The role of phospholipases in atherosclerosis

Citation for published version (APA):

Ghesquiere, S. A. I. (2006). *The role of phospholipases in atherosclerosis*. [Doctoral Thesis, Maastricht University]. Universitaire Pers Maastricht. <https://doi.org/10.26481/dis.20060607sg>

Document status and date:

Published: 01/01/2006

DOI:

[10.26481/dis.20060607sg](https://doi.org/10.26481/dis.20060607sg)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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- The final published version features the final layout of the paper including the volume, issue and page numbers.

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The role of phospholipases in atherosclerosis

The studies presented in this thesis were performed at the Department of Molecular Genetics, Cardiovascular Research Institute Maastricht, Maastricht University (CARIM), The Netherlands

The role of phospholipases in atherosclerosis

Proefschrift

Ter verkrijging van de graad van doctor aan de
Universiteit Maastricht, op gezag van de Rector Magnificus,
Prof. Mr. G.P.M.F. Mols, volgens het besluit van het College
van Decanen, in het openbaar te verdedigen op
woensdag 7 juni 2006 om 14.00 uur

door

Stijn Albert Irena Ghesquiere

Geboren te Leiden op 29 september 1973

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The studies presented in this thesis were supported by the Dutch Organization for Scientific Research (NWO 902-26-194). Printing of this thesis was financially supported by the Dr. Ir. van de Laar Stichting, Heerlen and Hope Farms / abdieten, Woerden.

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-- J R R Tolkien

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Chapter 1

General introduction

History

Atherosclerosis is progressive disease, which is characterized by thickening and loss of elasticity of the arterial walls. The underlying initiating biological mechanism is the accumulation of low-density lipoprotein (LDL) in the arterial wall. This triggers an inflammatory reaction, followed by lesion formation and eventually a whole myriad of processes takes place, leading to the development of a complicated disease. While atherosclerosis is often considered a 'modern' disease as a result of our life style and feeding habits, a short introduction through the discovery of atherosclerosis teaches us that it is in fact not a 'new' disease.

Throughout a large part of history, atherosclerosis was not recognized as a life threatening disease. Pathological complications seldom develop before the fourth decade of life, an age seldom exceeded (1, 2), which made atherosclerosis a rather rare disease. However, a better control of infectious diseases over the last century resulted in increased life expectancy. In combination with changes in dietary habits, this resulted in an increased prevalence of cardiovascular failures. At a certain point, atherosclerosis became epidemic in western societies and developed into a major cause of death. However, atherosclerosis is not a recent phenomenon. Atherosclerotic lesions have been discovered in 3500-year-old Egyptian mummies (3). In the antiquity, Hippokrates (460-377 b.c.) mentions sudden death, probably caused by myocardial infarction (4). Erasistratos (~300 b.c.) described claudication intermittent, a condition originating in peripheral artery disease. Despite all this, understanding of the origin, the pathology and its importance in the circulatory system was very limited.

The centuries between the ancient Greeks and the late medieval times are marked by absence of new discoveries. No significant progression was made in the scientific understanding until the onset of the Renaissance. At that time, more attention

Fig. 1. Anatomy of the arterial wall

was given to the anatomy of the human body, and this quickly led to new insights. In 1575, Fallopius described the degeneration of vessels to bones in his '*Lectiones de partibus similaribus humani corporis*' (5). In fact he discovered arterial calcification, a feature of advanced atherosclerotic lesions. Understanding of the circulatory system in those days was rather limited. Most attention was given to the venous system, as the arterial system shrinks when blood pressure drops after death. This changed when William Harvey described the mechanism of blood circulation with arterial and venous vessels in 1628 (6). Interestingly, he mentions the presence of thrombi in the vessel walls of the arterial system, a feature of severe atherosclerosis.

In the 18th century, discoveries in the field of the vascular system gained pace. Morgagni (1761) observed calcifications in coronary arteries (7). In 1774, Albrecht van Haller found calcified lesions in an abdominal aorta (8). Towards the end of the century, chest pain was connected to coronary sclerosis by Parry (1799) (9) and Jenner (1799) (10), although the exact cause of pain wasn't discovered until 1880, when Potain explained angina pectoris as result of myocardial ischemia, due to reduction in blood flow to the heart muscles.

In the 19th century, focus shifted from anatomical observations to the etiology of atherosclerosis. Already in 1852, Virchow (11) postulated atherosclerosis as an inflammation of the vessel walls. The connection with food composition was made in the early decades of the 20th century. It was observed that rabbits fed on meat and eggs developed atherosclerosis (12, 13). Protein was considered the food component causing atherosclerosis. In 1913, Anitschkow established the connection with cholesterol (14). Moreover, he described cholesterol laden cells as main component of the lesions. He described what we now refer to as 'foam cells'.

For a large part during history, the formation of atherosclerosis was considered an inevitable result of wear and tear in the circulatory system. The 'tubes' were simply worn out when aging sets in. It was not until the last decades of the 20th century that arteries were not regarded as merely rigid, non-active structures, but as dynamic, living tissue. 'The response to injury' hypothesis, formulated by Ross and Glomset in 1976 (15, 16), marked the start of a new era in atherosclerosis research and understanding. Elevated levels of blood cholesterol had been associated with heart attacks (17) since the early seventies, but the mechanism that transported cholesterol into the vessel walls and its relation to plaque formation was not known. This changed once the function of low-density lipoprotein (LDL) in the lipid transport was discovered by Brown and Goldstein (18). LDL turned out to be responsible for cholesterol and cholesteryl ester transport in the body, and the link between LDL and atherosclerosis was quickly made. Although LDL is capable to accumulate in the vessel wall (19), it was known that the native LDL did not trigger macrophage foam cell formation and inflammation associated with atherosclerosis (20). LDL oxidation and enzymatic modifications render the particles pro-atherogenic (21, 22). Amongst the most potent enzymes capable to modify LDL are the phospholipases, which have been shown in atherosclerotic lesions and inflammatory diseases (23, 24).

Today, the development of atherosclerosis is considered a complex process, influenced by numerous factors and based on interaction between vessel wall and

lumen. Attention for atherosclerosis and its etiology increased, and this is reflected in the increasing number of publications. The annual number of articles published on atherosclerosis doubles every decade (25).

Anatomy of an artery

Basic knowledge of the structure and biology of the normal artery is essential for the understanding of the development and progression of atherosclerosis. Normal arteries have three distinct layers. At the inside there is the tunica intima. At birth, the tunica intima is a rather thin layer. At the lumen side it consists of an endothelial monolayer. Directly below is a collagen V and laminin rich layer, containing the internal elastic lamina. This lamina or layer consists of elastic fibers and functions as a barrier for cells and macromolecules between the intima and the underlying media (26).

The endothelium is in close contact with the blood and has many functions. It is a selective barrier between blood and tissues. The endothelial cells are closely interconnected with tight junctional complexes. At points in the circulation with irregular hemodynamics, like in artery branches and curvatures, the endothelial layer is less structured and more permeable. These sites are more prone to develop atherosclerosis (27). The intima is a biological active tissue that plays a role in inflammation, blood flow regulation, vascular modelling and thrombus formation. During adulthood, the intima becomes more complex in structure. Smooth muscle cells become part of the intima and different types of collagen (I and III) are

Lumen

Fig. 2. Onset and development of atherosclerosis
LDL accumulated in the vessel wall and becomes modified by reactive oxygen species (ROS), phospholipases and other processes. Monocytes enter the vessel wall and differentiate into macrophages. Continuous uptake of modified LDL leads to foam cell formation and inflammatory processes induce smooth muscle cell migration to form a stabilizing fibrous cap. Finally, cell death leads to the development of a necrotic core and progression into an advanced plaque.

deposited. This results in diffuse intimal thickening in many human arteries when aging. The tunica media lies in between the intima and the adventitia. This layer is rich in smooth muscle cells, embedded between elastin-rich extracellular matrix components. Arteries undergo large pressure changes during systole and diastole. The elasticity of the media is essential to buffer these pressure fluctuations. Arteries close to the heart have a thick, well-organized media, compared to the thinner media of less pressurized arteries. Below the tunica media, at the outside of the vessel lays the tunica adventitia. This layer with loosely arranged collagen fibrils, contains relatively few cells. However often ignored in studies, the adventitia is important as supportive tissue. In large arteries, the adventitia contains small arteries (vasa vasorum) that provide oxygen and nutrients for the artery.

Development of atherosclerosis

Accumulation of low-density lipoprotein (LDL) particles in the intima of the vessel wall is one of the earliest events marking the development of an atherosclerotic lesion (28). In the initial phase, LDL diffuses passively through the endothelial layer and enters the intima where it can accumulate (19). Once entrapped, various processes change the properties of the LDL particles. In the first place, LDL particles tend to attach to extracellular matrix components. This induces conformational changes, leading to aggregation and prolonged retention time (29, 30). Once bound to the extracellular matrix, LDL is susceptible to oxidation and enzymatic modifications (21, 30-32). Many of these modifications are pro-atherogenic (33). Initial LDL oxidation is promoted by reactive oxygen species released by nitric oxide metabolism (34). During the course of atherosclerosis, various enzymes produced by inflammatory cells induce further oxidation. Myeloperoxidase (35), 15-lipoxygenase (36) and NADPH oxidase (37) are the most important enzymes capable to induce LDL oxidation. Sphingomyelinase enzymatically modifies sphingomyelin, an abundant phospholipid in the membrane of LDL particles. The phosphorylcholine moieties are removed from the ceramide backbone. As result, the increased ceramide content induces LDL aggregation (38) and prolonging retention time in the vessel wall. Once entrapped, the LDL is continuously modified.

The secreted phospholipases A_2 (sPLA₂) are very potent modifiers of LDL particles in the vessel wall. sPLA₂ consist of a large group of related enzymes, from which several are present in atherosclerotic lesions. Phospholipases hydrolyse phospholipids and release free fatty acid (FFA) and lysophospholipids (lysoPL) (39). One of the released FFA is arachidonic acid (AA), providing a substrate for the production of a whole range of prostaglandins, leukotrienes and thromboxanes (40-42). Many of these molecules modulate inflammation and thus affect the course of atherosclerotic plaque formation. LysoPLs have various inflammation mediating functions that can affect lesion progression (43). Not only does phospholipase activity release inflammatory modulators, it also degrades and depletes the phospholipid content of LDL, thus inducing conformational changes, again promoting aggregation and enhancing retention in the vessel walls (28).

LDL modification and degradation in the vessel wall initiates an inflammatory response. Monocytes are amongst the first inflammatory cells attracted. Endothe-

Fig. 3. Phospholipase A_2 structure

The enzymatic active cavity of the phospholipase A_2 molecule is surrounded by an interfacial-binding surface (IBS). The IBS contains cationic, aromatic and aliphatic residues and determines the substrate preference. Differences between phospholipases mainly depend on the ability to bind to the substrate and the properties of the enzymatic cavity. In the active site, Ca^{++} , a water molecule and a His-Asp dyad participate in the actual enzymatic activity: the hydrolysis of the sn-2 ester of a phospholipid.

route using the scavenger receptors, SR-A and CD36, that has no feedback mechanism, resulting in continuous ingestion and transgression into lipid laden foam cells (20). With increasing numbers of monocytes entering and transforming into macrophages and foam cells, thickening of the vessel wall begins and a so-called 'fatty streak' is formed. These fatty streaks further evolve into complex atherosclerotic plaques. Under the influence of inflammatory mediators and oxidized LDL, the macrophages secrete chemoattractant molecules, like platelet-derived growth factor (PDGF) (47). This triggers smooth muscle cells to migrate from the tunica media into the intima (48, 49). The invading SMC's are non-contractile in contrast to normal vascular smooth muscle cells. In the intima, replication further contributes to a slow, but steady increase in smooth muscle cells content of the lesion (47, 50, 51). Smooth muscle cells contribute to the growth in plaque volume due to their potent production of extracellular matrix components (52). A considerable amount of advanced plaque volume consists of interstitial collagen (type I and III), proteoglycan (versican, decorin, biglycan) produced by these SMCs (53). In complex lesions, the smooth muscle cells release cytokines and bone morphogenic proteins, stimulating calcification of the plaque (54). This process of vascular calcification is similar to the formation of skeletal bones, albeit less organized (55). At a certain point during the growth of the atherosclerotic lesion, diffusion as

lial cells produce and present vascular cell adhesion molecule 1 (VCAM-1) and selectins in the lumen of the vessel (44). Monocytes adhere to these molecules and are slowed down in a rolling motion along the vessel wall. Once the monocytes are stopped, they must receive a signal in order to migrate through the endothelial layer. This essential signal is delivered in the form of chemoattractant cytokines or chemokines (45). Monocyte chemoattractant protein-1 (MCP-1) is one of the primary signaling molecules, produced in response to modified LDL (45). Once inside the intima, the recruited monocytes differentiate into macrophages and take up LDL particles (46). Native LDL is only slowly taken up by macrophages through the LDL receptor. Intracellular cholesterol content downregulates this expression, reducing uptake and thus allowing complete processing and removal of lipoproteins and cholesterol without becoming 'overloaded'. However, modified LDL follows another uptake

means of oxygen and nutrient transport is no longer sufficient and becomes a limiting factor in plaque growth. The formation of new blood vessels, or angiogenesis, takes place. Endothelial cells migrate into the lesion under influence of growth factors and develop small intra-plaque vasculature (54, 56, 57). These newly formed micro-vessels, however, are prone to rupture, often resulting in haemorrhage and thrombus formation inside the plaque.

Cell death is a serious complication in the progression of atherosclerosis. Cell migration and division is followed by death of increasing numbers of SMCs and macrophages (58). Although the exact mechanism is still controversial, programmed cell death or apoptosis is considered to be chiefly responsible. Apoptosis in the plaque is provoked by various inflammatory cytokines excreted by the macrophages and T-lymphocytes (59). Besides active cell death, the cytotoxic effect of oxidized LDL also contributes to death of macrophages inside the lesion (60). The remnants of the decaying smooth muscle cells and macrophages accumulate and develop into an unstructured necrotic core. This necrotic core is enriched with free cholesterol released from death foam cells (61, 62).

The stability of an atherosclerotic plaque is affected by the size and consistency of the lipid core, the thickness of the fibrous cap and the level of inflammation

Fig. 4. Structure of LDL

Low density lipoproteins (LDL) are cholesterol carriers of the lipid transport system in the human body. A single apoB-100 protein embedded in a lipid monolayer surrounds a core, filled with mainly cholesteryl esters and triglycerides. The surface monolayer consists of phospholipids and a few triglycerides and cholesterol esters. The structure and properties of LDL depends on both the conformation of the apoB protein and the lipid composition.

and repair in the plaque (63). A typical stable plaque consists of a small lipid core, covered by a thick collagen rich cap. Inflammation is limited and the endothelium at the luminal side is not disrupted. The fibrous cap is essential to protect the plaque against haemodynamic forces and reduces the risk on erosion and rupture. The development of a large necrotic core and increased free cholesterol enhances plaque fluidity (64). As result of this, biomechanical forces start to concentrate on the shoulder regions, common sites for rupture (65). The thickness and consistency of the fibrous cap determines the resistance to plaque rupture. In atherosclerosis, there is a balance in production and degradation of cap components. Thinning of the cap is caused by the activity of metalloproteinases MMP-1 and MMP-2 secreted by macrophages (66). Macrophages are in fact the primary cells responsible for breakdown of the structural important collagen components of the cap (67). In contrast, smooth muscle cells contribute to cap thickening as they produce extracellular matrix components (52). In lesions with relative limited quantities of SMC's, a thin cap develops, resulting in rupture prone lesions (68). If plaque stability is impaired, erosion and rupture can readily occur. Plaque erosion is a superficial process in which thrombus formation occurs due to damage of the lesion's surface. However, the molecular basis of plaque erosion is not well understood. Defects in the endothelial barrier due to apoptosis and matrix degradation, as well as formation of cholesterol crystals in the plaques (69) can induce cap erosion. Rupture of atherosclerotic plaques is better defined and occurs when a plaque is unable to cope with the haemodynamic forces (70). Rupture immediately results in thrombus formation. This causes either a local occlusion of the artery, or a downstream obstruction of the vessel if the thrombus detaches and is transported with the circulation. Plaque rupture is the principal cause of death in acute myocardial infarctions, accounting for a proximal 70% of fatal myocardial infarctions and sudden coronary deaths (71).

Development of atherosclerosis is not a constant process, but is characterized by a step-wise growth. Periods of exacerbation are followed by quiescent periods. Infections, plaque disruptions and intra-plaque haemorrhages enhance temporary eruption of lesion formation (72, 73). Symptoms of atherosclerosis rarely develop before the fourth decade of life. During early development of atherosclerotic lesions, the vessel functions are hardly impaired. In the first place, growth of the plaque is directed to the periphery of the artery. Secondly, remodelling of the artery ensures rapid accommodation. As a result of this adaptive mechanism a decrease in lumen size is prevented (47). However, when the capacity to compensate is exceeded, the lumen narrows and arterial stenosis develops (74, 75). In general, lumen reductions from 60 percent and more have the potency to become pathological. Even then, physical activity is often necessary to provoke symptoms. Typical examples are angina pectoris and claudicatio intermittens. In the first case, stenosis of the coronary arteries reduces the blood flow to the heart muscles, resulting in acute chest pain under physical exercise. In the latter case, stenosis in the arteries supplementing the leg muscles produces a similar ischemic condition during walking. These are relative benign symptoms of progressive atherosclerosis. Myocardial infarction, unstable angina and stroke are severe complications that often appear suddenly and are life-threatening.

Despite the complexity of atherosclerosis, continuous progress is made in unravelling its complex pathogenesis. Understanding the mechanisms behind the onset and development of atherosclerosis is essential for developing future therapeutic interventions.

Aim of this thesis.

The relation between LDL modification and atherosclerosis is complex, and its mechanism is poorly understood. The objective of this thesis is to elucidate the role of phospholipases in lipid modification and atherosclerosis. Phospholipases are a large group of enzymes, of which many have been reported in relation to inflammation and atherosclerosis. An extensive overview of the phospholipases and their functions is given in **chapter 2**.

For more than a decade, it is known that phospholipase IIA plays a role in inflammation (24). The enzyme was identified in human atherosclerotic lesion (23). The development of a transgenic mouse model (76), over-expressing human sPLA₂ IIA, delivered a new tool to study the role of sPLA₂ IIA in inflammation. This general over-expression mouse model expressed the enzyme indifferently in all organs. On a high fat diet, development of significant atherosclerotic lesions was observed (77). However, the lipid profile in this model is very pro-atherogenic, with decreased HDL and elevated LDL levels, and signs of general inflammation. To study the local role of sPLA₂ IIA in the development of atherosclerotic lesions, this model is unsuitable. We therefore needed a more refined model with specific expression at the lesion site. A new transgenic mouse model was made, with macrophage specific over-expression. Bone marrow transplantation to the LDL receptor knockout mouse model allowed us to examine the role of sPLA₂ IIA in lesion formation in detail. **Chapter 3** contains the results and discussion of our findings in our novel mouse model.

While group IIA has little impact on intact cell membranes and native LDL, group X is known as a very potent modifier of eukaryotic phospholipids (78). It has been discovered that sPLA₂ X modified LDL induces potent foam cell formation in macrophages (79). Combined with the reports of sPLA₂ X being present in atherosclerotic lesions of APOE knockout mice (79), sPLA₂ X became an interesting candidate for more detailed examination. An animal model over-expressing sPLA₂ X at the site of the atherosclerotic lesions was needed to reveal the role of group X in the inflammation and atherosclerosis. In a first step, we created a macrophage specific sPLA₂ X construct, similar to our approach for the sPLA₂ IIA mouse model. Murine macrophage cell lines were transfected and selected for stable expression of human sPLA₂ X. These cell lines allowed us to perform in vitro experiments related to foam cell formation and inflammatory regulation. In **chapter 4**, these experiments are described in detail. The same construct was used to make a transgenic mouse. Unfortunately, the viability of these mice was extremely low, proving it hard to establish sPLA₂ X mouse lines. Nevertheless, we carefully examined the mice we obtained and found very interesting initial clues about the role of sPLA₂ X in lung pathology (**chapter 5**).

As mentioned before, the phospholipases are a large and diverse group of enzymes. Recent discoveries show specific expression and biological functions for

the various phospholipases. Many of the phospholipases mediate inflammation and modulate lipid accumulation. However, little is known about their role and presence in the different stages of atherosclerosis. We therefore decided to look at the expression patterns of the most prominent phospholipases in human plaques. Early lesions were compared with advanced stable and unstable plaques. To verify our results and to examine macrophage specific expression we performed in-vitro experiments with human macrophages under inflammatory conditions. At the same time we also looked at mice lesions and collected data from in vitro experiments with murine macrophages. We believe that combining these data can contribute to a better understanding of the role of phospholipases in atherosclerosis and inflammation. **Chapter 6** describes the outlines, results and discussion of this study.

Finally, in **Chapter 7** the results of our experiments and future perspectives are discussed.

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Chapter 2

The role of phospholipases A₂ in lipid modification and atherosclerosis

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Cardiovascular Toxicology. 2005;5(2):161-82.

Abstract

Phospholipases have received wide attention as it has become clear that several isoforms of the phospholipase family play a role in onset and progression of atherosclerosis. The release of free fatty acids (FFA) and lysophospholipids (lysoPL) provide metabolites for various inflammatory pathways, and this has been considered the main mechanism of phospholipase-driven inflammation. However, generation of FFA and lysoPL are only part of the story. The induction of low-density phospholipoprotein (LDL) aggregation and accumulation, receptor binding, co-regulation with cyclooxygenase (COX) and lipoxygenase (LO) pathways, internalization through heparan sulfate proteoglycan (HSPG) shuttling, and crosstalk between phospholipases all play a role in atherosclerosis.

Group IIA phospholipase has long considered a key enzyme in the initiation of various inflammatory diseases, but new data also indicates a role in the subsequent resolution of inflammatory processes. Recently, secreted group V and group X phospholipase and platelet activating factor acetylhydrolase (PAF-AH) are also recognized as important enzymes in atherosclerosis, modifying LDL and leading to lipid accumulation.

The phospholipases and their function in atherosclerosis are not fully understood. Future investigations can deliver better insight in the complex role of these enzymes. The present review summarizes the current state of phospholipase research related to atherosclerosis.

Introduction

Atherosclerosis is a progressive disease of the large arteries. Accumulation of low-density lipoprotein (LDL) and inflammation in the intima of vessel walls is a crucial event in the early onset and the progression of plaques (1). It is widely accepted that LDL needs to be either enzymatically modified or oxidized to become atherogenic (2). Modified LDL attracts monocytes, which differentiate into macrophages and transform into foam cells after uptake of the LDL. In addition, inflammatory mediators are released from the modified lipids, accelerating the inflammatory response (3).

Several enzymes modify LDL to such an extent that they become pro-atherogenic. In particular, phospholipases (PLA₂) have received wide attention. The catalytic activity of phospholipases -hydrolysis of phospholipids- results in the formation of free fatty acids (AA) and lysophospholipids (lysoPL) (4). AA provides a substrate for the production of pro-inflammatory mediator products such as prostaglandins, thromboxanes and leukotrienes (5-8). Lysophospholipids also have various biological functions that play a role in atherosclerosis. Both the release of inflammatory metabolites and the modification of lipoproteins create pro-atherogenic conditions. LDL aggregates, fuses and accumulates in the vessel walls due to phospholipase activity. Accumulation and retention in the vessel wall is a strong pro-atherogenic condition (1). Besides their enzymatic properties, phospholipases A₂ act through phospholipase A₂ receptor (PLA₂R) binding (9). This activates the mitogen activated protein kinase (MAPK) pathway (10) and released chemokines (11). The receptor pathway also activates intracellular phospholipase, resulting in

the production of pro-inflammatory and vaso-regulating mediators (12).

