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Fibromodulin and Dystrophin in Atherosclerosis: Novel roles for extracellular matrix in plaque development

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Novel roles for extracellular matrix in plaque development

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Faculty opponent

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Abstract

Cardiovascular disease represents nearly half the cases of noncommunicable diseases worldwide and is the leading global cause of death. The main underlying cause is atherosclerosis, and in atherosclerotic plaque progression the structure, composition and integrity of a dynamic extracellular matrix (ECM) is one very important factor. This thesis discusses the importance of the connection between the ECM and cells for atherosclerotic lesion development. It also tests the hypothesis that changes in this connection – whether through a modified collagenous ECM, or modification in a cellular protein directly linking cells to ECM components – have a significant impact on atherosclerotic plaque structure and stability.

Fibromodulin is a small leucine-rich repeat proteoglycan of the ECM involved in the regulation of collagen fiber synthesis. In the fibrous murine atherosclerotic plaque, we found collagen fibrils synthesized in the absence of fibromodulin to be thicker and more heterogeneous, compared to fibrils generated in the control mouse. Murine plaques with an inflammatory phenotype, with a fibromodulin-deficient ECM, were also smaller with decreased lipid accumulation, whereas cell proliferation was increased. In addition, we show, for the first time, that high fibromodulin expression in the most stenotic region of a human carotid artery plaque is found in plaques from symptomatic patients, and in patients with diabetes. Fibromodulin expressed in human plaques co-localize with Oil Red O-staining and correlates with the area stained for lipids (quantified as percentage). Fibromodulin also correlates with the pro-inflammatory cytokines MIP-1β and sCD40L, as well as with VEGF and inversely with the anti-inflammatory cytokine IL-10.

Dystrophin, and the dystrophin-glycoprotein complex (DGC), link the actin cytoskeleton to the basement membrane. This thesis provides novel data on dystrophin deficiency, as illustrated by the mdx mouse. The absence of dystrophin stimulates neointimal hyperplasia, but inhibits atherosclerotic lesion development driven by lipid-retention. In the mdx-mouse, laminin expression is decreased in the endothelium of atherosclerotic lesions with an inflammatory phenotype, suggesting that altered endothelial cell function and an abnormal basement membrane may be a possible explanation for the attenuated plaque development.

In summary, this thesis shows that collagen structure and remodeling affects the growth, development and composition of atherosclerotic lesions, on the one hand promoting mechanical stability, and on the other hand affecting lipid accumulation and inflammation. In addition, growth of vascular lesions can be inhibited or promoted by a functional connection between cells and the ECM mediated by dystrophin and the DGC.

Key words: Atherosclerosis, Fibromodulin, Dystrophin, Carotid artery, Lipid accumulation, Restenosis, Plaque

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Fibromodulin and dystrophin in atherosclerosis

Novel roles for extracellular matrix in plaque development

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For my family

"What I find fantastic is the notion that there are answers beyond the realm of science. The answers are there, you just have to know where to look."

Dana Scully ("Pilot", The X-files)

Table of contents

Concerning extracellular matrix

Introduction

All stages of atherosclerotic plaque progression are governed by the structure, composition and integrity of a dynamic extracellular matrix – from the initial retention of lipids, through plaque growth and inflammation to the final disruption with development of symptoms.

In one individual, atherosclerotic plaques may be present at many different locations, in several vessels. The question is why a certain plaque suddenly ruptures. From a clinical perspective, we wonder: How are these plaques identified in the patients before rupture? From a pre-clinical perspective, we rather ask: How can we direct plaque growth to slow down, or at least stick to a more stable phenotype?

This thesis is based on the hypothesis that the future propensity of a plaque to destabilize may be determined already as its extracellular matrix is being synthesized, and with this work – composed around four papers – I do my best to explore atherosclerosis in light of the involvement of the extracellular matrix. The sections on fibromodulin focus on abnormal extracellular matrix in atherosclerosis, while the sections on dystrophin look into abnormal connections between cells and the extracellular matrix in atherosclerosis. I have sought to weave together the themes of atherosclerosis and extracellular matrix, and my hope is that this text is accessible to members of both fields.[†]

…The matrix is everywhere.

Morpheus

[†] For the full atherosclerosis/extracellular matrix experience, I recommend – as soundtrack – the album ‘Go’ (by Jónsi), which has been playing in the background during much of my writing.

Original papers

This thesis is based on the following original papers (I-IV):

I. Shami A, Gustafsson R, Kalamajski S, Krams R, Segers D, Rauch U, Roos G, Nilsson J, Oldberg Å, Hultgårdh-Nilsson A**. Fibromodulin deficiency reduces low-density lipoprotein accumulation in atherosclerotic plaques in apolipoprotein E-null mice**, *Arteriosclerosis Thrombosis, and Vascular Biology*. 2013 Feb;33(2):354-61. Epub 2012 Nov 29.

II. Shami A, Asciutto G, Bengtsson E, Tengryd C, Nilsson J, Hultgårdh-Nilsson A, Gonçalves I. **Expression of fibromodulin in carotid atherosclerotic plaques is associated with a stable plaque phenotype.** Manuscript.

III. Rauch U, Shami A, Zhang F, Carmignac V, Durbeej M, Hultgårdh-Nilsson A**. Increased neointimal thickening in dystrophin-deficient mdx mice**, *PLoS One*.2012;7(1):e29904.

IV. Shami A, Knutsson A, Murugesan V, Rauch U, Bengtsson E, Tengryd C, Durbeej M, Hultgårdh-Nilsson A. **Dystrophin deficiency reduces atherosclerotic plaque development in ApoE-null mice.** Manuscript.

Abbreviations

α-SMA	Smooth muscle α-actin
ADAM	A disintegrin and metalloproteinase domain
ADAMTs	ADAM-related metalloproteinases with a thrombospondin domain
AGEs	Advanced glycation end products
ApoB/E/C	Apolipoprotein A/E/C
CD	Cluster of differentiation
C/N-terminus	Carboxy or COOH/amino or NH_4 terminus
CLL	Chronic lymphocytic leukemia
CS/DS/HS	Chondroitin/dermatan /heparan sulfate
DGC	Dystrophin-glycoprotein complex
DMD	Duchenne muscular dystrophy
EC	Endothelial cell
ECM	Extracellular matrix
ER	Endoplasmatic reticulum
FACIT	Fibril-associated collagen with interrupted triple helices
GAG	Glycosaminoglycans
HDL	High-density lipoprotein
ICAM	Intercellular cell adhesion molecule
IDL	Intermediate-density lipoprotein
IFP	Interstitial fluid pressure
IGF	Insulin-like growth factor
IL	Interleukin
LDL/oxLDL	Low-density lipoprotein/oxidized LDL

LPL	Lipoprotein lipase
LRR	Leucine-rich repeat
MCP-1	Monocyte chemoattractant protein-1
Mdx	X-chromosome-linked muscular dystrophy
MI	Myocardial infarction
MIP-1β	Macrophage inflammatory protein-1β
MMP	Matrix metalloproteinase
NEFA	Non-esterified fatty acid
m/siRNA	Messenger/small interfering ribonucleic acid
NO/NOS	Nitrogen oxide/nitrogen oxide synthase
PDGF	Platelet-derived growth factor
PRELP	Proline/arginine-rich end leucine-rich repeat protein
RAG2	Recombination activating gene-2
sCD40L	Soluble CD40 ligand
SLRP	Small leucine-rich repeat proteoglycan
SMC	Smooth muscle cell
TIA	Transient ischemia attack
TGF-β	Tumor growth factor-β
TIMP	Tissue inhibitor of MMPs
UGC	Utrophin-glycoprotein complex
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein

Background

In this chapter I will present the background to the papers included in this thesis – the essentials of both the field of atherosclerosis and the field of extracellular matrix (ECM).

Atherosclerosis

Cardio- and cerebrovascular disease represent nearly half the cases of noncommunicable diseases worldwide and is the leading global cause of death. One underlying cause is atherosclerosis;[1, 2] fibrotic, calcified and/or fatty plaques of the innermost layer of arteries. A multitude of risk factors that promote atherogenesis have been identified, including elevated plasma cholesterol, hypertension, diabetes, smoking, older age and genetic predisposition.[3]

Atherosclerotic plaques preferentially develop in blood vessel regions with low shear stress and flow stagnation or in regions with turbulent blood flow, appearing for example in vessel bifurcations.[4] Plaques develop over decades, and their presence may go unnoticed until a disruption of the plaque leads to thrombus formation and symptoms. The nature of the symptoms depends greatly on the location of the plaque. For example, acute myocardial infarctions (MIs) commonly result from thrombosis in coronary arteries,[5] while carotid artery atherosclerosis is most often responsible for strokes caused by emboli traveling to the brain.[6]

Symptoms of thrombi formed in association with carotid artery plaques tend to be hemispheric, such as ipsilateral visual loss (*amaurosis fugax*), or contralateral weakness or numbness of the body.[4] Transient ischemic attacks (TIAs) may result from emboli to the brain, presenting with loss of consciousness, weakness, numbness, difficulties in forming or finding words and visual field defects, followed by complete recovery.[6] Classification of cerebrovascular events as TIA requires that symptoms resolve within 24 hours, but patients having suffered from TIA have an increased risk of recurrent cerebrovascular events, such as stroke.[4, 7] In symptomatic patients, especially those with greater than 70% stenosis, this risk may be lowered, by, for example, carotid endarterectomy – plaque removal by surgery.[8]

Worldwide, around 15 million people suffer strokes each year. Roughly one third do not survive, and another third are permanently disabled.[9] As many as almost one tenth of individuals over the age of 65 may have asymptomatic carotid artery atherosclerosis with a stenosis degree of at least 50%.[10]

Patients suffering from diabetes experience marked increases in both the initial risk for and the severity of micro- and macrovascular complications, including development of atherosclerosis and resulting vascular events, such as stroke and MI.[2, 11, 12] The incidence, and mortality, from all forms of cardiovascular disease is 2-8 fold higher in the presence of diabetes,[13] and stem from, for example, generation of advanced glycation end products (AGEs), an altered lipid profile and increased oxidative stress.[14]

The healthy artery

The artery wall consists of three layers, each with a distinct function and, consequently, distinct components such as cells and ECM (figure 1). The layers are separated by fenestrated elastic membranes, and together they make up a vessel with the correct elasticity and compression. The elasticity is required by the vessel for strength and resilience, in order to accommodate blood flow and changes in blood pressure.[15]

The tunica intima is the innermost layer, directly in contact with the vessel lumen. It is composed of endothelial cells (ECs) surrounded by a basement membrane and an underlying subendothelial ECM, consisting of laminin, fibronectin, collagen type IV and VIII, and hyaluronan.[16] Hyaluronan is needed for the ECM to be able to endure compression, and collagen types IV and VIII form networks used as permeability barriers and anchoring sites for substrates in basement membranes.[15]

Layers of smooth muscle cells (SMCs) make up the middle layer, the tunica media. Like ECs, SMCs are also surrounded by a basement membrane and an ECM, though in this case consisting of collagen types I, III, V and XVIII, fibronectin, proteoglycans, and elastic elements.[16] Collagen types I and III provide tensile strength,[15] and it is through the contractile abilities of the SMCs that blood pressure and flow is controlled.[16]

The adventitia is the outermost layer, and consists of collagens types I and III, fibroblasts and vasa vasora, the latter providing nourishment to the vessel wall.[16]

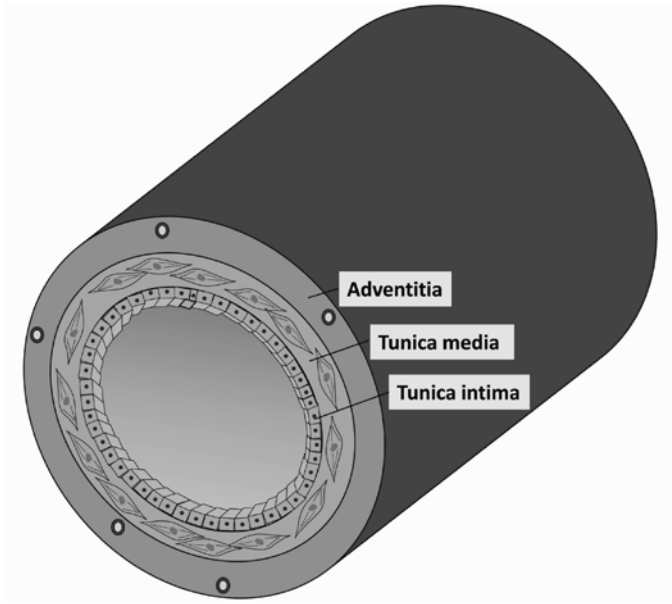

Figure 1:
Cross-section of a healthy artery wall.

Theories on atherogenesis

The response-to-retention model of atherogenesis was presented in 1995 by Tabas and Williams.[17] The model concludes that the central initiating event (and pathogenic process) in atherosclerosis plaque development is subendothelial retention of apolipoprotein B (ApoB)-containing lipoproteins. Lipoproteins bind to proteoglycans of the ECM in regions of the arterial wall that are lesion-prone, but still healthy in the sense that they are lesion-free. Retained lipoproteins are susceptible to oxidation; they become modified and are taken up by foam cells.[18] A chronic and maladaptive macrophage- and T cell-dominated inflammatory response induces changes in SMC phenotype, which further contributes to atherosclerotic plaque growth. The hypothesis that retention of lipoproteins, rather than changes in influx or efflux, is the major factor in atherosclerosis, has been tested by, for example, the use of labeled lipoproteins.[19]

Competing hypotheses include the response-to-injury hypothesis by Ross and Glomset, which has its basis in endothelial denudation, injury and/or activation,[20-22]

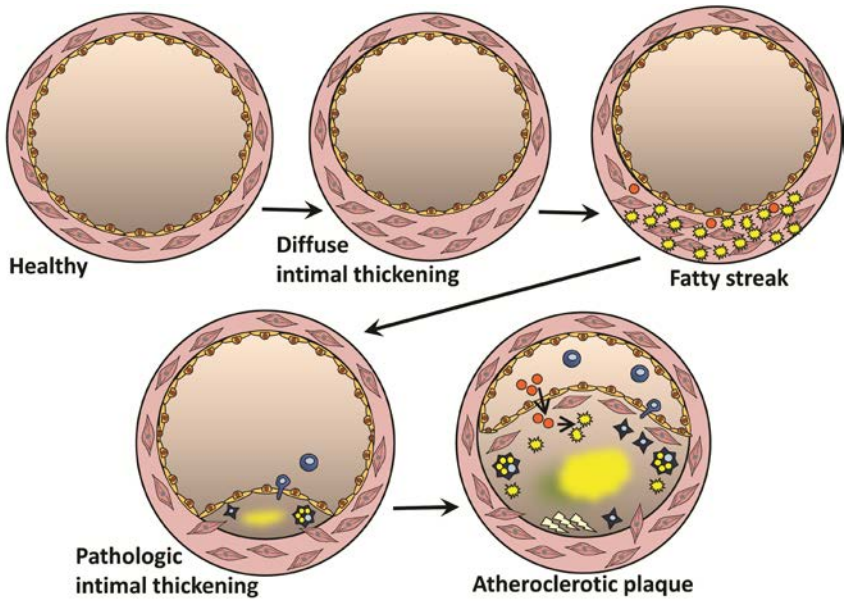

Figure 2:
Lesion progression from diffuse intimal thickening to atherosclerotic plaque.

and, the lipid oxidation hypothesis, that – as the name implies – proposes that the oxidation of lipids is the key process in atherosclerosis development.[23, 24]

Though the topic of this thesis is atherosclerosis, most aspects of this body of work are infused with, and based on, concepts of matrix biology. The development of atherosclerosis as described in this thesis will mainly follow the response-to-retention hypothesis, as I have found this theory to be superior in illustrating the key position the ECM plays in atherogenesis.

Changes in arteries preceding plaque development

Diffuse intimal thickenings appear from an early age – sometimes as early as during fetal development – and increase with age (figure 2). They are present in arteries that are considered to be atherosclerosis-prone,[25] and, initially, the thickened intima consists mainly of SMCs, elastin and proteoglycans.[26] Nakashima et al. examined coronary arteries from autopsied subjects and found that the earliest stage of human coronary atherosclerosis is a fatty streak that develops through extracellular deposition of ApoB-containing lipids in the outer layer of areas characterized by this type of diffuse intimal thickening.[26] The initial accumulation of

lipids takes place in the deep layer of the thickened intima[27] before foam cells are involved, [28, 29] and the resulting fatty streak can be defined as a lesion that contains lipid deposits, but remains non raised.[30] As lipids continue to accumulate, the lesion expands into a so called pathologic intimal thickening, which is a pre atheromatous lesion with a pool of extracellular lipids, covered by a layer of SMCs and lipid-laden macrophages.[27]

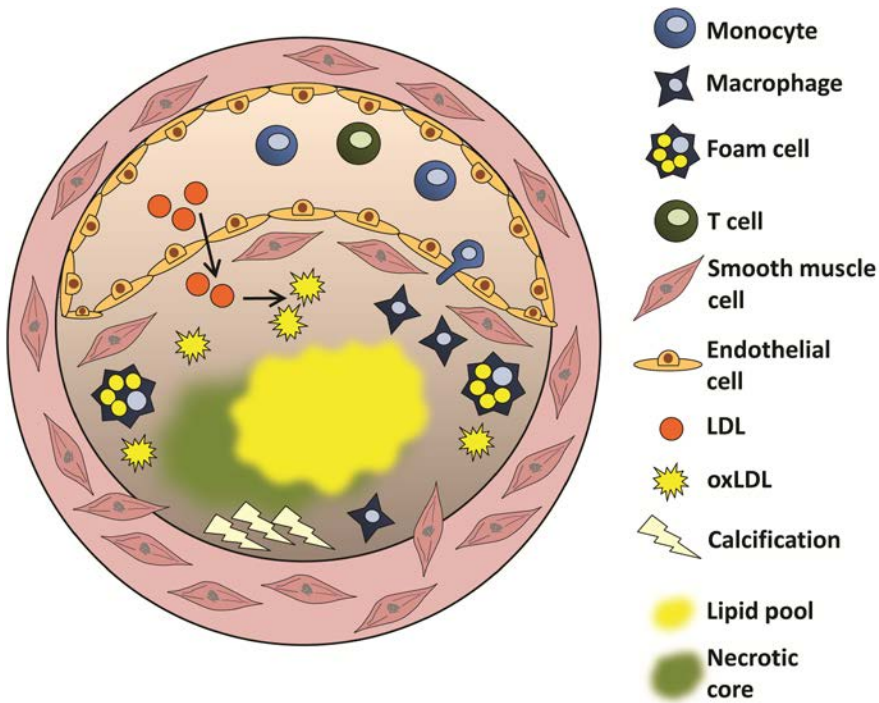

Figure 3
An advanced atherosclerotic plaque.

The atherosclerotic plaque

As plaque growth progresses past the fatty streak, the typical atherosclerotic plaque consists of cells, connective tissue elements, lipids and debris (figure 3).[31] A core region with foam cells, extracellular lipids and, in advanced plaques, regions of necrosis is surrounded by a fibrous cap of collagen-rich ECM and ECM-producing SMCs. The most vulnerable regions are the shoulder regions, where inflammatory and immune cells primarily infiltrate the lesion, and through which

the plaque grows.[32] The major cause of cardio- and cerebrovascular events, such as MI and stroke, is the formation of a thrombus – most often caused by plaque rupture[33] or erosion[27, 34].

As lipids bound to ECM proteoglycans[35] are being subsequently oxidized, an inflammatory response is initiated in the vessel wall. Plaque growth is promoted as the endothelium is activated by released phospholipids. [35, 36] Expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1)[37], intercellular adhesion molecule-1 (ICAM-1)[38, 39], and selectins E and P,[40] as well as inflammatory genes are increased in the activated endothelium,[41] attracting monocytes and lymphocytes to the lesion. Attached cells become stimulated by chemokines released from the underlying intima to migrate.[42-44] Macrophage colony-stimulating factor, whose expression is promoted by low-density lipoprotein (LDL) oxidation, [45] induces monocyte differentiation into macrophages.[46] Macrophage differentiation is associated with up-regulation of pattern recognition receptors, including scavenger receptors and toll-like receptors.[47, 48]

Infiltrated macrophages take up modified lipids, such as oxidized LDL (oxLDL).[49] Lipids build up within macrophages, which develop into foam cells and accumulate within the lesion.[18] Together with ECs, foam cells produce cytokines and growth factors that activate the vascular SMCs in the tunica media of the vessel wall. A phenotypic switch is induced in activated medial SMCs that dedifferentiate from their specialized, contractile state to gain the abilities to migrate into the intima, proliferate and synthesize massive amounts of ECM components.[50, 51]

In advanced atherosclerosis, neovascularisation often occurs within the plaques. Angiogenesis – endothelial proliferation and sprouting – usually originates in vasa vasorum in the adventitia, and growths through media, into the base of plaque. New vessels are fragile, leaky, and express adhesion molecules, such as VCAM-1, all features that promote intra-plaque hemorrhage and facilitate extravasation of plasma proteins and inflammatory cells.[52, 53] Association of intra-plaque hemorrhage with risk of future neurological events in carotid artery plaques has been reported.[54]

Plaque rupture

The fibrous cap in advanced atherosclerotic plaques is in danger of disruption, an event that exposes components of the plaque core to the blood,[55] resulting in a coagulation cascade and thrombus formation. Thrombi are commonly formed by plaque rupture, but can also be the result of erosion or even the presence of calcified nodules (figure 4).

