The pro-atherogenic role of intimal hyperplasia

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" If I have seen further it is by standing on the shoulders of Giants"

Sir Isaac Newton

Abstract

Atherosclerosis is a leading cause of mortality worldwide, and results from accumulation of plasma lipoproteins, mainly low-density lipoproteins (LDL), in the sub-endothelial layer of the arterial wall. In this thesis, I investigated how structural changes of the vessel wall can make the vessel more prone to developing atherosclerotic lesions.

Project 1: Accelerated atherosclerosis occurs following vascular interventions, such as percutaneous coronary intervention and implantation of saphenous vein grafts. However, the cause of the accelerated atherogenesis is not known. We found that intimal hyperplasia induced by vascular interventions makes the vessel wall highly susceptible to LDL retention and accelerated atherosclerosis by a mechanism that can be targeted by glycosaminoglycan (GAG)-binding antibodies.

Project 2: Cadmium is an important risk factor for atherosclerosis, but the underlying mechanism for how cadmium increases the risk of atherosclerosis is unclear. We observed: (1) increased expression of perlecan and the GAG-chain modifying enzyme CHST3 in arteries following local exposure to cadmium; and (2) increased LDL-binding in proteoglycans isolated from cells cocultured with cadmium. Finally, we showed that local cadmium exposure increased LDL retention in the arterial wall.

Project 3: Immunofluorescence microscopy is a method used to study the spatial location of proteins in tissues and cells. Here we present an enhanced multi-fluorescence set-up based on condensed filter sets that are more specific for each fluorochrome and allow for a more efficient use of the light spectrum.

Keywords: Atherosclerosis, Vascular intervention, Multi-color microscopy, Cadmium, Intimal hyperplasia

Sammanfattning på svenska

Åderförkalkning är en av de ledande dödsorsakerna i världen. Sjukdomen utvecklas under lång tid, men startas av att LDL—även kallat de ”onda kolesterolet”—ackumuleras i kärlväggen. I den här avhandlingen undersöks huruvida strukturella förändringar i kärlväggen resulterar i ökad inbindningen av LDL partiklar samt om kadmium ökar denna LDL inlagring.

Projekt 1: En accelererad åderförkalkningsprocess kan uppstå till följd av kärlinterventioner, såsom ballongvidgning och bypass kirurgi. De bakomliggande orsakerna till det snabba förloppet vid accelererad åderförkalkning är inte kända. I projekt 1 visar vi att en glatt muskelcellsrik förtjockning som bildas efter kärlintervention, så kallad intimal hyperplasi, resulterar i en kärlvägg med hög benägenhet att binda LDL. Vi visar även att det går att blockera inbindningen av LDL till kärlväggen genom att störa den elektrostatiska interaktionen som finns mellan kärlväggen och LDL partikeln.

Projekt 2: Kadmiumexponering ökar risken för att utveckla åderförkalkning, men man känner inte till hur. Vi fann att lokal stimulering av kärlväggen med kadmium gav ett ökat uttryck av proteoglykanproteinet perlecan, samt enzymet CHST3 som gör proteoglykaner mer negativt laddade. Vidare fann vi att odlade celler som stimulerats med kadmium producerade proteoglykaner med ökad förmåga till att binda LDL. Slutligen visade vi att LDL-inbindning till kärlväggen ökade till följd av lokal kadmiumstimulering.

Projekt 3: Fluorescensmikroskopi används för att studera lokaliseringen av proteiner i vävnader och i celler. Vi utvecklade ett förbättrat ljusfiltersystem i mikroskopet som möjliggör att fler proteiner (upp till 6 proteiner) kan visualiseras samtidigt i ett och samma prov. Det här kan jämföras mot standard fluorescensmikroskopi som högst kan visualisera 4 proteiner samtidigt.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Intimal hyperplasia induced by vascular intervention causes lipoprotein retention and accelerated atherosclerosis in mice**

Siavash Kijani, Ana Maria Vázquez, Malin Levin, Jan Borén and Per Fogelstrand

Submitted

II. **Non-toxic cadmium accelerates subendothelial retention of atherogenic lipoproteins in humanized atherosclerosis–susceptible mice**

Siavash Kijani, Göran Bergström, Malin Levin, Lars Barregård, Björn Fagerberg, Per Fogelstrand and Jan Borén.

Manuscript

III. **Filter-Dense Multicolor Microscopy**

Siavash Kijani, Ulf Yrlid, Maria Heyden, Malin Levin, Jan Borén, Per Fogelstrand.

PLoS ONE, March 2015

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ABBREVIATIONS

apoB	Apolipoprotein B
apoA	Apolipoprotein A
apoE	Apolipoprotein E
***ApoE*$^{-/-}$**	ApoE knockout
ASCVD	Atherosclerosis cardiovascular disease
bFGF	Basic fibroblast growth factor
CABG	Coronary artery bypass graft
CHST3	Carbohydrate (chondroitin 6/keratan) sulfotransferase 3
CS	Chondroitin sulfate
ddPCR	Droplet digital PCR
DIT	Diffuse intimal thickening
DS	Dermatan sulfate
ECM	Extracellular matrix
FDMM	Filter–dense multicolor microscopy
GAG	Glycosaminoglycan
HIF-1A	Hypoxia-inducible factor 1-alpha
HS	Heparan sulfate
HA	Hyaluronan
KS	Keratan sulfate
LDL	Low–density lipoprotein
***Ldlr*$^{-/-}$**	LDL receptor knockout
LPL	Lipoprotein lipase
NO	Nitric oxide
PCI	Percutaneous coronary intervention

PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
PDGF	Platelet-derived growth factor
RT-PCR	Real time-PCR
SLRP	Small Leucine–rich Proteoglycan
SVG	Saphenous Vein Graft
TGF-β1	Transforming growth factor beta 1
VEGF	Vascular endothelial growth factor
VLDL	Very low–density lipoprotein
VSMC	Vascular smooth muscle cells

Introduction

The cardiovascular system

The cardiovascular system supports the tissues in the body with oxygen and nutrients. It is also an important transport system for components of the immune system and the endocrine system. The cardiovascular system consists of the heart that pumps the blood and vessels that transport the circulating blood. There are three main types of vessels; arteries, veins and capillaries. The arteries are high-pressure vessels that carry oxygenated blood from the heart to tissues, and the veins are low-pressure vessels that carry the blood back to the heart to be re-oxygenated via the lung circulation. The exchange of oxygen and nutrients from blood into tissues occurs via the capillaries in the tissues. The capillaries are very small in size and abundant, which creates a large "exchange surface" between the blood and the tissue.

Composition of the vessel wall

The vessel wall of arteries and veins consists of three layers separated by fenestrated elastic membranes. The outer layer (*tunica externa* or *adventitia)* is a collagen-rich connective tissue that contains fibroblasts, leukocytes, nerves, and lymph vessels. The adventitia gives support to the vessel and anchors it to the surrounding tissue.

The middle layer (*tunica media* or *media*) consists of vascular smooth muscle cells (VSMC) that run perpendicular to the long axis of the vessel. The VSMCs are responsible for the muscle-tonus of the vessel wall.

The inner layer (*tunica intima or intima*) consists of a monolayer of endothelial cells and sub-endothelial matrix. The endothelium is positioned as the interface between the blood and the vessel wall, and is an important regulator of the function of the

vessel by releasing vasodilatory factors such as nitric oxide, prostacyclin and endothelium derived hyperpolarizing factor, as well as vasoconstrictive factors such as thromboxane and endothelium-1[1].

Diffuse intimal thickening forms following accumulation of VSMC

In some vessels, the intima is thickened due to accumulation of VSMCs. This is called Diffuse Intimal Thickening (DIT). DIT is not a pathological state, but rather a natural adaptation over-time to hemodynamic forces from the blood. Interestingly, atherosclerosis cardiovascular disease (ASCVD) develops preferentially in vascular beds that forms DIT[2,3].

Vascular smooth muscle cells

VSMCs exists in a range of phenotypes in the arterial wall[4]. In the media, VSMCs exist mostly in the contractile phenotype, while intimal VSMCs have a more synthetic phenotype. The contractile phenotype contain high number of contractile filaments[5], which enable VSMCs to contract and relax to alter the luminal diameter. However, VSMCs can undergo a phenotypic modulation to a synthetic phenotype. In the synthetic phenotype, VSMCs contain more organelles and express significantly more proteins that are secreted into the surrounding ECM[5]. Also the synthetic phenotype has higher grow rate and higher migratory activity compared to contractile VSMCs[6]. However, it is important to remember that VSMCs are not either synthetic or contractile; rather they exist on a sliding scale between the two phenotypes. There are protein markers that are specific to contractile VSMCs, but markers for synthetic VSMC are rare. Instead, it is gradual downregulation of contractile VSMC markers that are associated with synthetic VSMC. See table 1 for examples of markers for VSMC and associated phenotype.

Marker	Phenotype association	Subcellular localization	Function
SM22α	c>s	Actin-associated	Regulation contraction
α-smooth muscle actin	c>s	Contractile filaments	Contraction
Smooth muscle myosin heavy chain	c>s	Contractile filaments	Contraction
Smoothelin	c	Actin-associated	Regulation contraction
SM-caloponin	c	Actin-associated/ cytoskeleton	Regulation contraction/signal transduction
CRBP-1	s>c	Cytoplasm	Retinoid transport and metabolism
Smemb	s>c	Contractile filaments	Contraction

Table 1. Adapted from Rensen et al[5]. Examples of VSMC markers and associated phenotypes; c=contractile phenotype, s=synthetic phenotype, > indicated phenotype preference.

Atherosclerosis

ASCVD has affected humans for eons. In fact, in 1853 Czermak J observed in Egyptian mummies the oldest known atherosclerotic lesions at that time. Later several studies confirmed the existence of atherosclerotic plaques in other Egyptian mummies, with the oldest documented case thus far dating back to 1550 to 1580 BCE[7].