Overview of phospholipases

Phospholipases A₂ are a diverse group of enzymes capable of hydrolyzing ester bonds of glycerophospholipids. The members of the A₂ subgroup hydrolyze glycerophospholipids at the sn-2 position. Classification is based on the structure, molecular weight, and the substrate preference (13, 14). Three main groups can be distinguished: the secreted PLA₂s (sPLA₂), the cytosolic PLA₂s (cPLA₂) and the CA²⁺ independent PLA₂s (iPLA₂), sometimes regarded as a subgroup of cPLA₂. The enzymatically active site of sPLA₂ is surrounded by the interfacial-binding surface (IBS) (15, 16). The IBS contains cationic, aromatic and aliphatic residues, determining the substrate preference (17-21). Binding to zwitterionic (e.g. phosphatylcholine) and anionic membranes (e.g. phosphatidylglycol) depends on the presence of aromatic residues, such as tryptophan (17, 21-23). In the active domain, CA²⁺, a water molecule and a His-Asp dyad participate in the hydrolysis of the sn-2 ester of phospholipids (15, 16).

sPLA₂ group IIA

sPLA₂ group IIA is present in spleen, thymus, intestines, tonsils, bone marrow, liver (24-27), prostate (28), cartilage (29) and placenta (30, 31). On cellular level, IIA can be found in smooth muscle cells (32), endothelial cells (33), neutrophils (34, 35), mast cells (36, 37), hepatic cells (38) and macrophages (39-43). It should be noted, however, that group IIA antibodies used in some experiments cross react with group V, which might invalidate some earlier findings (44, 45). Elevated levels of group IIA in plasma and serum are found in sepsis (46), chronic rheumatic arthritis (47), and coronary heart diseases (48). In arteries, group IIA is associated with smooth muscle cells in the intima. Group IIA is present in atherosclerotic lesions, where it is located in macrophage rich areas, lipid atheromas, and damaged intima, associated with collagen fibers (41-43). The enzyme binds extracellular matrix components such as glypican (49), decorin (49) and biglycan (50), allowing accumulation in the vessel wall (51) and exerting enzymatic activity on LDL particles. Clinical studies indicated that elevated group IIA concentration in the plasma is related to increased risk for coronary heart diseases (52). Various inflammatory pathways (38, 53, 54) regulate group IIA. Secretion is induced by interleukin-1 β (IL-1 β) and tumor necrosis factor (TNF) (53, 55). Studies with transgenic mice indicate a minor importance for IIA in AA and eicosanoid formation (56-58). Group IIA activity on mammalian cell membranes is low due to inability to bind phosphatidylcholine (57, 59, 60). The enzyme plays a role in bacterial host defense (61) and functions as the primary anti-bacterial defense in eye fluid (62). The mice strain C57BL/6, 129/sv and B10.RIII are natural "knockouts" for group IIA due to a frameshift mutation (63). However, no obvious phenotype is present in these mice strains, except for the finding that group IIA is involved in prevention of intestinal neoplasm formation (64), although there is no firm evidence for this (65, 66).

To elucidate the role of phospholipase group IIA in inflammation, mouse models

have been created. The sPLA₂ IIA over-expressing transgenic mouse model (56) uses the human sPLA₂ IIA gene with its natural promoter. Interestingly, these mice already developed atherosclerotic lesions on a normal chow diet (67). However, LDL levels are increased, while HDL levels are lowered, resulting in a concomitant reduction in paraoxonase activity. The systemic changes observed in this model increase atherosclerosis susceptibility. Recently, Webb et al. (68) avoided the systemic effects by transplanting transgenic bone marrow to lethally irradiated LDL-receptor knockout mice (LDLR^{-/-}). On a mild fatty diet, the mice showed a 73% increase in lesion size at the aortic root area. We performed similar experiments with macrophage specific transgenic group IIA mice and found a 2.5 fold increase in atherosclerotic lesion size. Interestingly the collagen content in the lesions is strongly increased (69).

Group IIA can be internalized through the heparan sulfate proteoglycan (HSPG) shuttling pathway (Fig. 1A), and induce the production of intracellular FFAs, which serve as precursor for various metabolic pathways (49, 57, 70-72). Redundancy in phospholipases exists as group IIA sPLA₂ functions can be taken over by group V (73).

sPLA₂ group V

The human group V is related to group IIA and is thought to share the same origin (74). The IBS binds efficiently to zwitterionic PC membranes (75). Group V releases AA and lysophosphatidylcholine from intact cell membranes (Fig. 1B) (57, 71, 76). The cationic charge facilitates cell membrane proteoglycans binding (Fig. 1C)(70). Group V hydrolysis of LDL is 30 times higher compared to group IIA (77). Group V exhibit antibacterial effects, albeit much smaller than those of sPLA₂ IIA (78). The enzyme is capable to follow the HSPG shuttling pathway (49, 57, 72).

Phospholipase V is highly expressed in the heart, the placenta and in lesser quantities in lung and liver (79, 80). Expression is regulated by pro-inflammatory stimuli such as lipopolysaccharide (LPS) (73). Group V is present in human and mouse atherosclerotic lesions (81) and is a potent modifier of LDL phospholipids. LDL particles decrease in size and their phospholipid content is reduced, rendering them susceptible to aggregation (81). In contrast with group IIA, no binding to the proteoglycans in the extracellular matrix has been described. Mouse peritoneal macrophages incubated with group V modified LDL show enhanced LDL uptake and develop into foam cells (81). The presence in atherosclerotic lesion and the increased foam cell formation, in combination with LDL aggregation and the resulting accumulation in the vessel wall, makes it interesting to study the role of group V in atherogenesis using an in vivo model.

sPLA₂ group VII (PAF-AH)

Platelet activating factor (PAF) is a potent lipid mediator promoting atherosclerosis through induction of superoxide anions in macrophages (82, 83). The PAF-hydrolyzing enzyme PAF-acetylhydrolase (PAF-AH, also known as lipoprotein-associated phospholipase A₂) is present in human plasma (84). PAF-AH is classified as group VII phospholipase. The primary sources of PAF-AH are hematopoietic cells (85). Bone marrow transplantation in a Japanese patient who received a trans-

plant from a donor lacking PAF-AH activity, resulted in complete abolition of the PAF-AH activity (86), supporting the role of the hematopoietic system. PAF-AH is expressed in differentiated macrophages, but not in monocytes (87). Despite its phospholipase activity, PAF-AH structure differs substantially from most phospholipases (88). PAF-AH is calcium independent, with preference for short acyl groups ($C < 6$) at the sn-2 position (89, 90). In this respect, it differs from other phospholipases, which do not show acyl length specificity. This prevents hydrolysis of lipoproteins and cell membranes (91). Three isoforms are known, two of which are present only in tissues and one of which is produced by macrophages and is present in plasma (92).

Oxidation of phospholipids results in shortened acyl groups. This makes them susceptible to PAF-AH hydrolysis (93-95). Therefore, PAF-AH may play a role in anti-oxidative scavenging (96). Besides phospholipase activity, PAF-AH shows trans-acetylase activity, transferring the acetate group from PAF to lyso-PC (97, 98).

PAF-AH in human plasma is associated with LDL and to a lesser extent with HDL, with the latter containing between 11 to 30% of the total amount (89, 99, 100). Within the LDL population, PAF-AH mainly associates with small, dense LDL particles (99). In mice, PAF-AH is mainly associated with HDL (101). It is important to realize that not all PAF-AH activity found in HDL is necessarily produced by PAF-AH. Recent publications indicate that other enzymes, such as paraoxonase 1 (PON1) (102) and lecithin-cholesterol acyltransferase (LCAT) (97, 103-106) exhibit PAF-AH-like activity.

The role of PAF-AH in atherosclerosis is ambiguous, since both pro- and anti-atherogenic properties have been reported. PAF-AH is capable of removing PAF-like oxidized phospholipids from the surface of oxidized LDL particles, resulting in a reduction of the pro-atherogenic properties (107). ApoE-deficient mice overexpressing human plasma PAF-AH develop less atherosclerosis due to reduced lipid accumulation, inhibition of neo-intima formation, and reduced macrophage attraction (108). However, the metabolites produced by PAF-AH activity are pro-inflammatory lysoPC and oxidized FFA (93-95, 109, 110). Moreover, increased levels of PAF-AH were shown to be a strong risk factor for coronary artery disease, although it is unclear whether this is cause or consequence (111). While the majority of the publications favor a protective role of PAF-AH in atherosclerosis (112), the production of pro-inflammatory molecules may have adverse long-term effects.

sPLA₂ group X

Structurally, group X contains sequences similar to both group IB and group IIA (113). Enzymatic activity is only present after proteolytic removal its propeptide (114, 115). Phospholipase group X is very active on both anionic and zwitterionic phospholipids and hydrolyzes phosphatidylcholine (59). Its catalytic efficiency resembles cobra venom sPLA₂ (116, 117). The hydrophobic nature of sPLA₂ X enhances its ability to reach phospholipids in cell membranes (75). Although both group X and group V are capable to bind intact cell membrane surfaces, group X is far more potent (118).

sPLA₂ X modified LDL induces formation of small intracellular lipid droplets in

peritoneal macrophages, leading to the formation of foam cells. The morphology of the lipid droplets differs from that of those seen in oxidized LDL loading of macrophages, and resembles that of acetylated LDL (119). The lipid content and the overall charge of the LDL particles is changed, but no fragmentation or aggregation of the apoB-100 molecules occurs (119). The uptake increase of the modified LDL is not induced by changes in LDL charge, but more likely due to conformational changes in the apoB-100 protein. In this manner, group X resembles group V LDL modifications. Aggregation of group X modified LDL has not been described, but can be expected.

Group X is expressed in spleen, thymus, and peripheral blood leukocytes, and in low quantities in lung, pancreas, and colon cells (113). In mice immunohistochemistry confirmed its presence in splenic macrophages (115). Atherosclerotic lesions of high fat-fed apolipoprotein E knockout mice show increased presence of group X in foam cells and vascular smooth muscle cells (119). Although the exact role of phospholipase group X in atherosclerosis remains to be elucidated, the properties described here strongly suggest a proatherogenic role for group X phospholipase.

Phospholipase A₂ interactions

Phospholipases induce intracellular release of AA through the heparan sulfate proteoglycan (HSPG) shuttling pathway (Fig. 1A). This involves binding of phospholipase to HSPG at the membrane surface near caveolae and subsequent internalization. Phospholipases capable of following the HSPG route are IIA and its close relatives IID and IIE, as well group V. HSPG molecules accumulate in caveolae of activated cells and play a role in transporting phospholipases into the cell and to subcellular compartments (49, 57, 70-72). Intracellular distribution allows phospholipases to contact specific substrates and release local FFA and lysoPL, precursors for the eicosanoid pathway (120). Not only does the HSPG pathway deliver intracellular lipid metabolites, it also inactivates sPLA₂ by degradation in the lysosomes (121). Phospholipase group V and X can act directly on intact cell membranes and release AA and lysoPL, bypassing the HSPG pathway (21, 71, 75, 77, 114, 115, 121-123). The neutral charge of group X prevents HSPG binding (71). Phospholipases can also bind to the phospholipase A₂ receptor (PLA₂R) (Fig. 1D) (9). PLA₂R is expressed in alveolar type II epithelial cells and in a subset of splenic lymphocytes. It is absent in peritoneal macrophages, although these express the closely related mannose receptor (10). In mice, group IB is a strong ligand for the PLA₂ receptor (124, 125) in contrast to group IIA (125). Binding to PLA₂R elicits lipid mediator production, cell proliferation (9, 126), activation of the mitogen-activated protein kinases (MAPK) pathway (10), and chemokine production (11). However, in mouse peritoneal macrophages, group IB induces hardly any FFA release (122). PLA₂R knockout mice are highly resistant to endotoxic shock. Survival rate after sublethal dose of LPS in combination with lethal sPLA₂ IB injection is prolonged. The decreased TNF and IL-1 β plasma levels in PLA₂R knockout mice indicate a potential role for PLA₂R in the onset and progression of endotoxic shock (127).

Group X has high affinity for the PLA₂R in mice, but requires its prepropeptide

Fig. 1. A: The heparan sulfate proteoglycan (HSPG) shuttling pathway. Phospholipase group IIA, IID, IIE, and V bind glypican molecules on the cell membranes, near caveolae of activated cells. Internalization occurs and transports the phospholipases to intracellular compartments. B: Direct enzymatic activity on intact mammalian cell membranes. Phospholipase groups IIF, III, V, and X are capable of binding and hydrolyzing intact membranes. This results in the release of FFA and lysoPL from the membrane. C: Heparan sulfate proteoglycan (HSPG) phospholipase accumulation. Binding of group IIA and V to heparan sulfate proteoglycan-rich areas on cell membranes allows local accumulation and facilitates enzymatic activity, resulting the release of FFA and lysoPL. D: Phospholipase receptor pathway. Binding of phospholipases to the phospholipase receptor elicits signaling and MAPK pathway activation. Internalization followed by phospholipase degradation aids in phospholipase clearance. E: Cytosolic and secreted phospholipase cross talk. Phospholipase receptor binding and metabolites from phospholipase activate the MAPK pathway. Subsequently, cytosolic group IV α is activated by phosphorylation and binds to the perinuclear membranes, where it releases AA and lysoPL. Calcium presence is essential for the cPLA₂ membrane binding.

to be removed. The same applies to group IB, which binds only in its active state (128). Clearance of group X in mice is provided through PLA₂R binding, internalization and degradation (129). In contrast to rodents, human PLA₂R ligands are not well defined. Group IB binds weakly to the human PLA₂R (10) and group IIA does not bind at all. It is not clear if human sPLA₂ X is a ligand for the human PLA₂R.

Interestingly, snakes have soluble PLA₂ receptors circulating in their blood as protection for leakage of venom phospholipases into circulation (130). A similar soluble receptor has been found in mice (131), binding and inhibiting sPLA₂ IB and X. There are no confirmations of the existence of soluble PLA₂R in humans (131), although a potential alternative splicing variant of PLA₂R in kidneys suggest this possibility (132).

For years it has been clear that there is a cross talk between secreted and cytosolic phospholipases (Fig. 1E). In macrophages, functional cPLA₂ α strongly enhances the enzymatic effects of group IIA (133, 134). Translocation of cPLA₂ α to perinuclear membranes provides coupling to 5-LO and COX pathways (135, 136). To fully exert its enzymatic activity, cPLA₂ α must be activated through phosphorylation by MAPK pathways (137, 138). Binding to the PLA₂R and subsequent activation of MAPK pathways provide a mechanism explaining the coupling between group IIA and cPLA₂ α (12). Indirectly, the production pro-inflammatory lipid metabolites descending from sPLA₂s AA release (12) can provide a coupling between sPLA₂s and cPLA₂ α . In neutrophils, leukotriene B₄ (LTB₄)-initiated cPLA₂ α activation and subsequent amplification of LTB₄ production has been described (139). Mice deficient for with cPLA₂ α show a reduced anaphylactic response and exhibit increased resistance to ischemic brain injury (140). Intestinal epithelium of cPLA₂ α knockout mice shows small ulcerations, indicating reduced production of protective PGE₂ (141). Activated macrophages and mast cells in cPLA₂ knockout mice produce only small amounts PGs and LTs upon activation (140), emphasizing the importance of cPLA₂ α in the PG and LT production. Phenotype of the knockout mice is similar to the phenotype of mice lacking a functional COX and 5-LO gene. However, prostaglandins, prostacyclins, and leukotrienes are still produced in cPLA₂ α knockout mice, indicating that other phospholipases A₂ play a role in the production of the precursor metabolite arachidonic acid.

Phospholipase metabolites and atherosclerosis

Cyclooxygenases and phospholipases

FFAs produced by phospholipase activity are metabolized into bioactive molecules, in particular eicosanoids. Eicosanoids regulate inflammation and play an important role in atherosclerosis (142, 143). Cyclooxygenase (COX) converts AA into prostaglandin H₂ (PGH₂). Further modification of PGH₂ results in the production of prostacyclins, thromboxanes (Tx_s), and prostaglandins (PG_s) (142, 144-146). The lipoxygenase (LOX) pathways metabolize AA into leukotrienes (LT_s) (Fig. 2). Three COX isoforms are known, which are regulated in different ways. COX-1 is constitutively expressed in various tissues, such as kidney, stomach, and plate-

lets (143, 147, 148). Peritoneal mouse macrophages are also known to express COX-1 (149-151). In general, COX-1 can be considered as a "housekeeping" enzyme that controls the production of AA metabolites necessarily to maintain physiological tissue integrity. It serves functions in the renal regulation, maintaining gastric mucosa, and hemostasis. Recently a splice variant of COX-1 was identified as COX-3 (152, 153).

In contrast with COX-1, COX-2 is inducible. It is expressed in response to hormones, growth factors, inflammatory cytokines such as IL-1 β , TNF and interferon- γ , and bacterial endotoxins (8, 143, 154). COX-2 is an important target in cancer research as various tumors, such as in colon, pancreas and lung, show expression of COX-2 (149, 155). The COX-2 promoter region contains elements that are recognized by peroxisome proliferator-activated receptors (PPARs). In turn PPARs can be activated by eicosanoids (156). COX-2 is found in SMC, macrophages, and endothelial cells in and near atherosclerotic lesions (157-159). The presence of COX-2 expression in atherosclerotic lesions (157, 158) and its observed elevated expression in destabilized plaques (160) relates it to the development of atherosclerosis.

Fig. 2. Arachidonic acid (AA) is transformed into PGH₂ by cyclooxygenase (COX) and to leukotriene A₄ by 5-lipoxygenase (5-LO). PG, prostaglandin; Tx, thromboxane; PGES, PGE-synthase; PGDS, PGD-synthase; PGFS, PGF-synthase; PGIS, PGI-synthase; LT, leukotrienes; LTA₄-H, LTA₄-hydrolase; 15-HETE, 15-Hydroxyeicosatetraenoic acid; 15-HpETE, 15-hydroperoxyeicosatetraenoic acid.

Prostacyclin and thromboxane

COX-2 determines the production rate of PGI₂ (prostacyclin) from the precursor PGH₂ by PGI synthase (PGIS) (161, 162). PGIS is widely expressed in tissues, most notably by endothelial cells (163-165). The expression of PGIS is regulated by proinflammatory cytokines as TNF (33, 166), IL-1 (167), and IL-6 (166, 168). In vessel walls, endothelial cells are the main prostacyclin producers. The thromboxane-producing pathway is regulated by COX-1 (169, 170), and inhibition of COX-2 does not affect thromboxane production (171). Prostacyclin plays a protective role in atherosclerosis and thrombosis through vasodilatation and inhibition of platelet aggregation and leukocyte adhesion (172-174). Thromboxane induces vasoconstriction and platelet aggregation (169, 175-180). The interplay between thromboxane and prostacyclin is crucial for platelets-vessel wall interactions (173) and epithelial damage repair (181, 182). Animal experiments with increased PGIS synthase show inhibition of neointimal formation in balloon-injured arteries (175).

Mice lacking the PGI₂ receptor exhibit increased platelet activation and vascular proliferation after injury of the vessel walls (182).

Blocking both the COX-1 and the COX-2 pathway reduces prostacyclin and thromboxane production. Classic, non-selective NSAIDs such as aspirin and naproxen, block the COX-1 and the COX-2 pathway. This results in gastrointestinal problems through the reduction of protective prostaglandins (183-185). Selective COX-2 inhibitors have been developed to bypass the detrimental effects (184, 185). However, COX-2 specific inhibitors influence the balance between the protective prostacyclin and the pro-thrombotic and vasoconstrictive thromboxane, increasing the risk for cardiovascular events (186). COX-2 selective inhibitors have become controversial due to increased cardiovascular risks associated with their use (184, 185, 187-189). Results from the Vioxx Gastrointestinal Outcomes Research (VIGOR) trial, which compared rofecoxib with naproxen, showed an alarming increase in acute myocardial infarctions (AMI). Initially, this was attributed to cardioprotective properties of naproxen, instead of cardiotoxic effects of rofecoxib (190-192). However, careful reexamination of available data revealed little protection provided by naproxen and indicates a detrimental effect of COX-2 inhibition (187, 189). Rofecoxib increases the risk for AMI by 3.7 fold (192) and Juni et al. (189) found a relative risk in the randomized controlled trials of 2.30 initially and 2.24 after a year. Little or no evidence indicated changes in the control groups that could explain the observed increased risk for AMI.

Although COX-2 inhibition might increase the risk for myocardial events, it is not necessarily pro-atherogenic. Inhibition of COX-2 is expected to decrease the production of pro-inflammatory prostaglandins, thus lowering the inflammatory processes involved in atherosclerosis. However, chronic inhibition of COX-2 in apolipoprotein E knockout mice does not alter lesion compositions (193) and has no effects on lesion development and progression (194). This indicated that other pathways might influence the onset and progression of inflammation in these mice. Experiments with COX-1 and COX-2 deficient mice (195) shows antagonistic effects of these enzymes. COX-1 deficient mice exhibit decreased response to angiotensin II, compared with the increased response in COX-2 deficient mice. Prostacyclin and Prostaglandin E₂ (PGE₂) production in kidneys is severely affected in COX-2 deficient mice, resulting in reduced renal vaso-regulation. COX-1 deficiency shows opposite effects possibly due to reduced thromboxane and PGF₂α production (195).

Despite its effects on PGE₂ and prostacyclin production, mice lacking a functional COX-2 gene, exhibit normal inflammatory responses after activation or arachidonic acid addition (196).

Prostaglandin E₂

PGE₂ is produced from PGH₂ by the PGE synthase enzymes cPGES, a cytosolic PGES (197), and the membrane-bound PGES (mPGES) (58, 198, 199). cPGES is constitutively expressed, and shows little variation after pro-inflammatory stimulation (197), while mPGES is induced by pro-inflammatory interleukines (200, 201). mPGES regulation is coupled with COX-2 expression (58, 202) and does not show COX-1 co-expression (58). In SMC and macrophages, PGE₂ is the main prostaglan-

din produced after activation and upregulation of mPGES and COX-2 (203, 204). Group IIA and V induce efficient production of prostanoids, while only minimal production is seen with group X (71). Despite its potent production of AA, group X induced only minimal PGE₂ biosynthesis and production of PGE₂ and TXA₂. However, addition of LPS, inducing COX-2 upregulation, resulted in strong eicosanoid production. sPLA₂ X does not regulate COX-2 expression directly, but COX-2 potentiates its effects.

Similar to prostacyclin, PGE₂ inhibits platelet activation and induces vasodilatation (205). It induces SMC proliferation (206) and upregulates MMP-2 and MMP-9 in macrophages (160, 207, 208). These MMPs facilitate macrophage migration and may decrease plaque stability (209-211). PGE₂ also induces the production of pro-inflammatory IL-6 (212). Despite its mainly pro-inflammatory properties, PGE₂ downregulates various inflammatory cytokines (213) and reduces chemokine production in macrophages through binding at the EP4 receptor (213).

Prostaglandin D₂ and F₂α

PGF₂α is biosynthesized from PGH₂, PHE₂, and PGD₂ through distinct pathways and promotes vasoconstriction (214-217). PGD₂ plays an important role in asthma (218) and allergic inflammatory reactions (219). It recruits eosinophils and Th2 cells in pulmonary inflammation (220), modulates pain perception (221), induces vasodilatation (222, 223) and reduces coagulation (224). PGD₂ metabolites inhibit NF-κB (225-227) and IKK2 (228) and activate peroxisome proliferator-activated receptor γ (PPARγ) (229), reducing inflammatory response.

Lipoxygenases and phospholipases A₂

The lipoxygenase pathway converts AA in precursor molecules for LT production. In humans 5-lipoxygenase (5-LO) and 15-lipoxygenase-1 (15-LO-1) are the most prominent lipoxygenases (230). The 5-LO pathway produces leukotriene A₄ (LTA₄), which is further metabolized to leukotriene B₄ (LTB₄) or other leukotrienes (LTC₄, LTD₄ and LTE₄). In mice the 12/15-LO is the ortholog to human 15-LO-1 (230). 5-LO expression is almost exclusively exhibited in myeloid descendant leukocytes (146). High levels of 5-LO are found in macrophages, dendritic cells, and mast cell within human atherosclerotic lesions (3, 231). 5-LO concentrations are positively correlated with the severity of the lesions (231). Products from 5-LO stimulate the expression and release of vascular endothelial cell growth factor (VEGF) (232). In apolipoprotein E / B100 double knockout mice, VEGF increases monocyte levels and lesion formation (233), linking the 5-LO pathway with atherosclerosis. Absence of 5-LO expression protects mice against atherosclerosis. Combined 5-LO and low-density lipoprotein receptor (LDLR) knockout mice show a remarkable decrease (>26x) in aortic lesions. 5-LO colocalizes with macrophages in lesions of LDL receptor knockouts. (234).

