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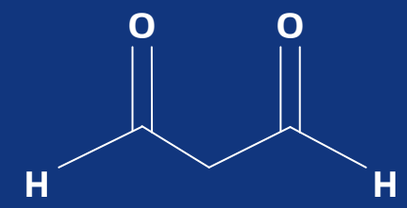

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Immune Responses Against Aldehyde Modified Extracellular Matrix in Atherosclerosis

Pontus Dunér

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Abstract

Atherosclerosis is a chronic inflammatory disease and the leading cause of myocardial infarction and stroke. Accumulation, aggregation and subsequent oxidation of LDL in the arterial wall are considered as key events in the development of atherosclerosis. The oxidation of LDL generates reactive aldehydes, including malondialdehyde (MDA) that modifies ApoB in LDL. Immune responses against MDA-modified epitopes on ApoB have been shown to be linked to atherosclerotic disease. This thesis is focused on the possibility that LDL oxidation results in the release of MDA that modifies surrounding extracellular matrix (ECM) proteins. These modifications may subsequently target immune responses against the plaque ECM and influence the atherosclerotic process. Our studies provide evidence for the presence of MDA-modifications on ECM proteins during atherosclerosis. We also show that antibodies against several MDA-modified ECM proteins are present in human plasma. A prospective clinical study showed that subjects that later suffered from acute cardiovascular events had significantly lower IgG and IgM antibody levels against MDA-modified fibronectin. These epidemiological results indicate that immune responses against MDA-modified fibronectin may have protective effects in atherosclerotic disease. To investigate the functional role of immune responses against modified matrix proteins in atherosclerosis, we immunized ApoE-/- mice with two different MDA-modified matrix proteins to which we had found antibodies in human plasma. MDA-modified fibronectin immunization significantly decreased the atherosclerotic plaque development in both aorta and in subvalvular lesions, while MDA-modified laminin resulted in increased plaque development. Finally we show that injection of Alum, an adjuvant commonly used in vaccines, results in LDL oxidation and aldehyde generation at the injection site.

Key words: Atherosclerosis, autoantibodies, plaque, oxidized LDL, malondialdehyde, extracellular matrix, fibronectin, laminin, T regulatory cells, Alum

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TO MY WIFE

Nobody has yet discovered, not even scientists, how much love the human heart may hold.

I'm yours forever

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Abbreviations

ApoB	Apolipoprotein B
Apoe$^{-/-}$	Apolipoprotein E deficient mice
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
FoxP3	Forkhead box P3
HSP	Heat-shock protein
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MHC	Major-histocompatibility-complex
MDA	Malondialdehyde
MMP	Matrix metalloproteinase
MI	Myocardial infarction
NKT	Natural killer T
NF-κB	Nuclear factor kappa B
oxLDL	Oxidized low density lipoprotein
SMC	Smooth muscle cell
SR	Scavenger receptors
TGFβ	Transforming growth factor-β
Th	T helper
TLRs	Toll like receptors
Tr1	T regulatory type 1
Treg	T regulatory
TNFα	Tumor necrosis factor alpha
VLDL	Very low-density lipoproteins

Introduction

According to the world health organization, cardiovascular diseases are the number one cause of disability and death throughout the world. An estimated 17.5 million people died from cardiovascular disease in 2005, representing about thirty percent of all deaths globally. Of these deaths, 7.6 million were due to myocardial infarction (MI) and 5.7 million due to stroke. If current trends are allowed to continue, it is predicted that by 2015 an estimated 20 million people will die from cardiovascular disease, mainly from MI or stroke. The underlying pathology is atherosclerosis, a chronic inflammatory disease that begins in fetal life, slowly progress and accelerates in adult life. Atherosclerosis develops inside the vascular wall of large and medium sized arteries and is the most common cause of cardiovascular diseases. The disease affects mainly the intimal layer of the vascular wall and is characterized by lipid accumulation, gathering of inflammatory cells and structural changes in the extracellular matrix (ECM). The development of the atherosclerotic lesion is mainly a consequence of lipid accumulation, subsequent oxidation and inflammation. As the atherosclerotic disease progress the changes in the wall results in growing atherosclerotic plaques and clinical complications may arise as a direct consequence of the growing plaques. At later stages, plaque size may reduce blood flow causing stenosis or the plaque may rupture leading to thrombosis and acute complications such as MI, heart failure, ischemic stroke or transient ischemia. There are many traditional risk factors, such as hypercholesterolemia, smoking, age, male gender, hypertension and diabetes. Even if elevated levels of lipids remains as a key risk factor in the development of atherosclerosis much attention is now focused on the role of the immune system. During all stages of atherosclerosis immune cells and immune components are present in the atherosclerotic plaques. There is strong evidence that major targets for these immune responses are modified endogenous structures and that immune responses against these epitopes drive inflammation in atherosclerosis. Discovering how specific immune cells and immune mechanisms affect the formation, growth and stability of the atherosclerotic plaque could provide new intervention targets.

The arterial vessel wall

The vessel wall in normal arteries consists of three distinct layers: intima, media and adventitia (Fig. 1A). The intima is the innermost layer of the artery and consists of the endothelial layer and the subendothelial connective tissue. The medial layer is separated from the intima by the internal elastic lamina and is made up of tightly arranged smooth muscle cells and elastic tissue. The adventitia is separated from the media by the external elastic lamina. The adventitia is mostly composed of connective tissue including collagens, elastic fibers, proteoglycans and glycoproteins but also of small blood vessels and nerve fibers that support the vessel. During atherosclerosis, structural changes result in atherosclerotic plaque development in the intima (Fig. 1B).

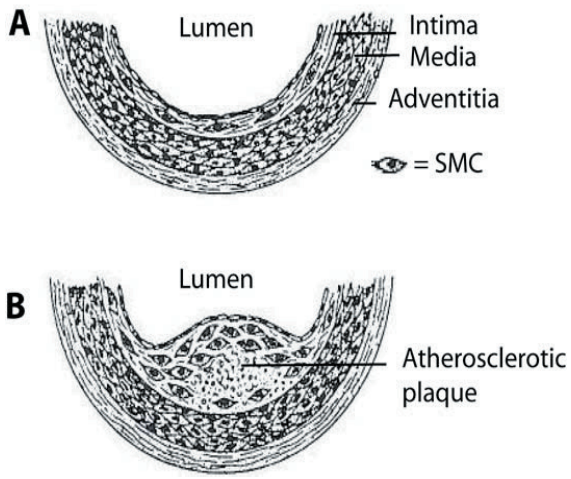

Figure 1: (A) The the different layers in the arterial wall. (B) The structural changes that occur during plaque development in atherosclerosis. Adopted from Thomas N. Wight (Extracellular Matrix, Volume 1, Tissue Function, 1996, edited by Wayne D. Comper)

Pathogenesis of atherosclerosis

There are many risk factors for developing atherosclerosis including high cholesterol levels in the blood, smoking, age, obesity, diabetes, hypertension, high blood pressure, lack of exercise and heritability. However, irrespective of the underlying risk factors atherosclerosis will commonly develop as a consequence of the immune response against lipid retention and oxidation in the vascular wall.

