The effect of hypoxia on macrophage proteoglycans: potential role in atherosclerosis

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A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in manuscript).

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Till Dan och Maximilian

för att ni finns

Abstract

A critical step in the development of atherosclerotic lesions appears to be the retention of low density lipoproteins (LDL) in the arterial wall, mediated by negatively charged proteoglycans (PG). Retained LDL is susceptible to modification and uptake by resident macrophages that are found in hypoxic sites in atherosclerotic lesions (with oxygen levels below 1%). PG are multifunctional proteins and in addition to their interaction with LDL, they bind and regulate the activity of growth factors and cytokines as well as cell migration and adhesion.

The aim of this thesis was to investigate how hypoxia affects PG synthesis in macrophages and the potential consequences on the atherosclerotic process. We found that expression of two large secreted PG, versican and perlecan, was increased in human monocyte-derived macrophages (HMDM) exposed to hypoxia (0.5% O_2) compared with cells in normal cell culture conditions (21% O_2). We found that the hypoxic induction of these two PG involved the hypoxia-inducible transcription factors HIF-1α and HIF-2α, and that HIF-1α and versican co-localized in macrophage-rich areas in human advanced atherosclerotic lesions. The negative charge of PG is on their attached glycosaminoglycans (GAG). We found that GAG secreted under hypoxic conditions bound LDL with higher affinity than GAG secreted under normal cell culture conditions, which could be due to the increased sulfation and size of GAG secreted in hypoxia. In contrast to the hypoxic induction of macrophage-secreted PG, hypoxia decreased the synthesis of the cell-associated heparan sulfate (HS) PG syndecan-1 as well as the HS GAG chains. The general motility of macrophages increased upon hypoxic incubation and was associated with the amount of HS GAG chains.

In conclusion, we found that hypoxia affects the synthesis of the extracellular matrix PG in macrophages with the potential to contribute to increased LDL deposition. Hypoxia also modulates the synthesis of cell-associated PG, with consequences for HMDM cell motility. These results are of importance to understand the role of macrophages in biological processes such as atherosclerosis.

Keywords: atherosclerosis, proteoglycans, glycosaminoglycans, macrophages, hypoxia, hypoxia-inducible transcription factor, LDL binding, cell motility

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Hypoxic regulation of secreted proteoglycans in macrophages[1]
Annika Asplund, Pia Stillemark-Billton, Erik Larsson, Ellen Knutsen Rydberg, Jonatan Moses, Lillemor Mattsson Hultén, Björn Fagerberg, Germán Camejo and Göran Bondjers
Glycobiology, 2009 Sep 11. Epub ahead of print

II. Macrophages exposed to hypoxia secrete proteoglycans for which LDL has higher affinity
Annika Asplund, Vincent Fridén, Pia Stillemark-Billton, Germán Camejo and Göran Bondjers
Manuscript

III. Hypoxia increases macrophage motility, possibly by decreasing the heparan sulfate proteoglycan biosynthesis[1]
Annika Asplund, Gunnel Östergren-Lundén, Pia Stillemark-Billton, Germán Camejo and Göran Bondjers
Journal of Leukocyte Biology, 2009. 86: 381-388

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Abbreviations

ADAMTS	A disentegrin and metalloprotease domain with thrombospondin motifs
Apo	Apolipoprotein
ATP	Adenosine triphosphate
CD	Cluster of differentiation
CS	Chondroitin sulfate
DS	Dermatan sulfate
EC	Endothelial cells
ECM	Extracellular matrix
EC-SOD	Extracellular superoxide dismutase
EXT	Exostoses
EXTL	Exostoses-like
EPO	Erytrophoetin
FFA	Free fatty acid
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GalNAc	N-acetyl-galactosamine
GF	Growth factor
GFP	Green fluorescent protein
GlcA	Glucuronic acid
GlcNAc	N-acetyl-glucosamine
GLUT	Glucose transporters
HIF	Hypoxia-inducible factor
HMDM	Human monocyte-derived macrophages
HS	Heparan sulfate
IdoA	Iduronic acid
IL	Interleukin
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
MCP-1	Monocyte chemoattractant protein -1
MCSF	Macrophage colony stimulating factor
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinases
NDST	N-deacetylase/N-sulfotransferases
NO	Nitric oxide
PDGF	Platelet-derived growth factor
PG	Proteoglycan
PMA	Phorbol 12-myristate 13-acetate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SMC	Smooth muscle cells
VEGF	Vascular endothelial growth factor

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Idel solsken gör öken

1 Introduction

1.1 Atherosclerosis

Atherosclerosis is the underlying pathology of cardiovascular disease -the leading cause of illness and death in industrialized countries. More than 1 million people suffer from cardiovascular disease in Sweden [1]. Atherosclerosis is characterized by a thickening of the arterial wall, leading to narrowing of the arteries and impairment or even occlusion of blood flow. Insufficient blood supply may cause ischemia and produce symptoms such as angina pectoris (chest pain). Total occlusion of an artery, as a result of plaque rupture, may cause myocardial infarction or stroke. The atherosclerotic process begins in childhood and may continue for decades without any symptoms. Diabetes, hypercholesterolemia, obesity, high blood pressure, old age and smoking are all risk factors for atherosclerosis. With an increasingly obese and ageing population, cardiovascular disease is likely to increase in the near future [2]. If this development is to be stopped, we need to further understand the cellular processes and underlying mechanisms of atherosclerosis, in order to identify more specific therapeutic targets.

1.1.1 The arterial wall and the atherosclerotic process

The wall of a normal artery has three distinct morphological layers. Closest to the lumen, below the endothelial cells (EC), is the *intima*. It is constituted by a thin layer of extracellular matrix (ECM) components, mainly collagen and proteoglycans (PG). Next to the intima is the *media* layer, which is composed of smooth muscle cells (SMC). The third layer is the *adventitia*, which contains connective tissue with small capillaries called the vasa vasorum. During plaque progression, the organisation of these three layers is disturbed due to thickening of the intima and disruption of the media.

Normally, circulating lipoproteins diffuse in and out of the arterial intima. A hallmark of atherosclerosis is the increased subendothelial accumulation of mostly low density lipoproteins (LDL), a retention process mediated by the ECM PG. Retained LDL particles are subjected to modifications that stimulate EC to synthesize chemotactic signals and surface markers that mediate the recruitment of circulating monocytes. Recruited monocytes differentiate into macrophages that take up modified LDL. An imbalance within the lipid metabolism generates lipid-loaded macrophages, so called foam cells. The aggregated foam cells are the first visible sign of a *fatty streak*. These cells express a number of cytokines, enzymes, growth factors and reactive oxygen species (ROS) that promote inflammation and LDL modification as well as proliferation and migration of SMC. The initiation

of the atherosclerotic process, with focus on the role of macrophages and PG, is illustrated in *Figure 1*.

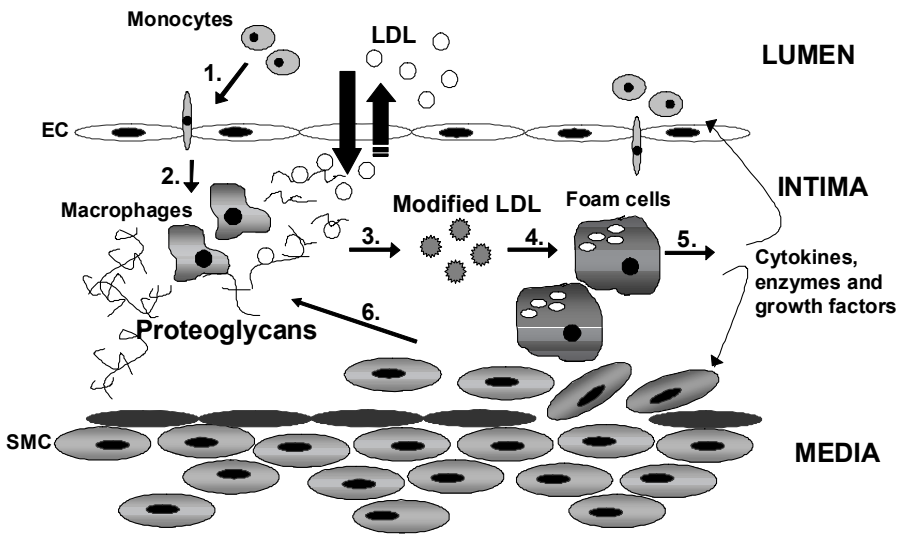

Figure 1. Macrophages in the atherosclerotic process. Circulating monocytes are recruited to the arterial wall **(1)** and differentiate to macrophages within the intima **(2).** LDL particles are retained within the intima by binding to proteoglycans **(3).** The modified LDL is taken up by macrophages, **(4**) resulting in lipid-loaded macrophages (foam cells) **(5).** They secrete molecules that will attract more monocytes to the intima as well as stimulate migration of SMC**.** Proliferated SMC secrete increased amounts of proteoglycans **(6)**, thus increasing the rate of LDL retention and macrophage uptake.

Eventually some foam cells burst and cell debris, toxic enzymes, lipids and cholesterol accumulate around them. With time, the lesion develops a core of necrotic cells covered by a supportive fibrous cap that narrows the lumen and impairs blood flow. Compensatory dilation of the vessel may delay symptoms of atherosclerotic disease for decades. However, plaque rupture may result in thrombus formation or embolism, producing acute symptoms of ischemia.

1.1.2 Mechanisms behind atherosclerosis

The mechanisms behind the formation of an atherosclerotic plaque are not fully understood. Two major theories are at the heart of this thesis:

In 1995, Williams and Tabas introduced the *response to retention* theory in a review article [3], summarizing findings that negatively charged PG within the intima mediate binding and retention of positively charged LDL [4-8]. This mechanism is

necessary for further modifications of LDL prior to macrophage uptake, foam cell formation and plaque progression. Extensive work has been done to confirm this theory. Today, it is generally accepted that PG play a key role in atherosclerosis [9].

The Anoxemia theory was presented as early as 1944 by Hueper [10]. This theory suggests that hypoxia plays a key role in atherosclerosis. It has been supported by the findings of hypoxic areas, with high concentrations of lactate and low ATP (adenosine triphosphate) and glucose, in atherosclerotic plaques. The cells of the intima depend on diffusion of oxygen through the arterial wall. Thickening of the wall decreases the diffusion capacity of oxygen, creating a hypoxic environment. Hypoxia has been found to mediate several processes important in the development of atherosclerosis [11, 12].

1.2 Proteoglycans

The ECM is mainly constituted by PG, hyaluronan, collagen, elastin, laminin and fibronectin. PG are negatively charged proteins with attached polyanionic sugar chains called glycosaminoglycans (GAG). Their charge and viscosity enables them to bind water and form a hydrated gel that mediates mechanical strength and support of tissues. PG regulate the activity of growth factors, cytokines and enzymes and are thus involved in the dynamic processes of cell signaling, adhesion, proliferation and migration [13, 14].

1.2.1 Structure and classification

Monosacharides and sulfate are metabolised by cells, converted to uridine diphosphate (UDP-sugars) and 3´phosphoadenosine 5´-phosphosulfate (PAPS) and transported to the endoplasmatic reticulum and Golgi apparatus where synthesis of GAG chains occurs. Serine residues of the PG core protein mediate the binding of a link region, consisting of three saccharides (Xylose-Galactose-Galactose) followed by glucuronic acid (GlcA). The link region serves as an attachment site and further elongation of the GAG chain. Depending on the combination of the amino sugar N-acetyl-glucosamine (GlcNac) or N-acetyl-galactosamine (GalNac) with either glucuronic acid (GlcA) or iduronic acid (IdoA), different types of chains are synthesized: chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) *(Figure 2)* [13, 15]. There is a fourth type of GAG chain, keratan sulfate (KS), composed by galactose and GlcNac. KS and its core protein, lumican, have been found in the arterial wall [16, 17]. However, the contribution of KS to atherosclerosis seems to be minor, and since macrophages do not express this PG, it will not be discussed further

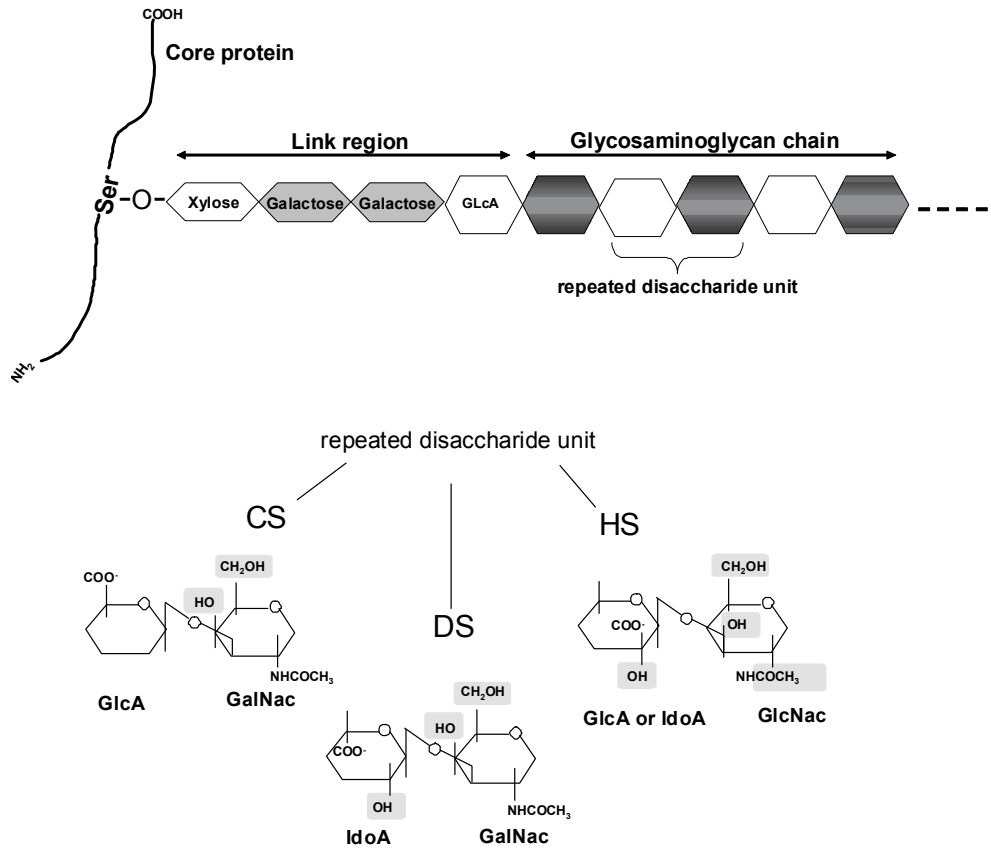

Figure 2. The general structure of a proteoglycan. Grey shading on disaccharide units indicates potential residues for sulfation.

