external carotid artery in 9 of 12 animals tested⁷³, indicating that the evaluation of constrictive remodeling in this mouse model warrants further investigation.

3. Animal models of atherosclerosis

Numerous animal species have been used to study the pathogenesis of atherosclerosis. Until the 1990s, research was restricted to relatively large animals, such as non-human primates^{42,74}, swine^{75,76}, rabbits^{77,78}, hamsters⁷⁹ and pigeons⁴³. Also, the balloon-injured rat was used, sometimes in combination with hypercholesterolemia, although those lesions rather reflect the process of neointimal hyperplasia than primary atherosclerosis.^{80,81} The large animal models present particular problems, like their non-reproducibility, expensive housing, and rather non-human like atherosclerotic lesions.

Since the 1990s, the mouse is evolving as an animal model for atherosclerosis. Since the development of transgene and knockout technology, mouse models have become very popular, since mice are relatively cheap, small, genetically homogeneous and can easily be used for studies of possible therapies that require large numbers of animals. Another advantage of the use of transgenic and knockout mouse models is the possibility of studying the effect of single gene mutations, for instance by cross-breeding several knockouts.

Before transgene technology, the only mouse model that was used in atherosclerosis research, was the C57Bl6 mouse.^{82,83} This mouse model exhibits some features of atherosclerosis when fed an atherogenic diet. Lesions of this mouse are small, and usually restricted to the root of the aorta, and associated with the cusps of the valves.^{82,83}

In 1992, the ApoE deficient mouse was developed, created by homologous recombination in embryonic stem cells.^{84,85} ApoE is one of the apolipoproteins that coats the surface and mediate the metabolism of lipoprotein particles. ApoE mediates the clearance of VLDL and LDL and chylomicrons from plasma. Consequently, mice deficient in ApoE exhibit high plasma cholesterol levels (predominantly VLDL and IDL) and spontaneously develop atherosclerotic lesions throughout the arterial tree from 10 wks of age.⁴¹ Upon feeding an atherogenic diet, lesion development is accelerated.⁴¹

In 1993, another atherosclerotic mouse model was developed, the LDL-receptor deficient mouse.⁸⁶ As in humans with this genetic defect (type III hypercholesterolemia), these mice exert high levels of total cholesterol, predominantly in the IDL and LDL fractions.⁸⁶ LDL-receptor deficient mice develop atherosclerosis upon atherogenic diet. Compared to ApoE^{-/-} mice, LDL-receptor deficient mice develop atherosclerosis more slowly. The first advanced atherosclerotic lesions are found at 8 months of age.²⁵⁷

In addition to these two basic hyperlipidemic mouse models, other variants have been developed. One of these mouse models is the ApoE*3Leiden mouse. The ApoE*3Leiden gene was originally discovered in a family exhibiting familial dysbetalipoproteinemia.⁸⁷ Compared to the normal ApoE gene, the ApoE*3Leiden gene contains a tandem duplication of codons 120-126 in the ApoE gene. The presence of a single allele for this mutation results in the development of familial dysbetalipoproteinemia in patients.^{88,89} The transgenic mouse model, carrying this human ApoE*3Leiden gene, exhibits high levels of cholesterol (predominantly VLDL

and IDL) and triglycerides.^{90,91} These mice develop initial and advanced atherosclerosis, but only after being fed an atherogenic diet.⁹² An important difference with the ApoE^{-/-} mouse is the fact that ApoE*3Leiden mice still carry their own functional ApoE gene. Therefore, it is postulated that their atherosclerotic lesions are still capable of reversed cholesterol transport.⁹⁰⁻⁹²

The hyperlipidemic mouse models as described above show many similarities with human atherosclerosis. First of all, they develop human-like advanced atherosclerotic lesions, containing a fibrous cap and a lipid core. Second, the cellular composition of their plaques resembles human atherosclerosis, since mouse atherosclerotic lesions contain macrophages, SMCs, T-lymphocytes, endothelial cells and fibroblasts. Third, as in humans, atherosclerotic lesions predominantly develop at aortic branchpoints and bifurcations. However, there are also major differences. Arterial geometry, flow- and shear- patterns structurally differ between mice and humans. Compared to human atherosclerotic plaques, mouse lesions show an increased cellularity, and an increased amount of macrophage derived foam cells. Ruptured atherosclerotic plaques, as well as plaque hemorrhage are not observed in any of the animal models described above. Consequently, complications of atherosclerosis like cerebral- and myocardial infarctions are very uncommon in these models. Therefore, extrapolation of mouse data to human coronary or peripheral atherosclerosis should be applied with caution.

This thesis

Although the 'response to injury' hypothesis is continuously being adapted to the newest insights in the pathogenesis of atherosclerosis, precise mechanisms responsible for lesion initiation and progression are still incompletely understood. Mechanisms are multiple, complex and the different mechanisms most likely form a network of events interrelating and influencing each other. Elucidation of these mechanisms in detail and their (inter)actions may provide new therapeutic targets for inhibiting initiation and progression of atherosclerosis, or for inhibiting the transformation of stable into unstable plaques.

This thesis focusses on two mechanisms that are considered to play a major role in the pathogenesis of atherosclerosis. The first mechanism is the balance between DNA-synthesis and apoptosis, and the second mechanism is the influence of chronic inflammation and the immune system. To provide the background for the experimental observations reported in this thesis, we will describe the two mechanisms below. Paragraph 4 focusses on the role of cell turnover (DNA-synthesis and apoptosis) during atherogenesis and paragraph 5 focusses on the role of inflammation, in particular the role of the CD40-CD40L system in atherosclerosis.

4. Cell Turnover

4.1 Introduction

DNA-synthesis (proliferation) and apoptosis are two important processes in embryonic development, normal growth and development and, when an organism has reached maturity, in cell repair processes. At that time, cell turnover occurs as a response to injury, cell death or mechanical deformation. The processes of DNA-synthesis and apoptosis are tightly regulated, and disturbances in the process can have disastrous effects. For example, congenital abnormalities are often the result of disturbances in DNA-synthesis and apoptosis. Furthermore, several diseases, like cancer and fibro-proliferative diseases, but also cardiovascular diseases, are characterized by an altered balance between DNA-synthesis and apoptosis.

4.2 DNA-synthesis

4.2.1 Cell cycle

To a great extent, the mechanism of cell division is comparable in all dividing cells, and has been conserved during evolution. The cell cycle is divided into five phases. It consists of the G₀ (gap₀), G₁- (gap₁), S- (DNA-synthesis), G₂- (gap₂) and the M-phase (mitosis and cytokinesis). G₀, G₁, S and G₂ are called the interphase. Quiescent cells are found in the G₀ phase and are in a state where mRNA and protein synthesis are minimal. When stimulated, cells enter the G₁ phase, in which the cell generates mRNAs and proteins that are necessary for DNA-synthesis. The S-phase is the period during which the genetic material is replicated, and G₂ the period during which the fidelity of DNA-replication is determined and errors are corrected. After the interphase, the cell proceeds to the M-phase, during which cells divide into two daughter cells (fig. 1.4).⁹³

4.2.2 Cell cycle regulators

The cell cycle is tightly regulated by the sequential formation, activation, and inactivation of a series of cell cycle regulatory proteins to minimize errors in the replication process. These proteins include a group of regulatory subunits called the cyclins, and a group of catalytic kinase subunits known as cyclin dependent kinases (cdk's). The enzymatic activity of the cdk is determined by the association with a cyclin. There are at least 7 cdk families, each of which associates with a distinct cyclin molecule. The resulting complexes have characteristic substrate specificities, and are expressed in different stages of the cell cycle, thereby driving the cell from one stage to another.

The cyclinB/cdc2 complex is the primary regulator of transition from G2 to the M phase. It is activated by a cdk activating kinase (CAK) and phosphatase (cdc25) that removes inhibitory phosphates. Activation of this complex results in chromosome condensation, nuclear membrane breakdown and spindle formation (fig. 1.4).

The regulation of the entry G1 to S is complex. Two cyclin/cdk complexes, cyclin D/cdk4 or -6, and cyclin E/cdk2, phosphorylate the retinoblastoma gene product (Rb), and alter the ability of Rb to bind with cellular proteins. One such protein is E2F, a transcription factor that heterodimerizes with other transcription products such as DP1, and activates several genes required for S progression, including thymidine kinase, DNA-polymerase α , c-myc, c-myb, and cdc2 (fig. 1.4).

The activity of cdk's is also regulated by cdk inhibitors. These low-molecular weight proteins either inhibit cdk activity broadly (p21^{Cip1/Waf1}, p27^{Kip1}, p57^{Kip2}) or selectively inhibit cyclin D/cdk4 or -6 complexes (p16^{INK4a}, p15^{INK4b}, p18). The best known member of this family is p21. It inhibits both cdk's and proliferating cell nuclear antigen (PCNA). P21 is induced by DNA damage through transcriptional activation by p53, and inhibits cell cycle progression at several points (fig. 1.4), and permits DNA repair to take place. P53 is seen as 'the guardian of the genome'. When DNA-damage takes place, p53 influences transcription to either halt cell cycle progression (by induction of p21), to permit repair of DNA, or, if the damage is too large, to induce apoptosis.⁹³

The behaviour of the cell in an organism is tightly regulated by its environment. Cell cycle processes are stimulated or inhibited by extracellular substances, like growth factors (PDGF, bFGF, EGF, TGF β , angiotensin II), hormones and cytokines (TNF α). After binding to their surface receptors, these substances induce several signalling pathways that convert extracellular signals into a specialized cell cycle action in the responding cell.⁹³

Fig. 1.4
The cell cycle: most important regulators

4.3 DNA-synthesis and vascular disease

4.3.1 DNA-synthesis and arterial injury

In 1973, Ross postulated in his 'response to injury' hypothesis that intimal changes during atherosclerosis and restenosis were the result of excessive DNA-synthesis of SMCs.^{28,94} This hypothesis was supported by observations in many animal models of arterial injury.

4.3.2 DNA-synthesis and animal models of arterial injury

One of the most widely used animal models of arterial injury, is balloon injury in the carotid artery of the rat. In this model, a catheter is inserted into the carotid artery, inflated and pulled back, thereby inducing endothelial denudation and medial damage.⁹⁵ Subsequently, 3 waves can be distinguished. The first response to balloon injury consists of medial SMC DNA-synthesis and begins ≈24 hours after the event. Four days thereafter, SMCs start migrating into the intima. Once arrived, SMCs replicate for weeks to months.⁹⁵⁻⁹⁸ In other models of arterial injury, like pigs,

rabbits and non-human primates, the same pattern was observed.⁹⁹ Animal models of vein graft arteriosclerosis, show a comparable SMC-rich neointima, and elevated levels of DNA-synthesis were also observed.^{100,101}

Factors inducing DNA synthesis *in vivo* after balloon angioplasty or graft procedures^{80,102}, are PDGF¹⁰³, FGF¹⁰⁴⁻¹⁰⁷, TGF β ¹⁰⁸, Angiotensin II¹⁰⁹, and ACE¹¹⁰ (table 1.1). Other molecules, gaining increasing interest in vascular disease are Gas6 (growth arrest specific gene 6) and its tyrosine kinase receptor Axl¹¹¹ (table 1.1). Gas6 was originally identified as a gene that was differentially expressed in serum-starved fibroblasts, and that acts as a survival factor.¹¹² However, Gas6 seems to affect cell turnover in a cell type dependent manner. For example, Gas6 is mitogenic in the presence of low serum for fibroblasts^{113,114} and Schwann cells¹¹⁵, but not for myeloid cells¹¹⁶, or endothelial cells¹¹⁷. Interestingly, increased expression of Gas6 and Axl was observed after arterial balloon injury in rats, and *in vitro* studies reveal that Gas6 acts as a chemoattractant for human and rat SMCs.^{118,119}

After binding of these growth factors to the receptor, an intracellular signalling pathway is elicited, thereby transducing signals into the nucleus to stimulate the cell cycle. Most of the growth factor receptors belong to the receptor tyrosine kinase family, with the MAP kinases ERK and JNK, c-fos and c-jun, as important downstream elements.¹²⁰ Expression or intervention studies involving (downstream) members of the receptor tyrosine kinase family in balloon injured animal models revealed a significant role for these molecules in SMC replication after arterial injury (table 1.1).¹²¹⁻¹²³ Furthermore, several transcription factors, important for DNA-synthesis, like NF κ B, AP1 and E2F are also activated after arterial injury (table 1.1).^{121,123-126}

Downstream, activation or inhibition of cell cycle regulators promoted or inhibited SMC DNA-synthesis. Cell cycle regulators that are known to be involved in neointima formation are p27, cdk2, cyclinA, p21, PCNA.¹²⁷⁻¹³² Cell cycle regulators are easy targets for the use of gene therapy.^{130,133,134} For example, adenovirus mediated expression of p21, and administration of antisense oligodeoxynucleotides for E2F successfully inhibited neo-intima formation in balloon injured rats, and in rabbit vein grafts (table 1.1).^{124,127,129,135}

4.3.3 DNA-synthesis in human neo-intima formation

In humans, acute arterial injury is induced by procedures aimed at restoration of adequate blood flow like PT(C)A, arterial or venous bypass graft surgery, and endarterectomies. Immediately after these procedures, blood flow is restored successfully. However, as a 'response to injury' a neointima develops, resulting in restenosis or re-narrowing of 20-60% of the arteries within 6 months, depending upon the type of revascularisation procedure and the artery involved.¹³⁶ The neointima is SMC rich, and resembles the lesions observed after experimental balloon angioplasty or graft procedures in animal models.¹³⁶

Human restenotic lesions show an increased expression of growth factors^{106,137,138}, and cell cycle regulatory proteins^{139,140}, that can induce SMC DNA-synthesis (table 1.1). In human restenotic lesions, DNA-synthesis is however rather

low, probably because lesions are investigated months after arterial injury. Therefore, an early proliferative response may have been missed.^{141,142} DNA-synthesis in these lesions was confined to the SMC.¹⁴¹ In humans, interventions with DNA-regulatory proteins have been performed.^{137,143} Although some were successful in inhibiting the process of neointimal formation, data on the level of DNA-synthesis in these lesions are scarce (table 1.1).¹⁴⁴ Inhibition of PDGF and bFGF after PTCA¹³⁷, as well as administration of oligonucleotides inhibiting E2F (E2F decoy) in human vein grafts were able to inhibit DNA-synthesis and thereby, restenosis or recurrent graft occlusion.¹⁴³

3.2.4 DNA-synthesis and primary atherosclerosis

As in restenosis, primary atherosclerosis results from an 'endothelial response to injury', followed by excessive DNA-synthesis. However, the pathogenesis of primary atherosclerosis differs compared to that of restenosis. Primary atherosclerotic lesions develop more slowly, and endothelial denudation is less rigorous. Furthermore, atherosclerotic lesions mainly consist of macrophage derived foam cells, and SMCs are present in a lower amount.¹³⁶

4.3.5 DNA-synthesis in animal models of primary atherosclerosis

Compared to balloon injured animals, DNA-synthesis in hypercholesterolemic animal models, such as rabbits and pigs, is lower, but still significantly elevated compared to the non-diseased arterial wall.¹⁴⁵⁻¹⁴⁷ In these animal models, DNA-synthesis was not confined to the SMC, but was predominantly observed in macrophage derived foam cells.^{145,147} In hypercholesterolemic rabbits, most DNA-synthesizing nuclei were present in the luminal layer, rich in macrophage derived foam cells, while no DNA-synthesizing cells could be observed in the lipid core region.^{145,147} Most DNA-synthesizing cells were found in early atherosclerotic lesions, and their number decreased in more advanced atherosclerotic lesions.¹⁴⁷

Factors known to induce or repress DNA-synthesis in the rat balloon injury model, like PDGF, TGF β , FGF, angiotensin II, receptor tyrosine kinase signalling molecules, transcription factors, and cell cycle proteins (p53, p21, p27, cdk2, cyclin A) were also expressed in atherosclerotic lesions (table 1.1).¹⁴⁷⁻¹⁵⁴ However, intervention studies with DNA-synthesis inhibiting agents are scarce, and mostly fail to reduce DNA-synthesis (table 1.1).

4.3.6 DNA-synthesis in human atherosclerosis

In human atherosclerotic lesions, DNA-synthesis is low (0-3.5%).^{141,142,155-159} This low level of DNA-synthesis might be expected in lesions that take many years to become clinically manifest. Most of the studies on DNA-synthesis during atherogenesis were performed on advanced atherosclerotic lesions. In these lesions, the cell types that exhibited DNA-synthesis were either the macrophage derived foam cell¹⁵⁵⁻¹⁵⁷ or the SMC.¹⁴¹ Interestingly, an *in vitro* study, using either fatty streak or fibrous plaque derived cells, showed that DNA-synthesis in fatty streak lesions was much higher¹⁶⁰, indicating an early proliferative response in atherosclerosis.

Table 1.1

In vivo expression and intervention studies of regulators of DNA-synthesis, with proven effects on DNA-synthesis levels, in vascular disease.

	Restenosis				Atherosclerosis				References
	Expression Animals	human	Intervention animals	human	Expression animals	human	Intervention animals	human	
<i>Growth factors</i>									
PDGF	+	+	+	+	+	+	NA	NA	103,106,137,138,181
FGF	+	+	+	NA	+	+	NA	NA	104-107,150
TGFβ	+	+	+	NA	+	+	NA	NA	108,138
Ang II	+	+	+	NA	+	+	NA	NA	109,110
ACE	+	+	+	NA	+	+	NA	NA	110
Gas6/Axl	+	?	NA	NA	NA	NA	NA	NA	113,119
<i>Signal transduction</i>									
MAPK: ERK/JNK	+	NA	+	NA	+	+	NA	NA	121-123,149
c-fos	+	NA	+	NA	NA	NA	NA	NA	121-123
c-jun	+	NA	+	NA	NA	NA	NA	NA	121-123
<i>Transcription factors</i>									
E2F	NA	NA	+	+	NA	NA	NA	NA	124,143
AP1	+	NA	NA	NA	NA	NA	NA	NA	121,123
NF-KB	+	NA	+	NA	+	+	NA	NA	125,126,151,152,162
<i>Cell cycle regulators</i>									
Cyclin A	+	+	NA	NA	NA	NA	NA	NA	127,139
Cyclin E	+	+	NA	NA	NA	+	NA	NA	139,163
Cdk2	+	+	+	NA	+	+	NA	NA	104,127,129,139,164
PCNA	+	+	+	NA	+	+	NA	NA	129,139
P21	+	NA	+	NA	NA	+	NA	NA	128,131,148
P27	+	NA	+	NA	+	+	NA	NA	127,131,132,163,164
P53	+	+	+	NA	+	+	+	NA	140,148,153,154,165,166

NA= not available

- + positive expression
- + positive result intervention study
- + DNA-synthesis promoting factor
- negative expression
- negative results intervention study
- DNA-synthesis inhibitor

The distribution of DNA-synthesizing cells in the atherosclerotic lesion is largely unknown, since most of the studies have been performed on atherectomy specimens, which lack decent lesion morphology. However, topographical analysis revealed that DNA-synthesis was tightly correlated with areas of structural repair and foam cell infiltration.^{155,157,158}

Also, in human atherosclerotic lesions enhanced expression of growth factors (PDGF, FGF, TGF β , AngII, ACE)^{106,110,138,161}, elements of the receptor tyrosine kinase pathway (dr. S. Heeneman, personal communication), transcription factors (NF κ B)¹⁶², and cell cycle regulatory molecules (cyclinE, cdk2, PCNA, p21, p27, p53)^{148,163,164} was observed. However, intervention studies with DNA-synthesis regulating agents in primary atherosclerosis are scarce, and data on its effects on DNA-synthesis are not available (table 1.1).

4.4 Apoptosis

The homeostasis of organisms requires a balance between the generation of new cells and cell death. Cells can die when they have sustained sufficient hypoxic, heat, oxidative, or ultraviolet damage that cannot be repaired. A cell can be killed if it becomes infected with a virus or another intracellular pathogen that destroys the cell or is recognized by the host's lymphocytes, which will kill the infected cell. In multicellular organisms, cell death is an essential component of normal development and cellular regulation. Abnormal regulation of cell death can lead to disorders such as cancer, lymphocyte depletion and atrophy or degeneration of tissues.¹⁶⁷

Two different forms of cell death can be distinguished: necrosis and apoptosis. Necrosis refers to a range of morphological changes resulting from the enzymatic digestion of the cell, the disruption of cellular membranes, and the denaturing of proteins that accompanies cell death. Necrosis is always accompanied by an inflammatory response (table 1.2).^{168,169}

Apoptosis, in contrast, is a programmed, highly selective mechanism of cell death, which can be completed in several hours. Morphologically, the cell shrinks and the chromatin becomes pyknotic and condensed into sharply delineated masses present at the edge of the nuclear envelope. Simultaneously with these changes, cell volume decreases, cytoplasmic organelles compact, cell density increases, and DNA is fragmented. Microvilli disappear, cytoplasmic blebs form at the cell surface, and portions of the cell bud to create apoptotic bodies. These apoptotic bodies may be phagocytized or remain free and undergo disruption by secondary necrosis. Apoptosis occurs without any signs of inflammation (table 1.2).^{168,169}

As described above, apoptosis is induced by a number of different stimuli, including DNA-damage, intracellular damage, growth factor withdrawal and cytokines. These stimuli operate in conjunction with other intrinsic factors that determine the cell's potential to undergo apoptosis. Cell type and basal conditions of the cell, including cell cycle and differentiation, markedly influence whether a cell will undergo apoptosis. Major regulators of apoptosis are indicated in table 1.3 and fig. 1.5.^{170,171}

Table 1.2
Features of apoptosis and necrosis

	Apoptosis	Necrosis
<i>Morphology</i>	Volume loss, chromatin	Increased volume, nucleus intact until
<i>Plasma membrane</i>	Remains intact	Early disruption
<i>DNA breakage</i>	Early, intranucleosomal pattern	Later, random DNA breakdown
<i>Mechanisms of DNA</i>	Gene activated, endonucleases	Random injury, ATP depleted
<i>Tissue reaction</i>	No inflammation, phagocytosis	Inflammatory response
<i>Calcium</i>	Moderate influx	Massive influx

Table 1.3
Some of the regulators of apoptosis

Apoptosis promoters	Apoptosis inhibitors
Fas	Bcl-2
TNF receptor	Bcl-x
Caspases	Angiotensin II
Bax	
Bad	
Bak	
Ceramide	
NO	
ANP	
Cholesterol oxides	
Angiotensin II	

Extracellular signals through the Fas receptor or TNF receptor, activation of TNF, IL1 and IFN γ , or intracellular signals related to p53 detection of irreparable DNA-damage lead to the activation of apoptotic genes. For example, Fas-induced apoptosis is mediated by the sphingomyelin pathway, which leads to ceramide accumulation, an important mediator of apoptosis (fig. 1.5). Other mediators, activated by the molecules described above, is the interleukin converting enzyme (ICE) family of proteases, also named the caspases. Activated caspases cleave specific proteins, activate the cell death pathway, and DNA-fragmentation occurs.^{170,171}

The bcl-2 protein is a membrane associated protein of the mitochondria, nuclear envelope and endoplasmatic reticulum. The bcl-2 family contains members that inhibit cell death (bcl-2 and bcl-x) and members that promote cell death (bax, bad, bak). Bcl-2 members associate as homodimers or heterodimers and the combined effects of the various dimers allow a fine level of control over cell turnover. When any of the death promoters exist as homo- or heterodimers, the cell dies by apoptosis. When a death promotor heterodimers with bcl-2 or bcl-x, cell death is prevented (fig. 1.5).^{170,171}

Fig. 1.5
Apoptosis: initiators and regulators
A short overview

4.5 Apoptosis and vascular disease

4.5.1 Apoptosis and arterial injury

In addition to DNA-synthesis, apoptosis is an important feature of the response to arterial injury. It counteracts the excessive proliferative response of SMC in media and neointima.

4.5.2 Apoptosis and animal models of arterial injury

Cell death in the intima was already postulated by Clowes et al in 1983, on the basis of the number of replicating cells and the actual cell number in the intima.⁸⁰ Animal models of arterial injury following balloon angioplasty have shown that apoptosis occurs within 30 minutes after injury in medial SMCs, and remains elevated until 30 days after injury in SMCs in the intima.^{172,173} Levels of apoptosis after balloon injury vary from 3-20%. Pro- and anti-apoptotic factors are also found in these models. For example, bcl-x, bcl-2, Fas, FasL, caspases, NO and p53 were present in neointimal lesions (table 1.4).¹⁷⁴⁻¹⁷⁹ Intervention studies in vivo are scarce, but show promising results (table 1.4). In this regard, administration of anti-sense oligonucleotides against the anti-apoptotic factor bcl-x induced selective apoptosis of neo-intimal cells and regression of neo-intimal thickening.¹⁷⁸ Furthermore, delivery of FasL into

the vessel wall induced apoptosis of Fas-expressing SMCs and inhibition of neointima formation.^{174,175}

4.5.3 Apoptosis and human neointima formation

In human restenotic lesions, and saphenous vein grafts, increased levels of apoptosis are also present.^{170,180-183} Interestingly, apoptotic cells were found in areas adjacent to areas of DNA-synthesis, indicating that apoptosis is topographically related with DNA-synthesis. Apoptosis was predominantly observed in SMCs. Data regarding the presence of apoptotic markers, or intervention studies in human restenosis are scarce (table 1.4).¹⁷⁶

4.5.4 Apoptosis and primary atherosclerosis

Cell death in human primary atherosclerotic lesions was already suggested by R. Virchow in 1858.²⁶ He stated that atherosclerotic lesions are formed by cell replication and then die. Since techniques have become available to visualize the apoptotic process, it is known that enhanced rates of apoptotic cell death are also found in human primary atherosclerotic lesions.^{172,180,184,185}

4.5.5 Apoptosis and animal models of primary atherosclerosis

In hypercholesterolemic animal models of atherosclerosis, such as the rabbit and the ApoE-/- mouse, elevated levels of apoptosis were also observed.^{145,186,187} Apoptotic nuclei were predominantly observed in macrophage derived foam cells, that were located at the medial site of the lesion.^{145,187}

Genes related to the apoptotic process (p53, bax, Fas, NO) were present in atherosclerotic lesions of these models (table 1.4).^{170,171,188} In vivo intervention studies revealed divergent results. Overexpression of Fas ligand in hypercholesterolemic rabbits resulted in accelerated atherosclerotic lesion formation.¹⁸⁹ On the contrary, the absence of p53 in ApoE-/- mice resulted in a decrease in lesion progression, but did not affect the level of apoptosis (table 1.4).¹⁵³

4.5.6 Apoptosis and human primary atherosclerosis

In human atherosclerotic lesions, apoptotic labeling indices of 1-40% have been reported and apoptotic nuclei are mostly confined to macrophage derived foam cells in the lipid core and in regions adjacent to the lipid core^{172,180,184} or SMCs in the fibrous cap^{6,190,191}.

Expression of genes involved in apoptosis (like Fas, FasL, bcl2, bax, caspases, NO) is enhanced in atherosclerotic lesions, and the expression pattern varies at the different stages of atherosclerosis (table 1.4).^{170,171,184,192-195} Bax, a pro-apoptotic protein and bcl-2, an anti-apoptotic protein are both present in human fatty streak lesions, while no TUNEL positivity (an indicator of apoptotic cells) was observed.¹⁹² During lesion progression, expression of the pro-apoptotic proteins bax and ICE increases, while expression of the anti-apoptotic protein bcl-2 is no longer observed. These phenomena are associated with an increased TUNEL-positivity.¹⁹²

Table 1.4

In vivo expression and intervention studies with proven effects on the level of apoptosis in vascular disease.