However, it was first when the western world underwent a drastic societal and economical changes during the 19th and 20th century that atherosclerosis became a widespread health problem. Driven by urbanization and industrialization, the living standard for large parts of the population significantly improved. This resulted in among other things to over nutrition and increased life expectancy. These two factors contributed to the epidemic in ASCVD seen during the 20th century[8].

For a long time, ASCVD primarily affected the population of the western developed world as account of higher living standards. However, the significant economic development and rapid urbanization in middle and low-income countries has resulted in a global increase in ASCVD[9]. In fact, mortality rates have increased and continue to do so in middle and low-income countries while they have declined in high-income countries[10]. Today, ASCVD accounts for 13,5 million deaths worldwide[9]. This places ASCVD as the largest single cause of mortality globally[9]. Furthermore, the number of projected deaths is expected to increase to over 23.6 million by 2030.

Multiple risk factors have been identified over the years to modulate the risk of atherosclerosis. These include raised apoB/apoA1 ratio, abdominal obesity, psychosocial factors, diabetes, hypertension, smoking, daily consumption of fruits and vegetables, alcohol consumption and regular physical activity[11]. Except for high levels of apoB lipoproteins, emerging evidence indicate that they are not causative[12]. Thus, they do not induce increased CVD risk in the absence of at least a mild hypercholesterolemia. Rather, they modulate the risk and thus lower the threshold for initiation of the atherogenesis. Therefore, pa-

tients affected by a number of these risk factors can reduce risk of developing ASCVD by LDL lowering intervention[13,14].

ASCVD is caused by atherosclerotic lesions, i.e. lipid-rich inflammatory lesions within the vessel wall. Development of atherosclerotic lesions is a slow process that in most cases takes several decades to manifest clinically. Large lesions can cause narrowing of the vessel lumen, resulting in reduced blood supply and hypoxia/ischemia in downstream tissues. This causes for example angina pectoris in the heart and intermittent claudication in the legs. Atherosclerotic lesions may also rupture and expose highly thrombogenic material to the bloodstream, which leads to acute thrombus formation and often a total blockage of the vessel at the site of the rupture[15]. Clinically, the patient will suffer ischemic events such as myocardial infarction in the heart or ischemic peripheral vascular disease in the legs. Finally, atherosclerotic lesions may also generate thrombi that are released into the blood stream causing blockage of smaller vessels further downstream. This is a common cause for stroke.

LDL accumulation in the arterial wall is the root cause of atherosclerosis

In the early 19th hundreds, pathologists began to investigate the gross morphological changes in the arterial wall. The French surgeon and pathologist Jean Lobstein was the first to coin the term "atherosclerosis" in 1829[16]. However, the pathological processes leading to atherosclerosis was for a long time unknown.

In 1856 Rudolf Virchow proposed that atherosclerosis might be caused by plasma components and that these components can induce the inflammatory response in atherosclerosis. Although this hypothesis is strikingly in agreement with current knowledge, it was at the time a controversial statement. The prominent pathologist C. Von Rokitansky instead proposed that atherosclerotic lesions were a result of changes in the vessel

wall, and even if he recognized inflammation, he considered it to be of a secondary nature.

In 1910, Adolf Windaus showed that atherosclerotic plaques consist of connective tissue and cholestrol[17]. Inspired by the work of Dr. Ignatowski, suggesting that rabbits develop atherosclerosis when feed non vegetarian food, Nikolai Anichkov published a milestone paper in 1913 showing that cholesterol alone can induce atheromatous changes in the vessel wall[18]. This was shown by feeding rabbits diet supplemented with cholesterol. The study established cholesterol as the first known risk factor for developing atherosclerosis, and it strongly suggested a causal relationship between blood cholesterol and atherosclerosis.

The Norwegian physician Carl Müller, working with patients with familiar hypercholesterolemia, was in 1939 one of the first investigators that associated elevated cholesterol levels in humans with increased risk of ASCVD[19]. However, at this time the nature of plasma cholesterol was not known. In 1949, Gofman et al isolated different lipoprotein fractions from humans using analytic ultracentrifugation[20]. Importantly, he was one of the first investigators too report that high levels of VLDL and especially LDL seemed to be associated with increased ASCVD[21]. This was later on supported by results from the Farmingham study[22].

In the late 1960s, 12 centers specialized in atherosclerosis were opened in the USA and Canada. The centers investigated if reduction of serum LDL cholesterol levels would lead to reduction of cardiovascular incidents. This was tested by administrating cholestyramine, one of the first cholesterol lowering drugs, to patients. The patients displayed a significant reduction in LDL cholesterol as well as a significant reduction in ASCVD events. Thus, for the first time reduction of LDL cholesterol levels was correlated to reduced risk of ASCVD events. Since then, several other lipid-lowering drugs have successfully decreased the number of ASCVD events in risk patients[23-25]. Furthermore, a strong correlation between plasma cholesterol levels and ASCVD is also seen in human genetic variants, where

genetic variants that increase plasma cholesterol cause increased risk ASCVD[26,27], and genetic variants that decrease plasma cholesterol levels cause reduced risk of ASCVD[28,29]. Thus, it is now well established that plasma cholesterol—in particular apoB-containing lipoproteins <70 nm in diameter—are the root cause of atherosclerosis.

LDL binds to the vessel wall through electrostatic interactions to proteoglycan glycosaminoglycans

Already in 1949 Mogen Faber, who studied human lesions, proposed that the affinity of cholesterol-transporting proteins for sulfate-containing glycosaminoglycans (GAGs) were involved in the accumulation of cholesterol in the arterial wall. GAGs are unbranched negatively charged sugar chains attached to a core protein. These complexes are called proteoglycans. Work from different groups then showed that LDL indeed can bind electrostatically to negatively charged GAGs, including GAGs isolated from human aorta[30-32]. More recently, mice deficient in GAGs have been shown to have delayed onset of atherosclerosis[33].

The structure of proteoglycans

All cell types typically found in the arterial wall, including endothelial cells and VSMCs, produce proteoglycans[34]. Proteoglycans are secreted to the surrounding ECM or anchored to the cell membrane[35,36]. They are involved in many functions in the body including hydration of tissue, stabilization and storage of growth factors, receptors and co-receptors as well as inhibiting cell signaling[35,37-41]. Studies have identified a number of proteoglycan species in the human arterial wall, such as biglycan, decorin, fibromodulin, prolargin(PRELP), lumican, perlecan, versican and aggrecan[42-48]. Perlecan and biglycan is also found in the mouse arterial wall[49].

Proteoglycans have two components, a core protein and one or more covalently attached glycosaminoglycan (GAG). The exception is Hyaluronan (HA) which lacks the core protein. GAGs are linear polysaccharides and carry a negative charge. Furthermore the GAGs can be divided into sulfated GAGs such as chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparin and heparan sulfate (HS), and non-sulfated GAGs such as HA. One core protein can also have two types of GAG chains, for example CS and DS. See table 2 for overview of proteoglycans.

Name	Number/type of GAG chains	Size core protein (kDa)	Location
SLRPs			
Biglycan	1-2 CS/DS	38	ECM
Decorin	1 CS/DS	36	ECM
Lumican	4 KS	37	ECM
Fibromodulin	4 KS	59	ECM
PRELP	KS	45	Cell membrane
Aggrecan family			
Aggrecan	100 CS/DS	208	ECM
Versican	12-15 CS/DS	265	ECM
Other			
Perlecan	1-3 HS	400	ECM, Basement-membrane

Table 2. Proteoglycans found in the arterial wall. SLRPs=Small Leucine–rich Proteoglycans CS=Chondroitin sulfate, KS=Keratan sulfate, HS= heparan sulfate, ECM=extra cellular matrix.

The CS, DS and HS GAGs chains contain disaccharide-repeating regions of acetylated amino sugar moieties. CS contain glucuronic acid and N-acetylgalactosamine. DS also contain a glucu-

ronic or iduronic acid and an N-acetylgalactosamine. HS contain glucuronic or iduronic acid and N-acetylglucosamine. KS contain galactose and N-acetylglucosamine[37,50]. The saccharides can also have sulfate substitutes in various positions.

The response-to-retention hypothesis

Based on the work done on LDL binding to the vessel wall, Kevin Williams and Ira Tabas proposed the *response-to-retention hypothesis of early atherogenesis* in 1995[51]. The response-to-retention hypothesis states that the initial event in atherogenesis is retention of apoB-containing lipoproteins, within the vessel wall. In the vessel wall, retained lipoproteins become modified and pro-inflammatory. If arterial lipoprotein accumulation remain high over a long period of time, the inflammatory response may escalate and turn into a maladaptive inflammation that leads to an atherosclerotic lesion[51].

Identifying the LDL GAG-binding site

The next task was to find the GAG-binding site(s) on the LDL particle. Camejo and others showed that the apoB100 lipoprotein that surround the LDL particle contain at least 8 clusters of positively charged amino acids with the potential to bind negatively charged GAGs[52-54]. However, these experiments were performed with delipidated apoB fragments.

To confirm which—if any—of the positively charged amino acid clusters on apoB100 was functional *in vivo*, Borén et al generated mice expressing mutant human apoB100[55]. Recombinant LDL expressed in these mice where isolated and their ability to bind proteoglycans were tested. The study identified residues 3359–3369 in apoB100 as the principal proteoglycan-binding site. Specifically, the positively charged arginine and lysine residues were critical for LDL binding to the proteoglycans. This amino acid cluster is called “Site B” and is located

in the C-terminus of apoB100. Interestingly, "Site B" is also the binding-site for the LDL-receptor[56].

Transgenic mice expressing mutant forms of recombinant apoB100 were used in atherosclerosis studies where the biological significance of the proteoglycan-binding ability for atherogenesis was tested[57]. This was the first time the "Response-to-Retention" hypothesis was experimentally verified *in vivo*.