Another 5-LO AA metabolite, LTA₄, is secreted from leukocytes and taken up by other cells, where it is transformed in LTB₄ and other leukotrienes (235-237). This amplifies leukotriene production in inflamed tissues. LTB₄ is a potent polymorphonuclear neutrophil leukocytes (PMNs) recruiting agent (146) and induces PMN and monocyte adherence to the vessel wall (238) through integrin changes

(239). Human endothelial cells showed increased leukocyte adherence (240-242). Blocking the LTB_4 receptor in mice results in reduced lesions, accompanied by reduced monocyte chemoattractant protein-1 (MCP-1) (243). The peptide-leukotrienes induce vasoconstriction and increase vascular wall permeability, release of pro-inflammatory cytokine IL-1 β (244, 245), and proliferation of SMC (246). The 12/15-LO pathway results in the production of 15-hydroperoxyeicosatetraenoic acid (15-HpETE) and 15-Hydroxyeicosatetraenoic acid (15-HETE), which both exhibit strong oxidative properties on cell membranes and LDL particles (5, 247, 248). Phospholipases work in synergy with the 12/15-LO pathway as they deliver the AA substrate and at least group IIA is up-regulated by the 12/15-LO metabolites (248). Cytosolic phospholipases are involved in the production of intracellular AA and the induction of group IIA regulation. The exact mechanism of the group IIA in this context has not been fully elucidated (248). Mice lacking 12/15-LO show reduced atherosclerosis (249, 250). 12/15-LO is essential for foam cell formation and endothelial activation (249).

LysoPC production and effects

Lysophospholipids (LPs) consist of a sphingoid backbone with a single acyl chain and a phosphate chain. The most common lysophospholipids are lysophosphatidic acid (LPA), sphingosine 1-phosphate (SP1), lysophosphatidylcholine (lysoPC), and sphingosylphosphorycholine (SPC). Lysophospholipids are not only metabolites of phospholipid degradation; they are also a key molecule in phospholipid synthesis. LysoPC up-regulates the adhesion molecules ICAM-1 and VCAM-1 in endothelial cells (251) and inhibit vasodilatation (252). Furthermore, lysoPC induces macrophage and SMC proliferation (6, 253) and increase monocyte (6, 254) and T lymphocytes attraction (255).

Oxidized LDL contains large quantities of lysoPC (256). This is the result of phospholipase-like activity exhibited in the LDL particles during oxidative modifications, which converts up to 40% of the phosphatidylcholine molecules (257). High levels of lysoPC are present in atherosclerotic lesions (258, 259). LysoPC stimulates IL-1 β production in macrophages (260).

Extracellular lysophospholipids are hydrolyzed by lysophospholipase D (lysoPLD) to LPA (261, 262). Intracellular LPA is produced from LP by several pathways, but likely not secreted (261). Intracellularly, LPA serves as substrate for glycerolipid and phosphatidylinositol synthesis (261). Extracellular LPA exerts various biological functions through binding to LP receptors (263). Four LPA (LPA1-4) receptors have been characterized (reviewed in [(264)]). A whole range of downstream signaling cascades are mediated by LPA signaling and lead to cell proliferation and differentiation, cell survival, and cell motility (265).

LDL aggregation and fusion

Aggregation, retention, and fusion of LDL in the vessel wall is important in the onset of early atherosclerosis (266). Lipoproteins found in atherosclerotic lesions are derived from circulating LDL entering the vessel wall (267, 268). The transformation of macrophages into foam cells is enhanced with modified and aggregated LDL (269, 270) (Fig. 3).

LDL incubated with mouse sPLA₂ group V (81) or bee group III readily aggregates. In the latter, LDL fusion is induced (271). Results from group X indicate that it does not cause LDL aggregation (119), despite its extensive enzymatic activity. However, it is suggested (81) that the absence of albumin in the medium in these experiments, prevented AA and lysoPC transfer out of the LDL particles, thereby inhibiting particle decrease and preventing ApoB-100 conformational changes, lipid reorganization, and subsequently aggregation (272, 273).

LDL binding to extracellular matrix components (EMC) is a key event in atherosclerosis. Glyco-amino-glycan (GAG) molecules in the arterial proteoglycan (PG) matrix bind LDL particles through ApoB-100 interaction (1, 274) and enhance accumulation in the intima. Binding to GAG induces irreversible changes in the apoB-100 molecule (275) and alters the lipid distribution within the LDL particle (276). Other elements from the EMC that interact with apoB-100 are biglycan and decorin (277). Group IIA effects are neglectable on non-modified LDL due to its low activity on the membranes (278). However, in the presence of decorin, group IIA becomes a very potent modifier of LDL (51). The combination binding of decorin to LDL (277) and decorin co-localization with collagen in atherosclerotic lesions (279) makes group IIA an important player in the development of atherosclerosis.

Phospholipases, sphingomyelinase (SMase) and LDL

Sphingomyelin is abundant in LDL (280). Subendothelial lipoprotein accumulation is associated with SMase activity (281-283). LDL modified with SMase aggregates, fuses, and shows increased affinity for binding to aortic proteoglycans (271, 284). At neutral pH conditions, SMase does not exert pro-atherosclerotic actions, unless the LDL particles are modified with group IIA phospholipase (282). In vitro experiments revealed increased susceptibility of SMase modified LDL particles to group V modifications (77). The combination of group IIA, SMase and group V action on LDL in the vessel walls might act synergistically and can thus lead to induction and progression of atherosclerosis.

sPLA₂ IIA and plaque composition

Although, phospholipase A₂ IIA in atherosclerotic lesions increases the size of the plaques (68), few detailed studies have looked at the effects on plaque composition. To study that in detail, we created a macrophage sPLA₂ IIA overexpression model with the myeloid-specific CD11b promoter (285) combined with the human genomic sPLA₂ IIA gene (69). Bone marrow was transplanted to LDLR^{-/-} mice to study atherogenesis. Group IIA induced remarkable increase in atherosclerosis. One of the main features of the lesions in the sPLA₂ IIA mice was increased collagen content. The combination of more collagen (i.e. cap thickening) and the absence of increased necrosis or apoptosis, suggests a more stable plaque development. It has not been described before that sPLA₂ IIA affects collagen production or fibrosis in general. There is no indication that changes in the number of SMC and fibroblasts attribute significantly to the increased collagen production. Gilroy et al. (286) described a role for phospholipase in resolution of inflammation.

Both group IIA and V are capable of inducing release of pro-resolving eicosanoids (286-288). Experiments with a carrageen-induced air pouch inflammation model in group IIA lacking C57BL/6 mice, revealed an increased number of inflammatory cells during resolution. This difference is not observed during the initiation of inflammation (286). This indicates that sPLA₂ II can reduce attraction of inflammatory cells and increase the pace of inflammatory resolution. Part of inflammatory resolution is the deposition of extra cellular matrix components (289). The role of phospholipase IIA in atherosclerosis thus appears to be more complicated than initially expected.

Fig. 3. LDL entering the vessel walls is oxidized and enzymatically modified. Modified LDL binds to the extracellular matrix. The enzymatic action of phospholipases induces LDL aggregation and releases AA and lysoPL. LysoPL provides monocyte chemotaxis and SMC proliferation. The aggregated LDL is taken up by macrophages. AA is released from the LDL particles and metabolized through COX, 5-LO, and 12/15-LO pathways, resulting in the production of PGE₂ and LTB₄. Inflammation increases due to the AA and 5-LO products. MMP production is increased and facilitates monocyte and macrophage motility in the lesion. The macrophages transform into foam cells after continuously uptake of modified LDL. The development from an early fatty streak into an atherosclerotic lesion is initiated.

Concluding remarks

It is clear that phospholipases are involved in various processes that are pro- as well anti-atherosclerotic. The release of arachidonic acid and lysoPC are key events leading to the production of various eicosanoids. Despite the fact that many phospholipase-induced effects are pro-inflammatory, the role of PAF-AH illustrates protective mechanisms. Mice overexpressing PAF-AH have reduced lipid accumulation, intima formation, and macrophage attraction (108).

The role of phospholipase group IIA in atherosclerosis and inflammatory diseases

has been intensively studied in the last decades. The complex mechanism behind phospholipase IIA is not completely understood. The recent finding of group IIA-induced collagen formation in atherosclerotic lesions (69) illustrates this. The primary role of phospholipase IIA appears to be protection against Gram-positive bacteria (290, 291). The expression and presence in atherosclerotic lesions might be the results of activation of defense pathways in macrophages and neutrophils. The role of infections in atherosclerosis should be considered in this context. However, group IIA can play a role in normal cell metabolism when taken up through the HSPG shuttle pathway, releasing phospholipid metabolites at specific cell compartments.

Recently, phospholipase group V and X and PAF-AH gathered more attention and are recognized as important enzymes in atherosclerosis (81). Future investigation will certainly shift the focus to a broader spectrum of phospholipases. Aggregation and retention of LDL particles are a known effect of phospholipase IIA. Similar results have been obtained for group V (81), and there are strong indications that group X will show similar effects. Phospholipases are pro-atherogenic because lipid accumulation in the vessel wall is key element in the onset of lesion formation (1).

Phospholipase A_2 receptor binding is another aspect of the phospholipase A_2 -mediated actions that has not been fully elucidated. The interspecies differences indicate the subtle differences between related phospholipases A_2 .

The redundancy of group IIA in mice, which has not been reported in humans, indicates that care should be taken to interpret the obtained results in mice. Nevertheless, animal models overexpressing group V, group X, and PAF-AH will be of great importance to elucidate the role of these phospholipases. In combination with models deficient in downstream pathways of phospholipase, metabolites could deliver new insights in the complex role of these enzymes. The distinction between inflammatory responses, lipid accumulation, and inflammation-resolving functions needs further clarification.

Therapeutic approaches should take in account the differences between the phospholipases to be successful and to avoid side effects that ameliorate the beneficial properties of putative phospholipase blockers.

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Chapter 3

Macrophage specific overexpression of group IIA sPLA₂ increases atherosclerosis and enhances collagen deposition

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Abstract

Atherosclerosis is a chronic inflammatory disease of the vessel wall, characterized by the accumulation of lipid-loaden macrophages and fibrotic material. The initiation of the disease is accompanied by accumulation of modified lipoproteins in the vessel wall. Group IIA secretory phospholipase A₂ (sPLA₂ IIA) is a key candidate player in the enzymatic modification of low-density lipoproteins. To study the role of sPLA₂ IIA in macrophages during atherogenesis, transgenic mice were generated using the human sPLA₂ IIA gene and the CD11b promoter. Bone marrow transplantation to LDL receptor-deficient mice was performed to study sPLA₂ IIA in atherosclerosis. After 10 weeks of high fat diet, mice overexpressing sPLA₂ IIA in macrophages showed 2.3-fold larger lesions as compared to control mice. Pathological examination revealed that sPLA₂ IIA-expressing mice had increased collagen in their lesions, independent of lesion size. However, smooth muscle cells or fibroblasts in the lesions were not affected. Other parameters studied, including T-cells and cell turnover, were not significantly affected by overexpression of sPLA₂ IIA in macrophages. These data clearly show that macrophage sPLA₂ IIA is a proatherogenic factor and suggests that the enzyme regulates collagen production in the plaque and thus fibrotic cap development.

Introduction

Atherosclerosis is a progressive disease of the large arteries. The accumulation of LDL in the intima of the vessel wall is a crucial factor in driving atherogenesis in both the early onset and subsequent progression to an advanced plaque (1). It is widely accepted that oxidized and enzymatically modified LDLs are pro-atherogenic (2). Modified LDLs attract monocytes, which subsequently differentiate into macrophages. The macrophages transform into foam cells as they take up the modified LDL particles. In addition, inflammatory mediators are released from the modified lipids, which contribute to an inflammatory response in the vessel wall (3,4).

Several enzymes are known to modify LDL particles. Group IIA secretory phospholipase A₂ (sPLA₂ IIA) has been detected in atherosclerotic lesions in CD68-positive macrophages (5-7). sPLA₂ IIA can hydrolyze LDL-associated phospholipids, resulting in the formation of the so-called 'bio-active' lipids, such as lysophospholipids and arachidonic acid. sPLA₂ IIA is a member of a large family of related enzymes that are able to catalyze the hydrolysis of phospholipids at the sn-2 ester bond, a process that results in the generation of free fatty acids and lysophospholipids (8). sPLA₂ IIA is widely expressed in the human body (9) and is considered to be important in the amplification of inflammation in many disease processes, including atherosclerosis (6,7,10,11). Furthermore, sPLA₂ IIA-modified LDL also show an enhanced affinity for proteoglycans (10,12,13) and glycosaminoglycan binding (14).

Recent experiments with phospholipase group V, which is potent modifier of LDL, showed reduction in LDL particle size and subsequent aggregation (15). Smaller LDL particles have an increased preference for binding to proteoglycans (13). Although sPLA₂ IIA exhibits low enzymatic activity on intact LDL particles, binding

to extracellular matrix components like decorin, versican and biglycan, enhances the hydrolytic activity dramatically (11,13,16). The interaction between LDL and proteoglycans changes the LDL properties, enabling human sPLA₂ IIA, which is normally weakly active on LDL, to induce LDL aggregation and fusion, further increasing binding to the proteoglycan molecules (17,18) and making it more pro-atherogenic.

Grass et al. (19) generated a transgenic mouse model to study the role of sPLA₂ IIA in inflammation. In this model, the human sPLA₂ IIA gene was used with its own promoter and regulatory elements, leading to a natural pattern of overexpression. Interestingly, these mice developed atherosclerotic lesions even on a normal chow diet (20). However, LDL levels in these mice were increased, combined with decreased HDL levels and a reduction in paraoxonase activity. All of the systemic changes observed in this overexpression model are known risk factors contributing to increased atherosclerosis, which makes this mouse model not very well suited to study the precise role of sPLA₂ II in the vessel wall on lesion formation. More recently, Webb et al. (21) used an elegant approach to avoid systemic effects by transplanting transgenic bone marrow from the above mentioned model to lethally irradiated LDL receptor knockout (LDLR^{-/-}) mice. After a period of a mild fatty diet, the mice showed a 73% increase in lesion size at the aortic root area. It was concluded that sPLA₂ IIA can contribute to atherosclerotic lesion development independent of the systemic lipoprotein metabolism. However, how sPLA₂ IIA affects the pathology of lesion development was not addressed in detail.

We have previously shown that sPLA₂ IIA is not detectable in monocytes or differentiated, unstimulated macrophages, but its expression is induced upon minimally modified LDL stimulation of the latter (5). To acquire a better understanding of the role of sPLA₂ IIA in lesion development and the effects on the lesion composition, we created a macrophage sPLA₂ IIA overexpression mouse model. In this mouse model, a selective expression was obtained by using the myeloid-specific CD11b promoter fused to the human genomic sPLA₂ IIA gene lacking the endogenous promoter. The CD11b promoter has been shown to drive transgene expression in granulocytes, monocytes, and macrophages (22). Bone marrow from these mice was transplanted to LDLR^{-/-} mice to study atherogenesis. Macrophage sPLA₂ was shown to strongly increase atherosclerosis and increase collagenous cap formation. Other inflammatory parameters such as T-cells, granulocytes, and cell turnover, were not affected.

Materials & Methods

Mouse models

A 3.8 kb fragment, containing the genomic sPLA₂ IIA gene, was isolated from vector pBG34:3.8 with *Hind*III and blunted. This fragment corresponds to the *Eag*I/*Not*I fragment described by Kramer et al. (23). The blunted *Hind*III fragment was ligated into a pB202 vector containing a 1.7 kb human CD11b promoter (22) (a generous gift from Dr. D. Tenen, Beth Israel Hospital, Boston, MA), which was cut with *Bam*HI and *Not*I and also blunted. Correct orientation was confirmed with digestion and sequencing from the CD11b promoter. To generate transgenic mice,

the pB202:sPLA₂ construct was partial digested with *Hind*III and *Sac*II restriction enzymes. The complete 5.6 kb fragment (CD11b + sPLA₂ IIA) was isolated with electrophoresis in agarose gel, followed by electroelution. Oocyte injection to generate transgenic mice was performed according to standard procedures (24). Two transgenic mice lines were obtained (line 1 and 2). Before their use in experiments, the transgenic mice were tested for presence of endogenous sPLA₂ IIA with Southern blot analysis using a ³²P-labeled probe.

Because of a mutation, C57Bl/6 mice are natural knockouts for mouse sPLA₂ IIA (25). The transgenic sPLA₂ IIA bone marrow donors were backcrossed 5 times with C57Bl/6 mice. LDLR^{-/-} mice have been described (26). They were backcrossed four times to C57Bl/6J mice and originally obtained from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were approved by the Committee for Animal Welfare of Maastricht University.

Northern blotting

To study the expression of the transgene, total RNA was isolated from various tissues and from thioglycollate-elicited and bone marrow-derived macrophages, which were isolated as described before (27). RNA Instapure System (Eurogentec S.A. Seraing, Belgium) was used to isolate RNA from the samples. When appropriate, the tissues were first frozen and mechanically pulverized. The RNA samples were separated by electrophoresis through a denaturing agarose gel (1% w/v) containing 7.5% formaldehyde and then transferred to Hybond N according to the manufacturer's recommendations. Hybridization was performed on the blots with ³²P-labeled human sPLA₂ IIA DNA probe at 54°C in hybridization mixture (50 % formamide, 1% SDS, 10% dextran sulphate, 5 x SSC, 1 x Denhardt's, 0.2 M Na₂PO₄, and 50 mg/ml sonicated salmon sperm DNA).

Phospholipase activity

Levels of sPLA₂ protein in cell lysates of peritoneal macrophages were assessed by measuring the hydrolysis of FFAs from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (Sigma), as described in detail before (28). Phospholipase activity in blood of the transplanted mice was measured in pooled plasma samples collected at week 8 after the start of the diet, using a photometric assay (no. 765001; Cayman Chemical Co., Ann Arbor, MI).

In vitro foam cell formation

Bone marrow macrophages were cultured as described before (27). LDL was isolated according to standard procedures and modified as described before (27). Cells were incubated for 24 h in culture medium with or without 25 µg/ml native LDL or 25 µg/ml Cu²⁺ oxidized LDL. Cholesterol esters were determined as described before (29).

Bone marrow transplantation

Thirty-four 9-week old female littermate LDLR^{-/-} mice were put in filter-top cages. The mice received acidified water supplemented with neomycin (100 mg/l) and polymixin B sulfate (60,000 U/l), during the week before transplantation. One day

before the actual transplantation, the mice were irradiated with a lethal dose of 10 Gy röntgen. The bone marrow of five female wildtype mice and five female transgenic sPLA₂ IIA littermates was collected and pooled. Bone marrow cells were derived by flushing the femur and tibia of the mice. For transplantation, 16 mice received wildtype bone marrow (wt-tp) and 18 mice received transgenic derived bone marrow (sPLA₂-tp). Bone marrow cells (10^7) were injected in the tail vein of each mouse.

Blood samples and analysis

Four weeks after transplantation, the mice were put on a high-fat diet containing 16% fat, 0.15% cholesterol, and no cholate (Hope Farms, Woerden, The Netherlands) for 10 weeks. At two time points (4 weeks and 8 weeks of diet), blood was collected from the mice after overnight fasting. Plasma cholesterol was determined with an enzymatic assay kit (catalog number 401; Sigma-Aldrich, Zwijndrecht, The Netherlands). The blood lipid profile was analyzed with pooled plasma collected after 8 weeks of diet. The samples were separated over an Superose 6PC 3.2/30 column in an AKTASystem chromatography system (Amersham Biosciences, Roosendaal, The Netherlands). In the collected fractions (50), the cholesterol levels were determined by adding Roche Cholesterol CHOL_PAP reagent (catalog number 1489232; Roche Diagnostics, Mannheim, Germany) and measured with plate reader (Bio-Rad laboratories, Veenendaal, The Netherlands). Paraoxonase activity was measured in the blood of 10 randomly selected mice (5 wt-tp and 5 sPLA₂-tp). The activity was determined as the rate of conversion of paraoxon into *p*-nitrophenol and diethylphosphate by hydrolysis (30,31). Serum of each mouse was incubated with 5 mM Diethyl *p*-nitrophenyl phosphate (Sigma-Aldrich Chemie GmbH, Steinberg, Germany). The reaction was kept at 37°C and the extinction rate was recorded at 450 nm with a microplate reader (BioRad Laboratories).

Atherosclerosis assessment and lesion analysis

After 10 weeks of high-fat diet the mice were killed. Atherosclerosis was analyzed as described before (27). Briefly, the animals were dissected and hearts and aortic arches were removed and bisected perpendicularly to the heart axis, just below the atrial tips. The tissue was frozen in tissue-tec (Shandon, Veldhoven, The Netherlands) with the base facing downward. Cryosectioning was performed from the atrioventricular area. Sections (7 µm) were made with an interval of 42 µm and collected on slides. The aortic lesion area was analyzed using serial sections with 42µm interval, beginning from the onset of the aortic valves until the valves had disappeared. The collected sections were stained with toluidin blue and digitally photographed and quantified using digital image software (Adobe Photoshop 6.0).

Pathology

All pathology measurements were done without knowledge of the genotype. Lesion sections from the aortic root were fixed in acetone and incubated with antibodies against various cell types and cell markers. The following antibodies were used: FA11 (macrophages), NIMP (granulocytes), 1A4 (smooth muscle

cells), ERTR7 (fibroblasts), KT3 (T-cells), Ki-67 (cell proliferation), and sPLA₂ IIA (a generous gift from Dr. Timo Nevalainen, Turku University, Turku, Finland). Terminal deoxynucleotidyl transferase end labeling (TUNEL) assay (Roche) was used to detect apoptosis. Sirius Red staining was performed to visualize collagen. The stained sections were all photographed in the same conditions with a digital microscope camera (Nikon DXM1200). To quantify the percentage of collagen within the lesions, the lesion area was selected manually and subtraction filters (red channel minus the green channel) were applied to select the stained pixels (Adobe Photoshop 6.0). The same filter preferences were used on all sections, and quantification was assessed blindly (i.e., without knowledge of genotype). To quantify necrosis, necrotic areas were measured using toluidin-stained sections as described before (27). Cellular density was determined by counting nuclei and measuring area. All data are expressed relative to the lesion area size. For statistical analysis, one observation was the average value per mouse.

Statistical analysis

All data were analyzed using Graphpad Prism 4.01. Groups were compared using Welch's corrected two-tailed, non paired t-tests and data are expressed as means $\pm$ SEM. Tests were considered statistically significant at $p < 0.05$.

Results

Generation and phenotyping of the sPLA₂ IIA transgenic mice

To assess the local, vascular role of sPLA₂ IIA in atherosclerosis, in the absence of systemic effects of sPLA₂ IIA, a macrophage overexpression mouse model was created. The human genomic sPLA₂ IIA gene was cloned behind the CD11b promoter (22) (Fig. 1A). Two transgenic mouse lines were successfully generated. The mRNA expression pattern was examined by Northern blot analysis. The mice of line 1 showed expression (Fig. 1B) in liver, spleen, lung, and isolated primary macrophages. A similar expression pattern was observed in line 2, but expression was lower (data not shown). Therefore, all following experiments described here were performed with line 1. Both bone marrow derived macrophages (not shown) and thioglycollate-elicited macrophages (Fig. 1B) exhibited high expression of sPLA₂ IIA. To test if the observed mRNA expression patterns translated into effective production and presence of the sPLA₂ IIA protein, immunohistochemistry using a rabbit anti-human sPLA₂ IIA polyclonal antibody was performed on different organs. The antibody used is known not to show cross-reactivity with endogenous mouse sPLA₂ IIA and is specific for the sPLA₂ of group IIA (32), confirmed by the lack of signal in wildtype tissues. Immunostainings (Fig. 1C) showed presence of sPLA₂ IIA in macrophages in different tissues. An abundance of positive stained cells were observed in the spleen of the transgenic mice. Lung showed a moderate level of positive cells, whereas liver had only a few cells. In all organs tested, wild-type mice remained negative for sPLA₂ IIA. Staining with an antibody directed against CD11b (Mac1) confirmed the myeloid-specific expression of the sPLA₂ IIA transgene (not shown). Finally, phospholipase activity was measured in cell lysates from thioglycollate-elicited macrophages. In cells from transgenic mice 12-18

ng sPLA₂ per mg protein was measured, whereas wildtype cells remained below detection level. In vitro foam cell formation (i.e. cholesterol ester accumulation) of wildtype or sPLA₂ transgenic bone marrow macrophages was not different after overnight incubation without LDL (0.47 ± 0.2 and 0.62 ± 0.1 $\mu\text{g}/\text{mg}$ protein, respectively), 25 $\mu\text{g}/\text{ml}$ native LDL (0.88 ± 0.1 and 0.83 ± 0.2 $\mu\text{g}/\text{mg}$ protein), or 25 $\mu\text{g}/\text{ml}$ oxidized LDL (5.2 ± 0.2 and 5.1 ± 0.2 $\mu\text{g}/\text{mg}$ protein).