A high level of cholesterol loaded low density lipoprotein (LDL) particles in plasma is the most important risk factor for development of atherosclerosis. However it is not the increased lipid levels in plasma that is the direct factor contributing to the disease. Lipids infiltrate, aggregate and oxidize in the arterial intima. LDL infiltration into the intima at a low rate is a normal process that will supply the cells of the arterial wall with cholesterol needed for maintaining diverse cell functions. However, at atherosclerosis prone sites characterized by increased endothelial cell permeability, usually at sites with disturbed flow, LDL may enter at a higher rate. If the infiltration rate exceeds the capacity of the tissue to eliminate these LDL particles they will interact with the ECM proteoglycans and aggregate in the intima[1, 2]. LDL is oxidized through the exposure to oxidative stress and obtains new oxidized epitopes that drives the inflammation in atherosclerosis. Oxidized low density lipoproteins (oxLDL) induce cells in the intima to produce cytokines and chemokines which activate the endothelial cell layer. Activated endothelial cells express adhesion molecules including E selectin, P selectin, vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 already at very early stages of atherosclerosis[3-5]. The expression of adhesion molecules results in leukocyte rolling along the vascular surface and adherence at the site of activation[6]. Once the leukocytes have adhered to the endothelial cells, chemokines including monocyte chemoattractant protein-1 produced in the underlying intima stimulate them to migrate through the inter-endothelial junctions and into the intima[7]. Mice deficient in chemokines and adhesion molecules responsible for leukocyte adhesion and migration develop less atherosclerosis[8-10].

In the intima monocytes differentiate into macrophages and up-regulate pattern recognition receptors, including scavenger receptors (SR) and toll-like receptors (TLRs) with the help of macrophage colony stimulating factor[11, 12]. Macrophages bind oxLDL with the help of SR promoting enodcytosis and degradation of oxLDL. Atherosclerotic apolipoprotein E knockout (*Apoe*$^{-/-}$) mice deficient in either SR-A or CD36 develop less atherosclerosis compared to *Apoe*$^{-/-}$ mice with normal receptor capacity[13, 14]. TLRs bind ligands, including oxLDL, and initiate a signaling cascade resulting in further

macrophage activation[15-18]. The TLR signaling pathway includes the adaptor protein MyD88 and *Myd88*$^{-/-}$*Apoe*$^{-/-}$ mice showed significantly less atherosclerosis compared to *Myd88*$^{+/+}$*Apoe*$^{-/-}$ mice[19]. If cholesterol derived from oxLDL uptake cannot be mobilized from the macrophages at a sufficient extent, it accumulates as cytosolic droplets. Finally, cholesterol loaded macrophages are transformed into foam cells. Foam cells lack the ability to migrate out of the atherosclerotic plaque and are one of the characteristic cell types in atherosclerotic plaques.

T cells, mostly CD4+ positive cells infiltrate the intima and are present in plaques[20]. Both activated macrophages and T cells produce inflammatory cytokines promoting smooth muscle cells (SMC) to migrate from the media into the intima. Inside the intima SMC starts to proliferate and produce ECM proteins forming a fibrous cap around the foam cell filled area[21].

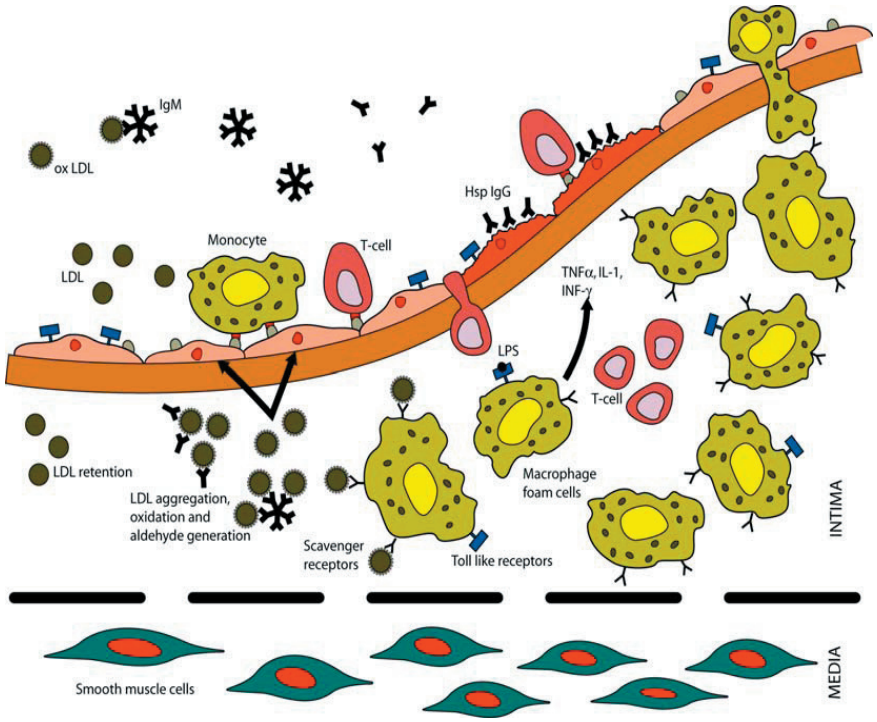

Figure 2: Immune responses during the pathogenesis of atherosclerosis. LDL aggregates and oxidizes in the ECM in the arterial intima, generating reactive aldehydes. Activated endothelial cells express adhesion molecules, resulting in leukocyte adhesion and infiltration. Monocytes differentiate into macrophages, which accumulate oxLDL and are transformed into foam cells. Activated macrophages and T cells produce inflammatory cytokines subsequently promoting SMC migration into the area. Kindly provided by Jan Nilsson.

T-cell mediated immunity in atherosclerosis

About ten percent of the cells in both murine and human atherosclerotic plaques are T-cells. Both $CD4^+$ and $CD8^+$ cells are represented in all regions of the atherosclerotic plaque, but there are significantly more $CD4^+$ cells in the fibrous cap[20]. Most of the $CD4^+$ cells isolated from human plaques express CD45RO, representing memory or effector T-cells[22]. Initially T-cell activation against atherosclerotic antigens probably takes place in the regional lymph nodes[23], possibly as a consequence of antigen presentation by dendritic cells that have migrated from the plaque to the lymph node. Activated T-cells may subsequently enter the plaque from the blood. Macrophages in the plaque present antigens to these T-cells which further increase inflammation in the plaque.