PG may be classified by their location: versican, perlecan, decorin and biglycan are *secreted* PG while syndecan and glypican are *cell-associated* PG *(Figure 3)*. They may also be classified by their GAG chains: the *chondroitin sulfate* PG and the *heparan sulfate* PG. Neither classification is perfect; since a specific PG can carry different types of chains, and perlecan for instance is secreted but referred to a *basement membrane* PG [14, 15, 18].

1.2.2 The chondroitin sulfate proteoglycans and their biosynthesis

Versican, decorin and biglycan are the major chondroitin sulfate (CS) PG within the arterial ECM (*Figure 3)*. Decorin and biglycan belong to the family of small leucine-rich PG and are constituted by small core proteins of ~40 kDa with 1-2 CS or DS chains attached [19].

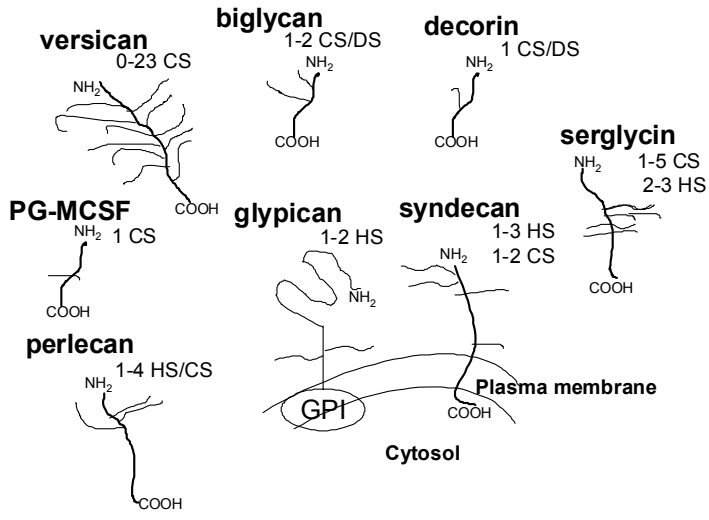

Figure 3. Location and structure of the proteoglycans investigated in this thesis.

Versican belongs to the family of hyaluronan-binding PG. Hyaluronan is a large unsulfated GAG chain [20] and together with versican it forms enormous networks within the ECM [14]. Versican is a large PG with up to 23 CS GAG chains attached. The size of the core protein and number of chains are dependent on alternative splicing of the versican mRNA transcript. Four different isoforms can be generated: V0, V1, V2 and V3 *(Table 1)* [21]. All isoforms contain an amino-terminal end (G1), known as the hyaluronan binding site, as well as a carboxyl-terminal end (G3). The middle regions of versican are encoded by two large exons called αGAG (exon 7) and βGAG (exon 8) and contain the attachment sites for the CS GAG chains [22]. V3 has no CS chains and should perhaps be regarded as a glycoprotein rather than a PG. The distribution and function of the different isoforms are not clear. Due to its high negative charge, versican plays an important role in the interactions and regulations of cytokines, enzymes, growth factors, lipoproteins and other extracellular matrix molecules within the arterial wall during the atherosclerotic process [9, 18, 23].

The PG form of macrophage colony stimulating factor (PG-MCSF) is secreted and carries one CS chain [24, 25]. This secreted PG has the capacity to mediate binding and uptake of LDL and to regulate cholesterol metabolism in macrophages [26, 27]. Serglycin is normally an intracellular PG but is secreted by macrophages. It can carry both CS and HS GAG chains. Although serglycin from monocytes is suggested to carry mainly CS chains [28]. It is involved in the formation, storage and delivery of secretory granules and is suggested to play an important role in the regulation of proteases and inflammatory mediators [29].

Versican isoforms	mRNA transcript	Core protein (kDa)	Number of CS chains
V_0	G1 aGAG ßGAG G3	370	17-23
V_1	G1 ßGAG G3	260	12-15
V_2	G1 aGAG G3	180	5-8
V_3	G1 G3	72	0

Table 1. The different versican isoforms.

The synthesis of CS GAG chains is dependent on the activity of specific enzymes. Several synthases are involved in the initiation and elongation steps that determine the number and size of the chains, which can contain up to 60 disaccharides. The 4-0- and 6-0-sulfotransferases catalyze the sulfation of position 4 and 6 on GalNac (positions marked in *Figure 2*) [15].

1.2.3 The heparan sulfate proteoglycans and their biosynthesis

Syndecan, glypican and perlecan are all heparan sulfate (HS) PG *(Figure 3)*. The syndecan family consists of four transmembrane PG (syndecan 1-4) and is involved in a wide variety of biological activities, including cell migration, adhesion and proliferation. The extracellular domain contains the HS GAG chains but syndecan-1 and -4 might also have CS chains near the cellular membrane. The HS GAG chains are known to mediate the binding and activity of several growth factors (GF) to their receptors, like fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). The extracellular domain can be proteolytically cleaved, generating free HS GAG chains that can bind and regulate cytokines and GF within the ECM. (For a review of syndecans, see [30]).

Glypican is anchored to the cell surface by a glycosyl-phosphatidylinositol (GPI), and six isoforms have been described. This PG is involved in cell-to-cell contact, adhesion and GF regulation and signalling [31].

Perlecan, a secreted PG, differs from other HS PG: its four GAG attachment sites may also carry CS chains. It has a large core protein of ~470 kDa and with GAG chains attached, this PG can reach a molecular weight of 800 kDa [14]. Perlecan binds and regulates several ECM components, including GF, and may function as a reservoir for GF. Due to its close association with the cell membrane, perlecan can also regulate GF signalling, just like the cell-associated PG [32].

HS GAG chains may contain as much as 150 disaccharides. The elongation of the chain is mediated by a family of five glycosyl transferases. Two of them, exostoses (EXT)1 and 2, are the main enzymes responsible for initiation and polymerization of the HS chain [33]. Less is known about the 3 other family members, exostoses-like (EXTL)1-3, although EXTL2 has been suggested to play a critical role in the initiation of HS synthesis [34, 35]. The HS chain can be modified in four different ways [33]: **1.** N-deacetylase/N-sulfotransferases (NDST) first deacetylate the aminogroup ($NHCOCH_3$) on a specific GlcNac and then catalyze a sulfation reaction. **2.** C5 epimerase catalyzes the epimerization of GlcA to IdoA, which results in an orientation of the carboxyl group below the hexose ring. **3.** IdoA and GlcNac can be subsequently sulfated on the C2 position by 2-O sulfotransferases. **4.** GlcNac may further sulfated by 3-0- and 6-0 sulfotransferases. Sites for potential sulfation are marked in *Figure 2*.

1.2.4 Modulation of proteoglycans

The normal turnover of PG is mediated by the families of MMP (matrix metalloproteinases) and ADAMTS (A disintegrin and metalloproteinase domain with thrombospondin motifs). Versican and perlecan core proteins can both be cleaved by specific enzymes of these families [36, 37]. Specific cleavage sites in versican V0/V1 have been described for ADAMTS-1 and -4 [38]. The cleavage (i.e. shedding) of the ectodomain of syndecans can be mediated by several MMP and ADAMTS-1 [39, 40]. The turnover of PG also includes cleavage and digestion of the GAG by enzymes of the endoglycosidase, sulfatase and exoglycosidase families [41]. One example is the heparanase, which is both intracellular and cell-associated, that digests HS GAG chains [42].

1.2.5 The role of proteoglycans in atherosclerosis

The high negative charge of PG, due to the numerous sulfate and carboxylic groups of their GAG chains, confers high affinity for the positively charged regions of the apolipoprotein B (apoB) within the LDL particle [43-45]. PG binding sites have been demonstrated within apoB [6, 46], and it has been shown that atherosclerosis is reduced in mice with defective PG-binding sites in apoB [47].

This PG-LDL interaction is the mechanism behind the *response to retention hypothesis* of atherosclerosis [48-50].

CS PG plays an important role in LDL binding [43] and regions with increased amounts of CS-rich PG (versican, decorin and biglycan) are associated with areas of LDL depositions in human lesions [51, 52]. Versican is considered the most atherogenic PG, but in the earliest stage of human lesions, it is biglycan that is suggested to be associated with apoB deposition [53]. HS and DS GAG chains are more negatively charged than CS [54]. However, versican has higher LDL-binding capacity than biglycan and decorin *in vitro* [55]. This may be explained by the high number of CS GAG chains on versican with a large number of binding sites for LDL.

Longer and more sulfated CS GAG chains have higher affinity for LDL *in vitro* [56-58]. Several proatherogenic factors induce changes within the GAG chains with effects on their affinity for LDL [9]. LDL has been shown to have higher affinity for GAG rich in 6-sulfated disaccharides compared to 4-sulfated [56, 59].

The PG-mediated retention of LDL makes the particles susceptible to modification and oxidation by enzymes and reactive oxygen species (ROS). The presence of PG is also important for the enzymatic activity of phospholipase A_2 towards LDL [60-62]. The PG-LDL complex induces LDL aggregation and unmasking of binding sites, which increases complex strength as well as macrophage uptake [8, 23, 63-69]. Interestingly, PG can distinguish between lipoprotein subclasses, leading to more effective macrophage uptake [66].

HS is the most negatively charged GAG chains. Although HS GAG chains bind and mediate LDL uptake by macrophages [54, 70-72], decreased levels of HS [73] as well as perlecan core protein [74] are associated with human atherosclerosis. The suggested anti-atherogenic effect of HS GAG chains in ECM may be due to their ability to inhibit SMC proliferation, possibly by binding and storage of fibroblast growth factor (FGF) [75]. However, in addition to its anti-atherogenic effect, perlecan has been shown to induce EC proliferation and angiogenesis [76]. Platelet-derived growth factor (PDGF) competes with LDL for binding sites on GAG, and GAG binding and storage of PDGF influences the proliferation of SMC [64, 77]. Cleavage of both versican and perlecan are suggested to promote SMC proliferation, possibly by release of GF bound to these PG [78]. Cell-associated HS is a co-receptor for several GF. Reduction of HS reduces FGF mediated nitric oxide (NO) release [39]. Several anti-atherogenic mechanisms are associated with NO, such as the vasodilatory effect and inhibition of SMC proliferation and platelet aggregation. Loss of NO activity seen in atherosclerosis could be associated with the anti-atherogenic characteristics of HS. Cell-associated HS GAG chains also play an important role in the regulation of the superoxide anion $O_2^{\bullet}$, which is associated

with atherosclerosis mainly by its capacity to oxidize LDL. EC-SOD (extracellular superoxide dismutase), an enzyme with antioxidant characteristics, catalyzes the reaction of $O_2^{\bullet}$ to H_2O_2 (hydrogen peroxide and oxygen) and thereby suppresses LDL oxidation. Since EC-SOD has a unique heparin binding domain, the HS GAG chains play an important role in determining the localization of this enzyme within the intima.

1.3 Macrophages

1.3.1 Macrophage origin, differentiation and phenotypes

Monocytes originate from pluripotent stem cells in the bone marrow. Their proliferation and differentiation is dependent on the growth factor MCSF. Several monocyte phenotypes have been identified. They are basically distinguished by their expression profiles of CD14 and CD16, their cytokine production and their phagocytic activity. Interestingly, inflammation, infection and high fat diet affect the numbers and types of monocytes released from the progenitor cells (for an updated review about monocyte development, see [79]). Monocytes and macrophages are parts of the innate immune system and phagocytose foreign pathogens and other substances. This process may be followed by secretion of pro-inflammatory mediators like cytokines that further enhance the immune response. Monocytes and macrophages are also antigen-presenting cells, of importance for the adaptive immune response by T-cells.

Monocytes circulate in the blood for 1-3 days and enter tissues in response to chemotactic signals, like the monocyte attracting protein-1 (MCP-1), and adhesion molecules on luminal EC, such as VCAM and P-selectin. In response to tissue inflammation, macrophages develop different phenotypes: classically activated macrophages (M1), which mainly secrete pro-inflammatory cytokines, and alternatively activated macrophages (M2), which mainly secrete anti-inflammatory cytokines [80]. In addition to inflammation, other factors can influence macrophage phenotype, such as excessive amounts of lipids (which induces macrophage foam cells in atherosclerosis), and hypoxia (which generates tumor associated macrophages in tumor tissues). Recent work has focused on identifying macrophage phenotypes in pathological conditions. However, the *in vivo* situation with multiple stimuli generates complex phenotypes such as macrophages in atherosclerotic lesions, which are exposed to lipids, hypoxia and inflammatory mediators.

1.3.2 The role of macrophages in atherosclerosis

Macrophages, like SMC and EC, are involved in the modification of LDL by secretion of ROS, NO and enzymes like myeloperoxidase, lipoxygenase and phospholipase A_2 [81-85]. Macrophages express numbers of scavenger receptors that mediate the uptake of several types of modified LDL, e.g. oxidated and enzymatically changed LDL. Macrophage degradation of LDL within the lysosome generates free cholesterol. An abundance of free cholesterol induces ApoE that mediates reverse cholesterol transport [86, 87]. However, an excessive amount of available LDL and uncontrolled uptake by scavenger receptors leads to an imbalance in lipid metabolism that generates intracellular accumulation of cholesterol esters in lipid droplets [88, 89]. Macrophage uptake of fatty acids followed by intracellular accumulation of triglycerides is also involved in foam cell formation [90, 91].

Foam cells secrete a number of pro-inflammatory mediators such as interleukins (IL-1, IL-6 and IL-8), MCSF and tumor necrosis factor (TNF)-α. These factors mediate recruitment of monocytes and T-cells [92, 93]. Foam cells also secrete GF, like PDGF, FGF and VEGF, that induce proliferation and migration of SMC and EC and lead to progression of the lesion [94].

Macrophages play a role in plaque rupture. They secrete MMP and ADAMTS that degrade collagen and PG in the fibrous cap that provides the mechanical strength of the plaque. For instance, macrophages have been shown to secrete ADAMTS-4, which specifically cleaves versican V0/V1 [95-97], and heparanase, which cleave HS GAG [98]. As previously mentioned, matrix degradation also has major consequences for the biological activity of PG in terms of storage and regulation of GF activity within the intima.

1.3.3 Role of macrophages in proteoglycan synthesis in atherosclerosis

Extensive work has been done to clarify the role of macrophages in lipid metabolism, inflammation and matrix degradation. However, their role in PG synthesis has received less attention.