	Restenosis				Atherosclerosis						
	Expression animals	human	Intervention animals	human	Apoptosis	Expression animals	human	Intervention animals	human	Apoptosis	References
Fas (ligand)	+	NA	+	NA	+	+	+	+	NA	±	174,175,189
TNF receptor	NA	NA	NA	NA	+	NA	NA	NA	NA	+	NA
P53	+	NA	NA	NA	?	+	+	+	NA	?	177,195
Caspases	+	NA	NA	NA	+	NA	+	NA	NA	+	177,184,193
Bax	+	+	NA	NA	+	+	+	NA	NA	+	176,177,188
Bad	NA	NA	NA	NA	?	NA	NA	NA	NA	?	NA
Bak	NA	NA	NA	NA	?	NA	NA	NA	NA	?	NA
NO	+	NA	+	NA	+	+	+	NA	NA	+	170,171,179
Bcl-2	+	NA	+	NA	-	+	+	NA	NA	-	176,177,188,192,194
Bcl-x	+	NA	+	NA	-	NA	NA	NA	NA	?	177,178

+ positive expression

+ positive results intervention study

+ apoptosis promoting factor

NA= not available

- negative expression

- negative results intervention study

- apoptosis inhibiting factor

Intervention studies, directly targeting apoptotic genes in primary atherosclerosis in vivo are not available. Whether inhibition of apoptosis is beneficial or detrimental during atherogenesis is still under debate. On the one hand, stimulation of apoptosis might reduce the extent of atherosclerosis. Furthermore, apoptosis of macrophages and T-lymphocytes might reduce inflammatory activity, and abolish their production of collagen degrading substances, thereby strengthening the fibrous cap.^{170,171} On the other hand, enhanced rates of apoptosis contribute to lipid core expansion, and formation of an instable plaque configuration.^{6,170,171,190,191} Furthermore, apoptosis of SMCs and fibroblasts in the fibrous cap may not only induce thinning of the fibrous cap, but also inhibit their collagen production, making the fibrous cap rupture prone.^{6,7,170,171}

5. Atherosclerosis: an inflammatory disease

5.1 Inflammation

Inflammation is a reaction of the body that is characterized by the migration and extravasation of inflammatory cells from the blood into extravascular tissues, and is accompanied by activation of the immune system. Inflammation is frequently an expression of the host's attempt to localize and eliminate metabolically altered cells, foreign particles, or antigens. Under normal conditions, the inflammatory response eliminates the pathogenic insult and removes injured tissue components. This process accompanies either regeneration of the normal tissue architecture and return to physiologic function, or the formation of scar tissue to replace what cannot be repaired.^{196,197}

The inflammatory response starts with the release of specific inflammatory mediators such as chemokines, cytokines, factors of the complement system, growth factors and vasoactive substances at the site of injury. These substances are able to recruit and guide inflammatory cells such as leukocytes, T-lymphocytes, monocytes and macrophages to the site of injury, make them remove injured tissue and induce a wound healing response (fig. 1.6).^{196,197}

The inflammatory reaction in general consists of several phases. First, vasoactive substances such as histamine induce vasodilation and increased vascular permeability, thereby facilitating influx of inflammatory mediators and cells. Furthermore, the expression of leukocyte adhesion molecules such as P- and E-selectin, ICAM and VCAM is upregulated to allow increased rolling and adhesion of inflammatory cells to the endothelium, and finally diapedesis of inflammatory cells from the endothelium into the tissue (fig. 1.6). Subsequently, inflammatory cells migrate towards the site of injury by chemotaxis, elicited by chemokines (MCP-1, IL8), cytokines (IL1, TNF α) and components of the complement system (C5a). When inflammatory cells arrive at the site of injury, macrophages remove damaged tissue by phagocytosis and initiate a reparative response. Activated macrophages and T-lymphocytes induce the expression of growth factors such as PDGF, FGF and TGF β , that induce proliferation and migration of (myo)fibroblasts and SMCs. These are able to synthesize collagen and other extracellular matrix elements, required for the induction of tissue fibrosis and the formation of scar tissue (fig. 1.6).^{196,197}

There are two forms of inflammation that interrelate with another: acute and chronic inflammation. Acute inflammation comprises the initial response to injury and is characterized by the accumulation of fluid and plasma components in the affected tissue, stimulation of platelets and the presence of granulocytes. Chronic inflammation occurs as a result of ongoing injury. It is characterized by the presence of macrophages, T-lymphocytes and plasma cells, and a fibroproliferative response. Besides its positive effects, the inflammatory response is also potentially harmful to the body. For example, the acute inflammatory reaction for removal of a pathogen can result in high fever, diarrhea, dehydration and death. Chronic inflammatory reactions can result in severe tissue dysfunction. Diseases that are the result of chronic inflammation include liver cirrhosis, rheumatoid arthritis, glomerulosclerosis, pulmonary fibrosis and chronic pancreatitis.^{196,197}

Fig. 1.6
Leukocyte adhesion, diapedesis and migration during the early inflammatory response

5.2 Key regulator of inflammation: CD40-CD40L system

CD40 is a 50kD integral membrane protein of the tumor necrosis factor receptor (TNF-R) family of receptors that includes TNF-R1, TNF-R2, CD30, CD27, Fas and others, whose tissue distribution was originally thought to be restricted to B-cells, dendritic cells and basal epithelial cells.¹⁹⁸ The ligand of CD40, CD40L (CD154, gp39, TBAM), is a 39 kD member of the TNF family, and was originally thought to be restricted to activated T-lymphocytes.¹⁹⁹ Similar to the TNFR-TNF binding, CD40 forms a trimer that binds CD40L at the interface between two monomers. Each CD40 trimer is able to bind 1 CD40L molecule. After binding, the CD40 associated factor (CRAF) associates with the cytoplasmatic tail of CD40, and signalling is elicited (fig. 1.7).²⁰⁰ Precise signalling mechanisms still remain to be elucidated.

The function of CD40-CD40L interactions was originally described to occur between T- and B-lymphocytes, comprising Th-cell driven activation, proliferation and differentiation of B-lymphocytes, isotype switching, primary T-lymphocyte response and thymic selection.^{199,201}

In vivo importance of CD40-CD40L interactions, was found in patients suffering from the X-linked hyper-IgM syndrome. These patients show defective antibody production, since they lack normal B-cell proliferation and differentiation, and lack circulating IgG and IgA, due to an absent isotype switching. Their symptoms are due to a dysfunctional CD40L on activated T-lymphocytes, caused by a mutation in the extracellular domain of CD40L.²⁰² Also mice with genetic disruption of the CD40-CD40L system show a defective immune-response. For example, CD40L^{-/-} mice cannot elicit an IgE inflammatory response to parasites, and fail to elicit eosinophilia after ovalbumin induced lung injury.²⁰³⁻²⁰⁶

Fig. 1.7
Structure of CD40, CD40L and their interactions

Nowadays, it is known that, except in T- and B-lymphocytes, CD40 and CD40L are also expressed in other cell types, like dendritic cells, monocytes, macrophages, endothelial cells and fibroblasts.²⁰⁷⁻²¹⁰ Furthermore, it was found that CD40-CD40L interactions are involved in many inflammatory pathways, like leukocyte-adhesion and migration, induction of chemokines, cytokines and activation of fibroblasts.²⁰⁷⁻²¹⁵

CD40-CD40L interactions are involved in a wide range of chronic inflammatory diseases, like allergic encephalitis, collagen induced arthritis, multiple sclerosis, SLE, Crohn's disease and in acute and chronic graft versus host disease.^{202,216-218} Treatment with an anti-CD40L antibody has shown promising results in animal models of these diseases.^{216,219-222} Blocking of CD40 permitted long term acceptance of skin and cardiac allografts in mice^{219,220}, and administration of an anti-CD40L antibody prevented experimentally induced allergic encephalitis and collagen induced arthritis in mice.^{216,217}

5.3 Atherosclerosis and inflammation

Table 1.5
Expression of inflammatory mediators in primary atherosclerosis

Inflammatory mediators	References
OxLDL	231,232
Adhesion molecules	
P-selectin	233-235
E-selectin	234,235
ICAM	233,235
VCAM	236
Chemokines	
MCP-1	237
CCR2	62
IL-8	62
GRO- α	62
CXCR-2	62
Cytokines	
Gm-CSF	238
IL-1	239,240
TNF α	239,240
IL-6	241,242
IFN γ	7
IL-10	243,244
Proteases	
MMP-1	245-248
MMP-2	245-248
MMP-3	245-248
MMP-9	245-248
TIMP	248

In addition to the chronic inflammatory diseases described above, atherosclerosis is also considered to be a chronic inflammatory disease. The latest modifications of the 'response to injury' hypothesis state that besides endothelial damage, activation of the endothelium and the subsequent upregulation of inflammatory pathways and the immune system, play a major role in the pathogenesis of atherosclerosis (see paragraph 2.2).³³

Atherosclerotic lesions have long been considered to consist of an accumulation of lipids in the arterial wall, accompanied by SMC migration and proliferation. However, inflammatory cells and mediators are a major component of

Table 1.6

Inflammatory mediators in atherosclerosis: Genetic interventions in mouse models of atherosclerosis.

Inflammatory	Mouse model	Effects	References
Adhesion molecules			
P-selectin	P-sel- <i>-/-</i> /ApoE- <i>-/-</i>	↓ plaque area	249-252
E-selectin	E-sel- <i>-/-</i> /ApoE- <i>-/-</i>	↓ plaque area	249-251
ICAM	ICAM1- <i>-/-</i> /ApoE- <i>-/-</i>		249,250
VCAM	Lethal	↓ plaque area	
Chemokines			
MCP-1	MCP-1 ↑/ApoE- <i>-/-</i>	↑ plaque area, ↑ lipid core, ↑ lesion progression	253
	MCP-1- <i>-/-</i> /ApoE- <i>-/-</i>	↓ plaque area	254
CCR2	CCR-2- <i>-/-</i> /ApoE- <i>-/-</i>	↓ plaque area, ↓ lesion	255
IL-8	NA		
GRO-α	NA		
CXCR2	CXCR-2- <i>-/-</i> /LDLR- <i>-/-</i>	↓ plaque area, ↓ lipid core, ↓ SMC proliferation	62
Cytokines			
gm-CSF	Op- <i>-/-</i> /ApoE- <i>-/-</i>	↓↓↓ plaque area,	52,256
	Op- <i>-/-</i> /LDLR- <i>-/-</i>	↓↓↓ plaque area, ↓ lesion progression	257
IL-1	NA		
TNFα	NA		
IL-6	IL-6 ↑/ApoE- <i>-/-</i>	↑ plaque area	258
IFNγ	IFNγ- <i>-/-</i> /ApoE- <i>-/-</i>	↓ plaque area, ↑ collagen	259,260
IL-10	IL-10- <i>-/-</i> /ApoE- <i>-/-</i>	↑ plaque area, ↑ T-lymphocytes, ↓ collagen	261,262
	IL-10 ↑/ApoE- <i>-/-</i>	↓ plaque area	261,262
Proteases			
MMP-1	NA		
MMP-2	NA		
MMP-3	NA		
MMP-9	NA		
TIMP	TIMP-1 ↑/ ApoE- <i>-/-</i>	↓ plaque area, ↑ collagen, ↑ SMC	263

the atherosclerotic lesion. Fatty streak lesions already contain a significant amount of activated macrophages and T-lymphocytes.²²³ During lesion progression, the inflammatory cell content even increases.^{4,33,39} Furthermore, all subsets of inflammatory mediators are expressed in atherosclerotic lesions (table 1.5) and their importance in atherosclerosis is confirmed by the effects on plaque development, progression and phenotype of genetic intervention studies in hyperlipidemic mice (table 1.6), thereby stressing the important role of inflammation during atherogenesis. Clinically, systemic inflammatory parameters, such as C-reactive protein, IL-6, fibrinogen, neopterin, sCD40L, serum amyloid A and sVCAM are elevated in plasma of patients suffering from coronary or peripheral atherosclerosis²²⁴⁻²²⁷, while plasma levels of the anti-inflammatory factors, such as

TGF β and IL-10, tended to be decreased in these patients.^{224,227,228} Furthermore, antibodies against pathogens like chlamydia trachomatis and CMV are also present in plasma and atherosclerotic plaques of patients suffering from vascular disease.^{140,229,230}

Inflammatory reactions in atherosclerosis

Both modified LDL and oxLDL are able to induce the expression of several leukocyte adhesion molecules in vitro and in vivo. After activation of the endothelium, P- and E-selectin, ICAM-1 and VCAM-1 are upregulated, thereby inducing selective adhesion and transmigration of monocytes and T-lymphocytes into the intima (fig. 1.8).²⁶⁴⁻²⁶⁶ Subsequently, monocytes differentiate into tissue macrophages by stimulation with gm-CSF, start to ingest lipids, and become foam cells.²⁶⁷⁻²⁶⁹

Recent discoveries have provided insights into the process of lipid accumulation into macrophages, leading to foam cell formation. We now recognize a number of macrophage scavenger receptors that preferentially bind the atherogenic oxLDL.²⁷⁰ Internalization leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters, resulting in foam cell formation. Scavenger receptors (SR) are divided into 6 classes, of which SRAI and II, CD36 and CD68, are considered to be involved in atherogenesis.²⁷¹ Besides cholesterol influx, efflux also occurs in foam cells. A protein known to be important in cholesterol-efflux is the cholesterol efflux regulatory protein (ABC). Its importance in atherosclerosis is stressed by the observation that patients with mutations in the gene encoding this protein (ABC1 gene) develop premature atherosclerosis (Tangier disease).^{272,273}

Substances responsible for progression of atherosclerotic lesions, are chemokines and cytokines. Chemokines (like MCP-1 and IL-8) and cytokines (like IL-1, TNF α and IFN γ) are strong chemotactic agents and induce the expression of leukocyte adhesion molecules, thereby recruiting more macrophages into the lesion.²⁷⁴⁻²⁷⁶ Furthermore, chemokines and cytokines are able to activate intra-plaque macrophages and T-lymphocytes, thereby augmenting the inflammatory response.^{239,240,274-276}

Activated plaque macrophages and T-lymphocytes exert a spectrum of pro-atherogenic actions, including scavenging and oxidizing LDL, induction of the expression of leukocyte adhesion molecules, induction of other chemokines and cytokines, proteolytic enzymes, growth factors, apoptosis and procoagulant molecules like tissue factor (fig. 1.8).^{7,33}

Growth factors (TGF β , PDGF, FGF)^{31,277-279}, proteolytic enzymes (MMP-1,-2,-3 and 9)²⁴⁵⁻²⁴⁸ and their inhibitors (TIMP-1,-2,-3)²⁴⁸, that can be induced by inflammation, are important regulators of collagen turnover during atherosclerosis. Growth factors induce the migration and proliferation of SMCs and (myo)fibroblasts into the atherosclerotic lesion. In addition to proliferation and migration, growth factors (especially TGF β and PDGF^{63,280-282}) also induce SMCs/fibroblasts to synthesize extracellular matrix. For example, TGF β induces the expression of fibronectin, collagen types I, III, IV and V, as well as glycosaminoglycans, proteoglycans and elastic fiber proteins.²⁸³ IFN γ inhibits collagen synthesis,

Fig. 1.8

Inflammation during atherogenesis. The upper panel shows the inflammatory response and mediators during lesion initiation. The lower panel shows a role of these mediators in plaque progression.

members of the matrix-metalloproteinase family degrade collagen, and tissue inhibitors of matrix metalloproteinases (TIMPs) prevent degradation.

When inflammation precedes, the balance between macrophage accumulation, collagen synthesis and degradation becomes disturbed. Procoagulant factors like tissue factor are formed in excess, the fibrous cap weakens, the lipid core expands, and the atherosclerotic lesion can rupture, and progress into a clinically significant stage.⁷

5.4 CD40-CD40L system in atherosclerosis

Until now, a key regulator of the inflammatory events during atherogenesis has not been defined. However, as described above, CD40-CD40L interactions are known

to be a key modulator of many inflammatory and immune reactions. Therefore, a role for CD40-CD40L interactions in atherosclerosis is proposed.

Increasing evidence suggests a role for CD40-CD40L signaling in atherosclerosis. Immunohistochemistry and in situ hybridization on advanced human atherosclerotic plaques revealed the presence of CD40 and CD40L in plaque macrophages, endothelial cells, T-lymphocytes and SMCs. Both CD40L and CD40 were predominantly present at the shoulder region.²⁸⁴ Furthermore, sCD40L was present in patients suffering from unstable angina.²²⁵ Interestingly, hyperlipidemic mice, containing initial atherosclerotic lesions that were treated with an anti-CD40L antibody for 12 wks, exhibited a profound reduction in atherosclerotic lesion area.²⁸⁵

5.4.1 Possible effects of CD40-CD40L interactions in atherosclerosis

Leukocyte adhesion molecules

As described above, in table 1.6 and fig. 1.8, leukocyte adhesion molecules like P- and E-selectin, ICAM and VCAM are crucial for the development and progression of atherosclerosis.³³ Activation of the CD40-CD40L pathway in vitro stimulates the expression of E-selectin, VCAM and ICAM in endothelial cells and monocytes^{209,211,215} and the expression of ICAM and VCAM in fibroblasts.^{209,210} Interestingly, hyperlipidemic mice, expressing initial atherosclerotic lesions show a decreased expression of VCAM after anti-CD40L antibody treatment.²⁸⁵

Chemokines

Chemokines include more than 30 homologous small polypeptides, that all have a role in the recruitment of leukocytes, and the migration, growth and activation of multiple cell types in inflammatory states including atherosclerosis (table 1.5 and 1.6).²⁷⁴ Activation of the CD40-CD40L system seems crucial for the expression of chemokines. For example, in vitro cell-cell contact between macrophages and cells engineered to express CD40L induce macrophages to express MCP-1, MIP-1 α , MIP-1 β and RANTES.²⁰⁸ Furthermore, platelet CD40L can induce HUVEC cells to express both MCP-1 and IL-8, all chemokines known to be involved in atherosclerotic lesion progression and initiation (table 1.5 and 1.6).²⁸⁶

Cytokines

Cytokines that are involved in atherosclerotic lesion progression, are also expressed after stimulation of the CD40-CD40L pathway. In SMCs and endothelial cells, stimulation induces the pro-atherogenic cytokines IL-1 β , ICE, and IL-6, but not TNF α .^{209,211,212,214,284} However, in macrophages, CD40 ligation induces the cytokines IL-1 β , IL-6, IL-12 as well as TNF α .^{207,215,284} Furthermore, CD40 ligation on fibroblasts induces production of IL-6.²¹⁰

Matrix metalloproteinases

CD40 ligation on human endothelial cells, SMCs and macrophages induces the expression and release of MMP-1 and MMP-3 from all three cell types, as well as MMP-9 from endothelial and SMCs.^{287,288} Moreover, MMP-2, present in endothelial

cells and SMCs in its latent form, is released in its activated form after CD40 ligation.^{287,289} CD40 ligation induces the expression of these enzymes to a greater extent than maximally-effective concentrations of TNF α or IL-1 β , established agonists of MMPs.²⁸⁷

CD40-CD40L interactions and plaque rupture

CD40-CD40L interactions are also postulated to play an important role in the development of plaque rupture. Not only are CD40 and CD40L abundantly present in the shoulder region of rupture prone lesions²⁸⁴, but stimulation of their signaling pathway also results in the upregulation of the expression of MMP-1,-2,-3 and -9^{287,288} and the procoagulant tissue factor in vitro.^{288,290} Furthermore, both activated platelets and fresh thrombi express high levels of CD40L.²⁹¹ Patients suffering from unstable angina show elevated levels of degranulated platelets and sCD40L plasma levels.²²⁵ Therefore, CD40L-CD40 signaling may induce a procoagulant stage, a feature favoring the development of plaque rupture and thrombosis.²⁹²

6. Hypothesis

As discussed in the previous paragraphs, atherosclerosis is a multifactorial disease, and two of its main mechanisms, important in its pathogenesis, are thought to be changes in cell turnover (DNA-synthesis and apoptosis) and chronic inflammation.

It is known that both DNA-synthesis and apoptosis are present in atherosclerotic plaques, and it is postulated that enhanced levels of apoptosis contribute to the development of an unstable atherosclerotic lesion. Cells reported to exhibit DNA-synthesis and apoptosis are the macrophage and the SMC. However, levels of DNA-synthesis and apoptosis in the different stages of plaque progression, as well as their topographical distribution, and cell-phenotype are largely unknown, since most of the work on cell turnover is focussed on advanced atherosclerotic lesions, and/or has used atherectomy specimens. In these small samples of atheromatous tissue, plaque morphology has been disrupted, and the precise location of DNA-synthesizing and apoptotic cells is difficult to determine.

Our first working hypothesis is that the level, topographical distribution and phenotype of DNA-synthesizing and apoptotic cells change during atherogenesis. We hypothesized that DNA-synthesis is confined to the SMC, and that its level is higher in early lesions than in advanced atherosclerotic lesions. Furthermore, we hypothesize that apoptosis occurs in SMCs and macrophages, and that it is an important feature of advanced atherosclerotic lesions. Subsequently, we hypothesize that genetic absence of Gas6, known as a survival factor suppresses DNA-synthesis and promotes apoptosis, thereby resulting in smaller atherosclerotic lesions in ApoE-/- mice.

The second main mechanism, important during atherogenesis, is the upregulation of inflammatory pathways. The key regulator of inflammation during atherogenesis is not known, although increasing evidence suggests a role for the CD40-CD40L system. Our second working hypothesis was that the CD40-CD40L system is a key regulator of the inflammatory pathways during atherogenesis.

Inhibition of CD40-CD40L signaling will therefore inhibit lesion initiation and lesion progression.

7. This thesis

In this thesis, we first characterized plaque phenotype and the diet dependent progression of atherosclerosis in ApoE*3Leiden mice (chapter 2). Subsequently, the level, distribution-pattern and cell phenotypes of DNA-synthesis and apoptosis of all stages of atherosclerosis of mice and man are described in chapters 2 and 3. To further characterize ApoE*3Leiden and ApoE-/- mice, we investigated the degree of vascular remodeling during in different stages of atherosclerosis (chapter 4). Chapter 5 describes the effect of genetic absence of Gas6 on cell turnover and plaque phenotype in ApoE-/- mice.

This brings us to the second hypothesis. Chapters 6 and 7 focus on the CD40-CD40L system. In chapter 6, the role of complete genetic disruption of CD40L on plaque progression and phenotype is described, while chapter 7 focusses on the effects of early and delayed anti-CD40L antibody treatment. In chapter 8, the findings of these studies and future directions are discussed.

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Chapter 2

Atherosclerosis in ApoE*3Leiden transgenic mice: from proliferative to atheromatous stage

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Abstract

This study documents (1) the progression of atherosclerosis along the entire arterial tree in ApoE*3Leiden mice after 1, 4, 6, 9, and 12 months of a high-fat/high-cholesterol (HFC) diet and (2) the amount and phenotype of DNA-synthesizing and apoptotic cells in different lesion types after 6 months of HFC diet. Diet duration was correlated with a craniocaudal progression of lesion development and with an increase in severity of the lesion. Typically, the lesions contained smooth muscle cells, macrophages, and T lymphocytes and were covered by an intact endothelium. Whereas DNA synthesis (BrdU uptake) was predominantly elevated in type II lesions ($8.6\pm0.8\%$ versus $1.0\pm0.2\%$ in the non-diseased arterial wall; $p<0.05$), apoptosis was found primarily in advanced lesions (type IV, $1.3\pm0.1\%$ and type V, $1.2\pm0.2\%$ versus $0.04\pm0.04\%$ in the non-diseased arterial wall; $p<0.05$). Cell phenotyping revealed that the majority of DNA synthesis and apoptosis was confined to the macrophage derived foam cell ($68.6\pm3.0\%$ and $82.2\pm4.6\%$, respectively).

This study shows that in ApoE*3Leiden mice, duration of an HFC diet is associated with (1) a craniocaudal progression of lesion development and (2) an increased complexity of atherosclerotic lesions. Furthermore, DNA synthesis is predominant in early lesions, whereas apoptosis is present mainly in more advanced lesions. Both parameters of cell turnover are confined primarily to the macrophage derived foam cell.

Introduction

One of the mutant apoE genes that is associated with familial dysbetalipoproteinemia in humans is the ApoE*3Leiden gene.^{1,2} To evaluate the phenotype in more detail, this gene has been introduced in mice, the ApoE*3Leiden mouse.^{3,4} The data published so far show that ApoE*3Leiden mice exhibit severe hypercholesterolemia when they are fed a high-fat/high-cholesterol (HFC) diet. However, atherosclerosis in these mice has been described only after relatively short periods of cholesterol feeding.^{3,5,6} To date, no detailed description of lesion morphology including data on cell turnover is available. Also, data on the progression of lesions along the entire arterial tree and long-term observations on the progression of plaque extent and complexity are lacking. This study was initiated to provide these data.

Methods

Mice

Transgenic mice expressing the human ApoE*3Leiden gene were generated and bred with C57Bl/6J females as described earlier.^{3,4} For the present study, 33 male and 27 female mice of the F10 generation were used.

Diet

Before entry into the study, animals were kept on standard mouse chow (SRM-A, Hope Farms). At the age of 37 ± 0.6 weeks (mean $\pm$ SEM), the animals were put on a diet containing 15% cacao butter, 0.5% cholate, 1% cholesterol, 40.5% sucrose, 10% corn starch, 1% corn oil, and 4.7% cellulose (Hope Farms)⁷ for 1 (n=4), 4 (n=9), 6 (n=12), 9 (n=12), and 12 (n=12) months. Control ApoE*3Leiden (n=6) and C57Bl/6J (n=6) mice received standard mouse chow for 9 months. To label DNA-synthesizing cells, 6 transgenic animals of the 6-month diet group received 5'-bromo-2'-deoxyuridine (BrdU, Serva; infusion rate, $13 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) by an osmotic minipump (Alzet 2001, Alza Corp) for the 7 days before they were euthanized. Pumps were placed subcutaneously between the shoulder blades under ether anesthesia. All mice were housed under standard conditions. The study was approved by the institutional committee for the welfare of laboratory animals of the University of Maastricht.

Tissue Handling

After completion of the diet according to protocol, each mouse was anesthetized with xylazine 0.0025 ml/g and ketamine 0.001 ml/g ip. Blood (0.5 to 1 ml) was collected from the caval vein for the assessment of lipid profile, and the arterial tree was perfused with 0.9% NaCl (3 minutes) and 10% phosphate-buffered formalin (pH 7.4, 3 minutes), both containing 20% nitroglycerin, through a catheter into the left ventricular apex. Subsequently, the complete arterial tree was taken out and fixed overnight in 10% phosphate-buffered formalin. All arterial segments indicated in fig. 1 were processed and embedded in paraffin, and $4 \mu\text{m}$ sections were stained with hematoxylin and eosin. Atherosclerotic lesions were classified according to the

guidelines given by the American Heart Association (AHA)⁸ by 1 investigator (E.L.), who was blinded to presence or absence as well as duration of diet and to genotype.

Assessment of Lipid Profile

Total serum cholesterol and triglycerides (but not free glycerol) were measured as described previously.³

Immunohistochemistry

Study 1: Diet-Dependent Lesion Progression

Because of its size and because all lesion types also developed in the aortic arch, we concentrated the immunohistochemical analyses on this segment. Parallel sections of the aortic arch were immunolabeled with either ED-1⁹ for the detection of macrophage derived foam cells, CD-3 (CD-3, polyclonal, 1:200, Dako A0452) for the detection of T-lymphocytes, α -smooth muscle actin (monoclonal, 1:500, FITC-labeled, Sigma Chemical Co) or desmin (monoclonal, 1:100, Organon MDEII, Eurodiagnostics) as smooth muscle cell markers, or factor VIII (polyclonal, 1:2000, Dako A0082) for the detection of endothelial cells. Immunostaining was performed according to conventional methods with biotin- or FITC- (α -smooth muscle actin) labeled secondary antibodies and 3,3'-diaminobenzidine (DAB) as a chromogen. Sections were counterstained with hematoxylin and mounted with coverslips. In all immunohistochemistry, sections treated without the respective primary antibody served as controls.

Study 2: Cell Turnover in Atherosclerotic Lesions

BrdU Staining.

After dehydration, the sections were incubated for 2 hours in 3% EDTA (Merck) at pH 7.2 to block nonspecific staining of calcium-containing vesicles.¹⁰ BrdU staining was performed as described previously¹¹, with DAB as the chromogen.

TUNEL Staining

Parallel sections were used for the TUNEL staining. After dehydration, sections were incubated with 3% citric acid to inhibit aspecific binding of the nucleotides to calcium-containing vesicles. TUNEL staining was performed as described previously.¹⁰ Amino-3-9-ethylcarbazole (AEC) was used as chromogen. Sections were counterstained with hematoxylin and mounted with coverslips. Negative controls included omission of TdT from the labeling mixture. Tonsils were used as a positive control.

Cell Phenotypes

Parallel sections of atherosclerotic lesions of the 6 APOE*3-Leiden mice were immunolabeled with ED-1, α -smooth muscle actin, CD-3, and factor VIII as described above.

Cell Counting

Tissue sections were investigated by light microscopy at *400 magnification with a standard field size. All nuclei of the respective lesion type were counted. Cells containing dark nuclear BrdU staining were considered to be DNA-synthesizing cells. Apoptotic nuclei were defined as TUNEL-positive nuclei in cells that showed morphological features of apoptotic cell death (cell shrinkage, aggregation of chromatin into dense masses, cell fragmentation).¹² The labeling index, defined as the total number of positive cells divided by the total number of cells, was calculated for the entire atherosclerotic lesion, including the underlying media. This was performed separately for the BrdU- and TUNEL-stained sections. Labeling indices were also determined in different sites of the lesion: the underlying media, the endothelial coverage, the shoulder region, the lipid core, and the fibrous cap. Furthermore, the distribution of the different immunophenotypes of BrdU- or TUNEL-positive cells was quantified. The immunophenotype of 89.1±1.4% of the DNA-synthesizing cells and 97.3±2.2% of the apoptotic cells could be determined by the above-described panel of antibodies (CD3, ASMA, FVIII, ED-1). All measurements were performed by 1 investigator (E.L.). Intraobserver variation was <10%.

Statistical Analysis

Study 1: Diet-Dependent Lesion Progression

Data are expressed as mean±SEM or as median values (AHA classification). All experimental groups were compared with the ApoE*3Leiden mice on normal chow. Atherosclerotic lesions of male and female mice in the different diet groups were compared by a non-parametric Mann-Whitney U test. Because no significance was found, atherosclerotic lesions of male and female mice were pooled. Cholesterol, triglyceride, and apoE levels were analyzed by the Kruskal-Wallis test, and Student's t-test was used for comparisons between the groups.

Study 2: Cell Turnover in Atherosclerotic Lesions

Data are expressed as mean±SEM. To test whether lesions of the same type in different mice and lesions of the same type at different sites of the arterial tree were comparable, a 1-way ANOVA was performed on both parameters.

This was performed for both BrdU- and TUNEL-stained sections. Because no significant difference was found, lesions of the different mice and at the different sites of the arterial tree were assumed to be comparable. Subsequently, a Mann-Whitney U test was used for comparisons between the lesion types. In all tests, the level of statistical significance was assumed to be at $p < 0.05$.

Results

Study 1: Diet-Dependent Lesion Progression

Cholesterol, Triglyceride, and ApoE Levels

In the ApoE*3Leiden mice on the HFC diet, all values except triglyceride levels after 1 month of HFC diet were significantly higher than in both control groups ($p<0.05$) (table 2.1). Human apoE plasma levels were elevated in all transgenic animals, confirming the expression of the ApoE*3Leiden gene.

Table 1
Cholesterol, Triglyceride and human ApoE levels in ApoE*3Leiden mice and C57Bl6 mice.

	Cholesterol (mmol/l)	Triglycerides (mmol/l)	Human ApoE (g/l)
ApoE*3, 1m HFC (n=4)	18.4 ± 1.4*	0.4 ± 0.1	0.4 ± 0.06*
ApoE*3, 6m HFC (n=12)	38.5 ± 5.3*	2.3 ± 0.2*	0.9 ± 0.11*
ApoE*3, 9m HFC (n=12)	43.8 ± 5.8*	2.0 ± 0.3*	1.0 ± 0.10*
ApoE*3, 12m HFC (n=12)	30.6 ± 3.9*	2.4 ± 0.3*	0.7 ± 0.08*
ApoE*3, 9m NC (n=6)	1.3 ± 0.03*	0.2 ± 0.05	0.1 ± 0.06
C57Bl/6J, 9m NC (n=6)	0.8 ± 0.11*	0.1 ± 0.04	0.0 ± 0.00

Values are mean±SEM. ApoE*3 indicates ApoE*3-Leiden transgenic mice; C57Bl/6J indicates C57Bl6J (wildtype) mice; m, months; HFC, high fat cholesterol diet; NC, normal chow. $p<0.05$ vs ApoE*3Leiden mice on normal chow.