Bone marrow transplantation, lipid analysis, and phospholipase activity

To test the effect of sPLA₂ IIA on atherogenesis, bone marrow of wildtype mice and transgenic littermates was transplanted to lethally irradiated atherosclerosis-susceptible LDLR^{-/-} mice. The resulting mice were all LDLR^{-/-} with either sPLA₂ IIA transgenic hematopoietic cells (sPLA₂-tp) or wildtype hematopoietic cells (wt-tp). Both groups were allowed to recover for 4 weeks prior to high-fat feeding for 10 weeks.

Plasma cholesterol levels in the blood of the mice were examined after 4 and 8 weeks of diet (Fig. 2A). After 4 weeks, the cholesterol levels in the sPLA₂-tp mice were slightly higher than in the wt-tp mice (26.8 ± 1.1 and 22.1 ± 1.0 mM, respectively; $p < 0.01$). After 8 weeks, cholesterol levels were further increased but were no longer significantly different between groups (37.2 ± 2.7 and 30.8 ± 2.7 mM for sPLA₂-tp and wt-tp respectively; $p = 0.10$). Paraonase activity in the blood of the

Fig. 1: Generation and characterization of CD11b-sPLA₂ IIA mice. A: Schematic representation of the CD11b-sPLA₂ IIA construct. The 5.6 kb fragment was used for oocyte injection. B: Northern blot analysis of liver, lung, and spleen (left blot) and thioglycollate-elicited macrophages (right blot) from line 2 mice hybridized with a human sPLA₂ IIA probe. tg, sPLA₂ IIA transgenic; wt, wildtype. C: Immunostaining of tissues with rabbit-anti-human sPLA₂ IIA serum. Liver, lung, and spleen from wildtype (wt) and transgenic (tg) mice are shown.

Fig. 2: Cholesterol levels and lipoprotein profile of sPLA₂ IIA and wildtype transplanted LDLR^{-/-} mice. A: Cholesterol levels in wildtype (wt-tp; closed squares) and sPLA₂ IIA (sPLA₂-tp; open circles) transplanted LDLR^{-/-} mice after 4 and 8 weeks of high-fat diet. B: Lipoprotein cholesterol profiles of wildtype (closed squares) and sPLA₂ IIA (open circles) transplanted LDLR^{-/-} mice after 8 weeks of high fat diet. The values represented in the graph show the cholesterol content in each fraction.

mice after 8 weeks on diet did not differ between the wildtype and the transgenic group (1.37 ± 0.11 and 1.39 ± 0.26 IU/ml, respectively). To examine the blood lipid levels in more detail, lipid profiles were generated using plasma samples from week 8 (Fig. 2B). No major differences in VLDL, LDL, and HDL fractions were observed between the wt-tp and sPLA₂-tp mice.

Phospholipase activity of the pooled plasma samples was measured at 8 weeks, and no significant difference was detected (22.4 ± 0.2 and 22.3 ± 0.7 $\mu\text{mol}/\text{min}/\text{ml}$ for wt-tp and sPLA₂-tp, respectively).

Effect of macrophage-specific sPLA₂ IIA overexpression on atherosclerotic lesion size

Bone marrow-transplanted mice were sacrificed after 10 weeks of high-fat diet. Analysis of atherosclerotic lesions was done at the aortic root, near the heart valves. Quantification of the lesion area revealed a 2.3-fold increase in lesion size (Fig. 3) in sPLA₂-tp animals compared to wt-tp. The sPLA₂-tp mice had an average lesion size of 130.0 ± 15.1 ($\times 1000 \mu\text{m}^2$; $n=18$), whereas the wt-tp mice had an area of 56.0 ± 11.3 ($\times 1000 \mu\text{m}^2$; $n=16$) ($P<0.001$). Regression analysis did not show a clear correlation between lesion area and cholesterol levels at either 4 weeks of diet ($R^2=0.11$, $p=0.25$ and $R^2=0.04$, $p=0.51$ for wt-tp and sPLA₂-tp, respectively) or 8 weeks of diet ($R^2=0.019$, $p=0.60$ and $R^2=0.16$, $p=0.12$ for wt-tp and sPLA₂-tp, respectively).

In addition to the quantitative approach of measuring the lesion area, the plaques were also studied in more detail for their composition. Representative lesions stained with toluidin blue are shown in Fig. 4A, and 4B. The major constituents of the lesions were macrosialin-positive macrophages (Fig. 4C, and 4D) while also some collagenous cap formation was also observed using Sirius red staining (Fig. 4E, and 4F). The presence of sPLA₂ IIA in the lesions was confirmed by immunostaining (Fig. 5). The sPLA₂-tp mice showed clear staining in the lesions, whereas the wt-tp mice remained negative. Interestingly, lesions from sPLA₂-tp mice were

positive in their macrophage areas, but the majority of the sPLA₂ IIA staining was in the media. This may indicate accumulation of macrophage-derived sPLA₂ in the medium.

In addition, the lesions were classified according to three different types as described before (27): 1) early lesions, with fatty streaks consisting of only foam cells; 2) moderate lesions, or foam cell lesions with a fibrotic cap; 3) severe lesions, with increased fibrosis and media involvement. Interestingly, although the lesions in the sPLA₂-tp mice were 2.3 fold larger, no significant shift towards a more severe phe-

nototype of the lesions was observed (early lesions: 19.5 and 15.7; moderate lesions: 65.9 and 74.5; advanced lesions: 14.6 and 9.8; for wt-tp and sPLA₂-tp, respectively; all as relative percentages of the total number of lesions). This demonstrates that the lesions in sPLA₂-tp mice are much larger but not more severe as compared to their controls.

Fig. 3: Atherosclerotic lesion size in sPLA₂ IIA and wildtype transplanted LDLR^{-/-} mice. Lesion area measurements of wildtype (wt-tp) and sPLA₂ IIA (sPLA₂-tp) transplanted LDLR^{-/-} mice. Symbols represent individual mice.

* $P < 0.001$.

Plaque phenotype

To characterize atherosclerosis in more detail different cellular parameters were determined. All were expressed relative to the size of the lesions.

Two factors important for plaque stability (i.e., necrosis and collagen content) were analyzed. Necrotic area did not differ between the two groups (Fig. 6A). Also in both groups, no clear difference in correlation between lesion size and necrosis could be found (Fig. 6B). Interestingly, collagen, as measured by Sirius red staining of the lesions, was 2-fold increased in sPLA₂-tp lesions ($6.5 \pm 1.5\%$ and $13.6 \pm 2.9\%$ for wt-tp and sPLA₂-tp, respectively; $p < 0.05$), even though the values were corrected for lesion size (Fig. 6C). Regression analysis confirmed this difference, showing correlation between lesion size and collagen content ($R^2 = 0.62$, $p < 0.0001$ and $R^2 = 0.73$, $P < 0.0001$ for wt-tp and sPLA₂-tp, respectively) with a 2-fold steeper slope for sPLA₂-tp mice (0.20 ± 0.02 and 0.38 ± 0.03 for wt-tp and sPLA₂-tp, respectively; $P < 0.001$) (Fig. 6D). These data show that overexpression of sPLA₂ in macrophages does not affect necrosis but results in atherosclerotic lesions with more collagen content.

Other cellular parameters were also studied. Smooth muscle cell (SMC) content was analyzed because increased collagen might be a reflection of increased SMC in the lesions. However, quantification of these cells after SMC α -actin staining did not reveal any differences between sPLA₂-tp and controls (Fig. 7A). Moreover, fibroblasts (ERT7 positive cells) also did not differ between groups ($4.6 \pm 1.1\%$ and $5.7 \pm 1.6\%$, for wt-tp and sPLA₂-tp, respectively). Next, T-cells were quantified. Although they appeared to be reduced (Fig. 7B) the difference did not reach statistical significance ($p = 0.10$). For cell turnover, proliferation (i.e., Ki-67 positivity),

and apoptosis (TUNEL staining) were examined. Proliferation appeared to be reduced in sPLA₂-tp mice, but the difference was only borderline significant ($p=0.06$; Fig. 7C). Finally, apoptosis was not changed in sPLA₂-tp group as compared With wt-tp mice (Fig. 7D).

Because changes in cell turnover (i.e. apoptosis, necrosis, and proliferation), and SMC or collagen content could not completely account for the 2.3-fold increase in total lesion size observed, macrophage density was determined. The increase in lesion size could be the result of either increased cell size or just increased cell number. Counting of the number of macrophages per selected area showed no differences in cell density between wt-tp and sPLA₂-tp (574.5 ± 42.8 and 548.2 ± 24.3

Fig. 4: Atherosclerotic lesions in sPLA₂, IIa and wildtype transplanted LDLR^{-/-} mice. Representative lesions from wildtype (A, C, E) and sPLA₂, IIa (B, D, F) transplanted LDLR^{-/-} mice stained with toluidin blue (A, B) for general cellular composition, macrophage (C, D) for macrophages and sirius red (E, F) for collagen.

Fig. 5: sPLA₂ IIA immunostaining. Immunostaining for human sPLA₂ IIA of lesions from wildtype (wt-tp) and sPLA₂ IIA (sPLA₂-tp) transplanted LDLR^{-/-} mice.

cells/ μm^2 , respectively), indicating that the size of the macrophages was not changed. Therefore, the increased lesion size is due to the increased number of macrophages and not due to their increased size.

Fig. 6: Necrosis and collagen in lesions from sPLA₂ IIA and wildtype transplanted LDLR^{-/-} mice. Necrosis (A, B) and collagen (C, D) were quantified in the lesions of wildtype (wt-tp) and sPLA₂ IIA (sPLA₂-tp) transplanted LDLR^{-/-} mice as described in Material and Methods. Both are expressed as a relative percentage of lesion area per animal (A, C) and as an individual area correlated to the lesion size (B, D). In A and C, each symbol represents one animal, and in B and D, each symbol represents one lesion. * $p < 0.05$.

Discussion

In this study, we examined the effects of macrophage sPLA₂ IIA on atherogenesis. We show that macrophage-specific overexpression strongly increases atherosclerotic plaque size without the normally associated lesion progression to a more severe phenotype. In addition, collagen deposition in the lesions was also increased even when corrected for lesion size. Remarkably, other cellular parameters were not affected by sPLA₂ IIA.

We could demonstrate that sPLA₂ IIA is present at the site of the atherosclerotic lesions of the mice transplanted with sPLA₂ bone marrow. Only minimal systemic effects on cholesterol levels were observed, with no change in circulating sPLA₂ or paraoxonase activity. Hence, we have generated an animal model to investigate the direct lesional role of sPLA₂ IIA in plaque development and progression. Quantification of the atherosclerotic lesions revealed a 2.3-fold increase in lesion size attributable to increased macrophage sPLA₂ IIA expression. Similar results, albeit less pronounced, have been reported by Webb et al. (21). Closer examination of the lesions in our experimental animals revealed interesting changes in plaque morphology in response to increased levels of sPLA₂. Both collagen content and increased macrophage numbers contributed to lesion enlargement in the sPLA₂-tp mice. Apoptosis and necrosis were not altered, and cell proliferation did not increase, indicating that the increased cell number in sPLA₂-tp mice must be attributed to increased monocyte infiltration in the vessel wall. It was recently shown that one of the products of sPLA₂ activity, lysophosphatidylcholine, can be chemotactic for monocytes (33), so this may account, at least in part, for the observed increased cell number and increased atherosclerosis. No other signs of increased inflammation could be measured. No changes were observed in T-cells, inflammatory cells that are often associated with atherosclerotic plaques. Also, granulocyte count (data not shown) did not reveal any differences.

Despite the cell-specific expression of the transgene, the sPLA₂ IIA produced in macrophages and monocytes proved to be sufficient to increase the cholesterol levels in mice after 4 weeks of diet. However, at later times, this difference was no longer significant. No significant correlation between lesion area and cholesterol levels at either 4 or 8 weeks of high-fat diet was found. Additionally, no clear change in the blood lipoprotein profile or in paraoxonase activity was detected. Paraoxonase levels have been previously described to be reduced in sPLA₂ transgenic animals, and it can act as a protective factor against lipoprotein modification (20). From these data, we conclude that total systemic effects of sPLA₂ IIA were rather limited in our experimental setup, and it is unlikely that they contributed significantly to the phenotype. This is in contrast with the drastic lipid changes observed by Ivandic et al. (20). They demonstrated that general overexpression results in decreased levels of HDL-cholesterol and increased levels of LDL/VLDL on chow diet, and even more prominent changes were observed on a high-fat diet. The high expression levels of sPLA₂ IIA in the liver of this mouse model (34) could contribute to these changes in lipid parameters.

Immunohistochemistry for sPLA₂ IIA revealed the presence of the enzyme in the media of the aorta near atherosclerotic lesions in the sPLA₂-tp mice. Nevalainen et al. (34) observed similar accumulation of sPLA₂ IIA protein in de media without

Fig. 7: Cellular characterization of lesions from sPLA₂ IIA and wildtype transplanted LDLR^{-/-} mice. Smooth muscle cells (SMC) (A), T-cells (B), proliferation (C), and apoptosis / terminal deoxynucleotidyl transferase end labeling (TUNEL) (D) were quantified in lesions of wildtype (wt-tp) and sPLA₂ IIA (sPLA₂-tp) transplanted LDLR^{-/-} mice and expressed relative to the lesion size. Each symbol represents one animal.

the presence of mRNA for sPLA₂ IIA. A mechanism for this specific localization is offered by Sartipy et al. (35), showing binding of secreted sPLA₂ IIA to such proteoglycan molecules as decorin and versican. We observed medial sPLA₂ IIA presence only near the lesions, suggesting the accumulation of diffused sPLA₂ IIA from within the lesion. sPLA₂ IIA has been shown to bind to a whole range of extracellular matrix components, such as glypican, a glycosulphatidylinosol (GPI)-anchored proteoglycan (36), decorin (36), and biglycan (12,14,16,35). Kovanen et al. (37) demonstrated an increased affinity of modified LDL to collagen as a result of decorin linkage. Moreover, Flood et al. (12) also demonstrated increased affinity for proteoglycans of sPLA₂ IIA-modified LDL. Interaction with the extracellular matrix components may result in rearrangement of the phospholipids in the LDL particles, which may increase the susceptibility to sPLA₂ IIA modifications, in accordance with results shown by Sartipy(11). These mechanisms in turn attribute to prolonged LDL retention and subsequent modifications (38) in the extracellular matrix, resulting in increased atherogenicity of the lipoproteins (39). Besides direct binding to the matrix, sPLA₂ IIA-modified LDL has been previously shown to aggregate more and fuse in presence of proteoglycans (17,40,41), which may also increase atherogenesis. The absence of clear inflammatory effects in the lesions of our sPLA₂-tp mice, despite the increased lesion size, may indicate that LDL retention in the vessel wall is an important factor in sPLA₂ IIA amplified atherosclerosis.

One of the main features of the lesions in the sPLA₂ IIA mice was increased collagen content. The combination of more collagen (i.e., cap thickening) and the absence of increased necrosis or apoptosis suggests a more stable plaque development attributable to the presence of sPLA₂ IIA. It has not been described before that sPLA₂ IIA affects collagen production or fibrosis in atherogenesis. However, using sPLA₂ IIA transgenic mice in a model of acute pancreatitis, Mayer et al. (42) also observed increased fibrosis in the pancreas of the transgenic animals, whereas fibrosis was absent in the wildtype mice. SMC and fibroblast contents were not changed in sPLA₂-tp mice, which may indicate that the difference in collagen in the lesions cannot be attributed to an increase in specific cell types producing collagen. The exact source of the collagen remains unclear, but it should be noted that collagen VIII can be produced by macrophages in atherosclerotic lesions (43). More collagen in atherosclerotic lesions can be the result of either increased overall production in the lesion or decreased degradation. Degradation may be affected by reduction in matrix metalloproteases (MMPs), which degrade collagen, or increases in tissue inhibitor of MMPs. However, no data are available on the effect of sPLA₂ IIA on these kinds of enzymes; future experiments should focus on this issue. In the lesions of our sPLA₂ IIA-transplanted mice, we found an increase in macrophages and foam cells but no clear signs of increased inflammation (i.e., changes in T-cells or granulocytes). Fibrosis or scar formation is often considered to be a final anti-inflammatory phase (44) and reflects the resolution of inflammation. Interestingly, using an air pouch model, Gilroy et al. (45) recently described a new role for phospholipases in inflammation. sPLA₂ IIA and group V were shown to be important in the resolution phase of inflammation, whereas these enzymes did not contribute to the initiation phase of inflammation. Both were capable of upregulating platelet-activating factor and lipoxin A₄, resulting in group IV cytosolic PLA₂-mediated release of proresolving eicosanoids (45-47). Moreover, they showed an increase number is inflammatory cells in the resolving phase in C57BL/6 mice, a natural knockout for sPLA₂ IIA compared with BALB/c animals, a difference not observed in the initiating phase (45). These results may indicate a role for sPLA₂ IIA in the termination of inflammatory responses. Our observed increased collagen in the atherosclerotic plaques may be in agreement with such an anti-inflammatory role. Combining our model with activation through other pro-inflammatory stimuli may shed light on the specific effects in the initiation and resolution of inflammation.

In conclusion, we have generated a novel mouse model to study the function of sPLA₂ IIA in macrophages and atherogenesis. We show that sPLA₂ IIA in macrophages is a proatherogenic factor. Our findings may also suggest an LDL-accumulation and retention-based mechanism as major cause for the effects of sPLA₂ IIA. Moreover, we found that overexpression of sPLA₂ IIA also affects collagen deposition in the plaque, which indicates a novel function of sPLA₂ IIA in the vessel wall. The exact mechanisms involved in sPLA₂ IIA induced collagen deposition remain speculative, and more detailed mechanistic studies are needed. Future studies may also focus on longer lasting atherosclerosis experiments to investigate how more advanced atherosclerosis is affected by sPLA₂ IIA and how plaque stability develops in the presence of increased levels of this enzyme.

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Chapter 4

Secretory Phospholipase A₂ group X expression mediates foam cell formation and inhibits lipopolysaccharide induced activation of murine macrophages.

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Ready for submission

Abstract

Phospholipases are a diverse group of enzymes capable of hydrolyzing phospholipids, releasing free fatty acids (FFA) and lysophospholipids (lysoPL). Several phospholipases have been shown to be involved in atherosclerosis in which they can modulate lesion formation by influencing inflammation and lipoprotein modification. However, the role of the distinct phospholipases in these processes is not fully elucidated. In this paper, we examined the role of secreted phospholipase A₂ group X (sPLA₂ X) in murine macrophages. sPLA₂ X is one of the most potent phospholipases in mammals, capable of hydrolyzing intact cell membranes and it has been reported in murine atherosclerotic lesions. Here we show that the sPLA₂ X gene is expressed in murine macrophages. We found that sPLA₂ X overexpression in macrophages strongly increases foam cell formation upon incubation with native LDL. Moreover, we could show that sPLA₂ X expression inhibits lipopolysaccharide-induced macrophage-activation, characterized by reduced cell adhesion, lower levels of TNF secretion and increased interleukin-10 production. These effects are likely mediated by increased production of two anti-inflammatory prostaglandins, prostaglandin E₂ and 15-deoxy- Δ 12,14-prostaglandin J₂. This study shows a potentially two-faced role for sPLA₂ X in atherosclerosis, with foam cell promoting capacities and also anti-inflammatory properties reducing macrophage activation.

Introduction

Atherosclerosis is a progressive disease of the large arteries. The accumulation of low-density lipoprotein (LDL) and the development of inflammation in the vessel wall are essential events in the early onset and progression of plaque formation (reviewed in (1)). Oxidation and enzymatic modifications highly contribute to LDL accumulation (2). LDL modifications result in the release of various inflammatory mediators and modulate inflammatory responses. Monocytes attracted to the site of inflammation differentiate into macrophages and subsequently transform into foam cells after uptake of the LDL (3). Phospholipases are known to modify LDL to the extent that they become pro-atherogenic. Recent mouse studies confirmed a pro-atherogenic role for group IIa secretory phospholipase A₂ (sPLA₂) (4-7).

The common property of the phospholipases is that they hydrolyze phospholipids at the sn-2 position, resulting in the formation of free fatty acids, such as arachidonic acid (AA), and lysophospholipids (lysoPL) (8). Classification of the phospholipases is based on structure, molecular weight and substrate preference (9). Three groups are distinguished: the secreted PLA₂s (sPLA₂), the cytosolic PLA₂s (cPLA₂) and the Ca⁺⁺ independent PLA₂s (iPLA₂). AA released by sPLA₂ activity is the substrate for the production of inflammatory products like prostaglandins, thromboxanes and leukotrienes. Besides enzymatic activity, phospholipase A₂ receptor (PLA₂R) binding is reported and results in the release of chemokines through the mitogen activated protein kinase (MAPK) pathway (10-12).

The potent group X sPLA₂ (sPLA₂ X), discovered by Cupillard et al. (13) is structurally related to both sPLA₂ group IB and IIA. sPLA₂ X displays potent activity on both anionic as well zwitterionic phospholipids and hydrolyzes phosphatidyl-

choline (14). Although comparable with group V in terms of cell binding, the hydrophobic nature of sPLA₂ X enables the enzyme to be far more potent (15, 16). Addition of sPLA₂ X to cell cultures elicits cellular AA release. This is in contrast with the weak activity of group IIA which needs HSPG-binding, internalization (HSPG-shuttling pathway) and coupling to cPLA₂ to produce AA (17). Human sPLA₂ X is present in many organs including spleen, thymus, peripheral blood leukocytes, testis, lung, pancreas and colon cells (13, 18). Expression of murine sPLA₂ X is found in brain, spleen, lung, testis, thymus and uterus (19). Group X protein has been shown to be present in macrophage foam cells and smooth muscle cells in the vessel walls of high fat-fed apolipoprotein E knockout mice (20). Mouse macrophages incubated with LDL modified by purified human sPLA₂ X readily transform into foam cells. The lipid droplet distribution and pattern of these foam cells resembles acetylated LDL loading and the cholesterol ester content increased vastly (20). Moreover, spleen macrophages are reported to contain sPLA₂ X (19), indicating a possible role for sPLA₂ X in inflammation or immune responses.

sPLA₂ X activity is capable of liberating large quantities of AA, the substrate in the production of inflammatory mediating prostaglandins. Therefore, in addition to its role in foam cell formation, we examined the effect of sPLA₂ X overexpression on macrophage-activation and found powerful anti-inflammatory effects demonstrated by reduced TNF and increased IL-10 levels, accompanied with an increase in prostaglandin E₂ (PGE₂) and 15-deoxy- Δ 12,14-prostaglandin J₂ (15d-PGJ₂) production. At the same time, we show that sPLA₂ X overexpression itself in unstimulated macrophages does not elicit an inflammatory response.

Material and Methods

Cell culture

The murine macrophage cell line RAW264.7 was cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine and 50 μ M β -mercaptoethanol, hereafter referred to as RAW medium. Experiments were performed in the same RAW medium but w/o β -mercaptoethanol. Bone marrow derived macrophages (BMM) were generated and cultured from C57/Bl6 mice as described before (21). After differentiation, cells were replated in R10 medium (RPMI 1640 medium, containing 10% heat inactivated FCS, 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine and 10 mM Hepes) supplemented with 15% L-929 cell conditioned medium (LCM) (22).

RNA isolation and PCR analysis

In order to analyze sPLA₂ X expression, RNA was isolated from C57/B6 mice BMM or RAW264.7 cells using Tri Reagent (Sigma), according to the manufacturers protocol. RNA concentration was measured using the nanodrop (Witec AG, Germany). 1 μ g total RNA was converted into cDNA. Oligo dT primers and RevertAid M-MuLV reverse transcriptase (Fermentas) were used according to the manufacturers protocol. Presence of sPLA₂ X expression was tested by PCR with appropri-

ate primers (forward: GGCTGTTATTGTGGCCTTGCT; reverse: TCCTGAGCCC-GAGAGTAGCA), which are selected in such way that an intron was included to prevent genomic amplification. Product length was checked on regular agarose gel electrophoresis. Quantitative realtime PCR (qPCR) with the same primers for murine sPLA₂ X was performed to quantify sPLA₂ X expression. For qPCR (TAQ-Man) analysis with 300 nM primer/ 200 nM probe sets, the qPCR Mastermix Plus kit (Eurogentec) was used on 10 ng cDNA. Analysis was performed on an ABI Prism 7700 Sequence Detector. The housekeeping genes cyclophilin A and β -Actin were used to correct for RNA concentration differences between the samples.