$CD4^+$ T-cells isolated from atherosclerotic plaques recognize protein antigens presented to them on the major-histocompatibility-complex (MHC) class II. $CD4^+$ T-cells reactive against oxLDL, heat shock protein 60 and *Chlamydia* have been detected in human atherosclerotic plaques[24-27]. T-cells that bind to their specific antigens express cell surface molecules and cytokines. Based on the expression pattern of these molecules T cells are divided into sub populations. T helper cells (Th) are categorized as Th1, Th2, Th3 or Th17 cells[28, 29]. Th1 cells are dominant in atherosclerotic plaque and are characterized by the interferon gamma (IFNγ) and interleukin-2 (IL) production, which generally stimulate inflammation and atherosclerosis. Th2 cells produce IL-4, IL-5, IL-10, IL-13 and help B-cells to produce antibodies, promoting pre-dominantly anti-atherosclerotic immune reactions. The Th17 cell population is highly pro-inflammatory, producing IL-17, IL-6, IL-23 and has been linked to atherosclerotic disease[30, 31]. Modulating the balance between Th cells may result in a different outcome of atherosclerotic disease. $CD8^+$ cells are mainly cytotoxic killer cells but they also produce tumor necrosis factor alpha (TNFα) and IFNγ. $CD8^+$ cells are restricted to antigens presented on MHC class I and mostly respond to viral antigens. Activation of $CD8^+$ cells in *Apoe*$^{-/-}$ mice may cause cell death and increased atherosclerosis[32]. A minor T-cell population in early atherosclerotic plaques is natural killer T cells (NKT) which recognize lipid antigens presented by CD1 molecules. Activation of NKT cells has been shown to increase atherosclerosis in *Apoe*$^{-/-}$ mice[33].

Regulatory T cells (Treg) are cells that have a crucial role by controlling the immune response against self and non-self antigens. Tregs may suppress the effector function of other immune cells through cell-to-cell contact or by the production of anti-inflammatory cytokines such as IL-10, transforming growth factor-β (TGFβ), IFNγ and IL-35[34-37]. Naturally occurring Tregs are $CD4^+$ cells that constitutively express high levels of CD25

and the transcription factor forkhead box P3 (FoxP3). Adoptive transfer experiments with $CD4^+CD25^+$ Tregs in *Apoe*$^{-/-}$ mice decreased atherosclerosis, suggesting a protective role for Tregs in atherosclerosis[38]. Accordingly, injection with anti-CD25 antibodies (which efficiently remove Treg function) increased atherosclerosis[38, 39]. Other types of Tregs are Tr1 and Th3 cells. Tr1 secrete large amounts of IL-10 which have been shown to have potent anti-inflammatory and anti-atherosclerotic effects[40]. Transfer and *in vivo* stimulation of ovalbumin specific Tr1 cells increased IL-10 production and reduced atherosclerosis in *Apoe*$^{-/-}$ mice[34, 41]. Th3 are regulatory T cells that secrete TGFβ. *Apoe*$^{-/-}$ mice with disrupted TGFβ signaling in T cells developed significantly more atherosclerosis compared to *Apoe*$^{-/-}$ mice with normal TGFβ signaling[42]. Moreover, injection with TGFβ blocking antibodies resulted in increased plaque size and less plaque stability[43].

Plaque stability and rupture

An advanced atherosclerotic plaque has a necrotic core in the center of the plaque, filled with dead cells, cell debris and extracellular lipids. The core is surrounded by foam cells and the death of foam cells play an important role in the growth of the core[44] The core is covered by the fibrous cap. Besides the necrotic core, plaques are composed of immune cells and ECM proteins (Fig. 2). Plaques may grow during many years causing reduced blood flow and stenosis. However it is the stability of the plaque rather than plaque size that is essential for the clinical outcome. Plaque rupture causes thrombus formation which can obstruct blood flow and cause MI or stroke. The vulnerability of atherosclerotic plaques is defined by the size of the lipid filled necrotic core, by the thickness of the fibrous cap covering the core and by the degree of inflammation within the plaque. Histopathological studies performed on human atherosclerotic plaques have revealed that unstable plaques are characterized by a core that occupies more than forty percent of the plaque area[45, 46]. The thickness and strength of the cap are important properties of plaque stability. Collagen provides tensile strength to the tissue and reduced collagen content increases the plaque vulnerability[47]. The rupture prone shoulder regions of the plaque are characterized by the accumulation of inflammatory cells, including macrophages, mast cells and T cells[48-50]. Macrophages and mast cells secrete proteolytic enzymes, including cystein proteases and matrix metalloproteinases (MMPs). Proteolytic enzymes, including MMP-1,-2,-3,-8,-9,-13 are present in human atherosclerotic plaques. Proteolytic enzymes may cause degrading of ECM proteins and thereby increasing

plaque instability[51-54]. Tissue inhibitors of MMPs and cystatin C suppress their proteolytic activity[55, 56]. T cells are able to promote MMP expression of other cells in the plaque by cell-to-cell contact or may inhibit collagen synthesis of SMC by secreting IFNγ[57, 58]. Moreover, a recent study has suggested that activated T-cells may limit collagen maturation in atherosclerotic plaques and thereby affect plaque stability[59].

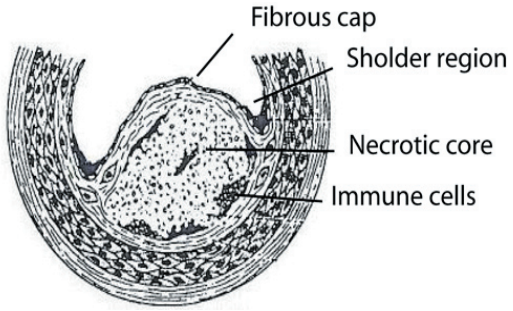

Figure 2: Advanced atherosclerotic plaque. Adopted from Thomas N. Wight

Lipid retention and oxidation

During atherosclerosis lipids accumulate within the arterial wall. Ionic interaction between positively charged regions of apolipoprotein B (ApoB) with negatively charged ECM proteins, mostly proteoglycans and to lesser extent collagen, fibronectin and laminin, is thought to mediate this process[60]. The primary binding sites on ApoB have been identified to the so called site B (residues 3359-3369)[61]. Binding of LDL to the ECM promotes retention of LDL in the intima. Several oxidant generating systems have been implicated in the oxidation of LDL *in vivo*, including myeloperoxidase, nitric oxide synthase and 15-lipoxygenase[62-66]. Oxidation of LDL leads to fragmentation of the LDL associated ApoB protein and peroxidation of polyunsaturated fatty acids, phospholipids and cholesterol esters in LDL resulting in the formation of reactive aldehydes including 4-hydroxynonenal and malondialdehyde (MDA).