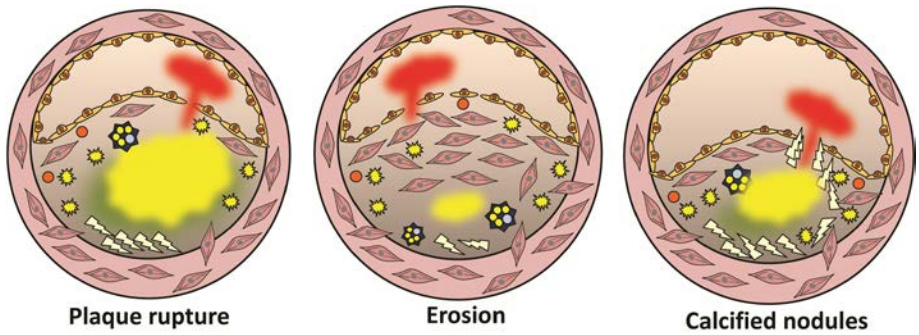

Figure 4:
Different modes of plaque disruption.

Plaque rupture occurs in so called vulnerable plaques, characterized by a thin fibrous cap covering a large necrotic core and a heavy infiltration of macrophages and T-lymphocytes.[27, 56] The fibrous cap consists of mainly type I collagen and few SMCs.[56, 57] Digestion of fibrillar collagen by matrix metalloproteinases (MMPs) is considered the main contributor to fibrous cap thinning, and for example MMP-1, -8 and -13 are found in association with cleaved collagen.[58, 59]

In contrast to plaque rupture, erosion results in a thrombus associated with a thick fibrous cap, and a matrix rich in proteoglycans and SMCs, but containing fewer inflammatory cells, less calcification and no endothelial lining.[27, 34, 56, 60] At the site of erosion, there have been both reports of lower numbers of SMCs[34] and of SMC clusters.[60] Eroded plaque tissue is associated with thrombi rich in versican, hyaluronan and type III collagen.[57] In a study on sudden coronary death, eroded, compared to ruptured, plaques occurred more often in younger individuals (<50 years) and in women, and they were also associated with smoking, especially in premenopausal women. Erosion was also linked to a lower degree of stenosis and total plaque burden.[27, 60]

Calcified nodules occur in heavily calcified arteries, and cause thrombi by breaking through the fibrous cap from within.[27] There is usually no collagen or endothelium overlying the nodules, which frequently are surrounded by fibrin.[27, 56]

Interestingly, an autopsy study found that the total number of affected vessels was highest in patients dying with stable plaques and healed MIs.[61] The number of affected vessels was intermediate in patients dying from plaque rupture and lowest in patients dying from plaque erosion.

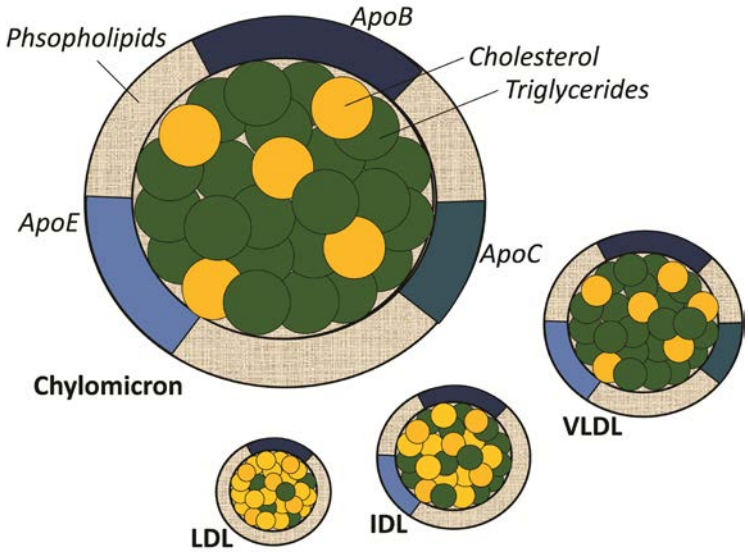

Figure 5:
Chylomicron and very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL).

A thrombus or ruptured plaque is not always fatal, and does not even always result in symptoms – or symptoms severe enough to be reported. It is possible for a disrupted plaque to stay unnoticed, and not cause clinical symptoms, while healing through formation of a "secondary cap" overlying the, old ruptured, one.[56, 62] A study of death due to coronary causes showed that among control subjects, having died from non-cardiac causes, 4% displayed acute coronary thrombosis, and 9% exhibited plaque rupture without thrombus.[62] The majority of patients with acute coronary syndrome have also been found to have one or more plaque ruptures in addition to the culprit lesion, often in another artery.[63]

Lipids and cholesterol – are eggs the bane of our existence?

Nikolaj Anitschkow discovered already in 1913 that rabbits fed a cholesterol-rich diet developed atherosclerosis,[64, 65] but lipoproteins were not isolated until 1950, by Gofman et al.[66]

Elevated plasma cholesterol drives atherosclerosis development, even though other risk factors are absent,[3, 67] as evidenced by patients with familial hypercholesterolemia who, even when no other risk factor of cardiovascular disease is present,

suffer from accelerated atherosclerosis development.[68] In addition, serum triglyceride levels,[69] reflecting very low density lipoprotein (VLDL) levels, is also a risk factor, as is the level of chylomicron remnants[70]. The common denominator of all these lipid forms is ApoB.[68]

As cholesterol and triglycerides are both hydrophobic molecules, they are insoluble in plasma. In order to be carried with the circulation, they are transported in lipoproteins (figure 5); representing a hydrophobic core that is covered by a hydrophilic coat with apolipoproteins, phospholipids and free cholesterol.[71] Every LDL particle thus carries one ApoB particle, wrapped around its surface. The human form exists in two isoforms: The full-size ApoB100 and the truncated ApoB48. ApoB100 is synthesized in the liver and is, therefore, also a part of the liver-derived VLDLs and intermediate density lipoproteins (IDLs). ApoB48 – representing the first 48% of the N-terminal amino acids due to messenger ribonucleic acid (mRNA) editing[72] – is only present on intestine-derived lipoproteins. This form is, therefore, in humans, only found on chylomicrons and their remnants. However, in mice, it is also found on VLDL, IDL and LDL.[73]

Chylomicrons transport dietary fat from the intestine. In the circulation, chylomicron particles are hydrolyzed, enabling fatty acids to enter tissues, such as muscle and adipose tissue. Resulting from lipolysis are high-density lipoprotein (HDL) particles formed by phsopholipids and chylomicron proteins, and chylomicron remnants that are taken up by LDL receptors and LDL receptor-related proteins in the liver.[74] HDLs recruit excess free cholesterol from peripheral tissues before returning to the liver,[75] where the cholesterol is excreted with the bile salts.[76]

In the liver, triglycerides and cholesterol esters are arranged into VLDLs that are released into the circulation to deliver lipids to other tissues. As these particles undergo lipolysis, their triglyceride levels go down, and they become IDLs (30% triglycerides remaining), that are either taken up by tissues or further lipolized to LDL (10% triglycerides remaining). In humans, the only major apolipoprotein remaining in LDL particles is ApoB100. LDL is taken up by the LDL receptor, but the kinetics of LDL uptake in this manner is slow, and, as a consequence, LDL has a longer half life in circulation compared to the other types of lipoprotein particles.[74]

Lipoproteins pass through the endothelium and enter the vessel wall. With sizes at around 15-25 nm and 25-35 nm, respectively, both LDL and IDL are well under the size limit of 70 nm for transcytotic vesicles.[68, 77] Additional means of lipoprotein transport across the endothelium include endocytosis[77] and passage through leaky junctions associated with dying or dividing cells.[78] Moreover, an increase in the rate of unidirectional LDL transport across EC monolayers as a result of monocyte migration has been reported.[79]

Oxidation of LDL promotes its uptake by macrophages.[80, 81] An initial mild oxidation of lipids is followed by oxidation of ApoB, rendering it recognizable to scavenger receptors.[82] In the lysosomes, oxLDL is hydrolyzed to free cholesterol and free fatty acids. Excess cholesterol can either be exported from the cell, or be re-esterified and accumulate in the cytosol as the lipid droplets that are so characteristic for foam cells.[83]

The lipid-rich core of atherosclerotic plaques is avascular, soft, hypocellular and poor in collagen. Lipids originate both from dead foam cells and, perhaps even more importantly, from retention through extracellular trapping and enzymatic processing, leading to aggregation, fusion and matrix binding of LDL particles.[84] Lipids are also derived from the membranes of red blood cells, present in the plaque as a result of intra-plaque hemorrhage.[85]

Statins lower circulating lipid levels and can also exhibit pleiotropic effects. In carotid artery plaques anti-inflammatory properties, decreased levels of MMP-2 and cell death, as well as increases in tissue inhibitor of MMP-1 (TIMP-1) and collagen content, have been found, suggesting that statins contribute to plaque stabilization.[86] In addition, statins have been reported to inhibit antigen-dependent T-cell activation,[87] enhance endothelial nitrogen oxide (NO) availability[88] and reduce platelet activity[89]. Moreover, statins have also been found to potentially stabilize plaques by dissolving cholesterol crystals.[90]

Finally, regarding the consumption of eggs[‡], the vast majority of the cholesterol in the blood stream (~80%) originates from endogenous synthesis, and not from the diet.[71] It also seems that the increase in plasma LDL resulting from dietary LDL is accompanied by an equal increase in HDL[91] – a lipoprotein mainly considered atheroprotective.[92] In addition, there are numerous epidemiological studies that failed to find any association between egg consumption and coronary heart disease (in non-diabetic individuals).[93, 94]

Cells of the atherosclerotic plaque

Though, somewhat controversially, some studies have suggested that a portion of plaque SMCs originate in the bone marrow, travelling with the blood to athero-

[‡] And, of course, other – less archetypal – cholesterol-rich foods…

sclerotic lesions,[95] the prevailing view still seems to be that SMCs primarily reach the neointima through migration from the media.[96-98] Macrophages secrete platelet-derived growth factor (PDGF) B-chain, a strong mitogen, throughout plaque development[99] and the specific localization of SMCs to the fibrous cap area may be due to cell sorting through cell surface cadherins and an endothelial-medial gradient of, for example, PDGF-BB, insulin-like growth factor 1 (IGF-1) and fibrin degradation products. [100, 101]

The endothelium is initially, though activated and dysfunctional, intact. Denuded (de-endothelialized) regions appear mainly in advanced plaques.[102] Endothelial NO synthase (NOS) synthesizes the vasodilator NO, suggested to be atheroprotective. In contrast, the inducible NOS of macrophages, with a higher capacity and powerful oxidative properties, is thought to be potentially proatherogenic.[3]

Key chemoattractants of monocytes are oxLDL and monocyte chemoattractant protein-1 (MCP-1), expressed by ECs, SMCs, and the macrophages themselves. There is a 20-fold up-regulation of its receptor on stimulated monocytes/macrophages. Once monocytes have differentiated to macrophages in the intima, they internalize lipoproteins through scavenger receptors, of which scavenger receptor type 1 and cluster of differentiation(CD)-36 have proved to be of great importance. The native LDL receptor responds to increased cellular cholesterol accumulation with down-regulation – however, a similar regulatory mechanism is lacking in scavenger receptors, resulting in the continuous lipid uptake that eventually leads to foam cell formation. The presence of macrophages promotes a vulnerable, thrombogenic plaque phenotype in several ways; in addition to making up the majority of the plaques' foam cells (with some contribution from SMC foam cells), they also promote and produce matrix-degrading enzymes, inflammatory cytokines and tissue factor.[3]

T-cells are often found in advanced atherosclerotic lesions.[103] Plaques contain mainly CD4+ cells reactive to, for example, oxidized LDL as antigen.[104] In addition, smaller populations of natural killer T cells, also recognizing lipid antigens,[105] and CD8+ T cells that may recognize viral antigens[106] also infiltrate plaques. T cells activate and stimulate macrophages and vascular cells, for example through interferon-γ, tumor necrosis factor, and interleukin(IL)-1, to produce additional inflammatory and cytotoxic molecules. [107, 108] T cell activity in the plaque is inhibited by atheroprotective TGF-β[109] and IL-10,[110]

Antibody-producing B cells are present, but not numerous, in lesions, and are considered to be atheroprotective.[111] More substantial numbers, may, however, be present in the surrounding adventitia.[112] However, though lymphocytes clearly have a role to play in existing plaques, it has been demonstrated through ApoE/ recombination activating gene-2 (RAG2) double-deficient mice that even a complete lack of lymphocytes does not prevent atherosclerosis from developing.[113]

Activated mast cells may be present in both plaque and adventitia, especially associated with vulnerable and rupture-prone lesions.[114, 115] Neutrophils are rare in less advanced lesions, but have been reported in connection with plaque rupture and erosion[116].

In more advanced plaques macrophages/foam cells, ECs and SMCs die through apoptosis, as well as necrosis, with many detrimental effects. Besides destabilizing the plaque core and fibrous cap,[117] cell death contributes to tissue factor activity and, therefore, the thrombogenicity of the plaque[118].

The extracellular matrix in atherosclerotic plaques

"What is the matrix? Control." [§]

In the ECM of atherosclerotic plaques, expression of basement membrane components, such as laminins[119, 120] and heparan sulfate,[121] is altered, and there is an increase in collagen types I and III, elastin, fibronectin and chondroitin and dermatan sulfate-containing proteoglycans.[16, 121, 122]

Stability of atherosclerotic lesions is greatly dependent on ECM.[123, 124] The principal initiating factor in plaque formation is binding of LDL to vascular wall proteoglycans,[125] and the subsequent vascular retention of LDL can be reduced in an altered ECM, such as in ApoE-deficient mice with ECM lacking fibromodulin (as we show in Paper I) or in mice with perlecan lacking heparan sulfate-side chains.[126]

The fibrous cap is formed and maintained mainly through synthesis of ECM components by SMCs, and provides structural stability.[127] Degradation of the fibrous cap ECM leaves the plaque in danger of rupturing.[128, 129]

The ECM also carries out many regulatory functions, on cells as well as on enzymes and lipids. Fibrillar collagen[130-132] and laminins[133, 134] promote the more differentiated state of SMCs, limiting proliferation, spreading and migration. A similar effect is observed in macrophages, with fibrillar collagen inhibiting MMP-9 production and LDL-uptake.[135, 136] Adhesion to collagen type I was also found to increase macrophage phagocytic activity,[137] and to stimulate uptake of acetylated

[§] Morpheus, again. (The Matrix)

LDL[136]. In fact, when comparing gene expression in monocytes/macrophages cultured on collagen or in suspension, 896 differentially expressed gene fragments were found – of those, 316 were induced specifically by adhesion to collagen (and not seen with fibronectin or laminin).[138]

ECM remodeling and MMPs

Remodeling of the artery wall can be expansive and outwards (preserving the lumen) or constrictive (narrowing the lumen).[3] A correlation has been reported between plaque size, expansive remodeling and a sizeable lipid-rich core, but no association was found between these features and plaque inflammation.[139] Increased collagen turnover and SMC migration, but not overall collagen fiber content, during flow-induced arterial remodeling in rabbits was reported by Sluijter et al.[140]

The metalloproteinases have a central role in the enzymatic ECM proteolysis that takes place during remodeling. There are three classes of metalloproteinases: MMPs, the ADAM family (a disintegrin and metalloproteinase domain) and the ADAMTS family of ADAM-related metalloproteinases with a thrombospondin domain. Of the two latter families, several members have been detected in the vessel wall, but not much is known about their role in atherosclerosis.[14]

Required for remodeling, MMPs contribute to progression of lesions by digestion of the fibrillar collagens of the plaque ECM. MMP activity is, in turn, regulated by the TIMPs. Collagenases MMP-1, -2, -8, -13 and -14 degrade fibrillar collagens,[141] and MMP1, -8 and -13 also cleave fibrillar collagen monomers. Collagen fragments denature into gelatin, which can be further degraded by MMP2 and -9.[16] In outwards remodeling, degradation of elastin, particularly by MMP-9 and -12, seems to have a significant role.[141]

Though MMPs have been extensively studied in transgenic mice as well as in human plaque tissue, there is variability in the results regarding the role of MMPs in plaque stability. MMP-2 and -9 have been deemed pro-atherogenic since in ApoE-mice deletions result in decreased plaque sizes, and in the case of MMP2 also in decreased accumulation of SMCs and macrophages.[142-144] However, regarding MMP9, the opposite was found in another study, reporting increased plaque size and macrophage content in MMP9-null mice.[145] No effects on plaque size were found with deletion of MMP13, though increased collagen content and fibril size and alignment in the fibrous cap was reported.[146] Plaques in ApoE-mice were smaller with a more fibrous phenotype after inhibition, but not deletion, of MMP12.[143, 147]

Increased collagen content, without effect on plaque size, was found in mice with a mutated collagenase cleavage site.[148] Thus, atheroprotective roles have also been

suggested for MMPs; Deletion of MMP3 resulted in larger plaques, with more fibrillar collagen.[149] However, neointima formation after carotid ligation was decreased in MMP3-null mice in another study, suggesting a role for MMP3 in restenosis development.[150] Interestingly, atherosclerosis was reduced when human MMP-1 was expressed in ApoE-deficient mice on a western diet – suggesting that neointimal remodeling by MMP-1 may actually be beneficial.[151]

In human carotid plaques, increased expression of MMP-1, -2, -3 and -9 have been described,[14] and expression is mainly found in the shoulder regions, colocalizing with macrophages.[152, 153] They have been linked to plaque instability; MMP-1 occurred within intraplaque hemorrhage,[154] and MMP-2 and -9 were expressed with IL-6 and -8 in carotid artery lesions.[155] Higher levels of MMP-8 and -9 have also been reported in unstable plaques and in symptomatic patients following stroke.[155-157] MMP-7 and -12 were found along the perimeter of the lipid core and near, but not associated with, cells of the fibrous cap.[158] A high MMP-12 expression in carotid artery plaques was associated with adverse clinical outcome after carotid endarterectomy.[159]

ECM calcification

Plaque calcification commonly occurs in plaques from individuals of both genders and prevalence increases with age.[160] However, the degree of plaque calcification in women compared to men has a 10 year delay, with equalization not until the eighth decade.[161] At one point in time, plaque calcification was considered so prevalent that it was deemed the only feature of atherosclerosis worthy of mention, at least according to whomever coined the expression 'åderförkalkning'[**]. Yet, it is not merely a passive and degenerative development; vascular calcification, just as calcification in bone tissue, is an actively controlled process.[162]

Under physiological conditions, the concentration of calcium and phosphate ions in both serum and tissue fluid is sufficient for biomineralization. However, hydroxyapatite formation is tightly regulated,[163] and initiation requires presence of crystallization nucleators and absence of mineralization inhibitors. Calcium deposits, lipids and several enzymes, many of which required to inhibit spontaneous calcium phosphate precipitation and nucleation, are contained in extracellular ma-

[**] Swedish. Directly translated simply as "vessel calcification" – the, rather oversimplified, way atherosclerosis was generally referred to in Sweden, in the olden days (and, sometimes, still).

trix vesicles.[164-167] Nucleators are later, and with the proper stimulus, formed through calcium-phosphate-lipid complexes within the vesicle membranes.[164]

Matrix vesicles have been identified in the tunica intima of arteries,[168] and macromolecular matrix proteins involved in biomineralization is expressed in calcified vessels.[169-171] Accumulation of calcium is promoted during atherogenesis by for example pro-inflammatory cytokines, tumor growth factor-β (TGF-β) and modified LDL.[172-175] Hydroxyapatite crystals are propagated along ECM fibers with the periodicity of negative charges on collagen,[176] and ECM composition is important in regulating this process.[16]

Calcification is driven by cells, and, though their origins are still unknown, in atherosclerotic lesions cells have been identified that produce mineralized matrix and undergo osteoblastic differentiation: pericytes in microvessels, pericyte-like, calcifying vascular cells in the aortic intima, SMCs in the media, and myofibroblasts in the adventitia.[170, 177-179] Unidentified cell colonies with osteoblastic or chondrogenic phenotype have been explanted from bovine aortic media.[170] Exposure to collagen type I *in vitro* has been shown to enhance calcification by these cells[180] and involvement of collagen type I has been suggested in commitment of intimal cells to the chondrogenic lineage[181].