Construct

Human sPLA₂ X cDNA (a generous gift from Dr. Cupillard) was isolated from a pRc/CMV vector (Invitrogen), containing the sPLA₂ X cDNA starting at the second methionine start site (met-32) (13). The met-32 fragment was selected using convenient restriction sites and inserted in a pcDNA 3 expression vector behind a CD68 promoter construct (2.9 kb CD68 + IVS-1 sequence (23)). The CD68 – sPLA₂ X in pcDNA3 plasmid was verified by sequencing the start and the end of the met-32 construct and boundaries of the CD68 promoter region. Additional verification was performed using various restriction enzymes and subsequent gel electrophoresis.

Fig. 1. Expression of sPLA₂ X in bone marrow derived and RAW264.7 macrophages. A: PCR with murine sPLA₂ X specific primers on cDNA isolated from bone marrow derived macrophages and RAW264.7 cell lines. RT negative controls (left) have been included to check for genomic DNA amplification. B: sPLA₂ X expression in RAW264.7 macrophages after stimulation with 10, 100 and 1000 ng/ml LPS after 4 and 24 hours.

Electroporation

For electroporation, 10 μ g CD68 – sPLA₂ X plasmid DNA was combined with 2 μ g of a Neomycin resistance plasmid and added to 9 x 10⁶ RAW264.7 cells resuspended in 500 μ l Optimem (Invitrogen) and electroporated with a BioRad Gene Pulser II at 1070 μ F and 280 Volt in 4 mm cuvettes. Following electroporation, the cells were replated in large flasks with RAW medium and allowed to recover for 48 h. The cells were selected with 0.1 μ g/ml Geneticin one week. Surviving cells were replated in 96 wells plates for clonal selection and individual colonies were selected and screened with PCR for presence of the human sPLA₂ X construct.

Northern blotting

To verify the expression of human sPLA₂ X in transfected RAW264.7 cells, RNA was isolated using Tri-

Reagent (Sigma) according to the manufacturers protocol. RNA concentration was measured using a smartspec 3000 photospectrometer (BioRad). The RNA samples were separated by electrophoresis through a denaturing agarose gel (1% w/v) containing 7.5% formaldehyde and then transferred to Hybond N according to the manufacturer's recommendations. Hybridization was performed on the blots with 32P-labeled sPLA₂ X fragment at 54°C in hybridization mixture (50 % formamide, 1%SDS, 10% dextranulphate, 5 x SSC, 1 x Denhardtts, 0.2 M NA₂PO₄, 50 mg/ml sonicated salmon sperm DNA).

Phospholipase activity

Phospholipase activity was tested in culture media from RAW264.7 controls and cells expressing hu-sPLA₂ X using a sPLA₂ assay kit (Cayman Chemical, Ann Arbor, MI, US) according to the manufacture's instructions.

LDL isolation and modification

Normal LDL was isolated from the serum of healthy blood donors (obtained from 'Stichting Sanquin', the Netherlands). The serum was brought to 1.21 g/ml density with KBr addition and placed in a NaCl density gradient and centrifuged for 18 hours with 134.000g at 4°C (Beckman SW28.1 rotor and 25x89mm Ultra-tubes). The LDL fraction (density 1.019 – 1.063 g/ml) was isolated and dialyzed against sterile PBS contain-

Fig. 2. CD68 sPLA₂ X construct, Northern blot and phospholipase activity assay. A: CD68 met-32 sPLA₂ X construct. The 3-kb CD68 promoter and IVS-1 preceding the methionin-32 humane sPLA₂ X fragment. This construct is used for electroporation into RAW264.7 macrophages. B: Northern blot analysis of RNA isolated from CD68 methionin-32 sPLA₂ X expressing RAW264.7 cell lines. Control cell lines (ctrl 1-3 on the left) have undergone electroporation, but failed incorporating the hu-sPLA₂ X construct. hu-sPLA₂ X positive cell lines (grX 1-3) are on the right side. C: Phospholipase activity assay. PLA₂ activity assays were performed on culture medium from the hu-sPLA₂ X expressing RAW264.7 cell lines and the negative control cell lines. The essay measures the PLA₂ activity as the rate (mmol/min/ml) in which the 1,2-dithio analog of diheptanoylphosphatidylcholine is hydrolyzed.

ing 10 μ M EDTA and stored under N₂ at 4°C. The concentration of LDL was quantified by protein determination (BioRad Protein Assay).

LDL loading and HPTLC

2x10⁶ cells were plated out in 6-well plates in serum free medium (Optimem, Invitrogen) constituted with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine. After a 4h period at 37°C, 25 μ g/ml LDL was added to the medium. The cells were incubated for 24 hours after which they were washed, lifted and resuspended in 300 μ l MilliQ and lysed by freezing, followed by passage through a 27G hypodermic needle. The lysates were diluted in MilliQ to 200 μ g protein in a total volume of 800 μ l. Chloroform, internal standard (20 μ g Cholesterolacetate/ml Chloroform), and additional water (1 ml) was added and mixed. After centrifugation at 3000 rpm for 10 minutes, the chloroform fraction was isolated and dried at 50°C under N₂. HPTLC plates (Alltech/Applied Science) were activated overnight at 130°C and prepared with Methanol / Chloroform (2:1), followed by intermediate drying at 130°C. A run with NaOH (10% in methanol) was performed and the plates were incubated o/n at 130°C. Lipid samples were dissolved in 50 μ l chloroform and applied in spots on the HPTLC plate. The chromatography was performed with a hexane, diethylether and 2-propanol mixture (90:10:3). Thereafter the plate was dried at 130°C and stained (H₂SO₄ dissolved MnCl₂ in 50% methanol) for 20 seconds and incubated at 130°C for 30 minutes.

Fig. 3. Cholesterol accumulation in RAW264.7 cell lines. The amount of cholesterol esters present in RAW264.7 cell lines transfected with CD68 hu-sPLA₂ X after 24h incubation without or with LDL.

The lysates were diluted in MilliQ to 200 μ g protein in a total volume of 800 μ l. Chloroform, internal standard (20 μ g Cholesterolacetate/ml Chloroform), and additional water (1 ml) was added and mixed. After centrifugation at 3000 rpm for 10 minutes, the chloroform fraction was isolated and dried at 50°C under N₂. HPTLC plates (Alltech/Applied Science) were activated overnight at 130°C and prepared with Methanol / Chloroform (2:1), followed by intermediate drying at 130°C. A run with NaOH (10% in methanol) was performed and the plates were incubated o/n at 130°C. Lipid samples were dissolved in 50 μ l chloroform and applied in spots on the HPTLC plate. The chromatography was performed with a hexane, diethylether and 2-propanol mixture (90:10:3). Thereafter the plate was dried at 130°C and stained (H₂SO₄ dissolved MnCl₂ in 50% methanol) for 20 seconds and incubated at 130°C for 30 minutes.

Fig. 4. Effect of hu-sPLA₂ X expression on cellular activation. Microscopic pictures of RAW264.7 cells not expressing (wt, ctrl) or expressing hu-sPLA₂ X (grX) were activated with LPS for 24h.

The HPTLC plate was scanned and the samples were quantified against the internal standard with the Bio-Rad Quantity One (version 4.2.3) software package. The LDL loading experiment was performed several times, with two different media (RAW medium and RAW medium with the heat inactivated fetal calf serum substituted with free fatty acid free bovine serum albumin). Results (data not shown) were similar in all experiments.

ELISA for cytokines and prostaglandins

5 x 10⁵ cells were plated in 24-well plates in 0.5 ml RAW medium. The cells were left to attach for 4 hours and then activated with LPS. The medium was analyzed for TNF and IL-10 after 4 hours and 24 hours using commercially available ELISA kits (Biosource Inc., Camarillo, CA). PGE₂ and 15d-PGJ₂ were measured with competitive immunoassay kits according to the manufactures instructions (Assay Designs, Ann Arbor, MI).

Results

We first examined sPLA₂ X mRNA expression in primary murine bone marrow derived and RAW264.7 macrophages and found sPLA₂ X expression in both (Fig. 1A). Control samples, without reverse transcriptase added remained negative, confirming mRNA specificity and excluding genomic amplification. To study the effects of inflammatory stimulation on sPLA₂ X mRNA expression, we incubated RAW264.7 macrophages with three different LPS concentrations (10 – 100 – 1000 ng/ml) and isolated mRNA at 4 and 24 hours. As the results show (Fig. 1B), sPLA₂ X expression did not change under the influence of LPS. A small, but non-significant ($p=0.2$) decline in sPLA₂ X expression after 24h 1000 ng/ml LPS activation was observed.

To study the function of sPLA₂ X in macrophages we overexpressed human sPLA₂ X (Fig. 2A) in murine RAW264.7 macrophages. The human CD68 promoter was selected for its ability to deliver high expression in mouse macrophages(23). The methionin-32 start site of the human sPLA₂ X gene has proven to deliver potent sPLA₂ X production (13) and was cloned behind the CD68 promotor. Stably transfected RAW264.7 cell lines were established and the expression of human sPLA₂ X was confirmed by Northern blotting (Fig. 2B). Clones overexpressing sPLA₂ X were selected for subsequent experiments and clones that were negative on the Northern blot were used as controls, alongside non-transfected cells referred to as wildtype cells. Next, we measured phospholipase activity in the medium of transfected cells and control cells (Fig. 2C). Phospholipase activity showed a 5-fold increase in cells expressing the sPLA₂ X construct compared to control cell lines. LPS treatment of the macrophages cells did not change sPLA₂ activity (data not shown).

To study the effects of sPLA₂ X overexpression on foam cell formation, we incubated RAW264.7 overexpressing sPLA₂ X with native LDL for 24h. Interestingly, a two-fold increase in cholesterol ester accumulation was observed in cells expressing sPLA₂ X compared to controls. These data show that sPLA₂ X expression in macrophages promotes foam cell formation.

Activation experiments were performed to investigate the role of sPLA₂ X in inflammation. Upon incubation with LPS (250 ng/ml) wildtype and control cell lines

Fig. 5. ELISA for TNF and IL-10 release during activation of RAW264.7 cells expressing hu-sPLA₂ X. RAW264.7 cells expressing hu-sPLA₂ X after 4 and 24h activation with LPS. A: Levels of TNF; B: levels of IL-10 measured by ELISA. The results of a wildtype (wt) cell line, a control cell line (ctrl), negative for hu-sPLA₂ X expression and two hu-sPLA₂ X expressing cell lines (grX) are depicted.

showed morphological characteristics of cellular activation (i.e. cell flattening, increased adherence, and extension of pseudopodia). Strikingly, this was not observed in macrophages overexpressing sPLA₂ X (Fig. 4). To explain the absence of activation features in LPS activated sPLA₂ X overexpressing cells we measured cytokines released by the cells. The macrophages were incubated with LPS for 4 and 24 hours. TNF levels were strongly induced after 4h LPS, but sPLA₂ X overexpressing cells showed considerable less TNF release compared to control and wildtype cells (Fig. 5A). The same observation was made after 24h (data not shown). Real-time quantitative PCR showed a similar effect on TNF gene expression (data not shown). In addition to TNF, the anti-inflammatory cytokine interleukin-10 (IL-10) was also measured. IL-10 levels were detectable after 24 hours of LPS and a two-fold increase was observed in the sPLA₂ X overexpressing cells compared with control and wildtype cells (Fig. 5B). These data show that sPLA₂ X expression results in a more anti-inflammatory phenotype after LPS activation of macrophages.

To investigate the prostaglandins that can be generated from the AA that is released by the action of sPLA₂ X, PGE₂ and 15d-PGJ₂ were measured upon activation

Fig. 6. PGE₂ and 15d-PGJ₂ release during activation of RAW264.7 cells expressing hu-sPLA₂ X. RAW264.7 cells expressing hu-sPLA₂ X after 4 and 24h activation with LPS. A: Levels PGE₂ and B: levels 15d-PGJ₂ with competitive ELISA. The results of a wildtype (wt) cell line, a control cell line (ctrl), negative for hu-sPLA₂ X expression and two hu-sPLA₂ X expressing cell lines (grX) are depicted.

with LPS (250 ng/ml). PGE₂ and 15d-PGJ₂ levels in non LPS-treated macrophages showed no differences between the cell lines (Fig. 6). After 4 hours LPS activation, PGE₂ levels increased, with more PGE₂ being produced by the overexpression cell lines. At 24 hours activation, PGE₂ levels in sPLA₂ X overexpressing macrophages increased more than two-fold compared to the wildtype and control macrophages. A similar pattern developed in 15d-PGJ₂, except that 15d-PGJ₂ levels did not show a potent induction after 4 hours. These results show that both PGE₂ and 15d-PGJ₂ levels increased under influence of LPS stimulation, and sPLA₂ X overexpression results in a strong increase in the release of these prostaglandins.

Discussion

sPLA₂ X has been implicated in atherosclerosis and immune functions (19, 20). We now demonstrate a two-faced function of macrophage-expressed sPLA₂ X. First, it can mediate the formation of foam cells by modification of native LDL resulting in enhanced uptake and cholesterol ester accumulation in macrophages. In addition, we show that activation of sPLA₂ X overexpressing macrophages results in an enhanced release of both PGE₂ and 15d-PGJ₂, which exert anti-inflammatory effects on macrophages. These data, combined with the previously described presence of sPLA₂ X in atherosclerotic lesions (20), make sPLA₂ X a potentially important player in atherosclerosis development.

The sPLA₂ X protein has previously been detected in macrophage derived foam cells (20) and spleen macrophages (19) by immunohistochemistry. However, as macrophages are capable of binding and internalizing sPLA₂ X protein (19, 24) it was not conclusive so far whether the enzyme originates from macrophages or from other cells. We now demonstrate sPLA₂ X mRNA expression in both C57/Bl6 bone marrow derived macrophages and the macrophage cell line RAW264.7 which implicates that macrophages are capable of producing sPLA₂ X themselves.

The regulation of the sPLA₂ X gene has not been fully elucidated, but we could not detect major changes in sPLA₂ X expression after LPS activation. In general sPLA₂ X production appears to be unaffected by inflammatory processes. For example, no differences have been reported in synovial lining cells and interstitial fibers between active and inactive RA sections (25) and in lung and diaphragm tissue during carageenin-induced rat pleurisy (18). However, no detailed investigations into the expression of sPLA₂ X in cultured cells treated with different inflammatory stimuli have been described. Also, analyses to characterize the promoter sequences of the sPLA₂ X gene have not been performed. Its strong capacity to release AA makes sPLA₂ X an interesting enzyme to study the role of phospholipase activity in inflammatory diseases such as atherosclerosis.

sPLA₂ X proved to be a potent modifier of LDL, which subsequently induces foam cell formation (20). Similarly, we observed a two-fold increase in cholesterol esters in macrophages overexpressing sPLA₂ X in the presence of native LDL. This confirms the potential role of sPLA₂ X in atherogenesis, as foam cell formation is a key factor in this disease (1, 26).

Our results show that sPLA₂ X overexpression alone is not sufficient to elicit an inflammatory response in macrophages. Both PGE₂ and 15d-PGJ₂ did not increase, despite the capacity of sPLA₂ X to release abundant quantities of AA, the substrate

for prostaglandin production. However, prostaglandin production is also dependent on the cyclooxygenases (27). Cyclooxygenases-1 and 2 (COX-1 and COX-2) convert AA into PGH_2 , which is the substrate for a whole myriad prostaglandins, each catalyzed by a specific prostaglandin synthases (28). COX-2 is induced in response to inflammatory stimuli, such as LPS (19, 29). In our experiments, the limited availability of COX-2 in unstimulated macrophages (30), and thus limited PGH_2 production, will restrain PGE_2 and 15-d-PGJ_2 production, even though sPLA_2 X overexpression delivers sufficient AA substrate. Only in combination with LPS COX-2 is induced and the AA generated by sPLA_2 X overexpression shows its anti-inflammatory potential. Both PGE_2 and 15-d-PGJ_2 levels increased significantly more in sPLA_2 X transfected macrophages compared to non-transfected, and this was accompanied by a strong anti-inflammatory change in cytokine production, with reduced TNF secretion and increased IL-10 production.

PGE_2 is a potent anti-inflammatory prostaglandin, that exerts its actions through specific PGE_2 receptors (31) and is the principal prostaglandin produced in LPS stimulated macrophages (32). PGE_2 is synthesized by membrane bound PGE -synthase in response to pro-inflammatory interleukines such as IL- 1β and TNF (33, 34). PGE_2 provides a feedback mechanism in macrophages that regulates and reduces inflammatory responses (35, 36). This is illustrated by the fact that LPS induced PGE_2 reduces TNF production, effectively limiting the inflammatory response (37). In the same way, it reduces the effects of macrophage activation by zymosan through down-regulation of TNF and IL-10 up-regulation (38). Whereas the anti-inflammatory properties of PGE_2 are well established, 15-d-PGJ_2 is emerging as an even more potent anti-inflammatory prostaglandin (39, 40). 15-d-PGJ_2 is a metabolite of prostaglandin D_2 (PGD_2), with no specific synthase known for its formation (28, 41). PGD_2 and 15-d-PGJ_2 are known to play a role in inflammatory resolution and are produced after the onset of PGE_2 production (42). This supports our observed delay 15-d-PGJ_2 in respect to PGE_2 . 15-d-PGJ_2 may exert its anti-inflammatory properties through inhibition of the NF- κB pathway (43-45). RAW264.7 macrophages have been reported to produce PGD_2 in response to LPS treatment, while COX-2 inhibition diminishes PGD_2 production (46).

Based on these anti-inflammatory properties of PGE_2 and 15-d-PGJ_2 we conclude that sPLA_2 X overexpression in macrophages provides increased AA quantities to the prostaglandin synthesis pathway, which depends on COX-2 and is activated under influence of an inflammatory stimulus like LPS. As such, sPLA_2 X provides an anti-inflammatory feedback mechanism through the production of anti-inflammatory prostaglandins, which reduce macrophage activation. This may have consequences for therapeutic interventions based on COX-2 suppression. COX-2 specific inhibition has the potency to eliminate the anti-inflammatory effects of sPLA_2 X in macrophages. In the light of the recent controversy around COX-2 selective inhibitors, its use in macrophage driven inflammatory diseases should be investigated more in detail. Certainly as COX-2 is the limiting factor in prostaglandin production and is expressed in chronic inflammatory diseases and during inflammatory resolution (42, 47).

Our results show that sPLA_2 X contributes to foam cell formation and at the same time reduces macrophage activation through enhancing PGE_2 and 15-d-PGJ_2 pro-

duction. Because both foam cell formation and inflammation are pivotal in this disease, the role of sPLA₂ X in atherosclerosis is ambiguous. Future in vivo experiments with mouse models overexpressing sPLA₂ X in atherosclerotic lesions may elucidate the role of this enzyme in atherosclerosis in more detail.

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Chapter 5

Phospholipase group X overexpression induces fatal pulmonary inflammation and protein accumulation in mice.

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In preparation

Abstract

Secreted phospholipases A_2 are a large family of enzymes, characterized by the hydrolysis of phospholipids at the sn-2 position, releasing free fatty acids (FFA) and lysophospholipids (lysoPL). Secreted phospholipase A_2 group X (sPLA $_2$ X) is one of the most potent phospholipase enzymes in mammals, capable of hydrolyzing intact cell membranes. sPLA $_2$ X has been shown in atherosclerotic lesions and lungs. In this paper, we studied the role of sPLA $_2$ X in vivo. We created a macrophage specific sPLA $_2$ X overexpression mouse model to investigate the role of sPLA $_2$ X in atherosclerosis. Our results showed a potential involvement in inflammatory lung disease. Mice expressing sPLA $_2$ X in their macrophages develop fatal lung pathology, with massive macrophage invasion and additional protein accumulation. This study emphasizes that phospholipase activity affects a broad range of organs. We conclude that more refined models with sPLA $_2$ X expression would be a valuable tool to elucidate the role of phospholipase activity in inflammation. Both vascular and pulmonary research can potentially benefit from future phospholipase models.

Introduction

Phospholipases are a large group of related enzymes. Typical is their hydrolysis of phospholipids at the sn-2 position, resulting in the liberation of free fatty acids and lysophospholipids, which are involved in various inflammatory processes (1). Phospholipases received a lot of attention in the last decades and are often associated with chronic and acute inflammation (2, 3). However, the exact role of phospholipases in inflammation and atherosclerosis is still not fully elucidated. In this study, our aim was to establish a novel model for phospholipase overexpression in atherosclerosis. For this, we developed a mouse model with macrophage specific overexpression of phospholipase A_2 group X (sPLA $_2$ X).

sPLA $_2$ X was discovered in 1997 (4) and is present in many organs, such as spleen, thymus, testis, and lung of humans, mice, and rats (4-6). *Interesting is the detection of sPLA $_2$ X in macrophages in atherosclerotic lesion of high fat-fed apolipoprotein E knockout mice and has a strong capacity to induce foam cell formation (7).* In general, sPLA $_2$ X is a very potent enzyme and, in contrast to most other mammalian phospholipases, sPLA $_2$ X acts on intact cell membranes (8, 9).

The regulation of the sPLA $_2$ X gene has not been fully elucidated, but in a previous study we could not detect major changes in sPLA $_2$ X expression after LPS activation (Ghesquiere et al. 2006, chapter 4). In general sPLA $_2$ X production appears to be unaffected by inflammatory processes. For example, no differences have been reported in synovial lining cells and interstitial fibers between active and inactive RA sections (10) and in lung and diaphragm tissue during carageenin-induced rat pleurisy (5). However, no detailed investigations into the expression of sPLA $_2$ X in cultured cells treated with different inflammatory stimuli have been described. Also, analyses to characterize the promoter sequences of the sPLA $_2$ X gene have not been performed.

We have previously shown (Ghesquiere et al. 2006, chapter 4) that sPLA $_2$ X overexpression in macrophages elicits an enhanced anti-inflammatory response after LPS

addition. In these macrophages, sPLA₂ X increased the production of arachidonic acid (AA), which is converted to prostaglandin E₂ and 15-deoxy- Δ 12,14-prostaglandin J₂, both known to exert anti-inflammatory properties in macrophages. The general phospholipase A₂ IIA (sPLA₂ IIA) overexpression (11) and a macrophages specific overexpression model (12), did not show generalized inflammation.

To study the role of sPLA₂ X in inflammation, we developed an in vivo mouse model with macrophages specific overexpression. Establishing an sPLA₂ X mouse model with macrophages specific expression proved difficult. We observed lung problems in the transgenic mice, with massive macrophage invasion, thickening of the interstitium and accumulation of proteins in the lungs.

Materials and methods

Construct

Human sPLA₂ X cDNA containing the sPLA₂ X cDNA starting at the second methionine start site (met-32) (4) (a generous gift from Dr. Cupillard), was isolated from a pRc/CMV vector (Invitrogen). The met-32 fragment was selected using convenient restriction sites and inserted in a pcDNA 3 expression vector behind a CD68 promoter construct (2.9 kb CD68 + IVS-1 sequence (13), a generous gift of Dr. D.R. Greaves). The CD68 - sPLA₂ X in pcDNA3 plasmid was verified by sequencing the start and the end of the met-32 construct and boundaries of the CD68 promoter region. Additional verification was performed using various restriction enzymes and subsequent gel electrophoresis.

Mouse

The complete 4.56kb fragment (CD68 + sPLA₂ X) was isolated with electrophoresis in agarose gel, followed by electroelution. Oocyte injection to generate transgenic mice was performed according to standard procedures (14) in C57BL/6J. Two transgenic mice were obtained. All experiments were approved by the local committee for Animal Welfare. The animals were held under pathogen-free conditions. Microbiological status was checked regularly by routine serological, bacteriological and histological procedures.

Transgenic mice were identified by polymerase chain reaction (PCR) with human sPLA₂ X specific primers. Additionally we performed southern blot analysis. Tail DNA was isolated, digested with *Bam*HI and labeled with ³²P tagged sPLA₂ X probe. To estimate the integrated copy number, linearized CD68 sPLA₂ X construct vector mixed in 1/1, 10/1, and 100/1 ratio with genomic DNA was included on the blot.

Histology

Whole mice were fixed with 10% formalin in PBS. After fixation, transversal section of the mice or of the organs were made and embedded in paraffin. Histological slides of three micron were stained with hematoxylin and eosin (H&E).