Atherosclerotic Lesions in ApoE*3Leiden Transgenic Mice

Atherosclerotic lesions developed in the aorta and large vessels in all ApoE*3Leiden mice on an HFC diet (fig. 2.1). Lesions developed in the proximal coronary arteries (fig. 2.2a), the aortic root, the aortic arch and its main branch points (fig. 2.2b), the thoracic aorta, the abdominal aorta, the renal artery branch points (fig. 2.2c), the abdominal aorta bifurcation, and the iliac artery bifurcations. Almost all lesion types were observed: initial lesions with isolated macrophage foam cells (type I), fatty streaks with mainly intracellular lipid accumulation (type II, fig. 2.3a), and intermediate lesions with type II changes and small extracellular lipid pools (type III). Also, more advanced lesions could be detected, such as an atheroma with type II changes and a core of extracellular lipid (type IV, figs. 2.2b and 2.3d) as well as fibroatheromata with a lipid core and a fibrotic layer (figs. 2.2a and 2.2c), or with multiple lipid cores and fibrotic layers (type Va), or mainly calcific (type Vb, figs. 2.2d and 2.3e) or fibrotic (type Vc) fibroatheromata. Type VI lesions with plaque rupture, thrombus formation, and hemorrhage were not observed.

In the ApoE*3Leiden mice on normal chow, only initial type I and II lesions were observed. Wild-type mice on normal chow did not develop atherosclerosis.

Immunophenotype

Macrophages were not present in the normal media but were found in the media and intima of all lesion types (fig. 2.3a). In advanced lesion types, lipid-filled macrophages were present primarily in the shoulder region of the lesion. T-lymphocytes were present primarily in advanced lesion types (IV to Vb), especially in the shoulder region and fibrous cap (fig. 2.3b).

Fig. 2.1
Progression of atherosclerosis along the entire arterial tree after 4 and 12 months of HFC diet in ApoE*3Leiden mice.

Both the media of non-diseased vessel segments and the non-diseased media below an atherosclerotic lesion showed no desmin-positive vascular smooth muscle cells (VSMCs) (fig. 2.3c and 2.3d). However, desmin-positive VSMCs were present in the intima and fibrous cap of advanced lesions (fig. 2.3d). All atherosclerotic lesions contained α -smooth muscle actin positive VSMCs, both in the non-diseased media below the lesion and in the lesion itself (fig. 2.3e). They were most numerous in the fibrous cap of advanced lesion types. The endothelial coverage of the lesions remained intact, even in very advanced lesions (fig. 2.3f).

Effect of Diet Duration on Lesion Progression

The variability in the development of atherosclerotic lesions between mice in 1 diet group was remarkably low, and diet duration seemed to predict lesion type and site (data not shown). After 1 month of HFC diet, initial type II lesions were observed only in the aortic root, the aortic arch and its main branches, the carotid artery bifurcations, and the thoracic aorta. Below the diaphragm, no lesions were observed.

After 4 months of HFC diet, initial type II lesions were present in the coronary arteries. Advanced atherosclerotic lesions (type IV) with a core of extracellular lipid were present in the aortic root, the aortic arch and its main branchpoints, and the right carotid artery bifurcation. Below the diaphragm, only initial lesions (type I and II) with foam cells in the subendothelium were observed (fig. 2.1).

Fig. 2.2

Photomicrographs of haematoxylin and eosin stained sections. A, Ascending aorta (AA); Heart (H); and coronary artery (CA). B, Ascending (AA) and descending (DA) aorta; brachiocephalic trunk (BCT); left common carotid artery (LCCA); and left subclavian artery (LSA). C, Abdominal aorta (AbA); renal artery (RA). D, Calcified core (arrow); lumen (L) of left common carotid artery; media (M); and intima (I).

After 6 months of HFC, advanced type IV lesions had developed in the coronary arteries (fig. 2.2a). Furthermore, type V lesions with a fibrous cap and a lipid core were present in the aortic arch and its main branches (fig. 2.2b). Type IV lesions had also developed in the abdominal aorta and renal artery branch points.

After a diet period of 9 months, severe calcification of the atherosclerotic lesions (type Vb) was observed in the aortic arch and its main branch points (fig. 2.2d). Type V lesions were present in the right carotid artery bifurcation. Type IV lesions had developed in the thoracic aorta, in the abdominal aorta, and at the renal artery branch points. However, in the abdominal aorta and the iliac artery bifurcations, only type II lesions could be observed.

After 12 months of HFC diet, lesions in the aortic arch showed calcification (type Vb), and type V lesions were observed at both the right and left carotid artery bifurcations. Moreover, 5 of 12 mice showed calcifications in their coronary arteries. Furthermore, at the abdominal aorta bifurcation and the iliac artery bifurcations, advanced lesions (type IV) had developed (fig. 2.1).

Fig. 2.3
Immunohistochemistry of atherosclerotic lesions in ApoE*3Leiden mice with different antibodies. A, ED-1; B, CD3; C, desmin; D, desmin; E, ASMA; F, factor VIII. L indicates lumen.

Fig. 2.4 A-C
Photomicrographs showing subsequent immunolabeling of a type II lesion (BrdU staining, ED-1 and ASMA).

Fig. 2.5 A-C
Photomicrographs showing subsequent immunolabeling of a type IV lesion (TUNEL, ED-1 and ASMA).

Study 2: Cell Turnover in Atherosclerotic Lesions

In total, 135 lesions were investigated throughout the arterial tree of the 6 ApoE*3Leiden mice fed an HFC diet for 6 months. BrdU- and TUNEL-positive nuclei were counted in all lesion types except in type I lesions, because the total number of nuclei in this lesion type was too small. Thirty non-diseased arterial segments served as controls.

DNA Synthesis

In all lesion types, DNA synthesis was significantly elevated compared with the nondiseased arterial wall (fig. 2.4). The highest BrdU labeling was found in type II (fatty streak) lesions ($8.6\pm0.8\%$ versus $1.0\pm0.2\%$ in the non-diseased arterial wall; $p<0.05$) (fig. 2.4a). With progression of severity of the lesion, DNA synthesis decreased to $5.8\pm0.7\%$ in complex type V lesions ($p<0.05$ versus type II).

Not only the level but also the site of DNA synthesis changed with progression of the lesion (fig. 2.4d). In type II lesions, most DNA-synthesizing cells were found in the media (77.9% of all DNA-synthesizing cells), whereas in type III, IV, and V lesions, only a small amount of DNA-synthesizing cells was found in the media (17.1%, 14.1%, and 12.1%, respectively; <0.05). With the development of a

shoulder region in type III and IV lesions, DNA synthesis shifted to that region (32.9% and 41.0% of all DNA-synthesizing cells, respectively) as well as to the endothelial cells covering the lesion (30.0% and 32.1%, respectively). The percentage of DNA synthesis of endothelial cells covering type III and IV lesions, was significantly increased compared with both type II lesions and the non-diseased arterial wall ($p < 0.05$). When atherosclerosis had progressed to advanced type V lesions with a fibrous cap and a lipid core, DNA-synthesizing cells were located in the shoulder region (36.2%) and fibrous cap (29.3%), whereas the fraction of DNA-synthesizing cells of endothelial cells covering the fibrous cap showed a significant decrease to 12.1% compared with type IV lesions ($p < 0.05$). DNA synthesis in the lipid core of type IV and V lesions was low (12.8% and 12.1% of all DNA-synthesizing cells, respectively).

Subsequent immunolabeling (fig. 2.4) showed that $68.6 \pm 3.0\%$ of the DNA-synthesizing cells were macrophage derived foam cells, $14.5 \pm 2.5\%$ VSMCs, $1.3 \pm 0.3\%$ T-lymphocytes, and $5.3 \pm 1.2\%$ endothelial cells of the endothelial coverage. In all lesion types, the majority of DNA-synthesizing cells were macrophage derived foam cells, but the ratio of macrophage derived foam cells to VSMCs decreased with lesion development. In early type II lesions, this ratio was 76.9, whereas in complex type V lesions, this ratio had decreased to 3.7 ($p < 0.05$).

Apoptosis

The distribution of apoptotic nuclei in the different lesion types differed from the distribution of DNA-synthesizing nuclei (fig. 2.5). In type II and III lesions, the apoptotic labeling index was not different from the apoptotic labeling index in the non-diseased arterial wall. The percentage of apoptotic nuclei was elevated only in type IV and V lesions ($1.3 \pm 0.1\%$ in type IV and $1.2 \pm 0.2\%$ in type V versus $0.04 \pm 0.04\%$ in the non-diseased arterial wall). The majority of apoptotic nuclei was located in the lipid core of these type IV and V lesions (81.9% and 79.5% of all apoptotic cells, respectively). Furthermore, only low levels of apoptosis were found in the fibrous cap (9.0% of all apoptotic cells).

Cell phenotyping revealed that the majority of apoptosis was confined to the macrophage-derived foam cells ($82.2 \pm 4.6\%$) (fig. 2.5). Apoptosis in other cell types was rarely observed.

Fig. 2.4 D-E (left)

D, Level and distribution of DNA-synthesizing cells in atherosclerotic lesion types. Ec indicates endothelial coverage; fcr, foam cell-rich area; sh, shoulder region; lc, lipid core; and fc, fibrous cap. * $p < 0.05$ vs non-diseased (ND), # $p < 0.05$ vs type II. E, Phenotypical distribution of DNA-synthesizing cells in atherosclerotic lesion types. MF indicates macrophage, EC, endothelial cell; and NC, not classified.

Fig. 2.5 D-E (right)

D, Level and distribution of apoptotic cells in atherosclerotic lesion types. * $p < 0.05$ vs ND, # $p < 0.05$ vs type II, & $p < 0.05$ vs type III. E, Phenotypical distribution of apoptotic cells in atherosclerotic lesion types. Abbreviations as in fig. 2.4.

Discussion

One of the most important steps in atherogenesis is the transition of a fatty streak into an atherosclerotic plaque. In the present study, it was demonstrated that fatty streaks were characterized by DNA synthesis with no detectable apoptosis. These lesions progress toward atherosclerotic plaques, which were characterized by the presence of both DNA synthesis and apoptosis. This finding is comparable to the classic studies of Virchow, who described atherosclerosis as a formative process, starting with "proliferation" and terminating with "fatty degeneration."¹³

So far, few human data on DNA synthesis during the entire process of atherogenesis are available.¹⁴ Orekhov et al¹⁴ report peak levels of DNA synthesis in lipid-rich lesions, whereas DNA synthesis in fibrous lesions is much lower. The majority of data on DNA synthesis in human atherosclerosis are confined to advanced lesions only. They show low levels of DNA synthesis (0% to 2%),¹⁵⁻¹⁸ but this level is significantly higher than in the non-diseased arterial wall.^{14,15,18,19} One study on DNA synthesis in early fatty-streak lesions also reports low values of DNA synthesis (0% to 2%).²⁰ DNA synthesis in these human lesions is confined primarily to either the macrophage-derived foam cell^{15,17,18} or the VSMC.¹⁶ As in human *in vitro* studies²¹ and rabbit models²²⁻²⁴, the ApoE*3Leiden mouse exhibits a DNA-synthesis peak in early lesion types. DNA synthesis in advanced lesion types is low, which is in accordance with the low levels of DNA synthesis found in advanced human atheromata.¹⁵⁻¹⁸ Interestingly, we observed 2 kinds of type II lesions. The early type II lesion contained macrophage derived foam cells that were located only in the media, whereas the late type II lesion contained macrophage-derived foam cells in the intima. We defined them as type IIa and IIb, respectively. In the early type IIa lesions, DNA synthesis was significantly elevated compared with the late type IIb lesions ($10.3 \pm 0.8\%$ in type IIa versus $6.0 \pm 1.3\%$ in type IIb). In both lesion types, the majority of DNA-synthesizing cells were macrophage-derived foam cells (91.1% and 94.2%, respectively). The fact that the intima of a non-diseased vessel in the mouse consists of only a very small subendothelial layer may explain our observation that early lesions contain medial macrophage-derived foam cells, whereas in advanced lesions, these macrophages are present in the intima. Thus, it seems that the first migrating macrophage derived foam cells are transported from the blood into the most luminal layer of the media. With the accumulation of increased numbers of macrophage derived foam cells (type IIb through Vb), a neointima develops.

Enhanced rates of apoptotic cell death have also been observed in advanced human atherosclerosis.²⁵⁻²⁸ In a recent study, we found that fatty streak lesions are characterized by very low levels of apoptosis, whereas advanced atherosclerotic lesions showed remarkable levels of cell death.²⁹ In human atheromata, the apoptotic labeling index varies from 0% to 40%, and apoptotic nuclei are present primarily in the lipid core and regions adjacent to the lipid core, but also in the fibrous cap.^{25,27,28} The majority of apoptotic nuclei have been found in macrophages^{25,27} and T- lymphocytes.²⁶ Also, in ApoE*3Leiden mice, apoptosis was confined to the advanced stages, albeit at fairly low levels. Moreover, apoptosis in the fibrous cap was a rare phenomenon. Whether this phenomenon can be an

explanation for the apparent plaque stability observed in these mice remains to be investigated.

One of the major differences of atherosclerosis in the ApoE*3Leiden mouse with human atherosclerosis is the absence of plaque rupture, thrombus formation, and/or hemorrhage in the atherosclerotic plaque.³⁰ We have no explanation for the apparent stability of the atherosclerotic plaques in the ApoE*3Leiden mouse. One of the possibilities is that the morphologically intact endothelial layer that covers all lesion types in the ApoE*3Leiden mouse prevents lesions from rupturing. Another explanation is the low level of apoptosis in the fibrous cap in these mice.

The data presented here may have clinical implications. It is known that treatment with HMG-CoA reductase inhibitors can slow the progression of atherosclerosis.³¹ One of the mechanisms that have been described is the inhibition of DNA synthesis in both VSMCs and macrophages.^{32,33} Our data imply that these agents may be most successful in slowing the progression of atherosclerosis when they are applied early in the disease process, when DNA synthesis is mostly elevated. Conversely, it may be more interesting to look at agents that modulate apoptosis. Because apoptosis is a late event in atherogenesis, inhibition of apoptosis may both slow the progression of atherosclerosis and be effective in preventing the conversion from stable into unstable atherosclerotic lesions.

In the present article, we demonstrate that ApoE*3Leiden mice show a highly reproducible diet- and time-dependent cranio-caudal progression of atherosclerosis extent and plaque complexity. We found a peak in DNA synthesis in early lesions and a peak in apoptosis in late lesions, which are both confined to macrophage derived foam cells. Diet dependency, predilection site, plaque composition, and cell turnover make the ApoE*3Leiden mouse a suitable model to study different aspects of early atherogenesis and the evaluation of pharmacological and non-pharmacological interventions.

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Chapter 3

Biphasic pattern of cell turnover characterizes the progression from fatty streaks to ruptured human atherosclerotic plaques

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Abstract

Objective: To study the amount and phenotype of DNA-synthesizing and apoptotic cells during atherogenesis.

Methods: Atherosclerotic lesions (n=76) obtained at autopsy (N=6) or vascular surgery (N=8) were classified (type I-VI; American Heart Association (AHA) classification), immunolabeled with MIB 1 or the TUNEL technique and double stained with cell-type specific antibodies. Subsequently, labeling fractions were quantified.

Results: In type II-VI lesions, intimal DNA-synthesis was increased compared to that of the non-diseased (ND) arterial wall. DNA-synthesis peaked in early type II lesions ($2.7 \pm 0.5\%$ vs $0.02 \pm 0.02\%$ in ND; $p < 0.05$), and declined to $0.7 \pm 0.2\%$ in type V lesions ($p < 0.05$). Interestingly, a second peak of DNA-synthesis of $1.7 \pm 0.1\%$ was observed in type VI (ruptured plaque) lesions. Double staining revealed that DNA-synthesis was mostly confined to the macrophage derived foam cell (51.9%). In type II lesions, 100.0% of all DNA-synthesizing cells were present in the intimal foam cell rich area, while in advanced type III, IV and V lesions, DNA-synthesis had shifted to the shoulder region (74.8, 78.5 and 68.1% respectively). In type VI lesions, DNA-synthesis was present in the area underlying the plaque rupture (52.7%). Apoptosis was only elevated in advanced type IV, V and VI lesions (0.8 ± 0.1 , 0.8 ± 0.1 and $1.1 \pm 0.1\%$, respectively, vs $0.0 \pm 0.0\%$ in ND) and was predominant in the lipid core (90.5% in type IV lesions; 54.2% in type V lesions) or equally divided between the lipid core and the region underlying the plaque rupture (31.8 and 34.6% in type VI lesions). In type III-VI lesions, 50.0, 38.9, 42.6 and 42.8% of the TUNEL positive cells were macrophages.

Conclusions: In stable atherosclerotic lesions, DNA-synthesis is an early event, while apoptosis is a late event. Ruptured plaques showed a second peak of cell turnover. Lastly, cell turnover is mostly confined to the macrophage derived foam cell.

Introduction

From previous studies, it is known that both DNA-synthesis and apoptosis are present in human atherosclerotic lesions¹⁻⁷, and may play an important role in lesion development and in the conversion from stable to unstable plaques.^{5,6,8,9}

The data published so far, show that DNA-synthesis in primary human atherosclerotic lesions is low (0-3.5%).^{1,5,7,10,11} This low level of DNA-synthesis might be expected in lesions that take many years to become clinically manifest. Topographical analysis in ruptured plaques reveals that DNA-synthesis is tightly correlated with areas of structural repair and foam cell infiltration.⁵ Cell phenotypes exhibiting DNA-synthesis in atherosclerotic lesions are either the macrophage-derived foam cell^{1,5,11} or the vascular smooth muscle cell.¹⁰

Enhanced rates of apoptotic cell death are also found in human atherosclerotic lesions.^{3,4,6,12,13} Apoptotic labeling indices of 0-40% have been reported and apoptotic nuclei are either mostly confined to macrophage-derived foam cells in the lipid core and in regions adjacent to the lipid core^{3,4,12} or to vascular smooth muscle cells in the fibrous cap.^{8,9,14} Enhanced rates of apoptosis in the lipid core and/or fibrous cap are postulated to be major risk factors for the transition from stable to unstable plaques.^{8,9,14}

There are several limitations of the majority of these human studies. First of all, they almost invariably focus on advanced lesion types and therefore may have missed an early peak in cell turnover. Second, most studies have used atherectomy specimens. In these small samples, the morphology of the atherosclerotic lesion has been disrupted and, therefore, the topographical localization of both DNA-synthesis and apoptosis is hard to determine.¹⁵

Since DNA-synthesis and apoptosis are thought to be important features in atherogenesis, and since human data on cell turnover in all of the different stages of atherosclerotic lesion development, including ruptured plaques, are not available, we investigated the amount, phenotype and topographical localization of both DNA-synthesis and apoptosis in type I-VI lesions.

Methods

Patient characteristics and tissue preparation

In total, 76 atherosclerotic segments of non-branching parts of the descending aorta were obtained during autopsy (Dept. of Pathology, Academic hospital Maastricht) (N=6) or vascular surgery (dept. of General Surgery, Academic hospital Maastricht) (N=8). Seven non-atherosclerotic segments of non-branching parts of the descending aorta of four of the six autopsy patients served as control tissue. All tissue samples were obtained at comparable hemodynamic locations. Autopsy specimens were obtained from 3 adult male patients (58.3±1.8 years old, range 55-61 years) and three adult female patients (74.3±11.2 years old, range 52-86 years). The causes of death of the autopsy patients was diverse (cardiovascular disease, cancer). Surgical specimens were obtained from six male patients (62.6±1.8 years old, range 56-68

years) and 2 female patients (66.5±0.5 years old, range 66-67 years). Patients undergoing vascular surgery all had symptoms of vascular occlusion. The investigation conforms with the principles outlined in the 'Declaration of Helsinki'.

Immediately after harvesting, the samples were fixed in 10% phosphate buffered formalin (pH 7.4) for 24 hours and processed for paraffin embedding. Sections (4µm) were cut and stained with haematoxylin and eosin. Only sections with an intact lesion morphology were selected. These selected lesions were classified I-VI according to the recommendations of the Committee on Vascular Lesions of the Council on Arteriosclerosis of the American Heart Association.¹⁶

Immunohistochemistry

DNA-synthesis

DNA-synthesis was detected by use of the antibody MIB 1 (Immunotech, France), targeting the Ki-67 antigen, which is expressed in all stages of the cell cycle except G₀ and the early phase of G₁. The Ki-67 antigen is mostly upregulated in the G₂ and M phases. Therefore, Ki-67 expression only correlates with DNA-synthesis and does not reflect DNA-repair.^{17,18}

After dehydration and blocking of endogenous peroxidase activity with 0.3% H₂O₂, the sections were placed in a 0.01 mol/L citrate buffer, pH 6.0 and boiled for 2x 3 min. in a microwave. After subsequent washings in H₂O and Tris Buffered Saline (TBS), the slides were incubated with the MIB 1 monoclonal mouse antibody for 60 min. at room temperature at a dilution of 1:50, followed by incubation with a biotinylated sheep anti mouse IgG (1:250, Amersham, Life Science, England) for 30 min. at room temperature. Sections were then labeled for 30 min. with an avidin-biotin peroxidase complex (Brunschwig, USA). 3,3'-Diaminobenzidine (DAB) was used as the chromogen. The sections were counterstained with haematoxylin and mounted with coverslips.

Phenotyping of DNA-synthesizing cells

To assess the phenotype of DNA-synthesizing cells, parallel sections were double labeled with MIB 1 and cell-type specific antibodies for macrophages (CD68), vascular smooth muscle cells (VSMC) (α-smooth muscle actin), endothelial cells (CD34) and T-lymphocytes (CD3). After performing the MIB 1 staining as described above, sections were washed with H₂O and TBS and incubated with one of the antibodies (CD68: mouse monoclonal, 1:500, Dako, Denmark; α-smooth muscle actin (ASMA): 1:500, mouse monoclonal, Dako, Denmark and CD3: 1:200, rabbit polyclonal, Dako, Denmark) for 45 min. at room temperature. Subsequently, sections were labeled for 30 min. with a biotin-labeled sheep anti-mouse IgG (1:250, Amersham, life Science, England) (CD68 and ASMA) or sheep anti-rabbit IgG (1:1000, Dako, Denmark) (CD3), followed by 30 min. incubation with an avidin-biotin alkaline phosphatase (1:200, Dako, Denmark) complex. Fast blue (Sigma, MO, USA) was used as the chromogen. CD34 immunostaining (1:400 Becton Dickinson, CA, USA) preceded the MIB 1 staining. No counterstaining was performed and sections were mounted with immunomount (Shandon, PA, USA).

Apoptosis

For the detection of apoptosis, the TUNEL-technique was used.^{6,19} After dehydration, proteinase K was applied (Boehringer Mannheim, Germany) (20 µg/ml) for 15 min. at room temperature. After washing, sections were placed for 2 min. in triton X-100 (0.1%)/ sodium citrate (0.1%) on ice, rinsed in Phosphate Buffered Saline (PBS, pH 7.2), incubated for 15 min. in PBS containing 0.3% H₂O₂, and incubated for 1 hour in 3% citric acid (pH 7.2) at room temperature. After 10 min. equilibration, sections were incubated with enzyme mix (0.03 U/µl TdT, 0.04 nmol/µl dig-dUTP, 1.5 mM CoCl₂) (Boehringer Mannheim, Germany) for 60 min. at 37°C. After blocking the reaction in stop/wash buffer and rinsing in PBS, sections were incubated with peroxidase labeled anti-digoxigenin (1:450, Boehringer Mannheim, Germany) for 30 min. DAB was used as the chromogen. The sections were counterstained with haematoxylin and mounted with coverslips.

Phenotyping of apoptotic cells

For the detection of the phenotype of apoptotic cells, double labeling with the TUNEL technique and antibodies against CD68, ASMA, CD34, and CD3 was performed at parallel sections. After the TUNEL technique, sections were incubated with one of the above described antibodies for 45 min. at room temperature. After washing in TBS, sections were labeled for 30 min. with a biotin-labeled sheep anti mouse IgG (1:250, Amersham, Life Science, England)(CD68 and CD34) or sheep anti-rabbit IgG (1:1000, Dako, Denmark) (CD3), followed by 30 min. incubation with an avidin-biotin alkaline phosphatase complex (1:200, Dako, Denmark). The alkaline phosphatase substrate kit (Brunschiwig, the Netherlands) was used as the chromogen. ASMA immunolabeling preceded the TUNEL technique. No counterstaining was performed and sections were mounted with immunomount.

Cell counting

Tissue sections were investigated by light microscopy at *400 magnification using a standard field size. All intimal nuclei (± 600-5000 nuclei) of the respective lesion type, and 10 fields of the underlying media (± 400 nuclei) were counted. Cells containing nuclear MIB 1 staining were considered to be DNA-synthesizing cells. TUNEL positive nuclei were only considered to be apoptotic nuclei when the cells also showed at least one of the morphological features of apoptotic cell death (cell shrinkage, aggregation of chromatin into dense masses and cell fragmentation).²⁰⁻²²

The intimal labeling index, defined as the total number of intimal positive nuclei divided by the total nuclear intimal number, was calculated for the entire atherosclerotic lesion. The medial labeling index was calculated as the number of medial positive nuclei divided by the total number of positive nuclei present in 10 standard medial fields. The labeling index was calculated for both MIB 1- and TUNEL-stained sections. To obtain further insight into the distribution pattern of DNA-synthesizing and apoptotic cells, labeling indices were also determined at different sites within the lesion: the endothelial coverage (ec), foam cell-rich area (fcr), shoulder region (sh), the lipid core and regions adjacent to the lipid core (lc), the fibrous cap (fc) and the area underlying the plaque rupture (rupt).

Double labeling of parallel sections was used to assess the phenotype of DNA-synthesizing and apoptotic cells. To calculate the different fractions of phenotypes, the number of MIB 1-positive or TUNEL-positive nuclei, also positive for one of the phenotype specific antibodies, was divided by the total number of MIB 1- or TUNEL-positive cells. With the above described panel of antibodies (CD68, ASMA, CD34, CD3), the immunophenotype of 72.1% of the DNA-synthesizing and 59.9% of the apoptotic cells could be determined. All countings were performed by one investigator. The intra-observer variation was less than 10%.

Statistics

To test whether lesions of the same type of different patients could be pooled, a one-way ANOVA was performed. This was performed separately for MIB 1 and TUNEL stained sections. Since no statistical significance was found ($p > 0.05$), lesions of one type, but of different patients, were assumed to be comparable.

A Mann-Whitney U test was used for comparisons between the lesion types. For comparisons within the lesion types, the Wilcoxon signed ranks test was used. In all tests, the level of statistical significance was assumed to be at $p < 0.05$. Data are expressed as $\text{mean} \pm \text{SEM}$.

Results

In total, 76 lesions ($n=8-12$ per lesion type) and 7 non-atherosclerotic segments (controls) of the non-branching parts of the descending aorta were investigated.

DNA-synthesis

Media

Medial DNA-synthesis in all lesion types was low ($0.06 \pm 0.03\%$), and not elevated in any of the lesion types as compared to the non-diseased (ND) arterial wall ($p > 0.05$). However, a small peak of $0.19 \pm 0.10\%$ (compared to $0.02 \pm 0.01\%$ ND) was observed in type I lesions ($p=0.31$). Medial DNA-synthesis was confined to the vascular smooth muscle cell (93.2%).

Intima

In all lesion types, except type I lesions, intimal DNA-synthesis was significantly elevated (fig. 3.1a), as compared to the non-diseased arterial wall. The highest MIB 1 labeling was found in type II (fatty streak) lesions ($2.7 \pm 0.5\%$ vs $0.02 \pm 0.02\%$ in the non-diseased arterial wall; $p < 0.05$) (fig. 3.1a). With progression of lesion severity, DNA-synthesis decreased to $0.7 \pm 0.2\%$ in advanced type V lesions ($p < 0.05$). Interestingly, a second peak of DNA synthesis of $1.7 \pm 0.1\%$ was observed in ruptured plaques (type VI lesions).

Not only the level, but also the site of DNA-synthesis changed with progression of the lesion (fig. 3.1a). In the early type II lesions, 100.0% of all DNA-synthesizing cells were found in the foam cell rich intimal area. With the development of a shoulder region in type III and IV lesions, DNA-synthesis shifted to that region (74.8% and 83.9%

Fig. 3.1

A, Level and localisation of DNA-synthesis in human atherosclerotic lesions. Abbreviations: ec, endothelial coverage; fcr, foam cell rich area; sh, shoulder region; lc, lipid core; fc, fibrous cap; rupt, rupture; and ND, non-diseased arterial wall. * $p < 0.05$.

B, Phenotype of DNA-synthesizing cells in human atherosclerotic lesions. Abbreviations: ASMA, α -smooth muscle actin; CD68, macrophages; CD34, endothelial cells; CD3, T-lymphocytes; and NC, not classified.

respectively; $p < 0.05$). In type V lesions, the majority of DNA-synthesizing cells (68.1%) was still present in the shoulder region, although the number had significantly decreased compared to that found in type III and IV lesions. This decrease is probably due to a shift of DNA-synthesis from the shoulder region to the fibrous cap in which 20.3% of all DNA-synthesizing cells was located. Type VI lesions showed a different pattern. The majority of DNA-synthesizing cells (52.7%) in the ruptured plaques was found in the area underlying the rupture, while only 33.7% of all DNA-synthesizing cells was located in the shoulder region and 15.1% in the fibrous cap. Interestingly, DNA-synthesis was low in the lipid core of type IV, V and VI lesions (5.4, 11.6 and 1.8% of all DNA-synthesizing cells respectively).

Double staining of all lesions revealed that 51.9 ± 4.3 % of the DNA-synthesizing cells were macrophage-derived foam cells, 3.0 ± 0.9 % vascular smooth muscle cells, 3.2 ± 1.0 % endothelial cells and 12.7 ± 2.6 % were T-lymphocytes (fig. 3.1b). In all stable lesion types (type II-V), the majority of DNA-synthesizing cells were macrophage-derived foam cells (68.6% (type II), 64.7% (type III), 63.6% (type IV) and 68.9% (type V)). However, in ruptured plaques, the majority of DNA-synthesis was almost equally divided between macrophages and T-lymphocytes (35.2 ± 3.6 % and 33.3 ± 5.8 %).