Malondialdehyde

Aldehydes are highly reactive and may covalently attach to nucleic acid, proteins or phospholipids. The most studied aldehyde product is MDA which is commonly used as a

marker of lipid peroxidation both in plasma and in atherosclerotic plaques. Plasma from atherosclerotic patients has been shown to contain higher levels of MDA compared to healthy subjects[67-70]. Moreover human atherosclerotic plaques contain more MDA compared to plaque-free arterial tissue[71, 72]. *Apoe*$^{-/-}$ mice have high levels of circulating MDA in plasma, as well as atherosclerotic plaques with MDA epitopes[73]. The main source of MDA is the peroxidation of polyunsaturated fatty acids, containing two or more double bonds. Several different hypotheses describing the *in vivo* formation of MDA have been proposed (Fig. 3A)[74-76]. MDA is in contrast to other aldehydes a hydrophilic aldehyde and may therefore diffuse out of the oxidizing LDL particle and interact with surrounding molecules. The MDA molecule reacts with primary amines on lysine, arginine and histidine to form adducts (Fig. 3B)[77, 78]. Oxidation of LDL *in vitro* results in the loss of lysine and histidine residues on ApoB and to the formation of covalently bound aldehydes, including MDA adducts[77]. Aldehyde modification of ApoB has been implicated in the conversion of LDL into an atherogenic form, that is recognized by macrophages and eventually give rise to the formation of foam cells[79]. Moreover, MDA forms adducts with ApoB fragments generating neo-epitopes and immune response against MDA-modified ApoB peptides have been linked to atherosclerotic disease both in humans and in mice[80-82]. MDA has also been proposed to cause intra- and inter-molecular cross-linking of proteins[83, 84] (Fig. 3C) and may contribute to the arterial stiffening of cardiovascular tissue[83].

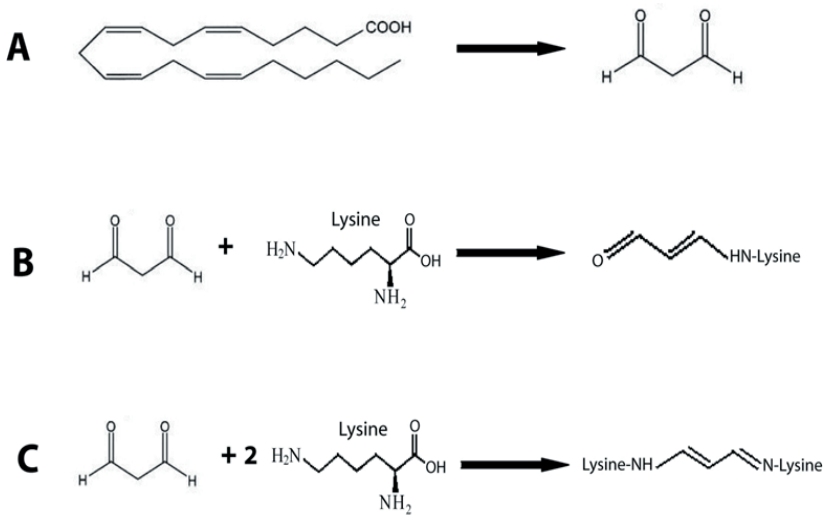

Figure 3: (A) The formation of MDA during the breakdown of fatty acid. (B) The formation of MDA adducts on lysine. (C) MDA induced cross linking.

The extracellular matrix in the vascular wall

Cells in the vascular wall are surrounded by a mixture of matrix molecules that interact to form the ECM. The ECM is composed by a variety of macromolecules, mainly produced locally by cells and assembled into a complex network, providing tensile strength, compressibility and elasticity to the wall

There are three main groups of macromolecules in the ECM, proteoglycans with the distinct polysaccharide chains, fibrous proteins including collagens, elastin and glycoproteins. The proteoglycans form the matrix ground structure into which the fibrous proteins are incorporated. Fibrous proteins have structural functions in the network and regulate cells through specific binding sites, like integrins. Collagen is the major component of vascular ECM[85]. Six different types of collagens have been identified in vascular tissue and the most predominant collagens are type I and type III. Collagens form distinct fibrils throughout all the layers of the wall and provide tensile strength to the tissue. Since collagens provide strength to the tissue, different distribution of collagens in atherosclerotic plaques may result in plaque instability and rupture[86]. Both type I and type III are present in atherosclerotic plaques and have the capacity to retain LDL in the vascular wall[87]. Elastin is another major structural component of the vascular wall[88]. It is a fibrous protein providing strength and elasticity to the tissue. Proteoglycans are hydrophilic macromolecules with the capacity to trap water and provide viscoelasticity and turgor pressure to the vascular wall. These molecules are also involved in vascular permeability and lipid retention[89, 90]. Moreover, proteoglycans interact with vascular cells, regulating adhesion, migration and proliferation. Major types of vascular proteoglycans identified in blood vessels include versican, decorin, biglycan and perlecan[91-96]. The final group of macromolecules present in the vascular ECM is glycoproteins, including fibronectin, laminin, thrombospondin, tenascin and osteopontin[85, 97-101]. Glycoproteins have the ability to interact with other ECM molecules, regulate the integrity of the vascular wall and bind to cells with specific cell-binding sequences.

Basement membranes are a highly specialized extracellular matrix structures beneath the endothelial cell layer in blood vessels and surround individual smooth muscle cells. They are thin protein sheets (50 nm thick) and the major components are the laminins, collagen type IV, nidogen-1 and the proteoglycan perlecan. The function of basement membranes is to provide structural support for cells, to separate tissue compartments and to provide a

barrier for cells and molecules. They also influence basic cell functions like proliferation, migration and differentiation.

Since lipids are accumulated and oxidized on ECM proteins in the intima, it is likely that reactive aldehydes formed during lipid oxidation will modify surrounding ECM proteins. MDA is a small soluble aldehyde that is able to diffuse away from the oxidizing lipids and thereby modify nearby targets. Screening of human plasma against antibodies that recognize MDA-modified ECM proteins (paper I and III) resulted in high antibody levels against MDA-modified fibronectin and laminin. The expression of fibronectin and laminin is up-regulated in atherosclerotic lesions and, interestingly, fibronectin is deposited at atherosclerosis-prone sites before others signs of atherosclerosis in mice[102]. Moreover, both and fibronectin and laminin have the capacity to bind LDL[60, 103, 104] making these proteins suitable candidate for MDA-modification.

Fibronectin

Fibronectin is a high-molecular weight glycoprotein that exists both as a soluble form in plasma and as an insoluble form in the tissue throughout the body. In tissues, fibronectin binds to other ECM components such as collagen, fibrin and proteoglycans and is assembled into the ECM network. Fibronectin interacts with cells trough integrin-recognition sequences, affecting adhesion, growth, migration and differentiation. Besides interactions with cells through cell binding sequences, fibronectin has a variety of functions, including heparin, collagen/gelatin and fibrin binding[105, 106]. Fibronectin exists as a dimer, consisting of two polypeptide chains linked together by disulfide bonds[107]. Each fibronectin monomer has a molecular weight of 230-250 kDa and contains type I, II, and III modules. The fibronectin protein is produced from a single gene, but alternative splicing results in the creation of several isoforms, including the extra domain A and B, which both appear to influence the development of atherosclerosis, and a variable V-region. Fibronectin is a very complex molecule and even if fibronectin has been extensively studied during the last three decades scientists are still discovering new biologically interesting sites.