Results

To establish sPLA₂ X transgenic mice, three oocyte injection rounds were performed with the CD68 sPLA₂ X construct (table 1). Only three founder mice, of a total of 55 born, were positive by PCR. The success rate of this construct (5.5%) was compared with mice produced at the same time, and in the same facility under the same conditions, but with SRA-PFAFH and SRA-mA20 constructs. The results show that these constructs yielded 18.6%, respectively 8.9% transgenic mice. Moreover, the

Table 1

Construct	number of mice born	average nest size	PCR positive mice	Transgenesis success rate	Transmission (F1 and F2)
CD68 sPLA ₂ X	55	4.1	3*	5.5 %	0/3
SRA PFAFH	43	5.1	8	18.6 %	7/8
SRA mA20	45	5.0	4	8.9 %	4/4

Table 1. Number of transgenic mice born after oocyte injection rounds. The number of born after CD68 sPLA₂ X, SRA-PFAFH, and SRA-mA20 construct injections in the oocytes. * two died at ~3 weeks of age.

founder mice from these latter constructs mostly transmitted the transgenes to F1 and F2 (7/8 and 4/4, respectively). For the sPLA₂ X transgenic mice, the first one (female, founder #1) died before three weeks of age and unfortunately no material was obtained for pathological examination. Later on, a second founder mouse (male, #2) was obtained. Southern blot was performed to check the transgene and to estimate the copy number (Fig. 1). Two bands were visible in the transgenic mouse, from which the small fragment (538 bp) corresponds with the *Bam*HI sites in sPLA₂ X and the promotor and is also present in the linearized construct vector. The Southern blot indicates that a single copy of the transgene was integrated

Fig. 1. Southern blot. BamHI digested mouse DNA and controls (CD68 sPLA₂ X construct material) blotted with sPLA₂ X probe. Southern blot from 4 mice is shown on the left, at the right are controls with different construct / genomic copy number ratios.

in the genome of the mouse. This animal showed very poor growth and died at 3 weeks of age after which total histopathology was performed. The animal showed severe lung pathology (Fig. 2) in combination with focal rhabdomyopathy. The cause of death was an interstitial pneumonia with severe infiltration of alveolar macrophages and edema (Fig. 2). In a small part of the serratus dorsalis muscle, rhabdomyopathy was observed characterized by necrosis of the myocytes, infiltration of macrophages and giant cell formation. No abnormalities were found in any other organs or muscles. The third oocyte injection round finally delivered one

Fig. 2. Microscopy of the lung. A: Normal lung of a control C57BL/6 mouse (200x). B: Interstitial pneumonia of a sPLA₂ X mouse with massive infiltration of macrophages and granulocytes. Alveoli are filled with protein-lipid material laden macrophages (arrows) (200x).

transgenic mouse that exhibited no external signs of pathology and reached adulthood. Total offspring of this mouse was 62, with only one animal being positive for the transgene, indicating very poor transmission of the transgene. The only F1 animal also did not transmit the transgene to its offspring, from a total of 16 pups, all were negative for sPLA₂ X. Both transgenic mice from this line (i.e. the F0 and the F1 animal) were finally sacrificed and their pathology was carefully investigated. The lungs of these mice showed increased macrophage infiltration and signs of diffuse lung inflammation, indicating a similar but much milder phenotype as was observed in founder #2.

Discussion

Our results show that macrophage specific overexpression of sPLA₂ X leads to severe lung pathology with massive macrophage invasion, interstitial thickening and edema. There were no signs of systemic inflammation, bacterial or viral infections in these animals held under SPF conditions.

We had limited success in our attempts to obtain viable transgenic sPLA₂ X mice (table 1). In the first place only a few transgenic mice were born, despite repeated oocyte injection rounds. This is possibly caused by the fact that sPLA₂ X induced prostaglandin production can affect embryogenesis (15-17). Alternatively, some transgenic mice might have died shortly after parturition and were eaten by the mother. Thirdly, from the transgenic mice we were able to obtain, two founders died at early age of which one showed severe lung pathology (Fig. 2), while the founder that survived also showed some lung defects, albeit less severe, after being sacrificed. Possibly, the surviving mice had little sPLA₂ X production in the lungs due to a low transgene copy number or due to integration in a less active region of the genome. In addition, this mouse may have been chimeric for the transgene since only one out of 62 offspring was transgenic. However, founder #2, which died at early age, had only a single transgene copy (Fig. 1). We hypothesize that the genetic background inhibited the expression of the transgene in the surviving founder and in one of the offspring. As such, most of the transgenic off-

spring might have died before or shortly after birth and skewed the transmission rate from the expected 1/1 to 1/62.

While we did observe lung pathology, no signs of general inflammation were observed. We hypothesize that sPLA₂ X activity in the lung degraded the surfactant in these mice, leading to respiratory distress and eventually death due to pulmonary failure. In the normal mouse, healthy lung, surfactant is degraded by lysosomal phospholipase A₂ (LPLA₂), a specialized enzyme used by alveolar macrophages to process surfactant in their lysosomes (18-20). Surfactant turnover in the lungs is a delicate balance between production by alveolar type II epithelial cells and clearance by alveolar macrophages (20). In our sPLA₂ X overexpression model, the phospholipids component of surfactant may have been degraded at an unusual high rate due to the sPLA₂ X activity in macrophages (21). As such sPLA₂ X overexpression can tip the surfactant balance towards shortage, a condition associated with inflammatory lung diseases (3, 22).

Additionally, sPLA₂ X releases free fatty acids and lysophospholipids from surfactant, which are metabolized into inflammatory mediators. As result of this, sPLA₂ X can either promote as well reduce inflammation, depending on the produced eicosanoids. In the case of increased 5-lipoxygenase (5-LO) production, lung injury is enhanced (23, 24), while the production of prostaglandin PGE₂ and 15d-PGJ₂ has the potency to reduce inflammation in macrophages and other cells (25-27)(Ghesquiere et al. 2006, chapter 4). However, current data is not conclusive whether increased phospholipase activity in the lungs induces or enhances inflammation.

The respiratory failure we observed in one of the sPLA₂ X mice shows some similarities with what is known as acute respiratory distress (ARD), a disease related to pulmonary inflammation and surfactant degradation, and associated with increased phospholipase activity (28). sPLA₂ group IIA activity has been proposed as the cause of ARD (28), but this enzyme shows little activity on lung surfactant (22) and probably has little contribution to the disease itself (29, 30). Potential alternative candidates involved in the onset and progression of ARD are phospholipase group V and X, both are known as potent modifiers of lung surfactant (22) and are present in alveolar macrophages, alveolar epithelium and interstitium (3).

We propose a positive feedback loop for sPLA₂ X induced lung pathology and ultimately respiratory failure. In the initial phase, sPLA₂ X produced by macrophages disturbs the surfactant balance due to increased surfactant phospholipid degradation. The reduction in surfactant results in lung injury. At the same time, inflammatory mediators are being produced. The injury and inflammatory mediators elicit an inflammatory response and additional macrophages are attracted to the lungs. These can worsen the problems, when the newly arrived macrophages start to produce sPLA₂ X, increasing the pressure on surfactant and enhancing inflammation. While sPLA₂ X overproduction in macrophages can limit their inflammatory responses (Ghesquiere et al. 2005, chapter 4), massive sPLA₂ X release in the lungs is not sustainable and has the capability to develop into acute lung failure, as we have observed in our mice. Our mice with limited sPLA₂ X production, showed indeed increased macrophages in the lungs, but did not develop lethal lung pathology.

Additionally to inflammation, we observed protein-lipid laden macrophages in

the lungs (Fig. 2). We hypothesize that partially degraded surfactant in the lungs is ingested by macrophages attracted to the area. Whereas native alveolar macrophages have the required mechanism to properly degrade surfactant (20), the invading macrophages might lack this mechanism, leading to intracellular accumulation of the remainders of surfactant. To some extent, this protein-lipid accumulation has similarities to pulmonary alveolar proteinosis (PAP), a rare human disease caused by alveolar surfactant protein accumulation due to insufficient surfactant degradation (31, 32). In this respect, our sPLA₂ X mouse model delivers a model for protein accumulation, additionally to the current granulocyte macrophage colony stimulating factor (GM-CSF) deficient mouse model (33, 34). In the GM-CSF model, incomplete macrophage maturation limits surfactant clearance (35). It is very interesting to see that both surfactant degradation and accumulation can develop into protein accumulation, but it remains to be seen if our novel sPLA₂ X model is a suitable animal model for PAP.

Our initial goal was to create a mouse model to study the role of sPLA₂ X in atherosclerosis. We have not been able to determine the effects on arterial lesion formation, but instead showed that a sPLA₂ X overexpression model offers an interesting opportunity to investigate the role of phospholipases in lung pathology. Because it was hard to establish a viable mouse model with our sPLA₂ X overexpression construct, we propose alternative options, such as the use less potent variants of the CD68 promotor (13), a longer, less active sPLA₂ X fragment (4), or restricting expression to alveolar macrophages using the LPLA₂ promotor to drive sPLA₂ X. In conclusion, a more subtle phospholipase group X overexpression model would deliver a valuable tool for phospholipase studies both in the field of atherosclerosis and pulmonology.

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Chapter 6

Phospholipase expression in different stages of human atherosclerotic lesions.

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In preparation

Abstract

Atherosclerosis is an inflammatory disease initiated by the accumulation of low-density lipoproteins (LDL) in the vessel wall of the arterial system. Native LDL is not retained in the vessel wall, unless it is oxidized and enzymatically modified. One of the most prominent enzymes responsible for LDL modification is the family of secreted phospholipases A₂ (sPLA₂). The sPLA₂ are a large group of enzymes, from which several members are present in atherosclerotic lesions. However, little is known about the role and expression of these phospholipases during human plaque development. We therefore investigated the expression patterns of phospholipase A₂ group IIA (sPLA₂ IIA) and X (sPLA₂ X) and platelet activating-factor acetylhydrolase (PAF-AH) in human atherosclerotic lesions. sPLA₂ activity releases free fatty acids (FFA), such as arachidonic acid (AA), and lysophospholipids. Because AA can be metabolized into inflammatory eicosanoids, we included enzymes as 5-lipoxygenase (5-LO), cyclooxygenase-2 (COX-2) and prostaglandin E₂ synthase (mPGES) in our analyses.

Arterial plaque material from 13 patients was collected and classified into an early (intimal thickening/xantoma), advanced (thick fibrous cap atheroma, fibrocalcified) and ruptured (luminal thrombus/intraplaque hemorrhage) group based on the Virmani classification. mRNA was isolated and the expression of selected genes was quantified by realtime PCR. Our results show a strong decrease in sPLA₂ IIA expression in ruptured lesions, indicating a role in lesion stability. Conversely, PAF-AH expression levels were increased in ruptured lesions. The expression of the potent sPLA₂ X enzyme did not differ between the early, advanced and ruptured lesions. In the prostaglandin synthesis pathway, we observed increased COX-2 expression in the ruptured lesions, which was not accompanied by increased mPGES expression indicating that possibly other prostaglandins are produced in these lesions. 5-LO expression levels increased strongly in advanced and ruptured lesions, which may implicate leukotrienes (LTs) in atherosclerotic plaque severity.

The role of phospholipases in atherosclerosis is more complex than initially thought and the effect of their presence in atherosclerotic lesions depends largely on the closely connected eicosanoid synthesis pathways.

Introduction

In the last decades, it has become increasingly clear that lipid modification is an integral feature of atherosclerosis (1). Accumulation of low density lipoproteins (LDL) in the vessel walls is a hallmark of atherosclerosis (2). Crucial for LDL retention in the vessel wall is enzymatic modification. Subsequently monocytes are attracted and stimulated (1, 3). These monocytes differentiate into macrophages and transform into foam cells through unrestricted LDL uptake (1). Amongst the key enzymes known to modify LDL particles in the vessel wall are the phospholipases. Recent in vivo studies showed various pro-atherogenic properties of these enzymes (4-7).

At least 10 mammalian phospholipases are known, classified according structure, molecular weight and substrate preferences (8). Three sub-families are dis-

tinguished: the secreted PLA_2 s (sPLA_2), the cytosolic PLA_2 s (cPLA_2) and the Ca^{++} independent PLA_2 s (iPLA_2). Phospholipases hydrolyze phospholipids at the sn-2 position, and release free fatty acids (FFA), often arachidonic acid (AA), and lyso-phospholipids (lysoPL) (9). AA is the key substrate for the production of inflammatory mediators like prostaglandins, thromboxanes and leukotrienes.

Phospholipase A_2 group IIA (sPLA_2 IIA) has been shown in various organs, such as spleen, thymus, intestines, tonsils, bone marrow, and liver (10-12). At cellular level, the enzyme is present in hepatic cells, endothelial cells, smooth muscle cells and macrophages (13-15). sPLA_2 IIA is often considered a marker for inflammatory processes, and elevated plasma and serum levels are found during sepsis (16) and coronary heart disease (17). Mouse models expressing sPLA_2 IIA showed increased lesion size (5, 6), accompanied by increased collagen content (6).

sPLA_2 group VII, also known as platelet activating-factor acetylhydrolase (PAF-AH) or lipoprotein associated phospholipase A_2 , is associated with LDL and HDL in human plasma (18, 19). The enzyme plays a role in membrane repair through removal of oxidized fatty acids (20). PAF-AH can potentially inhibit LDL accumulation through LDL repair (20), but the released oxidized FFA can enhance inflammation (21). PAF-AH is expressed in macrophages (22), and is involved in anti-oxidative scavenging (21). Overexpression in mice results in reduced atherosclerosis susceptibility (23), but human PAF-AH activity is associated with increased coronary artery disease (CAD) (19, 24, 25). However, it is not fully elucidated whether the association of PAF-AH with CAD is cause or consequence. Despite this, recent cross-sectional studies (26) show that PAF-AH activity indicates increased risk of CAD independently of other established risk factors.

Structurally, phospholipase group X (sPLA_2 X) is related to both sPLA_2 group IB and IIA. sPLA_2 X is one of the most potent phospholipases (27, 28). In contrast to sPLA_2 IIA, sPLA_2 X elicits high AA release in cell cultures and does not bind

Fig. 1. Overview of the eicosanoid pathways and the production of oxidized free fatty acids. Phospholipase and eicosanoid pathways. Phospholipids are hydrolyzed by secreted phospholipases A_2 (sPLA_2) group IIA, V, and X resulting in the release of arachidonic acid (AA). AA is transformed into PGH₂ by cyclooxygenase (COX) and to leukotriene A₄ and B₄ by 5-lipoxygenase (5-LO). PG, prostaglandin; PGES, PGE-synthase; PGDS, PGD-synthase; 15d-PGJ₂, 15-deoxy- Δ 12,14-prostaglandin J₂; LT, leukotrienes; 15-HETE, 15-Hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroperoxyeicosatetraenoic acid. Additionally, PAF-AH liberates oxidized free fatty acids (ox-FFA) from oxidized phospholipids (ox-PL).

to extracellular matrix components (29). Phospholipase group X has been found in macrophage foam cells and vascular smooth muscle cells in the vessel wall of high fat-fed apolipoprotein E knockout mice (30). Recently we found sPLA₂ X expression in murine macrophages and have shown that sPLA₂ X overexpression induced potent foam cell formation (Ghesquiere 2006, chapter 4).

Phospholipase activity results in the release of free fatty acid, which are rapidly transformed into eicosanoids (31, 32). The cyclooxygenase (COX) enzymes are responsible for the initial step in eicosanoids production (33-35). Two major COX isoforms are known, with COX-1 being constitutively expressed, while COX-2 is inducible (32). Hormones, growth factors, inflammatory cytokines and bacterial endotoxins regulate COX-2 expression (32). Because COX-2 is expressed in atherosclerotic lesions (36, 37) and destabilized plaques (38) we included COX-2 in our study.

The lipoxygenase pathways convert AA in precursor molecules for leukotriene (LT) production. In humans 5-lipoxygenase (5-LO) and 15-lipoxygenase-1 (15-LO-1) are the most prominent lipoxygenases (39). The 5-LO pathway produces leukotriene A₄ (LTA₄), which is further metabolized to leukotriene B₄ (LTB₄) or other leukotrienes (LTC₄, LTD₄ and LTE₄). Previous studies showed a correlation between lesion severity and 5-LO concentrations (40) and it has been proposed that LTs produced through the 5-LO pathway trigger arterial wall inflammation and remodeling during atherosclerosis development (41). As such we decided to include this enzyme in our study.

Currently, little is known about the relation between phospholipases and atherosclerotic lesion severity. We therefore analyzed the expression of a set of carefully selected genes in plaque material obtained from patients with atherosclerosis. We categorized the lesions according to their severity. Three groups were created: early lesions, advanced lesions and ruptured lesions. mRNA was isolated from the material and quantitative real-time PCR was performed for the selected genes.

Material and Methods

Tissue was obtained from the Maastricht Pathology Tissue Collection (MPTC). Collection, storage and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (<http://www.fmwv.nl>). Tissues were processed as described previously (42). Tissue sections were snapfrozen for RNA isolation. Adjacent sections were fixed and embedded in paraffin. Based on haematoxylin-eosin stained sections (4 µm), the vessel wall was classified and classified according to Virmani et al. (43). Lesions with intimal thickening, intimal xanthoma, and pathological intima thickening were combined in a group of early lesions. Thick fibrous cap atheroma's, and fibrocalcified lesions were categorized as advanced plaques. Lesions with a luminal thrombus of intraplaque hemorrhage were labeled as ruptured.

The early and advanced lesion group contained material from 5 patients and the ruptured lesion group contained the material from 3 patients. Total RNA was isolated using Tri Reagent (Sigma), according to the manufacturers' protocol. RNA concentration was measured using the nanodrop (Witec AG, Germany). 0.5 µg total RNA was converted into cDNA. Oligo dT primers and RevertAid M-MuLV

reverse transcriptase (Fermentas) were used according to the manufacturers protocol. TAQMan analysis with SYBR Green was performed with the qPCR Master-mix Plus kit for SYBR green (Eurogentec) on 10 ng cDNA. The following primers were used:

sPLA ₂ IIA	F: GGCACCAAATTTCTGAGCTACA R: CCTTATCACACTCACACAGTTGACTTC
PAF-AH	F: AGCAACGGTTATTTCAGACTCTTAGTG R: ACTTCATCACCCAGTGGAAACA
sPLA ₂ X	F: TGGCAGTGCGTCAATCAGA R: TTGCACAACAGTTCTTGGCATT
5-LO	F: CGGCGATGTCGAGGTTGT R: TTTACGTCGGTGTTGCTTGAGA
COX-2	F: TCCCTCCCTTACCATCAAATCA R: TCCGCCGTAGTCGGTGTAC
mPGES	F: ACACGGAGGCCCCAGTA R: TAGTCGAAGCCCAGGAAAAGG

The primers were verified not to amplify genomic DNA material. Analysis was carried out on an ABI Prism 7700 Sequence Detector. The housekeeping genes cyclophilin-A, B-actin, and gapdh were used to correct for RNA concentration differences between the samples. All housekeeping genes gave the same results, and as such cyclophilin-A is used for the data presented in this paper. Expression levels in early, advanced, and ruptured lesions, obtained from the qPCR, were analyzed with the non-parametric Mann-Whitney test, significance levels of $p < 0.05$, in GraphPad Prism version 4.01 (GraphPad Software, CA, USA)

Results

Phospholipids are converted into a whole range of eicosanoids (Fig1.). In the production of these inflammatory mediators, the role of phospholipase activity is pivotal, as this delivers the AA substrate for to the prostaglandin and lipoxygenase pathways. We also examined the expression of COX-2 and mPGES in the prostaglandin pathway and 5-LO in the lipoxygenase pathway. PAF-AH is a special case due to its ability to remove oxidized free fatty acid moieties from phospholipids in oxidized LDL (oxLDL) and exhibiting sn-2 phospholipase activity.

The sPLA₂ IIA expression in ruptured lesions (Fig. 2A) is very little compared to advanced lesions (0.16 compared to 1.91, respectively; $p = 0.036$) and shows a large variation in the early lesions (SD=2.72 with an average of 2.22). PAF-AH (Fig. 2B)

Fig. 2. Relative expression levels of phospholipases in the lesions. 2A: secreted phospholipase A₂ group IIA (sPLA₂ IIA); 2B: platelet activating factory acetylhydrolase (PAF-AH) and 2C: secreted phospholipase A₂ group X (sPLA₂ X). * = $p < 0.05$.

Fig. 3. Relative expression levels of enzymes in the arachidonic acid processing pathway. 3A: 5-lipoxygenase; 3B: cyclooxygenase-2 (COX-2) and 3C: membrane bound prostaglandin E_2 synthase (mPGES). * = $p < 0.05$.

showed considerably (15-fold increase, $p=0.036$) more expression in ruptured lesions compared to early lesions. In advanced lesions PAF-AH levels were not significantly increased ($p=0.22$), with an expression level intermediate of early and the ruptured lesions. Phospholipase A_2 group X (Fig. 2C) did not reveal differences in expression levels and thus no relation with plaque severity could be observed. Little sPLA $_2$ X expression was present in all the lesions.

We investigated several enzymes downstream the eicosanoid pathways, which use the AA produced by phospholipase activity as substrate. The most remarkable results were in the 5-LO expression (Fig. 3A). 5-LO expression was increased in both the advanced and the ruptured lesions; with advanced lesions showing 5-LO expression levels two-fold of those measured in the early lesions ($P=0.032$). Ruptured lesions exhibited a three-fold increase compared to the early lesions ($P=0.036$). In addition, COX-2 expression in ruptured lesions (Fig. 3B) was 1.5-fold increased compared with the advanced lesions ($p=0.036$), but did not differ between early and advanced, and between early and ruptured lesions. Furthermore, the mPGES expression levels (Fig. 3C) showed no significant changes between the three groups, but we could observe a trend toward lower expression in advanced and ruptured lesions.

Discussion

The results show a strong reduction in sPLA $_2$ IIA expression in ruptured lesions, while PAF-AH expression on the other hand increased significantly in the ruptured lesions. Phospholipase group X did not show considerable changes in expression between the early, advanced and ruptured lesion groups. In the phospholipase related pathways, the potent increase in 5-LO expression was remarkable. The increase in COX-2 expression was not accompanied by an increase in mPGES expression, indicating that other prostaglandins than PGE $_2$ are activated.

The sPLA $_2$ IIA results are intriguing. The expression of the enzyme drops dramatically in ruptured lesions. We have previously shown that sPLA $_2$ IIA overexpression in atherosclerotic lesions improves plaque stability through increased collagen content (6). We therefore hypothesize that lesions low in sPLA $_2$ IIA expression have the tendency to become prone to rupture due to low collagen levels in these lesions. While not quantified, early and advanced lesions in our study that exhibited high sPLA $_2$ IIA expression all have a thick, fibrous cap and a stable phenotype. It has been previously shown that sPLA $_2$ IIA is associated with collagen fibers in

the vicinity of macrophage rich areas, lipid atheromas and damaged intima (15, 44). At the same time, sPLA₂ IIA is associated with inflammatory diseases (16, 45, 46) and sPLA₂ IIA plasma levels have been proposed as a marker for acute myocardial infarction (17). However, plasma levels are not necessarily related to sPLA₂ IIA presence in the atherosclerotic lesions, but are rather the result of a systemic inflammatory reaction to plaque rupture. As such, high sPLA₂ IIA plasma levels are indicative for acute coronary events as a result of plaque rupture, while sPLA₂ IIA in the lesions may have a preventive effect. Unfortunately the total number of patients in our study is too small to draw solid conclusions, but with the current knowledge therapeutic strategies involving sPLA₂ IIA inhibition are not recommended. Prior to such interventions a better understanding of the relation between sPLA₂ IIA and plaque composition is required.

The dramatic increase in PAF-AH in the ruptured lesion compared to the early lesions supports the earlier established association between PAF-AH, CAD, and atherosclerosis (19, 24, 25). However, these associations are made between PAF-AH plasma levels and CAD, and do not related CAD with expression and production in atherosclerotic lesions. PAF-AH is located on circulating LDL particles (26, 47), and thus accumulation of these particles in the vessel wall, as seen in atherosclerosis (2), can transport PAF-AH into the lesions. We now show that PAF-AH in atherosclerotic lesions can be produced locally, independent of plasma levels. Whether PAF-AH reduces lesion formation is not clear. PAF-AH can mediate and eventually enhance inflammation in atherosclerotic plaques through the release of oxidized phospholipids (21, 48), but is also capable to enhance anti-oxidative scavenging (21). Our results, in combination with the results published by other groups, show a potential active role of PAF-AH in atherosclerotic progression and rupture rather than being a mere risk marker, creating an opportunity for therapeutic strategies.