Fig. 3.2
A, Level and localisation of apoptosis in human atherosclerotic lesions. Abbreviations: ec, endothelial coverage; fcr, foam cell rich area; sh, shoulder region; lc, lipid core; fc, fibrous cap; rupt, rupture; and ND, non-diseased arterial wall. * $p < 0.05$.
B, Phenotype of apoptotic cells in human atherosclerotic lesions. Abbreviations: ASMA, α -smooth muscle actin; CD68, macrophages; CD34, endothelial cells; CD3, T-lymphocytes; and NC, not classified.

Apoptosis

Media

Medial apoptosis was a rare phenomenon. In fact, the medial apoptotic labeling fraction was low in all lesion types ($0.01 \pm 0.01\%$) and did not increase during atherogenesis.

Intima

While intimal DNA-synthesis was an early event, apoptosis predominantly occurred in late stages of the disease (fig. 3.2a). In type I, II and III lesions, the fraction of apoptotic cells in the intima was not different from that in the non-diseased arterial wall. However, the apoptotic labeling fraction was significantly elevated in advanced type IV, V and VI lesions ($0.8 \pm 0.1\%$ in type IV, $0.8 \pm 0.1\%$ in type V and $1.1 \pm 0.1\%$ in type VI vs $0.0 \pm 0.0\%$ in ND). In type IV lesions, apoptosis was mostly confined to the lipid core (90.5% of all apoptotic cells) (fig. 3.2a). In type V lesions, the apoptotic labeling fraction in the lipid core significantly decreased to 54.2% ($p < 0.05$ compared to type IV lesions), while 27.7% of all apoptotic cells were found in the fibrous cap. In ruptured plaques (type VI lesions), apoptosis was equally divided between the lipid core (31.8%), fibrous cap (24.3%) and the area underlying the rupture (34.6%).

Double staining failed to identify the phenotype 41.8% of the apoptotic nuclei. It showed however that $37.8 \pm 4.4\%$ of all apoptotic nuclei were macrophages, $17.0 \pm 3.3\%$ vascular smooth muscle cells, $1.6 \pm 0.8\%$ endothelial cells and $1.8 \pm 0.9\%$ T-lymphocytes (fig. 3.2b). In advanced lesion types (type III-VI), the majority of identified TUNEL-positive cells were macrophages (50.0, 38.9, 42.6 and 42.8% of all apoptotic cells respectively). The vascular smooth muscle cell was another prominent TUNEL- positive

celltype (23.6, 19.2 and 20.1% of all apoptotic cells in type IV, V and VI lesions, respectively).

Discussion

This is the first study describing DNA-synthesis and apoptosis during several stages of atherosclerosis in humans. The low, but significantly elevated levels of DNA-synthesis that we found in advanced stable human atheromata are in accordance with other published data.^{1,5,7,10,11,23} As described in the present study, the presence of a peak in fatty streaks (type II) is a remarkable feature of the pattern of DNA-synthesis during atherogenesis in humans. This early peak is in accordance with the peak in DNA-synthesis that has been observed in human in vitro studies²⁴ and in several hypercholesterolemic animal models.²⁵⁻²⁷ Recently, Orekhov et al.⁷ also described enhanced proliferation in lipid rich lesions, while much lower levels of DNA-synthesis, were observed in fibrous lesions. A possible explanation for the occurrence of this early peak is the mitogenic effect of shortly oxidized LDL²⁸, that is present in macrophage derived foam cells in these early lesions.

While DNA-synthesis is an early phenomenon in atherogenesis, apoptosis shows the opposite. As our results show, apoptosis is confined to advanced stages of atherosclerosis and is not elevated in early type I, II and III lesions. Apoptosis in the intima of advanced lesion types has already been reported previously, but its level shows remarkable variations (0-40%).^{3,4,12-14,29} The high levels of apoptosis might reflect non-specific staining of calcium containing vesicles³⁰, the detection of both apoptosis and oncosis¹⁴, and staining of RNA-synthesizing nuclei²² by the TUNEL technique. In the present study, we therefore applied very strict criteria for the TUNEL method and the detection of apoptotic nuclei (pretreatment with citric acid, optimization of the enzyme concentration and apoptotic morphology).^{22,30} Independent from our study, Kockx et al., using the very same criteria, found comparable levels of apoptosis in human lesions. They also reported that apoptosis was predominantly confined to advanced stages of the disease.⁶

Most TUNEL positive nuclei were located in macrophages and vascular smooth muscle cells, as demonstrated by double labeling with CD68 and α -smooth muscle actin. However, a significant fraction of TUNEL-positive cells could not be identified by the panel of antibodies used. This could reflect a loss of specific markers during apoptosis.^{3,4,6}

In a recent study, we investigated DNA-synthesis and apoptosis in type II-V lesions in ApoE*3Leiden mice. Interestingly, in this atherosclerotic mouse model, we observed the same pattern of cell turnover during atherosclerotic lesion development as in humans.²⁷ In accordance with the data described here ApoE*3Leiden mice also show a peak in DNA-synthesis in early type II lesions and a decrease with lesion progression. Furthermore, apoptosis in atherosclerotic lesions in ApoE*3Leiden mice is also confined to advanced lesion types.²⁷

Our data show that both parameters of cell turnover are increased in ruptured plaques (type VI). The majority of both DNA-synthesis and apoptosis in these lesions is present at the site of the plaque rupture, and cell turnover was mostly confined to inflammatory celltypes (macrophages and T-lymphocytes). Thus cell turnover, and especially apoptosis, may play an important role in the transition from stable to unstable plaques. Morphological characteristics of atherosclerotic lesions prone to rupture are an extensive lipid core, loss of vascular smooth muscle cells of the fibrous cap, macrophage infiltration into the shoulder region of the fibrous cap and thinning of the fibrous cap.^{8,31} These phenomena can, at least partially, be explained by the different patterns of cell turnover we observed during atherogenesis.

In accordance with other publications, we found that the majority of apoptosis in human atheromata occurs in macrophage derived foam cells in the lipid core and in regions adjacent to the lipid core.^{3,4,6,12,13,29} Strongly oxidized LDL, that is abundantly present in the lipid core of atherosclerotic lesions, may be one of the factors that induce apoptosis in this region. Indeed, several in vitro studies have reported that, strongly oxidized LDL is capable of inducing apoptosis in macrophages. Moreover, macrophages are able to oxidize LDL which may induce self inflicted apoptosis.^{28,32,33} The loss of cellular mass due to apoptosis in the lipid core and adjacent regions may contribute to further expansion and softening of the lipid core and may make the lesion more prone to rupture.^{8,9}

The vascular smooth muscle cell is the other cell type that shows elevated levels of apoptosis in advanced atherosclerotic lesions. Elevated levels of apoptosis in vascular smooth muscle cells not only results in a decreased content of vascular smooth muscle cells in the fibrous cap, but also reduces the population of cells that are able to synthesize extracellular matrix proteins. Both these factors contribute to thinning of the fibrous cap and therefore, to a decreased stability of the atherosclerotic plaque.^{14,34,35}

The present study suggests, that cell turnover mechanisms may provide targets for therapeutical interventions in atherosclerosis. First of all, inhibition of DNA-synthesis may slow the progression of atherosclerosis. According to our findings, inhibitors of DNA-synthesis may be most effective, when applied early in the disease process (early DNA-synthesis peak). Inhibition of DNA-synthesis of advanced atherosclerotic lesions may even be undesirable, since it has been postulated that proliferation of vascular smooth muscle cells is part of a repair process and contributes to the maintenance of lesion stability.^{36,37}

A different approach could be to interfere with the apoptotic process. Stimulation of apoptosis in atherosclerotic lesions may decrease lesion size. However, extensive apoptosis may make the lesion more vulnerable to rupture. Thus, since there appears to be such a delicate balance between the cell turnover mechanisms in atherosclerotic lesions, the effect of interventions, affecting parameters of cell turnover, is rather unpredictable.

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Chapter 4

Both compensatory enlargement and stenosis develop in ApoE^{-/-} and ApoE*3Leiden transgenic mice

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Submitted

Abstract

Atherosclerotic mouse models develop little ischemic organ damage, and no infarctions, despite the presence of large atherosclerotic lesions. Therefore, one might speculate that luminal changes do not follow atherosclerotic lesion development. Since one phenomenon that may explain a discrepancy between luminal changes and lesion size is vascular remodeling, we measured parameters of vascular remodeling in the carotid arteries (CA), thoracic aorta (TA), and abdominal aorta (AA) of ApoE^{-/-} and ApoE*3Leiden mice, two well known mouse models of atherosclerosis. Atherosclerotic lesions were classified (AHA II-V), and plaque thickness, compensatory enlargement vs constrictive remodeling, lumen diameter, stenosis and media thickness were measured, relative to the non-diseased arterial wall.

In CAs, plaque thickness increased during atherogenesis. Concomitant to this increase, CAs showed compensatory enlargement (ApoE^{-/-}: +55%, ApoE*3Leiden +38%). Regression analysis revealed a positive correlation between plaque and lumen area (ApoE^{-/-}: $R=0.95$, ApoE*3Leiden: $R=0.90$). Medial thinning and elastolysis were also observed. During atherogenesis, lumen diameter decreased (ApoE^{-/-}: -69%, ApoE*3Leiden: -40%) and stenosis >70% developed. TA and AA showed similar features, but neither developed a progressive decrease in lumen diameter nor stenosis >70%.

In CA, TA and AA of ApoE^{-/-} and ApoE*3Leiden mice, atherogenesis is associated with compensatory enlargement, medial thinning and elastolysis. A progressive decrease in lumen diameter and stenosis >70% only occur in CA. Vascular remodeling is more prominent in ApoE^{-/-} mice.

Introduction

For a long time, the arterial system was considered to be a rigid tube that showed a decrease in lumen diameter in response to a growing plaque mass.¹ However, in 1987, Glagov, and many others thereafter, showed that coronary arteries developed compensatory enlargement in response to plaque growth.²⁻⁶ Later it was shown, that besides compensatory enlargement, constrictive remodeling may develop.⁷ The mode of remodeling (compensatory vs constrictive) may vary strongly over the length of one artery, and between different arteries.⁸ Clinically, constrictive remodeling is associated with ischemic organ complications.⁹

Although enlargement and constrictive remodeling have extensively been reported in different animal models after balloon angioplasty, like the baboon^{10,11}, the rat, the rabbit¹² and the pig¹³, an animal model of *de novo* atherosclerosis that shows constrictive remodeling with significant stenosis and ischemic organ complications is still lacking.⁹ Hypercholesterolemic Macaque monkeys, rabbits and pigs all show arterial compensatory enlargement, while high gradestenosis or a decrease in lumen diameter are hardly observed.^{10,11}

Also ApoE^{-/-} mice, the most frequently used atherosclerotic mouse model, show compensatory enlargement, and, when fed an extremely high cholesterol diet, or during infusion of angiotensin II, even aneurysm formation.¹⁴⁻¹⁷ Some ApoE^{-/-} mice do develop significant stenosis in the external carotid artery, however without evidence of ischemia.¹⁵

Since parameters of vascular remodeling during lesion progression in atherosclerotic mouse models are largely unknown, we investigated these parameters in all stages of atherosclerosis, in 3 differently sized vessels, in 2 different hypercholesterolemic mouse models, the ApoE^{-/-} mouse¹⁸, and the ApoE*3Leiden transgenic mouse.¹⁹⁻²¹ Our data indicate that both mouse strains show compensatory enlargement, medial thinning and elastolysis in the carotid arteries, the thoracic and the abdominal aorta. Interestingly, the carotid arteries showed a decrease in lumen diameter and stenosis >70%, especially in type IV and V lesions. However, constrictive remodeling and ischemic organ damage did not develop.

Materials and Methods

Mice

Data of the present study were obtained from ApoE^{-/-} mice (n=14) and ApoE*3Leiden mice (n=45), fed an atherogenic diet for 4-12 months. For validation purposes (see results section), wild type (C57Bl6) mice on a normal chow (n=6) or atherogenic diet (n=6) were used.

Tissue handling

After the experimental period, mice were sacrificed and tissue was processed as described previously.²¹ In summary, the arterial tree was perfused under standardized pressure (100mmHg) with PBS (3 min) and 10% phosphate buffered

Fig. 4.1

A, Figure showing a Lawson stained section of a TA containing a type III lesion. Measurement points for reference values and plaque values are indicated. IEL, internal elastic lamina; EEL, external elastic lamina.

B, Figure showing measurement area, lumen area and the area between both IEL, used for regression analysis.

formalin (3 min) both containing 0.1 mg/ml sodium nitroprusside, through a catheter introduced into the left ventricular apex. Subsequently, the entire arterial tree was excised and formalin fixed for 24 hours. Both CAs, the TA and AA were embedded longitudinally in paraffin and 4µm sections were cut. Five sections of the CA, TA and AA (16µm apart) were stained with haematoxylin and eosin and classified from II-V according to the AHA classification.²²

Morphometry

For morphometry, 5 sections of CA, TA and AA, consecutive to the HE-stained sections, were stained Lawson (a modified elastica von Giesson staining).

Morphometric parameters were determined using a microscope coupled to a computerized morphometry system (Quantimet 570, Leica, the Netherlands).

Parameters were measured at the center of the lesion as well as at the non-diseased part of the same vessel (100 μ m proximal and distal of the atherosclerotic plaque). These measurements were taken as reference values (fig. 4.1a).

Plaque thickness at the center of the lesion, plaque area, lumen area, and the area between both internal elastic laminae were measured as described in figure 4.1a and 4.1b. Compensatory enlargement/constrictive remodeling was defined as the relative increase/decrease of the distance between both internal elastic laminae compared to reference values. Relative lumen diameter was defined as the distance between both internal elastic laminae minus plaque thickness, compared to reference values. Media thinning/thickening was defined as the relative decrease/increase of the media underlying the plaque (distance between internal elastic lamina and external elastic lamina) compared to reference values. The amount of medial elastolysis was defined as the number of elastin breaks in the media underlying the atherosclerotic lesion. The percentage stenosis was defined as plaque diameter divided by the distance between both internal elastic laminae. Post-stenotic dilatation was defined as the difference of the distance between both elastic laminae of the reference value sites distal and proximal from the atherosclerotic lesion.

Lipid profile

Plasma cholesterol and plasma triglyceride levels were determined in duplicate using a colorimetric assay (CHOD-PAP 1442341 and GPO-PAP 701912 respectively, La Roche).

Statistics

Values are given as mean $\pm$ SEM. Relative increases and decreases were obtained by dividing the value of the center of the lesion by the mean of both reference values. Arteries in which it was impossible to obtain proper reference values, as well as arteries containing 2 opposite lesions in one segment were discarded. Differences during lesion progression were tested by a one way ANOVA. For the analysis of differences between the same lesion types between both mouse models, a Mann-Whitney U test was used. Regression analysis was performed to test the correlation between plaque area, lumen area, and the area between both IEL. To test whether vascular remodeling was an age dependent effect, reference values, as well as plaque values of mice of the different diet groups (4, 6, 9, 12 months) were compared by a 2-way ANOVA. The level of statistical significance was assumed to be at $p < 0.05$.

Results

General

Mean body weight did not differ between the mouse strains. Survival rates were 92% for ApoE^{-/-} mice, 96% for ApoE*3Leiden mice, and 100% for C57Bl6 mice. Histological analysis of heart, brain, lungs, kidneys and liver (HE stained sections) revealed no ischemic damage. In one ApoE^{-/-} mouse, an apical myocardial infarction caused by LAD occlusion was observed, and in one ApoE*3Leiden mouse, cerebral infarction was observed. In ApoE*3Leiden mice, cholesterol and triglyceride levels were elevated compared to ApoE^{-/-} mice (cholesterol: 34.0 ± 0.6 mmol/l vs 24.0 ± 1.2 mmol/l; triglyceride levels: 2.0 ± 0.2 mmol/l vs 0.9 ± 0.1 mmol/l). In C57Bl6 mice, these levels were low (normal chow: cholesterol 0.6 ± 0.3 mmol/l, triglycerides 0.1 ± 0.0 mmol/l; diet 0.9 ± 0.4 mmol/l and 0.1 ± 0.0 mmol/l, respectively).

For validation of the measurements, reference values of ApoE^{-/-} and ApoE*3Leiden mice were compared with remodeling parameters obtained from non-diseased arteries of C57Bl6 mice on normal chow diet or on atherogenic diet. No significant differences were found between reference values of arteries of atherosclerotic and non-diseased mice. Also, remodeling parameters between C57Bl6 mice on normal chow and atherogenic diet were not different. To investigate whether remodeling was an age dependent effect, reference values, as well as plaque values of mice of the different diet groups (4, 6, 9, 12 months diet) were compared. Age dependent remodeling was not observed.

In total, 367 atherosclerotic lesions (=1835 slides) of three different sized arteries were analyzed (ApoE^{-/-} CA n=37; TA n=43; AA n=99; ApoE*3Leiden CA n=98; TA n=40; AA n=50).

TA had the largest diameter (ApoE^{-/-} $689 \pm 21 \mu\text{m}$, ApoE*3Leiden $629 \pm 19 \mu\text{m}$), followed by the AA ($479 \pm 13 \mu\text{m}$ and $541 \pm 17 \mu\text{m}$) and the CA ($285 \pm 9 \mu\text{m}$ and $289 \pm 7 \mu\text{m}$).

Vascular Remodeling

Carotid arteries

Plaque thickness increased during the progression from type II to type V lesions (ApoE^{-/-}: $+804 \pm 89\%$, ApoE*3Leiden: $+547 \pm 113\%$) (fig. 4.2a). Compared to ApoE*3Leiden mice, the increase in plaque thickness was significantly higher in ApoE^{-/-} mice, especially the thickness of type IV and V lesions (fig. 4.2a).

The increase in plaque thickness was associated with the development of compensatory enlargement. Compensatory enlargement (from type II-V lesions) was $+55\%$ in ApoE^{-/-} mice, and $+38\%$ in ApoE*3Leiden mice (fig 4.3a). In ApoE^{-/-} mice, compensatory enlargement was more prominent than in ApoE*3Leiden mice. Statistically significant differences between both mouse strains were observed in type IV and V lesions (fig. 4.3a). Regression analysis revealed a high correlation between plaque area and compensatory enlargement (ApoE^{-/-} mice $R=0.95$; ApoE*3Leiden mice $R=0.90$) (fig 4.4ab).

Despite the development of compensatory enlargement, lumen diameter decreased and stenosis $>70\%$ developed. In ApoE^{-/-} mice, the decrease of lumen diameter was more prominent compared to ApoE*3Leiden mice (ApoE^{-/-} type II:

Fig. 4.2 (left)

Plaque thickness in all atherosclerotic lesion types in *A*, the carotid arteries, *B*, thoracic aorta and *C*, abdominal aorta of ApoE^{-/-} and ApoE^{*3}Leiden mice. Data are compared to the respective lesion type between both mouse models (* $p < 0.05$). To evaluate plaque thickness during atherogenesis within a mouse strain, a one-way ANOVA was performed ($\dagger p < 0.05$). Data reveal an increase in plaque thickness during atherogenesis in all three vessels.

Fig. 4.3 (right)

Compensatory enlargement in all atherosclerotic lesion types in *A*, the carotid arteries, *B*, thoracic aorta and *C*, abdominal aorta of ApoE^{-/-} and ApoE^{*3}Leiden mice. Data are compared to the respective lesion type between both mouse models (* $p < 0.05$), or to reference values ($\dagger p < 0.05$). To evaluate compensatory enlargement during atherogenesis within a mouse strain, a one-way ANOVA was performed ($\ddagger p < 0.05$). Data reveal the presence of compensatory enlargement in all three vessels during atherogenesis, most prominently in ApoE^{-/-} mice.

Fig. 4.4
Regression analysis, revealing a positive correlation between plaque area and the area between both internal elastic laminae (IEL), in *AB*, the carotic arteries, *CD*, thoracic and *EF*, abdominal aorta of ApoE^{-/-} and ApoE*3Leiden mice, indicating compensatory enlargement.

-19±3%, type V lesions: -88±9%; ApoE*3Leiden type II: -23±2%, type V -63±5%) (fig 4.5a). Stenosis >70% were observed in advanced type IV and V lesions, and predominantly in ApoE^{-/-} mice (type IV: ApoE^{-/-} 90±4% vs ApoE*3Leiden 71±4% and type V: 91±5% vs 73±4%) (fig. 4.6a). Despite the development of stenosis, significant post-stenotic dilatation was not observed. Regression analysis revealed no correlation between plaque area and lumen area (ApoE^{-/-} R=0.21; ApoE*3Leiden R=0.56). Constrictive remodeling (defined as negative compensatory enlargement) did not occur (fig. 4.3a).

Fig. 4.5 (left)

Relative lumen diameter in all atherosclerotic lesion types in the carotid arteries (a), thoracic aorta (b) and abdominal aorta (c) of ApoE^{-/-} and ApoE^{*3}Leiden mice. Data are compared to the respective lesion type between both mouse models (* p < 0.05), or to reference values († p < 0.05). To evaluate relative lumen diameter during atherogenesis within a mouse strain, a one-way ANOVA was performed (‡ p < 0.05). The decrease in lumen diameter during atherogenesis was most prominent in the carotid arteries of ApoE^{-/-} mice.

Fig. 4.6 (right)

Percentage stenosis in all atherosclerotic lesion types in the carotid arteries (a), thoracic aorta (b) and abdominal aorta (c) of ApoE^{-/-} and ApoE^{*3}Leiden mice. Data are compared to the respective lesion type between both mouse models (* p < 0.05). To evaluate the progression of stenosis during atherogenesis within a mouse strain, a one-way ANOVA was performed (‡ p < 0.05). Significant stenosis was defined as stenosis >70%. Data reveal that significant stenosis only develops in type IV and V lesions of the carotid arteries, and most prominent in ApoE^{-/-} mice.

Fig. 4.7

Relative media thickness in all atherosclerotic lesion types in *A*, the carotid arteries, *B*, thoracic aorta and *C*, abdominal aorta of ApoE^{-/-} and ApoE^{*3}Leiden mice. Data are compared to the respective lesion type between both mouse models (* $p < 0.05$) or to reference values († $p < 0.05$). To evaluate the progression of stenosis during atherogenesis within a mouse strain, a one-way ANOVA was performed (‡ $p < 0.05$).

Fig. 4.8

Medial elastolysis and its distribution among the different elastic laminae (IEL= internal elastic lamina, EL2= the second elastic lamina from the luminal site, EL3= the third elastic lamina from the luminal site, EL4= the fourth elastic lamina from the luminal site, EEL= external elastic lamina) in all atherosclerotic lesion types and the non-diseased arterial wall (ND) in *A*, the carotid arteries, *B*, thoracic aorta and *C*, abdominal aorta of ApoE^{-/-} and ApoE^{*3}Leiden mice. Data are compared to the respective lesion type between both mouse models (* $p < 0.05$) or to the non-diseased arterial wall († $p < 0.05$). To evaluate the progression of stenosis during atherogenesis within a mouse strain, a one-way ANOVA was performed (‡ $p < 0.05$).

Also, the media underlying the plaque changed. First of all, in both mouse models, the media hypertrophied in early lesions (type II: ApoE^{-/-} +64±17%, ApoE*3Leiden +31±11%), and atrophied in type V lesions (type V: ApoE^{-/-} -29±7%, ApoE*3Leiden -17±6%)(fig. 4.7a). Second, medial elastin breaks were observed. Interestingly, the number of medial elastin breaks, as well as the distribution pattern differed between both mouse models. In ApoE^{-/-} mice, the mean number of elastin breaks was 4.1±0.6, while it was only 1.9±0.2 in ApoE*3Leiden mice, compared to 1.1±0.3 and 0.6±0.4 in the non-diseased parts (fig 4.8a). Furthermore, in ApoE^{-/-} mice, breaks were distributed equally throughout the lesion, while in ApoE*3Leiden mice, breaks were confined to the IEL (37.8% in IEL in ApoE^{-/-} vs 70.7% in ApoE*3Leiden mice) (fig. 4.8a).

Thoracic and Abdominal aorta

Although an increase in plaque thickness, compensatory enlargement, as well as a positive correlation between plaque area and compensatory enlargement (TA: ApoE^{-/-} R=0.74 ApoE*3Leiden R=0.84, AA R=0.78 and R=0.94) were also observed in TA and AA (figs. 4.2bc, 4.3bc and 4.4c-f), a progressive decrease in lumen diameter, as well as stenosis >70% did not occur (figs. 4.5bc and 4.6bc). No significant differences between ApoE^{-/-} and ApoE*3Leiden mice were found. In TA and AA, regression analysis revealed no negative correlation between plaque area and lumen diameter (TA ApoE^{-/-} R=0.32, ApoE*3Leiden R=0.57 and AA R=0.42 and R=0.56). Also in TA and AA, medial changes were observed in both mouse models. In ApoE^{-/-} mice, progressive medial thinning occurred (TA -28±9% in type V, AA -18±3% in type V), while in ApoE*3Leiden mice, the media hypertrophied in all lesion types (TA +46±10%, AA +30%±12, both in type V) (fig. 4.7bc). The pattern of elastolysis equaled that in the CA (fig. 4.8bc).

Discussion

This manuscript is the first to provide data on parameters of vascular remodeling in three different sized arteries of two hypercholesterolemic mouse models, in all atherosclerotic lesion types. The main findings are that 1) both mouse models develop compensatory enlargement, correlated with plaque area, 2) only carotid arteries are able to develop a progressive decrease in lumen diameter and stenosis >70%, 3) medial hypertrophy is a feature of early atherosclerosis, while medial thinning with elastin breaks are observed in advanced lesions, and 4) the development of constrictive remodeling is lacking.

Vascular remodeling is an adaptive process that occurs as a response to chronic changes in hemodynamic conditions.^{3,23} When a human atherosclerotic plaque develops, compensatory enlargement, as well as post-stenotic dilatation occurs as an attempt to maintain pre-existing flow and shear stress.^{4-6,23,24} In early stages of atherosclerosis, even overcompensation of the lumen was observed.^{5,23,25} When atherosclerotic lesions progress, the capacity of the arterial wall to compensate for the increase in plaque thickness decreases. At this stage, compensatory enlargement is inadequate^{4,26,27}, and a correlation between plaque

area and lumen loss can be found.²³ Additionally, constrictive remodeling, with a decrease in lumen diameter has been reported.^{8,28}

Consistent with data found in humans and atherosclerotic animal models^{12,14-16,29}, we demonstrate compensatory enlargement in advanced atherosclerotic lesions of both ApoE^{-/-} and ApoE*3Leiden mice. However, overcompensation of the lumen in early lesions and the development of post-stenotic dilation was not observed in either of the 2 mouse models. As in other animal models of *de novo* atherosclerosis, ApoE^{-/-} and ApoE*3Leiden mice failed to develop (inadequate), constrictive remodeling. However, in contrast to other atherosclerotic animal models, both mouse models did develop significant stenosis in the common carotid arteries, but not in the thoracic or abdominal aorta. These data are consistent with the data of Seo et al., who observed significant stenosis in peripheral vessels (popliteal and external carotid arteries) of 9 out of 12 ApoE^{-/-} mice.¹⁵ The difference in remodeling we observed between the different sized arteries can be due to the smaller vessel diameter of the carotid artery. In the carotid artery, vessel diameter is twice as small, while maximal plaque thickness is equal to that in the thoracic or abdominal aorta. This suggests that the vessel stenoses twice as fast in carotid arteries compared to the thoracic or abdominal aorta. A reason for this phenomenon might be the different structural composition and function of the carotid artery, which is a musculo-elastic vessel with a relative thin media, and the thoracic or abdominal aorta, which is rather a thick walled conduit vessel.

Until now, the mechanisms of vascular remodeling are poorly understood. Postulated mechanisms are changes in shear stress, shear stressed induced NO-production, endothelial dysfunction, hypertension, and the activation of matrix degrading proteins.⁹

During atherogenesis, shear stress changes, and as a response, the arterial endothelium adapts by inducing the chronic expression of vasodilatory substances like nitric oxide (NO).^{30,31} Consequently, the artery dilates and thereby compensates for the initial lumen loss. On the other hand, atherosclerosis induces endothelial dysfunction, causing an impaired endothelial dependent relaxation, which contributes to inadequate vasodilation, and inadequate compensatory enlargement.³²⁻³⁴ In addition, it is reported that a decreased blood flow causes constrictive remodeling.^{24,30} These features also apply, albeit to a lesser extent in ApoE^{-/-} mice. Although ApoE^{-/-} mice develop compensatory enlargement, ApoE^{-/-} mice show a diminished vasodilatation response to acetylcholine, and to NO.^{16,35-37} The severity of endothelial dysfunction in ApoE^{-/-} mice varies in the different studies, and seems to be positively correlated with age, and atherogenicity of the diet.^{16,35-38} Furthermore, the mouse hyperdynamic circulation may result in a continuous release of vasodilatory factors, that might prevent the development of constrictive remodeling.³⁹

In humans, hypertension is also correlated with an increase in arterial diameter, and could thereby contribute to compensatory enlargement.⁴⁰ However, vice versa, the stiffness of a chronic dilated artery could facilitate the manifestation and maintenance of systolic hypertension.⁴⁰ Interestingly, hypertension and increased

arterial wall stiffness were also observed in ApoE^{-/-} mice, possibly contributing to dilation of the arteries.^{35,38}

Another mechanism, responsible for remodeling is the involution of the support structure of the arterial wall.^{9,41,42} During atherogenesis, collagen, as well as elastin is degraded. This often results in an atrophic, elastolytic media and a decreased collagen content of the adventitia. As a response, the artery enlarges, often resulting in aneurysm formation.^{41,43} ApoE^{-/-} mice fed a severe atherogenic diet, or after 1 month of angiotensin II infusion, develop medial elastolysis with aneurysm formation.^{14,17} Degradation of collagen and elastin is mediated by plasmin dependent activation of matrix metalloproteinases (MMPs), especially MMP-3, -9, -12 and -13, which are produced by infiltrating macrophages.¹⁴ Other observations support the important role of MMPs in aneurysm formation are that administration of an MMP inhibitor in rats and targeted disruption of MMP-9 in mice suppress the development of experimental abdominal aortic aneurysms.^{44,45} Also in the present study, medial elastolysis was observed in ApoE^{-/-} and ApoE*3Leiden mice. In ApoE^{-/-} mice, the level of elastolysis was elevated compared to ApoE*3Leiden mice, and elastin breaks were found throughout the media, whereas in ApoE*3Leiden mice, elastolysis was confined to the IEL. This might explain the more prominent vascular remodeling in ApoE^{-/-} compared to ApoE*3Leiden mice.