Fibronectin in atherosclerosis

Fibronectin plays a key role in wound healing[108, 109] and is expressed locally to support the creation of new tissue. In early stages of atherosclerosis there is increasing amounts

of fibronectin expressed under the affected endothelial layer. In more advanced plaques there is a wider distribution and fragmentation of fibronectin[110-112], indicating dysregulation of the response to injury. It has also been shown *in vivo* that fibronectin enhances shear-stress induced nuclear factor kappa B (NF-κB) activation of endothelial cells[102]. NF-κB regulates adhesion and infiltration of leukocytes into the intima, which are hallmarks of atherosclerosis.

The levels of plasma fibronectin in patients suffering from coronary syndromes have been studied in several clinical studies. The picture is so far unclear, several studies have pointed towards increased fibronectin levels in plasma from patients suffering from coronary diseases compared to controls[113-116], while other studies have showed no[117] or an inverse correlation[118]. However, these studies have been performed in relatively small patient cohorts and larger studies are needed before any clear conclusion can be made.

The EDA domain is included into the fibronectin structure by alternative splicing during embryogenesis, wound healing[119] or cellular damage. The EDA domain has been shown to be a Toll-like receptor 4 ligand[120] and is expressed in both human and murine atherosclerotic plaques[121, 122]. Atherosclerotic *Apoe*$^{-/-}$ mice deficient of the EDA domain developed significantly less atherosclerosis[122, 123]. In human plaques EDA has been shown to be associated with a stable plaque phenotype[121].

The alternatively spliced EDB domain is less studied in the context of atherosclerosis. The EDB domain is virtually undetectable in normal healthy tissue but is inserted into fibronectin by alternative splicing during angiogenesis and tissue remodeling, which are characteristic features of advanced atherosclerotic plaques. One study has showed the expression of the EDB splice form both in murine and human atherosclerotic plaques[124].

Laminin

Laminins has important structural functions in the matrix. They bind to cell receptors tightly connecting the basement membrane to the endothelial cell layer. Laminins are a family of large glycosylated heterotrimeric proteins with a multidomain structure. Each laminin protein consists of α, β and γ chains resulting in many isoforms. According to literature there are currently five α, four β and three γ chains that have been identified. These chains can combine to form 15 different isoforms[125]. Laminins is a major component of all basement membranes which are sheet-like extracellular structures present in most organs[126, 127]. Laminins contains multiple cell binding sites, including the

biological important RGD and YIGSR sequences, and serves as important cell binding structures[128, 129].

Laminin in atherosclerosis

Laminins is diffusely distributed in normal and atherosclerotic intima, mostly concentrated in the endothelial basement membrane[101]. Laminins has been linked to several diseases, but the role of laminin in atherosclerotic disease has only been sparsely studied. Laminins interacts with arterial SMC through several receptors, inhibit proliferation of SMC and retain these cells in a contractile phenotype[130, 131]. Disruption of the normal laminin expression pattern or modification of existing laminin proteins could possible disturb the regulation of SMC function and influence disease progression. Moreover, endothelial cells attach to laminins trough several binding sites including the SIKVAV sequences. The lysine "K" residue of the SIKVAV sequence could possibly be modified by MDA and modulate endothelial cell-binding capacity. Endothelial cell dysfunction is an important hallmark of atherosclerosis.

Models to study atherosclerosis

Murine models

Animal models are needed to study pathogenic and protective mechanisms in atherosclerosis. Several mouse knockout models that develop hypercholesterolemia and atherosclerosis have been developed. Most commonly used are *Apoe*$^{-/-}$ mice, which spontaneously develop hypercholesterolemia and atherosclerosis and LDL receptor knockout mice, which develop hypercholesterolemia when fed high fat diet.

High fat diet enhances the formation and progression of atherosclerotic lesions. The most widely used diets are the standard chow diet and the Western type diet. The chow diet contains 4–6% fat and <0.02% cholesterol. The Western diet is a high fat diet and contains 21% fat and 0.15–0.2% cholesterol. Various modifications in the amount of fat or cholesterol content are used.

The assessment of atherosclerotic lesions is made in the aortic root by *en face* measurements of the entire aorta. Histological analysis of the aortic root allows characterization of size, composition and complexity of plaques. However, the aortic root only shows the atherosclerotic development at one site while *en face* analysis of the aorta reflects the atherosclerotic burden at large, with limited possibilities of composition analysis. The major limitation of these mouse knockout models is the absence of plaque rupture in mice, which is the primary reason of clinical events in humans.

Apoe knockout mice

Apoe is a glycoprotein produced mainly in the liver and is an important part of most lipoproteins. Lipoproteins transport cholesterol and other fats in the bloodstream. Apoe functions as a ligand for LDL receptor and is essential for the normal metabolism of lipoproteins. Apoe is a major protein component of a specific type of lipoprotein called very low-density lipoproteins (VLDL). VLDL has the important function to remove excess cholesterol from the blood and carry it to the liver for processing. Impaired cholesterol clearance in *Apoe*$^{-/-}$ mice dramatically increases cholesterol levels in plasma from 150-200 to 500-600 ug/dL. High fat diet further increases the cholesterol levels to about 1500 ug/dL. The major advantage of *Apoe*$^{-/-}$ mice is that they develop progressive atherosclerotic lesions similar to human plaques[132, 133]. A disadvantage is that the

lipoprotein profile is characterized by increased levels of VLDL rather than LDL as in human patients. All our mice experiments were conducted with *Apoe*$^{-/-}$ mice on C57BL/6 background and high fat diet were used to increase the atherosclerotic burden.

Human model

The subjects included in the clinical studies were born between 1926 and 1945 and recruited from the Malmö Diet and Cancer (MDC) study cohort. Fifty percent of those who entered the MDC study between November 1991 and February 1994 were randomly invited to take part in a study in the epidemiology of carotid artery disease[134]. Eighty-five cases of acute coronary heart events, that is, fatal or nonfatal MI or deaths resulting from coronary heart disease were identified during follow-up in 2001. Participants who had a history of MI or stroke prior to enrolment were not eligible for the present study. Risk factors for cardiovascular disease and carotid intima-media thickness were assessed as previously described[135].

Two controls, matched for age, sex, smoking habits, presence of hypertension, and month of participation in the screening examination and during the follow up were chosen for each case. Only one control was available for seven cases and no controls for one case. This case was excluded from analysis. Due to limited plasma material three cases and one control were excluded from the present study. Thus, the study population consisted of 223 subjects, 75 cases and 148 controls, ages 49 to 67 (median 61 years) years at baseline. Blood sampled were taken after overnight fastening and plasma were stored at -80°C until further assayed. The ethical committee of Lund University, Sweden approved the study.

Aim of thesis

The aims of this thesis were:

- To explore the possibility that LDL oxidation causes MDA-modifications of ECM proteins inside the arterial wall during atherosclerosis.

- To investigate how such MDA-modifications affect the functional ability of ECM proteins.