Macrophages express syndecan, glypican, serglycin, versican, perlecan and the PG form of MCSF [26, 99-103]. Serglycin is suggested to be the major secreted PG synthesized by human and mouse macrophages [104, 105]. Interestingly, inflammatory mediators increase the secretion of serglycin-related CS GAG chains in macrophages [106]. The role of serglycin in the regulation of proteases and inflammatory mediators indicates that this PG may play at role in atherosclerosis. The PG distribution in early stages of human intimal thickening was recently

reviewed [9], and versican, biglycan, decorin and lipoproteins are identified in the absence of macrophages. These results indicate that macrophages do not contribute to the PG synthesis in early lesion development. Perlecan is present in macrophage-rich areas of advanced human lesions, while decorin is found predominantly around foam cells [51]. Versican staining has been observed in areas with macrophages and SMC. Versican has not been demonstrated in the macrophage-rich fibrotic core. This is probably one reason why versican synthesis during lesion progression has generally been attributed to SMC [36, 51, 52]. However, gene array analysis of versican expression patterns in different human tissues indicates that myeloid cells, monocytes and hole blood have the highest expression of versican, see *Figure 4* (adapted from [107, 108]). Consistent with this finding, macrophages were recently suggested to play an important role in versican accumulation and plaque progression in mice [109].

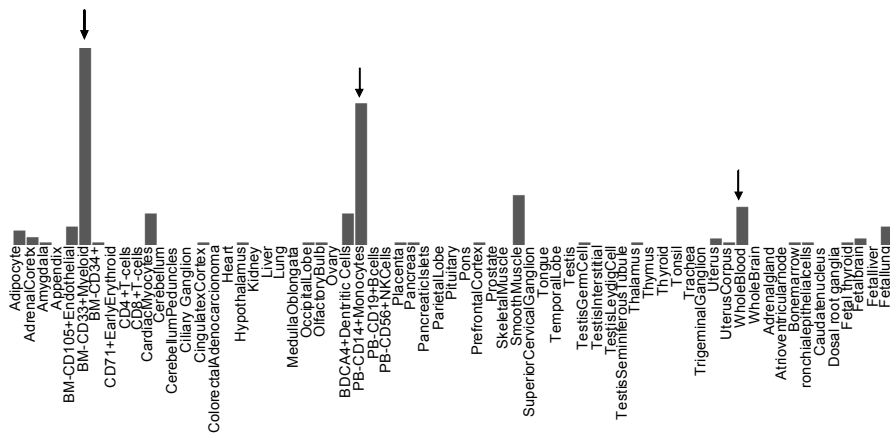

Figure 4. Gene array analysis of versican expression patterns in different human samples.

Macrophages can synthesize CS GAG chains with high capacity to mediate LDL binding [110, 111]. The macrophage secreted PG form of MCSF has also been shown to bind LDL *in vitro* [26], but its presence in atherosclerotic lesions has not yet been demonstrated. Aviram and co-workers have shown that both macrophage-secreted and cell-associated PG can facilitate LDL binding, aggregation and uptake [67, 112-114]. Macrophage cell-associated HS GAG chains also mediate uptake of native as well as oxidized LDL by binding to the bridging molecule lipoprotein lipase and the enzyme phospholipase A_2 [70, 72].

1.4 Hypoxia

Cells within the arterial intima depend on diffusion of oxygen from the luminal blood flow or from the vasa vasorum. The diffusion capacity for oxygen is normally ~100-300 μm within tissues [115]. During lesion development, an increasing number of cells will be located too far from the blood stream to receive enough oxygen. The oxygen tension is 20 to 70 mmHg (2.5-9%) in healthy tissues but reduced to below 10 mmHg (<1% oxygen) in pathological tissues [116]. Since a number of processes are influenced by low oxygen concentrations (i.e. hypoxia), it is important to consider the oxygen concentrations in *in vitro* experiments to obtain valid results regarding cellular functions in atherosclerosis.

1.4.1 Cellular adaption to hypoxia

ATP is the most important source of cellular energy. It is generated by glycolysis, the citric acid cycle and oxidative phosphorylation. Oxidative phosphorylation, which occurs in the inner mitochondrial membrane, is the most efficient process for generation of ATP. 80-90% of all intracellular glucose is normally used for ATP production. Numerous genes are induced in hypoxic cells to promote anaerobic production of ATP, save oxygen and increase oxygen delivery to the cell: glucose transporters (GLUT) and glycolytic enzymes (to increase the capacity of glycolysis); lactate dehydrogenase (LDH) and pyruvate dehydrogenase 1 (to inhibit the entrance of pyruvate into the citric acid cycle) [117]; vascular endothelial growth factor (VEGF) (stimulates angiogenesis); and erytrophoetin (EPO) (stimulates production of red blood cells). For a complete list of hypoxia-induced genes involved in oxygen homeostasis, see [118].

1.4.2 HIF, the main transcription factor in hypoxia

Hypoxia-inducible factor (HIF), identified in 1992 [119], is the major transcription factor mediating cellular adaptation to hypoxia [120, 121]. It is a heterodimer whose regulation is controlled by the α subunit, since the β subunit —also known as ARNT (arylhydrocarbon receptor nuclear translocator)—, is constitutely expressed. At normal tissue oxygen pressure, proline residues on amino acids 402 and 564 within the oxygen dependent degradation domain (ODD) are hydroxylated by prolylhydroxylase (PHD). Von Hippel Lindau tumor suppressor protein (VHL) binds to the hydroxylated prolines and acts as a substrate for the E3 ubiquitin ligase complex, that marks the HIF-1α subunit for proteasome degradation. However, in hypoxic conditions, PHD is phosphorylated and thereby inactivated, generating stable HIF-1α that translocates to the nucleus and dimerizes with HIF-1β. This complex, together with co-activators, bind to the hypoxia response element (HRE)

RCGTG (where R is A or G) on the DNA segment [122]. This binding mediates transcription of genes involved in the cellular adaption to hypoxia. The regulation of HIF is illustrated in *Figure 5*. For additional details, see review [123].

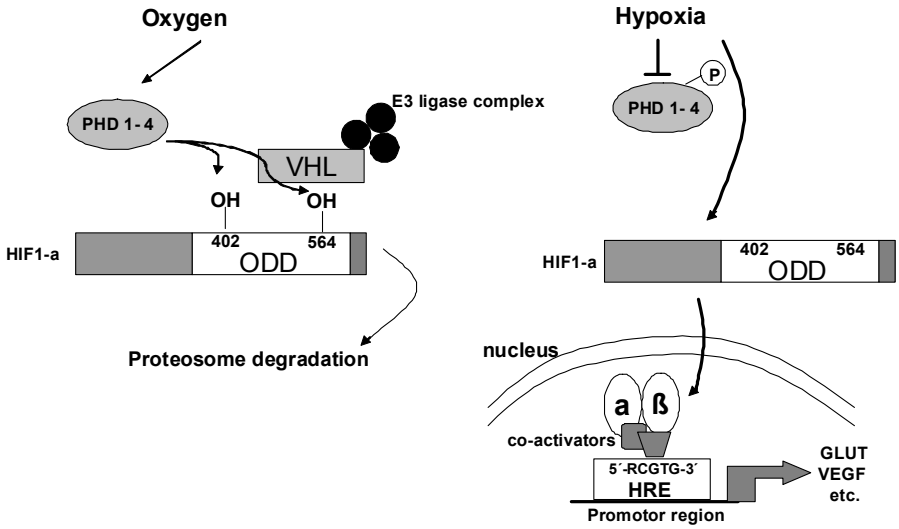

Figure 5. Regulation and activation of the transcription factor hypoxia-inducible factor (HIF).

There are three isoforms of the α subunit, HIF-1α, HIF-2α and HIF-3α. They all have the capacity to bind and form a complex with the β subunit. HIF-1α is the best characterized isoform, although HIF-2α (also known as EPAS-1) has been found to have a similar structure and to regulate the same genes. However, different functions are suggested for the two proteins [124-126]. HIF-1α is thought to play a major role in regulation of genes of importance to the acute response to hypoxia (glucose transporters and glycolytic enzymes), whereas HIF-2α is involved in the more long-term adaptive response to hypoxia (VEGF and EPO) [122].

Oxygen is the main regulator of HIF activity but other pathways, not dependent on oxygen, have been described. One of them involves RACK1. This protein inhibits the heat shock protein HSP90, which normally binds and stabilizes HIF-1α. RACK1 also competes with the HSP90 binding site to promote HIF-1α ubiquitination and degradation [127]. Other enzymes that mediate ubiquitination of the α subunit independent of oxygen are the E3 ligases HAF (hypoxia-associated factor) and Int6 (integration site 6) [128, 129]. ROS levels are generally proportional to the oxygen concentration. Interestingly, ROS increases in response to hypoxia,

which has been shown to be important for stabilization of both HIF-1α and HIF-2α [130].

1.4.3 The role of hypoxia in atherosclerosis

Hypoxic regions have been demonstrated in both animal and human atherosclerotic lesions, as well as high levels of lactate and low levels of glucose and ATP [131-134]. Macrophages are found in these hypoxic areas [131, 133], where they interestingly enough, co-localized with HIF [134]. These areas become hypoxic not only because of decreased availability of oxygen, but also because of the increased oxygen demand of foam cells. Foam cells have been found to be hypoxic even when located within oxygen diffusion distance [134, 135].

Inflammation is a hallmark of atherosclerosis. The hypoxic induction of inflammatory mediators in macrophages is well documented [116, 136]. Hypoxia contributes to oxidation of LDL [137, 138] and induces intracellular accumulation of lipids and cholesterol [90, 139]. The hypoxia-induced angiogenesis is associated with atherosclerotic plaque progression and rupture [12]. Macrophages are trapped in hypoxic areas of the lesion, but the reason for this is unclear. It has been shown, however, that macrophage migration decreases in response to hypoxia [140], possibly because of hypoxic induction of MIF (macrophage migration inhibitory factor) [141] or changed levels of specific MMP [142].

A secondary effect of cell metabolism in hypoxia is the increased production of lactate [133], which generates an acidic environment with consequences for several pH-dependent processes. As early as 1980, low pH was shown to be important for the interaction between LDL and PG [43]. This phenomenon was recently investigated and it was found that the binding of LDL to PG increased with lower pH [143]. Interestingly, it was also shown that pre-modification of LDL, mediated by low pH, further increased LDL binding to PG. Considering that macrophages are strongly associated with hypoxic areas in atherosclerotic lesions, it is somewhat surprising that the effect of hypoxia on PG and its potential role in LDL retention, has only been investigated in SMC [144].

2 Aims of the thesis

Proteoglycans, macrophages and hypoxia are all key players in the development of atherosclerosis. The role of human macrophages in proteoglycan expression and biosynthesis under pathophysiological oxygen conditions (i.e. hypoxia) has not been investigated. The general objectives of this thesis were to investigate the effects of hypoxia on macrophage proteoglycan synthesis and their potential implications for atherosclerosis.

Specifically, the following questions were addressed:

- Does hypoxia influence the synthesis of macrophage-secreted proteoglycans and if so, are these changes regulated by the transcription factor *hypoxia-inducible factor (HIF)*? (Paper I)
- Does versican co-localize with HIF-1α and macrophages in human advanced atherosclerotic lesions? (Paper I)
- Do the effects of hypoxia on macrophage proteoglycan biosynthesis have consequences for LDL affinity? (Paper II)
- Does hypoxia affect the synthesis of macrophage cell-associated proteoglycans and does it influence general cell motility? (Paper III)

3 Methodological considerations

A detailed description or original references for each method that have been used are described in each paper.

3.1 Macrophage culture and differentiation

Most of the experiments in this thesis were performed on human monocyte-derived macrophages (HMDM). They were isolated from *buffy coat*, prepared in the blood bank of the hospital by centrifugation of whole blood. A *buffy coat* is a concentrate of leukocytes (80% lymphocytes and 20% monocytes) and small amounts of red blood cells and plasma. For isolation of monocytes, we used the Ficoll-Paque solution [145].

When the isolated monocytes (with some contamination of lymphocytes) are incubated in serum-free conditions, cells adhere to the plastic surface within 1-3 hours, while the lymphocytes stay in solution and can be eliminated by repeated washing with PBS (phosphate-buffered saline). One reason to use a serum-free medium in this step is to avoid potential activation of monocytes by lymphocytes due to the presence of alloantigens in pooled serum [146]. Monocytes were

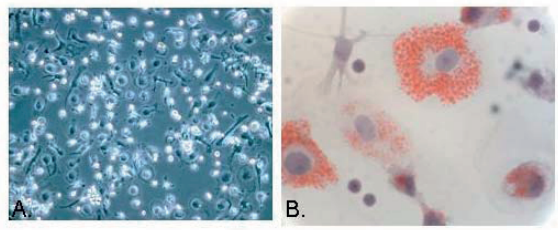

Figure 6. Human monocyte-derived macrophages after 7 days of differentiation in the presence of 10% human serum**. A.** Light microscopy (20x). **B.** Lipid staining with Oil Red O and hematoxylin (40x).

differentiated into macrophages during 7 days of culture in presence of 10% human serum. The medium was changed every third day (three times in total), and cells were washed three times in PBS upon every change. HMDM from representative donors are shown in *Figure 6*. This procedure results in a cell culture with >95% positive staining for the macrophage marker CD68 with minor contamination of lymphocytes (most likely T/B-cells). Potentially, this method of monocyte differentiation could allow circulating stem cells to differentiate into SMC [147]. To confirm that the PG from this cell culture originated from macrophages and not from SMC, we assessed the presence of the SMC marker SM-alpha actin and

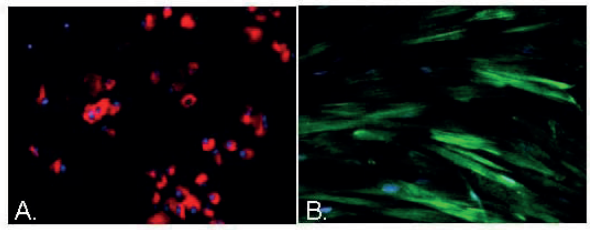

Figure 7. Staining for SM-alpha actin (green fluorescence) and CD68 (red fluorescence) in **A.** HMDM culture and **B.** SMC, used as positive controls. Nuclei staining with DAPI (blue).

CD68. We found no indication of SMC contamination in our macrophage culture (*Figure 7*).

HMDM are present in atherosclerotic plaques and seemed to us the most relevant cell type to study *in vitro* in this context. Differentiation of HMDM in presence of serum is an established method. We exclusively used human serum to avoid potential activation of human cells by animal proteins in bovine serum. A disadvantage of the method is the variation in attachment efficiency between donors. In approximately one out of five donors, we found detachment of cells within the first 3-5 days of culture. Individual responses of cells to the human serum might explain this [148]. To avoid this problem, others have used a serum-free medium -specific for macrophages- in combination with granulocyte-macrophage colony-stimulated factor (GM-CSF). This method results in a more homogeneous cell preparation without loss of donors. However, the PG synthesis might be stimulated by unknown components of this medium, and GM-CSF has been shown to induce versican expression in a dose-dependent manner [149]. Increased knowledge about macrophage differentiation in response to different stimuli and in specific pathological conditions will hopefully generate more precise methods in the future.