This finding presented a problem since mice only carries apoB48-containing lipoproteins that lack SiteB, but they still develop atherosclerosis at elevated lipoprotein levels[58]. In fact, studies showed that apoB48-containig lipoproteins bound to proteoglycans with similar affinity as apoB100 containing lipoproteins[59].

This promoted the discovery of the amino acid clusters 84 to 94 called Site B-1b on the apoB48 protein[60]. The Site B-1b is also present on the apoB100 protein but it is masked by the C-terminus end. This explains atherogenesis in apoE deficient mice on high-fat diet, since these mice express >90% apoB48-containing lipoproteins[61]. In addition, it is possible that this binding site (in addition to apoE) is functional in binding of chylomicron remnants to artery wall proteoglycans.

Lipoproteins bound to the arterial wall are subjected to proinflammatory modifications

Once bound to proteoglycans in the arterial wall, the LDL particles are subject to a number of modifications. The LDL-modifications trigger an inflammatory response, including infiltration of macrophages that start to phagocytose modified LDL.

The modifications are caused by LDL binding itself to proteoglycans, which leads to conformational changes and LDL aggregation; and by enzymes and reactive oxygen species that are present in the vessel wall, which leads to chemical changes of the LDL surface and further LDL aggregation. Enzymes involved in this process includes secretory sphingomyelinase (S-

SMase) and non-pancreatic secretory group V phospholipase-A2 (PLA2-V)[62-64].

Continuous LDL retention causes a maladaptive inflammatory response

Continuous accumulation of LDL particles in the arterial wall may lead to an escalated inflammatory response that intensifies LDL retention due to the release of so called bridging molecules. Bridging molecules are primarily released by activated macrophages and causes a strong hydrophobic binding of LDL. Secreted bridging molecules bind to proteoglycan GAG chains via electrostatic interactions and bind to LDL particles via hydrophobic interactions. Hence, they serve as a binding bridge between proteoglycans and LDL-particles. At this stage of atherogenesis, macrophages turn the inflammatory reaction into a maladaptive inflammatory response where they do more harm than good. Two main types of bridging molecules are described to be active during this stage; lipoprotein lipase (LPL) and Apolipoprotein E (apoE).

LPL—is normally found in the lumen of capillaries, anchored via electrostatic interactions to proteoglycans (heparin sulfate) on endothelial cells. The normal function of LPL is to hydrolyze and transport triglycerides from lipoproteins in circulation to cells in tissues. However, LPL can also be secreted by macrophages and VSMC in the later stages of atherogenesis[65-69]. In the vessel wall LPL causes a strong hydrophobic LDL binding[70-74].

ApoE—is an apolipoprotein involved in transport and clearance of chylomicron remnants and VLDL lipoproteins via the LDL receptor and the LRP pathway[75-77]. It contains two negatively charged binding sites that interact with proteoglycan GAG chains[78,79]. Minimal amount of apoE is detected in normal intima, but large deposits has been detected in atherosclerotic intima where they also co-localized with the proteoglycan biglycan[80].

Vascular interventions induce accelerated atherosclerosis

Atherosclerosis can also develop following vascular interventions such as percutaneous coronary intervention (PCI) and bypass surgery with saphenous vein-grafts (SVGs)[81,82]. However, in contrast to native atherosclerosis, the atherosclerosis formed following vascular interventions develops at a significantly accelerated timescale. In PCI, the first atherosclerotic lesions are observed after one year and the overall prevalence in drug-eluting stents is 30%[81,83,84]. In SVG, the first atherosclerotic lesions is also observed one year following surgery, and half of all SVGs fail within 10 years as a result of atherosclerosis[85,86]. Interestingly, just like in native athero-prone arteries, the new atherosclerosis is preceded by the formation of a VSMC-rich intima, called intimal hyperplasia.

Intimal hyperplasia is formed as a result of a healing response to vascular injury[87,88]. Vascular injury activates VSMCs in the vessel wall, leading to a phenotypic switch of VSMCs towards a more synthetic phenotype[5]. The activation is caused by the mechanical injury itself, which damages the basal membrane that surround VSMCs, and by growth factors and cytokines released by recruited leukocytes and damaged VSMCs. VSMCs then start to migrate and proliferate in the intima, forming an intimal thickening consisting of accumulated VSMCs and ECM[89].

Loss of endothelial cells play a key role in the intimal growth following vascular interventions. Normally the endothelium have a vaso-protective function by secreting a number of growth-inhibitory and anti-inflammatory factors[5]. However, when the endothelium is damage this protection is lost. This is especially true in PCI were a complete loss of the endothelium is observed[81,83]. This further exacerbates the growth of the intimal hyperplasia as the exposed subendothelium will be covered by platelet components that releases growth factors promoting migration and proliferation of VSMC[90].

SVGs differs from PCI in that the intimal hyperplasia forms after endothelial regeneration[91]. In SVG, additional factors influ-

ence the growth of the intimal hyperplasia. One such factor is the transient ischemia the vein graft experiences during explantation. During transient ischemia, the endothelial cells reduces the production of anti-proliferative factors such as prostacyclin, adenosine and NO[92]. SVGs will also experience a sudden and pronounced increase in wall stress as the vein is implanted and exposed to the higher arterial pressures. This results in increased production and release of a number of growth factors such as PDGF, endothelium-1 and bFGF. Damaged endothelial cells and VSMCs releases significant amounts of bFGF, which is a potent mitogen[93]. Observations have also been made in human subjects where elevated serum levels of growth factors such as PDGF A, PDGF B and bFGF are measured following PCI[94].

To summarize, the intimal hyperplasia formed by vascular interventions may be a pro-atherogenic environment with VSMCs secreting large amount of ECM proteins, including hyperelongated proteoglycans (discussed below). In addition, intimal hyperplasia recruits leukocytes that may lower the threshold for initiation of atherogenesis by secreting bridging molecules[51,70]. Furthermore, intimal hyperplasia also provides a larger intima in which LDL can be trapped. Hence, multiple factors may contribute in creating an athero-prone environment during formation of intimal hyperplasia that leads to accelerated atherogenesis.

Hyperelongated proteoglycans displays increased LDL-binding

VSMCs can produce proteoglycans with hyperelongated GAG chains that have increased LDL binding[5]. The expression of core proteins and enzymes involved in the GAG chain biology are regulated by independent biochemical possesses[95]. Interestingly, growth factors that are released during the vascular remodeling stage have also been found to regulate expression of both core proteoglycans as well as enzymes involved in the GAG chain biology. For instance, Schönher et al. showed that

treatment of arterial VSMC with either PDGF or TGF-β1 had different effects on the core proteins biglycan and decorin; two proteoglycans implicated in native atherosclerosis[96,97]. Treatment with PDGF or TGF-β1 increased incorporation of [^{35}S] sulfate to the GAG chain of biglycan with 3.3 and 2.9 fold respectively. However, no increase of [^{35}S] sulfate incorporation was detected in decorin. TGF-β1 stimulation but not PDGF also resulted in increased biglycan mRNA expression. However, no increase in expression of decorin was detected following TGF-β1 or PDGF stimulation. The lab of Peter Little confirmed these results and also showed that PDGF stimulates versican synthesis[98-100]. Furthermore, proteoglycans isolated from PDGF inhibitor treated VSMC showed significantly reduced LDL retention[101-103].

GAG chain synthesis is regulated by a number of enzymes in Golgi

Stimulation of cells with growth factors modulate expression of enzymes controlling proteoglycan GAG chain synthesis. The length and sulfation of GAGs is regulated by enzymes in the Golgi apparatus, where they act on the proteoglycan core protein. For simplicity, we will only describe CS and HS GAG chain synthesis. The GAG chain synthesis is divided into three stages; initiation of the GAG chain, divergence to CS or HS GAG chain and finally elongation and sulfation.

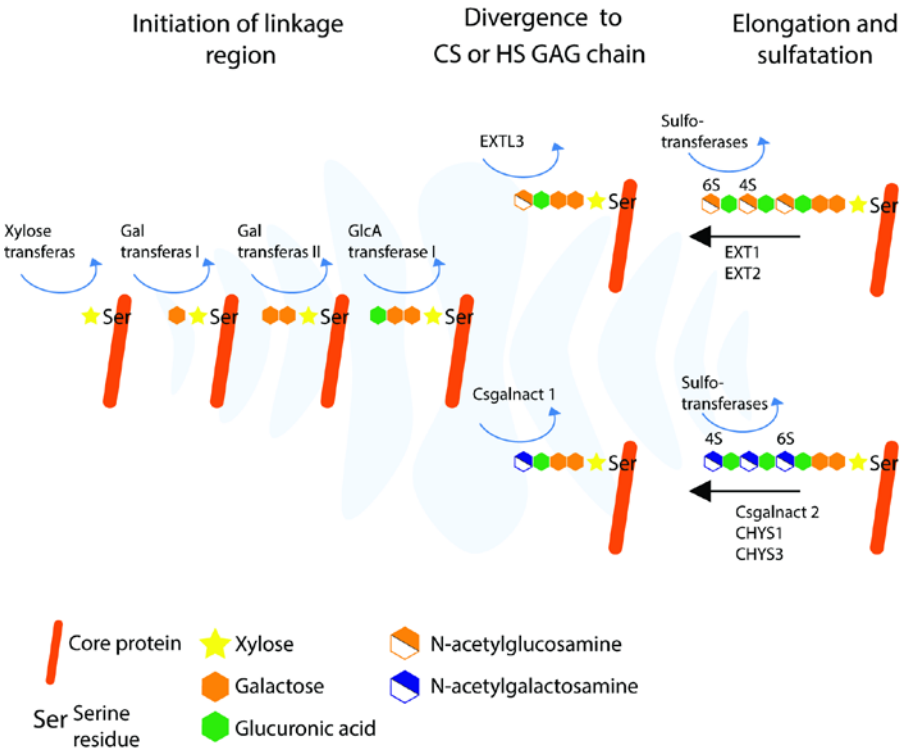

Figure 1. Overview of proteoglycan post-translation process resulting in chondroitin sulfate or heparan sulfate GAG chains

(1) Initiation: In the first stage, xylose transferase attaches xylose to the serine residue on the proteoglycan core protein. The

serine residues are located in a motif with the sequence Ser-Gly-x-Gly (x = any amino acid). Then Gal transferase I and II attaches two galactose monosaccharides to the xylose. GlcA transferase I attaches a glucuronic acid completing the tetrasaccharide linkage region[34,104,105].