Calcium content is often viewed as a common component of plaques with a stable phenotype: calcified plaques are less likely to cause symptoms in patients,[182-184] and calcification is inversely correlated to macrophage infiltration.[183] However, calcium content is also associated with stenosis severity,[185-187] and an estimate of total plaque burden, as composite calcium scores are predictive of future cardiovascular events.[188] Therefore, while a particular plaque, proving to be rich in calcium, might be considered more stable at a certain point in time than a plaque containing a large lipid pool,[185, 189] it may also indicate a large general plaque burden in this patient.

Finally, the location of calcium deposits in the plaque also determines their contribution to plaque stability. For example, increased peak stress has been reported in connection with calcification in a thin fibrous cap, compared to calcification in the lipid core.[190] This can be illustrated by the part played by calcified nodules in plaque rupture.[27] Calcium deposits in the plaque core, on the other hand, may provide mechanical stability.[163, 191]

Elastin

Elastin is insoluble, amorphous, and hydrophobic. In the vessel wall, elastin is organized in a network of extensively cross-linked and covalently bound polymers of tropoelastin molecules. Elastic lamellae are regularly arranged around the vessel lumen as concentric cylinders, providing elasticity, tensile strength and stability.[192]

Elastin is a highly stable molecule that undergoes little turnover. During atherosclerotic plaque progression, elastic fibers are modified due to strong inflammatory presence. Elastase activity is increased within the plaque,[193, 194] and calcium and lipids are deposited, promoting proteolytic degradation. As a result of the slow turnover, damage to elastin stays virtually unrepaired,[195] and, though increased elastogenesis has been reported in plaques[193] it is ineffective and results in immature cross-linked elastin.[196]

Elastases are found in the serine-, cysteine- and metallopeptidase (MMP-2, -7, 9 and -12) families, and are expressed by different cell types during plaque development, such as SMCs and macrophages.[194, 197]

The peptides generated through elastin degradation are known as elastokines, and have a biologically active role during plaque generation. Elastokines belong to the matrikines, a family encompassing several matrix fragments with cytokine-like activities,[198] and levels are increased in blood from atherosclerotic patients.[199] Elastokines interacts with cells through the elastin receptor complex, located in the plasma membranes,[198] and may, for example, promote additional elastolytic activity. They also attract monocytes through chemotaxis,[200] regulate inflammatory response of monocytes,[201] promote differentiation of fibroblasts to cells of an osteoblast-like phenotype,[202] and stimulate neoangiogenesis[203, 204].

Arterial proteoglycans

Proteoglycans are implicated in atherosclerosis development. They consist of a core protein with covalently bound long-chain carbohydrates – glycosaminoglycans (GAGs). GAGs are repeating disaccharide units carrying negatively charged sulfate and carboxy groups. They can be divided into different categories: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin, keratan sulfate, and hyaloronan. In the extracellular space of arteries, versican (large, CS-containing), biglycan and decorin (contain CS and CS/DS, respectively) are found. Perlecan is another large HS-containing protein that resides in subendothelial matrix. Syndecans and glypicans (HS-containing) are pericellular, existing in SMC and EC plasma membranes.[68] The human intima is rich in versican,[205] while in mouse lesions, perlecan is more abundant.[206]

Arterial proteoglycans are produced by SMCs, ECs and macrophages, and expression patterns are altered in atherosclerosis.[68] Proteoglycan synthesis is regulated by several factors, including other ECM components, TGF-β1, PDGF, oxLDL, and non-esterified fatty acids.[207-209] The effects of some factors, such as TGF-β1 and PDGF are additive.[207]

Lipoprotein retention in the arterial wall

Compared with the LDL receptor, cell surface proteoglycans bind LDL with lower affinity, but instead possess a higher capacity for binding, for example, small dense LDL.[210, 211] Ionic binding connects positively charged amino acid groups on ApoB to negatively charged groups on GAGs.[212] A key site for proteoglycan binding is the, so called, site B, located in the C-terminal half of ApoB100. Positively charged arginine and lysine residues in this region have been found critical for binding CS/DS proteoglycans.[213] The site's main importance is in the initiation phase of early atherosclerosis development, and the size of lesions in transgenic mice with non-functional site B is initially reduced. However, lesion growth eventually "catches up" to the that which is generated in mice with a functional site B.[35] The finding that ApoB48 is the major ApoB-form in the atheroprone ApoE-null mice[214] and that ApoB48 and ApoB100 are equally atherogenic, despite site B not being present in ApoB48, indicated existence of one or more additional proteoglycan binding sites.[215] As it turns out, another proteoglycan binding site is located in the N-terminus of ApoB, though it is masked by the C-terminus in LDLs containing apoB100.[216] Since the GAG-binding ability of LDL is increased after treatment with phospholipase A2[217], additional sites, such as site A, located in close proximity to site B, are also proposed to be functional in modified LDL.[218]

As atherosclerotic lesions progress, the content and size of proteoglycans are modified;[205, 219] the presence of oxLDL (and other products from LDL oxidation) both increase the length of GAG chains and their affinity for native LDL.[220] Studies have both showed decreased[221] and increased – especially by mildly oxidized LDL[222] – binding to proteoglycans by oxLDL.

Infiltrated macrophages further increase LDL retention by secretion of bridging molecules, such as lipoprotein lipase (LPL),[223] an enzyme shown to increase lipoprotein binding to cells as well as matrix components.[224, 225] LPL is normally bound to proteoglycans, but also possesses binding sites for lipoproteins. It has been observed in atherosclerotic lesions, and functions through its lipolytic activity, in addition to serving as a bridge between ApoB-containing lipoproteins and proteoglycans.[225, 226] LPL is also produced by SMCs.[227] Furthermore, hepatic[228] and endothelial[229] lipases have been identified, and all forms may be involved in lipid retention in atherosclerosis.

ECM components other than proteoglycans are also involved in lipoprotein retention. LDL has been found to interact with elastin.[230] Collagen types I and III bind native and oxLDL,[231] a process that may be aided by associated small leucin-rich proteoglycans (SLRPs)[232]. It has also been hypothesized that LDL-binding sites of collagen and fibronectin are masked by overlying HS proteoglycans, and that loss of HS by heparanase digestion may expose these sites.[233]

Atherosclerotic ECM in diabetes

In the presence of diabetes, production of advanced glycation end products (AGEs) is promoted by chronically elevated glucose levels in plasma. The AGEs are proteins, lipids and nucleic acids that have undergone irreversible cross-linking with sugars, which can aggravate vascular injuries such as atherosclerosis.[234] In the ECM, AGEs preferentially form on proteins with a low turnover rate. Presence of AGEs alters properties of ECM components, such as collagen and elastin.[235, 236] Increased stiffness in vessels is for example caused by AGE cross-linking of collagen type I and elastin, which increases the area of the ECM.[235, 237] In collagen type IV, AGEs can disrupt binding involving the non-collagenous domains, resulting in disruption of self-assembly and overall basement membrane structure.[238] In addition, AGEs can alter cellular properties through modification of intracellular proteins and through interaction with the AGE receptor.[239] Resulting pro-atherogenic events include chemotaxis of monocytes, increased expression of pro-inflammatory cytokines and adhesion molecules by the endothelium, and transition of the endothelium from an anticoagulant to a procoagulant state.[239, 240]

One reason behind increased atherosclerosis in insulin resistance and diabetes is changes in circulating lipoproteins and chronic high levels of albumin-bound non-esterified fatty acids (NEFA).[241] Accelerated intimal thickening associated with insulin resistance and diabetes,[242] include excessive ECM production. Exposure of NEFAs to human arterial SMCs *in vitro* was found to upregulate mRNA for the proteoglycans versican, syndecan and decorin, and increase the length of GAG-chains. The resulting ECM bound LDL with higher affinity.[208] In addition, upregulation of mRNA for enzymes required for GAG synthesis increased chondroitin sulfate:heparan sulfate ratio and the negative charge of the proteoglycans – effects that all are blunted in the presence of insulin.[243]

The mouse as a model for atherosclerosis

Animal models are a very useful, often invaluable, aid in order to explore mechanisms behind human disease and then to test possible cures and treatments. As the bulk of the work behind this thesis has its basis in the world of rodents, I would like to devote a section to briefly describe the development, along with some advantages and limitations, of mice as a model for human atherosclerosis. As the ApoE-knock out is our mouse of choice, the main focus will be on this model.

The first report of experimental atherosclerosis was by Ignatowski in 1908.[244] It described a thickening of the intima with formation of large, clear cells in the aortas of rabbits fed a diet rich in animal proteins. Through the years, many different

species have been used in atherosclerosis research: monkeys, rabbits, dogs, swine, guinea pigs, and hamsters – today, the mouse is the most widespread. There are many advantages to the mouse model, compared to other species previously used and, in some aspects, even to human studies, considering the great ability to control the environment and diet in studies involving mice, not to mention the myriad of genetic experiments that science, ethics and common sense prohibits in humans. In comparison to other candidate animals, mice have a shorter generational time (~9 weeks), smaller size, a more cost-effective maintenance, and faster progression of disease. The mouse genome is also completely mapped,[245] making genetic manipulation – insertion/deletion/mutation of specific gene/s/ – common available.

Mice do not spontaneously develop atherosclerosis; in fact, they are highly resistant to atherosclerosis. The one exception seems to be the C57Bl/6 strain.[246] While humans carry approximately 75% of plasma cholesterol on LDL, mice carry cholesterol mostly on HDL, which is associated with an atheroprotective profile, and as a result, mice on a regular chow diet do not develop atherosclerosis.[246]

Dietary manipulation was the earliest strategy to induce atherosclerotic lesions in mice. Standard chow for lab mice is a low-fat diet with only 5-6% fat (w/w) and 0.02-0.03% cholesterol. The atherogenic diet was first introduced in the 1960s[246] and consisted of 30% fat, 5% cholesterol and 2% cholic acid. The diet did result in vascular lesions forming in C57Bl/6 mice, though with limited resemblance to the human condition. As the atherogenic diet is very toxic, it was later modified by Paigen to a new diet regimen containing 15% fat, 1.25% cholesterol, 0.5%cholic acid.[247] Lesions then develop, though small (even after several months), and mainly restricted to the aortic root. Lesions induced by the Paigen diet do not advance beyond the early fatty streak stage and SMC involvement is scarce. In addition, the diet is still unphysiological and inflammatory.[246, 248]

A diet more physiological than the Paigen diet has since been developed[246, 249] – referred to as a western-type diet, as it was similar to an average American diet, consisting of 21% fat, 0.15% cholesterol, and no cholic acid. Today, this is the most commonly used atherogenic diet, and the one used by us in Papers I and IV (sometimes also referred to as a "fatty diet".)

ApoE-deficient mice

In 1992 the first transgenic mouse model for atherosclerosis was presented,[249, 250] a mouse in which ApoE had been inactivated. ApoE is a 34 kDa glycoprotein. It is a high affinity ligand to the LDL- and chylomicron receptors and facilitates lipid removal and uptake of chylomicrons and ApoE-containing particles in the liver.[214]

Development is normal in ApoE-deficient mice, but clearance of chylomicrons and VLDL is defective, resulting in lipoprotein particles being kept in circulation, and, thus, accumulation of atherogenic lipid remnants and hyperlipidemia.[246, 251] Plasma cholesterol and triglyceride levels are increased, while HDL levels are decreased, and cholesterol transport shifts from HDL to LDL.[246, 249] Plasma cholesterol increase 5 to 8-fold on chow, and 15 to 18-fold on a western diet. While ApoE-deficient mice spontaneously develop atherosclerosis on a standard chow diet, plaque progression is quicker and more severe in mice on a western-type diet.[252]

Lesion progression is similar to larger animal models, as well as humans[252], with the same types of cells involved[252, 253] and preferential lesion development at vascular branch points. There is an early monocyte adhesion to the endothelium (5-6 weeks), followed by formation of fatty streaks with foam cells and migrating SMCs (6-10 weeks). These lesions progress to more advanced plaques, often containing a necrotic core surrounded by collagen-and elastin-containing ECM, a fibrous cap-like structure, and even calcified foci in older mice.[246, 252, 254] In addition, lesions contain oxidation-specific epitopes, and antibodies against these oxidized LDL-epitopes are present in plasma.[255]

Other mouse models

The LDL receptor-deficient mouse – as the name implies – has an induced disruption of the LDL-receptor gene, resulting in defective uptake of LDL and IDLs. Lipoprotein abnormalities are milder than in the ApoE-mice, with a modest plasma cholesterol increase (~2-fold on chow). The LDL receptor-mouse is, however, very responsive to a fatty diet, upon which a 15-fold increase in blood cholesterol is reached.[256] Large, complex lesions are developed in the aortic root and throughout the aorta as a response to a western-type diet.[257]

The ApoE/LDL receptor-deficient mouse was the first double knock-out atherosclerosis model. A severe hypercholesterolemia and hyperlipidemia results in atherosclerotic lesion development even without a fatty diet.[258, 259]

A transgenic mouse model expressing human ApoB produces both full length ApoB-100 and the truncated version, ApoB48, in the liver. LDL is only mildly increased, but there is a distinct LDL peak (in contrast to the normally found HDL peak).[260, 261] and lesions are developed in response to a western-type diet.[262]

There are a number of variants of the ApoB transgenic mouse model, such as mice expressing exclusively ApoB48 or -100, on an ApoE-deficient background. The cholesterol level of $ApoB^{100/100}/ApoE^{-/-}$ is much less than either control $ApoE^{-/-}$ or $ApoB^{48/48}/ApoE^{-/-}$, while atherosclerosis is worsened on an LDL receptor-deficient background (even with a regular chow diet).[215, 263]

Mouse models and plaque rupture

Among mouse models, it is rare to find a model able to replicate the morphology of the more unstable/vulnerable plaques (in danger of rupture), exhibiting, for example, the intra-plaque hemorrhage and thinning of the fibrous cap that appear in humans. Lack of thrombus formation may be explained by the differing vessel morphology and shear pressure in mice and humans; with the decreased vessel diameter in mice, the surface tension increases exponentially.[246] The Holy Grail of sorts is a mouse model actually exhibiting natural plaque rupture and thrombus formation, and a number of models that include some of these features have been proposed.

In some models plaque rupture is achieved mechanically, for example using blunt forceps to cause disruption of lesions in the abdominal aorta,[264] or through inserting a microsurgical needle in the luminal surface.[265] In perivascular collar-induced atherogenesis in ApoE-deficient mice, plaque disruption was reported when the mice were challenged with lipopolysaccharides, stress, or both.[266]

Intraplaque hemorrhage and spontaneous plaque rupture is also described in brachiocephalic arteries of older, chow-fed ApoE mice, 24-60 weeks of age,[267] and there is a similar report of ApoE-deficient mice fed a western-type diet for 14 months.[268] Finally, Calara et al., reported intraplaque hemorrhage in both older ApoE- and LDL receptor-deficient mice fed a cholesterol enriched diet.[269]

Limitations

Though there are many advantages using mice as model organisms in atherosclerosis research, there are also limitations that are important to keep in mind when planning experiments and interpreting results. For example, the small size and short generational time of mice – often the very reason this species is chosen in the first place – present a substantial difference compared to humans. In addition, the smaller size may pose a problem in performing certain surgical manipulations, though, judging from experience from our own, and other labs, the possibilities available through microsurgery should not be underestimated. One does, however, often curse the diminutive size of murine atherosclerotic lesions, which limits the pool of analysis methods that are possible, often excluding, for example, the use of Western blots and primary culture of plaque cells.

One substantial difference is the one between mouse and human LDL. Mouse LDL contain ApoE, while human LDL do not, and as a result, in humans, direct binding of ApoB-100 to proteoglycans is more important than the use of bridging

molecules, an issue addressed by the transgenic mouse expressing human ApoB-100.[35, 74]

Plaque morphology and characteristics in mice are somewhat different from those of human plaques; the primary cause for plaque development in mice may be inflammation, which is not necessarily the case in humans. There is also a difference in lipid profiles, as cholesterol in mice is mainly carried on HDL, rather than LDL as in humans. Histologically, foam cell accumulation, reminiscent of a fatty streak do appear in mice, but is not preceded by intimal hyperplasia as in atherosclerosis-prone arteries in humans.[246] The human normal arterial intima contains a population of mesenchymal cells – most probably SMCs – which expands throughout life, and do not always develop into atherosclerotic lesions. The only exceptions among the animal models are swine[270] and primates,[271] in which neointimal thickening occurs.

Lipid retention is reported to be a common feature of atherosclerotic lesion development in mice and humans. However, the principal proteoglycan in this process in mice is perlecan, while versican is more prominently featured in the human process.[206]

Finally, even with congenic strains, there is a prominent variability in lesion area between mice, and even more so when mice are put on fatty diets.[272] However, this may mainly be problematic for data interpretation, as human lesions also vary greatly in composition and size.

A broader look at extracellular matrix

The purpose of this thesis is to further explore the function and significance of ECM in atherosclerosis. By either altering the ECM, or disrupting proper cell contacts with a proper ECM, we have identified some clues about processes important for plaque development and phenotype. We worked with two proteins – one important for proper synthesis of the ECM component collagen, and one important in the connection between SMCs and the basement membrane. I will dedicate the following chapter to delving further into the finer points of ECM structure.

Collagen

Collagen is the most abundant protein of the ECM, as well as the body (of mammals) as a whole; it represents around a third of all proteins. So far 28 different kinds of collagens have been identified, with type I being the most common.[273]

Collagen often exists as fibrils and both form the shape of tissues and give strength. Collagen fibrils are millimeters in length, with a range in diameter from only a few to several hundred nanometers. Fibril bundles are organized into lager fibers that in turn are gathered into even larger, higher-order structures in tissues. The arrangement of fibril bundles and fibers depends on the tissue, and can be represented by parallel bundles, such as in tendons and ligaments, orthogonal lattices, such as in the cornea, or concentric waves, as is found in bone.[274]

Collagens can be divided into two categories: Fibrillar and non-fibrillar collagens. The fibrillar collagens, types I, II, III, V, XI, XXIV and XXVII function as a scaffold, providing structural and mechanical support to tissues and acting as substrate for cellular and ECM molecule attachment.[275] The nonfibrillar collagens – 'Fibril-associated collagen with interrupted triple helices', the FACIT collagens – aid in the association between fibrillar collagens and other ECM components.[276] Among the non-fibril forming collagens, there are also network forming collagens, such as collagen type IV in basement membranes, as well as transmembrane collagens, endostatin-producing collagens and beaded-filament-forming collagens.[275]

In many tissues, including tendon, bone and skin, the major collagen is type I, while type II is the major collagen in cartilage. Often is more than one type of collagen found within the fibrils; in for example skin, types I and II collagen can both be found.[274]

Collagen fibers are trimeric molecules, with each chain consisting of repeats of Gly-X-Y triplets, where X and Y may represent any amino acid residue, but often turn out to be proline and hydroxyproline, respectively. Each chain is a left-handed helix that intertwines with two others to make up a right-handed triple-helix – whether it is homo- or heterotrimeric depends on the collagen type.[277] In fibrillar collagen, the triple-helical domain is flanked by globular domains without the Gly-X-Y repeat structure – one at each terminus. It is not until after these regions have been removed through proteolytical cleavage that triple-helices are able to assemble into higher-order fibers.[274]

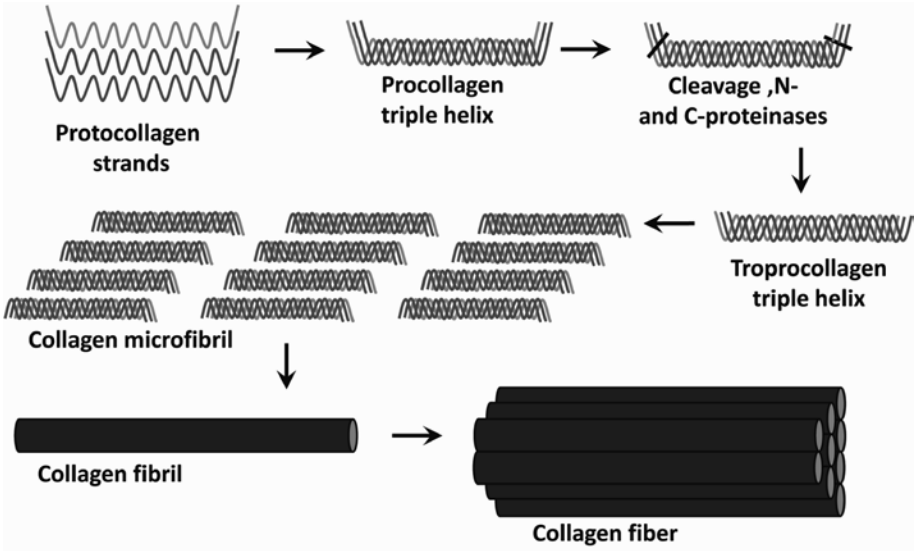

Figure 6:
Collagen fibrillogenesis.