- To determine if immune responses against MDA-modified epitopes in the ECM are associated with atherosclerotic disease.

- To investigate how immune responses against MDA-modified ECM epitopes affect atherosclerosis.

- To investigate how Alum adjuvant affects atherosclerosis.

Present investigation and discussion

Extracellular matrix proteins may be MDA-modified during LDL oxidation

The first aim of study I was to investigate the possibility that LDL oxidation results in aldehyde-modifications of surrounding matrix proteins. Immune responses against specific epitopes including MDA-modified ECM proteins would represent a strong indication that these epitopes occur *in vivo*. With the help of ELISA we detected the existence of autoimmune responses in normal human plasma against several different MDA-modified ECM proteins including collagen type I, collagen type III, fibronectin, tenascin-C (paper I, figure 1) and laminin (paper III, figure 1). Immune responses against MDA-modified fibronectin were the most abundant and accordingly we decided to focus paper I on this protein. Interestingly, there were no autoantibodies against the proteoglycans biglycan and decorin. Proteoglycans have the highest affinity to interact with LDL and should therefore be particularly exposed to MDA.

LDL particles are entrapped on ECM proteins and oxidation of LDL results in the release of highly reactive aldehydes including soluble MDA. It is possible that oxidized LDL-derived aldehydes may react also with ECM proteins in the arterial wall. Using an *in vitro* model we demonstrated that oxidation of LDL results in MDA-modification of fibronectin. The fibronectin protein was kept separate from the oxidizing LDL by a dialysis membrane. Accordingly, only free MDA molecules were able to diffuse through the membrane and react with fibronectin (paper I, figure 2). The fact that free MDA was able to diffuse away from the oxidizing LDL and react with fibronectin, demonstrated that ECM proteins are possible targets for MDA-modifications. Finally, to determine that MDA-modification of fibronectin takes place also *in vivo* we analyzed human atherosclerotic plaques. Western blot and immunohistochemical analyses demonstrated the presence of MDA-modified fibronectin in atherosclerotic plaques (paper I, figure 2). Taken together, these observations demonstrate that oxidation of LDL in the vascular wall may result in MDA-modifications of surrounding ECM proteins, such as fibronectin, that MDA-modified fibronectin is present in atherosclerotic plaques and that immune responses against several MDA-modified ECM proteins are present in human plasma.

Functional importance of MDA-modification of ECM proteins

In paper I we also tried to asses the consequences of MDA-modification of ECM proteins. A MDA molecule has two reactive sites and could possibly react with two different proteins and result in cross-linking of proteins in the ECM. When we analyzed MDA-modified fibronectin formed by LDL oxidation *in vitro* we detected the formation of fibronectin complexes with higher molecular weight, compared to native fibronectin molecules (paper I, figure 2). Cross-linking of fibronectin molecules could affect the biological role of fibronectin, such as wound healing and thrombosis. Moreover, since ECM provides physical properties like strength and elasticity to the vascular wall, cross-linking could also have severe impact on the vessels ability to function normally. Cross-linking has been shown to be important in the later stages of diabetes mellitus and results in increased arterial stiffening of cardiovascular tissue[136]. The precise mechanism behind cross-linking are not clear but advanced glycation end products and MDA molecules have been proposed to mediate cross-linking of collagens[83, 136].

MDA-modification of ECM proteins will not only affect the mechanical properties of the ECM, but could also modify different cell-matrix interactions. Many of these interactions are mediated through a family of cell surface receptors named integrins. Cell-binding sites containing lysine, histidine or arginine residues are modulated by MDA-modifications which affect their properties. Fibronectin contains the biologically important arginine-glycine-aspartic acid (RGD) sequence which is vulnerable to modification by MDA at the arginine residue. Many cell types interact with the fibronectin RGD site including monocytes and macrophages. Macrophages are one of the characteristic cell type in atherosclerotic plaques and we chose to use macrophages as a model to analyze how MDA-modification of fibronectin affects protein-cell interactions. Fibronectin fragments have been shown to stimulate macrophages to secrete TNFα, MMP-9 and platelet-derived growth factor-BB, however after MDA-modification of fibronectin macrophages almost completely lost their ability to respond to fibronectin (paper I, figure 4). MDA-modification of fibronectin did not seem to induce a macrophage inflammatory response although macrophages lost their ability to respond to fibronectin. If the ECM looses its normal ability to support basal cell function it could have devastating effects on plaque progression and stabilization. To conclude, we show that MDA-modification of ECM proteins could affect the biological functions of proteins and could also change vascular mechanical properties by inducing cross-linking.

Immune responses against MDA-modified fibronectin in humans

Atherosclerosis is as disease primarily driven by inflammation and the key antigen in atherosclerosis is oxLDL. Much focus has been put to identify immune responses against different epitopes on oxLDL and their association with atherosclerosis. Several studies have reported that elevated levels of autoantibodies against oxidized LDL are predictive for the severity and progression of the disease[137, 138]. However, other studies have showed no or an inverse association between antibody levels and the extent of atherosclerosis[139, 140]. Accordingly, the results have so far been inconclusive and other clinically useful markers are needed. The observations that human atherosclerotic plaques contain MDA-modified fibronectin and that antibodies against several MDA-modified ECM proteins are present in human plasma suggest that immune responses against the ECM exist in atherosclerosis. In paper I we analyzed if there are any association between immune responses against MDA-modified fibronectin and atherosclerotic disease. We speculated that immune responses against the plaque ECM could increase inflammation and possibly play a role in the progression and stability of plaques. We conducted a prospective clinical study and measured IgG and IgM antibody levels in plasma from 75 individuals that subsequently developed acute MI or sudden cardiac death and 148 matched controls. To our surprise we found that the group that later suffered from acute cardiovascular events had significantly lower IgG and IgM antibody levels against MDA-modified fibronectin compared to controls (paper I, figure 3). The result are in line with previous clinical studies demonstrating an inverse association between antibody levels against ApoB peptides and the risk of developing acute cardiovascular events[81]. This protective effect could have several explanations. If MDA-modifications of ECM proteins have a negative impact on the proteins biological function, as discussed above, immune responses against these epitopes could help to remove damaged tissue and restore normal ECM functions. Antibodies could also bind to specific epitopes and silence inflammatory responses by blocking receptor recognition and binding to the damaged tissue thereby reducing inflammation.

Immune responses against MDA-modified ECM proteins in mice

The presence of IgG antibodies against MDA-modified fibronectin in human plasma indicates the involvement of an adaptive T cell-mediated immunity. In paper II and III, we aimed to investigate the functional importance of adaptive immune responses against different MDA-modified EMC proteins in atherosclerosis. We used the murine

atherosclerotic *Apoe*$^{-/-}$ model and immunized mice with MDA-modified fibronectin (paper II) or MDA-modified laminin (paper III) to induce immune responses against these epitopes. The immune responses were analyzed by measuring specific antibody levels and by characterizing the effect on specific immune cells. By measuring the development and composition of atherosclerotic plaques in aorta and the subvalvular region we determined the impact on atherosclerosis.