(2) Divergence to chondroitin sulfate or heparan sulfate: In the second stage, enzymes will commit the tetrasaccharide linkage region to either heparan sulfate or chondroitin sulfate. EXTL3 catalyzes formation of heparan sulfate by transferring N-acetylglucosamine to the linkage region, while Csgalnact 1 catalyzes formation of chondroitin sulfate by transferring of N-acetylgalactosamine to the linkage region[34,104,105].

(3) Elongation and sulfation: In the third stage, enzymes specific for either heparan or chondroitin sulfate elongates and sulfates the GAG chains. It is during this step that sulfotransferase enzymes transfers sulfate groups to the growing GAG chain and results in a negatively charged molecule[34,37,106-109].

A summary of some key enzymes involved in the post-translational modification can be found in table 3.

Gene/name	Description	Function
Initiation and/or elongation of chondroitin sulfate or heparan sulfate GAG chains		
Csgalnact 1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	Initiates CS chain synthesis of the common linkage region
Csgalnact 2	Chondroitin sulfate N-acetylgalactosaminyltransferase 2	Elongates CS chains
CHSY1	Chondroitin sulfate synthase 1	Elongates CS chains
CHSY3	Chondroitin sulfate synthase 3	Elongates CS chains
EXTL3	Exostoses (multiple)-like 3	Initiates HS synthesis of the common linkage region
EXT1	Exostoses (multiple) 1	Elongates HS GAG chains
EXT2	Exostoses (multiple) 2	Elongates HS GAG chains
Sulfotransferases		
CHST11	Carbohydrate sulfotransferase 11	Transfer sulfate molecule to CS chains
CHST13	Carbohydrate (chondroitin 4) sulfotransferase 13	Transfer sulfate molecule to CS chains
CHST3	Carbohydrate (chondroitin 6/keratan) sulfotransferase 3	Transfer sulfate molecule to CS chains
CHST15	Carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	Transfer sulfate molecule to CS chains
HS6ST	Heparan sulfate 6-O-sulfotransferase 3	Transfer sulfate molecule to HS chains

Table 3. Overview of selected enzymes involved in the post-translational modification of GAG chains.

Cadmium exposure increases the risk for developing atherosclerosis

Several studies have identified cadmium exposure as a risk factor for native atherosclerosis[110-115]. It has also been shown that administrating cadmium to *ApoE*$^{-/-}$ mice via drinking water increased atherosclerosis[116]. Thus, cadmium is an environmental factor associated with increased risk for developing atherosclerosis. However, the underlying causes is not known.

Cadmium is released into the environment following burning of fossil fuels. However, the most relevant source of cadmium exposure in humans are through cigarette smoke and diet[112,116]. After inhalation or ingestion, cadmium is transferred to the serum (serum cadmium concentration have been estimated to be in the range of 0.2–20 nmol/l[117]). Cadmium is transported in the circulation in the form of free ions or bound to proteins such as albumin or metallothioneins. It is then absorbed by cells in target organs (liver, kidneys and testis) via soluble carriers, calcium and manganese channels and iron transporters[118-120]. Furthermore, the metal also seems to accumulate in the aortic vessel wall of smokers, with concentration up to 20 μmol/L[121].

The precis role of cadmium in atherosclerosis and how it modulates the risk is still not known. It is difficult to say if increase in cardiovascular disease in humans following cadmium exposure is a direct effect on the arterial wall or caused by systemic factors. Interestingly, studies have shown that cadmium stimulation of cultured cells increases production of high weight proteoglycans as well as production of proteoglycan core proteins biglycan and decorin[122,123]. Furthermore, it has been shown that cadmium is accumulated in the vessel wall and thus may wield pro-atherogenic effects directly on the vessel wall.

METHODOLOGICAL CONSIDERATIONS

Surgery–induced intimal hyperplasia

In project 1, we studied if intimal hyperplasia formed following vascular interventions is a factor that can accelerate atherogenesis. In order to use genetically modifies mice, we set up a mouse model of intimal hyperplasia. We chose to use a carotid angioplasty model that mimics balloon angioplasty, since it triggers intimal hyperplasia by a combination of endothelial and VSMC injury[125]. These are the most important factors for the formation of intimal hyperplasia following PCI and vein graft bypass surgery in humans[87,88].

During the surgical procedure, the endothelium is denuded by careful scraping of the inner vessel surface using a nylon wire. In the next step the medial VSMCs are subjected to a stretch injury by pressurizing the vessel for 20 seconds at 120 kPA. The pressure is about nine times the blood pressure normally found in mice and applied using an angioplasty pressure device filled with saline. To stimulate growth of intimal hyperplasia the blood flow is reduced by ligating three of the four branches at the carotid bifurcation, leaving the thyroid artery as outflow tract. This results in a 90% reduction of blood flow. Two weeks after surgery a VSMC-rich intima is formed in the distal half of the carotid artery.

The carotid angioplasty model is technically challenging due to the small size of the mouse carotid artery. For a skilled technician, the procedure can take anywhere between six months to a full year to learn. The inflatable device used is adapted for human use, where pressures range from 1500–3000 kPa. This means that it is difficult to deliver the exact pressure since the pressure gauge is not well adapted to display the smaller pressure used in the mouse carotid angioplasty. This is a critical step since the vessels of mice are thin (only 3–4 VSMCs thick)

and minor differences in delivered pressured can kill all VSMCs in the media or not cause any VSMC at all.

Compared to an angioplasty procedure done in humans, no stent is implanted and the pressure is applied using saline instead of an inflated balloon. In vein grafting, the vein is subjected to a raise in lumen pressure when transferred from the low pressure vein system to the high pressure arterial system. However, the vein graft is subjected to a constant increase in pressure and not a short burst of increased pressure. In any case, in our mouse model and in both human vascular interventions there is a clear VSMC injury.

A common alternative method to induce intimal hyperplasia in mice, is placing an over-sized perivascular collar around the carotid artery[126,127]. In this procedure, the cause of the neointimal formation is not known and does not inflict a clear injury to the media VSMCs or the endothelial cells, making it less than ideal to study intimal hyperplasia caused by vascular intervention. Furthermore, the resulting intimal hyperplasia keeps growing until occlusion and does not mature. A constrictive collar can also be used that causes turbulent blood flow. However the constrictive collar only results in minor formation of intimal hyperplasia and is used as a model to induce atherosclerosis rather than intimal hyperplasia.

Models of hypercholesterolemia in mice

We used different mouse models of hypercholesterolemia to achieve diverse levels of total cholesterol (2–20 mmol/L) when studying atherogenesis in intimal hyperplasia. We used wild-type mice (Jackson Laboratories, Bar Harbor, ME), homozygous *APOB100* transgenic mice (*APOB100$^{Tg/Tg}$*)[128] and LDL receptor-deficient mice (*Ldlr$^{-/-}$*, Jackson Laboratories)[129]. All strains were on a C57Bl/6 background.

APOB100$^{Tg/Tg}$ and *Ldlr$^{-/-}$* mice have elevated total cholesterol levels of ~5–6 mmol/L[128,129] on chow, while wild-type mice have ~2 mmol/L. Switching diet to western diet results in a to-

tal cholesterol level of ~10 mmol/L in *APOB100$^{Tg/Tg}$* mice and ~20 mmol/L in *Ldlr$^{-/-}$* mice. Most cholesterol found in both *APOB100$^{Tg/Tg}$* and *Ldlr$^{-/-}$* are carried by apoB lipoproteins, while wild-type mice carry most cholesterol by apoA lipoproteins (HDL).

APOB100$^{Tg/Tg}$ mice over-express the human form of apoB100 apolipoprotein, and thus have the site B site to mediate lipoprotein retention. *Ldlr$^{-/-}$* mice express mouse apoB100, which also contain Site B. However, most of the mouse ApoB100 mRNA are spliced in liver to apoB48 which lack Site B. Instead, Site B-1b becomes exposed to mediate lipoprotein retention[60]. Furthermore, since the *Ldlr$^{-/-}$* mouse is unable to clear LDL particles with the LDL-receptor, the particles will stay in circulation longer and accumulate apoE on the lipid surface[129]. This makes the LDL particles more atherogenic, because apoE contains a GAG-binding site very similar to Site B.

The formation of intimal hyperplasia following vascular injury involves recruitment of leukocytes and migration/proliferation of VSMCs. This active phase levels off over time and turns into a mature non-proliferating intimal thickening dominating by VSMCs. Consequently, the lipoprotein retention properties may change over time following vascular injury. Since *APOB100$^{Tg/Tg}$* and *Ldlr$^{-/-}$* both have constant elevated LDL levels, it is difficult to determine at what stage the retained LDL was accumulated in the vessel wall. To investigate whether a mature intimal hyperplasia also promotes lipoprotein retention, we used a new virus-based model to induce hypercholesterolemia that allows onset of hypercholesterolemia in adult wild-type mice. In this model hypercholesterolemia is induced by a single retro-orbital injection of adeno associated virus encoding gain-of-function proprotein convertase subtilisin/kexin type 9 (PCSK9)[130]. PCSK9 is a protein expressed in liver that binds to the LDL receptor and targets it for destruction[131]. Mice injected with PCSK9 virus display a lipid profile similar to *Ldlr$^{-/-}$* mice because of depletion of the LDL receptor. Using this method, we could induce hypercholesterolemia in wild-type mice once a mature intimal hyperplasia first had been formed. We also in-

jected labeled human LDL, into mice with mature intimal hyperplasia. However, *ex vivo* isolation and labeling of LDL may affect the properties of the LDL particles. Furthermore, injection of non-self LDL particles will trigger an inflammatory response.