Collagen synthesis and assembly

As collagen mRNAs is translated, it is translocated into the lumen of the rough endoplasmatic reticulum (ER), where the procollagen monomers undergo many posttranslational modifications.

Prolyl-4-hydroxyase converts proline to hydroxyproline.[278] Ascorbic acid is needed as cofactor, and in its absence most procollagen cannot leave the ER, resulting in halted synthesis of collagen fibrils[††].[274] The hydroxylation stabilizes the structure of the triple-helix and increases the denaturation temperature.[279] Hydroxyproline coordinates the network of water molecules within the collagen triple-helix to form water bridges within and between collagen chains.[280, 281] Some hydroxylysine residues are further modified through glycosylation, with addition of galactose or glucosylgalactose,[282] by the enzymes hydroxylysyl galactosyltransferase

[††] Which is what happens in people suffering from scurvy, vitamin C deficiency – but I digress…

and galactosylhydroxylysyl glucosyltransferase,[283] which also contributes to the stabilization of the triple-helical structure.[284]

The folding of three polypeptide chains into a triple-helix (figure 6) begins at a small triple-helical nucleation point at the C-terminus and propagates towards the N-terminus, in a process that can be likened to the closing of a zipper.[285] The nucleation point is formed by intra- and interchain disulfide bonds catalyzed by protein disulfide isomerase, a subunit of prolyl-4-hydroxylase.[286] Folding and trimerization of procollagen of fibrillar collagens take place in the ER lumen, with the aid of many molecular chaperones and enzymes.[287] Triple-helices are then transported to the Golgi apparatus, where lateral aggregation of procollagen trimers into bundles is promoted by decreasing volume of the Golgi cisternae.[288]

N- and C-propeptides are removed by procollagen N- and C-proteinases.[289] N-proteinases are members of ADAMTS family[290, 291] and C-proteinase is a member of the tolloid family of zinc metalloproteinases[292]. The C-propeptides are important for association between monomeric procollagen chains,[293] and they help determine chain selectivity[294]. Removal of the C-propeptide decreases critical concentration for collagen assembly, and, thus, triggers self-assembly of collagen into fibrils.[295] This process is likened by Prockop to the process of crystallization: it occurs spontaneously, but requires critical concentration of monomer in solution, it is driven by large entropy changes from the loss of water from surface of monomer, and fiber formation has both a lag period and propagation phase.[296]

Procollagen is secreted by fibroblasts and other collagen-producing cells. The plasma membrane becomes highly convoluted during early collagen deposition; cross-sections show fibrils embedded in narrow channels surrounded by cell membrane.[297] The growth of fibrils in the ECM proceeds through accumulation and lateral (side-by-side) and linear (end-to-end) fusion,[298] with increases in fibril size and formation of crosslinks largely determining the strength of the resulting ECM.[274] The enzyme lysyl oxidase induces stabilizing intra- and interchain covalent crosslinks[299] through activation of lysine and hydroxylysine residues.[300]

Maturation of higher-order fibers continues by specific patterns of alignment. A banding pattern of 4.4 segments, with collagen molecules (each ~300 nm) quarter-staggered results in both overlap and a gap region, referred to as D-periodicity of 67 nm. Continued assembly with increased length and thickness, is controlled by tissue-specific factors to ensure correct conformation for future function.[301]

The process of collagen modification through glycation increases with age, and the crosslinks acquired through several AGEs contribute to the increasing stiffness of collagen that occurs with advancing age.[273]

Regulation by, and interaction with, fibril-associated molecules

SLRPs are located on the surface of fibrils, and regulate fibrillogenesis through, for example, stabilization and control of fibril sizes.[302-305] I will go into further detail on these proteins later in the section.

Collagen type I interacts with collagen type III during fibrillogenesis in tendons, and the interaction is thought to modulate the size of collagen type I.[288, 306]

The FACIT collagen family, includes for example collagens type IX, XII, and XIV, in which multiple collagenous domains are separated by non-collagenous segments.[274] The FACIT collagens may promote tissue integrity and plasticity,[274] with differing specificities. Type IX collagen is associated with the surface of type II collagen.[307, 308] Type XII and XIV are associated with the surface of type I fibrils,[309] and are thought to stabilize fibril structure[310] and limit fibril diameter[311], respectively. They also interact with other collagen-associated molecules, such as SLRPs,[276] perlecan[312] and N-proteinase[313].

Basement membranes

Basolateral to cell monolayers are basement membranes made up of 50-100 nm layers of specialized ECM,[16, 119] containing four primary components: laminins, collagen type IV, nidogen and perlecan. These sheet-like, cell-adherent membranes are vital for tissue organization and differentiation, and, underlying epithelia as well as vascular endothelia, they can surround individual cells or functional units. Attachment to cells seems to be mainly through laminins to cell surface sulfated glycolipids and transmembrane receptors.[314]

Basement membranes mediate specialized attachments between tissues. They also separate cell monolayers from underlying connective tissue, provide structural support for cells and influences and modifies cellular behavior via outside-in signaling.[314]

Self-assembly drives basement membrane formation. Collagen type IV suprastructure and laminin polymers make up the basic framework, with nidogen and perlecan bridging the networks, increasing their stability and influencing structural integrity (figure 7).[315] Cell surfaces, on which basement membranes form, are suggested to provide a nucleation surface for laminin, with critical, and likely tissue-specific, surface molecules marking the appropriate surfaces.[314] Dystroglycan, appearing later in this chapter, has been suggested to be one such anchoring molecule.[316]

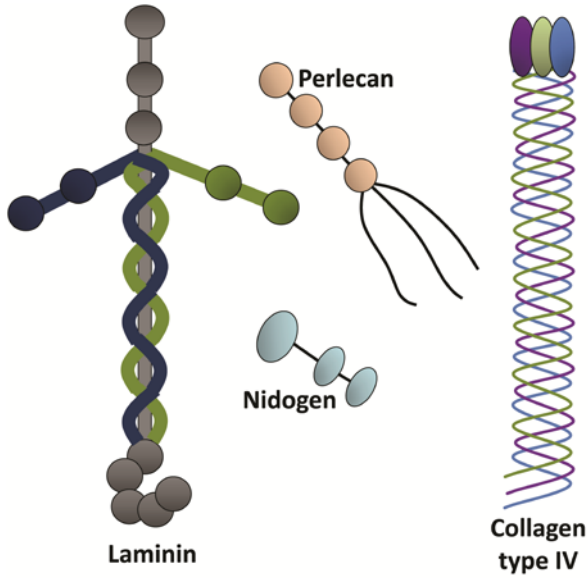

Figure 7:
The primary components of basement membranes (schematic view).

Basement membranes are coupled to the cells through integrins and dystroglycan of the plasma membranes. These proteins participate in basement membrane regulatory function as both signaling receptors and as direct links to the cytoskeleton.[314]

Basement membrane remodeling after damage includes *de novo* deposition of proteins, self-assembly and network formation. During this process cell behavior is influenced by the altered basement membrane composition as well as by exposed cryptic binding sites which promote tissue repair processes, such as immune cell recruitment and fibroblast activation.[317-320]

Laminin

Laminin is the most abundant non-collagenous protein in the basement membrane.[315] It exists as α-β-γ heterotrimers; a middle chain (α) with two arms (β and γ) usually depicted as a cross-like formation. In all, there are eleven different laminin chains – α1-5, β1-3, γ1-3. The monomer grouping is based on sequence identity and protein domain organization. Expression of isotypes varies depending on cell type.[314]

The N-terminal region of each subunit ("the arms and top part of the cross") usually begins with a globular domain, followed by a rod-like region of epidermal growth factor repeats as well as two additional globular domains.[314] Ionic interac-

tions in the Golgi apparatus favor βγ dimer formation. The heterotrimer is then stabilized and secreted upon incorporation of the α-chain.[321] Laminin next self-assembles into a network through globular domain VI of each chain.[322] Laminin trimers bind to each other through the N-terminal globular domain at the end of each arm.[323, 324]

Laminin seems to be essential for organization of basement membrane assembly. Purified laminin can alone assemble sheet-like ECM on cell surfaces *in vitro*[323] and basement membranes fail to form in a given tissue after tissue-specific elimination of laminin expression, while progressing normally in non-mutated tissues.[325, 326] In contrast, the main effects of type IV collagen, nidogens and perlecan may be to enhance membrane stability.

Type IV Collagen

Type IV collagen makes up around 50% of basement membranes. It provides structural integrity through collagenous domains of repetitive Gly-X-Y. Flexibility is brought by short sequence interruptions, and, being a nonfibrillar collagen, collagen type IV also contains globular and rod like, non-collagenous (NC) domains.

Collagen type IV protomers form in the Golgi apparatus, and, after secretion, six protomers interact to self-assemble into a hexamer.[327, 328] N-terminals of different collagen type IV chains spontaneously interact to form a multimeric complex. This self-assembled collagen type IV network is stabilized by covalent crosslinks involving disulfide and lysyl oxidase,[329-331] as well as through lateral associations between parallel collagenous filaments.[332] The network can extend indefinitely through C-terminal globular domains,[333] and is thought to provide protection against mechanical stress.[314]

Nidogen

Nidogens (or entactins) are glycoproteins, constituting 2-3% of basement membranes.[334] The core protein is made up of three globular domains with intervening rod-like segments,[335] and is thought to stabilize and tether the laminin/collagen type IV networks; the third globular domain binds strongly to laminins[336] while the second binds to collagen type IV, perlecan and fibulins[337].

Nidogens do not self-assemble, instead they are secreted as single molecules that integrate and bind to the laminin polymer and type IV collagen suprastructure.[338]

Perlecan

The perlecan of basement membranes is a large heparan sulfate proteoglycan,[339] assembling in a way similar to nidogen.[315] It binds numerous basement membrane components, including nidogen, integrin α2β1 and α-dystroglycan. Through its N-terminal HS-chains it binds laminins and collagen type IV, and through this region perlecan may also bind/sequester growth factors.[314]

Heterogeneity

Basement membrane composition is diverse, tissue specific and dynamic –there are 15 possible laminin heterotrimers and 6 possible collagen protomers, as well as additional splice variants. Differentially regulated genes drive tissue-specific up- and down-regulation and posttranslational modifications modulate function and binding affinities to other basement membrane components.[315]

Basement membrane specificity is also derived from additional components, including agrin, fibulin, type XV and XVIII collagens and osteonectin,[314] allowing it to perform highly specialized functions in different tissues.

"The puppet master" – the ECM controls cell behavior

Like chocolate chips in a cookie, cells are surrounded by the ECM (though, of course, in a slightly more organized manner). The ECM affects and controls cell behavior – different components have different effects, and even differing states of one particular component may result in different manners of cell regulation. For example, SMC proliferation is inhibited by components of a "normal, healthy" ECM/basement membrane, such as heparin[340] and heparan sulfate proteoglycans, including syndecan and perlecan in basement membrane, as well as laminin.[133, 134, 341]

Collagen exerts differential effects on cells depending on its fibrillar structure. Monomeric collagen type I has been shown to stimulates both SMC proliferation[130] and migration[130-132] *in vitro*, while the polymeric (fibrillar) form had inhibitory effects. Specifically, fibrillar collagen was found to up-regulate cyclin-dependent kinase-2 inhibitors through regulatory effects in early integrin signaling.[130]

The monomeric/polymeric state of type I collagen affects macrophages as well as SMCs. Seeding on fibrillar type I collagen inhibited spreading and synthesis of MMP-9, whereas the monomeric form promoted it.[135] In addition, both phagocytic activity[137] and LDL-uptake[136] have been reportedly influenced by type I collagen.

SLRPs

The SLRPs are a structurally and functionally related group of proteins. They are involved in ECM organization and affect cell behavior, such as adhesion, often through interactions with collagen.[342, 343] Kalamajski and Oldberg[342] suggest that the SLRPs may regulate intermolecular cross linking of collagen, either by inferring a specific cross-linking pattern or providing sterical hindrance, thus selecting the collagen lysine residues available for lysyl oxidase.

The SLRPs belong to the leucin-rich repeat (LRR) superfamily, but with a protein core mass averaging at around 40 kDa, they are – as the name implies – smaller than other LRR family members, such as versican and aggrecan with masses greater than 200 kDa.[344] Five different classes have been determined by sequence identity: class I includes biglycan, decorin and asporin, class II includes fibromodulin, lumican, keratocan, proline/arginine-rich end leucine-rich repeat protein (PRELP) and osteoadherin, class III includes epiphycan, osteoglycin and opticin, class IV includes chondroadherin, and class V includes podocan.[345] Since they are found in clusters on different chromosomes, they appear to have arisen from several duplication events.[344]

The structure of the LRR-protein ribonuclease inhibitor has been visualized through X-ray crystallography, and the final shape of the core protein, made up of alternating α-helices and β-sheets stabilized by hydrogen bonds, can be likened to the bent shape of a horseshoe.[346] Extrapolating from this structure, a similar configuration was suggested for the SLRPs, with the inner curvature able to accommodate a single collagen triple helix.[347] However, it has later been reported that the decorin core protein is less curved than the ribonuclease inhibitor. The variation, at least in part, is due to the shorter LRRs in decorin (21 or 24 amino acids in comparison to 28/29 in the ribonuclease inhibitor), and the inter-repeat angles suggest more of a “banana”-like shape for proteins with relatively short LRRs.[343]

The SLRPs share one common, 20-29 amino acid long, LRR domain consisting of 10-11 internal tandem repeats. The motif LxxLxLxxNxL is included, where x represents any amino acid and L (leucine) may be substituted by other hydrophobic amino acid residues, such as isoleucine or valine.[343]

The N-terminal domains are the least conserved, but all contain four cysteine residues that form intrachain disulfide bonds[348], unique for the SLRPs and also appearing in the C-terminus.[348] The core protein is often modified by carbohydrates, for instance by GAG chains in decorin and biglycan, and tyrosine-sulfate residues in fibromodulin and lumican. Polyanionic properties are derived from these negatively charged residues,[303] and the core protein and the glycosylation state together determine function. For example, SLPRs may accumulate growth factors within the ECM through interaction with GAG chains.[349]

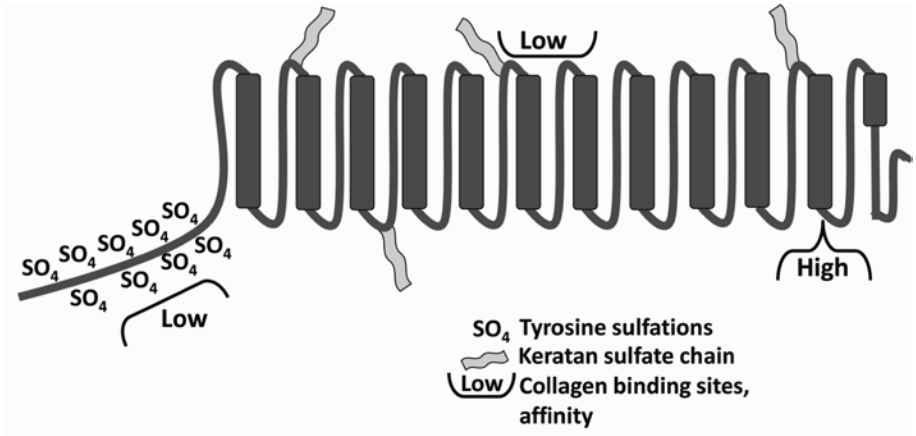

Figure 8:
The structure of fibromodulin.

Fibromodulin

The general characteristics of fibromodulin

Fibromodulin (figure 8) is a 59-kDa SLRP. It was initially purified from bovine articular cartilage, and is present in all cartilage, representing 0.1-0.3% of the wet weight. Fibromodulin also appears in variable amounts in other connective tissues, such as tendon, ligaments and sclera.[350] In mature tissues fibromodulin is mainly expressed in tissues exposed to tensile stress, such as tendons and cartilage.[350, 351] The molecular weight of fibromodulin is found to be, at least in part, dependent on tissue – it has been reported as 59 kDa in cartilage[350], 40 kDa in dental tissue and 52 kDa in alveolar bone[352].

In terms of amino acid composition, 14% of all residues are represented by leucine. Oldberg et al. determined the primary structure of fibromodulin from bovine tracheal chondrocyte mRNA – and found an overall 90% sequence homology to human fibromodulin.[351, 353] A 93% homology exists between bovine and murine fibromodulin.[303] Similar to other SLPRs in size and general structure with cysteine residues in conserved positions, fibromodulin is made up of 375 amino acids, with a predicted molecular weight of 42.2 kDa. The weight was originally found to be 59 kDa through analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE – this discrepancy can explained by anomalies in electro-

phoretic behavior due to carbohydrate substitution. Around 10-11 repeats of ~23 residues make up 60% of the core protein, the region located in the middle of the protein, flanked by non-repeat domains.[351]

Fibromodulin has 9 potential sites for O-sulfation of tyrosine residues in the N-terminus of the bovine form, and 11 sites in the human form.[354] Presence of tyrosine sulfate residues is confirmed in chondrocytes, sclera, tendon, fibroblasts and a fibroblast cell line. Only the N-terminus can carry sulfate substituents, which, adjacent to an acidic residue, can be modified by tyrosylprotein sulfotransferase.[355]

The N-terminal tyrosine sulfate domain is highly anionic, and has been found to bind the heparin-binding PRELP, basic fibroblast growth factor-2, MMP13, chondroadherin, IL-10 and the NC4 domain of collagen type IX , though neither fibronectin nor antithrombin III. The interaction involves the sulfations and it has been suggested that tyrosine sulfate residues sequester enzymes, growth factors and cytokines to specific structures in the tissue, and interact with for example PRELP and chondroadherin bound to a neighboring collagen fibril.[356]

Fibromodulin contain Asn-X-Ser/Thr sequences; potential sites for N-glycosylation, through which they are substituted with keratan sulfate chains.[351] Four sites seem to serve as acceptors for keratan sulfate,[355, 357] and substitutions are found in fibroblasts in sclera and tendon[355], but not skeletal tissue (at least of rodent origin).[358] Keratan sulfate chains are attached to the core protein via N-glycosidic linkage to asparagine.[351] Plaas et al. found keratan sulfate present at four out of five potential consensus sites for N-glycosylation in the LRR domain.[357] Differing keratan sulfate capping structures have been reported,[359] suggesting that cartilage may contain discrete populations of fibromodulin that may in turn have different roles in tissue.