In paper II, immunization with MDA-modified fibronectin significantly inhibited the atherosclerotic plaque development in aorta and subvalvular lesions compared to Alum control mice. There was also reduced macrophage staining of the remaining plaques, indicating less inflammatory activity. Immunization resulted in high levels of MDA-modified fibronectin antibodies, primarily of IgG1 isotype reflecting a Th2-type immune response. Immunization with MDA-modified fibronectin was also associated with the activation of T regulatory cells. In paper III we immunized *Apoe*$^{-/-}$ mice with MDA-modified laminin. Immunization significantly increased the atherosclerotic plaque development in both aorta and in subvalvular lesions compared to the Alum control. Immunization resulted in MDA-modified laminin antibodies, again primarily of the IgG1 isotype. MDA-modified laminin immunization also resulted in the activation of effector T cells and reduced levels of protective regulatory T cells.

The two studies both induced immune responses against the immunization antigen although they resulted in completely different outcomes in respect to atherosclerotic disease. Both MDA-modified fibronectin and laminin immunization resulted in an antibody response, almost exclusively of the IgG1 isotype, reflecting activation of a Th2-type immune response. However, IgG1 antibody levels against MDA-modified fibronectin were about fifty times higher compared to the IgG1 antibody levels in the MDA-modified laminin (paper II, figure 3 and paper III, figure II). Th2 (IgG1) isotype antibodies have been shown to reduce atherosclerosis in several studies[80, 141] and the difference in antibody levels could explain the difference in atherosclerotic disease. One other observation that could explain the different atherosclerotic outcome is the effect on immunosuppressive regulatory T cells. Tregs plays an important role in controlling effector T cell function and have previously been shown to reduce the development of atherosclerosis[39, 40, 42]. The study on MDA-modified laminin showed a significant increase of inflammatory Th17 effector T cells and a reduction in regulatory T cells (paper III, figure 3). MDA-modified fibronectin on the other hand induced regulatory T cells (paper II, figure 5) and the differences in protective regulatory capacity could explain the effect on atherosclerosis.

Fibronectin and laminin expression in atherosclerotic plaques differ. Laminin is mostly concentrated in the endothelial basement membrane while fibronectin is distributed in large areas of the plaque. Directing immune responses towards different areas in the plaque may have completely different effects on inflammation and atherosclerosis.

Two other possibilities that may be of importance for the athero-protective effect observed in the MDA-modified fibronectin study are the unexpected decrease of fibronectin and cholesterol levels in plasma. The changes in fibronectin levels could be due to the induction of cross-reacting antibodies towards fibronectin resulting in an elimination of fibronectin from plasma. However, the finding that plaques from immunized mice did not contain less fibronectin argues against the possibility that the atheroprotective effects observed is explained by immune responses against plaque fibronectin. The lowering of plasma cholesterol is an additional factor that may contribute to the inhibition of atherosclerosis observed in immunized mice. Several clinical studies have investigated the association between plasma fibronectin and cholesterol levels[113, 142, 143]. Fibronectin levels were positively correlated with LDL cholesterol, total cholesterol and were negatively correlated with high-density lipoprotein cholesterol levels. The mechanism behind these associations is still unclear, but interactions of fibronectin with lipoprotein receptors may be involved[144]. However the immune responses induced in paper II were present before the cholesterol lowering took place. The reduced level of cholesterol is likely to have some athero-protective effects in the MDA-modified fibronectin study.

To conclude, immune responses induced against the MDA-modified form of fibronectin and laminin have opposite result on atherosclerotic disease.

The effect of Alum adjuvant on atherosclerosis

The aluminum hydroxide adjuvant Alum, which is the most common adjuvant used in vaccines today, has been shown to have athero-protective effects in itself. Such properties make Alum a suitable adjuvant in developing vaccines for atherosclerosis. In paper IV we set out to characterize the immune pathways mediating the Alum effect on atherosclerosis. We injected wild type and *Apoe*$^{-/-}$ mice with Alum or PBS and assessed the immune response.

The result demonstrated that Alum have a modest pro-inflammatory effect in wild type mice and had no effect on regulatory T cells. Hypercholesterolemic *Apoe*$^{-/-}$ mice, on the

other hand, showed less activated T cells and a marked increase in regulatory T cells (paper IV, figure 1). It has been shown that Alum potently induces oxidative stress and lipid peroxidation[145-148]. Western blot analysis demonstrated that the injection site in *Apoe*$^{-/-}$ mice contained oxLDL and MDA-modified ApoB peptide seven days after injection of Alum (paper IV, figure 6). The tissue at the injection site in the hypercholesterolemic *Apoe*$^{-/-}$ animals was rich in LDL particles which probably are oxidized by Alum. These findings suggest that in *Apoe*$^{-/-}$ mice LDL is oxidized subcutaneously at the Alum injection site and could serve as the antigen that induces the activation of Tregs.

In the studies in paper II and III we used Alum as adjuvant. Surprisingly, immunization with native fibronectin or laminin resulted in antibodies directed against the MDA-modified form of the proteins. Since Alum precipitate recovered from the immunization site contained considerable amounts of oxLDL and MDA-modified ApoB peptide (paper IV, figure 6) it is likely that other native proteins also are modified as a result of Alum injection. To further investigate the possibility that native proteins injected together with Alum result in MDA-modification at the injection site we analyzed the aluminum precipitate from the injection site after native fibronectin immunization. Both Western blot and fluorescence measurement of aldehydes groups revealed presence of MDA-modified fibronectin at the injection site. Collectively, these results strongly indicate that native protein used together with Alum in *Apoe*$^{-/-}$ mice result in MDA-modified protein *in vivo* and is therefore not suitable as a control.

Conclusion and future perspective

Our studies provide evidence for the presence of MDA-modifications on ECM proteins during atherosclerosis. We also demonstrate that these MDA modifications may be a consequence of LDL oxidation. Immune responses against several MDA-modified ECM proteins are present in human plasma. A prospective clinical study showed that the group that later suffered from acute cardiovascular events had significantly lower IgG and IgM antibody levels against MDA-modified fibronectin compared to the control group. These epidemiological results suggest that immune responses against MDA-modified fibronectin may have protective effects in atherosclerotic disease, or could serve as a clinical marker. Induction of immune responses against MDA-modified fibronectin in *Apoe*$^{-/-}$ mice significantly decreased the atherosclerotic plaque development in both aorta and in subvalvular lesions. On the other hand immunization of *Apoe*$^{-/-}$ mice with another modified ECM protein, MDA-modified laminin, had the opposite effect resulting in increased plaque development. Finally we show that Alum, an adjuvant which is commonly used in vaccines, results in LDL oxidation and aldehyde generation at the injection site.

In a future perspective these observations have several potentially important implications. Cardiovascular diseases are a heavy burden both for the individual and for the society at large and new clinical useful targets for treatment and diagnostic tools are needed.