The monocyte-like cell line THP-1 is derived from a patient with acute monocytic leukemia [150]. These cells have capacity to differentiate into macrophages [151] and are frequently used as an *in vitro* macrophage model, making it easier to obtain large numbers of cells and avoid biological variation. It was recently demonstrated, however, that gene expression profiles and differentiation pathways of THP-1 macrophages and HMDM are quite different [152]. Thus, results obtained from THP-1 cells can not be directly applied to primary macrophages. Well aware of this, we verified that the hypoxic effects on PG mRNA expression were similar in HMDM and THP-1 cells, and concluded that THP-1 cells could be used as a model for HMDM in our experimental setup.

3.2 Hypoxic incubation

The oxygen concentration in pathological tissues, like atherosclerotic plaques, is <1% [116]. We used an oxygen concentration of 0.5% in our hypoxic experiments. The oxygen concentrations in healthy tissue do not exceed 9% O_2. However, most cell culture experiments are performed in 21% oxygen (oxygen concentration in air). To be able to compare our results with published data, we used 21% O_2 for our "normoxia" experiments, although ~9% O_2 would have been more accurate. However, results from our versican mRNA expression experiments have indicated that changes in response to hypoxia take place at oxygen concentrations less than 2%, rending the question of 9% or 21% insignificant in our experimental setup (*Paper I*).

In our hypoxic experiments, the medium was placed in a glass bottle sealed with a rubber plug prepared with two syringes. One syringe was connected to a plastic tube for medium transfer and elimination of oxygen. Oxygen was eliminated from the medium by pre-equilibration with 100% N_2 followed by equilibration with 0% O_2 and 5% CO_2 to obtain the correct pH and CO_2. Tests performed in our laboratory indicate that equilibration with 0% O_2 yields an oxygen concentration of ~2% in medium. However, the oxygen concentration of the medium decreases over time inside the hypoxic incubator. This time-dependent decrease of oxygen has recently been studied in our laboratory using an oxygen probe: approximately 3-4 hours of hypoxic incubation is needed to reduce the oxygen concentration of the culture medium from 18% to ~1%. This is consistent with the findings of others [153]. The rate of decrease is dependent on the number of cells, the volume of the medium and probably also the cell type. We used a hypoxic chamber with a constant flow of 100% N_2 to generate a working condition with an oxygen concentration of ~1%. With our method, cells were immediately exposed to hypoxia, which is important when incubating cells for a short period of time. One could argue, however, that slow adaptation of cells to declining oxygen concentrations is more physiological.

3.3 Cell viability

We used two different methods to analyze the potential cytotoxic effects of hypoxia on macrophages. A classical assay is to measure cell leakage of lactate dehydrogenase (LDH). We found LDH leakage into the culture medium after 48 hours of incubation in hypoxia and concluded that exposing the cells to hypoxia for 24 hours did not affect cell viability (*Paper III*). This method could be questioned, however, since hypoxia itself induces lactate production without effects on cell viability. Therefore, we also studied cell viability using the trypan blue exclusion test and found no differences between hypoxic cells and cells incubated

in normal conditions (*Paper I, supplemental data*). These results are consistent with published data on macrophage viability in hypoxia [90].

3.4 Immunosorbent protein assay

We developed two different immunosorbent assays for detection of versican, based on the binding of radioactively labelled versican to its antibody (*Paper I*). In the first assay, cells were incubated in the presence of ^{35}S–methionine, leading to radioactive labelling of all proteins produced. To get around the problem of GAG-chains attaching to the polysorb NUNC plate, leading to nonspecific signalling, GAG chains were eliminated by chondroitinase and heparinase treatment. Plates were coated with the versican antibody or PBS (negative control) and washed with Tween in PBS. Samples were applied without blocking, since all blocking solutions that we tested generated nonspecific binding of radioactively labelled proteins. After washing and addition of scintillation liquid, we measured the amount of versican protein by detecting the signal from ^{35}S–methionine. In the second assay, we quantified versican protein by its metabolically labelled GAG chains. Samples from cell cultures incubated in the presence of [^{35}S]-sulfate and D-[6-^{3}H]-glucosamine were applied on a maxisorb NUNC plate coated with the versican antibody or PBS (negative control). This plate was blocked before sample application to avoid nonspecific binding. Protein bound to the versican antibody was detected by measuring both [^{35}S]-sulfate and D-[6-^{3}H]-glucosamine. In both assays, the signal was normalised to total cellular protein. The principles for the two assays are illustrated in *Figure 8*.

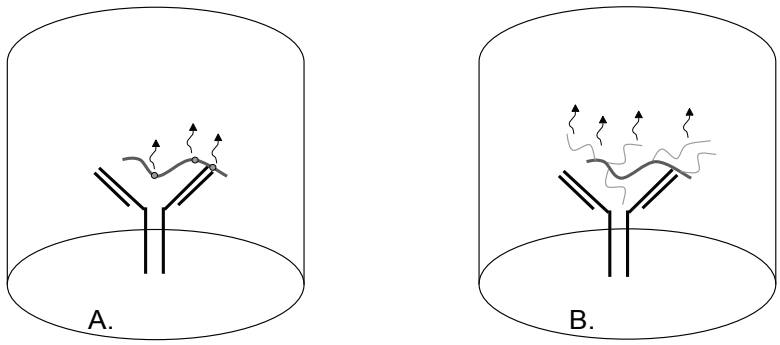

Figure 8. Illustration of the principles of the immunosorbent protein assays for versican detection. **A.** Detection of protein labelled versican. **B.** Detection of GAG labelled versican.

Both assays have limitations. For one thing, methionin is not the most common amino acid. Also, although the antibody is developed to recognise all the versican isoforms, potential degradation and variation of the isoforms may result in errors in quantitatation. Potential protein degradation during the experiment could have secondary effects on amino acid pool sizes. Drawing conclusions about quantitative changes in core protein synthesis based on GAG chains also has its difficulties. Differences between samples may be affected by potential changes in size and sulfation of the GAG chains rather than changes in the amount of core protein itself.

3.5 siRNA knockdown of HIF

RNA (ribonucleic acid) interference is an intracellular process that controls the activity of a specific gene. It is mediated by the binding of a complementary small interfering RNA (siRNA) sequence to messenger RNA (mRNA). siRNA is a very useful tool for studying the function of a specific gene [154]: Synthetically produced doubled-stranded siRNA fragments of 20-25 nucleotides, complementary to the mRNA sequence of the target gene, are transferred into the cell. The siRNA fragment is enzymatically cleaved and the antisense strand binds to its target mRNA sequence. The binding mediates mRNA degradation [155]. To transport the siRNA across the cell membrane, we decided to transfect our cells by electroporation by using the Amaxa system. Since transfection efficiency and stability is difficult to achieve in primary cells, we used THP-1 cells. We found it was necessary to split the cells (1:8) 3-4 days prior to transfection to put them in an activated state. We observed ~90% efficiency when cells were transfected with the green fluorescent protein (GFP) (*Figure 9*).

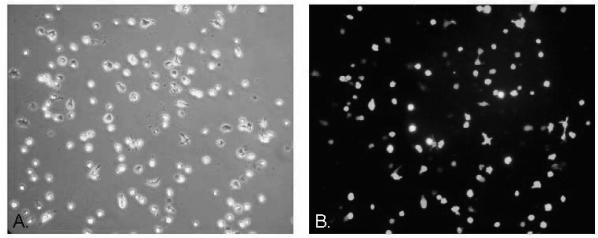

Figure 9. THP-1 cells, 20 hours after transfection with green fluorescent protein (GFP). **A.** light microscopy and **B.** green fluorescence 20x.

THP-1 monocytes are generally differentiated into macrophages for 72 hours in presence of 100 nM Phorbol 12-myristate 13-acetate (PMA). Since PMA itself has been found to affect the expression of several genes [152] and because of the short-term effects of siRNA, we used a PMA concentration of 10 nM and shortened the

incubation time to 20 hours. There were no visible differences in cell adhesion or morphology between the two PMA concentrations. Following activation by electroporation, cells attached to the culture dishes. When PMA was added 4 hours after transfection, the cells started to differentiate. The timing was crucial –if PMA was added earlier or later, cells detached.

4 Results and discussion

This thesis focuses on the impact of hypoxia on macrophage PG and on the potential involvement of the transcription factor hypoxia-inducible factor (HIF). We also investigate effects of hypoxia on GAG biosynthesis with implications for LDL affinity and cell motility. Below is a schematic illustration of the different components and mechanisms investigated in this thesis (*Figure 12*).

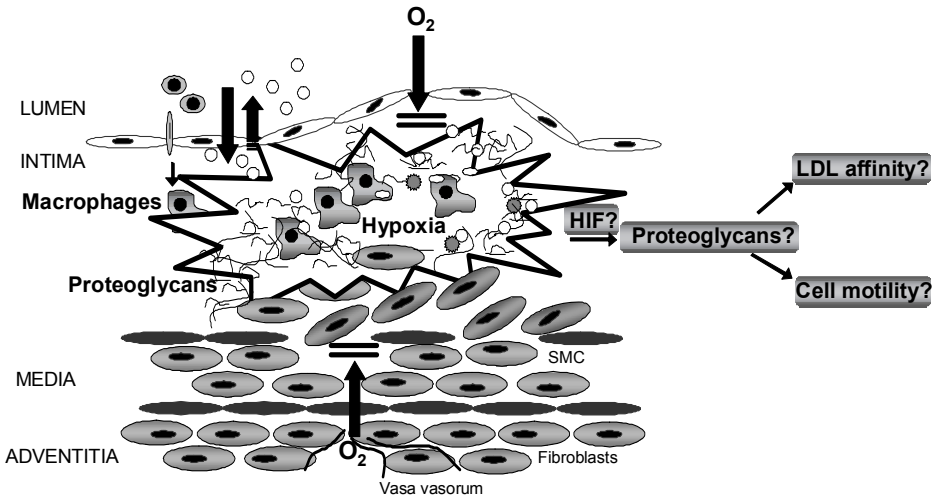

Figure 12. An overview of the different components of the atherosclerotic process investigated in this thesis.

4.1 The effect of hypoxia on proteoglycan core proteins in macrophages

4.1.1 The effect of hypoxia on secreted proteoglycans

In paper I, we analyzed the effects of hypoxia on the mRNA expression of the core proteins of several secreted PG in HMDM (*Figure 13)*. Interestingly, the most dramatic effect was found on versican. This large PG is postulated to contribute to retention of LDL and to modulate the function of cytokines, GF and enzymes in human atherosclerotic lesions [48, 49, 78, 156, 157]. Increased versican expression in ischemic heart has been associated with infiltrating monocytes in studies on both rat and human [149, 158]. Using an antibody that recognises all versican splice variants (like the primers and probe used to analyze mRNA expression of the core

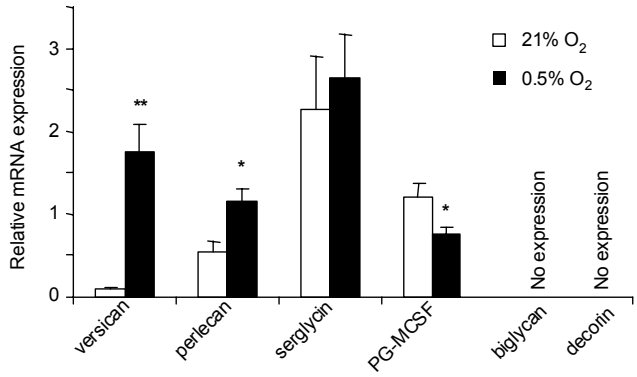

Figure 13. HMDM mRNA expression of secreted PG after 24 hours exposure to hypoxia compared to normal cell culture conditions. Values are means ± SEM, n= 5-9, *P<0.05, **P<0.01.

protein) [159], we performed western blots on HMDM cell lysates. We detected protein products with sizes corresponding to the three known versican isoforms: V0 (~370 kDa), V1 (~260 kDa) and V2 (~180 kDa) [22, 160] (*Paper I*). V2 is thought to be expressed mainly in the central nervous system, but has also been reported in human and bovine aorta [22, 161]. Our results indicate that V2 might be a product of resident macrophages. We also detected bands of ~130 and ~98 kDa. They do not correspond to V3 (with a predicted size of ~72 kDa) or to the incomplete splicing products described for V1 and V3, which generate products that are 2 kDa larger for each isoform [159]. Versican isoforms have not previously been described in HMDM, but expression of V0, V1 and V3 in ischemic rat heart was associated with infiltrating monocytes [149].

We were not able to demonstrate correlation of the increased versican mRNA expression with protein levels, neither by using western blots nor by measuring the methionine-labeled core protein bound to the versican antibody. However, in an immunosorbent assay, using the versican core protein antibody, we found an increased signal from metabolically labelled GAG. We speculate that this difference is related to increased incorporation of tracers into GAG (where every disaccharide may be labelled), compared to methionine-protein labelling (where only a fraction of the amino acids is radioactive). However, it is also possible that potential hypoxia-induced changes in GAG size or sulfation may influence the signal from metabolically labelled GAG chains. Changes in the specific activity of the precursors, due to potential protein turnover, may have effects on labelled amino acid pool sizes. Up to 70% of macrophage-secreted GAG has been reported to be free rather than bound to a core protein [104, 162], suggesting that rapid degradation of PG core proteins may occur. Macrophages produce several MMP

and ADAMTS [95, 163] that mediate versican degradation [38, 164]. Hypoxia has been shown to increase MMP synthesis in macrophages that might influence our results [165, 166]. Pulse chase experiments may clarify potential effect of hypoxia on versican core protein degradation.

Co-localization of macrophages, hypoxia and HIF in human atherosclerosis was recently demonstrated [134]. Co-localization of versican with HIF and macrophages has not previously been shown. In an immunohistochemistry experiment, using the versican antibody, we found that versican co-localized with HIF-1α and macrophage-rich areas in advanced human carotid lesions (*Paper I*). As mentioned in the introduction, versican presence in atherosclerotic plaque has been demonstrated before. In these studies, SMC, macrophages and versican were located in the same areas of the lesion. Since versican was absent in the macrophage-rich fibrotic core, versican production in plaques has generally been attributed to SMC [36, 51, 52]. However, one might speculate that the absence of versican in the fibrotic core may be due to degradation after interaction with lipoproteins [36, 38]. Our data indicate that areas where HIF, macrophages and versican co-localize are not rich in SMC. A recent study suggests an important role for macrophages in versican synthesis in atherosclerosis in mice [109], a finding that supports our results in humans. However, to establish the relative contributions of macrophages and SMC to the versican content in lesions requires an experimental approach beyond immunohistochemistry.