Lumican[360] is a close homologue to fibromodulin with ~50% identity.[361] Lumican appear as a keratan sulfate proteoglycan primarily in the cornea, and exists as a classical glycoprotein in skin and cartilage.[362] Thus, glycosylation of lumican appears to be tissue-specific, developmentally regulated[363] and dependent on age[364]. Keratan sulfate is not required for binding to collagen,[365] and, in cartilage, the size of the keratan sulfate decrease with age.[364]

Mice deficient in fibromodulin (and other SLRPs)

Fibromodulin-deficient mice have no obvious phenotype. They are viable, fertile, and have normal body weight and life-span. Structure of the heart, liver, lung, kidney, skin, and cartilage is normal, but tendons and ligaments are weakened.[366] Fibromodulin-deficient mice are predisposed to ectopic tendon ossification and develop osteoarthritis.[366-368] Morphological changes in collagen fibers provided

information on fibromodulin's role in collagen fibrillogenesis – which is more closely discussed in following sections.[366]

Lumican-deficient mice have opaque corneas, which progress with age, and fragile skin.[369] Collagen fibers are irregular, thicker, and loosely packed, especially in the cornea.[369, 370]

Double knockout mice lacking both fibromodulin and lumican are viable and fertile, but have a reduced body weight, bowed legs, gait abnormalities.[371, 372] and a thinner sclera.[373]

Biglycan[374] and decorin[375, 376] are CS/DS proteoglycans: biglycan usually carries two sulfations, while decorin only carries one.[344] Biglycan is involved in regulation of skeletal growth, and knockout mice grow more slowly and develop osteoporosis.[377]. Collagen fibril diameter in tendons and patella is decreased, while an increase is found in bone and skin dermis.[368, 378] Biglycan also binds α-dystroglycan[379] and α- and γ-sarcoglycan[380] (of the dystrophin glycoprotein complex in the sarcolemma), and mild muscular dystrophy is present in mice with biglycan deficiency[381]. In decorin-deficient mice,[382] similar defects in collagen fibrils are evident as seen with biglycan-deficiency, with the additional tissue-specific variation in range, size, orientation and distribution profiles.[378, 382, 383] These mice also have fragile skin with reduced tensile strength, similar to the human condition Ehlers-Danlos syndrome.[382]

Biglycan/decorin double knockout mice have a more severe phenotype in bone and collagen fibril ultrastructure. They are not fertile, and are born by heterozygous parents in lower than expected Mendelian frequencies.[378]

Collagen binding and fibrillogenesis

Collagen binding

Fibromodulin interacts with collagen type I and II and delays fibrillogenesis, even in small concentrations (<5% w/w).[384] Fibromodulin binds to collagen through the core protein, through which it also binds collagen type XII.[276] Hedbom et al. has reported that fibromodulin does not bind denatured collagen.[304]

A collagen-binding site is located in the C-terminus of the LRR domain, specifically in the β-sheet-loop region of LRR 11, mediated by glutamic acid-353 and lysine-355, which are both essential for collagen binding. An additional, weaker, collagen-binding site is found in LRRs 5–7, possibly mediated through glutamic acid-251.[385] Lumican binds collagen through aspartic acid-213 in LRR 7, located to the β-sheet loop region of the LRR domain[385] and in a similar region as the

decorin collagen-binding site[386]. Fibromodulin competes with lumican for collagen binding through the low affinity collagen-binding site in LRR 5-7, but not through the high affinity site in LRR 11.[387]

Fibromodulin is thought to replace collagen-bound lumican during the process of fibril growth, possibly through an increase in the ratio of fibromodulin/lumican synthesis.[303] This suggests a potential role for the SLRPs in the cross-linking of collagen fibrils – both fibromodulin and lumican may regulate the initial cross-linking, but only fibromodulin is capable of further cross-linking as fibrillogenesis progresses, possibly by connecting two collagen units through simultaneous binding, utilizing both binding sites.[387]

A third collagen binding site was recently reported, as the negatively charged N-terminus also was found to interact with collagen type I. Simultaneous binding to two collagen molecules can only be achieved by full-length fibromodulin, and not by a truncated protein missing the N-terminal region. This region was also found to accelerate collagen fibril formation *in vitro*, and promote a more organized fibril structure.[388]

Immunoreactivity, visualized by electron microscopy, can be seen close to, or at, the surface of collagen fibrils in cartilage. Fibromodulin presence is lowest close to cells, and highest in the interterritorial matrix. Distribution along fibrils is highly variable, likely an effect of functions such as the regulation of fibrillogenesis. Fibromodulin preferentially binds to the gap region of assembled collagen fibrils, and dose-dependent effect of fibromodulin levels on collagen fibril mean diameter has been found.[302]

Collagen fibrillogenesis

Collagen fibrils in the fibromodulin-deficient tendon are abnormal. Fibrils are less organized, with unevenly distributed cells and a reduction in endotendon tissue. There is a 25-55% reduction in fiber bundles, and fibrils are irregularly shaped, on average thinner, and contain a higher proportion of thin fibrils compared to the wild type. 10-20% of collagen bundles are abnormal in the heterozygote.[366]

In tails and the Achilles tendon, fibromodulin is present in the collagen bundles of tendons, with a weaker presence in dermis. Lumican appear preferentially in the dermis and peritendon tissue. There is an increased staining of lumican in fibromodulin-deficient mice, and staining also shows increased expression in the collagen fiber bundles. On a protein level, decorin expression remains unchanged, but lumican expression is increased 2-fold in tails and 3 to 5-fold in tendon. mRNA expression is, however, decreased, perhaps due to negative feedback derived from the dramatic increase in protein levels.[366] A similar increase in fibromodulin levels is not present in lumican-deficient mice.[372]

Fibromodulin's role in collagen fibrillogenesis is thought to be in the regulation and stabilization of the initial assembly of collagen monomers into short, small-diameter intermediates and the beginning of fibril growth. Fibromodulin also regulates growth of intermediate fibrils into mature large diameter fibrils.[371]

While lumican expression peaks ~8 days postnatally, followed by a dramatic decrease, fibromodulin peaks at 14 days postnatally, followed by a slow and slight decrease until 1month postnatally, and then ending in a dramatic decrease. Expression peaks suggests that lumican may be active during early fibrillogenesis, whereas fibromodulin functions through all stages, but is more relevant in the later stages.[371]

Ezura et al[371] compared collagen fibrils in lumican-, fibromodulin- and lumican/fibromodulin-deficient tendons. The primary alterations in collagen appeared to be of four categories:

- Irregular fibril profiles present in all three genotypes by the age of 3 months – very mild in lumican deficiency, intermediate in fibromodulin deficiency and very severe in double-deficient mice.
- A shift to increased diameters of fibril intermediates during early development was present in all genotypes. The premature generation of heterogeneous population in the double-deficient tendons may be explained by either an additive effect of lacking both lumican and fibromodulin, or a premature entry into the growth phase during the initial fibril assembly due to absence of a regulatory block.
- A large number of small-diameter (64 nm) fibrils were found in later stages of development in double-deficient mice, indicating a defect in entering and progressing through the fibril growth phase.
- A shift towards smaller fibrils 10 days postnatally. In lumican-deficient mice, fibril size returns to a wild type-like phenotype after 1-3 months, but the shift remained in fibromodulin- and double-deficient mice, The defect in transition through later growth stages during maturation of fibrils, again indicating that mainly fibromodulin is required for regulation of the later stages.

As a result, the following model of collagen fibril growth is hypothesized: Regulation takes place through changing expression patterns of lumican and fibromodulin, with lumican decreasing to barely detectable levels as fibromodulin increases. The stage-specific function likely stems from differential expression and synthesis patterns. The initial step in fibrillogenesis – assembly of monomeres into intermediates – and stabilization of the resulting intermediates (~64 nm) in order to

mediate fibril growth is regulated by both lumican and fibromodulin, while the later stages are regulated solely by fibromodulin.[371]

Other effects of fibromodulin

Calcification

Fibromodulin has a role in mineralization and calcification; it is expressed in mouse chondroblasts and osteoblasts during fetal bone development[389] as well as in adult bone[390]. In calcified tendinopathy in rat, fibromodulin was found in chondrocyte-like cells both in uncalcified matrix and calcified deposits.[391] Finally, increased fibromodulin expression was found during mineralization *in vitro*[392] as well as in tooth maturation.[393] and dentin mineralization. Enamel formation has been shown to be delayed in fibromodulin deficient mice.[394]

Hormonal control

Collagen remodeling, and fibromodulin expression, occur in a hormone-dependent manner. In ovariectomized mice, Markiewicz et al[395] observed a reduced expression of fibromodulin mRNA. Whereas no qualitative difference was seen in collagen fibrils, there was an increase in freshly synthesized fibers, suggesting a role for estrogen in regulating organization and stability of collagen fibrils.

Direct regulation of fibromodulin by estrogen and progesterone has also been reported. During the menstrual cycle, fibromodulin expression in the myometrium is higher in the secretory, progesterone-dominated phase than in the proliferative, estrogen-dominated phase.[396] The role of fibromodulin may be in myometrium remodeling, as, in mice, fibromodulin mRNA expression was higher in diestrous (progesterone dominance) than estrous (estrogen dominance). Estrogen appear to negatively regulate fibromodulin expression, since ovariectomy resulted in an increase in fibromodulin mRNA compared to estrous, while estrogen-treatment lead to a reduction of fibromodulin mRNA.[397]

Other interactions

Fibromodulin was recently reported to enhance angiogenesis (*in vivo*, *in ovo* chick embryo). Fibromodulin also accelerated cell adhesion, spreading, actin stress fiber formation, and tube-like structure network establishment in human umbilical vein endothelial cells *in vitro*.[398] Moreover, fibromodulin expression has been found, by mass spectrometry, in carotid and thoracic artery intimal hyperplasia, though it was present in similar amounts in the atherosclerosis-prone carotid artery, and in the atherosclerotic-resistant thoracic artery.[399].

Fibromodulin binds TGF-β (1, 2 and 3) through a conserved region of the core protein. Slight differences between the TGF-β binding properties of decorin, biglycan and fibromodulin may indicate differing regulation of TGF-β sequestering and/or activities.[400] Fibromodulin is reportedly regulated by TGF-β in granulation tissue (through expression by fibroblasts)[401] and in intervertebral disc during development.[402]

Collagen-bound fibromodulin is cleaved by MMP-13. The cleavage site is located in the N-terminus, and the N-terminal fragment is lost upon cleavage, while the larger fragment is retained in cartilage – and presumably still bound to collagen fibrils.[403] This cleavage of fibromodulin has been suggested by Monfort et al. to represent an early critical event in cartilage degradation in for example osteoarthritis.[404] Fibromodulin is also suggested to actually protect collagen from collagenase degradation by MMP1 and -13.[405]

A role for fibromodulin in promoting pluripotency in stem cells[406] and helping to maintain/organize the endogenous stem cell niche in tendon[407] has also been proposed.

Fibromodulin expression is induced by reactive oxygen species in the liver,[408] and by ultraviolet (UV) radiation *in vitro*; UVC[409] as well as UVA and UVB[410] irradiation.

Fibromodulin and pathologies

Wound healing

Fibromodulin affects ECM structure and remodeling in the process of wound healing[411]. In fibromodulin-deficient mice, the area of fibrosis is increased. The resulting scars are larger with disordered collagen fibers that are irregular in size and shape. Fibromodulin is also downregulated in, for example, hypertrophic scarring[412]

Fibromodulin and cancer

Several studies have shown fibromodulin to have an active role in the progression of cancer. Its expression is enhanced in metastatic melanoma (along with biglycan)[413], and Mikaelsson et al[414] found fibromodulin to be the most overexpressed gene in B-cell chronic lymphocytic leukemia (B-CLL). The ectopic expression is speculated to facilitate the interaction between B-CLL cells and the microenvironment; an interaction often essential for cancer cell survival. Spontaneous expansion of CD8+ T cells recognizing fibromodulin peptides in these pa-

tients was reported by Mayr et al., proposing fibromodulin to be a novel tumor-associated antigen.[415] In addition, Choudhury et al. later found that apoptosis in CLL cells, but not B cells, from healthy donors, was increased upon introduction of small interfering RNA (siRNA) to fibromodulin *in vitro*.[416]

In patients with colorectal cancer fibromodulin has been detected in the desmoplastic stroma surrounding the tumor. ECM of this kind is of high density and is induced through contact between colorectal cancer cells and surrounding fibroblasts, exhibiting unorganized collagen fibers and proteoglycans.[417] This is corroborated by a study by Oldberg et al.[418] in which fibromodulin-deficient mice were found to exhibit thinner and fewer collagen fiber bundles in tumor stroma, as well as a decreased density of the collagen scaffold. The accompanying lowering of the interstitial fluid pressure (IFP) may be of importance in the delivery of anti-cancer drugs, which can be restricted by the high IFP normally found in tumor stroma.

Other pathologies

Fibromodulin expression is upregulated in osteoarthritis,[419] and fibromodulin cleavage in the breakdown of rheumatoid or osteoarthritic cartilage may precede the major collagen destruction in this tissue.[403, 420]

Fibromodulin has also been found to promote liver fibrosis: higher fibromodulin levels were found in livers from patients suffering from cirrhosis. Fibromodulin was bound to collagen type I, but did not protect collagen fibers from degradation by MMP13. Over-expression of fibromodulin resulted in an increased expression of collagen type I and smooth muscle α-actin (α-SMA) in hepatic stellate cells and addition of recombinant fibromodulin promoted proliferation, migration, chemotaxis of these cells.[408] An increased fibromodulin expression is also found in bleomycin-induced lung fibrosis in rat.[421]

Enhanced fibromodulin expression is associated with both normal aging[422] and progeria[423]. In addition, structural changes in fibromodulin associated with aging are found in articular cartilage.[364]

Fibromodulin is directly associated with induction of inflammation, and has been shown to bind complement cascade component C1q, complement inhibitor factor H and complement inhibitor C4b-binding protein.[424-426] The simultaneous binding to complement inhibitors was found to regulate the ability of fibromodulin to activate the classical and alternative pathways of the complement system.[424]

The majority of studies focuses on the expression of fibromodulin – increased or decreased, present or absent – with only a few studies delineating effects of mutations. Nevertheless, point mutations in the LRR domain is reportedly linked with

high myopia.[427] Fibromodulin also seems to be involved in fibrillogenesis in post-natal development of the limbus – the transition between corneal and scleral stromas – during early corneal development. Its pattern overlaps with lumican, suggesting a cooperation. Fibromodulin expression may in this case be triggered by a change in mechanical stress[428] – perhaps somewhat reflective of what happens with fibromodulin in the atherosclerotic plaques of Papers I and II of this thesis.

Dystrophin: Connecting the cell to the extracellular matrix

Cells have several ways in which they bind to, associate with and communicate with the ECM. While the previous section explored the architecture of the extracellular environment itself, this section addresses one of these connections: dystrophin and the dystrophin-glycoprotein complex (DGC; figure 9). Dystrophin – or rather, the lack of dystrophin – has previously been (and still is) thoroughly investigated as it is an essential element in Duchenne muscular dystrophy (DMD).

Dystrophin is located at the cytoplasmic face of the sarcolemma,[429-431] and is thought to function as a stabilization against mechanical forces during muscle contraction and stretch. This conclusion is drawn from several observations in DMD patients and dystrophin-deficient mice, showing that sarcolemmal fragility[432-434], muscle weakness [435, 436] and necrosis [437] are all exacerbated by mechanical stress,[434, 438, 439] but improved by muscle immobilization,[440, 441] and corrected in dystrophin-deficient mice by transgenic expression of full-length dystrophin.[436]

The dystrophin gene is located to the X-chromosome, and DMD is caused, through recessive inheritance, by a defective copy of this gene.[442] Dystrophin is, thus, absent in muscle tissue in DMD-afflicted humans[429-431, 443] as well as in mice with the mdx mutation[442], a commonly used animal model for DMD. DMD affects one in 3500 male births.[444] Progressive muscle wasting, with clinical onset at 3-5 years of age, shortens life expectancy to 20-30 years due to respiratory failure.[442, 445] Becker muscular dystrophy is the same disease in a milder form, resulting from different mutations in the dystrophin gene, leading to low-level expression of a truncated dystrophin protein.[446, 447]

The dystrophin protein is made up of four major domains. At the N-terminal a pair of calponin homology modules form an actin binding domain.[448] It is followed by 24 triple helical spectrin-like repeats interspersed with four putative hinge do

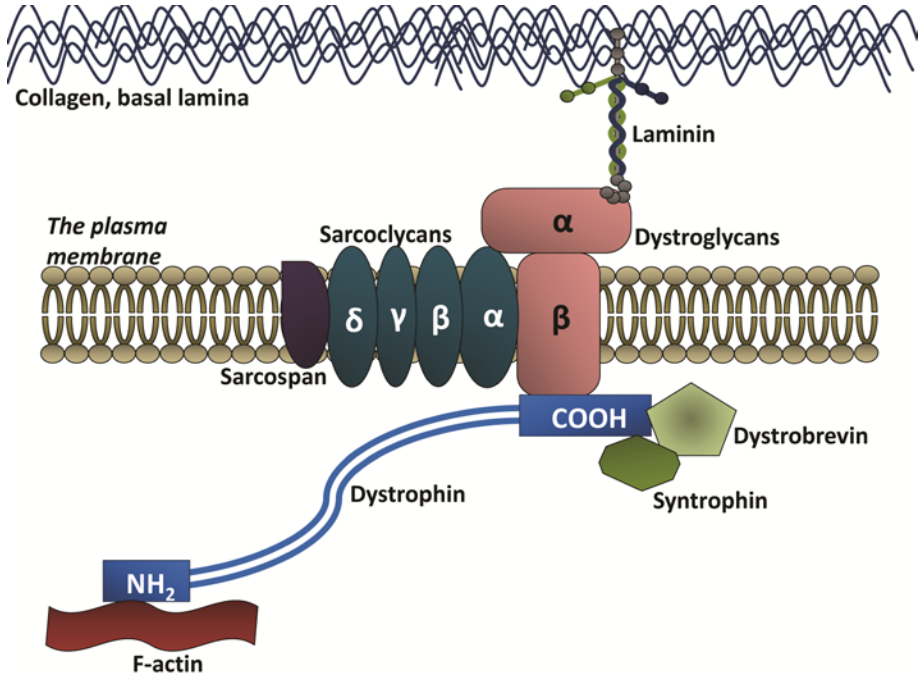

Figure 9
Schematic overview of the DGC.

mains – this is the largest domain,[448] and it gives dystrophin its elongated, flexible rod shape. Next is a cysteine-rich domain consisting of four modules: two EF hand-like motifs,[449] a WW domain[450] and a putative zinc finger ZZ domain,[451] which together is essential for the binding of dystrophin to β-dystroglycan of the DGC[452, 453]. The final domain is the C-terminal, with which it associates with the DGC.[454]

The 79 exons[455, 456] of the dystrophin gene translates into a 427 kDa protein in skeletal muscle.[442, 449] Through differential promoter usage of four internal promoters it may also appear as several short isoforms; Dp260, Dp140, Dp116, Dp71 (based on protein weights).[457] Dp260 is primarily found in the other plexiform layer of the retina.[458]Dp116 and Dp140 are mainly expressed in peripheral[459] and central nervous system and kidney[460], respectively. Dp71is ubiquitously expressed in many tissues,[461, 462] such as cardiac muscle,[463] retinal blood vessels,[457], liver, testis, lung, kidney – but it is not found in skeletal muscle.[464]

Utrophin[465] is a close homologue to dystrophin, and the two proteins are suggested to stem from an ancient gene duplication event.[466] Like dystrophin, it contains an N-terminal actin-binding domain, a central spectrin-like repeat region, and a C-terminal domain that binds the DGC.[467] Utrophin is expressed throughout the sar-

colemma in fetal and regenerating muscle, but is down-regulated at birth, and later restricted to myotendinous and neuromuscular junctions in normal adult muscle.[468] It is, however, expressed in nearly all non-muscle tissues, such as large and small arteries and veins,[469] pulmonary endothelial cells [470], the nervous system,[471] platelets,[472] heart, liver, testis and kidney.[462] Compared to dystrophin, utrophin has a different mode of binding to actin filaments and β-dystroglycan[473] and the two proteins do not compete for binding sites. Dystrophin binds actin filaments through two separate low-affinity sites and utrophin through one contiguous actin-binding domain.[474] The stronger, more extensive lateral association with actin filaments carried out by utrophin[475] is suggested to allow for a less elastic response of the actin-utrophin-sarcolemma linkage to muscle stretches, compared to a linkage involving dystrophin.

The dystrophin-glycoprotein complex

Dystrophin is a part of, and interacts with, the DGC; a large, hetero-oligomeric complex of membrane-associated and cytosolic proteins. Included in the DGC are also the dystroglycans (α, β), sarcoglycans (α, β, γ, δ), sarcospan, the syntrophins,[454, 476-478] and the dystrobrevins[479]. The DGC binds both actin and laminin, providing a link between the actin cytoskeleton and the basement membrane of the ECM.[480] The function of this connection is thought to protect skeletal muscle fibers from contraction-induced damage.[439, 480, 481]

The glycoproteins α- and β-dystroglycan are encoded by a single transcript, and the resulting propeptides are proteolytically processed into two non-covalently associated units – one extracellular unit (156 kDa), and one (43 kDa) single-pass transmembrane unit.[481] As stated in a previous section, β-dystroglycan binds to dystrophin (and utrophin)[482] through the cysteine-rich C-terminal domain.[483] This binding anchors the DGC to the sarcolemma, where it participates in interactions with several ECM components. Its function depends on glycosylation sites, which are regulated in a tissue-specific manner.[484] Dystroglycans are broadly expressed in both developing[485] and adult tissues, including non-muscle tissues.[486].