Whether elastin degradation in the media is the cause for the development of compensatory enlargement, or the consequence, is still not clear. In favor of a causative role of elastin degradation are the recently published results of a study that showed that administration of a serine elastase inhibitor completely reverses fatal pulmonary remodeling in rats.⁴⁶ In the present study, an increased amount of elastin breaks was already present in initial lesions, before significant compensatory enlargement develops, also indicating a causative role for media degeneration in the development of compensatory enlargement. The results of an elastin inhibitor in atherosclerosis will be awaited.

In conclusion, both ApoE^{-/-} mice and ApoE*3Leiden mice are appropriate small animal models for studying vascular remodeling, especially in the carotid artery. For the investigation of the regulation of remodeling, ApoE^{-/-} mice are more appropriate, since in this model, compensatory enlargement, stenosis, medial atrophy and elastolysis are most prominent. However, direct extrapolation to the human condition should be performed with caution, because some human features of vascular remodeling (such as post-stenotic dilation and constrictive remodeling) are not observed in ApoE^{-/-} or ApoE*3Leiden mice.

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Chapter 5

Gas6^{-/-}/ApoE^{-/-} mice develop a collagen-rich, differently organized plaque phenotype, that is prone to intra-plaque hemorrhage

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Submitted

Abstract

To investigate the role of Gas6 (growth arrest specific gene 6) in atherosclerosis, Gas6^{-/-}/ApoE^{-/-} mice were generated. Gas6^{-/-}/ApoE^{-/-} (n=20) and ApoE^{-/-} (n=24) mice were sacrificed after 23 wks on a normal chow or after 15 wks on a severely atherogenic diet. The aortic arch was taken out for analysis. Plaque area and the number of plaques per aortic arch did not differ between both genotypes. Initial lesions of the non-diet and diet group showed a 7* and 4* increase in collagen content, and a 4.8* and 4.1* increase in SMC content, respectively. Effects of Gas6 deficiency in advanced lesions were more pronounced. In the non-diet group, Gas6^{-/-}/ApoE^{-/-} mice showed a 21.2% and 30.1% decrease in macrophage and lipid core content. This was not observed in the diet group. Gas6^{-/-}/ApoE^{-/-} mice showed an increase in the number of lipid cores per lesion (2.1 vs 1.2 in ApoE^{-/-}, non-diet group; and 3.2 vs 2.0 in ApoE^{-/-}, diet group). Collagen content, as well as SMC content increased 2.2* and 3.6* in the non-diet group, and 1.3* and 2.8* in the diet group. In ApoE^{-/-} mice, SMCs were confined to the shoulder and fibrous cap region, while in Gas6^{-/-}/ApoE^{-/-} mice, a significant amount of SMCs was also present in the lipid core region (23.6% vs 0.8% ApoE^{-/-} in the non-diet, and 19.4% vs 2.7% in ApoE^{-/-} in the diet group). In the diet group, a significant decrease (2.3*) in T-lymphocyte content occurred. Interestingly, severe intraplaque bleeding, associated with iron deposition and fibrin formation, was observed in 11.1% of Gas6^{-/-}/ApoE^{-/-} mice of the non-diet, and in up to 50% of the advanced lesions of the diet-group. In ApoE^{-/-} mice, this phenomenon was only observed in 4% of the advanced plaques of the diet group. In conclusion, absence of Gas6 results in the development of a collagen/SMC rich, differently organized plaque phenotype, containing multiple lipid cores and prone to intraplaque hemorrhage.

Introduction

Gas6 (growth arrest-specific gene 6), was originally identified as a product of a gene, isolated by subtractive hybridization on the basis of preferential expression in the G₀ phase of the cell cycle.¹ Further analysis revealed that Gas6 is a new member of proteins possessing γ -carboxyglutamic acid (GLA), that shares a 44% sequence homology with the anticoagulant factor protein S.² However, Gas6 does not exert the anti-coagulant function of protein S, most probably since it lacks the two thrombin cleavage sites of protein S.

Gas6 binds to members of the Axl family of receptor tyrosine kinases including Axl, Sky and Mer, a family initiating several signaling cascades that determine a wide variety of phenotypic responses.³ Gas6 is expressed in many tissues, including lung, intestine, liver and the endothelium, and the expression of Axl, Sky and/or Mer is often overlapping.⁴ Therefore, it is postulated that Gas6 may exhibit diverse functions through three distinct receptors in a cell- and tissue-dependent manner.

The biological actions of Gas6 are largely unknown. Most of the studies reported, indicate that Gas6 acts as a survival factor.^{1,5-7} Gas6 exerts ambivalent effects on cell turnover in the different cell types. For example, Gas6 is mitogenic in the presence of low serum for fibroblasts^{5,6}, Schwann cells⁸, and enhances the mitogenic activity of thrombin in VSMCs^{2,9}, but it is not mitogenic for myeloid cells¹⁰, or endothelial cells.¹¹ A role for Gas6 in apoptosis is also postulated, since Gas6 plays a role in the cognition and clearing of dying cells by forming a bridge between phosphatidylserine on the surface of apoptotic cells and Axl on phagocytotic cells.¹²

Recently, P. Carmeliet et al. reported that Gas6^{-/-} mice are protected against venous and arterial thrombosis and collagen-induced thrombo-embolism, due to an impaired platelet aggregation. Gas6^{-/-} mice did, however, not suffer spontaneous bleeding and had a normal bleeding time.¹³

Interestingly, increased expression of Gas6 and Axl was observed after arterial balloon injury in rats, and in vitro studies reveal that Gas6 acts as a chemoattractant for human and rat VSMCs.^{9,14} Furthermore, Gas6 inhibits granulocyte adhesion to TNF α stimulated endothelial cells¹¹, and enhances the upregulation of scavenger receptor A, thereby contributing to foam cell formation in VSMCs in vitro.¹⁵

The phenomena described above play an important role in cardiovascular diseases, predominantly in atherosclerosis, suggesting an important role for Gas6 in atherogenesis also. Therefore, we generated mice deficient in Gas6 and ApoE. Mice were fed a normal chow diet or, to stress the phenotype, a severely atherogenic diet, containing 2% cholesterol and 0.5% cholate. Here we report that Gas6^{-/-}/ApoE^{-/-} mice develop a collagen rich, but disorganized plaque phenotype, and when stressed, massive intraplaque hemorrhage.

Materials and methods

Mice

Inactivation of the *Gas6* gene (*Gas6*^{+/-}) in embryonic stem (ES) cells was achieved by replacement of exons 1 and 2 with a neomycin phosphotransferase cassette. Correct targeting was confirmed by Southern blotting. Targeted ES cells were used for generation of chimeric mice and subsequent germline transmission. Apparently healthy *Gas6*^{+/-} and *Gas6*^{-/-} mice were born according to Mendelian inheritance. To generate *Gas6*^{-/-}/*ApoE*^{-/-} mice, *Gas6*^{-/-} mice were backcrossed to C57Bl6 mice, and crossed to *ApoE*^{-/-} mice.

Experimental protocol

Gas6^{-/-}/*ApoE*^{-/-} (n=12) and *ApoE*^{-/-} mice (n=12) of the non-diet group were fed a normal chow diet for 23 wks, while the diet group, consisting of n=8 *Gas6*^{-/-}/*ApoE*^{-/-} and n=12 *ApoE*^{-/-} mice, received a severely atherogenic diet (2% cholesterol, 0.5% cholate) for 15 wks from the age of 5 wks. Two weeks before the end of the experimental period, 5'-bromo-2'-deoxyuridine (BrdU, Serva Heidelberg, Germany, infusion rate 13 mg/kg.day) was infused using osmotic minipumps (Alzet, 2001) in n=5 animals per group. After the experimental period, mice were sacrificed, the arterial tree was excised, and the tissue was processed as described earlier.^{16,17}

Lipid Profile

Plasma cholesterol levels were determined in duplicate by using colorimetric assays (CHOD-PAP 1442341; Boehringer Mannheim).

Histomorphometry

For histo-morphological analysis, the aortic arch, including its main branchpoints, was used (fig 5.2a). Atherosclerotic lesions were divided into initial lesions (fatty streak lesions) and advanced lesions (with a lipid core and/or fibrous cap), according to the AHA classification.¹⁸ Additionally, advanced lesions of the diet-group were subdivided into small (<75,000µm²) and large (>75,000µm²) advanced lesions. Analysis of histo-morphological parameters was performed as described earlier.^{16,17}

(Immuno)histochemistry

For gross histological analysis and morphometry, sections were stained with haematoxylin and eosin (HE) and Lawson solution. Sirius red staining was performed to detect collagen, and Perls iron staining was used to detect iron.

Sections were immunolabeled with α-smooth muscle actin (ASMA^{FTC}, monoclonal 1:3000, Sigma Chemical Co) as a marker for smooth muscle cells and fibroblasts, ED1 (1:10) for the detection of macrophages, CD3 (CD3 polyclonal, 1:200, Dako A0452) for the detection of T-lymphocytes, anti-fibrin (goat monoclonal mouse antibody; 1:800, P. Carmeliet), anti-*Gas6* and anti-*Axl* (both 1:100, P. Garcia de Frutos), anti-BrdU (Mas 250b, Harlan sera labs) for the detection of DNA-synthesizing cells and TUNEL (Boehringer Mannheim) for the detection of apoptosis as described previously.^{16,17}

Statistical analysis

Data are expressed as mean $\pm$ SEM. Gas6^{-/-}/ApoE^{-/-} mice were compared with ApoE^{-/-} mice, using a non-parametric Mann-Whitney U test. The level of statistical significance was set at $p < 0.05$.

Results

General

Body weight, as well as heart weight did not differ between both mouse models. Gross histological analysis (HE staining) did not reveal any abnormalities in liver and spleen, especially no signs of bleeding.

In the non-diet group, cholesterol levels did not differ (Gas6^{-/-}/ApoE^{-/-}: 7.5 ± 0.4 mmol/L, ApoE^{-/-}: 7.0 ± 0.3 mmol/L). In the diet group, cholesterol levels were significantly decreased in Gas6^{-/-}/ApoE^{-/-} mice (30.3 ± 3.3 mmol/L vs 39.3 ± 1.9 mmol/L, $p < 0.05$).

Gas6 and Axl immunolocalisation

Gas6 was present in the atherosclerotic lesion as well as in the normal vessel wall. In the normal vessel wall, Gas6 was confined to the endothelium, whereas in the atherosclerotic lesion, Gas6 immunoreactivity could be observed in the endothelium, plaque SMCs and in some, but not all, macrophages.

Axl immunoreactivity was confined to the atherosclerotic lesion. Axl was present at the endothelium, in all macrophages, and in some of the plaque SMCs.

1. Non-diet group

In total, 49 lesions in the aortic arch of Gas6^{-/-}/ApoE^{-/-} ($n=12$) and 49 lesions of ApoE^{-/-} mice ($n=12$) were analyzed. Total plaque area ($174,057 \pm 119,453 \mu\text{m}^2$ in Gas6^{-/-}/ApoE^{-/-} vs $122,161 \pm 50,971 \mu\text{m}^2$ in ApoE^{-/-}), as well as individual initial ($11,873 \pm 1,595 \mu\text{m}^2$ vs $9,862 \pm 1,640 \mu\text{m}^2$ in ApoE^{-/-}) and advanced plaque area ($46,734 \pm 9,223 \mu\text{m}^2$ vs $79,111 \pm 13,830 \mu\text{m}^2$ in ApoE^{-/-}), or the number of plaques (2.9 ± 0.6 vs 2.8 ± 0.8 in ApoE^{-/-}) and lipid cores (0.7 ± 0.4 vs 1.1 ± 0.4) per aortic arch did not differ between both genotypes.

Initial lesions

Besides a 7 fold increase in collagen-content (fig 5.1a), absence of Gas6 showed no profound effects on initial lesion morphology. Relative ASMA content (fig 5.1b), macrophage content (72.8 ± 2.6 vs $67.9 \pm 2.9\%$) as well as T-lymphocyte content (fig 5.1e) were not different after genetic disruption of Gas6.

Advanced lesions

In contrast to initial lesions, advanced lesions of Gas6^{-/-}/ApoE^{-/-} mice exhibited a completely different plaque composition compared to ApoE^{-/-} mice. Collagen content and ASMA content increased 2.2 fold and 3.6 fold (fig 5.1a-b). However, this did not result in an increased fibrous cap thickness. In Gas6^{-/-}/ApoE^{-/-} mice, ASMA-positive cells were distributed throughout the lesion (38.6% of all ASMA positive cells in the shoulder region, 23.6% in the lipid core region, 37.8% in the

Fig. 5.1
Graphs showing plaque characteristics of *Gas6*^{-/-}/*ApoE*^{-/-} and *ApoE*^{-/-} mice of the non-diet and diet groups showing (a) collagen content, (b) ASMA content, (c) lipid core content, (d) the number of lipid cores per lesion, (e) T-lymphocyte content and (f) the degree of intra-plaque hemorrhage.

fibrous cap region), while in ApoE^{-/-} mice, ASMA positive cells were confined to the shoulder region (36.3%) and fibrous cap region (62.9%). Furthermore, polarization microscopy revealed the presence of increased amounts of immature collagen in Gas6^{-/-}/ApoE^{-/-} mice.

In Gas6^{-/-}/ApoE^{-/-} mice, macrophage content ($42.8 \pm 5.1\%$ vs $54.1 \pm 3.0\%$ in ApoE^{-/-}) and lipid core content decreased (fig 5.1c), while T-lymphocyte content was not affected (fig 5.1e). Interestingly, besides a decrease in lipid core content, the number of lipid cores was significantly increased in Gas6^{-/-}/ApoE^{-/-} mice (fig 5.1d). These results reveal an inhibitory effect of Gas6 deficiency on macrophage infiltration and lipid core formation, although fibrous cap and lipid core seem to develop in a differently organized way.

2. Diet group

In the diet group, 72 lesions of Gas6^{-/-}/ApoE^{-/-} mice and 121 lesions of ApoE^{-/-} mice were analyzed. As in the non-diet group, no differences were found in total plaque area ($1,205,779 \pm 146,540 \mu\text{m}^2$ vs $1,152,157 \pm 136,035 \mu\text{m}^2$ in ApoE^{-/-}), individual initial ($32,742 \pm 18,233 \mu\text{m}^2$ vs $20,452 \pm 4,232 \mu\text{m}^2$ in ApoE^{-/-}) and advanced ($185,431 \pm 20,232 \mu\text{m}^2$ vs $191,076 \pm 17,398 \mu\text{m}^2$ in ApoE^{-/-}) plaque area or the number of plaques (7.2 ± 0.8 vs 6.8 ± 0.6 in ApoE^{-/-}) and lipid cores (6.1 ± 0.7 vs 5.3 ± 0.4 in ApoE^{-/-}) per aortic arch.

Initial lesions

As observed in the non-diet group, initial lesions of the diet group showed a 4.0 fold increase in collagen content and in addition, a 4.1 fold increase in SMC content (fig. 5.1a-b). Other parameters, like macrophage content ($65.3 \pm 6.7\%$ vs $68.0 \pm 3.7\%$ in ApoE^{-/-}) and T-lymphocyte content did not differ between both genotypes (fig 5.1e).

Advanced lesions

Advanced atherosclerotic lesions of the diet group were divided into small (AS) and large (AL) atherosclerotic plaques. As in the non-diet group, lesions exhibited a 0.9 (AS) and 1.3 (AL) fold increase in collagen content and a 2.2 (AS) and 1.6 fold (AL) increase in ASMA content (fig 5.1a-b and fig 5.2b-e). Also in the Gas6^{-/-}/ApoE^{-/-} diet group, ASMA positive cells were distributed equally throughout the lesion (AS: 54.6% shoulder, 32.1% fibrous cap, 13.3% lipid core; AL 36.1%, 44.4% and 19.4% respectively), while in ApoE^{-/-} mice, hardly any ASMA positive cells could be observed in the lipid core region (AS: 1.3%, AL: 2.7%) (fig 5.2d-e). Again, polarization microscopy revealed an increased presence of immature collagen in Gas6^{-/-}/ApoE^{-/-} mice.

Although Gas6 deficiency in the non-diet group inhibited lipid core formation and macrophage influx, these effects were probably masked by the severely atherogenic diet (fig. 5.1c). However, the number of lipid cores/lesion in advanced plaques of the diet group increased even more (fig. 5.1d and fig 5.2f-g). Interestingly, in both small and large advanced lesions of the diet group, T-lymphocyte content decreased 2.6 and 2.2 fold respectively (fig. 5.1e).

These results reveal that absence of Gas6 results in a differently organized plaque phenotype that is rich in collagen and SMCs.

Fig. 5.2

Histological characteristics of *Gas6*^{-/-}/*ApoE*^{-/-} and *ApoE*^{-/-} mice. (a) HE staining of a longitudinal section of the aortic arch, including its main branchpoints (brachiocephalic trunk, left carotid artery and left subclavian artery) of an *ApoE*^{-/-} mouse. (b and c) Sirius Red staining of an advanced lesion of a *Gas6*^{-/-}/*ApoE*^{-/-} and *ApoE*^{-/-} mouse, revealing increased collagen content in the *Gas6*^{-/-}/*ApoE*^{-/-} mouse. (d and e) ASMA staining of an advanced lesion a *Gas6*^{-/-}/*ApoE*^{-/-} and *ApoE*^{-/-} mouse, revealing increased ASMA immunoreactivity in *Gas6*^{-/-}/*ApoE*^{-/-} mouse. Interestingly, in the *Gas6*^{-/-}/*ApoE*^{-/-} mouse, ASMA positive cells were distributed throughout the lesion, while in the *ApoE*^{-/-} mouse, ASMA positive cells were confined to the fibrous cap. (f and g) HE staining of an advanced lesion a *Gas6*^{-/-}/*ApoE*^{-/-} and *ApoE*^{-/-} mouse, revealing a differently organized atherosclerotic lesion, with an increased number of lipid cores in the *Gas6*^{-/-}/*ApoE*^{-/-} mouse.

Fig. 5.3

(a) HE staining revealing severe intra-plaque hemorrhage at the shoulder region of an advanced atherosclerotic lesion of a Gas6^{-/-}/ApoE^{-/-} mouse. (b) Intraplaque hemorrhage in detail, the individual erythrocytes can be appreciated. (c) Perl's iron staining iron deposition. (d) Fibrin staining showing fibrin formation.

Cell turnover parameters

Since it is postulated that Gas6-Axl interactions play a role in survival, mitogenic and apoptotic activities, we investigated the level of DNA-synthesis and apoptosis in advanced and initial atherosclerotic plaques.

Interestingly, absence of Gas6 did not affect cell turnover parameters to a large extent. In initial and advanced lesions of the non-diet group, levels of DNA-synthesis (initial lesions: $5.9 \pm 2.1\%$ vs $6.3 \pm 3.3\%$ in ApoE^{-/-}; advanced lesions: $3.1 \pm 1.7\%$ vs $2.9 \pm 1.4\%$ in ApoE^{-/-}; $p > 0.05$) and apoptosis (initial lesions: $0.4 \pm 0.2\%$ vs $0.4 \pm 0.3\%$ in ApoE^{-/-}; advanced lesions: $1.3 \pm 0.7\%$ vs $1.2 \pm 0.2\%$ in ApoE^{-/-}; $p > 0.05$) did not differ between both genotypes. In the diet group, no differences in cell turnover parameters were observed in initial (DNA-synthesis: $6.8 \pm 2.4\%$ vs $8.7 \pm 2.8\%$ in ApoE^{-/-}; Apoptosis: $0.3 \pm 0.3\%$ vs $0.5 \pm 0.2\%$ in ApoE^{-/-}; $p > 0.05$) and small advanced lesions (DNA-synthesis: $6.2 \pm 2.0\%$ vs $4.2 \pm 0.8\%$ in ApoE^{-/-}; Apoptosis: $0.5 \pm 0.2\%$ vs $0.8 \pm 0.3\%$ in ApoE^{-/-}; $p > 0.05$) of both mouse models. Only in advanced large atherosclerotic lesions, absence of Gas6 induced a significant increase in DNA-synthesis ($5.6 \pm 0.8\%$ vs $3.9 \pm 0.4\%$ in ApoE^{-/-}) and a decrease in

apoptosis ($0.8 \pm 0.1\%$ vs $1.3 \pm 0.2\%$ in ApoE $^{-/-}$; $p < 0.05$). This probably reflects the repair process after intra-plaque hemorrhage.

Intra-plaque hemorrhage

The most remarkable finding of this study was the occurrence of intraplaque hemorrhage. In Gas6 $^{-/-}$ /ApoE $^{-/-}$ mice of the non-diet group, this was observed in 3 out of 27 advanced plaques, while in ApoE $^{-/-}$ mice, this phenomenon was not observed at all. In the diet group, massive intraplaque hemorrhage occurred in 3 out of 16 initial lesions, in 4 out of 23 small advanced plaques, and in 15 out of 30 large advanced plaques (fig. 5.3a and 5.3b). Furthermore, >60% of the intraplaque hemorrhages was associated with fibrin and iron deposition (fig. 5.3c and 5.3d).

Discussion

This manuscript is the first to describe the *in vivo* role of Gas6 during atherogenesis. It reveals that absence of Gas6 results in the development of a collagen rich, but differently organized plaque phenotype. The most remarkable finding of this study is the occurrence of severe intraplaque hemorrhage, associated with iron deposition that, to our knowledge, has never been described before in mice.

In humans, intraplaque hemorrhage is associated with an unstable plaque phenotype, prone to rupture and responsible for most of the acute complications of atherosclerosis.^{19,20} Other parameters of an instable plaque phenotype are a reduced collagen/VSMC content, large lipid cores and massive T-lymphocyte infiltration.^{18,21} Interestingly, these phenomena were not observed in Gas6 $^{-/-}$ /ApoE $^{-/-}$ mice. In these mice, collagen content even increased, and when stressed, T-lymphocyte content decreased, indicating a rather stable plaque phenotype. However, although collagen and VSMC content were increased, they did not cause an increase in fibrous cap thickness. Further analysis revealed that increased amounts of immature forms of collagen were present, and that VSMCs were distributed equally throughout the entire lesion, indicating rather a disorganized plaque phenotype. In addition, lipid cores of Gas6 $^{-/-}$ /ApoE $^{-/-}$ mice were composed of multiple small lipid cores.

Postulated mechanisms of the development of the disorganized plaque phenotype, and subsequent intraplaque hemorrhage are a decreased cell-cell adhesion and a disturbance in tissue organization. The receptor for Gas6, the tyrosine kinase receptor Axl, is a transmembrane protein, whose extracellular domain resembles that of many cell adhesion molecules.²² In insect cells and mammalian cell lines *in vitro*, overexpressing of the murine homologue of Axl, ARK results in a the formation of cell-aggregates.²³ Furthermore, myeloid cells expressing Axl exhibit increased aggregation in response to Gas6 treatment, indicating a role for Gas6-Axl interactions in cell-cell adhesion.²⁴ It is also reported that Gas6 plays a role in leukocyte-endothelial cell interactions. Although Gas6 did not affect adhesion of granulocytes to resting endothelial cells, it inhibited granulocyte adhesion to TNF α activated endothelial cells.¹¹ However, to obtain this effect, high doses of Gas6 were required and the relevance of this study remains questionable.¹¹

During embryogenesis, Axl is expressed from embryonic day 12.5, and is found particularly in mesodermal structures.²⁵ It might therefore play a role in the development and maintenance of mesenchymal organ structure.

Besides impaired cell-cell adhesion, and disturbances in tissue organization, impaired coagulation could also contribute to intra-plaque hemorrhage observed in Gas6^{-/-}/ApoE^{-/-} mice. Interestingly, Gas6 antibodies significantly inhibit platelet aggregation *in vitro*. Furthermore, Gas6^{-/-} mice are protected against venous and arterial thrombosis and against fatal collagen-induced thrombo-embolism.¹³

DNA-synthesis and apoptosis, as well as migration of VSMCs are thought to be important features in atherosclerosis and restenosis.^{16,26,27} In these processes, expression of growth factors or vasoactive substances, that signal through receptor tyrosine kinases, such as PDGF, FGF, angiotensin II and thrombin play a major role.²⁸ It is therefore postulated that Gas6, a ligand for the receptor tyrosine kinase Axl, can exert mitogenic, survival, apoptotic or migratory properties in cardiovascular diseases. Here, we report that absence of Gas6 exerts only little effects on cell turnover parameters. Gas6^{-/-}/ApoE^{-/-} mice exhibit a slight, but significant increase in DNA-synthesis and a decrease in apoptosis, but only in the advanced large lesions of the diet-group. Furthermore, the increased SMC content observed in plaques of Gas6^{-/-}/ApoE^{-/-} mice might reflect increased migration of SMCs.

Other studies, performed after balloon injury in rats, showed that both Gas6, and its receptor Axl were upregulated, with peak expression 7 and 14 days after arterial injury.¹⁴ Gas6 did not result in increased VSMC proliferation, but it induced Axl-mediated chemotaxis of VSMCs.^{9,14} In contrast with these results, we show that Gas6 deficiency might induce an increased VSMC migration. This discrepancy can be explained by the fact that the pathogenesis of primary atherosclerosis and restenosis is different.

Another feature, important in the pathogenesis of atherosclerosis, is the increased uptake of oxLDL by macrophages and VSMCs, resulting in foam cell formation.²⁰ The receptor, responsible for the uptake of oxLDL is the scavenger receptor.²⁹ In literature, it is reported that Gas6 induces the upregulation of scavenger receptor A in VSMCs *in vitro*, thereby postulating a role for Gas6 in foam cell formation.¹⁵ Consistent with these results, we report that absence of Gas6 reduces lipid core content and macrophage content in mice fed a normal chow diet.

In conclusion, we report that Gas6 plays an important role during atherogenesis, and since its absence induces a differently organized, hemorrhage prone plaque phenotype, we postulate that Gas6 is responsible for the maintenance of cell-cell contact and cell organization in the atherosclerotic lesion. In addition, Gas6 deficiency might induce impaired platelet aggregation in atherosclerotic lesions, and might consequently induce severe intraplaque hemorrhage. To our knowledge, this is the first atherosclerotic mouse model that exhibits intra-plaque hemorrhage. Although even in these mice, rupture of the fibrous cap with luminal thrombus formation did not occur, this animal model may provide clues that are also important in human plaque destabilisation.

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Chapter 6

Requirement for CD154 in the progression of atherosclerosis

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Abstract & Introduction

Atherosclerosis is a systemic disease of the large arteries, and activation of inflammatory pathways is important in its pathogenesis.¹ Increasing evidence supports the importance of CD40-CD154 (=CD40L) interactions in atherosclerosis^{2,3}, interactions originally known to be essential in major immune reactions⁴ and autoimmune diseases.⁵ CD40 is present on atheroma-derived cells in vitro and in human atheromata in situ.⁶ Ligation of CD40 on atheroma-associated cells in vitro activates the production of chemokines⁶, cytokines⁶, matrix metalloproteinases^{7,8}, adhesion molecules^{9,10} and tissue factor⁷, substances responsible for lesion progression and plaque destabilization.¹ Administration of antibody against CD154 to low-density lipoprotein receptor-deficient mice has been shown to reduce atherosclerosis and decrease T-lymphocyte and macrophage content; however, only initial lesions were studied.³ Here, we determined the effect of genetic disruption of CD154 in ApoE^{-/-} mice in both initial and advanced atherosclerotic lesions. Plaque area was reduced 5.5 fold. In contrast to previous reports, initial lesion development was not affected. Advanced plaques in CD154^{-/-}/ApoE^{-/-} mice had a less lipid-containing, collagen-rich, stable plaque phenotype, with a reduced T-lymphocyte/macrophage content. These data indicate that CD40-CD154 signaling is important in late atherosclerotic changes, such as lipid core formation and plaque destabilization.

Methods

Mice

CD154^{-/-} mice were generated at Yale University School of Medicine. ApoE^{-/-} mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Both strains of mice were bred into a C57BL/6 background. Mice deficient in both CD154 and ApoE were obtained by mating CD154^{-/-} and ApoE^{-/-} mice. PCR analysis was used to confirm the absence of both genes.

Primer sequences were: 1) 5'-CCTAGCCGAGGGAGAGCCG-3'; 2) 5'-TGT-GACTTGGGAGCTCTGCAGC-3'; and 3) 5'-GCCGCCCGACTGCATCT-3'. Primers 1 and 2 amplify a 155-base-pair wild-type band; primers 1 and 3 amplify a 245 base-pair mutated band. The male progeny of these matings were used here.

Tissue processing

Mice were killed at 23±0.1 weeks of age (both initial and advanced lesions are present at this time), after a 24-hour fast. Blood (0.5-1 ml) was obtained from the inferior caval vein to assess lipid profiles. The arterial tree was perfused, through a catheter inserted into the left ventricular apex, for 3 min with phosphate-buffered saline (PBS), and for 3 min with 10% phosphate-buffered formalin, pH 7.4, both containing 0.1mg/ml sodium nitroprusside (Sigma). The aortic arch, including its main branchpoints (brachiocephalic trunk, left common carotid artery and left subclavian artery) as well as the thoracic and abdominal aorta, were removed and fixed in 10% phosphate-buffered formalin. All vessels were processed and embedded longitudinally in paraffin. The entire aortic arch was then cut into

approximately 40 sections, and a series of 20 sections 4 μ m in thickness, which represented the central area of the arch with an intact morphology of the complete arch, was used for analysis. Plasma cholesterol and plasma triglyceride levels were determined in duplicate using colorimetric assays (CHOD-PAP 1442341 and GPO-PAP701912, respectively; Boehringer).