The effects of hypoxia on perlecan has been investigated only in EC, where decreased expression was observed [167]. This is consistent with the findings of reduced perlecan expression in human atherosclerotic lesions [74]. In HMDM, however, we found that hypoxia increased the mRNA expression of perlecan core protein (*Figure 13*). We detected perlecan protein in cell lysates by western blot, but we did not find increased protein levels in hypoxic samples. Following injury to the arterial intima, perlecan synthesis has been detected after 2 weeks [168, 169]. This is possibly related to macrophage infiltration and hypoxia. Altered expression of perlecan core protein is suggested to promote angiogenesis by stabilization of new blood vessels, a process that is associated with hypoxia [76].

The expression of serglycin was not significantly elevated in response to 24 hours of hypoxia. It would be interesting to extend the time of exposure, since serglycin is considered the major secreted PG in the human monocytic cell line U-937 (in normal cell culture conditions) and is influenced by inflammatory mediators [29]. Interestingly, the PG form of MCSF was the only PG found to be decreased in response to hypoxia. Expression of decorin and biglycan in HMDM has not previously been investigated. We found no mRNA expression of these PG (SMC were used as a positive control). This is consistent with results obtained in monocytes [101]. However, decorin co-localizes with foam cells in human

atherosclerotic lesions [51] which might indicate that the expression of decorin might be affected by foam cell formation in hypoxia.

4.1.2 The effect of hypoxia on cell-associated proteoglycans

Hypoxia has been found to induce syndecan expression in EC, fibroblasts and cardiomyocytes [170], but its effect on syndecan expression in HMDM has not been reported. We found that mRNA expression of the cell-associated HSPG syndecan-1 and -4 core proteins decreased in hypoxia (Paper III), unlike the secreted PG described above. Syndecan-1 has been identified as the major cell-associated HSPG in macrophages [171] and is found in arterial atherosclerotic lesions [172]. We found, in HMDM, that this 90 kDa syndecan-1 protein was decreased in response to hypoxia, consistent with the mRNA results. Most of the biological activity of syndecan is associated with their HS GAG chains. Therefore, the decrease in cell-associated HSPG in response to hypoxia will be further discussed in section 4.2.2.

4.2 The effect of hypoxia on macrophage proteoglycan biosynthesis —biological implications

4.2.1 Hypoxia induces changes in secreted GAG resulting in increased affinity for LDL

Increased retention of lipoproteins in the arterial wall is a result of both quantitative and qualitative changes in secreted PG. Structural changes in GAG biosynthesis, resulting in more negatively charged PG, are associated with increased affinity of LDL for GAG chains [56-59]. Macrophages synthesize more sulfated GAG compared to monocytes, and inflammatory mediators increase the sulfation of secreted CS GAG chains in both monocytes and macrophages [106, 173]. Macrophage-secreted GAG chains bind LDL [27, 67, 114]. Effects of hypoxia on GAG biosynthesis and the consequences on the interaction with LDL have been reported only for human SMC [144] and in porcine EC [174].

We isolated secreted GAG chains from HMDM, incubated in hypoxia and normal cell culture conditions for 24 hours in presence of [^{35}S]-sulfate and D-[6-^{3}H]-glucosamine. We demonstrated that HMDM exposed to hypoxia secreted GAG chains for which LDL had a higher binding affinity (*Paper II*). An increased ratio of [^{35}S] to [^{3}H] was found after hypoxic incubation, suggesting that hypoxia induces an increase in the relative number of sulfate groups per GAG chain. This may explain the increased affinity of LDL for these GAG. The increased negative charge of

GAG secreted from hypoxic cells was confirmed with ion exchange chromatography in additional HMDM donors.

The increased negative charge of GAG in response to hypoxia may potentially also be due to elongation of the chains or a shift in the position of the sulfate groups from position 4 to 6 [56-58, 71, 175, 176]. We found a modest shift towards longer GAG chains by size-exclusion chromatography in response to hypoxia. We confirmed that a majority of secreted CS GAG chains were 4-sulfated [104] and found that hypoxia increased the proportion of 6-sulfated disaccharides.

The observed increase of GAG sulfation and size in response to hypoxia may contribute to the observed increase of versican GAG in the immunosorbent assay (*Paper I*). However, this factor alone cannot explain the 3- to 7-fold increase of the signal.

Low pH, associated with hypoxia, increases the LDL affinity for PG [43, 177]. We speculate that the acidic environment *in vitro* might further increase the affinity of LDL for the more atherogenic GAG chains secreted by macrophages in hypoxia.

In our experiments on GAG biosynthesis, we isolated and analyzed the total amount of secreted GAG chains (CS, DS and HS) without the possibility to determine their PG source. We speculate that versican, with its large number of attached CS chains and its increased expression in hypoxia, may be the major source of LDL binding GAG chains isolated from hypoxic cell culture medium. However, perlecan, serglycin and the PG form of MCSF might also have contributed.

4.2.2 Hypoxia reduces cell-associated HS GAG chains, with consequences for general cell motility

HS chains can bind and mediate LDL uptake [54, 70-72]. However, atherosclerosis in humans is associated with a decrease in HS GAG [74, 178]. We observed a shift towards less HS GAG chains in cell lysates after 24 hours of exposure to hypoxia. In addition, we found decreased mRNA expression of all enzymes involved in the biosynthesis of HS GAG chains (*Paper III*). These results are in line with our findings of a hypoxia-mediated decrease of HSPG syndecan-1 and -4 core proteins.

Secreted perlecan may be associated with the cell surface, as mediated by integrins. Perlecan GAG may therefore be found in cell lysates and not only in the medium. As previously mentioned, we found an increased expression of perlecan in response to 24 hours of hypoxia. This is inconsistent with the decrease in HS GAG chains and mRNA expression of HS enzymes described above. However, the effect of hypoxia on perlecan core protein mRNA expression was larger than the effect

on syndecan, indicating that even small changes in syndecan are of importance for the total GAG in cell lysates. High turnover rate (4-24 hours) of cell-associated PG [179, 180], compensatory effects among different HS PG and the ability of perlecan to carry both HS and CS on their GAG attachment sites may all affect the composition of isolated GAG from cell lysates.

Macrophages accumulate in hypoxic areas of atherosclerotic lesions. This may be due to metabolic changes and low levels of ATP in hypoxic macrophages. A more specific regulatory mechanism has been suggested: induction of MIF (macrophage migration inhibitory factor) in response to hypoxia [141]. The roles of syndecan on cell migration and adhesion are mediated by their HS GAG chains. We found that the general motility of HMDM increased after 24 hours of hypoxia compared to normal cell culture conditions. Removal of additional HS GAG chains from hypoxic macrophages further increased the general cell motility (*Paper III*). This is comparable to the role of cell-associated HS PG in the motility of tumor cells: Reduced syndecan or HS GAG, which correlates with increased cell motility and migration, is associated with metastasis and poor prognosis in certain cancers. Hypoxia is associated with cancer progression and enzymes involved in the HS biosynthesis (i.e. exostoses) are considered to be tumor supressor genes [181-183].

Increased general motility of macrophages within hypoxic areas of lesions may facilitate uptake of surrounding lipids and increase the rate of foam cell formation. However, cell motility is only one of several potential functions that may be influenced by the decrease in cell-associated HS PG in hypoxia. The assumed anti-atherogenic effect of HS in atherosclerosis is generally attributed to their relative decrease of binding sites for GF [75]. Interestingly, another potential anti-atherogenic effect of HS may be mediated by EC-SOD. As described in the introduction, cell-associated HS is a ligand for the enzyme EC-SOD that regulates the levels of $O_2^{\bullet}$, thereby preventing oxidation of LDL [184]. Reduced EC-SOD activity is found in human atherosclerotic lesions [185], consistent with our results of decreased macrophage synthesis of syndecan and HS GAG chains in hypoxia.

4.3 Potential mechanisms involved in regulation of macrophage proteoglycans expression in hypoxia

4.3.1 The role of HIF

Hypoxia-inducible factor (HIF) is the main transcription factor that mediates cellular response to hypoxia. HIF co-localize with hypoxia and macrophages in human atherosclerotic lesions [134]. As previously described, we found that HIF-1α co-localized with versican in macrophage-rich areas in human atherosclerotic lesions. We also found potential binding sites for HIF-1α in the sequences of

versican, perlecan (*Paper I*) and syndecan-1 (*Paper III*). We hypothesized that HIF is involved in the hypoxia-induced changes in macrophage PG expression. siRNA-knockdown experiments suggested that both HIF-1α and HIF-2α were involved in the hypoxic up-regulation of versican in macrophages. The effect was additive when HIF-1α and HIF-2α were both targeted. Only knockdown of HIF-1α had an effect on perlecan mRNA expression, and no effects were found on syndecan or the enzymes involved in HS biosynthesis.

To our knowledge, there are no reports of a role for HIF in the regulation of PG in macrophages. However, HIF-2α expression has been shown to correlate with versican expression in stem cells [186]. Involvement of HIF-1α has been demonstrated in aggrecan, collagen and GAG synthesis in chondrocytes [187-189] and EC [190]. HIF-1α has been shown to mediate induction of several enzymes involved in the HS biosynthesis in EC [35, 190].

HIF-1α and HIF-2α have been shown to regulate overlapping sets of genes, but individual gene regulation is reported as well as compensatory mechanisms [191]. Induction of HIF-1α has been reported to start immediately upon cell exposure to 1% oxygen, whereas HIF-2α reaches maximum induction after 48 hours [192]. These effects have been observed in neuroblastoma cells, where HIF-1α induced early VEGF expression and HIF-2α became more important after some time [193]. The suggested role for HIF-1α in the acute response to hypoxia, compared to the more long term response described for HIF-2α, might be involved in our results on versican and perlecan mRNA expression over time (*Paper I*): we found a relatively late induction of versican (maximum changes after 24 hours) compared to perlecan (12 hours). This mechanism could also be due to a secondary regulation, mediated by HIF.

4.3.2 The role of glucose

Glucose consumption is increased in hypoxic cells. We hypothesized that decreased mRNA expression of syndecan in response to hypoxia may be due to insufficient glucose content of the cell culture medium. To study this possibility, we incubated HMDM with twice the amount of glucose present in RPMI 1640 medium (22 v.s 11 mM). We found no effects on mRNA expression of syndecan. Interestingly, versican expression was further increased in hypoxic cells in the presence of high glucose concentrations (*Figure 14*). This effect was not seen in control experiments with mannose.

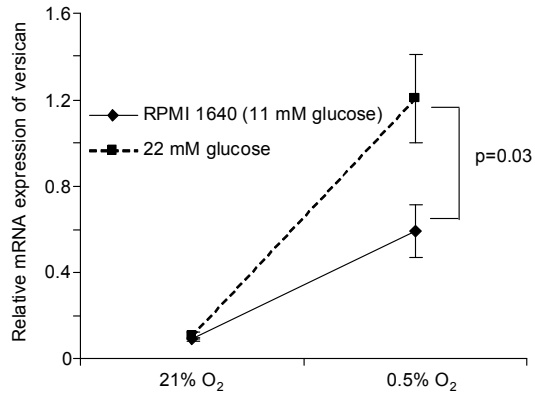

Figure 14: Versican mRNA expression in HMDM in presence of 11 mM glucose and 22 mM glucose after 24 hours incubation in hypoxia and normal cell culture conditions (Mean ± SEM of n=6).

4.3.3 Summary of potential mechanisms of versican regulation

Our results indicate that the hypoxic induction of versican expression in macrophages involves transcriptional activity of HIF. However, this regulation may be secondary to the multiple metabolic effects mediated by HIF. Hypoxic induction of versican seems to be dependent on the availability of glucose. This is interesting, since hyperglycemia is a risk factor for cardiovascular disease. No effects of glucose on macrophage PG synthesis have been found in normal oxygen concentrations [194]. This is consistent with the result of our experiment, where effects were seen only in hypoxia (*Figure 14*).

High concentrations of circulating free fatty acids (FFA) are a risk factor for type 2 diabetes, a condition strongly associated with atherosclerosis. Cells within the arterial intima are exposed to these high concentrations. Earlier studies from our group have shown that expression of versican and perlecan is induced in SMC exposed to high concentrations of FFA [195, 196]. FFA-induced versican expression in SMC is suggested to be mediated by the accumulation of diacylglycerol, which stimulates the phosphorylation of several protein kinase C isoforms [196]. We have analyzed versican mRNA expression in HMDM after incubation with 800 μM linoleate for 24 hours in normal cell culture conditions. In response to this high concentrations of linoleate, we found a 4.5 ± 1.0 fold increase in mRNA expression of versican (mean ± SEM of n=7). A consequence of hypoxia is a reduced mitochondrial capacity to oxidize fatty acids [158], which leads to their intracellular accumulation. Macrophages have been shown to accumulate increased amounts of lipids in hypoxic conditions due to uptake of fatty acids [90]. Our results on FFA-induced versican expression in normal cell culture conditions

lead us to speculate that excess intracellular fatty acids in hypoxic macrophages may increase versican expression even further. However, the combined effect of FFA and hypoxia on versican synthesis in macrophages is yet to be determined.

5 Conclusions and future perspectives

PG play a fundamental role in mediating accumulation of lipoproteins and regulation of GF and enzymes within the arterial intima during the atherosclerotic process. Macrophages, one of the most abundant cell types within the atherosclerotic plaque, are located in hypoxic areas, but little was known about macrophage PG synthesis in hypoxic conditions. In this thesis, I have presented results on the effects of hypoxia on macrophage PG syntheses that indicate a novel role for macrophages in atherosclerosis.

The results presented show that human macrophages exposed to hypoxia increase the expression of the large secreted PG versican and perlecan. The results further suggest that hypoxic induction of these PG involves HIF signalling. A role for hypoxic macrophages in versican synthesis is also indicated by the finding that versican co-localizes with HIF-1α in macrophage-rich areas of human advanced atherosclerotic lesions. In addition to the hypoxic induction of secreted PG core proteins, we found that macrophages in hypoxia secrete more negatively charged GAG chains with increased affinity for LDL. Secreted PG contribute to the pathology of atherosclerosis in several ways, but the purpose of the hypoxic induction of these PG in macrophages is not known. It might be a response to metabolic challenges. Versican and CS GAG have been suggested to play a role in protecting cells from ROS-induced injury upon oxidative stress [197-199], a condition present in hypoxic cells. Furthermore, oxidative stress has been shown to increase macrophage synthesis of a CS PG with capacity to bind LDL [112]. Increased perlecan synthesis has also been found in the arterial intima upon injury [168, 169]. HIF signaling is suggested to protect cells from ROS-induced apoptosis in hypoxia since HIF-1α-deficient mouse embryo fibroblasts in hypoxic culture undergo cell death upon increased ROS levels [200].