The sarcoglycans, α- (50 kDa[487]), β- (43 kDa[488]), γ- δ-(both 35 kDa[489, 490]), are single pass transmembrane proteins. Together, they compose a stable tetrameric complex, suggested to strengthen the interaction of β-dystroglycan with α-dystroglycan and dystrophin. They stem from related, but distinct, genes, and mutations in one gene results in loss of entire complex. The smaller sarcospan (25 kDa)[491] is stably associated with the sarcoglycan complex.

The dystrobrevins and syntrophins – 88 and 59 kDa respectively[479, 492, 493] – are cytoplasmic proteins, binding to each other and the C-terminal of dystrophin.[493] α-dystrobrevin also interacts with the sarcoglycan complex.[494]

Many binding partners to the DGC have been described. Laminin is an extracellular ligand for α-dystroglycan,[480, 481] High affinity binding has also been described between agrins, neurexins and perlecan to α-dystroglycan, the different associations dependent on oligosaccharide modifications. [495, 496] Biglycan binds the core protein of α-dystroglycan via its CS chains.[495, 496] Binding often takes place in order to couple DGC with other structural elements of muscle; such as synemin[497], syncoilin[498, 499], myospryn[500], γ-filamin[501], and cytokeratins 8 and 19[502].

Utrophin can interact with the DGC in a manner similar to dystrophin, forming the utrophin-glycoprotein complex (UGC).[503] A particular UGC exists in ECs, which may have a role in mechanotransduction through association with endothelial NOS in caveolar domains.[504] In SMCs, an UGC and DGC have been described to interact with caveolin-1 in caveolae,[505, 506] but the UGC has also been shown to localize to non-caveolae lipid raft domains.[505]

Mice deficient in DGC components

Muscular dystrophy is evident in mice deficient in dystroglycan.[507, 508] Loss of any of the sarcoglycans result in limb-girdle muscular dystrophy in humans, appearing as progressive muscular dystrophy in mice.[509]

A progressive, though mild, myopathy has been reported in mice deficient in α-dystrobrevin,[510] whereas none is seen in mice deficient in syntrophin, suggesting a function as modular adaptors (or scaffolds) to anchor, and regulate surface expression of ion channels and signaling molecules to the DGC.[511 512] Neither humans, nor mice, exhibit any myopathy as a result of mutations in sarcospan.[513]

The mdx mouse

The dystrophin-deficient mdx (X-chromosome-linked muscular dystrophy) mouse – explored in Papers III and IV of this thesis – has slightly reduced viability and fertility, and the creatinine kinase plasma level is elevated.[514] The loss of dystrophin is due to a point mutation – a substitution – in exon 23[515], causing a premature stop codon that terminates translation of the polypeptide chain.[516] With the exception of rare revertant myofibers, there is no detectable dystrophin expression found in the skeletal muscle of mdx mice.[442] On the other hand, expression of the short, C-terminal isoform Dp71 is unaffected.[457, 517] Mdx mice develop muscular dystrophy, though, in most skeletal muscles, with the exception of the diaphragm,

in a form that is milder than DMD[518] in that the initial dystrophic muscle regenerate to some extent.[519]

Dp71 mRNA does not contain the region of dystrophin mRNA containing the unfortunate stop codon that causes the mdx mutation. Thus, it contains the C-terminal, the cysteine-rich domains, and seven additional N-terminal amino acids – with some possible modifications produced by alternative splicing in the C-terminal domain. Lacking in Dp71 are the spectrin-like repeats along with the actin-binding N-terminal domain. Dp71 is expressed in brain, liver, and smooth muscle of the stomach, both in wild type and mdx mice – however, Dp71 is undetectable in both wild type and mdx skeletal muscle.[461]

The rest of the DGC components – the dystroglycans, sarcoglycans, syntrophin and dystrobrevins – are reportedly downregulated in the mdx mouse [503, 522]In contrast, other molecules have been found to be upregulated in mdx mice, likely due to compensatory cytoskeletal remodeling, such as γ-actin,[523] α7β1-integrin,[524, 525], biglycan,[379] and utrophin [475, 526, 527].

While the natural upregulation of utrophin in mdx muscle fails to reach an expression level sufficient to compensate for the deficiency of dystrophin,[474] transgenic overexpression of utrophin [528] – and also α7-integrin[529] – do rescue the dystrophic phenotype.

Mice deficient in utrophin display no obvious morphological defects; they breed normally and have a normal lifespan.[520] In contrast, mice lacking both dystrophin and utrophin exhibit a severe muscular deficiency phenotype, similar to DMD.[520, 521]

Dystrophin in blood vessels

Dystrophin is normally expressed in the tunica media of blood vessels, but is absent from vessels in mdx mice.[530-532] Expression has been described in both large arteries and large veins, but only in small arteries – the common denominator being that all are vessels with contractile properties, suggesting active involvement by dystrophin in the mechanical resistance of muscle membranes.[469] In the carotid artery, dystrophin is expressed by both vascular SMCs as well as ECs.[533] The DGC of vascular SMCs has a specific profile of proteins, containing dystrophin, α- and β-dystroglycan, ε-, β-, δ-, and γ- or ζ-sarcoglycan and sarcospan.[534, 535] Integrity of the cytoskeleton is preserved in mdx SMCs *in vitro*, but cell contractile ability differs, with contractions being less stable over time.[527]

Biomechanical properties are altered in mdx arteries – vessels are distended less, and there is greater circumferential and axial stiffness.[536] Loufrani et al. has inves-

tigated the role of dystrophin in vascular function,[533] using the mdx mouse, and found changes in the vessel wall; wall-to-lumen ratio was larger, and compliance and distensibility was less – in contrast, wall thickness and diameter was similar in wild type and mdx mice. In most respects, vascular reactivity, including blood pressure, was normal in the mdx mouse. However, flow (shear stress)-induced dilation is strongly reduced in the carotid and mesenteric arteries, representing compliance and resistance arteries, respectively. Since pressure (tensile stress)-induced tone is unaffected, the defect specifically concerns mechanotransduction of shear stress at the surface of ECs, and not pressure exerted on the whole vessel wall. Thus, endothelium-dependent vasorelaxation induced by flow (shear stress) is strongly attenuated in the mdx mouse.[533] Tronc et al. found flow-induced NO to be the main determinant of vascular remodeling in large compliance arteries, such as the carotid artery.[537] Therefore, the decreased ability of ECs to produce NO in response to shear stress (and thus, achieve vessel dilation) suggests a lesser ability to adapt to increased blood flow in tissue, such as skeletal muscle, where a metabolic need requires higher blood flow supply. [533, 538]

Both vascular and dystrophic phenotypes were improved in transgenic mdx mice that expressed dystrophin in smooth muscle only. Vasoconstriction induced by norepinephrine increased with smooth muscle dystrophin-expression – dystrophin-expression in SMCs may therefore facilitate autocrine regulation of NO, a hypothesis strengthened by the presence of neuronal NOS protein in the aorta of both wild type and transgenic mdx with SMC-dystrophin expression, but not in the original mdx mouse.[532]

Main methods

Smooth muscle cell isolation and ECM production in vitro

Similarl to when SMCs dedifferentiates from a contractile to a synthetic phenotype during the development of atherosclerotic and restenotic lesions, a phenotypic modulation takes place as SMCs are isolated and cultured *in vitro*. Reduction in expression levels of contractile proteins such as α-SMA is accompanied by an increase in proliferation and synthesis of ECM components. In addition, production of their own mitogens is taking place in these synthetic SMCs.[539]

In preparation of the *in vitro* experiments in papers I and III, we isolated vascular SMCs from mouse aortas. Entire aortas – from the aortic arch to the aortic bifurcation (into iliac arteries) were dissected out. After removal, the aortas were thoroughly cleaned from the surrounding adventitia to avoid contamination of SMC culture with fibroblasts. Only the medial and intimal layers of the vessel then remained, and as endothelial cells require specialized cell culture medium (and supplements), they do not survive the isolation process which includes plating with Ham's F-12 medium. This leaves the SMCs as the only viable cell type left at the end of the isolation procedure. To ensure continued good health, F-12 medium was supplemented with 10% serum, as well as with antibiotics. Ascorbic acid, used as a co-factor in hydroxylation of lysine and proline during collagen synthesis[540, 541], was added in excess every two to three days.

Isolated SMCs from wild type and fibromodulin-null mice were left to grow for several days. By extracting the cells with Triton X-100 and NH_4OH,[126] we were able to use the underlying intact ECM for experiments. We assessed the effects of collagen structure on lipid uptake and cytokine production by seeding macrophages on ECM synthesized by wild type and fibromodulin-deficient SMCs, and adding oxLDL.

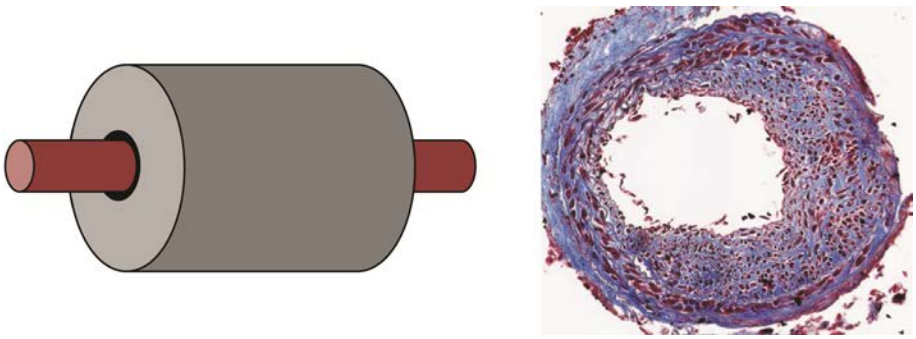

Figure 10:
The non-occlusive cuff injury, with resulting lesion visualized by a Masson's trichrome stain.

Injury-inducing casts and lesion formation

In paper III, we wanted to study neointima formation, and especially the role of SMCs in this process. To generate neointimal lesions in mice a non-occlusive plastic cuff was placed around the right common carotid artery (the left being used as uninjured control) for three weeks (figure 10). The resulting neointima is rich in SMCs and ECM, but there is no deposition of lipids,[542, 543] and the inflammatory component varies with different experimental timepoints.[544] Involvement of T and B cells in neointima formation have been shown previously using this model. Neointima formation was increased in immune-deficient Rag-1 knock-out mice, while reconstitution with B cells from wild type mice reduced neointima formation in these mice compared to unreconstituted mice.[545] Additionally, presentation of lipid antigens through the Cd1d-natural killer T cell pathway has been proposed to modulate vascular repair responses, as neointima formation was reduced in mice lacking Cd1d.[546] In contrast, though carotid artery injury resulted in mobilization of regulatory and Th1 T cells, neointima formation was not affected in mice lacking regulatory T cells or antigen presentation to CD4+ or CD8+ T cells.[544]

For experiments that included the non-occlusive cuff, we used 4-5 month old wild type and mdx mice that were not bred on an atherosclerosis-prone background such as apoE- and LDL receptor deficiency, and the mice were given regular chow.

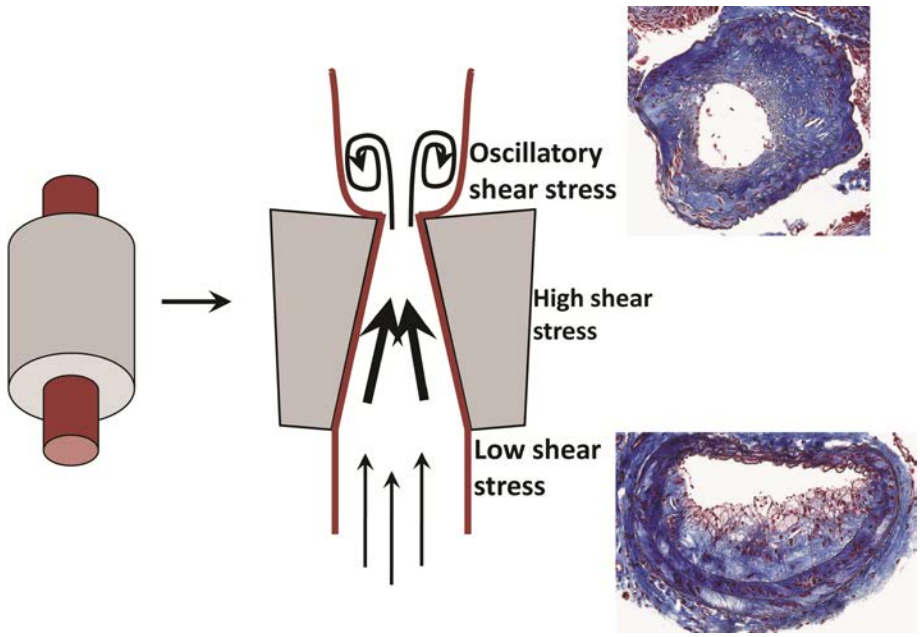

Figure 11:
The shear stress-modifying constrictive cast, with resulting lesions visualized by a Masson's trichrome stain.

In papers I and IV, we wished to study neointima formation in the context of atherosclerosis development. To induce development of lesions resembling the inflammatory and lipid-rich plaques found in the human disease, we used a cast model that modified shear, rather than mechanical, stress (figure 11).[547]

Shear stress is a parallel frictional drag force on the endothelium, which affects endothelial gene expression through activation of shear-stress response promoter elements. Blood flow rate, vessel radius and fluid viscosity together determine the size of the force, which is expressed as dynes/cm^2. Normal shear stress plays a role in vascular function through anti-inflammatory, antithrombotic, anticoagulative, profibrinolytic and anti-hypertrophic effects, but the modified shear stress that appears with disturbed blood flow is involved in the formation of atherosclerotic lesions.[548] Plaques have been reported to preferentially develop under conditions of low shear stress, appearing, for instance, in the inner curvature of coronary arteries, and through oscillatory shear stress, occurring near bifurcations.[549] A decrease in shear stress is associated with reduced vasodilation and endothelial repair accompanied by an increase in reactive oxygen species, leukocyte adhesion and permeability of lipoproteins.[548]

We induced shear stress-modification *in vivo* through the use of a perivascular constrictive, tapered cast. The cast was placed around the right common carotid artery. As was also the case using the non-occlusive cuff, the placement around the straight part of the artery, where lesions are not prone to form spontaneously, allowed us to induce alterations in a controlled manner. Stenosis is stimulated by the cast; shear stress is lowered upstream from the cast, increased within the cast, and oscillatory downstream from the cast.[547] Cheng et al. found atherosclerotic lesions of a vulnerable phenotype to form in areas with low shear stress, while more stable lesions formed in areas with oscillatory shear stress.[547]

In preparation for the experiments involving this cast, mice were started on a Western diet (21% fat, 0.15% cholesterol) at 16 weeks of age. Cast placement took place two weeks later, and carotid arteries (injured arteries, as well as uninjured control arteries) were recovered after an additional 12 weeks – along with heart, descending aorta and blood, which were also saved for experiments.

Murine tissues

Carotid arteries were either snap-frozen or placed in Histochoice for fixation before paraffin-embedding. Hearts and descending aorta were fixed with Histochoice; hearts were later frozen, while aortas were used for flat preparations and Oil Red O-staining of lipids. Plasma was frozen.

Histology

Serial sections of frozen and paraffin-embedded carotid plaques were 8 and 5 µm thick, respectively. Plaque composition was analyzed by histological and immunohistochemical methods as well as through immunofluorescence. The size of the carotid plaques formed by either cast model is only around one 10^{th} of a mm^3 at best, which is too small to quantify through Western blot, and, at least in our hands, too small to homogenize in order to perform enzyme-linked immunosorbent assay, ELISA. Analyses based on histology have therefore proved to be the most efficient and accurate way to study plaque components.

The content of different plaque components was quantified using sections collected in close proximity to the cast. Quantification of size is represented by a mean value of several sections that were collected at the most stenotic region of the plaque, 15 µm apart.

Collagen (or rather ECM, with collagen being the most abundant component) and lipids were identified through histological staining. Masson's trichrome and Sirius red were used in staining for collagen, and Oil Red O was used to stain for lipids.

Other plaque components were visualized using immunohistochemistry and immunofluorescence, depending on which antibody was used and which method produced stainings of the best quality. The choice to use frozen or paraffin-embedded tissue was also made on similar premises, and either type of treatment of tissues presents with advantages as well as limitations. The main point of contention is that antibodies often seem to function better on frozen tissue, for example requiring less antigen epitope retrieval for successful staining, while tissue is more completely preserved through dehydration and subsequent paraffin-embedding, resulting in more complete and better looking sections (whereas frozen section frequently give a rather squashed appearance). In only a few instances is the choice made for us, such as with Oil Red O-staining which must be performed on frozen sections, as the alcohol used for dehydration is a solvent for lipids.

Figure 12:
A frozen human carotid plaque, cut into sections.

Human tissues: The Carotid Plaque Imaging Project

Analysis of human plaque tissue was possible due to the Carotid Plaque Imaging Project, CPIP; a biobank for blood and plaque tissue collected from patients undergoing carotid endarterectomies performed at the Vascular Department of Skåne University Hospital in Malmö.

Patients included in Paper II had undergone operation in 2004 to 2011, and was enrolled after giving informed consent. On the day before surgery, blood samples were taken, and patients underwent ultrasound examination of carotid arteries. Additionally, the patients were assessed by a neurologist. The patients were oper-

ated when having experienced neurological symptoms and carotid artery stenosis degree of >70%. Patients without neurological symptoms were operated when having a stenosis degree >80%.[550, 551] Absence of symptoms were defined as patients with no *amaurosis fugax*, transient ischemic attacks or strokes in the 6 months preceding surgery.

Information was gathered concerning medical treatments and clinical risk factors. Later analysis of postoperative cardiovascular events was performed through the Swedish national health register of hospitalizations,[552] with clarifications gained through telephone interviews and reviews of medical charts as needed.

Plaque tissue was immediately snap-frozen in liquid nitrogen at the operation room, directly after removal from the patient (figure 12). Plaques were weighed and photographed. One portion (one millimeter thick) from the most stenotic region of the plaques was saved for histological analyses and a similar, adjacent, portion was saved for RNA analysis. The remainder of the plaques was homogenized[553] for analysis of plaque proteins, lipids and other components.

Plaque tissue used for histological studies was treated and analyzed in a similar way as the frozen murine plaque tissue used for this purpose. Cytokine assessment was performed on plaque homogenate supernatants.

Results of the present studies

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny ...'"

Isaac Asimov

Aims

The studies presented in this thesis address the role of the ECM in vascular lesion development through the following questions:

- Is fibromodulin expressed in atherosclerotic plaques, and – if so – is it associated with a more or less stable plaque phenotype?
- Is dystrophin expressed in neointimal hyperplasia and/or atherosclerotic plaques?
- Is the ECM of vascular lesions altered as a result of fibromodulin or dystrophin deficiency?
- Does fibromodulin or dystrophin deficiency affect plaque formation and/or composition?
- Is fibromodulin a possible biomarker? As measured in human carotid plaques, may it be of use to predict total plaque burden and/or future events?

Paper I

In Paper I, we tested the hypothesis that fibromodulin affects the development of atherosclerotic plaques – possibly through its role in the regulation of collagen fiber structure. To achieve this, we compared fibromodulin-deficient apoE mice with apoE mice with normal expression of fibromodulin (referred to as ApoE- versus ApoE/fibromodulin-deficient mice).

Previous studies show effects on collagen structure in fibromodulin-deficient mice, such as altered collagen fibril thickness. Therefore, we had a special focus on effects on plaque stability. For example, a finding of thinner collagen fibrils – similar as in fibromodulin-deficient tumor stroma,[418] a type of pathological tissue with many similarities to plaque tissue – might indicate that fibromodulin deficiency has a destabilizing effect in the atherosclerotic plaque. Interestingly, what we found instead was that fibromodulin deficiency in atherosclerotic plaque tissue results in collagen fibrils that are thicker than those in plaques from control mice. In addition, the total plaque burden, as assessed by flat preparation and Oil Red O-staining of whole aortas, was smaller in fibromodulin-deficient apoE-mice.

The main part of this study was an investigation of plaques induced in the common carotid artery through the shear stress-modifying cast. We found the more inflammatory low shear stress plaques to be smaller in fibromodulin-deficient mice. Plaque sizes were similar in the more fibrous oscillatory shear stress regions of arteries from ApoE- and ApoE/fibromodulin-deficient mice. This was somewhat surprising, as low shear stress plaques actually contain less collagen than plaques from the oscillatory shear stress region. Though, on the other hand, lower collagen content is not necessarily synonymous with lower collagen synthesis – if collagen breakdown is increased, a higher degree of remodeling (with accompanying fibromodulin expression) may actually be taking place in plaques in the inflammatory low shear stress region.