Phospholipase A₂ group X expression levels did not significantly differ between the early, advanced and ruptured lesions. Data available about the role of sPLA₂ X in inflammation is still not conclusive. Our previous in-vitro experiments revealed that the expression of sPLA₂ X is hardly affected by lipopolysaccharide (LPS) stimulation (Ghesquiere et al. 2006, chapter 4) and there are strong indications that sPLA₂ X is constitutively expressed under most circumstances. For example in human synoviocytes sPLA₂ X expression remains constant despite cell activation (49). Similar in rat models for ear edema and carageenin-induced pleurisy (50), no changes in sPLA₂ X expression occurred. However, strong individual differences in sPLA₂ X expression levels have been observed in human joints (49) and in a small explorative pilot experiment, we found large differences between mice in macrophage sPLA₂ X expression (data not shown). This suggests that sPLA₂ X can be under influence of environmental factors or the presence of genetic variations in expression levels. Although various cell types continuously produce sPLA₂ X in small quantities (50, 51), the potent enzymatic activity of the enzyme can severely affect the course of inflammation. Overexpression of sPLA₂ X in murine macrophages inhibits LPS induced inflammation through the production of anti-inflammatory prostaglandins (Ghesquiere et al 2006, chapter 4), while mice overexpressing the enzyme in the macrophages develop severe lung pathology (chapter 5).

The exact role of sPLA₂ X in atherosclerosis might not be fully elucidated at the moment; it is at least known to induce potent foam cell formation (30)(Ghesquiere et al. 2006, chapter 4). We recommend more detailed population studies on sPLA₂ X in relation to atherosclerosis and inflammation.

The lipoxygenase pathways convert AA, liberated by phospholipase activity, in precursor molecules for leukotriene (LT) production. The increased 5-LO expression levels observed in this study are supported by previous studies, showing a relation between lesion severity and 5-LO concentrations (40). High levels of 5-LO have been shown in macrophages and mast cells within atherosclerotic lesions (1, 40). While the role of 5-LO has not been fully elucidated, the leukotrienes produced in the 5-LO pathway are responsible for various pro-atherogenic processes. Leukotrienes induce the release of vascular endothelial cell growth factor (VEGF) (52), enhance the recruitment of neutrophil leukocytes (PMNs) (35) and induce monocyte adherence to the vessel wall (53). The importance of 5-LO in atherosclerosis is emphasized by the observation that mice lacking 5-LO expression are protected against atherosclerotic lesion formation (54). Similarly, blocking LTB₄, a metabolite from the 5-LO pathway, in mice reduces lesion formation (55). In a human population study, however, increased atherosclerosis was found in individuals with low 5-LO expression (56). The authors attribute this paradoxical finding to redundancy and interaction between the eicosanoid pathways as proposed by Serhan (57). Our data confirms the pro-atherosclerotic role of 5-LO.

Cyclooxygenase is a key enzyme in the regulation of inflammatory processes. The production of eicosanoids depends on the conversion of arachidonic acid to prostaglandin H₂ (34, 35). Increased COX-2 levels are often associated with increased inflammation and have been shown in atherosclerotic lesions (36, 37). During acute inflammation, COX-2 delivers the substrate for the production of PGE₂, a potent anti-inflammatory prostaglandin, that exerts its actions through specific PGE₂ receptors (58). PGE₂ is the principal prostaglandin produced in LPS stimulated macrophages (59) and reduces macrophage activation (60, 61).

Our results did not show elevated mPGES expression in advanced and ruptured lesions, while COX-2 expression increased in ruptured lesions. This can imply that additional PGH₂ is being produced under influence of COX-2, while the production of PGE₂ is not augmented. It could well be that other prostaglandin synthases are being upregulated and as such play a role in lesion development. Unfortunately, we have not been able to examine the expression of the whole range of prostaglandin synthases. For future research we would definitely recommend further exploration in this field. Genetically engineered mouse models for the prostaglandin synthases would deliver a valuable research-tool.

The number of genes explored in this study is far from complete. Future investigations should also include the secreted phospholipase A₂ group V (sPLA₂ V) and the cytosolic phospholipase group A₂ IVa (cPLA₂a). The human group V is closely related to group IIA (62), but is capable of acting on intact cell membranes (63, 64) and is enzymatically more potent (65). sPLA₂ V expression is induced during inflammation (66) and is potentially involved in atherosclerosis (67). cPLA₂a is important through its interaction with other phospholipases, and its capability to regulate inflammation (68). Downstream the phospholipid hydrolysis pathway,

other prostaglandin synthase genes as PGDS and PGIS are excellent candidates to include. This pilot study definitely reveals interesting findings for future research. The effects of sPLA₂ IIA on plaque stability, the effect of PAF-AH expression in the lesion and the absence of mPGES expression, while COX-2 is elevated are worth to investigate in more detail and with a larger patient population.

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Chapter 7

General discussion

Atherosclerosis is progressive disease, initiated through the accumulation of low-density lipoprotein (LDL) in the arterial wall (1). A crucial step is the oxidative and enzymatic modification of these LDL particles. Once modified, LDL is taken up by macrophages and promotes foam cell formation, a hallmark of atherosclerosis (2). Amongst the most potent enzymes capable to perform LDL modification are the phospholipases, which have been shown in atherosclerotic lesions and inflammatory diseases (3, 4). Several members of the phospholipases have been found in atherosclerotic lesions. This thesis is focused on the role of two members of the phospholipase A₂ gene family: phospholipase A₂ group IIA (sPLA₂ IIA) and group X (sPLA₂ X).

Phospholipase IIA and atherosclerosis

sPLA₂ IIA is associated with many inflammatory diseases, in particular coronary heart diseases (5). The enzyme is present in atherosclerotic lesions, but shows little activity on intact cell membranes (6, 7) and LDL particles (8). Nevertheless, the interaction between sPLA₂ IIA, LDL, and extracellular matrix components can induce LDL aggregation and promote atherosclerotic lesion formation (9-11).

We have been able to establish the pro-atherosclerotic properties of sPLA₂ IIA. We found a 2.5 fold increase in lesion size due to sPLA₂ IIA overexpression (chapter 3). However, the role of sPLA₂ IIA in lesion progression is complex. Phospholipase IIA does not only enhance lipid retention and plaque formation in the vascular system, we also found increased collagen content in atherosclerotic lesions. We hypothesize that sPLA₂ IIA modulates the extracellular matrix balance either through binding newly formed matrix components and subsequently reducing their degradation rate, or through inducing elevated matrix component production. In the latter case, phospholipid derived inflammatory mediators are likely involved. Primary candidate is the production of anti-inflammatory prostaglandin D₂ (PGD₂). PGD₂ is involved in inflammatory resolution, which is associated with collagen deposition (12, 13). The role of sPLA₂ IIA in lesion stability is supported with our findings in human atherosclerotic lesions. Ruptured lesions showed little sPLA₂ IIA expression compared to early and advanced lesions. We hypothesize that low levels of sPLA₂ IIA result in reduced collagen content and thus make these lesions prone to rupture. As such sPLA₂ IIA (over)expression does not enhance lesion severity, despite its contribution to plaque size, and has an ambiguous role in atherosclerosis.

The limited catalytic activity of sPLA₂ IIA on mammalian membranes suggests that this enzyme might have little impact on inflammation. However, sPLA₂ IIA can bind to the phospholipase receptor, activate MAPK, which activates intracellular production of AA and lysophospholipids by cytosolic PLA₂ (cPLA₂) (14, 15). As such, sPLA₂ IIA can indirectly induce the formation of inflammatory mediators, through a functional coupling between these phospholipases (16). Nevertheless, sPLA₂ IIA does have a catalytic function as it shows potent activity on bacterial membranes, rich in phosphatidylglycol (17, 18). As such the primary function of sPLA₂ IIA might be situated in the field of bacterial host defense. Tear fluid (19) for example, contains large amounts of this enzyme. The fact that IIA is often related to acute infections (sepsis (4)) makes its anti-bacterial function quite plausible. The

presence of sPLA₂ IIA in atherosclerosis might very well be a side effect of its role in the innate immune system. Pathways activated during bacterial infections are also present in atherosclerotic lesion formation. One of the prominent cell types involved is the macrophage, for which sPLA₂ IIA is an effective tool during host bacterial defense. An anti-bacterial response in atherosclerosis is not always without merits. Infections are involved in atherosclerosis and can induce exacerbation in plaque growth (20-23) and sPLA₂ IIA expression in atherosclerotic lesion is correlated with the presence of microbial pathogens (24).

Phospholipase A₂ group X and atherosclerosis

In contrast to sPLA₂ IIA, sPLA₂ X exhibits potent enzymatic activity on intact cell membranes (6, 25) and is in fact one of the most potent phospholipases in the human body with a catalytic efficiency comparable to cobra venom sPLA₂ (6, 26). Phospholipase group X is present in many organs and tissues throughout the human body. Phospholipase group X expression and regulation is not fully understood, but appears not to be altered during inflammation (27, 28). Its constitutive expression pattern indicates a primary function in delivering eicosanoid substrate to the cell. In our experiments with murine macrophages, we were unable to show changes in sPLA₂ X expression after LPS induced inflammation (Ghesquiere et al. 2005, chapter 4). The involvement of sPLA₂ X in atherosclerosis has not been fully elucidated, however, sPLA₂ X is present in macrophage foam cells in the vessel wall of high fat-fed apolipoprotein E knockout mice (29). We have shown sPLA₂ X expression in macrophages. sPLA₂ X renders LDL particles pro-atherogenic as our results have shown (chapter 4). Nevertheless, we have been unable to find differences in sPLA₂ X expression between early, advanced and ruptured human plaques (chapter 6). While the exact role of sPLA₂ X is not fully elucidated, our results indicate that sPLA₂ X is an important enzyme for the delivery of eicosanoid substrate. More detailed studies on the expression of this enzyme are necessary. The potent enzymatic activity of sPLA₂ X combined with absence of extracellular matrix binding makes this enzyme an interesting tool to study the pure enzymatic component of phospholipase activity in atherosclerosis. sPLA₂ IIA, for example, shows little activity on intact cells and native LDL, but is capable to modulate collagen deposition. It is not clear to which extent the enzymatic activity is responsible for the collagen increase and the retention of LDL. sPLA₂ X could potentially help to distinguish the enzymatic property from other effects. A similar strategy would be to alter the enzymatic domain on sPLA₂ IIA, by removing the His-Asp dyad in the enzymatic cavity. Making use of the natural occurring sPLA₂ IIA knockout mouse strains, such as C57/B6, holds little chance for success as sPLA₂ IIA functions can be taken over by the closely related sPLA₂ group V (30). Phospholipase A₂ group V (sPLA₂ V) is, similar to sPLA₂ IIA, known to bind heparan sulfate proteoglycan (25), a component of the extracellular matrix. Additionally, sPLA₂ IIA expression in atherosclerotic lesion is correlated with the presence of microbial pathogens (24), with little expression in pathogen free mice. Even under optimal conditions, the natural knockout model is not an optimal model to study atherosclerosis and exhibits almost no abnormalities (31).

We choose to create a mouse model with macrophage specific sPLA₂ X overexpres-

sion with the macrophage specific CD68 promotor. Unfortunately, our attempts have been hampered by the difficulty to obtain viable mouse lines. A potentially more successful approach might be the use of a less potent variant of the CD68 promotor (32), or to use a longer sPLA₂ X cDNA fragment, which is known to result in less potent sPLA₂ X production (33). Similarly, another promotor can be used as well; good candidates are the CD11b and the SR-A promotor. An additional option is to use an inducible promotor to drive sPLA₂ X expression, followed by bone marrow transplantation to limit sPLA₂ X expression to the myeloid system.

Phospholipases in the lung

Fortunately, our sPLA₂ X model delivered a limited number of mice that lived long enough to study its phenotype. In the lungs (chapter 5) we observed macrophage infiltration, indications of surfactant accumulation, and thickening of the alveolar walls with reduction of the alveolar lumen. There were no signs of infection in these mice and the areas around the bronchioli and trachea remained unaffected. As such a macrophage specific sPLA₂ X overexpression model did inadvertently deliver a model for lung pathology. A stable surfactant balance requires phospholipase activity for clearance of surfactant (34). Lysosomal phospholipase A₂ (LPLA₂) is considered to be responsible for surfactant clearance, although LPLA₂ knockout mice do not develop lung pathology (35). Redundant phospholipase activity in the lung delivered by sPLA₂ group V and X shows to be sufficient to maintain adequate clearance (35). A phospholipase often associated with lung pathology is group IIA. During acute respiratory distress (ARD), a potentially lethal pulmonary condition related to various infectious diseases, sPLA₂ IIA production in the lung increases considerably (36). Nevertheless, it is more likely that sPLA₂ V is the primary phospholipase responsible for ARD symptoms (37). This is supported by the absence of lung pathology in both our macrophage specific sPLA₂ IIA model and the general sPLA₂ IIA model (38). Similar to sPLA₂ IIA, sPLA₂ V is present in the lung and is up-regulated during inflammation (30, 39), but instead is a potent modifier of lung surfactant (37). We believe that a murine sPLA₂ X overexpression model can contribute to a better understanding of the role of phospholipases in the lung. A valuable addition would be a mouse model for LPLA₂ overexpression. Eventually, one can consider using the LPLA₂ promotor to deliver alveolar macrophage specific expression of sPLA₂ X.

PAF-AH and atherosclerosis

PAF-AH removes oxidized phospholipids from membranes, including LDL particles (40, 41). Elevated plasma levels of PAF-AH are associated with coronary artery diseases (CAD), but it is not clear if this is cause or consequence (42). In our research, we have found a dramatic increase in PAF-AH expression in the ruptured lesions compared with the early lesions. Apparently, PAF-AH expression is associated with unstable plaques. The exact mechanism is unknown, but the preference of PAF-AH for oxidized phospholipids has the potential to release large amounts of oxidized free fatty acids in the atherosclerotic lesions, thus promoting inflammation and the production of oxidative molecules (41, 43). Our re-

sults, in combination with the results published by other groups, show a potential active role of PAH-AH in atherosclerosis rather than being a mere risk marker. This creates an opportunity for therapeutic strategies focussed on the inhibition of PAH-AH activity. In our research group, we have been engineering a macrophage specific PAH-AH overexpression model. Unfortunately, our attempts have been unsuccessful so far, largely due to the low expression delivered by the selected promotor.

Phospholipases and inflammation

In vitro sPLA₂ X overexpression revealed anti-inflammatory activity in macrophages (chapter 4). As our results show, the pro-inflammatory TNF levels were significantly reduced as result of sPLA₂ X overexpression, while the anti-inflammatory interleukine-10 (IL10) response increased. This observation was accompanied by an increase in prostaglandin E₂ (PGE₂) and 15-Deoxy-Delta 12,14-prostaglandin J₂ (15d-PGJ₂) production, both known to reduce inflammatory response in macrophages. In particular the increase of the anti-inflammatory 15d-PGJ₂ is interesting, as 15d-PGJ₂ is nowadays recognized as a potent anti-inflammatory prostaglandin (44, 45). In the sPLA₂ X induced anti-inflammatory response, cyclooxygenase-2 (COX-2) plays an important role. COX-2 is the rate limiting enzyme in the production of prostaglandins during inflammation (46, 47), which explains why in absence of inflammation, sPLA₂ X does not enhance the production of prostaglandins. Based on our results, we conclude that increased AA production by sPLA₂ X enhances an inflammatory feedback mechanism that reduces macrophage activation (48, 49). PGE₂ and PGD₂/15d-PGJ₂ knockout and overexpression mouse models would be valuable to investigate the role of phospholipase activity in atherosclerotic lesions, in particular the regulation of inflammation.

Our findings could have serious implications for the use of COX-2 inhibiting therapies in atherosclerosis. COX-2 specific inhibition has the potency to eliminate the anti-inflammatory effects of phospholipase activity, and could thus increase macrophage activation. In the light of the recent controversy around COX-2 selective inhibitors, a better understanding of the prostaglandin pathway activation is vital.

Phospholipases during the course of atherosclerosis

We investigated the role of several phospholipases in human atherosclerotic plaques and made interesting observations. Future research should include more genes and a larger research population. Excellent genes for inclusion are phospholipase group V and cytosolic phospholipase IVa (cPLA₂a). The first because sPLA₂ V is present in atherosclerotic lesions and has been shown to induce foam cell formation (50), the latter for its known modulation of inflammation through interactions with other phospholipases (14, 51). More enzymes involved with the eicosanoids production, such as prostaglandin D₂ and 15-lipoxygenase, should be included in future investigations.

Therapeutic strategies

The involvement of phospholipases in various inflammatory diseases, including atherosclerosis, makes them a potential target for therapeutic intervention. However, the role of phospholipases is ambiguous. While some phospholipases are usable as marker for inflammatory processes, this does not always imply direct involvement. Care must be taken to distinguish cause and consequence.

It has repeatedly been shown that PLA₂ IIA plasma levels increase during acute and chronic inflammation. Blocking the enzyme offers a credible therapeutic option, but sPLA₂ IIA has anti-bacterial properties and as such plays an important role in the innate immunity. Reducing the activity of sPLA₂ IIA might therefore have adverse effects during bacterial infections. Our results confirm that sPLA₂ IIA plays a role in atherosclerosis and can enhance lesion formation. However, sPLA₂ IIA inhibition might reduce plaque stability and increase the risk to plaque rupture. In specific cases, such as acute respiratory distress (ARD), sPLA₂ IIA inhibition might be beneficial. High levels of sPLA₂ IIA in the lungs can exacerbate the symptoms.

High PAF-AH plasma levels are associated with increased risk for CAD. Additionally, our results show that the PAF-AH expression in ruptured lesions is strongly increased. As such, reducing PAF-AH levels in both the plasma and the atherosclerotic lesions might offer a good opportunity to prevent CAD and plaque rupture. However, at the moment, it is unclear if PAF-AH increase is cause or consequence (42). As such, we recommend transgenic models with cell specific PAF-AH overexpression to further investigate the role of PAF-AH in atherosclerosis.

The biological effects of phospholipase activity are closely connected with the eicosanoid pathways. Recent discoveries have shown that both pro- and anti-inflammatory mediators are produced from the same precursor molecules. Developing successful phospholipase based therapies require selective, local inhibition of specific phospholipases, possibly in combination with monitoring and directing the related eicosanoid pathways.

At present, however, the therapeutic strategies involving "phospholipases" do not use the potential that is offered by these pathways. In part, this is due to limitations of our current knowledge. One of the challenges for the future is to get a better understanding of lipid interactions in complex diseases such as atherosclerosis. Technological advantages have made it possible to investigate which genes are involved during various stages of atherosclerosis. This work, and the work from other groups, showed that the expression of several phospholipases is regulated during the formation of atherosclerotic lesions.

The combination of in vitro work, animal models and population studies reveal the functions of enzymes, such as phospholipases. However, to fully understand plaque development it is equally important to understand the environment in which the products of the expressed genes operate. The activity of phospholipids, for example, depends on factors as phospholipid composition, interactions with charged compounds, and presence of oxidized lipid components. Additionally, phospholipase activity results in the production of a whole range of eicosanoids, which interact with plaque components and modulates inflammation.

It would be of great value to track lipid pathways in the atherosclerotic plaque and

make so called 'lipid-signatures'. Combining lipid data with gene expression patterns should allow to detecting subtle variations in gene expression that amplify atherosclerotic plaque development. With the link between the common lipid profiles and genetic expression patterns established, it becomes possible to develop new therapeutic strategies that channel lipid pathways to the most beneficial outcome.

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Summary

Atherosclerosis is an inflammatory disease of the large arteries, characterized by the accumulation of modified low-density lipoproteins (LDL). Native LDL is not retained in the vessel wall, unless it is oxidatively and enzymatically modified. Amongst the most prominent enzymes responsible for LDL modification are the secreted phospholipases A₂ (sPLA₂), which hydrolyse phospholipids at their sn-2 position, releasing free fatty acids and lysophospholipids. As such, phospholipase activity can modify phospholipids in the LDL particles enhancing their pro-atherosclerotic properties. At the same time, the generated FFA and lysoPL products are converted into potent inflammatory mediators, which can also highly affect atherogenesis.

Several members of the phospholipases A₂ family have been shown in atherosclerotic lesions. In this study, the main focus was given to sPLA₂ group IIA (chapter 3 and 6), sPLA₂ group X (chapter 4, 5 and 6), and sPLA₂ group VII or platelet activating factor acetylhydrolase (PAF-AH) (chapter 6). Additionally we investigated enzymes crucial in the processing of the arachidonic acid, which is liberated as result of phospholipase activity (chapter 6).

To study the role of sPLA₂ IIA in macrophages during atherogenesis, transgenic mice were generated using the human sPLA₂ IIA gene and the CD11b promoter (chapter 3). We performed bone marrow transplantations to LDLR deficient mice to study sPLA₂ IIA in an atherosclerotic susceptible mouse model. After 10 weeks on a high fat diet, the mice overexpressing sPLA₂ IIA in macrophages showed 2.3 fold larger lesions as compared to controls. Examination revealed that sPLA₂ IIA expressing mice had increased collagen in their lesions, independent of lesion size. However, smooth muscle cells or fibroblasts in the lesions were not affected, neither did we find significant changes in T-cell quantities and cell turnover as result of sPLA₂ IIA overexpression. Our data clearly show that macrophage sPLA₂ IIA promotes lesion growth, but at the same time enhances collagen production in the plaque and thus fibrotic cap development, which suggests improved plaque stability. Another indication that sPLA₂ IIA contributes to plaque stabilisation was obtained in human atherosclerotic lesions (chapter 6), where sPLA₂ IIA expression was remarkably decreased in ruptures lesions when compared to advanced and early lesions.

In chapter 4 and 5 we studied the involvement of macrophage sPLA₂ X in atherosclerosis and inflammation. sPLA₂ X is much more active on cell membranes and LDL particles than sPLA₂ IIA. We have been able to show sPLA₂ X expression in murine macrophages. To investigate the role of sPLA₂ X in inflammation and atherosclerosis, we generated a macrophage specific construct, in analogy with our sPLA₂ IIA construct used in chapter 2. This time, however, we used the macrophage specific CD68 promoter instead of the CD11b promoter.

First, we used the CD68 sPLA₂ X construct to transfect RAW264.7 cell lines and we obtained several stable cell lines expressing the sPLA₂ X construct. Prior to performing experiments, phospholipase activity was measured to confirm the production of enzymatically active sPLA₂ X. We found that sPLA₂ X overexpression in macrophages strongly increased foam cell formation upon incubation with native LDL, which indicates that sPLA₂ X is capable to contribute to atherosclerotic lesion formation. The next step was to use these macrophage cell lines for

activation experiments (chapter 4). Our results revealed a novel role for sPLA₂ X, as sPLA₂ X overexpression inhibited lipopolysaccharide-induced macrophage activation, characterized by reduced cell adhesion, lower levels of TNF secretion and increased interleukin-10 production. These effects are likely mediated by increased prostaglandin E₂ and 15-deoxy- Δ 12,14-prostaglandin J₂ production, as our results show. Our experiments show a potentially two-faced role for sPLA₂ X in atherosclerosis, with promoting foam cell formation and at the same time reducing the inflammatory response in macrophages.

Besides in vitro experiments with sPLA₂ X overexpression, we decided to examine the role of sPLA₂ X in atherosclerosis formation with a transgenic mouse model. Unfortunately, it proved hard to establish viable mouse lines with the CD68 sPLA₂ X construct, despite numerous attempts. Only a few transgenic mice were born, which either died within weeks after birth, or failed to transfer the sPLA₂ X gene to their offspring. Nevertheless, we took the opportunity to examine the transgenic mice we managed to obtain. In the mice that spontaneously died we observed severe lung pathology, with massive macrophage invasion and additional protein and lipid accumulation. The mice that failed to transfer the sPLA₂ X gene showed much milder lung pathology, but also exhibited increased macrophage infiltration. We could not find signs of infections in our mice that could have produced this pathology.