We found a decreased synthesis of syndecan and cell-associated HS GAG chains in response to hypoxia, consistent with the proposed decrease in HS GAG in human atherosclerosis. Interestingly, increased general motility of hypoxic macrophages might involve hypoxic-induced reduction in HS biosynthesis. We speculate that this mechanism may facilitate uptake of surrounding lipids and increase the rate of foam cell formation. It would be exciting to evaluate the hypoxia-induced effect on decreased levels of syndecan-1 and HS GAG chains on the binding of EC-SOD, which may have consequences for LDL oxidation. In several other cell types, syndecan is induced in response to vascular injury, indicating an important biological role of syndecan in tissue remodeling, vascular repair and inflammation [39].

According to our results from studies in hypoxic conditions, the role of macrophages in PG remodelling in atherosclerotic lesions might have been

underestimated. This could be due to the fact that *in vitro* studies are normally performed in 21% O_2. Our results support the conclusion in recent reviews [11, 12, 142] that hypoxia is a more realistic condition for *in vitro* experiments when studying macrophage function in atherosclerosis.

Macrophage recruitment and the role of macrophages in lipid storage, inflammation and matrix degradation have all been therapeutic targets in the different stages of plaque progression [201]. Our results suggest that macrophages in hypoxic regions of lesions may alter the matrix in ways that increase their contribution to development of atherosclerosis. These novel properties of hypoxic macrophages may be susceptible to therapeutic modulation and thus lead to more efficient therapies.

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7 References

1. RIKS-HIA, *SEPHIA Årsrapport*. 2007.
2. Rosengren, A., *Declining cardiovascular mortality and increasing obesity: a paradox*. CMAJ, 2009. **181**(3-4): p. 127-8.
3. Williams, K.J. and I. Tabas, *The response-to-retention hypothesis of early atherogenesis*. Arterioscler Thromb Vasc Biol, 1995. **15**(5): p. 551-61.
4. Camejo, G., E. Hurt, M. Thubrikar, and G. Bondjers, *Modification of low density lipoprotein association with the arterial intima. A possible environment for the antiatherogenic action of beta-blockers*. Circulation, 1991. **84**(6 Suppl): p. VI17-22.
5. Camejo, G., A. Lopez, F. Lopez, and J. Quinones, *Interaction of low density lipoproteins with arterial proteoglycans. The role of charge and sialic acid content*. Atherosclerosis, 1985. **55**(1): p. 93-105.
6. Camejo, G., S.O. Olofsson, F. Lopez, P. Carlsson, and G. Bondjers, *Identification of Apo B-100 segments mediating the interaction of low density lipoproteins with arterial proteoglycans*. Arteriosclerosis, 1988. **8**(4): p. 368-77.
7. Hurt, E., G. Bondjers, and G. Camejo, *Interaction of LDL with human arterial proteoglycans stimulates its uptake by human monocyte-derived macrophages*. J Lipid Res, 1990. **31**(3): p. 443-54.
8. Hurt-Camejo, E., G. Camejo, B. Rosengren, F. Lopez, C. Ahlstrom, G. Fager, and G. Bondjers, *Effect of arterial proteoglycans and glycosaminoglycans on low density lipoprotein oxidation and its uptake by human macrophages and arterial smooth muscle cells*. Arterioscler Thromb, 1992. **12**(5): p. 569-83.
9. Tannock, L.R. and V.L. King, *Proteoglycan mediated lipoprotein retention: a mechanism of diabetic atherosclerosis*. Rev Endocr Metab Disord, 2008. **9**(4): p. 289-300.
10. Hueper, W., *Atherosclerosis: the anoxemia theory*. Arch Pathol 1944. **38**: p. 173-205.
11. Hulten, L.M. and M. Levin, *The role of hypoxia in atherosclerosis*. Curr Opin Lipidol, 2009.
12. Sluimer, J.C. and M.J. Daemen, *Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis*. J Pathol, 2009. **218**(1): p. 7-29.
13. Gandhi, N.S. and R.L. Mancera, *The structure of glycosaminoglycans and their interactions with proteins*. Chem Biol Drug Des, 2008. **72**(6): p. 455-82.
14. Iozzo, R.V., *Matrix proteoglycans: from molecular design to cellular function*. Annu Rev Biochem, 1998. **67**: p. 609-52.
15. Prydz, K. and K.T. Dalen, *Synthesis and sorting of proteoglycans*. J Cell Sci, 2000. **113 Pt 2**: p. 193-205.
16. Funderburgh, J.L., M.L. Funderburgh, M.M. Mann, and G.W. Conrad, *Arterial lumican. Properties of a corneal-type keratan sulfate proteoglycan from bovine aorta*. J Biol Chem, 1991. **266**(36): p. 24773-7.
17. Onda, M., T. Ishiwata, K. Kawahara, R. Wang, Z. Naito, and Y. Sugisaki, *Expression of lumican in thickened intima and smooth muscle cells in human coronary atherosclerosis*. Exp Mol Pathol, 2002. **72**(2): p. 142-9.
18. Kjellen, L. and U. Lindahl, *Proteoglycans: structures and interactions*. Annu Rev Biochem, 1991. **60**: p. 443-75.
19. Iozzo, R.V., *The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins*. J Biol Chem, 1999. **274**(27): p. 18843-6.
20. Laurent, T.C. and J.R. Fraser, *Hyaluronan*. FASEB J, 1992. **6**(7): p. 2397-404.
21. Wight, T.N., *Versican: a versatile extracellular matrix proteoglycan in cell biology*. Curr Opin Cell Biol, 2002. **14**(5): p. 617-23.
22. Dours-Zimmermann, M.T. and D.R. Zimmermann, *A novel glycosaminoglycan attachment domain identified in two alternative splice variants of human versican*. J Biol Chem, 1994. **269**(52): p. 32992-8.
23. Jackson, R.L., S.J. Busch, and A.D. Cardin, *Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes*. Physiol Rev, 1991. **71**(2): p. 481-539.
24. Price, L.K., H.U. Choi, L. Rosenberg, and E.R. Stanley, *The predominant form of secreted colony stimulating factor-1 is a proteoglycan*. J Biol Chem, 1992. **267**(4): p. 2190-9.
25. Suzu, S., T. Ohtsuki, N. Yanai, Z. Takatsu, T. Kawashima, F. Takaku, N. Nagata, and K. Motoyoshi, *Identification of a high molecular weight macrophage colony-stimulating factor as a glycosaminoglycan-containing species*. J Biol Chem, 1992. **267**(7): p. 4345-8.

26. Chang, M.Y., K.L. Olin, C. Tsoi, T.N. Wight, and A. Chait, *Human monocyte-derived macrophages secrete two forms of proteoglycan-macrophage colony-stimulating factor that differ in their ability to bind low density lipoproteins.* J Biol Chem, 1998. **273**(26): p. 15985-92.
27. Suzu, S., T. Inaba, N. Yanai, T. Kawashima, N. Yamada, T. Oka, R. Machinami, T. Ohtsuki, F. Kimura, S. Kondo, and et al., *Proteoglycan form of macrophage colony-stimulating factor binds low density lipoprotein.* J Clin Invest, 1994. **94**(4): p. 1637-41.
28. Kolset, S.O. and L. Zernichow, *Serglycin and secretion in human monocytes.* Glycoconj J, 2008. **25**(4): p. 305-11.
29. Kolset, S.O. and H. Tveit, *Serglycin--structure and biology.* Cell Mol Life Sci, 2008. **65**(7-8): p. 1073-85.
30. Bernfield, M., M. Gotte, P.W. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, and M. Zako, *Functions of cell surface heparan sulfate proteoglycans.* Annu Rev Biochem, 1999. **68**: p. 729-77.
31. Filmus, J. and S.B. Selleck, *Glypicans: proteoglycans with a surprise.* J Clin Invest, 2001. **108**(4): p. 497-501.
32. Knox, S.M. and J.M. Whitelock, *Perlecan: how does one molecule do so many things?* Cell Mol Life Sci, 2006. **63**(21): p. 2435-45.
33. Whitelock, J.M. and R.V. Iozzo, *Heparan sulfate: a complex polymer charged with biological activity.* Chem Rev, 2005. **105**(7): p. 2745-64.
34. Kim, B.T., H. Kitagawa, J. Tamura, T. Saito, M. Kusche-Gullberg, U. Lindahl, and K. Sugahara, *Human tumor suppressor EXT gene family members EXTL1 and EXTL3 encode alpha 1,4- N-acetylglucosaminyltransferases that likely are involved in heparan sulfate/ heparin biosynthesis.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7176-81.
35. Kitagawa, H., H. Shimakawa, and K. Sugahara, *The tumor suppressor EXT-like gene EXTL2 encodes an alpha1, 4-N-acetylhexosaminyltransferase that transfers N-acetylgalactosamine and N-acetylglucosamine to the common glycosaminoglycan-protein linkage region. The key enzyme for the chain initiation of heparan sulfate.* J Biol Chem, 1999. **274**(20): p. 13933-7.
36. Halpert, I., U.I. Sires, J.D. Roby, S. Potter-Perigo, T.N. Wight, S.D. Shapiro, H.G. Welgus, S.A. Wickline, and W.C. Parks, *Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme.* Proc Natl Acad Sci U S A, 1996. **93**(18): p. 9748-53.
37. Whitelock, J.M., A.D. Murdoch, R.V. Iozzo, and P.A. Underwood, *The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases.* J Biol Chem, 1996. **271**(17): p. 10079-86.
38. Sandy, J.D., J. Westling, R.D. Kenagy, M.L. Iruela-Arispe, C. Verscharen, J.C. Rodriguez-Mazaneque, D.R. Zimmermann, J.M. Lemire, J.W. Fischer, T.N. Wight, and A.W. Clowes, *Versican V1 proteolysis in human aorta in vivo occurs at the Glu441-Ala442 bond, a site that is cleaved by recombinant ADAMTS-1 and ADAMTS-4.* J Biol Chem, 2001. **276**(16): p. 13372-8.
39. Alexopoulou, A.N., H.A. Multhaupt, and J.R. Couchman, *Syndecans in wound healing, inflammation and vascular biology.* Int J Biochem Cell Biol, 2007. **39**(3): p. 505-28.
40. Rodriguez-Manzaneque, J.C., D. Carpizo, C. Plaza-Calonge Mdel, A.X. Torres-Collado, S.N. Thai, M. Simons, A. Horowitz, and M.L. Iruela-Arispe, *Cleavage of syndecan-4 by ADAMTS1 provokes defects in adhesion.* Int J Biochem Cell Biol, 2009. **41**(4): p. 800-10.
41. Schwartz, N., *Biosynthesis and regulation of expression of proteoglycans.* Front Biosci, 2000. **5**: p. D649-55.
42. Vreys, V. and G. David, *Mammalian heparanase: what is the message?* J Cell Mol Med, 2007. **11**(3): p. 427-52.
43. Camejo, G., F. Lalaguna, F. Lopez, and R. Starosta, *Characterization and properties of a lipoprotein-complexing proteoglycan from human aorta.* Atherosclerosis, 1980. **35**(3): p. 307-20.
44. Camejo, G., T. Linden, U. Olsson, O. Wiklund, F. Lopez, and G. Bondjers, *Binding parameters and concentration modulate formation of complexes between LDL and arterial proteoglycans in serum.* Atherosclerosis, 1989. **79**(2-3): p. 121-8.
45. Olsson, U., G. Camejo, S.O. Olofsson, and G. Bondjers, *Molecular parameters that control the association of low density lipoprotein apo B-100 with chondroitin sulphate.* Biochim Biophys Acta, 1991. **1097**(1): p. 37-44.

46. Boren, J., K. Olin, I. Lee, A. Chait, T.N. Wight, and T.L. Innerarity, *Identification of the principal proteoglycan-binding site in LDL. A single-point mutation in apo-B100 severely affects proteoglycan interaction without affecting LDL receptor binding.* J Clin Invest, 1998. **101**(12): p. 2658-64.
47. Skalen, K., M. Gustafsson, E.K. Rydberg, L.M. Hulten, O. Wiklund, T.L. Innerarity, and J. Boren, *Subendothelial retention of atherogenic lipoproteins in early atherosclerosis.* Nature, 2002. **417**(6890): p. 750-4.
48. Camejo, G., U. Olsson, E. Hurt-Camejo, N. Baharamian, and G. Bondjers, *The extracellular matrix on atherogenesis and diabetes-associated vascular disease.* Atheroscler Suppl, 2002. **3**(1): p. 3-9.
49. Khalil, M.F., W.D. Wagner, and I.J. Goldberg, *Molecular interactions leading to lipoprotein retention and the initiation of atherosclerosis.* Arterioscler Thromb Vasc Biol, 2004. **24**(12): p. 2211-8.
50. Tannock, L.R. and A. Chait, *Lipoprotein-matrix interactions in macrovascular disease in diabetes.* Front Biosci, 2004. **9**: p. 1728-42.
51. Evanko, S.P., E.W. Raines, R. Ross, L.I. Gold, and T.N. Wight, *Proteoglycan distribution in lesions of atherosclerosis depends on lesion severity, structural characteristics, and the proximity of platelet-derived growth factor and transforming growth factor-beta.* Am J Pathol, 1998. **152**(2): p. 533-46.
52. O'Brien, K.D., K.L. Olin, C.E. Alpers, W. Chiu, M. Ferguson, K. Hudkins, T.N. Wight, and A. Chait, *Comparison of apolipoprotein and proteoglycan deposits in human coronary atherosclerotic plaques: colocalization of biglycan with apolipoproteins.* Circulation, 1998. **98**(6): p. 519-27.
53. Nakashima, Y., H. Fujii, S. Sumiyoshi, T.N. Wight, and K. Sueishi, *Early human atherosclerosis: accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration.* Arterioscler Thromb Vasc Biol, 2007. **27**(5): p. 1159-65.
54. Iverius, P.H., *The interaction between human plasma lipoproteins and connective tissue glycosaminoglycans.* J Biol Chem, 1972. **247**(8): p. 2607-13.
55. Chang, M.Y., S. Potter-Perigo, C. Tsoi, A. Chait, and T.N. Wight, *Oxidized low density lipoproteins regulate synthesis of monkey aortic smooth muscle cell proteoglycans that have enhanced native low density lipoprotein binding properties.* J Biol Chem, 2000. **275**(7): p. 4766-73.
56. Camejo, G., G. Fager, B. Rosengren, E. Hurt-Camejo, and G. Bondjers, *Binding of low density lipoproteins by proteoglycans synthesized by proliferating and quiescent human arterial smooth muscle cells.* J Biol Chem, 1993. **268**(19): p. 14131-7.
57. Mourao, P.A., S. Pillai, and N. Di Ferrante, *The binding of chondroitin 6-sulfate to plasma low density lipoprotein.* Biochim Biophys Acta, 1981. **674**(2): p. 178-87.
58. Sambandam, T., J.R. Baker, J.E. Christner, and S.L. Ekborg, *Specificity of the low density lipoprotein-glycosaminoglycan interaction.* Arterioscler Thromb, 1991. **11**(3): p. 561-8.
59. Cardoso, L.E. and P.A. Mourao, *Glycosaminoglycan fractions from human arteries presenting diverse susceptibilities to atherosclerosis have different binding affinities to plasma LDL.* Arterioscler Thromb, 1994. **14**(1): p. 115-24.
60. Rosengren, B., H. Peilot, M. Umaerus, A.C. Jonsson-Rylander, L. Mattsson-Hulten, C. Hallberg, P. Cronet, M. Rodriguez-Lee, and E. Hurt-Camejo, *Secretory phospholipase A2 group V: lesion distribution, activation by arterial proteoglycans, and induction in aorta by a Western diet.* Arterioscler Thromb Vasc Biol, 2006. **26**(7): p. 1579-85.
61. Sartipy, P., G. Bondjers, and E. Hurt-Camejo, *Phospholipase A2 type II binds to extracellular matrix biglycan: modulation of its activity on LDL by colocalization in glycosaminoglycan matrixes.* Arterioscler Thromb Vasc Biol, 1998. **18**(12): p. 1934-41.
62. Sartipy, P., B. Johansen, G. Camejo, B. Rosengren, G. Bondjers, and E. Hurt-Camejo, *Binding of human phospholipase A2 type II to proteoglycans. Differential effect of glycosaminoglycans on enzyme activity.* J Biol Chem, 1996. **271**(42): p. 26307-14.
63. Alves, C.S. and P.A. Mourao, *Interaction of high molecular weight chondroitin sulfate from human aorta with plasma low density lipoproteins.* Atherosclerosis, 1988. **73**(2-3): p. 113-24.
64. Fager, G., G. Camejo, U. Olsson, G. Ostergren-Lunden, F. Lustig, and G. Bondjers, *Binding of platelet-derived growth factor and low density lipoproteins to glycosaminoglycan species produced by human arterial smooth muscle cells.* J Cell Physiol, 1995. **163**(2): p. 380-92.
65. Hakala, J.K., K. Oorni, M.O. Pentikainen, E. Hurt-Camejo, and P.T. Kovanen, *Lipolysis of LDL by human secretory phospholipase A(2) induces particle fusion and enhances the retention of LDL to human aortic proteoglycans.* Arterioscler Thromb Vasc Biol, 2001. **21**(6): p. 1053-8.