In addition, we found evidence for decreased lipid accumulation in the vessel wall of fibromodulin-deficient mice. Lipid content was decreased in both low shear stress plaques in the carotid artery and in lesions in the aortic root. Additionally, in low shear stress plaques, the total amount of macrophages – the primary cell in which lipids accumulate – was also lower. To test the hypothesis that the fibromodulin-deficient ECM can affect lipid accumulation, we compared lipid uptake in a macrophage cell line grown on wild type and fibromodulin-deficient ECM *in vitro* and found decreased accumulation of oxLDL in macrophages grown on fibromodulin-deficient ECM. In this *in vitro* model, we also analyzed whether the altered ECM had an impact on cytokine production and found increased levels of

IL-6 and -10 in macrophages cultured on fibromodulin-deficient ECM in the presence of oxLDL.

In this study, we also showed that the altered – fibromodulin-deficient – ECM affects cell proliferation and collagen turnover in plaques. The rate of proliferation was more than doubled in carotid plaques from fibromodulin-deficient mice – both from low and oscillatory regions. As the rate of apoptosis was unchanged, this also resulted in an increased cell density in these plaques. When we analyzed tissue turnover through quantitative real-time PCR, we found an increase in mRNA expression of procollagen chain 1α2 in low shear stress lesions in apoE/fibromodulin-deficient mice – a similar increase was found in oscillatory shear stress lesions. Expression of lysyl oxidase, the main collagen cross-linking enzyme,[554], and urokinase plasminogen activator receptor-associated protein, in internalizing receptor that binds degraded collagen fragments[555], was also increased in plaques from low and oscillatory shear stress regions of fibromodulin-deficient mice, respectively.

With paper I, we provide evidence that collagen fiber structure affects plaque development. While collagen provides mechanical stability, this study shows that collagen may also promote lipid-accumulation, and that this ability is dependent on actions of fibromodulin during collagen fibrillogenesis. Finally, this study also confirms that collagen fibril integrity can modulate cell proliferation.

Paper II

The results on plaque material from mice in Paper I encouraged us to follow up this study with an investigation on human carotid plaques, obtained from patients undergoing endarterectomy. In Paper II we analyzed expression of fibromodulin in plaques from patients either having suffered a previous cerebrovascular event, namely stroke, transient ischemic attack or *amaurosis fugax* (symptomatic patients) or not (asymptomatic patients). We also explored the association of fibromodulin expression with plaque composition.

After immunohistochemical staining of 152 plaques with an antibody recognizing fibromodulin, we found fibromodulin expression of varying intensity in all plaques. Most often we found expression in the fibrous cap and shoulder regions of the plaque, and it was particularly strong at sites with inflammation. In contrast, we only rarely found fibromodulin expression in the outermost regions of the plaque; these regions represent the interface between the plaque and the underlying media. In most plaques, fibromodulin colocalized with lipids, and, though to a lesser degree, with macrophages.

Fibromodulin expression was higher in plaques from patients with symptoms compared to asymptomatic patients. Among the symptomatics, we found an inverse correlation between fibromodulin expression and the time between clinical events to surgery – that is, fibromodulin content was higher, the closer to the clinical event surgery took place.

Next, we compared fibromodulin expression among patients with and without diabetes. We found a higher fibromodulin content in plaques from patients who suffered from diabetes than in plaques from non-diabetics, and within the diabetic patient group fibromodulin content was, again, higher in plaques from symptomatic patients. In fact, when comparing plaques from symptomatics and asymptomatics within the *non*-diabetic patient group, there was no difference in fibromodulin expression.

We found fibromodulin expression to be associated with an inflammatory plaque phenotype. Fibromodulin content correlated with lipid content, as well as with decreased smooth muscle cell content – measured histologically and immunohistochemically, respectively. We also compared the expression of fibromodulin with that of cytokines measured in plaque homogenate, and found correlations between the content of fibromodulin and the pro-inflammatory cytokines macrophage inflammatory protein-1β (MIP-1β) and soluble CD40 ligand (sCD40L), as well as with vascular endothelial growth factor (VEGF). There was also a negative correlation between fibromodulin expression and levels of the anti-inflammatory cytokine IL-10 in the plaque.

Finally, we assessed the incidence of post-operative cardiovascular events occurring in the, on average, three years following endarterectomy. For this purpose, fibromodulin expression was divided into tertiles, and we found a trend – though non-significant – among the patients in the highest tertile, in comparison with patients in the two lower tertiles, towards an increased risk of developing post-operative cardiovascular events.

Paper II confirms the association between fibromodulin, collagen and plaque structure, especially in terms of lipid accumulation; while absence of fibromodulin, in Paper I, resulted in a more stable plaque phenotype, we found an association between a high expression of fibromodulin in human plaques with a more vulnerable plaque phenotype.

Paper III

Dystrophin, via the DGC, connects the cytoskeleton with the extracellular environment – this is achieved through binding components of the basement membrane. Loss of this connection in skeletal muscle results in muscular dystrophy, with progressive muscle wasting and a severely shortened life span due to respiratory failure.[445] Dystrophin and the DGC is also present in SMCs of the arterial wall, and is a useful tool to explore if and how a connection between cells and the basement membrane figures into neointima formation and atherosclerotic plaque development.

Paper III, the first of the two papers on dystrophin, focused on neointima formation as a response to vessel wall injury. The carotid periadventitial cuff injury model was used, in which neointimal lesions are mechanically induced and, at the timepoint used, consist almost solely of SMCs and ECM. In human terms, it closely resembles the type of neointimal hyperplasia that develops as a result of restenosis.

The results demonstrate that the size of the neointima was larger in carotid arteries from mdx mice, compared to wild type mice, as was the intima/media ratio. Lesions from mdx mice also exhibited an increased proliferation rate, whereas no difference could be detected *in vitro* in primary SMCs from wild type and mdx mice. However, an increase in migratory capacity was found in SMCs from mdx mice *in vitro*. We also compared expression of dystrophin mRNA in primary SMCs of contractile and synthetic phenotypes, and found a dramatic reduction of dystrophin expression after the phenotypic switch; mRNA expression of dystroglycan and β-sarcoglycan followed a similar pattern. Permanent adaptive molecular changes during the development of mdx smooth muscle may thus compensate for the lack of dystrophin, which may facilitate proliferation and migration *in vivo*, but not proliferation *in vitro*. In addition, the increase in proliferation in mdx lesions *in vivo* may stem from a more general loss of dystrophin and, thus, altered stimuli from cell types other than SMCs, not present *in vitro*.

We also assessed expression of other DGC components and binding partners in uninjured carotid arteries, as well as in lesions, through immunofluorescence. In the tunica media of uninjured vessels, laminin α2 chain coincided with α-SMA, and was present as basement membrane-like structures, surrounding each SMC, with expression similar in wild type and mdx mice. Presence of β-sarcoglycan was also similar in the media of wild type and mdx mice, while dystroglycan was consistently observed only in the media from wild type mice.

Expression of laminin α2 chain present in injured arteries and in the media was similar in wild type and mdx mice. However, while laminin α2 chain was found lining the luminal border of the neointima in wild type mice, positive immunoreactivity was found throughout the neointima in the mdx mice. Only weak and inconsistent immunoreactivity for β-sarcoglycan and dystroglycan was seen in the media and neointima in mice of both genotypes.

In Paper III we provide novel evidence that the connection between cells and the ECM, mediated by dystrophin, plays a role in neointima development induced by vascular injury.

Paper IV

In paper IV we set out to assess whether dystrophin and the link between cytoskeleton and ECM have a role in the development of atherosclerotic lesions, and, if so, whether this occurs in a manner that is similar to the effects delineated in Paper III (that is, larger neointimal lesions in mdx mouse). In order to achieve lesions that more closely resemble human atherosclerotic lesions we cross-bred mice with the mdx mutations and atherosclerosis-prone apoE mice to generate mice double-deficient in dystrophin and apoE. Mice was given a fatty diet and, as in Paper I, a shear stress-modifying periadventitial cast was placed around the common carotid artery in order to induce plaques with differing phenotypes.

In apoE/mdx mice there was a decrease in total plaque burden, as assessed through Oil Red O-stained flat preparations of aortas. There was also an overall decrease in the size of low shear stress-plaques in these mice, as well as in total macrophage and collagen content. In addition, the expression of two markers associated with a differentiated phenotype in SMCs –α-SMA and myosin heavy chain – was increased in the low shear stress plaques in apoE/mdx mice.

Comparing cytokine content in plasma, we also found that several inflammatory cytokines, as well as the atheroprotective IL-13, to be down-regulated in the apoE/mdx mouse.

As dystrophin connects the cytoskeleton with the basement membrane, we next investigated how the loss of this connection affects basement membrane synthesis and/or assembly in an atherosclerotic plaque. In order to analyze the plaque content of laminins we performed immunofluorescent staining using an antibody that recognized several laminin chains (α1, β1 and γ1), that together represent the vast majority of the laminin isoforms. Plaques from the low, but not oscillatory, shear stress regions in apoE/mdx contained less laminin compared to plaques from mice expressing dystrophin. In both genotypes, positive immunoreactivity was found in

the media and in the plaque regions corresponding to the endothelial basement membrane and the area directly underneath, where it appeared to surround both SMCs and foam cells.

We also explored expression patterns of additional key components in the cytokeleton-basement membrane connection; β-dystroglycan is a member of the DGC and utrophin is a homologue to dystrophin. The laminin α2 chain binds to cells via the DGC, and integrin α7 is an alternative receptor for laminin, found to be upregulated in mdx mice.

We found immunoreactivity for laminin α2 chain and β-dystroglycan to be weaker in plaques, and media connecting to plaques, from the apoE/mdx mouse – especially in the low shear stress region. Immunoreactivity for utrophin was stronger in plaques from both low and oscillatory shear stress regions in apoE/mdx mice, whereas expression of integrin α7 appeared to be similar in plaques from both genotypes.

With Paper IV we show, for the first time, that dystrophin and the DGC are involved in atherosclerotic plaque development, and that the absence of dystrophin result in plaques of reduced size and with altered basement membranes.

Discussion

Collagen fibril integrity and atherosclerosis

In Paper I, we report that abnormal collagen synthesis – due to lack of fibromodulin, possibly resulting in faulty crosslinking – alters plaque development. The study is followed up by Paper II, where we confirm the connection between fibromodulin and plaque phenotype and clinical events in humans.

An ever present question in researching atherogenesis is how we might push existing plaques towards more stable phenotypes. Paper I, with its main finding of attenuated plaque growth coupled to decreased lipid accumulation could perhaps be a step in this direction – though attempting to directly replicate the results in humans through gene therapy to block fibromodulin-expression might not be the most convenient – or safe – therapy. The key interest and value from an atherosclerosis point of view may instead lie in assessing all the effects of our knock out-mouse (together and separately), and consider how beneficial effects may be achieved – and how adversary effects may be avoided – using a different, and more accessible therapeutic strategy. Such a strategy might be as straightforward as blocking fibromodulin through medicines, or it might involve another drug target (or even gene therapy target), completely.

Considering the collective results from Paper I, alteration of collagen, being such a basic building block, has many diverse effects in the plaque. Our main focus has been on how cell behavior is affected, but if we further explored the overall ECM composition of the fibromodulin-deficient plaques, we would probably be very likely to find differences here as well. Potential alterations in expression levels, or localization, of other ECM components may represent a “middle man” of sorts, that, could participate in implementing the changes brought about by the abnormal collagen fibrils, perhaps through specific cell contact or by further altering binding among ECM components.

Intuitively, the tendency towards increased cell and collagen turnover in fibromodulin-deficient plaques seems reasonable, as a response to an environment where something is amiss. As a consequence, in striving to rectify the situation, cells react by trying to heal a perceived wound with increased proliferation and increased synthesis of collagen (and perhaps other ECM components, as of yet un-

measured). This would also explain the increased collagen degradation, as the collagen produced may constantly be perceived as faulty.

Does fibromodulin function directly or by proxy?

In Paper I, we found that retention of lipids in vascular ECM was reduced, and macrophages grown *in vitro* on an ECM lacking fibromodulin had reduced capacity to take up oxLDL. This effect can be interpreted either as fibromodulin having a direct, anti-atherogenic role in lipid retention, or as the absence of fibromodulin being secondary to the effect inferred by the altered collagen matrix.

Though we have seen evidence that fibromodulin is expressed in the plaque and by SMCs *in vitro*, we do not know whether fibromodulin remains bound to collagen after synthesis is complete. In addition, we have yet to elucidate whether fibromodulin is capable of directly binding LDL, and while this potential binding may contribute to the overall lipid-binding in a plaque, it is not clear whether fibromodulin is present in a quantity large enough to make a contribution so significant that loss of fibromodulin results in effects as marked as those we report in Paper I.

If indeed the main effect of fibromodulin in atherosclerotic plaques is in ensuring correct collagen synthesis, the effects of fibromodulin deficiency seen in the mouse study most likely have a basis in the resulting abnormal collagen fibers. In the human carotid artery plaques included in Paper II, fibromodulin could therefore be seen as an essential component in the process of an ongoing collagen synthesis and/or remodeling. This may also be reflected by the high fibromodulin expression in plaques from diabetic patients, found in Paper II. Altered ECM remodeling in diabetes has been previously reported by other studies,[556-558] and the enhanced fibromodulin expression may indicate an increase in collagen synthesis, possible as a compensatory response to signals generated by the abnormal ECM-remodeling. However, I in no way want to exclude the possibility that fibromodulin also has direct effects – in atherogenesis as well as in other healthy and/or pathological states.

While it is still not known whether fibromodulin (or the other SLRPs) remains associated with collagen fibers after fibrillogenesis is completed, or if it is mainly induced as part of remodeling response, functions outside of fibrillogenesis have been identified: fibromodulin is cleaved by MMP13 with the larger fragment remaining bound to collagen, with a possible function as a shield from cleavage.[403] Fibromodulin also binds additional ECM components, such as collagen XII, PRELP and heparin[356] which may imply roles both in further cross-linking and maintaining of ECM stability, as well as in sequestering of growth factors and other bioactive molecules.

In addition, fibromodulin is known to bind and activate the classical and alternative pathways of the complement system,[424] an ability that has been speculated to have an impact on the inflammatory response in joints[425] and may possibly affect inflammation in atherosclerotic plaques, as well. This is corroborated with our, still unpublished, finding, of a higher expression of the complement cascade component C3b in low shear stress-plaques of in ApoE/Fibromodulin-deficient mice.

The influence of fibromodulin on collagen synthesis in atherogenesis

The atherosclerotic plaque is an environment that is not only highly inflammatory, but also rich in lipids – perhaps the largest difference in comparison with tendon collagen synthesis, as well as other relevant pathological conditions, such as osteoarthritis. The presence of lipids is also a major difference in comparing collagen fibrils in the atherosclerotic plaque and tumor stroma, perhaps the pathological system most closely resembling the plaque among the conditions studied so far. Both plaques and tumors represent a somewhat closed off environment built through enhanced ECM-deposition and mutinous cell behavior. There is inflammation and a failure for the development to be halted by immune cells. As growth advances, neoangiogenesis commences in both structures to provide oxygen and nutrients.

So why is it, that dysfunctional cross-linking due to lack of fibromodulin results in thinner fibrils in the experimental carcinoma, and thicker fibrils in the atherosclerotic plaque? A comparison of the type of inflammation present in both instances may provide some clues – it may be that cytokine and chemokine profiles and the overall presence of inflammatory and immune cells have a regulatory or limiting/stimulating effect on fibromodulin during collagen fibrillogenesis.

Differing effects of immune response could also influence the collagen producing ability of plaque cells. Perhaps it is not the overall speed of collagen production, but the production speed of each cell, that may possibly be of relevance – total collagen production would be similar in a large number of cells with constant, but quite slow, collagen production and a smaller number of very active cells sporadically synthesizing collagen, but the latter scenario may be quicker or less synchronized in terms of each new collagen fibrils. This could possibly affect availability of sites for cross-linking and abnormal fusion of fibrils. In addition, there could be a feedback loop in effect; altered collagen may influence cytokine and chemokine secretion by macrophages. The resulting cytokine profile in turn affects collagen-production in SMCs and macrophages themselves, furthering and enhancing the initial effects of the altered collagen.

The difference between plaques and tumors that stands out is, again, the presence of modified lipids in the atherosclerotic plaque, which provide an additional stimulant for collagen-producing cells. In the atherosclerotic model, the presence of lipids seems to be vital for detecting functional differences induced by the abnormal collagen. Consequently, when applying the non-constrictive cuff model of paper III to fibromodulin-deficient mice, neither a difference in plaque size, nor in cell proliferation or density could be established *in vivo* (unpublished data).

So, is the effect on lipid accumulation due to the effect on cell behavior in the plaque, or in the binding of oxLDL to the ECM – or perhaps both? Results from our *in vitro* experiments conducted with macrophages cultured on wild type or fibromodulin-deficient ECM (Paper I) implies a principal effect on the uptake by cells, but unfortunately we have so far been unable to establish a functional model for measuring lipid-binding to fibromodulin-null ECM. An interesting speculation is that an LDL-binding site may be lost or weakened in altered fibrils. Collagen is not normally considered a major LDL binding partner in lipid retention – but its significance could increase if an abnormal structure results in an alternate manner of binding to associated proteoglycans which hides previously active LDL-binding regions. If so, the question becomes: Is there a collagen site that is rendered unavailable or altered in the absence of fibromodulin? Can this site be blocked *in vitro* or *in vivo*? May the site be selectively blocked in plaques, and would this replicate the effect on lipid accumulation observed in ApoE/fibromodulin-deficient mice?

Dystrophin and contact between cells and basement membranes

In Papers III and IV, we further explored ECM influence and cell behavior in atherosclerotic lesion formation by cutting a route of communication between cells and the basement membrane that is dependent on dystrophin and the DGC. In Paper III, we found that dystrophin deficiency resulted in increased neointimal hyperplasia, while, as reported in Paper IV, the development of plaques of a vulnerable phenotype (low shear stress plaques) was attenuated in the absence of dystrophin. However, exactly how the lost connection with laminin via the DGC delays plaque development is not yet clear.

The endothelial basement membrane plays a significant role for EC function, and one hypothesis is that a functional DGC is required for proper connections between ECs and laminin. This may also help explain the different effects on lesion formation demonstrated by the two studies. In the non-occlusive cuff model the endothelium is still dysfunctional, but as there is no fatty diet or atherogenic mutation in effect, and there are less lipids and inflammatory cells circulating with the blood stream, and therefore the non-activated endothelium may not be a signifi-

cant contributor in the resulting neointimal hyperplasia. Rather, it is likely an effect – enhanced proliferatory or migratory stimuli – on the SMCs that drive lesion formation. In contrast, ECs have a key role in the development of atherosclerotic plaques, for example through upregulation of adhesion molecules and its involvement in lipid transport across the endothelium. If proper function of these two processes is disturbed in dystrophin-deficient artery, the result could be both a reduction in lipids entering the plaque and a less prominent upregulation of adhesion molecules on the endothelium, leading to less monocyte/leukocyte infiltration from circulation, which in turn would affect SMCs through reduced stimulus for phenotypic modulation.

In Paper IV, we found reduced laminin content in plaques of a vulnerable phenotype (low shear stress plaques). Laminin normally promotes a more differentiated SMC phenotype, and reduced laminin content in the basement membrane may result in SMCs that are more prone to undergo a phenotypic switch – this might contribute to the increased neointimal hyperplasia and proliferation seen in Paper III. Additionally, this effect seemed to be present also in atherosclerotic plaques, though not in the case of proliferation. However, cell density was increased in the atherosclerotic plaques, which may be caused by increased migration by medial SMCs. Such an increase might lead to a shift in the ratio of SMCs and macrophages in atherosclerotic plaques, and, in fact, we did see an increase in the relative content of SMCs in these plaques (though the differentiated phenotype suggests that a lower degree of stimuli may be present once the SMCs reached the plaque).

Additionally, in Paper IV, we found that the cytokine profile, measured in plasma, was altered in that cytokine secretion of several pro-inflammatory, and atheroprotective, cytokine was decreased, suggesting and systemic effect in ApoE/mdx macrophages resulting in less efficient cytokine production or secretion. If this is the case, inflammatory cells in this mouse may be less able to respond to inflammatory stimuli. Increased inflammation of the arteries due to fatty diet is one reason behind plaque development in ApoE-null mice, and a reduction in inflammatory response may contribute to the smaller plaques. Exploration of additional organ systems, such as assessing the populations of inflammatory cells in the spleen, is necessary to verify and further analyze this effect.