Immunizing mice with GAG-binding idiotypic antibody

We immunized the mice with an idiotypic antibody called chP3R99. In this section, this antibody will be referred to as Ab1. The epitope–binding site of Ab1 contains an arginine motif at the heavy chain complementary determining region 3, with the amino acid sequence R98-X-X-R100a, where X can be any amino acid. This motif binds sulfated proteoglycan GAGs[132]. Subcutaneous injection of Ab1 induces an immune response against the GAG-chain binding site that causes production of anti-idiotypic antibody (Ab2). Ab2 has a complementary region that mirrors the GAG-chain binding site found on the Ab1. Ab2 will thus not bind to the GAG chains. However, the anti-idiotype Ab2 will also elicit production of anti-anti-idiotype antibody (Ab3) that will have the same region found on Ab1. Thus, Ab3 will have the ability to bind to GAG chain. This cascade will then continue repeat itself and produce Ab_n. chP3R99 have previously been successfully used to treat atherosclerosis in *ApoE*$^{-/-}$ mice[133]. A major advantage with initiating this cascade is that the effect is long lasting and thus may be a feasible treatment for humans. Indeed, anti-idiotypic antibodies is currently being tested as a cancer vaccine in humans[134].

Applying perivascular gel to administrate non-toxic levels of cadmium to the arterial wall

In paper 2, we investigated whether local cadmium exposure of athero-prone arteries resulted in increased accumulation of atherogenic LDL particles. This is of interest because several

epidemiological studies have shown that cadmium is a risk factor for ASCVD[110,113,116,121].

Cadmium was administered through a perivascular gel that was applied around the arterial wall to locally target the vessel. As discussed in the introduction, a VSMC-rich intimal thickening precedes lipid accumulation in human atherogenesis. As such, we used the carotid angioplasty to induce a VSMC-rich intima in mice. The gel was applied directly after the carotid angioplasty. The cadmium is released at a slow rate for as long as the gel is present around the vessel, which is around one week. However, the vessel wall is known to bind cadmium[121] and hence the cadmium–treated vessel will most likely contain elevated cadmium levels for many weeks.

Gene expression analysis using digital droplet PCR

We used droplet digital PCR (ddPCR, Bio-Rad) to analyze gene expression, instead of real time-PCR (RT-PCR) which is more commonly used. ddPCR offers significant advantages over RT-PCR including higher sensitivity and resolution[135-137]. However, ddPCR cost more per sample compared to qPCR when considering consumables and regents[136]. It also involves some more steps, which results in longer experiments as well as and more specialized equipment (droplet generator, thermal cycler and droplet reader). Despite the higher cost, there are significant advantages with using ddPCR over RT-PCR.

For instance, RT-PCR measures relative expression while ddPCR measures absolute gene expression. This results in significantly more accurate readings and less variations[137]. Consequently, significantly less tissue material is needed to analyze gene expression in ddPCR compared to RT-PCR. Furthermore, RT-PCR cannot detect gene expression differences between samples smaller than twofold. In contrast, ddPCR is able to detect differences of 30% or even less.

Immunohistochemistry

The central principle of immunohistochemistry is the detection of epitopes using antibodies in for instants tissue samples and cell cultures. The antibodies are raised in a number of species including, goat, rabbit, donkey, mouse, rat and guinea pig.

There are several ways to visualize antibodies. In this thesis, we mainly used antibodies conjugated to fluorochromes. Fluorochromes are small organic molecules that contain one or more aromatic ring. They consist typically of between 20-100 atoms and weight between 0.2 to 1 kDa. They absorb light energy (excitation light) and within nanoseconds emits light energy at a higher wavelength (emission light).

The filter cube in the microscope contains two light filters; one excitation filter and one emission filter. The excitation filter determines the wave length interval of the light that activates the fluorochrome. The emission filter determines at what wave length interval the emitted light from the fluorochrome shall be collected. The emitted light is then collected by a camera or the human eye.

A limiting factor in immunofluorescence microcopy is the number of fluorochrome channels (filter cubes) that can be combined. In this thesis, we designed a new setup of filter cubes that allowed us to simultaneously combine up to 6 channels. In practice, we stained for 5 protein markers and used one channel for cell nuclei staining.

Primary antibodies can emit a signal directly if they are directly conjugated with a fluorochrome. However, a secondary step is often used to amplify the signal. This is achieved by using a secondary antibody raised against the species of the primary antibody (and conjugated to a fluorochrome). If the primary antibody is biotinylated, a streptavidin conjugated to a fluorochrome can also be used. To study multiple markers in a sample, several antibodies from different species have to be used.

Sometimes good antibodies might be limited to certain species. Thus if two primary antibodies are made in the same species

and used in the same step, the secondary antibody will label both and it will not be possible the separate the signal from respective antibody. Then directly conjugated antibodies or biotinylated antibodies are needed to avoid antibody cross–binding.

AIM AND KEY RESULTS

Paper 1: Intimal hyperplasia induced by vascular intervention causes lipoprotein retention and accelerated atherosclerosis in mice

Aim

Accelerated atherosclerosis has been reported as a major cause of failure after vascular interventions such as angioplasty[81,83,84] and vein grafting[81]. In both cases, formation of intimal hyperplasia following the vascular intervention precedes the accelerated atherosclerosis[85,86]. The aim of this study was to test whether formation of intimal hyperplasia following vascular intervention promotes lipoprotein retention and atherosclerosis.

Key Findings

We surgically induced intimal hyperplasia in the distal half of the right carotid artery of mice using a procedure that mimics balloon angioplasty. The proximal half of the carotid artery was used as a control (**Figure 2A**). Three weeks following the carotid angioplasty a mature intimal hyperplasia was formed with no proliferation of VSMC (proliferation index: 0.00±0.02, n=6) and a recovered endothelium. Scattered macrophages were found in both the intimal hyperplasia and the injured underlying media. We first investigated whether vascular remodeling following carotid angioplasty increased lipoprotein retention in the arterial wall of $APO100^{Tg/Tg}$ mice, which have a mild hypercholesterolemia (total cholesterol levels: 5.0 ± 0.2 mmol/L). We observed significant retention of apoB-containing lipids within the intimal hyperplasia and the underlying media following carotid angioplasty compared to the proximal uninjured media in the same vessel (**Figure 2B left pictures and Figure 2C**). Furthermore, no apoB was detected in wild-type mice with intimal (**Figure 2B right pictures and Figure 2C**). Thus, carotid angioplasty induced apoB lipoprotein retention in the intimal hyperplasia and the underlying media already at moderately

elevated cholesterol levels. On a cellular level LDL retention was located in the ECM around SMCs in both the intimal hyperplasia and the media (**Figure 2D**). Leukocytes were found both in intimal hyperplasia and the media layer.

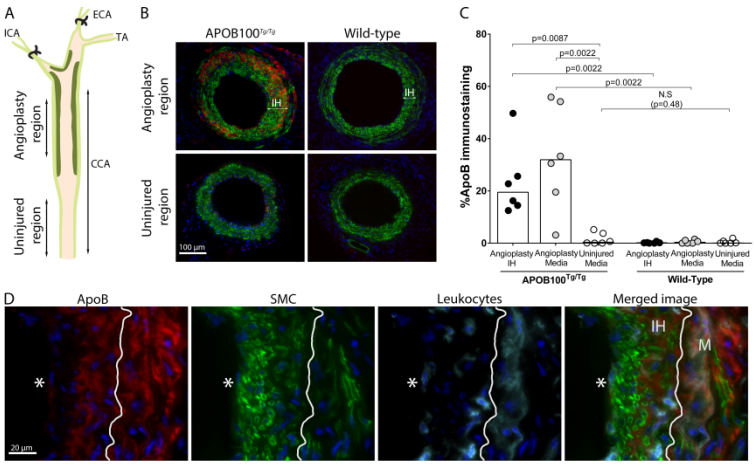

Figure 2. Vascular retention of apoB-containing lipoproteins following carotid angioplasty in mice with and without mild hypercholesterolemia. Carotid angioplasty was performed on *APOB100$^{Tg/Tg}$* mice and wild-type mice, both fed chow diet. Three weeks after surgery, carotid arteries were harvested, sectioned and multi-immunostained for apoB, VSMCs (α-actin), leukocytes (CD18) and cell nuclei (DAPI). (A) Schematic illustration of the common carotid artery (CCA) after carotid angioplasty, depicting the angioplasty region and the uninjured region. Dark green=intimal hyperplasia. ICA=internal carotid artery, ECA=external carotid artery, TA=thyroid artery. (B) Representative pictures of the angioplasty region (upper panels) and uninjured proximal region (lower panels) from an *APOB100$^{Tg/Tg}$* mouse (left panels) and a wild-type mouse (right panels). Red=apoB, green=SMCs, blue=nuclei, IH=intimal hyperplasia, n=6. (C) Quantification of apoB-positive area in intimal hyperplasia (IH) and media in the angioplasty region and the proximal uninjured region as indicated. Data was analyzed using Mann-Whitney rank sum test. $p<0.05$ is regarded significant, n=6 in each group. (D) Representative pictures of a carotid artery of an *APOB10$^{Tg/Tg}$* mouse, three weeks after surgery, multi-stained for apoB (red), SMCs (green), leukocytes (cyan) and nuclei (DAPI). IH=intimal hyperplasia, M=media, * =lumen, n=6.