66. Hurt-Camejo, E., G. Camejo, B. Rosengren, F. Lopez, O. Wiklund, and G. Bondjers, *Differential uptake of proteoglycan-selected subfractions of low density lipoprotein by human macrophages.* J Lipid Res, 1990. **31**(8): p. 1387-98.
67. Maor, I., T. Hayek, M. Hirsh, T.C. Iancu, and M. Aviram, *Macrophage-released proteoglycans enhance LDL aggregation: studies in aorta from apolipoprotein E-deficient mice.* Atherosclerosis, 2000. **150**(1): p. 91-101.
68. Oorni, K., M.O. Pentikainen, M. Ala-Korpela, and P.T. Kovanen, *Aggregation, fusion, and vesicle formation of modified low density lipoprotein particles: molecular mechanisms and effects on matrix interactions.* J Lipid Res, 2000. **41**(11): p. 1703-14.
69. Owens, R.T. and W.D. Wagner, *Proteoglycans produced by cholesterol-enriched macrophages bind plasma low density lipoprotein.* Atherosclerosis, 1991. **91**(3): p. 229-40.
70. Boyanovsky, B.B., P. Shridas, M. Simons, D.R. van der Westhuyzen, and N.R. Webb, *Syndecan-4 mediates macrophage uptake of group V secretory phospholipase A2-modified LDL.* J Lipid Res, 2009. **50**(4): p. 641-50.
71. Edwards, I.J. and W.D. Wagner, *Distinct synthetic and structural characteristics of proteoglycans produced by cultured artery smooth muscle cells of atherosclerosis-susceptible pigeons.* J Biol Chem, 1988. **263**(20): p. 9612-20.
72. Hendriks, W.L., H. van der Boom, L.C. van Vark, and L.M. Havekes, *Lipoprotein lipase stimulates the binding and uptake of moderately oxidized low-density lipoprotein by J774 macrophages.* Biochem J, 1996. **314 (Pt 2)**: p. 563-8.
73. Hollmann, J., A. Schmidt, D.B. von Bassewitz, and E. Buddecke, *Relationship of sulfated glycosaminoglycans and cholesterol content in normal and arteriosclerotic human aorta.* Arteriosclerosis, 1989. **9**(2): p. 154-8.
74. Tran, P.K., H.E. Agardh, K. Tran-Lundmark, J. Ekstrand, J. Roy, B. Henderson, A. Gabrielsen, G.K. Hansson, J. Swedenborg, G. Paulsson-Berne, and U. Hedin, *Reduced perlecan expression and accumulation in human carotid atherosclerotic lesions.* Atherosclerosis, 2007. **190**(2): p. 264-70.
75. Segev, A., N. Nili, and B.H. Strauss, *The role of perlecan in arterial injury and angiogenesis.* Cardiovasc Res, 2004. **63**(4): p. 603-10.
76. Bix, G. and R.V. Iozzo, *Novel interactions of perlecan: unraveling perlecan's role in angiogenesis.* Microsc Res Tech, 2008. **71**(5): p. 339-48.
77. Fager, G., G. Camejo, and G. Bondjers, *Heparin-like glycosaminoglycans influence growth and phenotype of human arterial smooth muscle cells in vitro. I. Evidence for reversible binding and inactivation of the platelet-derived growth factor by heparin.* In Vitro Cell Dev Biol, 1992. **28A**(3 Pt 1): p. 168-75.
78. Kenagy, R.D., A.H. Plaas, and T.N. Wight, *Versican degradation and vascular disease.* Trends Cardiovasc Med, 2006. **16**(6): p. 209-15.
79. Auffray, C., M.H. Sieweke, and F. Geissmann, *Blood monocytes: development, heterogeneity, and relationship with dendritic cells.* Annu Rev Immunol, 2009. **27**: p. 669-92.
80. Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati, *The chemokine system in diverse forms of macrophage activation and polarization.* Trends Immunol, 2004. **25**(12): p. 677-86.
81. Buttery, L.D., D.R. Springall, A.H. Chester, T.J. Evans, E.N. Standfield, D.V. Parums, M.H. Yacoub, and J.M. Polak, *Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite.* Lab Invest, 1996. **75**(1): p. 77-85.
82. Folcik, V.A., R.A. Nivar-Aristy, L.P. Krajewski, and M.K. Cathcart, *Lipoxygenase contributes to the oxidation of lipids in human atherosclerotic plaques.* J Clin Invest, 1995. **96**(1): p. 504-10.
83. Heinecke, J.W., *Pathways for oxidation of low density lipoprotein by myeloperoxidase: tyrosyl radical, reactive aldehydes, hypochlorous acid and molecular chlorine.* Biofactors, 1997. **6**(2): p. 145-55.
84. Napoli, C., F. de Nigris, and W. Palinski, *Multiple role of reactive oxygen species in the arterial wall.* J Cell Biochem, 2001. **82**(4): p. 674-82.
85. Wright, G.W., C.E. Ooi, J. Weiss, and P. Elsbach, *Purification of a cellular (granulocyte) and an extracellular (serum) phospholipase A2 that participate in the destruction of Escherichia coli in a rabbit inflammatory exudate.* J Biol Chem, 1990. **265**(12): p. 6675-81.
86. Basu, S.K., J.L. Goldstein, and M.S. Brown, *Independent pathways for secretion of cholesterol and apolipoprotein E by macrophages.* Science, 1983. **219**(4586): p. 871-3.

87. Mazzone, T. and C. Reardon, *Expression of heterologous human apolipoprotein E by J774 macrophages enhances cholesterol efflux to HDL3.* J Lipid Res, 1994. **35**(8): p. 1345-53.
88. Brown, M.S., Y.K. Ho, and J.L. Goldstein, *The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters.* J Biol Chem, 1980. **255**(19): p. 9344-52.
89. Warner, G.J., G. Stoudt, M. Bamberger, W.J. Johnson, and G.H. Rothblat, *Cell toxicity induced by inhibition of acyl coenzyme A:cholesterol acyltransferase and accumulation of unesterified cholesterol.* J Biol Chem, 1995. **270**(11): p. 5772-8.
90. Bostrom, P., B. Magnusson, P.A. Svensson, O. Wiklund, J. Boren, L.M. Carlsson, M. Stahlman, S.O. Olofsson, and L.M. Hulten, *Hypoxia converts human macrophages into triglyceride-loaded foam cells.* Arterioscler Thromb Vasc Biol, 2006. **26**(8): p. 1871-6.
91. Mattsson, L., H. Johansson, M. Ottosson, G. Bondjers, and O. Wiklund, *Expression of lipoprotein lipase mRNA and secretion in macrophages isolated from human atherosclerotic aorta.* J Clin Invest, 1993. **92**(4): p. 1759-65.
92. Linton, M.F. and S. Fazio, *Macrophages, inflammation, and atherosclerosis.* Int J Obes Relat Metab Disord, 2003. **27 Suppl 3**: p. S35-40.
93. Nathan, C.F. and S. Tsunawaki, *Secretion of toxic oxygen products by macrophages: regulatory cytokines and their effects on the oxidase.* Ciba Found Symp, 1986. **118**: p. 211-30.
94. Takahashi, K., M. Takeya, and N. Sakashita, *Multifunctional roles of macrophages in the development and progression of atherosclerosis in humans and experimental animals.* Med Electron Microsc, 2002. **35**(4): p. 179-203.
95. Jonsson-Rylander, A.C., T. Nilsson, R. Fritsche-Danielson, A. Hammarstrom, M. Behrendt, J.O. Andersson, K. Lindgren, A.K. Andersson, P. Wallbrandt, B. Rosengren, P. Brodin, A. Thelin, A. Westin, E. Hurt-Camejo, and C.H. Lee-Sogaard, *Role of ADAMTS-1 in atherosclerosis: remodeling of carotid artery, immunohistochemistry, and proteolysis of versican.* Arterioscler Thromb Vasc Biol, 2005. **25**(1): p. 180-5.
96. Newby, A.C., *Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability.* Arterioscler Thromb Vasc Biol, 2008. **28**(12): p. 2108-14.
97. Wagsater, D., H. Bjork, C. Zhu, J. Bjorkegren, G. Valen, A. Hamsten, and P. Eriksson, *ADAMTS-4 and -8 are inflammatory regulated enzymes expressed in macrophage-rich areas of human atherosclerotic plaques.* Atherosclerosis, 2008. **196**(2): p. 514-22.
98. Sasaki, N., N. Higashi, T. Taka, M. Nakajima, and T. Irimura, *Cell surface localization of heparanase on macrophages regulates degradation of extracellular matrix heparan sulfate.* J Immunol, 2004. **172**(6): p. 3830-5.
99. Clasper, S., S. Vekemans, M. Fiore, M. Plebanski, P. Wordsworth, G. David, and D.G. Jackson, *Inducible expression of the cell surface heparan sulfate proteoglycan syndecan-2 (fibroglycan) on human activated macrophages can regulate fibroblast growth factor action.* J Biol Chem, 1999. **274**(34): p. 24113-23.
100. Kulseth, M.A., S.L. Mustorp, L. Uhlin-Hansen, F. Oberg, and S.O. Kolset, *Serglycin expression during monocytic differentiation of U937-1 cells.* Glycobiology, 1998. **8**(8): p. 747-53.
101. Makatsori, E., F.N. Lamari, A.D. Theocharis, S. Anagnostides, A. Hjerpe, T. Tsegenidis, and N.K. Karamanos, *Large matrix proteoglycans, versican and perlecan, are expressed and secreted by human leukemic monocytes.* Anticancer Res, 2003. **23**(4): p. 3303-9.
102. Manakil, J.F., P.B. Sugerman, H. Li, G.J. Seymour, and P.M. Bartold, *Cell-surface proteoglycan expression by lymphocytes from peripheral blood and gingiva in health and periodontal disease.* J Dent Res, 2001. **80**(8): p. 1704-10.
103. Saphire, A.C., M.D. Bobardt, Z. Zhang, G. David, and P.A. Gallay, *Syndecans serve as attachment receptors for human immunodeficiency virus type 1 on macrophages.* J Virol, 2001. **75**(19): p. 9187-200.
104. Uhlin-Hansen, L., T. Wik, L. Kjellen, E. Berg, F. Forsdahl, and S.O. Kolset, *Proteoglycan metabolism in normal and inflammatory human macrophages.* Blood, 1993. **82**(9): p. 2880-9.
105. Zernichow, L., M. Abrink, J. Hallgren, M. Grujic, G. Pejler, and S.O. Kolset, *Serglycin is the major secreted proteoglycan in macrophages and has a role in the regulation of macrophage tumor necrosis factor-alpha secretion in response to lipopolysaccharide.* J Biol Chem, 2006. **281**(37): p. 26792-801.
106. Uhlin-Hansen, L., T. Eskeland, and S.O. Kolset, *Modulation of the expression of chondroitin sulfate proteoglycan in stimulated human monocytes.* J Biol Chem, 1989. **264**(25): p. 14916-22.