Interestingly, the somewhat unexpected result of increased lesion size after mechanical injury, and decreased lesion size caused by lipid-retention is mirrored in mice deficient in caveolin-1,[559-561] a key structural protein in caveolae in endothelial cells and SMCs. Caveolae are specialized microdomains, present as invaginations in plasma membranes of terminally differentiated cells,[562] involved in vesicular and cholesterol trafficking and signal transduction, and, in the endothelium, involvement of caveolae has been demonstrated in the uptake and transcytosis of oxLDL. Signaling complexes assemble through the aid of scaffold formed by

caveolin proteins, and SMC caveolae are reportedly enriched in dystrophin.[563-565] A hypothesis derived from this similarity is that one effect of dystrophin-deficiency is that cells may not be able to form caveolae in a normal way, in turn resulting in reduced lipid influx in the endothelium and increased migratory capacity in SMCs.

Does the novel function for dystrophin in vascular lesion formation have an impact in DMD patients?

Considering the dystrophin model from a clinical perspective, the results may not benefit patients suffering from DMD as they, with the aid of current therapies, do not reach an age where they are likely to experience cardiovascular events caused by atherosclerotic plaque disruption. In contrast, patients inflicted by Becker muscular dystrophies frequently have an almost normal lifespan, and might thus be candidates for surgical interventions such as angioplasty and stenting, mimicked by our non-occlusive carotid artery cuff-model, or experience clinical symptoms due to plaque-related thrombus formation. In light of the increased neointimal hyperplasia delineated in Paper III, these patients may be more prone to developing restenosis post-surgery, and – following more extensive verification of our results – might even benefit from monitoring of carotid artery plaque burden, and use of prophylactic drug therapy (possibly in a similar lipid-lowering regime as is routinely given to diabetics) to avoid future vascular events.

Despite indications from Paper IV that plaque burden is decreased with dystrophin deficiency, it is unclear what the effect might be on plaque stability, since there was no indication that the relative lipid retention or collagen content were altered. Also, we do not know whether the decreased integrity of the endothelial basement membrane could be detrimental to plaque stability. Therefore, there is a risk that, while lipid-rich atherosclerotic plaques may be smaller in patients with Becker muscular dystrophy, they may also be less stable – and size is not the only factor affecting whether plaques are prone to rupture.

ECM alterations through dystrophin and fibromodulin – do the results overlap?

Aside from potential effects through the protein itself, the fibromodulin model mainly showed effects on collagen type I, located in the ECM outside of the basement membrane. In contrast, the results from the dystrophin model seem to be concentrated on the basement membrane. Do the effects ever converge? Are certain effects propagated from the basement membrane to the plaque ECM, and/or

vice versa? Surprisingly, the answer seems to be 'no'. Despite carrying out very similar analyses in both mouse models, the only similarity is a decreased total plaque burden in descending aortas of both models (on an ApoE-null background). Intimal hyperplasia was increased in mdx mice, but not fibromodulin-null mice (unpublished data). Focusing on the results obtained by the shear stress-modifying cast, lipid accumulation was decreased in low shear stress-plaques in fibromodulin-deficient, but not dystrophin-deficient, mice. Increased content of differentiated SMCs was found in dystrophin-deficient, but not fibromodulin-deficient, mice. In addition, only fibromodulin-deficient mice exhibited an increased proliferation rate. While systemic inflammation was unchanged by fibromodulin deficiency, a decrease in plasma cytokines was found in dystrophin-deficient mice.

Taken together, the altered collagen fibrils caused by loss of fibromodulin seems to have a very specific, localized effect on cell behavior, such as lipid uptake and proliferation in atherosclerotic lesions, while the lost connection with basement membranes, due to dystrophin-deficiency, appear to in turn have a general effect on the very integrity of basement membranes. This is very consistent with the overall phenotype of each knock out-mouse. Effects of fibromodulin and collagen remodeling, as deduced from fibromodulin-null mice, seem to be quite contained to specific tissues such as tendons in the healthy state, and induced during remodeling in tissue healing and regeneration. The much more conspicuous phenotype of the dystrophin-deficient mdx mouse – with muscular dystrophy – seemingly appears in connection with muscle cells and basement membranes with altered functions throughout the body. The different extent in the two phenotypes nicely illustrates how specialized the ECM, indeed, is, in comparison to a defect originating from within multiple cell types, which hits a much broader target.

Strengths and limitations

The studies presented have both strengths and limitations to consider. From a clinical point of view, fibromodulin and dystrophin may appear to be quite unattractive as biomarkers – from the point of view of basic science, they are valuable tools to explore features of a modified ECM in plaque development. Any potential beneficial effects from a deficiency will likely have to be duplicated using an alternate strategy in order for them to have therapeutic value.

Concerning the conclusions derived from Papers I and IV. When using ApoE-null mice one can never exclude the possibility that results may be specific to the ApoE-deficient background, and that additional or differing effects might be seen with human lipoproteins and their mode of binding to the ECM.

When working with mouse models, there is always the additional question of accuracy since plaque rupture, a vital end point of the atherosclerotic process, in the vast majority of studies is completely absent. However, a strength of the mouse studies is the quite consistently large cohorts used, while, in contrast, one possible weakness of the human study in Paper IV may be the relatively small patient group available.

There is also the question of causality. Especially when comparing results obtained in mouse models and human plaque material, we do not know whether our results reflect direct functions of fibromodulin, or whether they are a reflection of a resulting ECM.

Conclusions

The four studies of this thesis conclude that:

- In the atherosclerotic plaque of a fibrous phenotype, collagen fibrils synthesized in the absence of fibromodulin are more heterogeneous, and, on average, thicker, compared to fibrils generated in the control mouse.
- In the atherosclerotic (murine) plaque of an inflammatory phenotype, a fibromodulin-deficient ECM results in decreased size and lipid accumulation, but increased cell proliferation.
- Higher fibromodulin expression , as measured immunohistochemically at the most stenotic region of a human carotid artery plaque, is found in plaques from patients with symptoms than in asymptomatics, and particularly in those with diabetes.
- Fibromodulin expression in human plaques co-localize with Oil Red O-stained plaque regions, and correlates with the content of lipids, the pro-inflammatory cytokines MIP-1β and sCD40L, and with VEGF and decreased levels of the anti-inflammatory cytokine IL-10.
- Dystrophin-deficiency, as illustrated by the mdx mouse, stimulates neointimal hyperplasia, but inhibits atherosclerotic lesion development driven by lipid-retention.
- In atherosclerotic lesions with an inflammatory phenotype, in the mdx mouse, expression of laminins is decreased in the endothelium, suggesting that altered endothelial cell function and an abnormal basement membrane may be a possible explanation for the attenuated plaque development.

In summary, the present studies show that collagen structure and remodeling affect the growth, development and composition of atherosclerotic lesions, on the one hand promoting mechanical stability, and on the other hand promoting lipid accumulation and inflammation. In addition, growth of vascular lesions can be inhibited or promoted by a functional connection between cells and the ECM mediated by dystrophin and the DGC, depending on the underlying pathology.

Concluding remarks and future perspectives

In this thesis, I discuss the importance of the connection between ECM and cells for atherosclerotic lesion development, and show that alterations in this connection – either through a modified collagenous ECM, or modification in a cellular protein directly linking cells to ECM components – have a significant impact on atherosclerotic plaque structure and, possibly, its stability.

All four papers explore novel involvement of fibromodulin and dystrophin in development of vascular lesions. Therefore, it is not possible to make an assessment whether our results are in concordance with similar studies. However, the basic concepts, such as effects of collagen structure and cell-ECM contact, are reflected in observations by others.

Much still remains to explore further in the future. The present studies raise questions closer to the nature of basic science, rather than therapeutic potential – at least for the time being. Questions of this kind tend to be of a lesser scope, and answers not too far away. Would the reduced lipid accumulation of fibromodulin-null plaques be associated with a lower prevalence of plaque rupture – if a functional mouse model for this could be developed? Does fibromodulin itself bind LDL, or is the effect we see an effect completely secondary to collagen structure? Exactly what (if any) role do fibromodulin play in complement activation *in vivo*? How does loss of dystrophin in an ApoE-null background result in reduction of systemic inflammation – are there any clues in the cells of the spleen, and could it be related to a shift in M1/M2 response in macrophages? Is dystrophin (or utrophin) expressed in human plaques?

I think, however, that it is important to also keep the larger questions in mind: May blocking fibromodulin in atherosclerotic plaques be of therapeutic use? Does the lack of fully functional dystrophin have an impact on vascular conditions in older patients suffering from Becker muscular dystrophy?

Summary in Swedish

Fibromodulin och dystrofin i ateroskleros; en populärvetenskaplig sammanfattning

Vad är ateroskleros?

Ateroskleros – det man tidigare kallade för åderförkalkning – är grundorsaken till hjärtinfarkt, stroke och kärlkramp. Ett aterosklerosplack är en förträngning i ett blodkärl, ofta vid en förgrening där blodflödet är turbulent. Placket börjar ofta växa redan i unga år genom att fett från blodet tar sig in i kärlväggen. Detta startar en kedja av inflammatoriska reaktioner, där inflammatoriska celler rekryteras från blodet och glattmuskelceller från kärlväggen. Ett typiskt plack består av dessa celler och fett, tillsammans med stabiliserande bindväv och ofta även hårda kalkansamlingar. Placket täcks av en fibrös kapsel – ett skyddande lock av bindväv – och med tiden kan en rutten, nekrotisk kärna bildas. En tunn fibrös kapsel och mycket fett tillsammans med en stor nekrotisk kärna är kännetecken för ett sårbart plack, som riskerar att brista och orsaka en farlig blodpropp.

Vilka symtom och komplikationer blodproppar orsakar beror mycket på vilket kärl som är drabbat. Antingen sker själva blockeringen i kärlet där placket finns, eller så bryts proppen loss, färdas med blodet och fastnar i ett mindre kärl – i hjärnan orsakar denna stroke och i hjärtat en hjärtinfarkt. Största delen av arbetet i denna avhandling är fokuserat på plack i karotisartärerna, det vill säga halsartärerna som förser huvudet och hjärnan med blod. När plack brister i en halsartär för blodet med sig proppen mot hjärnan. Komplikationer leder oftast till stroke – antingen är de stora och omfattande, eller mindre, med symtom som snabbt går över. Ett exempel på en sådan mindre stroke är en så kallad TIA-attack, då symtomen går över eftersom proppen spontant löses upp.

Aterosklerosplack utvecklas ofta i flera decennier innan de brister, och symtom börjar ge sig till känna. Denna avhandling är en pusselbit av många i ett stort forskningsfält som försöker besvara spörsmålen: Vad är det som händer i placket, som får det att brista? Kan man påverka plackets stabilitet och därmed hindra att det brister?

Bindväv: Både stomme och dirigent

Fibromodulin och dystrofin – resten av titeln på denna avhandling – är gäster från ett annat forskningsfält, matrixbiologi. Matrixbiologin fokuserar på kroppens bindväv. För att få en uppfattning om bindvävens uppgifter skiftar vi nu uppmärksamheten till hur våra kroppar är uppbyggda.

Våra kroppar består av flera biljoner celler som arbetar tillsammans. De är som små maskiner som var och en (eller gruppvis) utför en syssla som behövs för att våra kroppar ska fungera. Vi har till exempel en sorts viljestyrda muskelceller som gör att ögonen följer texten när vi läser, en annan sorts muskelcell i våra blodkärl som, utan att vi märker det, hjälper till att reglera blodtrycket. Men cellerna är inte bara associerade med varandra, utan de omges också av bindväv (även kallat extracellulärt matrix) – ett nätverk av fibrer, membran och smådelar. Å ena sidan kan man se bindväven som en byggnadsställning som ger stadga och som cellerna vilar mot – lite som hur havet omger fiskar. Men bindväven är mer komplex än så, bindväven kring cellerna är faktiskt lika specialiserad som cellerna själva! Beroende på bindvävens struktur och beståndsdelar skickar den olika signaler till cellerna om hur de ska bete sig, och den kan också gömma undan eller förstärka de signaler som cellerna skickar till varandra.

Bindväven kontrollerar även att cellerna håller sig till rätt uppgift. Det finns till exempel specialiserad bindväv kring muskelcellerna i blodkärlet som ser till att de behåller sin förmåga att dra ihop sig och slappna av, beroende på om blodflödet behöver minskas eller ökas. I ett blodkärl med aterosklerosplack påverkas bindväven av fettet och inflammationen som ansamlas – vissa fibrer bryts ner och fibrer av en annan typ byggs upp, och många av smådelarna byts ut. Man kan tänka sig en klädd julgran, som byts ut mot en tall med lite glitter och kulor kvar, men också durkslag, prästkragar, pennor och kaffekokare. Precis som att kaotiskt beteende skulle utbryta bland de förvånade gästerna på den julfesten, påverkar det cellerna i blodkärlet och placket när bindväven förändras. En del av muskelcellerna i blodkärlets vägg kommer till exempel att sluta se efter blodflödet och istället krypa in i placket, börja föröka sig och förvandlas till små bindvävsmaskiner som hjälper placket att växa. Den nya plackbindväven har dessutom nya egenskaper som antingen kan förvärra eller stabilisera placket. Det är dessa nya egenskaper som bindväven får i placket, som vi är extra intresserade av; vilka nya egenskaper har positiva effekter och vilka har negativa effekter? Tänk om vi kunde styra egenskaperna till att sakta ner placktillväxten och hindra att placket går sönder och orsakar en blodpropp!

Fibromodulin och dystrofin behövs för att bindväv ska fungera korrekt

Vi har undersökt bindvävens betydelse på två olika sätt. Dels har vi siktat in oss på en av bindvävens smådelar (en av julgranskulorna) som kontrollerar produktionen av bindvävens kollagenfibrer (själva julgranen). Den här lilla bindvävskomponenten heter fibromodulin och dess uppgift är alltså att ”modulera fibrerna”.

Vi har också undersökt ett av sätten som cellerna har kontakt med bindväven – ett protein som sitter på insidan av cellens omgivande hölje, och binder samman cellskelettet med cellytan, som i sin tur kontaktar bindväven utanför. För att hålla oss till julfesten – liksom gästernas armmuskler gör det möjligt att sträcka ut en hand och hantera julgranen (må den nu vara normalt klädd eller smyckad med diverse hushållsgeråd), ges cellerna möjlighet att interagera med omgivningen. Proteinet heter dystrofin – namnet kommer ifrån ordet ”muskeldystrofi”, muskelförtvining, som syftar på den sjukdom som drabbar människor som har fel på det här proteinet.

För att sammanfatta så här långt: Bindväven reglerar om placket är stabilt eller inte. Vi har undersökt hur placktillväxt påverkas; både av produktionen av fibrerna i bindväven och beroende på cellernas kontakt med bindväven.

Mindre ateroskleros i möss som saknar fibromodulin

Vi har jämfört hur plack bildas i vanliga möss med möss som dels saknar fibromodulin och därför producerar en defekt sorts fibrer, kollagenfibrer, och dels med möss som saknar dystrofin, och därför har sämre kontakt mellan cellerna och bindväven.

I mössen jämförde vi plack som ser olika ut – en sort som innehåller väldigt mycket bindväv och liknar stabila plack hos människor, och en annan sorts plack som istället innehåller mycket inflammation och fett, och liknar de placken som riskerar att brista och orsaka symtom. I mössen som saknar fibromodulin var de inflammatoriska placken mindre än i de vanliga mössen och de innehöll också mindre fett.

En möjlig förklaring kan vara att de onormala kollagenfibrerna påverkar cellernas förmåga att ta upp fett i möss utan fibromodulin. Denna hypotes kunde vi bekräfta genom experiment i ett cellodlingssystem, där mindre fett ansamlades i inflammatoriska celler som växte på onormala kollagenfibrer.

Fibromodulin har även en roll i ateroskleros hos människor

Som nästa steg undersökte vi om fibromodulin även kan ha betydelse i plack hos människor. Vi analyserade därför plack som sparats från så kallade endarterektomier, vilket är en sorts operation där besvärliga plack i halsartären tas bort. Placken kan antingen ha orsakat symtom hos patienten, exempelvis stroke, eller så har de upptäckts på något annat sätt och varit så stora att kirurgerna valt att ta bort dem trots att de inte ännu orsakat symtom.

Vi undersökte tvärsnitt av den delen av placken som tog upp störst plats i kärlet (det vill säga, hindrade blodflödet mest). Fibromodulin uttrycktes i plack både från patienter med och utan symtom, och ofta fanns fibromodulin på samma plats som fettet i placken.

Vi upptäckte också att det fanns mer fibromodulin i placken från de patienter som hade upplevt symtom från sina plack. Dessutom jämförde vi mängden fibromodulin i plack från patienter med och utan diabetes, och fann att det fanns mer fibromodulin i plack från diabetiker. Det visade sig även att det fanns mer fibromodulin i plack med mycket fett och inflammatoriska cytokiner. Placken med mycket fibromodulin innehöll också färre glattmuskelceller som producerar den stabiliserande bindväven.

Våra resultat från mössen och de humana placken passar bra ihop: Avsaknad av fibromodulin i mössen ledde till mindre inflammatoriska plack, med mindre fettinnehåll – snällare plack – medan mycket fibromodulin i plackvävnad från människor var associerat till mer aggressiva plack, som orsakade symtom.

Fibromodulins största roll i placken tycks vara att säkra produktion av korrekta kollagenfibrer i bindväven. Man kan därför även tolka våra resultat som att mycket fibromodulin är en signal om att det pågår en hög grad av kollagenfiberproduktion i ett sådant plack. Rent intuitivt är detta logiskt; man kan tänka sig att nyproduktion av bindväv är aktiv som en reparationsmekanism efter att ett aggressivt plack brustit. Man har i andra studier sett att bindvävsproduktion hos diabetiker inte är lika effektiv som hos icke-diabetiker. Den extra höga kollagenfiber-produktionen (och fibromodulin-innehållet) vi såg i plack från diabetiker skulle därför kunna vara ett försök till att kompensera för en abnorm bindväv.

Dystrofin har olika roller i restenos och ateroskleros

När vi undersökte dystrofin och cellernas kontakt med bindväven tog vi också hjälp av musmodeller. Först utforskade vi dystrofins roll i restenos – en komplikation som kan drabba patienter vars plack opereras ut. Restenos innebär att en störning i kärlets läkningsprocess gör att kärlväggen tjocknar så mycket att det åter igen bildas en förträngning som hindrar blodflödet. Vi inducerade en liknande

restenos-reaktion i halsartären hos vanliga möss och hos möss som saknar dystrofin – så kallade mdx-möss. Mdx-mössen utvecklade mer restenos jämfört med de vanliga mössen. Vi såg också att takten på celldelningen i restensosen var högre. När vi odlade celler från vanliga möss och mdx-möss, såg vi att cellerna från mdx-mössen också var mer rörliga.

Normalt skickar bindväven i friska kärl signaler till cellerna i kärlväggen. Signalerna hämmar cellernas aktivitet, och de låses fast i sin normala funktion att förse kärlen med kontraktila egenskaper. Den ökade aktiviteten vi såg i mdx-mössens kärlceller skulle kunna bero på att cellerna har sämre kontakt med bindväven, och därför missar dessa hämmande signaler. Dessa resultat visar att dystrofin, vid denna typ av skada, har en skyddande roll i kärlväggen, och att risk för restenos kan vara förhöjd hos människor som lider av muskeldystrofi orsakad av defekt dystrofin.

Vi undersökte även om dystrofin har en roll i utvecklingen av inflammatoriska och stabila aterosklerosplack. För dessa experiment använde vi samma musmodell som i fibromodulinstudien. Lite överraskande visade det sig att, även om restenosskadorna var större i mdx-mössen, så var de inflammatoriska placken mindre. Avsaknad av dystrofin ledde också till att det fanns mindre av en specialiserad sorts bindväv som finns ytterst på placken, närmast blodflödet. Vi håller nu på att undersöka mekanismerna bakom detta fynd.

Restenos och aterosklerosplack bildas genom olika mekanismer – en stor skillnad är till exempel att aterosklerosplack i grunden bildas genom att fett ansamlas i kärlväggen och aktiverar inflammatoriska celler, medan restenos beror på en mekanisk störning som främjar aktivering av bindvävsproducerande glattmuskelceller. Våra resultat tyder på att den förlorade kontakten mellan cellerna och vissa bindvävskomponenter i sin tur leder till att den specialiserade bindväven som omger cellerna i kärlväggen blir bristfällig. En konsekvens av detta skulle kunna vara en felaktig aktivering av cellerna, vilket kan ha stora effekter på den efterföljande inflammationen och bindvävsproduktionen.

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