To test whether there is a sustained lipoprotein retention when the active remodeling process is over, we induced hypercholesterolemia in wild-type mice three weeks after injury by a single injection of PCSK9 virus followed by a switch of diet to western diet[130] three weeks after surgery. Four days after virus injection (three days after western diet switch), pronounced apoB lipoprotein retention was detected in intimal hyperplasia and the underlying media of angioplasty treated carotids. Again, no apoB lipoproteins were detected in the proximal uninjured sec-

tions of the carotid artery (**Figure 3A and 3B**). Taken together, vascular intervention is a potent inducer of atherogenic lipoprotein retention, and the pronounced retention remains after the initial healing response.

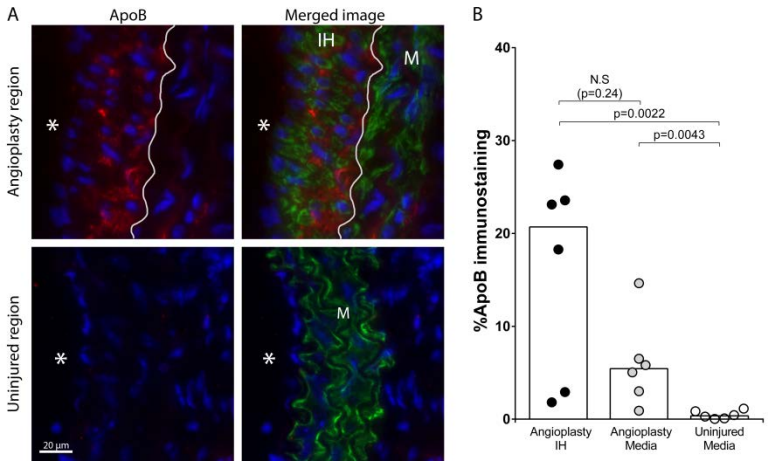

Figure 3. Retention of apoB lipoproteins in carotid arteries with mature intimal hyperplasia. Carotid angioplasty was performed in wild-type mice. Three weeks after surgery, hypercholesterolemia was induced by injection of PCSK9 virus and a switch of diet to western diet. Four days after injection (three days on western diet), the carotid arteries were harvested, sectioned and immunostained for apoB (red), SMCs (α-actin, green) and cell nuclei (DAPI, blue). (A) Tissue sections from the angioplasty region (upper panels) and uninjured proximal region (lower panels) from a representative carotid artery. IH=intimal hyperplasia, M=media, * =lumen, n=6. (B) Quantification of apoB-positive area in intimal hyperplasia (IH) and media of the angioplasty region and the proximal uninjured region as indicated. Data was analyzed using Mann-Whitney rank sum test. $p<0.05$ is regarded significant, n=6 in each group.

Using an *in vitro* LDL-binding assay, we found that LDL binding to the vessel wall could be blocked by a positively charged peptide corresponding to the main proteoglycan-binding sequence on the LDL particle (Site B), but not by a neutrally charged control peptide (Site B KE, **Figure 4A**). Furthermore, enzymatic digestion of GAG chains caused a drastic reduction in binding of LDL to the vessel wall in both the intimal hyperplasia and the media (**Figure 4B**). Furthermore, the lipoprotein retention in intimal hyperplasia was blocked by immunization with a GAG-binding antibody.

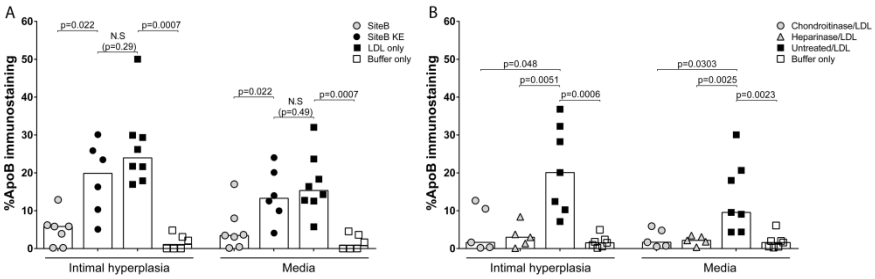

Figure 4. LDL binding to the vessel wall in vitro following treatment with Site B peptide or enzymatic digestion of proteoglycan GAG chains. Tissue sections of carotid arteries from wild-type mice with intimal hyperplasia were incubated with human LDL. Bound LDL was detected using anti-apoB antibody. (A) LDL binding to tissue sections pre-incubated with positively charged SiteB peptide (gray circles) or neutrally charged SiteB KE peptide (black circles). Black squares=no pre-treatment. White squares=no LDL incubation. (B) LDL binding to tissue sections pre-treated with the GAG-degrading enzymes chondroitinase (gray circles) or heparinase (gray triangles). Black squares=no pre-treatment. White squares=no LDL incubation. Data was analyzed using Mann-Whitney rank sum test. $p<0.05$ is regarded significant, n=6 in each group.

Finally, we investigated whether hypercholesterolemia would trigger formation of atherosclerotic lesions in *Ldlr*$^{-/-}$ mice. Three weeks after surgery, the diet was switched to western diet to induce a more extensive hypercholesterolemia. Already after five weeks on western diet, atherosclerotic lesions were formed in the intimal hyperplasia. The atherosclerotic lesions were complex with multi-layered capsule formation and large foam cells between the fibrous layers (n=4, **Figure 5, top middle panel**). Interestingly no atherosclerotic lesions were detected in the medial layer, despite significant LDL retention. Furthermore, no lesions were detected in the proximal uninjured regions of the same vessels subjected to low blood flow (n=4, **Figure 5, bottom middle panel**) or in mice that remained on chow diet (n=6, **Figure 5, right panels**).

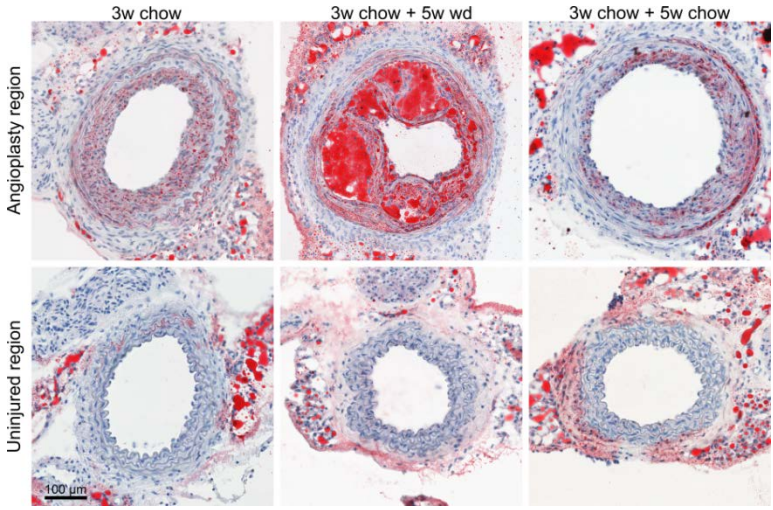

Figure 5 Intimal hyperplasia triggers rapid formation of complex atherosclerotic lesions in *Ldlr*$^{-/-}$ mice with hypercholesterolemia. Carotid angioplasty was performed on *Ldlr*$^{-/-}$ mice that were fed chow diet. Three weeks after surgery (3w chow, left panels, n=4), the diet was switched to western diet for 5 weeks (3w chow + 5w wd, middle panels, n=4) or remained on chow diet (3w chow + 5w chow, right panels, n=6). The vessels were sectioned and stained for lipids with Oil Red O (red). Nuclei are stained blue. Upper panels: representative sections from angioplasty regions, lower panels: representative sections from uninjured regions of the same arteries.

Conclusions

In this project, we provide evidence that formation of intimal hyperplasia following vascular intervention makes the vessel wall highly susceptible for retention of atherogenic lipoproteins, primarily through electrostatic binding to proteoglycan GAGs in the ECM. Furthermore, the lipoprotein retention in intimal hyperplasia can be targeted by immunization with idiotypic GAG-binding antibodies and by positively charged peptide. Such strategies that targets the vessel wall ECM may potentially be used to slow down the atherogenic response following PCI and bypass surgery.

Paper 2: Non-toxic concentrations of cadmium accelerate subendothelial retention of atherogenic lipoproteins in humanized atherosclerosis-susceptible mice

Aim

General exposure to cadmium through diet and cigarette smoke have been linked to ASCVD. However, the mechanism for how cadmium increases the risk for ASCVD is still unclear. In this project, we tested whether cadmium acts locally on the arterial wall to increase the LDL-proteoglycan binding and thereby promote the subendothelial retention.

Key Findings

Carotid angioplasty was performed to trigger formation of intimal hyperplasia, which turns athero-resistive arteries into athero-prone arteries. We applied a perivascular gel with and without cadmium around the injured carotid arteries directly after surgery. We then analyzed expression of selected genes. We observed significant increase in expression of proteoglycan core protein perlecan and in sulfotransferase carbohydrate (chondroitin 6/keratan) sulfotransferase 3 (CHST3) (**Table 4**).

Name/Gene	Description	Ratio target *vs.* housekeeping gene		
		Cadmium	*Control*	*P-value*
Proteoglycans				
Bgn	biglycan	11.2	9.9	0.52
Hspg2	perlecan (heparan sulfate proteoglycan 2)	1.3	0.71	**0.02**
Initiation and/or elongation of chondroitin sulfate or heparan sulfate GAG chains				
Csgalnact 1	chondroitin sulfate N-acetylgalactosaminyltransferase 1	0.49	0.72	0.27
Csgalnact 2	chondroitin sulfate N-acetylgalactosaminyltransferase 2	1.4	1.3	0.67
CHSY1	chondroitin sulfate synthase 1	3.9	3.7	0.76
CHSY3	chondroitin sulfate synthase 3	0.49	0.49	0.99
EXTL3	exostoses (multiple)-like 3	1.9	2.1	0.74
EXT1	exostoses (multiple) 1	4.1	4.3	0.65
EXT2	exostoses (multiple) 2	2.6	2.7	0.82
Sulfotransferases				
CHST11	carbohydrate sulfotransferase 11	0.44	0.40	0.56
CHST13	carbohydrate (chondroitin 4) sulfotransferase 13	0.0093	0.0090	0.91
CHST3	carbohydrate (chondroitin 6/keratan) sulfotransferase 3	0.41	0.30	**0.04**
CHST7	carbohydrate (N-acetylglucosamino) sulfotransferase 7	0.062	0.053	0.47
CHST15	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	0.64	0.59	0.73
HS6ST	heparan sulfate 6-O-sulfotransferase 3	0.59	0.56	0.79

Table 4. Effect of cadmium on the expression of genes encoding proteoglycan-related proteins in the carotid artery wall of wild-type mice with surgically induced intimal hyperplasia (n=5-12).