Histology and morphometry

For histological analyses, (four sections, 20 μ m apart) were stained with hematoxylin and eosin (fig. 6.1). Atherosclerotic lesions of both CD154-/-/ApoE-/- and ApoE-/- mice were then analyzed and classified as either initial or advanced lesions, based on histological criteria, defined by the American Heart Association.¹² The number of atherosclerotic plaques, the presence or absence of lipid cores and calcification, and the total number of cells per lesion were also determined. Lawson staining (modified Elastica von Giesson, Verhoef staining), which exclusively stains the elastic laminae, was used to delineate the internal elastic lamina, to permit an easier distinction between media and intima. Four Lawson-stained sections (20 μ m apart) were used for morphometrical analysis. All morphometric parameters are determined using a microscope coupled to a computerized morphometry system (Quantimet 570, Leica, the Netherlands). The aortic arch region was defined as the aortic root to approximately 1.5 mm caudal from the left subclavian artery branchpoint. Main branchpoints were also included into the measurements: the brachiocephalic trunk, left common carotid artery and left subclavian artery. Plaque area, excluding the underlying media, was determined for each individual plaque. The relative lipid core content was determined by dividing lipid core area by plaque area. The relative fibrous cap thickness was calculated by dividing fibrous cap thickness at the center of the lesion by lesion thickness. The ratio of fibrous cap to lipid core was calculated by dividing fibrous cap thickness by lipid core thickness, both determined at the center of the atherosclerotic lesion. The number of cells per mm² lesion area was also determined. The relative collagen area (that is, the percentage of area that stained positive for Sirius Red) was determined for each atherosclerotic plaque, using the same morphometry system (fig. 6.2a and b).

Immunohistochemistry and morphometry

Sections were immunolabeled with α -smooth muscle actin (ASMA monoclonal antibody, 1:500 dilution; Sigma) as a marker for vascular smooth muscle cells and fibroblasts; ED-1 (1:10 dilution; ref. 20) to detect macrophages; CD3 polyclonal antibody (A0452, 1:200 dilution; Dako, Carpinteria, California) to detect T-lymphocytes; and TdT-mediated dUTP nick end labeling (TUNEL) to detect apoptosis (Boehringer) as described.¹³ Lectin staining (GSI-lectin, 1:500 dilution; Sigma) was done to detect neovascularization. To determine the relative amounts of T-lymphocytes, macrophages and apoptotic cells in an atherosclerotic plaque, the number of CD3-, ED-1- or TUNEL-positive cells in the entire lesion was divided by the total number of cells in the entire atherosclerotic plaque. To assess the distribution pattern of macrophages, T-lymphocytes and apoptotic cells, labeling indices were also determined at the endothelial coverage, shoulder region, lipid core and fibrous cap. To determine the degree of neovascularization, the number of capillaries positive for lectin was divided by the respective plaque area. The relative

area of the atherosclerotic plaque positive for α -smooth-muscle actin was determined by dividing the ASMA-positive area by total plaque area, using the morphometry system. All measurements were made by one investigator (E.L.); intraobserver variation was less than 10%.

Statistics

Data are expressed as mean $\pm$ SEM. CD154 $^{-/-}$ /ApoE $^{-/-}$ mice were compared with ApoE $^{-/-}$ mice by a non-parametric Mann-Whitney U-test. Data were considered statistically significant at $p < 0.05$.

Results and discussion

To obtain more insights into the involvement of CD40-CD154 signaling in atherogenesis, we compared initial and advanced lesion development in CD154-deficient mice bred on an ApoE-deficient background ($n=12$) with that in ApoE $^{-/-}$ mice ($n=10$). We used C57BL6 ($n=5$) and CD154 $^{-/-}$ ($n=6$) mice as controls. Mice were killed at 23 ± 0.1 weeks of age, as the presence of both initial and advanced lesions has been described in the aortic arch at that time.¹¹

We analyzed 52 atherosclerotic lesions in the aortic arches of CD154 $^{-/-}$ /ApoE $^{-/-}$ mice ($n=12$) and 77 atherosclerotic lesions in the aortic arches of ApoE $^{-/-}$ mice ($n=10$) (figs. 6.1-6.3). There were no lesions in the ApoE $^{+/+}$ control mice. Initial lesions were defined as fatty streaks, containing macrophage-derived foam cells with intracellular lipid accumulation, whereas advanced lesions contained extracellular lipid, a lipid core and/or a fibrous cap.¹²

The total plaque area of the aortic arch was 5.5-fold less in CD154 $^{-/-}$ /ApoE $^{-/-}$ mice ($70,467 \pm 7,788 \mu\text{m}^2$ compared with $387,900 \pm 78,077 \mu\text{m}^2$ in ApoE $^{-/-}$) (fig. 6.1a and b). Furthermore, two aortic arches of CD154 $^{-/-}$ /ApoE $^{-/-}$ mice showed complete absence of atherosclerotic lesions, whereas lesion development was not absent in any of the ApoE $^{-/-}$ mice. Although the number of both initial lesions per aortic arch (2.4 ± 0.72 compared with 5.6 ± 1.15 in ApoE $^{-/-}$; $p < 0.05$) and advanced lesions per aortic arch (1.38 ± 0.43 compared with 2.33 ± 0.53 in ApoE $^{-/-}$; $p < 0.05$) were less in CD154 $^{-/-}$ /ApoE $^{-/-}$ mice, individual plaque area was less only in advanced atherosclerotic lesions of CD154 $^{-/-}$ /ApoE $^{-/-}$ mice ($p < 0.05$) (fig. 6.3a). The number of lipid cores per mouse was only 0.6 in CD154 $^{-/-}$ /ApoE $^{-/-}$ mice, whereas it was 2.0 in ApoE $^{-/-}$ mice ($p < 0.05$). We did not find calcification in any of the atherosclerotic lesions of CD154 $^{-/-}$ /ApoE $^{-/-}$ mice, whereas four lesions in ApoE $^{-/-}$ mice had calcified areas.

Further analysis of initial lesions in the aortic arch demonstrated no substantial differences between CD154 $^{-/-}$ /ApoE $^{-/-}$ mice and ApoE $^{-/-}$ mice (fig. 6.3a-d), except for a 76.6% reduction in relative T-lymphocyte content ($p < 0.05$) (fig. 6.3b). To further substantiate the lack of effect of CD154 disruption in lesion initiation, we also analyzed lesions in both the descending thoracic aorta and abdominal aorta. As expected^{11,13}, only initial lesions were present in these vessels. As with the results in the aortic arch, there were no differences in initial lesion development between CD154 $^{-/-}$ /ApoE $^{-/-}$ and ApoE $^{-/-}$ mice (data not shown). These data show a limited effect of CD154 signaling disruption on initial lesion morphology.

Fig. 6.1

Atherosclerotic lesions stained with haematoxylin and eosin. *A* and *C*, CD154-/-/ApoE-/- mice; *B* and *D*, ApoE-/- mice. *A* and *B*, longitudinal section of the aortic arch, including the brachiocephalic trunk, left carotid artery and left subclavian artery. Both the number of lesions and lesion area are smaller in the CD154-/-/ApoE-/- mouse (*A*), than in the ApoE-/- mouse (*B*). *C* and *D*, Advanced atherosclerotic lesion, containing a lipid core and a fibrous cap. The lipid core area is less, and relative fibrous cap thickness is greater in the CD154-/-/ApoE-/- mouse (*C*), than in the ApoE-/- mouse (*D*).

In contrast to the lack of differences in initial lesions, advanced lesions in CD154-/-/ApoE-/- mice had several differences compared with advanced atherosclerotic lesions in ApoE-/- mice. Plaques not only were smaller, but also were of a different composition (fig. 6.1c and d). The relative lipid core content in CD154-/-/ApoE-/- mice was 54% less than that in ApoE-/- mice ($p < 0.05$). Relative fibrous cap thickness was 5-fold more ($p < 0.05$) (fig. 6.2a and b). The ratio of fibrous cap to lipid core was 0.23 ± 0.10 in ApoE-/- mice and 4.42 ± 3.21 in CD154-/-/ApoE-/- mice ($p < 0.05$). Both relative collagen (figs. 6.2a and b and 6.3c) as well as the content of vascular smooth muscle cell/myofibroblast (ASMA) were considerably greater (3.4-fold and 5.8-fold, respectively) in CD154-/-/ApoE-/- mice. Advanced atherosclerotic lesions in CD154-/-/ApoE-/- mice contained 50.2% fewer macrophages and 71.6% fewer T-lymphocytes than those in ApoE-/- mice ($p < 0.05$) (figs. 6.2c and d and 6.3b and d). In both genotypes, macrophages were distributed throughout the plaque, whereas T-lymphocytes were mainly located

Fig. 6.2

Atherosclerotic lesions stained with Sirius Red and an antibody against CD3. *A* and *C*, CD154^{-/-}/ApoE^{-/-} mice. *B* and *D*, ApoE^{-/-} mice. *A* and *B*, Sirius Red staining of the lesions in Fig. 6.1 *C* and *D*, showing a higher relative collagen content in the CD154^{-/-}/ApoE^{-/-} mouse (*A*), than in the ApoE^{-/-} mouse (*B*). *C* and *D*, CD3 staining of an advanced atherosclerotic lesion.

in the shoulder region ($80.6 \pm 11.2\%$ in CD154^{-/-}/ApoE^{-/-} and $80.0 \pm 5.5\%$ in ApoE^{-/-}). Cell density, as well as the degree of neovascularization, did not differ between ApoE^{-/-} and CD154^{-/-}/ApoE^{-/-} mice ($p > 0.05$). These data show that genetic disruption of CD40-CD154 signaling results in a lipid-poor, collagen-rich, stable lesion morphology in advanced atherosclerotic lesions.

The plasma cholesterol and triglyceride levels of CD154^{-/-}/ApoE^{-/-} and ApoE^{-/-} mice were higher than those of either CD154^{-/-} or C57Bl6 mice ($p < 0.05$). However, there were no differences between CD154^{-/-}/ApoE^{-/-} and ApoE^{-/-} mice (cholesterol: 14.18 ± 1.42 mmol/l and 13.92 ± 0.77 mmol/l, $p > 0.05$; triglycerides: 3.13 ± 0.31 mmol/l and 2.29 ± 0.21 mmol/l, $p > 0.05$).

Thus, our data indicate that CD40-CD154 interactions are essential in the progression from initial to advanced atherosclerotic lesions, but seem to be not required for the initiation of atherosclerosis or lipid metabolism. Furthermore, late atherosclerotic changes, such as macrophage accumulation, lipid core formation and destabilization of the atherosclerotic plaque, all seem to depend on CD40-CD154 interactions.

A reduced plaque area as well as a decreased T-lymphocyte and macrophage content has been shown before in atherosclerotic plaques after inhibition of CD40-CD154 signaling.³ However, our study showed a more profound reduction of the progression of atherosclerosis and a completely different plaque phenotype. These

differences can be explained as follows. The previous study inhibited the CD40-CD154 system for only 12 weeks by administering an antibody directed against CD154 (ref. 3), whereas we used mice with a completely disrupted CD154 gene expression. Furthermore, in our study, we used the ApoE^{-/-} mouse as a model of atherosclerosis, whereas the low-density lipoprotein receptor-deficient mouse was used before.³ The ApoE^{-/-} mouse develops atherosclerosis more aggressively, and advanced atherosclerotic lesions are already present at the age of 15 weeks, even with a normal diet.¹¹ In low-density lipoprotein receptor-deficient mice, atherosclerotic lesions progress very slowly and advanced fibrofatty lesions are not seen until 8.5 months of high-cholesterol feeding.¹⁴ The previous study³ was concentrated on the effects of inhibition of the CD40-CD154 system in low-density lipoprotein receptor-deficient mice after 12 weeks of high-cholesterol feeding, so it focused on initial atherosclerotic lesions only. In contrast, we specifically sought to determine the effects of complete inhibition of the CD40-CD154 system on both initial and advanced lesions of ApoE^{-/-} mice, and we found the most prominent effect on advanced lesion progression and composition.

Both CD40 and CD154 are present on several cell types, including activated T-lymphocytes, macrophages, vascular smooth muscle cells and endothelial cells in the atherosclerotic plaque *in vivo*.^{3,8} Stimulation of the CD40-CD154 pathway in these cell types *in vitro* results in the activation of different pro-atherogenic pathways.^{6,8} The absence of CD154 may inhibit several of these pathways.

As CD154 is present on T-lymphocytes, but only when they are activated³, and as CD154 is required for T-cell priming¹⁵, and intimal T-lymphocytes are activated¹, it is likely that T-lymphocytes in CD154^{-/-}/ApoE^{-/-} mice are not activated and are not able to activate pro-atherogenic pathways, which, as our data indicate, is more important for lesion progression than for lesion initiation. Activated T-lymphocytes can induce the expression of several cytokines important in lesion progression and plaque destabilization.¹ However, in double-knockout studies, the effects of interventions in the immune system are heterogenic. Like the lesions in CD154^{-/-}/ApoE^{-/-} mice, the atherosclerotic lesions in ApoE^{-/-}/interferon- γ deficient mice have a collagen-rich, lipid-poor stable plaque phenotype¹⁶, whereas ApoE^{-/-} mice deficient in both T-lymphocytes and B-lymphocytes have a reduced plaque area of initial atherosclerotic lesions, but fail to show a plaque phenotype different from that of normal ApoE^{-/-} mice.¹⁷

The reported distribution of CD154 in the plaque^{3,8}, the heterogeneous outcome of several interventions in the immune system^{16,17} and the results of our study indicate involvement of the CD40-CD154 pathway in atherosclerosis between cell types other than T-lymphocytes and B-lymphocytes. Another cell type considered important during atherogenesis is the macrophage.¹ Plaque macrophages either induce the expression of or are activated by most of the pro-atherogenic chemokines, cytokines, adhesion molecules and proteolytic enzymes in atherosclerotic plaques. Furthermore, macrophages can express tissue factor and are able to oxidize lipids, all factors essential for lesion formation and progression.¹ Substantial evidence indicates CD40-CD154 interactions are important in these processes.^{2,3,9,18} Therefore, inhibition of T-lymphocyte and macrophage functions

Fig. 6.3

Quantification of plaque characteristics of CD154^{-/-}/ApoE^{-/-} and ApoE^{-/-} mice. A, individual plaque area, B, T-lymphocyte content, C, collagen content, and D, macrophage content were measured in initial and advanced lesions of CD154^{-/-}/ApoE^{-/-} mice (white), and ApoE^{-/-} mice (black).

dependent on CD40-CD154 interactions might cause the different plaque phenotype in CD154^{-/-}/ApoE^{-/-} mice.

The different, more stable plaque phenotype in CD154^{-/-}/ApoE^{-/-} mice emphasizes the importance of this pathway in plaque destabilization. Our study has shown that complete absence of CD154 during atherogenesis resulted in an increased extracellular matrix component (possibly by a decreased degradation by matrix metalloproteinases⁷), increased fibrous cap thickness, a decreased content of inflammatory cells and a decreased lipid core content, all factors that are favorable for plaque stability. Another important factor inducing plaque instability is apoptosis in the lipid core and fibrous cap.^{13,19} Although the degree of apoptosis was not different for ApoE^{-/-} and CD154^{-/-}/ApoE^{-/-} mice (initial lesions, $0.7 \pm 0.7\%$ and $0.8 \pm 0.6\%$; advanced lesions, $1.1 \pm 0.2\%$ and $1.2 \pm 0.3\%$), the distribution pattern differed. In CD154^{-/-}/ApoE^{-/-} mice, apoptotic cells were distributed throughout the plaque, whereas in ApoE^{-/-} mice, apoptosis was confined mostly to the center of

the plaque (62.8% in ApoE^{-/-} mice compared with 19.7% of all apoptotic cells in CD154^{-/-}/ApoE^{-/-} mice; $p < 0.05$), which may make lesions of CD154^{-/-}/ApoE^{-/-} mice less rupture-prone.

Thus, interruption of CD40 signaling may provide a therapeutical target in atherogenesis. Antibodies directed against CD154 or CD40 might not prevent the initiation of atherosclerosis, but may be beneficial in inhibiting lesion progression and in increasing the stability of the atherosclerotic plaque, thereby decreasing the acute manifestations of atherosclerosis.

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Chapter 7

Both early and delayed anti-CD40L antibody treatment induces a stable plaque phenotype

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Abstract

In the present study, we investigated the role of the CD40L-CD40 pathway in a model of progressive atherosclerosis. ApoE^{-/-} mice were treated with an anti-CD40L antibody or a control antibody for 12 wks. Antibody treatment started early (age 5 wks) or was delayed until the establishment of atherosclerosis (age 17 wks). In both the early and delayed treatment groups, anti-CD40L antibody did not decrease plaque area or inhibit lesion initiation or age-related increase in lesion area. The morphology of initial lesions was not affected, except for a decrease in T-lymphocyte content. Effects of anti-CD40L antibody treatment on the morphology of advanced lesions were pronounced. In both the early and delayed treatment groups, T-lymphocyte content was significantly decreased. Furthermore, a pronounced increase in collagen content, vascular smooth muscle cell/myofibroblast content, and fibrous cap thickness was observed. In the delayed treatment group, a decrease in lipid core and macrophage content occurred. Interestingly, advanced lesions of anti-CD40L antibody-treated mice exhibited an increased transforming growth factor β , immunoreactivity, especially in macrophages.

In conclusion, both early and delayed treatment with an anti-CD40L antibody do not affect atherosclerotic lesion initiation but do result in the development of a lipid-poor collagen-rich stable plaque phenotype. Furthermore, delayed treatment with anti-CD40L antibody can transform the lesion profile from a lipid-rich to a lipid-poor, collagen-rich phenotype. Postulated mechanisms of this effect on plaque phenotype are the down-regulation of pro-inflammatory pathways and upregulation of collagen-promoting factors like transforming growth factor β .

Introduction

Increasing evidence suggests a central role for the CD40L-CD40 signaling pathway in several immunogenic and inflammatory processes, including atherosclerosis. The interaction between CD40L (CD154, gp39) and CD40, members of the tumor necrosis factor (TNF) and TNF-receptor family, respectively, was originally thought to be restricted to T- and B-lymphocytes.¹ However, this interaction is now found to play an important role in several autoimmune diseases, including the X-linked hyper-IgM syndrome², collagen-induced arthritis³, allergic encephalitis and multiple sclerosis⁴, and acute and chronic graft versus host disease.^{5,6}

An important role for CD40L-CD40 signaling in atherosclerosis has been reported.⁷⁻⁹ In atherosclerotic plaques of mice and humans, CD40L and CD40 are present on vascular smooth muscle cells (VSMCs), endothelial cells, macrophages, and T lymphocytes.^{8,9} In vitro stimulation of CD40L-CD40 signaling in atheroma-derived cells¹⁰ results in the activation of proatherogenic pathways, like the production of chemokines¹⁰, cytokines¹⁰, matrix metalloproteinases^{9,11}, tissue factor¹¹, and leukocyte adhesion molecules.¹²⁻¹⁴

Recently, we reported an important role for CD40L-CD40 interactions in the progression of atherosclerosis by using mice deficient in CD40L and apoE. We showed a dramatic decrease in plaque area in CD40L^{-/-}/apoE^{-/-} mice compared with control apoE-deficient animals. Moreover, advanced atherosclerotic lesions of these mice showed a lipid-poor collagen-rich stable plaque phenotype, with reduced macrophage and T-lymphocyte content.⁷ Furthermore, administration of an anti-CD40L antibody to LDL-R^{-/-} mice, when started early in the development of atherosclerosis, inhibited lesion initiation.⁸

In this study, we investigated further the role of the CD40L-CD40 pathway in atherosclerotic plaque development and progression. An anti-CD40L antibody was administered to apoE^{-/-} mice for 12 wks, either at the onset of atherosclerosis (early treatment) or after the development of advanced plaques (delayed treatment). Anti-CD40L antibody treatment affected neither plaque area nor the age-related increase in plaque area. The most prominent effect of anti-CD40L antibody treatment in both treatment groups was the development of a lipid-poor, collagen-rich stable plaque phenotype, a phenotype similar to that in CD40L^{-/-}/apoE^{-/-} mice.⁷

Because most acute complications of atherosclerosis, like myocardial infarction and cerebrovascular accidents, are the result of a rupture of an unstable lipid-rich, collagen-poor lesion¹⁵, anti-CD40L antibody treatment may prevent the acute complications of advanced atherosclerosis.

Methods

Mice

ApoE^{-/-} mice (Iffa Credo), on a normal chow diet, received either a hamster anti-CD40L antibody or a hamster control IgG, generously provided by Biogen Inc. (Cambridge, MA, USA), at 500 µg per mouse by i.p. injection once per week for 12 wks. The early treatment group started at 5 wk of age (n=9 anti-CD40L, n=8 control), when hardly any atherosclerotic lesions were present. The delayed treatment group

(n=8 anti-CD40L, n=9 control) started at 17 wks of age, the time point at which advanced atherosclerotic plaques have developed.

Lipid Profile

Plasma cholesterol and plasma triglyceride levels were determined in duplicate by using colorimetric assays (CHOD-PAP 1442341 and GPO-PAP 701912, respectively; Boehringer Mannheim).

Histomorphometry

Atherosclerotic plaques were divided into initial and advanced lesions. Initial lesions were defined as fatty streaks containing macrophage-derived foam cells with intracellular lipid accumulation (AHA type II) or pools of extracellular lipid (AHA type III), whereas advanced lesions contained extracellular lipid, a lipid core (AHA type IV), and/or a fibrous cap (AHA type Va-c).¹⁶ Tissue processing, histological classification, and morphometry were performed as described previously.^{7,17}

Immunohistochemistry

Sections were immunolabeled with α -smooth muscle actin ((ASMA)^{FITC} monoclonal, 1:3,000; Sigma) as a marker for vascular smooth muscle cells and fibroblasts, ED-1²⁰ (1:10) for the detection of macrophages, CD3 (CD3 polyclonal, 1:200; Dako A0452) for the detection of T lymphocytes, anti-transforming growth factor β 1 (Bionostics, Wyboston, Bedfordshire, U.K.) for the detection of transforming growth factor (TGF) β 1, BrdUrd (Mas 250b, Harlan Laboratories, Haslett, MI) for the detection of DNA-synthesizing cells, and terminal deoxynucleotidyltransferase-mediated UTP end labeling (Boehringer Mannheim) for the detection of apoptosis, as described previously.^{7,17}

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Anti-CD40L antibody-treated apoE^{-/-} mice were compared with control-treated apoE^{-/-} mice. Anti-CD40L antibody-treated apoE^{-/-} mice of the delayed treatment group were also compared with control-treated 17-wk-old apoE^{-/-} mice to investigate plaque progression after treatment. For all analyses, a nonparametric Mann-Whitney U test was used. The level of statistical significance was set at $p < 0.05$.

Results

General

No significant differences in age, body weight, cholesterol, or triglyceride levels were observed between anti-CD40L antibody- and control-treated animals. Macroscopic and histological analysis of lungs, liver, intestine, spleen, and kidneys revealed no pathology in either anti-CD40L antibody- or control-treated mice. One mouse of the early anti-CD40L antibody treatment group and one mouse of the delayed treatment group died during the experiment.

Early Treatment

In total, 57 atherosclerotic lesions in the aortic arch of the anti-CD40L antibody-treated ($n=8$) and 49 lesions of control-treated animals ($n=8$) were analyzed. Total plaque area ($24,625 \pm 11,290 \mu\text{m}^2$ per aortic arch, anti-CD40L vs. $32,485 \pm 15,061 \mu\text{m}^2$ per aortic arch, control) and individual initial and advanced plaque area did not differ between the treatment groups (fig. 7.1a). Furthermore, no differences between the treatment groups could be observed in the number of initial (4.6 ± 0.7 anti-CD40L and 4.0 ± 0.8 control) or advanced (2.5 ± 0.5 vs. 2.4 ± 0.7) plaques, or in the number of lipid cores (0.8 ± 0.4 vs. 0.9 ± 0.3) or chondrocyte-containing plaques (0.4 ± 0.1 vs. 0.3 ± 0.1) per aortic arch.

Initial Lesions

Detailed histomorphological analysis revealed that no parameters except T-lymphocyte content differed between anti-CD40L antibody and control-treated groups. The relative T-lymphocyte content was $0.4 \pm 0.2\%$ in the anti-CD40L antibody-treated group, whereas it was $4.4 \pm 1.3\%$ in the control-treated group ($p < 0.05$) (fig. 7.1b). Relative macrophage content and collagen content, as well as ASMA content, did not differ between the treatment groups (fig. 7.1c–f). Also, no differences in cell number ($7,941 \pm 1,416 \mu\text{m}^2$ in anti-CD40L vs. $5,718 \pm 1,016 \mu\text{m}^2$ in control), DNA synthesis ($3.7 \pm 1.6\%$ vs. $13.6 \pm 5.8\%$), or apoptosis ($0.3 \pm 0.2\%$ vs. $0.4 \pm 0.3\%$) could be observed ($p < 0.05$). The ratio between type II and type III lesions did not differ between both treatment groups (6.4 ± 2.4 vs. 3.6 ± 1.8) ($p < 0.05$).

To substantiate further the lack of effect of early treatment on lesion initiation, lesions of descending thoracic and abdominal aorta were also analyzed. As expected, only small initial lesions were present. No differences in initial lesion development were found between the treatment groups.

Advanced Lesions

In contrast to initial lesions, analysis of advanced atherosclerotic lesions revealed several differences in plaque composition between the treatment groups. First of all, a 48% reduction in T-lymphocytes was observed in the anti-CD40L antibody-treated mice ($p < 0.05$) (fig. 7.1b). Furthermore anti-CD40L antibody treatment resulted in an 81.2% increase in collagen content and a 325% increase in ASMA content ($p < 0.05$) (fig. 7.1e and f). Thus, fibrous caps were significantly thicker in anti-CD40L-treated animals (37.9 ± 7.2 vs. $15.2 \pm 0.6 \mu\text{m}$) ($p < 0.05$). No differences could be observed in macrophage content, lipid core content (fig. 7.1c and d), and chondrocyte content. Also, total cell number ($4,161 \pm 485$ vs. $5,886 \pm 2,632 \mu\text{m}^2$), as well as the fraction of DNA-synthesizing ($2.9 \pm 1.2\%$ vs. $3.2 \pm 0.9\%$) or apoptotic ($1.2 \pm 0.3\%$ vs. $3.2 \pm 0.9\%$) cells did not differ.

The ratio between type IV and V lesions did not differ between anti-CD40L antibody- and control-treated mice (1 ± 0.3 vs. 1 ± 0.5) ($p < 0.05$). However, subdivision of type V lesions into either type Va (lipid rich) and type Vc (fibrous), revealed that in the anti-CD40L antibody-treated group, 50% of type V lesions were confined to type Vc (fibrous), whereas in the control-treated group, most of type V lesions were type Va (lipid-core rich) (87.5%) ($p < 0.05$).

Fig. 7.1 Quantification of plaque characteristics of both early and delayed anti-CD40L antibody- and control-treated apoE^{-/-} mice. A, Individual plaque area; B, T-lymphocyte content; C, macrophage content; D, lipid core content; E, collagen content; F, ASMA content. * $p < 0.05$, anti-CD40L antibody vs. control; # $p < 0.05$, 17-wk control treatment group (age at which delayed treatment started) vs. delayed anti-CD40L antibody treatment group or delayed control treatment group.

Delayed Treatment

Seventy-six atherosclerotic lesions of the anti-CD40L antibody-treated group (n=8 mice) and 87 lesions of the control-treated group (n=8) were analyzed. Concordant with the results of the early treatment group, no differences could be observed in total plaque area ($430,947 \pm 64,377 \mu\text{m}^2$ vs. $414,133 \pm 64,158 \mu\text{m}^2$) (fig. 7.2a and b), individual initial or advanced plaque area (fig. 7.1a), the number of either initial (5.0 ± 0.5 vs. 6.0 ± 0.5 per aortic arch) or advanced (4.3 ± 0.7 vs. 4.6 ± 0.7 per aortic arch) plaques, and the number of chondrocyte-containing lesions (0.3 ± 0.3 vs. 0.6 ± 0.4) between anti-CD40L antibody- and control-antibody-treated animals ($p < 0.05$). However, delayed treatment with anti-CD40L antibody decreased the number of lipid cores (2.7 ± 0.7 vs. 3.9 ± 0.5 per aortic arch) ($p < 0.05$).

Initial Lesions

As in the early treatment group, detailed histo-morphological analysis showed a reduction (67%) in T-lymphocyte content after anti-CD40L antibody treatment ($p < 0.05$) (fig. 7.1b). Macrophage content (fig. 7.1c), collagen content (fig. 7.1e), total cell number ($4,355 \pm 677$ vs. $4,337 \pm 872 \mu\text{m}^2$), DNA synthesis ($4.5 \pm 1.8\%$ vs. $5.2 \pm 1.2\%$), apoptosis (0.1 ± 0.1 vs. 0.5 ± 0.2), and the ratio between type II and III lesions were not affected (7.8 ± 1.0 vs. 2.6 ± 0.9) after anti-CD40L antibody treatment ($p < 0.05$).

Advanced Lesions

As in advanced lesions of the early treatment group, lesions of the anti-CD40L antibody-treated mice of the delayed treatment group also exhibited a decrease in T-lymphocyte content (58%), and increases in collagen content (64.2%), ASMA content (269.2%), and fibrous cap thickness (54.4%) were observed ($p < 0.05$) (fig. 7.1b–f, fig. 7.2c–h). However, in the delayed treatment group, changes in plaque phenotype were more pronounced. Macrophage and lipid core content had decreased significantly compared with the control treated animals (26% and 48%) ($p < 0.05$) (fig. 7.1c–d, fig. 7.2c, d, i, and j). Also, the chondrocyte positive area of chondrocyte containing lesions was significantly enlarged ($19.4 \pm 4.6\%$ in anti-CD40L vs. $6.8 \pm 1.2\%$ in control) ($p < 0.05$). The ratio between type IV and type V lesions did not differ between both treatment groups (0.4 ± 0.2 in anti-CD40L antibody vs. 0.9 ± 0.4 in control) ($p < 0.05$). However, in anti-CD40L antibody treated animals, 79.2% type V lesions were type Vc (fibrous), compared with 20.8% in control-treated animals.