Neo-epitopes created by MDA-modification of ECM proteins in atherosclerotic tissue represent potential antigens in future therapeutic vaccines for atherosclerosis. However, since the immune responses against MDA-modified fibronectin and MDA-modified laminin resulted in opposite effects on atherosclerotic disease, more animal studies are needed to conclude which proteins may be used in possible future treatments. MDA-modifications have also been linked to diabetic disease, where MDA causes cross-linking and arterial stiffening. It is also possible that while antibodies against some modified ECM proteins, like MDA-modified fibronectin are protective in atherosclerotic disease, antibodies against other modified matrix proteins could fuel the disease process. New epidemiological studies on antibody levels against MDA-modified ECM proteins in humans should include both different types of ECM proteins and subjects with other diseases than atherosclerosis such as diabetes.

The fact that MDA-modifications of ECM proteins occur in atherosclerotic tissue also suggest that MDA-modified ECM proteins represent possible clinical markers for plaque rupture. Plaques that are about to, or recently have ruptured, could release MDA-

modified ECM proteins into the blood. Plasma samples from patients suffering from acute CHD events should be analyzed against different MDA-modified ECM proteins. Another possibility is to dissect stable and unstable atherosclerotic plaques and investigate if the amount of MDA-modified ECM proteins is associated with plaque stability.

Populärvetenskaplig sammanfattning

Åderförkalkning och immunsvar mot aldehyd-modifierade vävnadsproteiner.
Ateroskleros eller som det vanligen kallas åderförkalkning, är en inflammatorisk, delvis autoimmun sjukdom i kärlväggen. Åderförkalkning är mycket vanlig i västvärlden och är den vanligaste orsaken till hjärtinfarkt och stroke. Uppskattningsvis dog 17,5 miljoner människor av hjärt-kärlkomplikationer under 2005, antalet drabbade stiger för varje år och om inget görs kommer denna siffra att stiga markant.

Transport av fetter och andra näringsämnen till olika delar av kroppen sker i blodet. Detta sker i artärerna som leder ut blodet från hjärtat till kroppens alla organ. Artärerna är elastiska och är uppbyggda av bindvävsproteiner och celler. Den främsta orsaken till att åderförkalkning uppstår är att fettpartiklar som transporteras i blodkärlen tränger ut i kärlväggen där de fastnar och oxiderar. Autoimmuna sjukdomar uppstår till följd av obalans i immunsystemet, där immunceller ser kroppens egna vävnader som ett hot och angriper dessa i ett försök att städa bort dem. Vid åderförkalkning ser immunförsvaret det oxiderade fettet som något främmande och immunceller tar sig in i kärlväggen för att ta bort det oxiderade fettet. Ansamlingen av immunceller i kärlväggen leder till en kraftig inflammation i området och då det hela tiden kommer att fortsätta tränga in fett som oxideras i kärlväggen, kommer inflammationen att fortgå. Ansamlingen av immunceller, oxiderade fetter och en förändring av omkringliggande bindväv resulterar i att det drabbade området expanderar och det bildas ett så kallat plack. Placken kan växa under många år och kan slutligen resultera i att blodflödet påverkas eller att placken brister. När plack brister leder detta till bildning av en blodpropp, proppen kan stoppa blodflödet och i sin tur kan ge upphov till hjärtinfarkt eller stroke. När fett oxideras skapas en rad biprodukter inklusive reaktiva aldehyder. Aldehyder kan modifiera peptider i det protein, ApoB, som finns i de flesta fettpartiklar. Immunsvar mot dessa ApoB-peptider har också visat sig vara skyddande mot åderförkalkning och vaccin baserat på vissa av dessa peptider håller på att tas fram.

Målsättningen med min avhandling var att undersöka om oxidering av fett leder till att reaktiva aldehyder, såsom malondialdehyd (MDA), förändrar omkringliggande vävnadsproteiner. Vi ville även undersöka om det finns immunsvar mot MDA-modifierade vävnadsproteiner och om immunsvar mot dessa påverkar sjukdomsförloppet.

I den första studien visade vi att det hos människa finns immunsvar i form av antikroppar mot en rad olika MDA-modifierade vävnadsproteiner, inklusive fibronektin och laminin. Vi visade också att dessa modifieringar kan uppstå när fett oxiderar. Vi genomförde en klinisk studie på hjärt-kärlsjuka patienter som inom en femårsperiod drabbades av akuta hjärt-kärlkomplikationer och jämförde dessa med friska kontroll personer. Resulatateten av dessa studier talar för att höga nivåer av antikroppar mot MDA-modifierat fibronectin har en skyddande effekt mot akuta hjärt-kärlkomplikationer.

I studie två och tre undersökte vi den funktionella betydelsen av immunsvar mot aldehyd-modifierat fibronektin och laminin i åderförkalkning. Vi immuniserade möss med MDA-modifierat fibronektin eller laminin, gav dem fet kost så att de utvecklade åderförkalkning och studerade hur immunförsvaret och sjukdomen påverkades. Immuniseringarna resulterade i höga antikroppsnivåer mot respektive MDA-modifierat protein, dock var nivåerna mot MDA-modifierat fibronektin betydligt högre. Immunisering med MDA-modifierat fibronektin minskade åderförkalkningen med över 70 % medan immunisering med MDA-modifierat laminin resulterade i en ökning på 60 %. Denna skillnad i åderförkalkning kan möjligen förklaras av olika antikroppsnivåer, men också i förekomsten av skyddande anti-inflammatoriska T-celler. Immunisering med MDA-modifierat fibronektin resulterade i en ökning av flera olika populationer av skyddande T-celler, jämfört med en betydande minskning efter immunisering med MDA-modifierat laminin.

Vid vaccinering används adjuvans som ökar immunförsvaret mot det ämne som ingår i vaccinet Ett av de vanligaste förekommande adjuvanterna i vacciner är Alum och det har i några studier visats att Alum i sig själv kan skydda mot ateroskleros I den fjärde studien undersökte vi hur Alum ger upphov till detta skydd. Vi visade att Alum som injicerats in i ett område som är rikt på fett gör att detta fett oxiderar och skapar MDA-modifiering av ApoB-peptider. Aluminjektionen resulterade också i en ökad förekomst av anti-inflammatoriska T-celler som har visats vara skyddande mot åderförkalkning.

För att summera:

- Vi visar för första gången att oxidering av fett kan resultera i att bindvävsproteiner blir MDA-modifierade.
- Immunsvar i form av antikroppar mot en rad olika MDA-modifierade bindvävsproteiner finns hos människa.

- Höga antikroppsnivåer mot MDA-modifierat fibronektin verkar vara skyddande vid hjärt-kärlsjukdom.
- Immunisering med MDA-modifierat fibronektin och laminin ger motsatt effekert på både immunsvar och åderförkalkning i mus.
- Alum injektion kan resultera i att fett oxiderar vid immuniseringsstället samt att detta ökar mängden anti-inflammatoriska T-celler.

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