CHST3 transfers sulfate molecules from 3'-phosphoadenyl-5'-phosphosulfate to chondroitin sulfate proteoglycans. We therefor investigated if cadmium stimulation of cultured VSMC would increase sulfation of proteoglycans by analyzing the incorporation of [^{35}S]sulfate in proteoglycans isolated from human arterial smooth muscle cells cultured in the presence or absence of cadmium chloride (2 µmol/L). We observed 44% increase in sulfate incorporation to proteoglycans isolate from the cells culture with cadmium compared to cells cultured in the absence of cadmium (**Figure 6A**). We also observed a significant increase in LDL-binding to proteoglycans isolated from VSMC incubated with cadmium (**Figure 6B**).

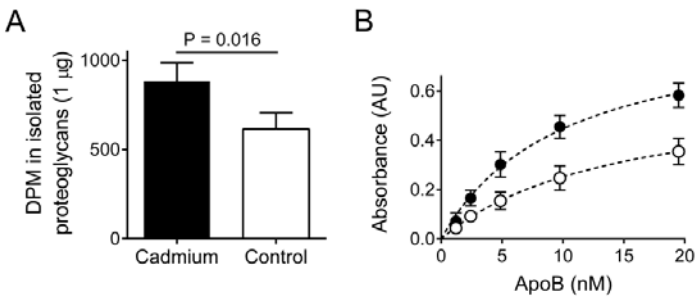

Figure 6 Cadmium treatment increases the sulfate content and human LDL binding affinity of proteoglycans isolated from human aortic smooth muscle cells. (**A**) Incorporation of [35S]sulfate in proteoglycans (1 µg) isolated from human aortic smooth muscle cells incubated in the presence or absence of 2 µM cadmium chloride for 48 h. B, Solid-phase assays of human LDL binding to proteoglycans isolated from human aortic smooth muscle cells incubated in the presence (●) or absence (○) of 2 µM cadmium chloride for 48 h. The results represent mean values (±SD) from 2 independent experiments (n=4 in each experiment) (A) or the mean of 2 independent experiments, each performed in triplicate (**B**). Kd 10.0 vs. 14.6 nM, p<0.001, for cells cultured in the presence or absence of cadmium, respectively.

Then we tested whether cadmium exposure would lead to increased subendothelial retention and accumulation of LDL *in vivo*. As above, we performed carotid angioplasty to trigger a intimal hyperplasia in *APOB100*$^{Tg/Tg}$ mice and applied a perivascular gel with and without cadmium. Two weeks after surgery, mice were sacrificed and lipoprotein content was analyses. We observed a significant increase in lipoprotein retention in the intimal hyperplasia of mice treated with cadmium compared control (**Figure 7**). Thus local cadmium exposure leads to significant increased accumulation of LDL within the ECM of intimal hyperplasia in mice with a human lipoprotein profile.

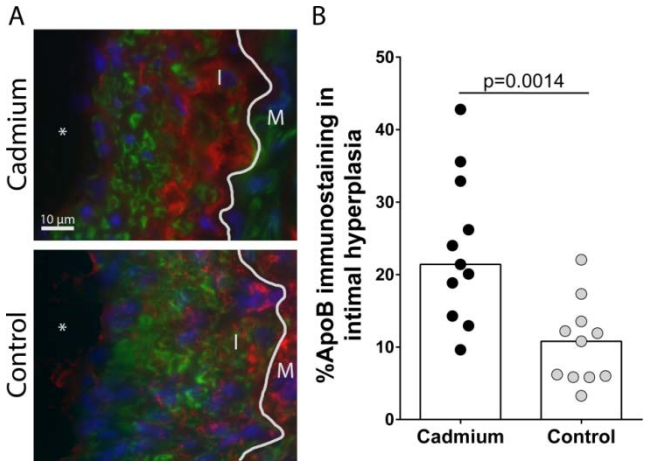

Figure 7. Local non-toxic cadmium exposure results in increased retention of atherogenic apoB-containing lipoproteins *in vivo*. (A) Representative carotid artery sections from humanized apoB100 transgenic mice 2 weeks after surgically induced intimal hyperplasia and exposure to cadmium chloride (80 ng in 20 µl gel). Sections were stained for apoB (red), vascular smooth muscle cells (green) and cell nuclei (blue). I, intimal layer; M, medial layer; white line, intima-media border. Scale bar = 10 µm. (**B**) Quantification of apoB-stained area in the intimal layer of each group (n = 11). Bars show medians. Data were analyzed using Mann-Whitney test.

Conclusions

Local cadmium stimulation of athero-prone carotid arteries leads to increased expression of perlecan and CHST3 and increased apoB lipoprotein retention.

Paper 3: Filter-Dense Multicolor Microscopy

Aim

To increase the number of fluorescence channels in multicolor microscopy by using the light spectrum in a more economical fashion.

Key Findings

We condensed the excitation and emission filter sets so that they were more specific for each fluorochrome and occupied a smaller portion of the available light spectrum. The filter sets were optimized for DAPI, 425 fluorochromes, 488 fluorochromes, Cy3 fluorochromes, 594 fluorochromes and PerCP.

The performance of the FDMM setup was verified by multi staining of cultured NIH cells, and mouse carotid endothelium. All channels used gave a specific signals with bleed through signals less than 40 times between channels (**Figure 8**).

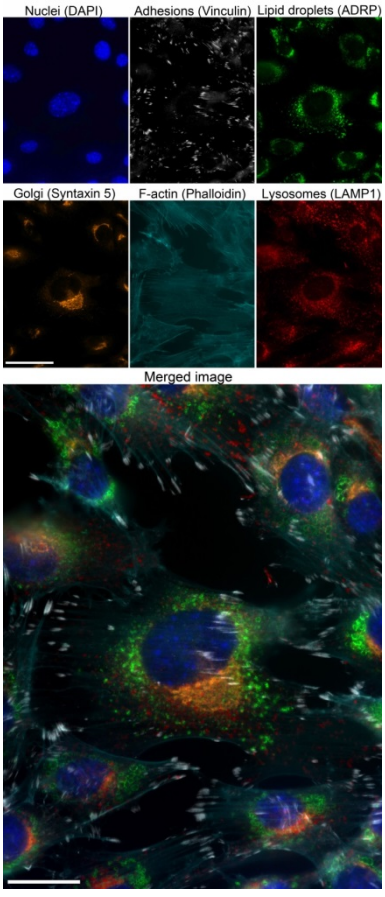

Fig 8. Six channel FDMM for subcellular staining of cultured NIH cells. Cultured NIH cells immunolabeled for different intracellular structures. Upper small pictures: Nuclei (DAPI channel, DAPI,12 ms), Focal adhesions (425 channel, anti-vinculin, 90 ms), Lipid droplets (488 channel, anti-ADRP, 50 ms), Golgi (Cy3 channel, anti-syntaxin 5, 10 ms), F-actin cytoskeleton (594 channel, phalloidin, 17 ms), and Lyzosomes (PerCP channel, anti-LAMP 1, 46 ms). Scale bar, 40 μm. Objective x63/1.4 Oil DIC. Lower large picture: Merged image of all channels. Scale bar, 20 μm

To demonstrate the advantage of more available fluorescence channels we designed an immunopanel that reveals the main cell types in mouse spleen. The immunopanel was applied on tissue sections of spleen from two different genetically modified mice, and the FDMM readily revealed morphological changes in each spleen, due proper to visualization of the relative location between several different cell types (**Figure 9**).

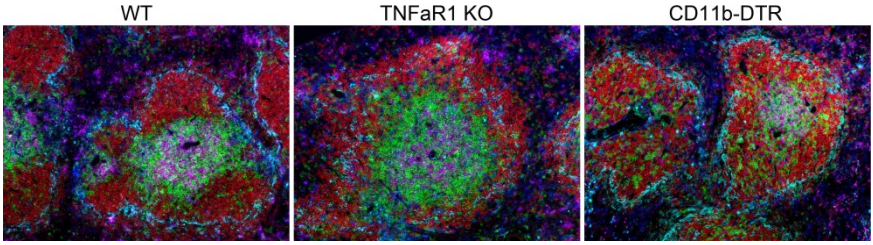

Fig 9. Multicolor antibody array for mouse spleen using FDMM. Tissue sections from a wild type spleen (WT), a TNFα-receptor 1 knockout spleen (TNFaR1 KO), and a spleen from a CD11b-DTR mouse (CD11b-DTR) were multi-immunolabeled with antibodies against marginal zone macrophages (anti-CD169, cyan), dendritic cells (anti-CD11c, blue), B cells (anti-B220, red), CD4+ T cells (anti-CD4, green), and CD8+ T cells (anti-CD8, magenta). Nuclei were stained with DAPI (not shown). Arrow in TNFα-receptor 1 knockout spleen indicates weak marginal zone structure (cyan). Arrowhead in CD11b-DTR spleen indicates CD4+ T cells (green) within the B cell follicles. All six channels are presented individually in S6 Fig. Scale bar, 100 μm.

We also designed an immunopanel to determine the phenotype of lipid-loaded leukocytes in a mouse atherosclerotic plaques, to demonstrate the need of more fluorescence channels for phenotyping of cell subsets.