Plaque Progression

To investigate the effects of delayed anti-CD40L antibody treatment on plaque progression, we also compared the delayed anti-CD40L antibody treatment group (age 29 wk) with the 17-wk-old control-treated group (the age at which delayed antibody treatment started). This revealed that anti-CD40L antibody treatment did not prevent the age-related increase in plaque area (fig. 7.1a) or the increase in the number of initial (4.0 ± 0.8 to 6.0 ± 0.5) or advanced (2.4 ± 0.8 to 4.6 ± 0.7) plaques ($p < 0.05$). However, several phenotypical changes occur with anti-CD40L antibody treatment. The percentages of T-lymphocytes decreased in both initial (56%) and

Fig. 7.2
Histological characteristics of delayed anti-CD40L antibody treatment. (A and B) Hematoxylin and eosin-stained longitudinal section of the aortic arch, including the brachiocephalic trunk, left carotid, and left subclavian artery (*25). Neither lesion area nor the number of lesions differed between anti-CD40L antibody- and control-treated mice. (C and D) Advanced atherosclerotic lesion, containing a lipid core and a fibrous cap. The relative lipid core area is less, and relative fibrous cap thickness is increased in anti-CD40L antibody (D) compared to control-treated mice (C). (E and F) Sirius red staining of advanced atherosclerotic lesions, showing a higher relative collagen content in the anti-CD40L antibody-treated mouse (F) than in the control-treated mouse (E). (G and H) ASMA staining of advanced atherosclerotic lesions, showing a higher relative VSMC/myofibroblast content in the anti-CD40L-treated mouse (H) than in the control-treated mouse (G). (I and J) ED-1 staining of advanced atherosclerotic lesions, showing a decreased relative macrophage content in the anti-CD40L-treated mouse (J) compared to the control-treated mouse (I).

Fig. 7.3

Anti-CD40L antibody treatment induces increased immunoreactivity of TGFβ1. (A and B) TGFβ1 immunostaining of advanced atherosclerotic lesions, showing an increased immunoreactivity of TGFβ in macrophages of the anti-CD40L antibody-treated mouse (B) compared with the control-treated mouse (A).

advanced lesions (59%) ($p < 0.05$) (fig. 7.1b), as did the macrophage content (30%) ($p < 0.05$) (fig. 7.1b and c). Relative to the 17-wk-old animals, anti-CD40L antibody treatment also completely inhibited lipid core expansion (fig. 7.1d). During anti-CD40L antibody treatment, collagen content, ASMA content, and fibrous cap thickness increased as compared with the 17-wk-old control-treated group (59%, 392%, and 183%, respectively) ($p < 0.05$) (fig. 7.1 e–f). These data reveal that anti-CD40L antibody treatment can modify the lesion profile from lipid-rich collagen-poor lesions into a lipid-poor, collagen-rich stable plaque phenotype.

TGFβ

Because TGFβ is known to reduce inflammation and induce tissue fibrosis and extracellular matrix synthesis¹⁸, we hypothesized that anti-CD40L antibody treatment induces up-regulation of TGFβ, thereby contributing to the stable atherosclerotic plaque phenotype. In support of that hypothesis, anti-CD40L antibody-treated mice showed an increased TGFβ1 immunoreactivity in macrophage-rich lesions of advanced lesions, whereas fibrous regions did not stain for TGFβ1 (fig. 7.3 a and b).

Discussion

The present study describes the effects of both early and delayed treatment with an anti-CD40L antibody in a mouse model of atherosclerosis. First, it reveals that interruption of the CD40L-CD40 pathway does not inhibit lesion initiation or age-related increase in lesion area, especially because anti-CD40L antibody treatment

affected neither initial lesion area nor phenotype in early and delayed treatment groups. Secondly, anti-CD40L antibody treatment results in the development of a lipid-poor, collagen-rich advanced stable plaque phenotype, with a reduced T-lymphocyte and macrophage content. The observation that the change of phenotype was most profound in advanced lesions of the delayed treatment group stresses the importance of CD40-CD40L signaling in late atherosclerotic changes.

The lipid-poor collagen-rich atherosclerotic plaque phenotype is concordant with the phenotype we observed after complete genetic disruption of CD40L-CD40 signaling.⁷ In the present manuscript, we show that anti-CD40L antibody treatment results in the development of a similar stable plaque phenotype. Moreover, we show that anti-CD40L antibody treatment is able to transform lipid-rich established atheromata into the lipid-poor collagen-rich stable plaque phenotype, implying a beneficial role for anti-CD40L antibody in the treatment of human atherosclerosis.

Activation of CD40L-CD40 signaling is associated with upregulation of proinflammatory pathways, important in the progression of atherosclerosis and plaque destabilization. First, after activation of CD40L-CD40 signaling, T-lymphocytes and macrophages produce several proatherogenic chemokines (MCP-1, IL-8, RANTES) and cytokines (TNF α , IL-6).¹⁰ Second, activation of the CD40-CD40L pathway in endothelial cells results in the upregulation of intercellular adhesion molecule, vascular cell adhesion molecule, and E-selectin¹²⁻¹⁴, and third, CD40-CD40L signaling induces the up-regulation of matrix metalloproteinases (MMP 1, 3, 6, 9).^{9, 11} These substances are associated with plaque macrophage recruitment, expansion of the lipid core, inhibition of collagen synthesis, and degradation of the fibrous cap *in vivo*.¹⁹ Inhibition of pro-inflammatory pathways might therefore have caused the observed lipid-poor, collagen-rich plaque phenotype. Furthermore, both activated platelets and fresh thrombi express high levels of CD40L²⁰, and T-cell-mediated activation of the CD40L-CD40 system results in the upregulation of tissue factor in macrophages and VSMCs.^{11, 21} Patients suffering from unstable angina show elevated levels of degranulated platelets and sCD40L plasma levels.²² Therefore, CD40L-CD40 signaling may induce a procoagulant stage, a feature favoring the development of plaque rupture and thrombosis.²³

Another postulated mechanism responsible for the change into a more stable plaque phenotype may be a different pattern of cell turnover. The influence of CD40L-CD40 signaling on cell turnover is, however, contradictory. It stimulates proliferation of B cells and Burkitt's lymphoma cells, whereas it inhibits proliferation of B-cell lymphoma cells.²⁴⁻²⁶ Furthermore, CD40 regulates apoptosis in a dual fashion, depending on the up-regulation of apoptosis resistance factors.²⁴ In the present study, anti-CD40L antibody treatment did not affect cell turnover, because total cell number and the level of DNA synthesis and apoptosis did not differ from control-treated animals. Alternatively, anti-CD40L antibody treatment might cause cell turnover of specific cell types (VSMC), thereby resulting in a different plaque phenotype. Selectivity of both DNA synthesis and apoptosis in atherosclerosis was shown before in ApoE*3Leiden mice, where both DNA-synthesis and apoptosis are confined to macrophage-derived foam cells.¹⁷ However, in the present study, too, DNA-synthesis and apoptosis were confined to the macrophage, making

involvement of the CD40L-CD40 system in cell turn-over during atherogenesis unlikely.

Interestingly, our study showed an increased immunoreactivity of TGF β 1 in lesions of anti-CD40L antibody-treated mice. Effects of TGF β on atherosclerosis are, however, contradictory. On the one hand, TGF β seems to have anti-atherogenic effects, because it prevents atherosclerosis in animals^{27, 28} and because patients with severe coronary artery disease express low levels of TGF β .²⁹ In addition, TGF β can stimulate the synthesis of several extracellular matrix proteins^{30,31}, inhibit the expression of matrix metalloproteases, enhance the expression of tissue inhibitors of matrix metalloproteinases (TIMPs)³², and induce tissue fibrosis³³. Therefore, up-regulation of TGF β might be responsible for the development of collagen-rich stable atherosclerotic plaques after anti-CD40L antibody treatment by stimulation of collagen production and inhibition of collagen degradation (down-regulation of MMPs and up-regulation of TIMPs). On the other hand, TGF β and its receptors were abundantly expressed in human fatty streaks and fibrofatty lesions³⁴, and TGF β induces the production of lipoprotein-trapping proteoglycans³⁵, indicating a stimulatory role of TGF β in the progression of atherosclerosis.

Although we and others report an increase of plaque collagen content after inhibition of CD40-CD40L signaling^{7, 36}, it has been shown that anti-CD40L antibody treatment prevents lung fibrosis after irradiation-induced injury.³⁷ Although we have no clear explanation for this phenomenon, the different tissue characteristics (artery vs. lung) and the different pathogenesis of the two models might play a role. In both models, inhibition of CD40-CD40L signaling results in a down-regulation of inflammation. However, in atherosclerosis, anti-CD40L antibody treatment results in the upregulation of TGF β , a molecule known to induce collagen synthesis, whereas in irradiation-induced lung injury, a down-regulation of TGF β is suggested.³⁷

In conclusion, we have shown that inhibition of CD40L-CD40 signaling in mice with advanced atherosclerotic lesions results in the development of a lipid-poor collagen-rich stable plaque phenotype. Therefore, treatment with an anti-CD40L antibody in humans might be beneficial, not by inhibiting lesion initiation or progression, but by transforming unstable plaques into stable atherosclerotic lesions, thereby reducing the acute complications of atherosclerosis.

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Chapter 8

General Discussion

8.1 Introduction

In this thesis, we characterized several hyperlipidemic mouse models and investigated the role of cell turnover and inflammation on atherosclerotic plaque progression and phenotype in these models. Cell turnover was also studied in human atherosclerotic plaques.

The main findings of these studies are that:

1. ApoE*3Leiden mice show a cranio-caudal atherosclerotic lesion progression, with plaque development predominantly at aortic branchpoints.
2. Both ApoE^{-/-} and ApoE*3Leiden mice develop arterial compensatory enlargement and carotid artery stenosis, but fail to develop constrictive remodeling.
3. In both ApoE*3Leiden mice and humans, DNA-synthesis is an early feature of atherosclerosis, while apoptosis is a late phenomenon. The cell-type that predominantly exhibits DNA-synthesis and apoptosis is the macrophage.
4. An intervention study with genetic inhibition of Gas6, a survival factor, does not exert effects on cell turnover, but results in the development of a differently organized plaque with a rupture prone plaque phenotype.
5. Inhibition of a major regulator of inflammatory and immune reactions, the CD40-CD40L system, results in the development of a lipid-poor, collagen-rich, stable plaque phenotype.

New insights into the pathogenesis of atherosclerosis

8.2 Cell turnover during atherogenesis. Is it important?

In 1858, R. Virchow, the founder of cellular pathology stated, that cell proliferation and cell death were important players in the pathogenesis of atherosclerosis.¹ Later, DNA-synthesis and apoptosis (programmed cell death) were observed in several model systems of atherosclerosis. However, even at present, the question whether cell turnover is a key event in atherosclerosis remains largely unanswered.

8.2.1 DNA-synthesis

Since the evolvement of the 'response to injury' hypothesis (chapter 1), atherosclerotic plaques were considered to arise by proliferation of cells within the arterial intima in response to injury.^{2,3} Evidence for this hypothesis was obtained from data of model systems of arterial injury, predominantly the balloon injured rat carotid artery.⁴ As the 'response to injury' hypothesis stated, arterial injury caused elevated levels of DNA-synthesis for weeks to months after the injury, resulting in neointima formation.⁵ Substances responsible for proliferation and neointima formation were identified, like PDGF, FGF and angiotensin II.⁶⁻⁸ Furthermore, intervention studies with their antagonists, as well as gene transfer studies with cell-cycle inhibitors (like p21,p27) were successful in inhibiting DNA-synthesis and in reducing neointima formation in the rat model.⁸⁻¹⁰

At that time, the rat arterial injury model was considered as a model system for the pathogenesis of primary atherosclerosis, but only little evidence for a role of

DNA-synthesis in primary atherosclerosis could be obtained. In fat-fed swine and rabbits, a transient increase in DNA-synthesis, with peak levels around 5% was observed^{11,12}, whereas studies in human primary atherosclerotic lesions show that levels of DNA-synthesis were extremely low (0-2%).¹³⁻¹⁷ Although interventions in model systems of arterial injury were successful, these positive results could not be duplicated in primary atherosclerosis. Thus, the role of DNA-synthesis in the pathogenesis of primary atherosclerosis remains questionable. However, there are several possible explanations for the apparent discrepancy in DNA-synthesis levels between arterial injury models and primary atherosclerosis.

First of all, in contrast to arterial injury models, atherosclerosis is a slowly developing disease, taking decades to become clinically manifest. Hence, the low level of DNA synthesis during primary atherogenesis as opposed to the process of neointima formation after balloon injury, may be explained by the rapidity of onset of neointima formation. This may require a short lasting burst of intensive DNA synthesis, while in the chronic process of atherogenesis, a low level DNA synthesis that is maintained for many years may contribute significantly to the progression of the disease.

Alternatively, it is possible that DNA-synthesis is important in some, but not all stages of atherosclerosis^{3,18}, for example during lesion initiation, or during plaque repair. Interestingly, in ApoE⁻³Leiden mice, as well as in humans, we observed a peak in DNA-synthesis in early type II (fatty streak) lesions.^{17,19} This peak of early DNA-synthesis was consistent with the transient peak observed before in the fat-fed swine and rabbit.^{11,12} This early peak in DNA-synthesis was not observed before in human atherosclerotic lesions. Since most of the studies focused on advanced atherosclerotic lesions¹³⁻¹⁶, an early proliferative peak may have been missed. In vitro studies revealed that macrophages obtained from human fatty streak lesions exhibited increased DNA-synthesis compared to cells obtained from advanced atheromata.²⁰

The question still remains, whether this early DNA-synthesis peak is important in the pathogenesis of atherosclerosis. The early DNA-synthesis peak might imply that proliferation is important in the early stage of atherosclerosis. Therefore, at least in theory, it can be postulated that administration of anti-proliferative drugs in atherosclerosis would be beneficial, when applied early in the disease, and maintained for a longer period of time. However, systemic administration of anti-proliferative drugs (for example cell cycle inhibitors) for a longer period most likely disturbs other (patho)physiological processes, like wound healing and cell regeneration. Vascular-specific administration of these drugs by such techniques as gene transfer may overcome this pitfall. Due to undesirable side-effects of the systemic approach, an antiproliferative therapy to halt the progression of primary atherosclerosis in the entire vasculature would be impractical. As stated before, several anti-proliferative drugs have been employed successfully in various animal models to prevent restenosis and hold considerable promise for clinical application. When applied locally at the site of vessel wall injury, systemic side effects are avoided to a great extent and the concentration of the drug in the vessel wall is increased significantly as compared to the concentration after systemic administration.

Another observation that suggests that low levels of DNA-synthesis do contribute to the progression of primary atherosclerosis, is that plaques contain a large monoclonal population of cells. This phenomenon was first observed by E. Benditt et al. in 1973.²¹ The studies were based on the hypothesis that one X-chromosome is randomly inactivated in early female development, and that all progeny from that cell retain the same inactivated X-chromosome.²¹ Female tissue is thus a mosaic of cells bearing either an inactive paternal or maternal X-chromosome. These properties make X-chromosome inactivation a very useful assay to distinguish polyclonal from monoclonal lesions. In advanced female human atherosclerotic lesions, SMCs exhibited the same X-chromosomal activation pattern, and were considered to be monoclonal.²¹ Several mechanisms for the development of monoclonality have been proposed. First of all, atherosclerosis could arise from a pre-existing clone of cells if the stimulus for proliferation is contained within a single patch of SMCs.²¹⁻²³ On the other hand, it is possible that plaque SMCs initially are polyclonal, but transform by a virus or chemical mutagen, thereby inducing selective proliferation of a single cell.²¹⁻²³ Lastly, and most likely, it is possible that SMCs of the normal arterial media are polyclonal, i.e. belonging to multiple lineages of which one is uniquely suited to form the plaque. In this view, monoclonality would result from the selection of a particular cell lineage either on the basis of proliferation, migration or survival.^{23,24} All the theories described above have one thing in common: monoclonality can only result from many generations of cell replication.²² Since atherosclerotic plaques take several decades to become advanced lesions, the relative low levels of DNA-synthesis might still be an important feature in the pathogenesis of atherosclerosis.

There are also arguments that support the view that the contribution of the low levels of DNA-synthesis to plaque progression and phenotype is limited. This is also reflected by the *in vivo* experiments described in this thesis.

Genetic disruption of Gas6, a survival factor, known to induce DNA-synthesis in serum starved fibroblasts²⁵, has no effects on the levels of DNA-synthesis in atherosclerotic plaques in the ApoE^{-/-} mouse (chapter 5). Furthermore, absence of Gas6 fails to inhibit lesion development or progression. Surprisingly, it induced the development of an altered atherosclerotic plaque phenotype. Atherosclerotic lesions of Gas6^{-/-}/ApoE^{-/-} mice were differently organized and exhibited an increased collagen deposition. The most remarkable feature of this phenotype, never observed before in ApoE^{-/-} mice, was the development of intraplaque hemorrhage, associated with fibrin and iron deposition. Although intraplaque hemorrhage does not reflect plaque rupture, the phenotype indicates that absence of Gas6 might induce plaque instability in mouse atherosclerotic lesions.

In two other studies (chapters 6 and 7), we report that inhibition of CD40-CD40L interactions resulted in the development of a lipid-poor, collagen-rich, stable plaque phenotype.^{26,27} One might expect that a change in DNA-synthesis would contribute to the observed phenotype. However, inhibition of CD40-CD40L signaling did not affect total plaque cell number nor plaque DNA-synthesis.

Other interesting findings of our cell turnover studies were the cell type that exhibits DNA-synthesis and the distribution of DNA-synthesis in the atherosclerotic

plaque. Although it was thought for a long time, that the SMC was the major cell type that exhibited DNA-synthesis in primary atherosclerosis², data obtained from our lab^{17,19}, and others^{13,16}, imply that the macrophage derived foam cell is the cell-type that predominantly exhibits DNA-synthesis. DNA-synthesis in SMCs was hardly observed, although our immunohistochemical stainings may not be completely conclusive. First, it cannot be excluded that some of the foam cells that are positive for our macrophage antibodies are of SMC origin, since SMCs can obtain macrophage markers during foam cell formation.²⁸ Second, some of the plaque SMCs have been missed, since they might not contain α -smooth muscle actin.

The data described in this thesis, and data described by others, imply other explanations than plaque progression, for the limited increase in DNA-synthesis in primary atherosclerosis. These include cell-repair instead of cell proliferation, and plaque repair instead of plaque growth. In vitro, it was shown that macrophages exposed to oxLDL, the cell type in which we predominantly observed DNA-synthesis, develop DNA-damage, and a mitogenic response.²⁹ Furthermore, in our mouse intervention studies, we saw no effects on total cell number, indicating that altered cell-division rates did not occur (chapters 5-7).^{26,27} The early proliferative peak observed in early atherosclerosis in ApoE*3Leiden mice, might very well be the result of a repair mechanism, especially since BrdU incorporation does not necessarily reflect cell division¹⁹, but may also indicate cell repair. Furthermore, we^{17,19}, and also others¹⁶ observe DNA-synthesis predominantly at sites with recent damage, such as plaque rupture, indicating plaque repair.

8.2.2 Apoptosis

The presence of apoptosis in the intima was first postulated by Clowes et al. (1983)³⁰ in the balloon injured rat carotid, on the basis of the level of DNA-synthesis and the actual number of cells. Later, when the techniques for the detection of apoptosis, such as DNA-laddering and the TUNEL technique had developed, increased numbers of apoptotic cells were observed in arterial injury models, as well as in primary atherosclerotic lesions of hyperlipidemic animal models and humans.³¹⁻³⁴

In this thesis, we confirmed the presence of apoptosis in atherosclerotic lesions of mice and humans by the TUNEL technique.^{17,19} As already observed by others^{31,33,34}, elevated levels of apoptosis were only present in advanced stages of atherosclerotic lesions. Furthermore, in ApoE*3Leiden mice, apoptosis was confined to macrophage derived foam cells in the lipid core area, whereas in humans, apoptosis was equally distributed between the macrophage derived foam cell in the lipid core area and the SMC in the fibrous cap.^{17,19}

However, caution should be paid to the interpretation of the TUNEL results. Although the TUNEL technique does stain apoptotic nuclei, it also stains necrotic cells, and cells undergoing RNA-splicing.³⁵ However, we only considered a cell to be apoptotic when it exhibits, besides TUNEL staining, morphological features of apoptosis (chapter 1). Furthermore the group of Kockx et al. applied a series of apoptotic markers on atherosclerotic plaques, and obtained comparable results.³⁶ Therefore, the data described in this thesis reflect the actual amounts of apoptosis.

As with DNA-synthesis, the exact role of apoptosis in the pathogenesis of atherosclerosis is not clear. Theoretically, apoptosis might be beneficial, by inducing regression of atherosclerotic lesions. However, since apoptosis is predominantly present in the lipid core and fibrous cap area, it might also be detrimental, and induce plaque instability. Apoptosis of macrophage derived foam cells in the lipid core area might contribute to lipid core expansion, whereas apoptosis of VSMC in the fibrous cap area might result in thinning of the fibrous cap, both features contributing to plaque rupture. The thinning of the fibrous cap is likely to be a result of a decreased VSMC density and a decreased cap extracellular matrix content.^{37,38}

In vivo experiments investigating whether apoptosis in atherosclerosis is beneficial or detrimental, are scarce and not conclusive. However, in arterial injury models, intervention studies with overexpression of apoptosis promoting agents (Fas, NO)^{39,40} and inhibition of apoptosis inhibiting agents (Bcl-2 and Bcl-x)⁴¹ revealed a decrease in neointima formation, indicating an important role for apoptosis in the pathogenesis of arterial injury.

Recently, Schneider et al. administered FasL to hypercholesterolemic rabbits, which contain primary atherosclerotic lesions. Surprisingly, levels of apoptosis were not affected, and atherosclerosis was even accelerated, due to an increased expression of VCAM-1, and an increased SMC proliferation, indicating other functions of FasL than apoptosis.⁴²

The role of other apoptotic mediators in atherosclerosis, is until now not confirmed by in vivo experiments. As can be concluded from the intervention studies performed in this thesis, the contribution of apoptosis in atherosclerosis seems to be limited. Genetic disruption of Gas6 resulted in a slight decrease of apoptosis in advanced atherosclerotic lesions, whereas the phenotypical characteristics indicate plaque instability. Furthermore, both genetic and pharmacological inhibition of CD40-CD40L signaling did not affect apoptosis levels, although decreased levels of apoptosis would be expected, since CD40L-inhibition induced an increased fibrous cap thickness, and a decreased lipid core content.^{26,27}

8.3 CD40-CD40L interactions

The CD40-CD40L system is an important regulator of inflammation and the immune system. Since inflammation is considered to play a major role in the pathogenesis of atherosclerosis (chapter 1), a role for CD40-CD40L interactions in atherosclerosis was postulated.

In this thesis, the proposed role for the CD40-CD40L system in atherosclerosis, described in chapter 1, is confirmed. In chapter 6, we describe the effects of genetic disruption of the CD40-CD40L system, and found that absence of the CD40L gene in ApoE^{-/-} mice resulted in a 5.5-fold decrease in atherosclerotic plaque area compared to normal ApoE^{-/-} mice.²⁶ Moreover, advanced atherosclerotic lesions in these mice showed a lipid-poor, collagen-rich, stable plaque phenotype, with a decreased macrophage and T-lymphocyte content. In a follow-up study (chapter 7), we were able to show that also pharmacological

interruption of CD40-CD40L signaling (anti-CD40L antibody treatment), induced the development of a lipid-poor, collagen-, SMC- and chondrocyte rich plaque phenotype, a phenotype quite similar to that in CD40L^{-/-}/ApoE^{-/-} mice.²⁷ This phenotype could even be established when antibody treatment was delayed until advanced plaques had developed. The group of Libby et al. showed similar results.^{43,44}

Since in humans, most of the complications of atherosclerosis are the result of plaque instability (chapter 1)⁴⁵, anti-CD40L antibody treatment might be beneficial in preventing the acute complications of atherosclerosis. It may not decrease plaque area or the number of lesions, but is likely to transform the lipid-rich, collagen-poor vulnerable plaque, into a more stable plaque phenotype. This would have profound effects on atherosclerosis related mortality and morbidity.

Several mechanisms for the development of this stable plaque phenotype have been proposed. First of all, in vitro data and immunohistochemical analysis revealed a downregulation of pro-inflammatory pathways, responsible for lesion initiation, progression and plaque destabilisation.⁴⁶ Furthermore, we described that anti-CD40L antibody treatment in mice induced the upregulation of TGF β .⁴⁷ TGF β promotes collagen synthesis, but also exerts other functions, like cell (trans)differentiation and the induction of wound healing, factors contributing to a stable plaque configuration.⁴⁸ However, precise mechanisms responsible for the development of this plaque phenotype still need to be elucidated.

8.3.1 Postulated mechanisms: preliminary results obtained from cDNA expression array analysis

To further elucidate factors that may play a role in the development of the CD40-CD40L inhibition induced stable plaque phenotype, cDNA expression array analysis (Atlas^{TM} mouse cDNA array 7741-1, Clontech, Palo Alto, USA) was performed on the atherosclerotic aortic arch of anti-CD40L antibody treated mice (n=3) and control treated mice (n=3). cDNA-array analysis is a method suitable for the rapid assessment of mRNA expression of a large number of genes.⁴⁹ This method has already been proven to be successful to evaluate disease related changes in gene expression in several other areas such as cancer⁵⁰, rheumatoid arthritis, inflammatory bowel disease⁵¹ and myocardial infarction⁵².

Analysis revealed that anti-CD40L antibody treatment induced differential mRNA expression patterns of genes with different functional subsets as described below.

1. Inflammation

As expected, anti-CD40L antibody treatment resulted in the downregulation of several pro-inflammatory genes, and especially those involved in macrophage and T-lymphocyte activation (table 8.1). For example, the expression of MIP⁵³, a chemokine that plays an important role in macrophage chemotaxis and activation, is completely blocked by anti-CD40L antibody treatment. Also thrombopoietin, IGF-1A³⁹ and IGFBP6³⁹, which induce enhanced expression of VCAM, IL1 β , IL6 and TNF α , show decreased expression⁵⁴, while expression of negative cytokine regulators, like NF-E2⁵⁵ and TGF β ⁴⁸ was increased.

Table 8.1
Expression pattern of genes involved in inflammation

Genes	Expression compared to control treatment
MIP	0.0
Thrombopoietin	-2.5
IGF-1A	-3.0
IGFBP-6	-2.7
NF-E2	2.2
TGF β	2.4
RAG-1	-3.6
Cathepsin D	-2.2
Prothymosin α	-6.3
LKLF	2.3
CTLA 4	2.1
IL6 receptor/gp130	3.9
GM-CSF	6.9
GM-CSF receptor	2.0
CD14	5.5
ICE	3.1
ACE	3.5
IRF 1	>> 50

The expression of RAG-1, a gene responsible for the activation and maturation of T- and B-lymphocytes⁵⁶ and for the assembly of immunoglobulins and T-lymphocyte receptors⁵⁷, was decreased after anti-CD40L antibody treatment, as well as the expression of cathepsin D and prothymosin α , genes involved in antigen presentation⁵⁸ and T-lymphocyte activation^{59,60}, respectively. Interestingly, LKLF⁶¹ and CTLA 4⁶², genes responsible for T-lymphocyte quiescence and inhibition of T-lymphocyte activation, displayed an enhanced expression.

Surprisingly, enhanced expression of pro-inflammatory genes such as the IL6 receptor/gp130⁶³, gm-CSF and the gm-CSF receptor⁶⁴, CD14⁶⁵, ICE⁶⁶, ACE⁶⁷ and IRF-1⁶⁸ after CD40-CD40L blockade was also observed. Although most of these genes contain besides pro-inflammatory, also anti-inflammatory properties^{63,69}, their enhanced expression might be the result of a positive feedback mechanism.

Interestingly, although cDNA-expression array analysis confirmed the importance of CD40-CD40L interactions in inflammation in atherosclerosis, it also revealed differential expression patterns of inflammatory modulators that were not known to be associated with CD40-CD40L interactions or atherosclerosis.

2. Cellular protection

One of the subset of genes that displayed an enhanced mRNA expression after anti-CD40L antibody treatment, were genes involved in cellular protection under stress (table 8.2). For example, HSP 84 and 86⁷⁰, and Net⁷¹, genes that stabilize intracellular processes under stress, were upregulated. Also glutathione peroxidase⁷², glutathione S-transferase⁷³ and NF-E2⁷⁴, genes that protect cells

Table 8.2
Expression pattern of genes involved in cellular protection

Genes	Expression compared to control treatment
HSP 84	27.3
HSP 86	2.1
Net	4.9
Gluthathione peroxidase	3.5
Gluthathione S-	2.2
NF-E2	2.2
MHR23b	2.3

against oxidative stress and exogenous chemicals, showed enhanced mRNA expression after anti-CD40L antibody treatment. We also observed upregulation of MHR23b, a gene responsible for the removal of damaged nucleotides.⁷⁵

Since an important feature of atherosclerosis is the increase in oxidative stress, predominantly due to increased levels of oxLDL¹⁸, these data reveal that inhibition of the CD40-CD40L system might increase the resistance of plaque cell types to oxidative stress.

3. (Trans)differentiation and phenotype

Plaque SMCs and macrophages undergo phenotypic modulation during atherosclerosis. Interestingly, anti-CD40L antibody treatment induced a differential gene expression pattern of genes involved in phenotypic modulation compared to normal plaque development (table 8.3).

Anti-CD40L antibody treatment induced upregulation of genes involved in cell differentiation in general such as HSP 86⁷⁶ and B-Raf⁷⁷, as well as hematopoietic stem cell differentiation such as NF-E2⁷⁸ and BKLf⁷⁹.

Furthermore, after anti-CD40L antibody treatment, genes encoding for different SMC phenotypical markers like sm-MHC⁸⁰, the ski-proto-oncogene⁸¹, TGFβ⁸² and EGR-1⁸³ were upregulated, while other SMC phenotypic modulators like the bFGF receptor⁸⁴ and IGF-1A³⁹, inhibin and its receptor follistatin⁸⁵ showed decreased expression. UBF-1, a transcription factor involved in SMC hypertrophy was downregulated.⁸⁶

Also genes inducing macrophage differentiation and maturation, such as IL6/gp130⁸⁷, CD14⁶⁵, EGR-1⁸⁸ and Flt-1⁸⁸ showed an increased expression after anti-CD40L antibody treatment, while the expression of cathepsin D and L, both involved in foam cell formation, was decreased.⁸⁹

TGFβ, a growth factor able to induce transdifferentiation of macrophages into a myofibroblast like phenotype⁹⁰, or of SMC into chondrocytes⁹¹, displayed an enhanced mRNA and protein expression after anti-CD40L antibody treatment.