While several phospholipases have been found in atherosclerotic lesions, such as sPLA₂ IIA and sPLA₂ X, little is known about the role and expression of these phospholipases during plaque development. We therefore investigated the expression patterns of sPLA₂ IIA, sPLA₂ X, and PAF-AH in human atherosclerotic lesions. In our analysis we included enzymes involved in pathways closely connected to phospholipase activity, such as 5-lipoxygenase (5-LO), cyclooxygenase-2 (COX-2) and prostaglandin E₂ synthase (mPGES) (chapter 6). Arterial plaque material from 13 patients was collected and classified into an early, advanced, and ruptured group. mRNA was isolated and the expression of selected genes was quantified by realtime PCR. Interestingly, our results showed a strong decrease in sPLA₂ IIA expression in ruptured lesions, indicating a role for this enzyme in lesion stability. While not quantified, the early, and advanced lesions exhibiting high sPLA₂ IIA expression levels had a thick, fibrous cap and a stable phenotype. This is nicely in line with our results with sPLA₂ IIA overexpression in mice (chapter 3), where we found increased collagen deposition due to sPLA₂ IIA overexpression. Conversely, PAF-AH expression levels were increased in ruptured lesions. Considering the previously established correlation between plasma PAF-AH and coronary artery disease, our results suggest that intra-plaque PAF-AH similarly contributes to lesion destabilisation. Our results, in combination with the results published by other groups, show a potentially active role of PAF-AH in atherosclerotic progression and rupture rather than being a mere risk marker. Interestingly, the expression of the sPLA₂ X did not differ between the early, advanced, and ruptured lesions. This is similar to what we have found in chapter 4, where we have shown that sPLA₂ X is not regulated by lipopolysaccharide (LPS) stimulation. There are strong indications that sPLA₂ X is constitutively expressed under most circumstances, although differences in basic expression levels between individuals can be of importance. As

such, sPLA₂ X expression patterns should be studied in larger population studies. Phospholipase activity results in the production of AA, which can be transformed and metabolised into a whole myriad of anti- and pro-inflammatory eicosanoids. As such, it is pivotal to understand which pathways are activated during and after phospholipase activity in the atherosclerotic plaque. Therefore we included several key enzymes in our study, COX-2 and mPGES from the prostaglandin synthesis pathway, and 5-LO from the leukotriene pathway. Our results are very interesting, as we observed increased COX-2 expression in the ruptured lesions, which did not come with increased mPGES expression. This might indicate that prostaglandins other than prostaglandin E₂ are produced in ruptured lesions. Because it is not clear to which extent this affects atherosclerosis and plaque stability, future research should expand to inclusion of other prostaglandin synthases, such as prostaglandin I₂ synthase (PGIS) and prostaglandin D₂ synthase (PGDS). The 5-LO expression levels in advanced and ruptured lesions were strongly increased, which confirms the involvement of leukotrienes in atherosclerotic plaque severity. This finding is supported by previous studies, where a correlation between lesion severity and 5-LO concentrations was found.

In conclusion: our results show that phospholipases, although they contribute to lesion growth, are not necessarily harmful due to their capacity to stabilize atherosclerotic lesions and reduce the detrimental inflammatory processes associated with atherosclerosis.

Samenvatting

Atherosclerose, ook wel bekend als 'aderverkalking', is de onderliggende oorzaak van veel hart- en vaatziekten. Kenmerkend aan deze aandoening is de stapeling van vetten en cellen in de vaatwand. Het gevolg hiervan is dat het vat nauwer wordt, hetgeen de bloedstroom kan belemmeren. Nog problematischer wordt het als de plaque scheurt en vervolgens stolsels gevormd worden die verstoppingen in het vaatstelsel kunnen veroorzaken. Vaak gebeurt dat in de coronaire vaten, waardoor de bloedvoorziening van de hartspeer in het gedrang komt, met een 'hartaanval' als gevolg.

Het onderzoek dat beschreven is in dit proefschrift richt zich op het ontstaan en de groei van atherosclerotische plaques. Het stapelen van vet, in de vorm van van LDL (low-density lipoproteins) in de vaatwand, en de opname ervan door macrofagen liggen aan de basis van plaquevorming. LDL deeltjes zijn verantwoordelijk voor vet- en cholesterol-transport van de organen naar de lever. Ze bestaan uit een eiwit, ingebed in een membraan van fosfolipiden, en zijn gevuld met cholesterol en lipiden. LDL deeltjes komen door diffusie in de vaatwand terecht en worden vervolgens opgeruimd door macrofagen. Door oxidatie en enzymatische activiteit kan het LDL gaan binden aan moleculen in de vaatwand en hoopt het LDL op. Daarnaast wordt de opname en verwijdering door macrofagen verstoord, en raken de macrofagen gevuld met vetdeeltjes en veranderen ze in 'schuimcellen'. Dit vormt de aanzet tot de vorming van een atherosclerotische plaque.

De voornaamste enzymen die LDL modifieren en LDL stapeling in de vaatwand en de vorming van schuimcellen veroorzaken zijn de secretore fosfolipasen. Deze enzymen breken fosfolipiden af die een hoofdbestanddeel vormen van celmembranen en LDL deeltjes. De hierbij vrijkomende vrije vetzuren (o.a. arachidonzuur) en lysofosfolipiden beïnvloeden ontstekingsprocessen, die dan weer een rol spelen in de ontwikkeling van atherosclerose.

In dit proefschrift wordt vooral de aandacht gegeven aan secretair fosfolipase-A₂ groep IIA (sPLA₂ IIA) (hoofdstuk 3 en 6) en groep X (sPLA₂ X) (hoofdstuk 4, 5 en 6). In mindere mate komt ook groep VII (hoofdstuk 6), ook wel bekend als (bloed)plaatjes activerende factor acetylhydrolase (PAF-AH) aan bod. Daarnaast hebben we in hoofdstuk 6 enzymen bestudeerd die arachidonzuur omzetten in ontstekingsmediatoren.

Om de rol van sPLA₂ IIA in atherosclerose te onderzoeken (hoofdstuk 3) hebben we een transgene muis gemaakt met het sPLA₂ IIA gen van de mens. Om de sPLA₂ IIA productie te beperken tot de atherosclerotische plaques is het gen voorzien van een macrofaag specifieke promotor. Vervolgens is het beenmerg van deze muizen getransplanteerd naar een muismodel voor atherosclerose: de LDL receptor deficiënte muis. De muizen werden vervolgens 10 weken op een vetrijk dieet gezet. Na afloop stelden we vast dat de muizen die sPLA₂ IIA beenmerg hadden gekregen gemiddeld 2.3 maal grotere atherosclerotische lesies hadden dan muizen die gewoon beenmerg ontvangen hadden. Niet alleen waren de lesies groter, ze bevatten in verhouding veel meer collageen. Dit is interessant omdat collageen een plaque sterker en stabiel maakt, waardoor deze minder snel scheurt. In het aantal gladde spiercellen, fibroblasten, T-cellen en celdelingen waren er geen verschillen. De resultaten van dit experiment laten zien dat sPLA₂ IIA de groei van atherosclerotische plaques bevordert, maar deze eveneens stabiel maakt. Dat

sPLA₂ IIA de stabiliteit van plaques beïnvloedt komt overeen met de vaststelling dat opengescheurde humane plaques aanzienlijk minder sPLA₂ IIA tot expressie brachten dan vroege en gevorderde lesies en daardoor mogelijk minder stabiel waren (hoofdstuk 6).

In hoofdstuk 4 en 5 worden de resultaten behandeld van onze experimenten met sPLA₂ X. Dit enzym is vele malen actiever op celmembranen en LDL dan sPLA₂ IIA. sPLA₂ X komt voor in atherosclerotische plaques en macrofagen. Door sPLA₂ X mRNA aan te tonen lieten we zien dat sPLA₂ X ook daadwerkelijk door macrofagen geproduceerd wordt. Om de rol van sPLA₂ X te kunnen onderzoeken werd het humane sPLA₂ X gen voorzien van een macrofaag specifieke CD68 promotor en ingebracht in muismacrofaag cellijnen, zodat deze grote hoeveelheden sPLA₂ X begonnen te produceren (hoofdstuk 4). Het eerste experiment met deze macrofagen bestond erin om LDL aan het kweekmedium toe te voegen. Hieruit bleek dat sPLA₂ X schuimcelformatie sterk bevorderde en zo kan bijdragen aan de vorming van atherosclerotische plaques. De volgende stap was het bestuderen van de ontstekingseigenschappen van deze sPLA₂ X macrofagen. Hiertoe werden de macrofagen gestimuleerd met lipopolysaccharide. De resultaten lieten zien dat sPLA₂ X de ontstekingreactie in macrofagen afremt. Zo was het TNF niveau lager dan in normale macrofagen, terwijl de interleukine-10 concentratie verdubbelde. sPLA₂ X bleek te zorgen voor een toename in de productie prostaglandin E₂ en 15-deoxy- Δ 12,14-prostaglandin J₂ in geactiveerde macrofagen. De toename van deze ontstekingsremmende prostaglandines verklaart de ontstekingsremmende werking van sPLA₂ X. Onze in vitro proeven tonen aan dat sPLA₂ X atherosclerose kan bevorderen door schuimcelformatie, maar daarnaast de ontsteking in atherosclerotische plaques kan remmen.

De logische volgende stap was het maken van een transgene sPLA₂ X muis in analogie met onze sPLA₂ IIA muis (hoofdstuk 5). Dit bleek moeilijker dan verwacht. Er werden slechts 3 transgene muizen geboren, waarvan er 2 op jonge leeftijd stierven. De overlevende muis gaf het gen nauwelijks aan het nageslacht. De longen van een van de gestorven muizen bevatte veel macrofagen, waren sterk oedemateus en vertoonden lipide- en proteïnestapeling. De transgene muizen die niet op jonge leeftijd stierven hadden een veel mildere vorm van longpathologie. We konden geen sporen van infectie vaststellen. Waarschijnlijk verstoorde sPLA₂ X de longsurfactant balans door afbraak van het surfactant. Dit veroorzaakte een ontstekingsreactie en stapeling van surfactant residuen. De conclusie is dat macrofaag specifieke sPLA₂ X overexpressie voor ernstige longproblemen kan zorgen via afbraak van surfactant.

Er is nog maar weinig bekend over het verband tussen de verschillende fosfolipasetypen en de ernst en samenstelling van een atherosclerotische plaque. Om dit beter in kaart te brengen hebben we de expressie van sPLA₂ IIA, sPLA₂ X en PAF-AH in humaan plaque materiaal gemeten (hoofdstuk 6). Atherosclerotisch materiaal van 13 personen werd verzameld en geclassificeerd in vroege, gevorderde en gescheurde plaques. Uit dit materiaal is RNA geïsoleerd en is vervolgens de expressie van de genen gemeten met realtime PCR. Onze resultaten laten zien dat sPLA₂ IIA expressie sterk afneemt in gescheurde plaques, hetgeen aangeeft dat dit enzym een rol speelt in plaque stabiliteit. Bovendien bleek dat

vroege en gevorderde plaques met een hoge sPLA₂ IIA expressie een stevigere structuur hadden. Het expressieniveau van PAF-AH daarentegen nam sterk toe in gescheurde plaques. De relatie tussen een hoge PAF-AH plasma waarden en coronair vaatlijden is al langer bekend, maar onze resultaten laten zien dat PAF-AH productie in de atherosclerotische plaque negatieve gevolgen kan hebben. Het lijkt erop dat sPLA₂ X productie niet beïnvloed wordt door de samenstelling van de plaque, aangezien er geen verschillen vastgesteld werden in vroege, gevorderde en gescheurde plaques. Er zijn aanwijzingen dat sPLA₂ X expressie per individu verschilt en zo een factor kan zijn in atherosclerose vorming. Hier is echter meer onderzoek voor nodig.

We hebben eveneens gekeken naar enzymen die het arachidonzuur, dat vrijkomt bij fosfolipase activiteit, omzetten in ontstekingsregulerende eicosanoiden. Cyclooxygenase-2 (COX-2) expressie was toegenomen in de gescheurde plaques, terwijl PGE₂ synthase (mPGES) expressie niet verhoogd was. Dit is interessant, aangezien COX-2 het substraat levert voor PGE₂ productie en deze twee enzymen vaak tegelijkertijd actief zijn. Waarschijnlijk spelen andere prostaglandine-synthases een belangrijke rol in gescheurde plaques en kunnen deze bijdragen aan het scheuren van de plaque. De expressieniveau's van 5-lipoxygenase (5-LO) waren toegenomen in zowel de gevorderde lesies als ook in de gescheurde lesies. Deze bevinding bevestigt de relatie tussen de ernst van de atherosclerotische plaque en de concentratie 5-LO.

De resultaten in dit proefschrift laten zien dat fosfolipasen kunnen bijdragen aan de groei van atherosclerotische lesies, maar eveneens de ernst van deze lesies kunnen verminderen door stabilisatie en het remmen van de ontsteking. Daarnaast laten onze resultaten zien dat de rol van fosfolipasen in het lichaam niet los kunnen worden gezien van de eicosanoïd routes.

Dankwoord Acknowledgements

Promoveren doe je niet alleen. Daarom wil ik alle collega's, kennissen, vrienden en familie die me de afgelopen jaren gesteund hebben bedanken. De moeilijkheid van een dankwoord schrijven is niemand te vergeten. Onvermijdelijk heb ik dat toch gedaan en daarom bedankt aan allen die ik vergeten ben!

Marten, mijn promotor, graag wil ik je bedanken voor het vertrouwen dat je in mij hebt gehad. Misschien hebben we elkaar niet altijd even goed begrepen, maar dat ligt achter ons. De afgelopen periode heb ik steeds het gevoel gehad dat je achter mij stond. Dat heeft mij veel geholpen en mij de moed gegeven mijn promotie te voltooien. Dank je Marten!

Beste Menno, je wist altijd het beste uit mijn resultaten te halen. Iedere keer als ik dacht dat er niets uit leek te komen, zag jij er toch nog iets in dat als basis kon dienen voor nieuwe experimenten. Je enthousiasme en optimisme heeft mij uiteindelijk veel geholpen. Ook waardeer ik dat ik altijd je kamer kon binnenlopen, ook als het je niet uitkwam. Dank je Menno dat je mijn co-promotor was!

Er is de afgelopen jaren veel veranderd bij Moleculaire Genetica. Toen ik eind 2000 als AIO begon waren we maar met handvol mensen. We konden een lab gebruiken bij Moleculaire Celbiologie en we hadden een paar zitkamers aan de de andere kant van de gang. Bij 'even naar het lab lopen' lag de nadruk dan ook op het lopen. Nu is de groep aanzienlijk groter. Samen met mijn mede-sardientjes heb ik met plezier de AIO kamer gevuld. Graag wil ik hen persoonlijk bedanken.

Daphna, rustig, vriendelijk en hulpvaardig, kortom ik heb je ervaren als een fijne collega.

Beste Iris, de tijd dat we samen op een kamer zatten is al weer jaren geleden. Het was vast wennen om met een slakkenman opgescheept te zitten. Bloemen op jou ijsdoos en een slak op de mijne, zo wisten van wat van wie was. Dank je voor die fijne tijd.

Kristiaan, jij als echte Belg op ons lab. Je hebt nu versterking van Pieter gekregen, misschien dat Patrick daar zijn pijlen op gaat richten, alhoewel... Ik ga je hier niet de zatte vriend noemen, aangezien ik je zo nog niet heb meegemaakt, maar toch bedankt ons ermee bekend te maken.

Mathijs, we wisten elkaars rommel goed in evenwicht te houden. Zo ook op de labtafel. Soms maakte ik me ongerust als er weer eens een ongedefinieerde vloeistof de labtafelgrens overschreed, maar dat zijn nu zorgen voor Pieter. Af en toe begon je te lachen achter je computer, dan wisten we dat je weer een goede cartoon op je scherm had staan. Het was fijn je als collega te mogen hebben, en wie weet kom ik je nog wel eens in een bos tegen, met een hele resem scoutjes in je kielzog.

Petra, jij was een bijzonder plezierige collega. Graag wil ik je bedanken voor de goede gesprekken die ik met je heb gehad, je vrolijkheid en natuurlijk ook voor mij goed op de hoogte te houden van het wel en wee van ex-studiegenootjes.

Pieter, er is te weinig tijd geweest om je goed te leren kennen, maar ik heb er alle vertrouwen dat je de Belgen eer aan zult doen. Maak je geen zorgen, dat kan ook zonder slakken, GSM's in de oven en experimenteren met droogijs.

Sander, jij mag niet ontbreken in dit dankwoord. Er hangen hier nog steeds satirische krantenknipsels op de deur, waarbij ik meteen ook aan je lach moet

denken. Misschien een variant op Andy Warholds techniek: "I leave little notes, so they'll remember me"? In ieder geval bedankt voor alles.

En natuurlijk ook Yana, bedankt. Mijn Russisch is nog steeds niet voldoende om er ook maar iets van te verstaan. Gelukkig ben je ook het Engels en het Nederlands machtig, waardoor een goed gesprek bij tijd en stond toch mogelijk was.

Naast mijn kamergenoten wil ik ook graag de volgende mensen bedanken voor hun steun en gezelligheid.

Danielle, je verhaal over katten die de restjes eieren opaten na het slachten, ik moet er nog om lachen. Je directe en open stijl hebben me meermaals aangenaam verrast!

Frank, je hebt me altijd opgewekt gegroet als we elkaar tegenkwamen. Kleine boompjes en Ajax, daar associeer ik je mee. Ik vermoed dat de eerste je minder in de steek lieten dan de laatste.

Guillaume, de liefhebber van Oosters schoon, timmerman en bovenal fijne collega. Ik kon altijd bij je terecht, en heb daar ook regelmatig gebruik van gemaakt. Dank je voor je steun en luisterend oor!

Beste Inge, eerst student, daarna metgezel op het lab en nu mijn paranimf. Ik heb je vaak teleurgesteld als ik weer eens niet wist welke band het nummer op de radio aan het spelen was. Voor mij was het altijd een geruststelling wanneer je een proef voor me uitvoerde. Immuno's, facsen, het is allemaal in goede handen bij jou. Het was fijn en gezellig om met je te mogen samenwerken!

Marion, vaak hebben we samen boven muis en microscoop gehangen. Dat waren fijne momenten waar ik met plezier aan terugdenk.

Monique, je 'Limburgs' toverde meermaals een glimlach op mijn mond. Ook je meezingen met de radio was aangenaam om te horen. Je aanpak en stijl heb ik als zeer leerrijk ervaren. Proeven in je handen leggen is zoiets als geld in een kluis stoppen, je hoeft je er geen zorgen meer om te maken. Het was prettig om met je samen te werken.

Patrick, het was fijn om samen met je experimenten te doen. Je was nooit te beroerd om alles extra goed uit te leggen en altijd bereid om te helpen. Dat je af en toe wat steken onder water gaf heb ik er graag bijgenomen.

Will, bedankt voor de fijne tijd. Hopelijk hebben we wat te vieren op 16 mei 2007!

Wilma, snel lopen over de gang met een bundel papieren onder de arm. Het is een van de eerste beelden die bij me opkomen als ik aan je denk. Graag wil ik je bedanken voor al je hulp.

Verder wil ik ook graag bedanken: Anne, Arjan, Bianca, Celine, Claudia, Debby, Elena, Ellen, Hanneke, Jan, Joost, Juliëtte, Le, Margot, Maurice, Mohammed, Nana, Olivier, Patrick J., Paula, Pierre & Annie, Raymond, Ronald, Ronit, Rori, Roy, Sofia, Stefan, Stefanie, Susan, Ton, Vivian, Wiel, Willem, Wino, Yana en Yvonne.

Beste Jorg alias JorgOS, de vele avonden filmpjes kijken, robotten, fantaseren, plannen maken en beestjes kweken, ik hoop dat we dat nog lang kunnen doen. De afgelopen jaren zijn al die dingen een aangename afwisseling geweest op het labwerk. Hoewel, een lab thuis zouden we ook kunnen gebruiken, niet?

Maarten, ik ben de tel kwijtgeraakt, maar de talrijke nerdetentjes voorzien van

de nodige maatschappelijke discussies en geleuter waren momenten waar ik naar uitkeek. Misschien dat de lotgevallen van Monster, triops en Pic er af en toe te veel aan waren. Ik wil je graag bedanken voor de steun ik ik van je heb gekregen.

Beste Peter, we kennen elkaar al lang en ik ben blij je nog met grote regelmaat te zien. Het is dan ook een eer voor mij dat je toegestemd hebt mijn paranimf te worden. De vrijdagavonden in Diepenbeek met Daniela, Alexander en Amélie zijn altijd fijne momenten geweest. Ik hoop dat onze vriendschap nog lang mag duren!

Veerle alias Veke, er is aardig wat veranderd in jou en mijn leven, maar gelukkig heeft dit alles ons dichterbij elkaar gebracht. Je woont en werkt nu vlakbij en dat maakt het gemakkelijk om even langs te komen, iets waar ik de komende jaren ook nog graag gebruik van wil maken. Dank je Veerle en ook jij Koen voor alle steun! Klaas, broertje of is het broer? Een huis, nieuwe baan, een leuke vriendin die weldra je vrouw zal zijn, er is voor jou in al die jaren het een en het ander veranderd. Het doet me altijd plezier je te kunnen spreken. Bedankt, en ook jij Els voor alle steun! Lieve Wouter en Catrien, als ouders hebben jullie mij alle mogelijkheden en prikkels geboden die ik nodig heb gehad om te geraken waar ik nu ben. Jullie steun heeft altijd veel voor me betekend. Naar Martenslinde gaan betekent nog steeds een beetje thuiskomen voor me, en dat zal ook altijd zo blijven. Bedankt voor alles wat jullie me hebben gegeven!

Jildou, mijn liefste, ik wil je graag bedanken voor alles wat je me gegeven hebt de afgelopen jaren: steun, liefde, vertrouwen en begrip. Dat alles heeft mij enorm geholpen om mijn promotieonderzoek te voltooien. Ik hoop nog lang bij je te mogen blijven!

Curriculum vitae

Publications

Curriculum vitae

Stijn Ghesquiere was born in Leiden on September 1973. He grew up in Martenslinde, a small town in Belgium. There he spends most of his time looking at everything that swam, crawled or flied at the countryside.

After completing secondary school at the St. Lambertuscollege in Bilzen in 1992, he moved to Leuven to study Chemistry. In 1993 he went to Maastricht where he started to study Biological Health Sciences at Maastricht University. Additionally he started with the study Medicine at the same university in 1995. He completing his internship at the department of Clinical Genetics in 1999 and obtained his MSc degree in both Biological Health Sciences and Medicine.

After a short intermezzo, in which worked as editor at a medical website company, he decided to apply for a PhD at the Faculty of Medicine at Maastricht University. At the Molecular Genetic Department in the lab of Martin Hofker he conducted research on the role of phospholipases in atherosclerosis. Through the use and development of transgenic mouse models, he tried to unravel the effect of phospholipase overexpression. In addition to this *in vivo* work, *in vitro* experiments and explorative studies with human material gave additional insights in the complex mechanism behind the development of atherosclerosis. While his understanding of the phospholipases and the onset of atherosclerosis grew considerable, he feels that only a few pieces of an immense puzzle have been discovered and put in the right place.

As a member of the Cardiovascular Research Institute Maastricht (CARIM) he obtained the CARIM certificate in 2004. In early 2006, he completes his PhD thesis and expects to finish the remaining experiments by the time he defends the thesis.

Parallel to his work at the university, he studied the biology of the Ampullariidae, better known as the apple snails. This resulted in the website Applesnail.net, which consumed much of his spare time. Despite receiving an offer for a PhD in the field of malacology at a lab in Chicago, he decided to keep it a hobby project.

Publications

Full papers

Ghesquiere, S.A., Gijbels, M.J., Anthonsen, M., van Gorp, P.J., van der Made, I., Johansen, B., Hofker, M.H., and de Winther, M.P. 2004. Macrophage-specific overexpression of group IIa sPLA₂ results in increased atherosclerotic lesion size and enhanced collagen deposition. *J Lipid Res* 46:201-210.

Ghesquiere SA, Hofker MH, de Winther MP.

The role of phospholipases in lipid modification and atherosclerosis. *Cardiovasc Toxicol.* 2005;5(2):161-82.

Submitted

Ghesquiere, S.A., van der Made, I., Vergouwe, M.N., Greaves, D.R., Hofker, M.H., and de Winther, M.P. 2006. Secretory Phospholipase A₂ group X expression mediates foam cell formation and inhibits lipopolysaccharide induced activation of murine macrophages.

Oral presentations

ZonMW Genetica Retraite (Kerkrade, 2000).

Annual Meeting of the European Lipoprotein Club (Tutzing, Germany, 2004).

Dutch Atherosclerosis Society (DAS, Ermelo, The Netherlands, 2004).