Conclusions

FDMM setup proved to be easy to use and could easily be applied to a standard fluorescence microscope, and most importantly; gave a specific signals for each of the fluorochromes used.

DISCUSSION AND FUTURE PERSPECTIVE

Atherogenesis in intimal hyperplasia

In humans, vessels subjected to vascular interventions; such as PCI and saphenous vein grafts, become susceptible to atherosclerosis. Compared to native atherosclerosis, this intervention-associated atherosclerosis occurs at an accelerated timescale. The mechanism(s) behind the accelerated timescale is not known, and may be due to multiple factors. In this thesis, I investigated if formation of intimal hyperplasia following vascular intervention intensifies lipoprotein retention and atherogenesis, and if local cadmium exposure can further promote lipoprotein retention in intimal hyperplasia.

In this thesis, the *in vivo* results are based on an angioplasty model that induces vascular remodeling with formation of intimal hyperplasia in the mouse common carotid artery. Normally this artery is resistant to atherosclerosis during hypercholesterolemia. However, when increasing the cholesterol levels following the carotid angioplasty the vessel became susceptible for lipoprotein retention and rapidly formed atherosclerotic lesions at aggravated hypercholesterolemia. Thus, the surgical intervention turned a highly athero-resistant vessel into a highly athero–prone vessel. This shows that changes in the vessel wall structure can drastically influence its susceptibility for atherosclerosis.

The formation of intimal hyperplasia may promote atherogenesis in several ways. The accumulated VSMCs in intimal hyperplasia are of a more synthetic phenotype that the original medial VSMCs. Stimulation of synthetic VSMCs with growth factors stimulate secretion of hypersulfated GAGs with high LDL-binding capacity. Moreover, the formation of intimal hyperplasia involves recruitment of leukocytes, which also may contribute to atherogenesis, for example by stimulating inflammation

and by secreting LDL-bridging molecules that potentiate LDL-retention. In this thesis, I show that electrostatic interaction between LDL and proteoglycan GAG chains among VSMCs is important for pronounced lipoprotein retention in injury-induced intimal hyperplasia. However, it cannot be excluded that other factors, such as inflammation also affects the atherogenesis following vascular interventions.

Interestingly, native atherosclerosis also develops preferentially in vessel segments that first form a thickened VSMC-rich intima, DIT[138]. However, as earlier mentioned native atherosclerosis develops at a much slower rate. Thus, the pro-atherogenic properties of DIT and injury-induced intimal hyperplasia seem to differ, although they share morphological similarities. However, this is not surprising given that intimal hyperplasia following vascular injury is formed during a much more active tissue remodeling process, which for example may stimulate the release of more sulfated proteoglycans.

Complex atherosclerotic lesions in vessels with intimal hyperplasia

Following carotid angioplasty, *Ldlr*$^{-/-}$ mice with elevated cholesterol levels developed complex atherosclerotic lesions with foam cell clusters deep inside the intima surrounded by high content of VSMCs and ECM. Interestingly, these lesions shared morphological similarities with native atherosclerotic lesions found in humans[2,138]. In contrast, the more common mouse models of native atherosclerosis, *Ldlr*$^{-/-}$ mice and *ApoE*$^{-/-}$ mice fed a western diet for between 12 to 20 weeks, form a different type of lesions. These lesions mainly consist of superficial depositions of fat and foam cells. Though given enough time, *Ldlr*$^{-/-}$ mice will eventually develop a fibrous cap consisting of muscle cells. To humanize mouse models of atherosclerosis, the intima preferably should contain VSMC as this result in lesions akin to that found in human. Moreover, the response to retention hypothesis underscores the role of VSMCs for production of LDL-binding proteoglycan in early atherogenesis[51]. In mice, initial

foam cell formation occurs in an intima only consisting of endothelium. Consequently, the initial intimal LDL retention is driven by endothelial-derived proteoglycans and not by VSMC-derived proteoglycans.

Cadmium further accelerated lipid retention in intimal hyperplasia

Prospective studies have associated lifelong cadmium exposure to increased risk of atherosclerosis[110-115]. However, it is not known whether cadmium increase risk for atherosclerosis by systemic effects or if it act directly on the arterial wall.

We show that vessel wall cadmium exposure results in increased retention of lipoproteins in intimal hyperplasia. The cadmium exposure was limited to the mouse common carotid artery following carotid angioplasty. The increased lipid retention in intimal hyperplasia was possibly a result of increased expression of the proteoglycan perlecan, and CHST3—an enzyme transferring sulfate molecule to the 6–position on N-acetylgalactosamine on the GAG chain. Cultured VSMC stimulated with cadmium also produced GAG chains with increased LDL-binding affinity. Thus, we show that cadmium can act directly on the VSMC found in the intimal hyperplasia to increase the atherogenicity.

As discussed above, intimal hyperplasia formed following carotid angioplasty contain more synthetic VSMC. Cadmium might stimulate these VSMC to produce more sulfated proteoglycan GAGs. Indeed, increased expression of sulfotransferase CHST3 points at that direction. Thus, cadmium probably acts on the VSMC found in the growing intimal hyperplasia and increases LDL-binding affinity of secreted proteoglycans.

Most studies have focused on investigating the role of cadmium exposure in native atherosclerosis. Interestingly, smokers display increased risk of vein graft failure[139]. This indicates that cadmium can stimulate VSMC to increase risk of both native

atherosclerosis and vascular intervention induced atherosclerosis.

A question not studied in this thesis is by which signaling pathways cadmium causes increased expression of perlecan and CHST3. *In vitro* studies have shown that cadmium upregulates Hif-1A and VEGF. These two markers are associated with hypoxia. It is tempting to speculate that cadmium actually causes a simulated hypoxic state in the intimal hyperplasia. It would be interesting to investigate if the increased lipoprotein retention in intimal hyperplasia following cadmium exposure could be targeting by inhibiting Hif-1A and VEGF.

The antibody chP3R99 as a potential vaccine against accelerated atherosclerosis following vascular intervention

Immunization with a GAG–binding idiotypic antibody could potentially be used to treat accelerated atherosclerosis following vascular interventions. We found that intimal hyperplasia following carotid angioplasty promoted lipoprotein retention through a mechanism mediated by proteoglycan GAGs. This lipoprotein retention to intimal hyperplasia could be targeted by immunizing mice with an idiotypic antibody that blocks the electrostatic LDL–GAG interaction. An interesting question not pursued in this thesis is whether we could retard or even potentially stop accelerated atherosclerosis by vaccination. Each year more than 1.9 million patients undergo vascular interventions such as PCI and CABG in the OECD countries alone (stats.oecd.org). However, 30% of all PCI will develop atherosclerosis within the first year[81,83,84], and 50% of all SVGs will fail within ten years because of accelerated atherosclerosis[85,86]. If vaccination would turn out to be a viable therapy against accelerated atherosclerosis following vascular intervention, then the clinical ramifications could be significant. Both in terms of cost for the healthcare systems, but especially for the patients. An advantage with antibody immunization is that it targets the vessel wall, and thus can be added on top of lipid-lowering drugs, which targets the blood cholesterol levels.

Filter-dense multicolor microscopy

In this thesis, we used multicolor microscopy to investigate the spatial location of different protein markers in tissue sections of carotid arteries. This was our most important tool that we used to obtain critical data in both project I and II. For instants, we used an immunopanel consisting of antibodies to detect endothelial cells (CD31), atherogenic lipoproteins (apoB), macrophages (CD18), proliferation cells (Ki67), bridging molecules (LPL) and cell nuclei staining (DAPI) in our standard panel. However, standard fluorescence microscopes are limited to a maximum of four channels, and most microscopes are only equipped with three channels.

One could possibly think that 2 trippel–stainings are basically the same as 1 sextuple–staining. However, the biological information lies in the relative location of proteins/cells to each other, and consequently staining with all antibodies in one and the same tissue section is critical. In fact, you need to perform 20 trippel-stainings in order to get the biological information from 1 sextuple–staining. Thus, expanding the number of antibodies during multi-immunostaining drastically increases the information, saves tissue sections, and saves time and reagents.

This spurred us to develop filter-dense multicolor microcopy (FDMM). To increase the number of channels in FDMM, we condensed filter sets for a suitable panel of fluorochromes. This resulted in a more conservative use of the light spectrum that in turn led to more fluorescence channels.

Once the FDMM system was up and running, we aimed to maximize the information of every sample by filling up the channels with relevant markers for studying the arterial wall biology. These markers might not have been crucial for the initial analysis, but when new questions arose, we often already had that information in our original raw data. Thus, the method enabled us to gather information that was useful for more than one single question.

FDMM offers significant advantages in other studies outside the scope of this thesis as well. For instance when investigating how different cells interact in multicellular structures such as in germinal centers,[140] or when subtyping cells such as leukocyte[141,142]. Due to the rapid development in biomedical science, the number of cell markers is constantly increasing, which in turn likely will continue the need for even more fluorescence channels. Although six channels is a big step forward, I am convinced that there will a demand for 10-12 channels in a few years, and that microscopy will become an alternative to flow cytometry with the advantage of also showing tissue architecture.

There are two strategies to further increase the number of channels. First, expanding the available light spectrum to also include the infra-red spectra would add two extra channels. This can easily be done by adding infra-red LEDs. Second, significant advantages have been made on the software side where bleed–through signals can be calculated and removed in in a post-analytical step. This is the technique used in flow cytometry to reach above four channels. Combining condensed filter sets with current software that removes bleed–through signals would at least double the number of FDMM channels.

Together with more fluorescence channels in the microscope, it is also important to improve the immunostaining procedures. Today immunohistochemistry is based on primary and secondary antibodies, and to multistain the animal species of the primary antibodies must differ. However, this makes it very difficult to combine many antibodies. In order to take multicolor microscopy to a new level, new staining systems that allow any combination of antibodies need to be developed.

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