Table 8.3

Expression of genes involved in (trans)differentiation and phenotype

Genes	Expression compared to control treatment
General	
HSP 86	2.1
B-Raf	3.1
NF-E2	2.2
BKLF	>>50
SMC phenotype	
SM-MHC	3.1
Ski-proto-oncogene	5.0
TGF β	2.4
EGR-1	>>50
BFGF	- 3.8
IGF-1A	- 3.0
Inhibin	- 2.3
Follistatin	- 2.3
UBF-1	- 9.1
Macrophage subset	
IL6 rec, gp 130	3.9
CD14	5.5
EGR-1	>>50
Flt-1	>>50
Cathepsin D	- 2.2
Cathepsin L	- 6.3

3. Extracellular matrix related mRNAs

According to the increase in plaque extracellular matrix (ECM) content observed after anti-CD40L antibody treatment^{26,27,44}, a different expression pattern of genes encoding for cytoskeletal proteins, genes responsible for the synthesis of ECM, and genes involved in extracellular matrix turnover was observed (table 8.4).

First of all, anti-CD40L antibody treatment resulted in the upregulation of zyxin, a cytoskeletal protein that is involved in α -actin filament assembly⁹² and EGR-1, a transcription factor responsible for the upregulation of fibronectin⁸³, while the expression of the gene encoding for vimentin⁹³, a gene associated with advanced atherosclerosis⁹⁴, was decreased.

Second, transcription factors (EGR-1 and NF1 β) and genes favoring the deposition of ECM (TGF β and ACE) displayed an enhanced expression after anti-CD40L antibody treatment.⁹⁵⁻⁹⁷

Table 8.4
Genes involved in extracellular matrix

Genes	Expression compared to control treatment
Zyxin	2
EGR-1	>>50
Vimentin	- 2.4
NF-1 β	>>50
TGF β	2.4
ACE	3.5
Cathepsin D	- 2.2
Cathepsin L	- 6.3
TIMP 2	5.0
TIMP 3	2.9

Besides the increased expression of ECM-synthesis promoting genes, genes involved in ECM degradation, like cathepsin D and L⁹⁸, were downregulated, while genes encoding for inhibitors of ECM degrading enzymes (TIMP 2 and TIMP 3) showed an increased expression.⁹⁹

These data suggest that anti-CD40L antibody treatment induces an increased ECM deposition, and a decreased degradation.

5. Embryonic genes

Upregulation of embryonic genes is a common feature of atherosclerosis. Interestingly, anti-CD40L antibody treatment revealed a number of embryonic genes that displayed an enhanced expression, especially genes that were involved in mesenchymal patterning, such as KIF3B¹⁰⁰, Flt-1¹⁰¹, and the ski-protooncogene (myoblast differentiation)⁸¹. Other embryonic genes that showed differential expression after treatment were LKLF (media and vessel stabilisation)¹⁰², NFE2 (hematopoietic development)¹⁰³, c-ErbA oncogene (myoblast differentiation) MLC fetal isoform (skeletal and cardiac muscle development) (table 8.5).¹⁰⁴

Table 8.5
Embryonic genes

Genes	Expression compared to control treatment
KIF3B	2.1
Flt-1	>>50
Ski-proto-oncogene	5.0
LKLF	2.3
NF-E2	2.2
c-ErbA oncogene	- 2.2
MLC fetal isoform	-14.3

6. Cell turnover

Table 8.6
Genes involved in cell turnover

Genes	Expression compared to control treatment
B-Raf proto-oncogene	3.1
Ski-proto-oncogene	5.0
Etoposide induced p53	2.5
ICE	3.1
IRF-1	>>50
EGR-1	>>50
CD14	5.5
Prothymosin α	- 6.3
BFGF	- 3.8
Tob anti-proliferative	- 7.7

Anti-CD40L antibody treatment resulted in a different expression of genes involved in cell turnover. However, a clear pattern could not be defined (table 8.6). Expression of genes, known to stimulate DNA synthesis (B-raf proto-oncogene¹⁰⁵, ski-proto-oncogene¹⁰⁶) as well as apoptosis inducing genes (etoposide induced p53 response factor¹⁰⁷, ICE⁶⁶, IRF1¹⁰⁸ and EGR-1¹⁰⁹, CD14⁶⁹) was increased after anti-CD40L antibody treatment, whereas the expression of other genes that exert the same function, was decreased (prothymosin α ¹¹⁰ and bFGF receptor¹¹¹ (proliferation), and Tob-antiproliferative factor¹¹² (inhibition cell cycle)).

7. Others

Table 8.7
Other genes

Genes	Expression compared to control treatment
Stat 1	2.1
Stat 3	2.3
Stat 6	>>50
PI3K	>>50
Gem induced	10.0
Insulin receptor	>>50
Serotonin receptor	>>50

Lastly, genes that exert a wide variety of functions, such as signal transduction molecules and transcription factors, showed a differential expression pattern after anti-CD40L antibody treatment (table 8.7).

8.3.2 Postulated mechanisms: Hypothesis

From the preliminary mRNA and protein expression data, we derived the following hypothesis for the development of CD40L inhibition induced stable plaque phenotype:

During atherogenesis, plaque cell types come into contact with a large variety of substances, such as chemokines, cytokines, MMPs and oxLDL, which induce changes in SMC and macrophage phenotype, contributing to the progression of atherosclerosis and destabilisation of the atherosclerotic lesion.^{3,18} SMCs switch from a contractile into a synthetic phenotype and gain a different subset of markers¹¹³, whereas macrophages ingest lipids and become macrophage derived foam cells, thereby gaining a different subset of phenotypical markers, like the scavenger receptor.⁴⁷

Our preliminary differential expression data suggest that inhibition of the CD40-CD40L system induced downregulation of inflammatory pathways (table 8.1). Furthermore, a different subset of genes involved in differentiation in general, and especially genes involved in the phenotypic modulation of SMCs and macrophages was observed (table 8.3). We therefore hypothesize that inhibition of CD40-CD40L (in)directly modulates SMC and macrophage phenotype. In our hypothesis, the different macrophage and SMC phenotype might be more resistant to oxidative stress (table 8.2), will increase extracellular matrix deposition and decrease extracellular matrix degradation (table 8.4), and will induce the expression of genes involved in mesenchymal patterning (table 8.5), all features favorable for the development of a stable atherosclerotic plaque phenotype (fig. 8.1).¹⁸

A candidate factor, that is important in modulating SMC and macrophage phenotype, that also acts as a collagen-promoting factor, is TGF β . Interestingly, many genes that are differentially expressed after anti-CD40L antibody treatment are positively correlated with the expression of TGF β . For example, EGR-1 induces the expression of TGF β ⁸³, as well as NF-E2¹¹⁴. Moreover, the ski-proto-oncogene interferes with TGF β signalling, and is able to inhibit the induction of TGF β as part of a negative feedback mechanism¹¹⁵. Moreover, we also observed an increased protein expression of TGF β in atherosclerotic lesions after CD40-CD40L inhibition.²⁷ The relation between the CD40-CD40L system and TGF β was also shown in rat cultured microglia, in which TGF β is able to downregulate CD40 expression.¹¹⁶ Therefore, we consider TGF β as one of the key factors that modulate the development of the stable plaque phenotype after CD40-CD40L inhibition.

To test the latter hypothesis, additional experiments need to be performed. For validation of the cDNA-expression array data, the experiment has to be repeated and RT-PCR analysis, RNA in situ hybridization, as well as immunohistochemistry and Western blotting, has to be performed. Intervention studies with candidate regulators in such model as the ApoE $^{-/-}$ mice will reveal their in vivo importance.

Fig. 8.1
Postulated mechanisms responsible for the development of the CD40L inhibition induced stable plaque phenotype.

8.4 Limitations of the study

The current data further elucidate the role of cell turnover, inflammation and the immune system in the pathogenesis atherosclerosis. Other factors that are important, especially genetic predisposition and the interplay between environmental influences and genetic background were not addressed. The majority of the conclusions are based upon data that were generated in mouse models of atherosclerosis and should be translated to clinical atherosclerosis with caution. Several differences between mouse models of atherosclerosis and human atherosclerosis need to be addressed.

Although atherosclerosis in mice develops more slowly than restenosis, the atherosclerotic process in mice is still rapid compared to the development of the disease in man. This may obscure some aspects of (early) plaque progression relevant to the human situation.

Inward remodeling was not observed in our mouse models and this may be related to different flow and shear conditions in mice as compared to men. The mouse cardiovascular system can be characterized as a high flow system in small caliber vessels and it is unknown whether the same shear conditions as in the human vasculature apply.¹¹⁷ Furthermore, our morphologic observations were performed in the large vessels of the mouse arterial system and therefore may not be totally applicable to the human coronary vasculature, where plaque rupture and thrombosis accounts for the majority of atherosclerosis related mortality and morbidity. The heterogeneity of vascular function that is encountered when studying different human vascular beds was not addressed.¹¹⁸

Although striking observations with respect to plaque phenotype were made, caution should be made linking lesion morphology to plaque vulnerability. This is also reflected in human atherosclerosis, for example, endothelial abrasion on plaques that have several morphologic characteristics of a stable plaque phenotype may still lead to thrombosis.¹¹⁹

Other (functional) aspects, important in the pathogenesis of atherosclerosis, should be taken into account as well. A number of key physiologic parameters such as systolic blood pressure, heart-rate, endogenous t-PA activity, PAI-1 levels (chapter 1), that act in concert to accelerate the progression of atherosclerosis, were not determined.

8.5 Anti-CD40L antibody treatment: a future therapy for atherosclerosis?

The current results corroborate the role of inflammation and immune activation in atherosclerosis. The contribution of cellular proliferation appears to be limited and therefore clinical application of anti-proliferative drugs seems more appropriate in the prevention of restenosis than to prevent cell turnover during early stages of atherogenesis.

The effect of inhibition of the CD40/CD40L system on plaque morphology would validate a clinical trial on patients presenting with thrombotic coronary syndromes appropriate. However, inhibition of this system might compromise the immune system of the patient. Therefore during clinical application of anti-CD40L antibody for the stabilization of ruptured coronary plaques, monitoring of the immunesystem would be necessary. To overcome this problem, vascular-specific administration by gene transfer may be needed.

Another approach to overcome the side effects of systemic CD40-CD40L inhibition, would be activation of the same cascade as CD40-CD40L inhibition, but through another target protein (eg. TGF β). To identify such a therapeutic target, further elucidation of the mechanism, responsible for the observed phenotype, is required. Therefore, validation of the cDNA-expression array results (paragraph 8.3), as well as additional experiments (ie micro-arrays, in vivo experiments) will be needed.

The preliminary results from analysis of the cDNA expression array point to a role for TGF β in the development of a stable atherosclerotic plaque phenotype. Interestingly, inhibition of TGF β has been shown to promote atherogenesis in mice¹²⁰, while the tamoxifen induced increase in TGF β , decreased plaque area in ApoE $^{-/-}$ mice.¹²¹ Furthermore, the protective effects of tamoxifen against cancer and cardiovascular morbidity in patients with breast cancer may be mediated through increases in serum TGF β levels.¹²² Therefore, a strategy of augmenting TGF β levels in the arterial wall potentially may have a profound effect on atherogenesis.

8.6 Concluding remarks

The role of cell turnover in primary atherosclerosis remains questionable. Although our data show a peak in DNA-synthesis in early lesions^{17,19}, and elevated levels of apoptosis in advanced atherosclerotic lesions^{17,19}, the contribution of DNA-synthesis and apoptosis in lesion initiation and progression seems to be limited. This is reflected by the negative results obtained from intervention studies aimed at altering the rate of cell turnover, and from our own studies described in chapter 5, 6 and 7. In these studies, absence of Gas6, as well as inhibition of the CD40-CD40L system^{26,27}, induced a different plaque phenotype but neither DNA-synthesis nor apoptosis were affected. These results indicate that other mechanisms are more important in regulating the plaque phenotype.

Since more and more studies showed a limited effect of cell turnover in atherosclerosis, the 'response to injury' hypothesis was modified. It is now stated that endothelial activation, and the consequent upregulation of inflammatory pathways and the immune system, rather than massive mechanical injury is important in atherosclerosis.¹⁸ Evidence for this hypothesis was obtained by the results of several intervention studies, aimed to intervene in inflammatory pathways (chapter 1, table 1.6). This is also reflected by the data in this thesis. Inhibition of the CD40-CD40L system, a key regulator of inflammation and the immune system resulted in the development of a collagen-rich, lipid-poor stable plaque phenotype.^{26,27} Although precise mechanisms are not known, preliminary results imply that, besides downregulation of inflammatory pathways, CD40-CD40L inhibition might (in)directly cause phenotypic modulation of SMCs and macrophages. A key regulator, responsible for the phenotypic modulation and the development of the stable plaque phenotype, might be TGF β .

In conclusion, this thesis provides interesting new insights into the pathogenesis of atherosclerosis. It reveals that the importance of cell turnover in atherosclerosis may be limited, while inflammatory pathways and the immune system seems to play a major role in atherosclerosis. The CD40-CD40L system is an interesting therapeutic target, since its inhibition results in the development of a lipid-poor, collagen-rich, stable plaque phenotype in mice. However, precise mechanisms, responsible for the development of this phenotype, and the exact role of CD40-CD40L interactions in human atherosclerosis remain to be elucidated.

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Summary

Samenvatting

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Curriculum vitae

Summary

Atherosclerosis is the leading cause of morbidity, disability and mortality in the developed world. Despite our familiarity with the clinical symptoms of the disease, its pathogenesis remains poorly understood. Two mechanisms that are considered to play a major role in atherosclerosis are changes in cell turnover (DNA-synthesis and apoptosis) and chronic inflammation.

In the present thesis we focused on these two mechanisms. In the first part we hypothesized that the level, distribution pattern and phenotype of DNA-synthesizing and apoptotic cells change during the diverse stages of atherosclerosis. In addition, we expected that Gas6 deficiency (a gene important in cell survival) would suppress DNA-synthesis and promote apoptosis, thereby resulting in smaller atherosclerotic plaques. The second part of this thesis focuses on the role of inflammation in lesion initiation and progression. Although many papers report an important role for the diverse inflammatory parameters in atherosclerosis, a key regulator of inflammation in atherogenesis is not known. We hypothesize that CD40-CD40L interactions, interactions important in immune- and inflammatory responses, play a key role in atherosclerosis. Therefore, we expected that inhibition of CD40-CD40L interactions would inhibit lesion initiation and progression.

In chapters 2 and 4, we characterized the initiation and progression of atherosclerotic lesions, as well as the effects of atherosclerosis on the entire vascular wall in two mouse models of atherosclerosis. The first part of chapter 2 describes the age and diet dependent progression of atherosclerosis in the ApoE*3Leiden mouse. We report that ApoE*3Leiden mice show a cranio-caudal lesion progression. Atherosclerotic lesions range from type I (a few macrophage derived foam cells) to type V (lesions containing a lipid core and a fibrous cap). Immunohistochemistry revealed the presence of macrophages, SMCs, endothelial cells and T-lymphocytes in these lesions. Ruptured plaques (type VI), or plaques containing intra-plaque hemorrhage were however not observed.

In chapter 4, we investigated the response of the vascular wall (vascular remodeling) to atherosclerotic lesion progression in ApoE*3Leiden mice and ApoE-/- mice. In both mouse models, the carotid arteries, thoracic and abdominal aorta show compensatory enlargement in response to plaque progression. Enlargement was predominantly observed in advanced type IV and V lesions, most prominently in ApoE-/- mice. Despite the presence of compensatory enlargement,

lumen diameter decreased and significant stenosis (>70%) developed, but only in the carotid arteries. Constrictive remodeling, as well as ischemic organ damage was not observed.

In chapters 2 and 3, we describe the level, distribution pattern and phenotype of DNA-synthesizing and apoptotic cells in atherosclerosis. Chapter 2 describes these phenomena in ApoE*3Leiden mice. In these mice, DNA-synthesis is highest in early type II lesions. Most DNA-synthesizing cells were located in the media. When atherosclerotic lesions progressed to type III-V lesions, DNA-synthesis shifted to the shoulder region, and its level decreased. Apoptosis was only observed in advanced atherosclerotic lesions. Levels were low, and apoptotic cells were predominantly observed in the lipid core and regions adjacent to the lipid core. In all lesion types, both DNA-synthesis and apoptosis were mostly confined to the macrophage.

Chapter 3 describes the same parameters in human atherosclerosis. Also in human atherosclerosis, DNA-synthesis peaked in early type II lesions to decrease in type V lesions. Interestingly, a second peak in DNA-synthesis was observed in type VI lesions (ruptured plaques). DNA-synthesis was mostly confined to the macrophage or, in type VI lesions, to macrophages and T-lymphocytes. As in mice, apoptosis was elevated only in advanced lesions in the lipid core or in regions adjacent to the lipid core. Most apoptotic cells were macrophages, although a significant amount were SMCs in the fibrous cap.

In conclusion, DNA-synthesis is an early phenomenon, whereas apoptosis is a late phenomenon of atherosclerosis. Furthermore, cell turnover is mostly confined to the macrophage.

In chapter 5, we describe the effect of deficiency of Gas6 on atherosclerosis. Gas6 is known as a survival factor, which inhibits apoptosis and stimulates DNA-synthesis in serum starved fibroblasts. Although we expected to find smaller lesions due to decreased levels of DNA-synthesis and elevated levels of apoptosis, none of this was true. Interestingly, Gas6^{-/-}/ApoE^{-/-} mice developed a differently organized atherosclerotic plaque phenotype. These atherosclerotic lesions consisted of increased amounts of collagen and SMCs. However, compared to ApoE^{-/-} mice, both SMCs and collagen fibers of Gas6^{-/-}/ApoE^{-/-} mice were distributed throughout the entire lesion. Furthermore, lesions of Gas6^{-/-}/ApoE^{-/-} mice were composed of multiple lipid cores. However, the most remarkable finding of this study was the development of intra-plaque hemorrhage in Gas6 deficient mice. Intra-plaque bleeding, associated with iron deposition and fibrin formation was observed in >50% of the advanced lesions.

The second part of this thesis focuses on the role of inflammation in atherosclerosis, especially on the role of the CD40-CD40L system. In chapter 6, we report that deficiency of the CD40L gene in ApoE^{-/-} mice results in a 5.5-fold decrease in plaque area. Furthermore, deficiency of CD40L caused the development of a lipid-poor, collagen- and SMC rich plaque phenotype, with a decreased T-lymphocyte content, features of a stable atherosclerotic plaque phenotype. In a follow-up study, described in chapter 7, we obtained similar results using a pharmacological approach. Although we did not observe a decrease in plaque area, both early and delayed treatment with an anti-CD40L antibody resulted in the same lipid-poor, collagen-rich, stable atherosclerotic plaque phenotype. Interestingly, anti-CD40L antibody treatment induced upregulation of TGF β .

In chapter 8, the findings of this thesis are discussed. The first part of chapter 8 discusses the importance of DNA-synthesis and apoptosis on plaque progression and plaque phenotype. The second part discusses the role of inflammation, and the role of the CD40-CD40L system in particular in atherosclerosis. Furthermore, several mechanisms, responsible for the CD40L inhibition induced stable plaque phenotype are postulated.

In conclusion, the role of cell turnover in primary atherosclerosis remains questionable. Although our data show an interesting pattern of cell turnover during atherogenesis, our intervention studies (both gas6 and CD40L inhibition) reveal that the contribution of cell turnover in lesion initiation and progression is limited. On the other hand, this thesis confirms the important role of inflammation (and especially the CD40-CD40L interactions) on plaque progression and phenotype. The CD40-CD40L system is an interesting therapeutic target, since its inhibition results in the development of a stable atherosclerotic plaque phenotype. However, precise mechanisms, responsible for the development of this phenotype still remain to be elucidated.

Samenvatting

Atherosclerose (aderverkalking) is een systemische aandoening van de grote slagaders die ieder mens in meer of mindere mate treft. In de Westerse maatschappij zijn de gevolgen van atherosclerose doodsoorzaak n°1. Klinische complicaties van atherosclerose omvatten o.a. het myocardinfarct, herseninfarct en klachten van perifere arterieel vaatlijden. Ondanks de klinische bekendheid met de gevolgen van atherosclerose, is de pathogenese van atherosclerose nog altijd slecht begrepen. Twee mechanismen waarvan men denkt dat deze een belangrijke rol spelen in de ontwikkeling en progressie van atherosclerose zijn "cell turnover" (DNA-synthese en apoptose) en chronische ontstekingsactiviteit.

In dit proefschrift zijn deze mechanismen verder onderzocht. In het eerste deel van dit proefschrift is de hypothese dat 'het niveau, het verdelingspatroon en het fenotype van DNA-synthetiserende en apoptotische cellen verandert in de verschillende stadia van atherosclerose' in zowel muizen als mensen getoetst. Verder worden de effecten van een genetische interventie met Gas6, een gen dat een belangrijke rol speelt bij cel overleving beschreven. In deze studie hadden we verwacht dat afwezigheid van Gas6 zou leiden tot een verminderde DNA-synthese en toegenomen apoptose, waarbij atherosclerose zou afnemen. Het tweede deel van dit proefschrift beschrijft de effecten van ontsteking in atherosclerose. Hoewel de belangrijke rol van chronische ontsteking in atherosclerose in vele artikelen beschreven wordt, zijn belangrijke regulatoren van de ontstekingscascade in atherosclerose nog grotendeels onbekend. In dit proefschrift hebben we de hypothese opgesteld dat CD40-CD40L interacties, een systeem bekend van zijn rol in het immuunsysteem, een sleutelrol spelen in atherosclerose. Daarom verwachtten we dat remming van CD40L zou resulteren in een vermindering van atherosclerose.

In hoofdstuk 2 en 4 worden plaque initiatie en progressie, alsmede de effecten van atherosclerose op de gehele vaatwand beschreven in 2 veelgebruikte muizenmodellen van atherosclerose. Het eerste deel van hoofdstuk 2 beschrijft de leeftijds- en dieet- afhankelijke progressie van atherosclerose in de ApoE*3Leiden muis. We laten zien dat ApoE*3Leiden muizen een cranio-caudale progressie van atherosclerose vertonen. Atherosclerotische plaques varieerden van type I lesies (enkele schuimcellen) tot type V lesies (met een necrotische kern en een fibreuze kap). Immunolabeling liet zien dat deze plaques waren opgebouwd uit macrofagen,

gladde spiercellen, endotheelcellen en T-lymfocyten. Plaque-rupturen, of intra-plaque bloedingen werden echter niet waargenomen.

In hoofdstuk 4 beschrijven we de effecten van atherosclerose op de gehele vaatwand (remodelering) in ApoE*3Leiden muizen en ApoE^{-/-} muizen. In beide modellen laten de carotiden, thoracale en abdominale aorta compensatoire vergroting zien als reactie op plaque ontwikkeling en progressie. Dit was met name het geval in gevorderde type IV en V lesies van ApoE^{-/-} muizen. Ondanks het ontwikkelen van compensatoire vergroting, nam de luminale diameter af en ontstonden er stenoses van meer dan 70% in de carotiden. Echter, vasculaire constrictie, noch ischemische schade werden waargenomen.

In hoofdstuk 2 en 3 beschrijven we de hoeveelheid, verdeling en het fenotype van DNA-synthetiserende en apoptotische cellen in de diverse stadia van atherosclerose. In hoofdstuk 2 worden deze parameters beschreven in de ApoE*3Leiden muis. In deze muizen werd een DNA-synthese piek waargenomen in vroege type II lesies (fatty streaks), waarbij de meeste DNA-synthetiserende cellen in de media waren gelegen. Vanaf de ontwikkeling van type III lesies verschoof de DNA-synthese naar het schoudergebied van de plaque, en daalde de hoeveelheid. Apoptose daarentegen werd alleen in de late lesies gezien. De hoeveelheid apoptotische cellen was laag, en deze cellen werden voornamelijk gezien in de necrotische kern en zijn aangrenzende gebieden. In alle typen plaques waren zowel DNA-synthese als apoptose voornamelijk beperkt tot de macrofaag.

Hoofdstuk 3 beschrijft dezelfde fenomenen in humane atherosclerose. Ook in humane atherosclerotische lesies werd een DNA-synthese piek waargenomen in type II lesies. Verder werd er een tweede piek in DNA-synthese gezien in type VI lesies (geruptureerde plaques). DNA-synthetiserende cellen waren m.n. macrofagen en, in de geruptureerde plaques ook T-lymfocyten. Net als in muizenplaques was apoptose alleen verhoogd in vergevorderde plaques. Het grootste gedeelte apoptotische cellen was van macrofaag origine en was gelegen in de necrotische kern. Echter, een aanzienlijk deel van de apoptotische cellen waren gladde spiercellen in de fibreuze kap.

In hoofdstuk 5 worden de effecten van Gas6-deficiëntie in atherosclerose beschreven. Gas6 is een 'survival factor' die apoptose remt, en DNA-synthese stimuleert. We hadden daarom verwacht dat gas6 deficiëntie in een atherosclerotisch muizenmodel zou leiden tot kleinere atherosclerotische plaques. Echter, niets bleek minder waar. Gas6^{-/-}/ApoE^{-/-} muizen hadden een vergelijkbaar

DNA-synthese en apoptose patroon, maar ontwikkelden een anders georganiseerde plaque. Plaques van Gas6-/-/ApoE-/- muizen bevatten meer gladde spiercellen, en meer collageen. Echter, gladde spiercellen waren door de gehele atherosclerotische plaque verspreid, en er werden jongere vormen van collageen waargenomen. Verder toonden de Gas6-/-/ApoE-/- muizen meerdere necrotische gebieden per lesie. Echter, de meest opmerkelijke bevinding van deze studie was het ontstaan van intra-plaque bloedingen in de Gas6-/-/ApoE-/- muizen. Deze intraplaque bloedingen waren geassocieerd met fibrine-formatie en ijzerdepositie, en werden in >50% van de vergevorderde lesies gevonden.

Het tweede deel van dit proefschrift beschrijft de rol van ontsteking in de atherogenese, met speciale aandacht voor het CD40-CD40L systeem. In hoofdstuk 6 beschrijven we dat deficiëntie van het CD40L gen in ApoE-/- muizen resulteert in een 5.5-voudige afname van plaque oppervlak. Niet alleen was het plaque oppervlak afgenomen, ook vertoonden deze plaques kenmerken van een stabielere plaque fenotype. Deze plaques hadden o.a. een kleine necrotische kern, en bevatten veel collageen en gladde spiercellen. In een farmacologische follow-up studie, beschreven in hoofdstuk 7, verkregen we soortgelijke resultaten. Alhoewel we geen afname van het plaque oppervlak zagen, resulteerde vroege en late anti-CD40L antilichaam behandeling in hetzelfde lipide-arme, collageen- en gladde spiercel-rijke atherosclerotische plaque fenotype. Verder vertoonden deze lesies een verhoogde expressie van TGF β .

In hoofdstuk 8 worden de bevindingen van dit proefschrift bediscussieerd. In het eerste deel wordt het belang van DNA-synthese en apoptose beschreven, terwijl het tweede deel zich richt op beschrijving van een eventueel toekomstige rol van anti-CD40L antilichaam behandeling in de toekomst. Verder wordt over het mechanisme nagedacht, aan de hand van preliminaire resultaten verkregen met een cDNA expressie array.

Concluderend: of DNA-synthese en apoptose een belangrijke rol spelen in atherosclerose blijft nog maar de vraag. Hoewel onze data een interessant patroon van DNA-synthese en apoptose laten zien, laten onze interventiestudies (zowel gas6 als CD40L inhibitie), slechts een beperkte rol voor DNA-synthese en apoptose in de initiatie en progressie van atherosclerose zien. Inflammatie (en met name het CD40-CD40L systeem) blijkt echter een veel grotere rol te spelen in plaque progressie en fenotype. Aangezien het CD40-CD40L systeem resulteert in de ontwikkeling van een

stabiel atherosclerotisch plaque fenotype, is dit systeem potentieel aantrekkelijk voor therapeutische doeleinden. Echter, het exacte mechanisme dat verantwoordelijk is voor de ontwikkeling van het stabiele plaque fenotype na CD40L inhibitie zal nog opgehelderd moeten worden.

Dankwoord

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Esther

Curriculum vitae

Esther Lutgens

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Education

1988-1994	Catholic Gymnasium Rolduc, Kerkrade, the Netherlands, <i>Cum Laude</i>
1994-1995	Propaedeuse Medicine, University of Maastricht, the Netherlands
1995-1998	Doctoral phase, Medicine, University of Maastricht, the Netherlands, <i>Cum Laude</i>
1998- March 2001	Internships, Medicine, University of Maastricht, the Netherlands

Research

1995-1996	Student-worker, dept. of General Surgery, University Hospital Maastricht, the Netherlands
1996-1997	Student-worker, dept. of Cardiology (Prof. dr. H.J.J. Wellens and dr. E.D. de Muinck; W. Pon Foundation), University of Maastricht, the Netherlands
1998-now	MD-PhD-student (Prof. dr. M.J.A.P. Daemen, Prof. dr. H.J.J. Wellens and dr. E.D. de Muinck; W. Pon Foundation), depts. of Pathology and Cardiology, University of Maastricht, Cardiovascular Research Institute Maastricht (CARIM), the Netherlands

Visits/Research other labs

1998	Visit Biogen Inc., Cambridge, MA, USA.
1998	Research in the Howard Hughes Institute of Immunobiology, (dr. RA. Flavell), Yale Medical School, New Haven, CT, USA.
1999	Research in the Center for Transgene Technology and Gene Therapy, KU Leuven, lab of Prof. dr. P. Carmeliet and Prof. dr. D. Collen.

Awards

1. Young Investigator Award, 6th Joint Meeting of the Belgian and Dutch Working Groups on Invasive Cardiology. January 8-9 1999, Maastricht, the Netherlands.
2. Dr. E. Dekker grant, the Netherlands Heart Foundation, March-July 1999. Research in the Center for Transgene Technology and Gene Therapy, KU Leuven, Prof. dr. P. Carmeliet and Prof. dr. D. Collen.
3. Young Investigator Award International Society for Heart Research, XXth European Section Meeting. June 21 1999, Maastricht, the Netherlands.
4. Prize for Best Hot Topic Presentation, 4th annual meeting of the European Council for Blood Pressure and Cardiovascular Research (ECCR). October 15-17 1999, Noordwijkerhout, the Netherlands.
5. New Investigator Travel Award, First Congress on Arteriosclerosis, Thrombosis and Vascular Biology. May 20-22 2000, Denver, Co, USA.
6. EVBA Fellowship, XIth international Vascular Biology Meeting. September 5-9 2000, Geneva, Switzerland.
7. Vasculaire Biologie prijs 1999/2000, Parke Davis. October 28 2000, Amsterdam, the Netherlands.

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