107. Nelander, S., E. Larsson, E. Kristiansson, R. Mansson, O. Nerman, M. Sigvardsson, P. Mostad, and P. Lindahl, *Predictive screening for regulators of conserved functional gene modules (gene batteries) in mammals.* BMC Genomics, 2005. **6**(1): p. 68.
108. Su, A.I., T. Wiltshire, S. Batalov, H. Lapp, K.A. Ching, D. Block, J. Zhang, R. Soden, M. Hayakawa, G. Kreiman, M.P. Cooke, J.R. Walker, and J.B. Hogenesch, *A gene atlas of the mouse and human protein-encoding transcriptomes.* Proc Natl Acad Sci U S A, 2004. **101**(16): p. 6062-7.
109. Seidelmann, S.B., C. Kuo, N. Pleskac, J. Molina, S. Sayers, R. Li, J. Zhou, P. Johnson, K. Braun, C. Chan, D. Teupser, J.L. Breslow, T.N. Wight, A.R. Tall, and C.L. Welch, *Athsq1 Is an Atherosclerosis Modifier Locus With Dramatic Effects on Lesion Area and Prominent Accumulation of Versican.* Arterioscler Thromb Vasc Biol, 2008.
110. Christner, J.E., *Biosynthesis of chondroitin sulfate proteoglycan by P388D1 macrophage-like cell line.* Arteriosclerosis, 1988. **8**(5): p. 535-43.
111. Edwards, I.J., H. Xu, J.C. Obunike, I.J. Goldberg, and W.D. Wagner, *Differentiated macrophages synthesize a heparan sulfate proteoglycan and an oversulfated chondroitin sulfate proteoglycan that bind lipoprotein lipase.* Arterioscler Thromb Vasc Biol, 1995. **15**(3): p. 400-9.
112. Kaplan, M. and M. Aviram, *Macrophage plasma membrane chondroitin sulfate proteoglycan binds oxidized low-density lipoprotein.* Atherosclerosis, 2000. **149**(1): p. 5-17.
113. Kaplan, M., K.J. Williams, H. Mandel, and M. Aviram, *Role of macrophage glycosaminoglycans in the cellular catabolism of oxidized LDL by macrophages.* Arterioscler Thromb Vasc Biol, 1998. **18**(4): p. 542-53.
114. Maor, I. and M. Aviram, *Macrophage released proteoglycans are involved in cell-mediated aggregation of LDL.* Atherosclerosis, 1999. **142**(1): p. 57-66.
115. Torres Filho, I.P., M. Leunig, F. Yuan, M. Intaglietta, and R.K. Jain, *Noninvasive measurement of microvascular and interstitial oxygen profiles in a human tumor in SCID mice.* Proc Natl Acad Sci U S A, 1994. **91**(6): p. 2081-5.
116. Lewis, J.S., J.A. Lee, J.C. Underwood, A.L. Harris, and C.E. Lewis, *Macrophage responses to hypoxia: relevance to disease mechanisms.* J Leukoc Biol, 1999. **66**(6): p. 889-900.
117. Brahimi-Horn, M.C. and J. Pouyssegur, *Oxygen, a source of life and stress.* FEBS Lett, 2007. **581**(19): p. 3582-91.
118. Semenza, G.L., *Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1.* Biochem J, 2007. **405**(1): p. 1-9.
119. Semenza, G.L. and G.L. Wang, *A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation.* Mol Cell Biol, 1992. **12**(12): p. 5447-54.
120. Semenza, G.L., *Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology.* Trends Mol Med, 2001. **7**(8): p. 345-50.
121. Semenza, G.L., *Regulation of physiological responses to continuous and intermittent hypoxia by hypoxia-inducible factor 1.* Exp Physiol, 2006. **91**(5): p. 803-6.
122. Wenger, R.H., D.P. Stiehl, and G. Camenisch, *Integration of oxygen signaling at the consensus HRE.* Sci STKE, 2005. **2005**(306): p. re12.
123. Semenza, G.L., *Hypoxia-inducible factor 1 (HIF-1) pathway.* Sci STKE, 2007. **2007**(407): p. cm8.
124. Hu, C.J., L.Y. Wang, L.A. Chodosh, B. Keith, and M.C. Simon, *Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation.* Mol Cell Biol, 2003. **23**(24): p. 9361-74.
125. O'Rourke, J.F., Y.M. Tian, P.J. Ratcliffe, and C.W. Pugh, *Oxygen-regulated and transactivating domains in endothelial PAS protein 1: comparison with hypoxia-inducible factor-1alpha.* J Biol Chem, 1999. **274**(4): p. 2060-71.
126. Wiesener, M.S., H. Turley, W.E. Allen, C. Willam, K.U. Eckardt, K.L. Talks, S.M. Wood, K.C. Gatter, A.L. Harris, C.W. Pugh, P.J. Ratcliffe, and P.H. Maxwell, *Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha.* Blood, 1998. **92**(7): p. 2260-8.
127. Liu, Y.V., J.H. Baek, H. Zhang, R. Diez, R.N. Cole, and G.L. Semenza, *RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O(2)-independent and HSP90 inhibitor-induced degradation of HIF-1alpha.* Mol Cell, 2007. **25**(2): p. 207-17.

128. Chen, L., K. Uchida, A. Endler, and F. Shibasaki, *Mammalian tumor suppressor Int6 specifically targets hypoxia inducible factor 2 alpha for degradation by hypoxia- and pVHL-independent regulation.* J Biol Chem, 2007. **282**(17): p. 12707-16.
129. Koh, M.Y., B.G. Darnay, and G. Powis, *Hypoxia-associated factor, a novel E3-ubiquitin ligase, binds and ubiquitinates hypoxia-inducible factor 1alpha, leading to its oxygen-independent degradation.* Mol Cell Biol, 2008. **28**(23): p. 7081-95.
130. Mansfield, K.D., R.D. Guzy, Y. Pan, R.M. Young, T.P. Cash, P.T. Schumacker, and M.C. Simon, *Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-alpha activation.* Cell Metab, 2005. **1**(6): p. 393-9.
131. Bjornheden, T., M. Levin, M. Evaldsson, and O. Wiklund, *Evidence of hypoxic areas within the arterial wall in vivo.* Arterioscler Thromb Vasc Biol, 1999. **19**(4): p. 870-6.
132. Cozzi, P.J., R.T. Lyon, H.R. Davis, J. Sylora, S. Glagov, and C.K. Zarins, *Aortic wall metabolism in relation to susceptibility and resistance to experimental atherosclerosis.* J Vasc Surg, 1988. **7**(5): p. 706-14.
133. Leppanen, O., T. Bjornheden, M. Evaldsson, J. Boren, O. Wiklund, and M. Levin, *ATP depletion in macrophages in the core of advanced rabbit atherosclerotic plaques in vivo.* Atherosclerosis, 2006. **188**(2): p. 323-30.
134. Sluimer, J.C., J.M. Gasc, J.L. van Wanroij, N. Kisters, M. Groeneweg, M.D. Sollewijn Gelpke, J.P. Cleutjens, L.H. van den Akker, P. Corvol, B.G. Wouters, M.J. Daemen, and A.P. Bijnens, *Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis.* J Am Coll Cardiol, 2008. **51**(13): p. 1258-65.
135. Bjornheden, T. and G. Bondjers, *Oxygen consumption in aortic tissue from rabbits with diet-induced atherosclerosis.* Arteriosclerosis, 1987. **7**(3): p. 238-47.
136. Murdoch, C., M. Muthana, and C.E. Lewis, *Hypoxia regulates macrophage functions in inflammation.* J Immunol, 2005. **175**(10): p. 6257-63.
137. Lobanova, E.M. and A.D. Tahanovich, *Hydrogen peroxide metabolism in alveolar macrophages after exposure to hypoxia and heat.* Physiol Res, 2006. **55**(5): p. 569-75.
138. Rydberg, E.K., A. Krettek, C. Ullstrom, K. Ekstrom, P.A. Svensson, L.M. Carlsson, A.C. Jonsson-Rylander, G.I. Hansson, W. McPheat, O. Wiklund, B.G. Ohlsson, and L.M. Hulten, *Hypoxia Increases LDL Oxidation and Expression of 15-Lipoxygenase-2 in Human Macrophages.* Arterioscler Thromb Vasc Biol, 2004.
139. Matsumoto, K., T. Taniguchi, Y. Fujioka, H. Shimizu, Y. Ishikawa, and M. Yokoyama, *Effects of hypoxia on cholesterol metabolism in human monocyte-derived macrophages.* Life Sci, 2000. **67**(17): p. 2083-91.
140. Turner, L., C. Scotton, R. Negus, and F. Balkwill, *Hypoxia inhibits macrophage migration.* Eur J Immunol, 1999. **29**(7): p. 2280-7.
141. Burger-Kentischer, A., H. Goebel, R. Seiler, G. Fraedrich, H.E. Schaefer, S. Dimmeler, R. Kleemann, J. Bernhagen, and C. Ihling, *Expression of macrophage migration inhibitory factor in different stages of human atherosclerosis.* Circulation, 2002. **105**(13): p. 1561-6.
142. Bosco, M.C., M. Puppo, F. Blengio, T. Fraone, P. Cappello, M. Giovarelli, and L. Varesio, *Monocytes and dendritic cells in a hypoxic environment: Spotlights on chemotaxis and migration.* Immunobiology, 2008. **213**(9-10): p. 733-49.
143. Sneck, M., P.T. Kovanen, and K. Oorni, *Decrease in pH strongly enhances binding of native, proteolyzed, lipolyzed, and oxidized low density lipoprotein particles to human aortic proteoglycans.* J Biol Chem, 2005. **280**(45): p. 37449-54.
144. Figueroa, J.E., Z. Tao, T.G. Sarphie, F.W. Smart, D.L. Glancy, and P. Vijayagopal, *Effect of hypoxia and hypoxia/reoxygenation on proteoglycan metabolism by vascular smooth muscle cells.* Atherosclerosis, 1999. **143**(1): p. 135-44.
145. Boyum, A., *Isolation of lymphocytes, granulocytes and macrophages.* Scand J Immunol, 1976. **Suppl 5**: p. 9-15.
146. Fisher DG, K.H., *Isolation of human monocytes*
Methods for studying mononuclear phagocytes, ed. E.P. Adams D, Koren HS (eds):. 1981, New Tork/London: Academic Press, Inc. 43-47.
147. Xie, S.Z., N.T. Fang, S. Liu, P. Zhou, Y. Zhang, S.M. Wang, H.Y. Gao, and L.F. Pan, *Differentiation of smooth muscle progenitor cells in peripheral blood and its application in tissue engineered blood vessels.* J Zhejiang Univ Sci B, 2008. **9**(12): p. 923-30.

148. Johnson, W.D., Jr., B. Mei, and Z.A. Cohn, *The separation, long-term cultivation, and maturation of the human monocyte.* J Exp Med, 1977. **146**(6): p. 1613-26.
149. Toeda, K., K. Nakamura, S. Hirohata, O.F. Hatipoglu, K. Demircan, H. Yamawaki, H. Ogawa, S. Kusachi, Y. Shiratori, and Y. Ninomiya, *Versican is induced in infiltrating monocytes in myocardial infarction.* Mol Cell Biochem, 2005. **280**(1-2): p. 47-56.
150. Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada, *Establishment and characterization of a human acute monocytic leukemia cell line (THP-1).* Int J Cancer, 1980. **26**(2): p. 171-6.
151. Tsuchiya, S., Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno, and K. Tada, *Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester.* Cancer Res, 1982. **42**(4): p. 1530-6.
152. Kohro, T., T. Tanaka, T. Murakami, Y. Wada, H. Aburatani, T. Hamakubo, and T. Kodama, *A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage.* J Atheroscler Thromb, 2004. **11**(2): p. 88-97.
153. Allen, C.B., B.K. Schneider, and C.W. White, *Limitations to oxygen diffusion and equilibration in in vitro cell exposure systems in hyperoxia and hypoxia.* Am J Physiol Lung Cell Mol Physiol, 2001. **281**(4): p. L1021-7.
154. Elbashir, S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl, *Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells.* Nature, 2001. **411**(6836): p. 494-8.
155. McManus, M.T. and P.A. Sharp, *Gene silencing in mammals by small interfering RNAs.* Nat Rev Genet, 2002. **3**(10): p. 737-47.
156. Llorente-Cortes, V., M. Otero-Vinas, E. Hurt-Camejo, J. Martinez-Gonzalez, and L. Badimon, *Human coronary smooth muscle cells internalize versican-modified LDL through LDL receptor-related protein and LDL receptors.* Arterioscler Thromb Vasc Biol, 2002. **22**(3): p. 387-93.
157. Wight, T.N. and M.J. Merrilees, *Proteoglycans in atherosclerosis and restenosis: key roles for versican.* Circ Res, 2004. **94**(9): p. 1158-67.
158. Gabrielsen, A., P.R. Lawler, W. Yongzhong, D. Steinbruchel, D. Blagoja, G. Paulsson-Berne, J. Kastrup, and G.K. Hansson, *Gene expression signals involved in ischemic injury, extracellular matrix composition and fibrosis defined by global mRNA profiling of the human left ventricular myocardium.* J Mol Cell Cardiol, 2007. **42**(4): p. 870-83.
159. Lemire, J.M., K.R. Braun, P. Maurel, E.D. Kaplan, S.M. Schwartz, and T.N. Wight, *Versican/PG-M isoforms in vascular smooth muscle cells.* Arterioscler Thromb Vasc Biol, 1999. **19**(7): p. 1630-9.
160. Schmalfeldt, M., M.T. Dours-Zimmermann, K.H. Winterhalter, and D.R. Zimmermann, *Versican V2 is a major extracellular matrix component of the mature bovine brain.* J Biol Chem, 1998. **273**(25): p. 15758-64.
161. Mazzucato, M., M.R. Cozzi, P. Pradella, D. Perissinotto, A. Malmstrom, M. Morgelin, P. Spessotto, A. Colombatti, L. De Marco, and R. Perris, *Vascular PG-M/versican variants promote platelet adhesion at low shear rates and cooperate with collagens to induce aggregation.* Faseb J, 2002. **16**(14): p. 1903-16.
162. Halvorsen, B., U.K. Aas, M.A. Kulseth, C.A. Drevon, E.N. Christiansen, and S.O. Kolset, *Proteoglycans in macrophages: characterization and possible role in the cellular uptake of lipoproteins.* Biochem J, 1998. **331 (Pt 3)**: p. 743-52.
163. Worley, J.R., M.D. Baugh, D.A. Hughes, D.R. Edwards, A. Hogan, M.J. Sampson, and J. Gavrilovic, *Metalloproteinase expression in PMA-stimulated THP-1 cells. Effects of peroxisome proliferator-activated receptor-gamma (PPAR gamma) agonists and 9-cis-retinoic acid.* J Biol Chem, 2003. **278**(51): p. 51340-6.
164. Westling, J., P.E. Gottschall, V.P. Thompson, A. Cockburn, G. Perides, D.R. Zimmermann, and J.D. Sandy, *ADAMTS4 (aggrecanase-1) cleaves human brain versican V2 at Glu405-Gln406 to generate glial hyaluronate binding protein.* Biochem J, 2004. **377**(Pt 3): p. 787-95.
165. Burke, B., A. Giannoudis, K.P. Corke, D. Gill, M. Wells, L. Ziegler-Heitbrock, and C.E. Lewis, *Hypoxia-induced gene expression in human macrophages: implications for ischemic tissues and hypoxia-regulated gene therapy.* Am J Pathol, 2003. **163**(4): p. 1233-43.

166. Watanabe, N. and U. Ikeda, *Matrix metalloproteinases and atherosclerosis.* Curr Atheroscler Rep, 2004. **6**(2): p. 112-20.
167. Li, Y.Z., X.H. Liu, and L.R. Cai, *Down-regulation of perlecan expression contributes to the inhibition of rat cardiac microvascular endothelial cell proliferation induced by hypoxia.* Sheng Li Xue Bao, 2007. **59**(2): p. 221-6.
168. Kinsella, M.G., P.K. Tran, M.C. Weiser-Evans, M. Reidy, R.A. Majack, and T.N. Wight, *Changes in perlecan expression during vascular injury: role in the inhibition of smooth muscle cell proliferation in the late lesion.* Arterioscler Thromb Vasc Biol, 2003. **23**(4): p. 608-14.
169. Nikkari, S.T., H.T. Jarvelainen, T.N. Wight, M. Ferguson, and A.W. Clowes, *Smooth muscle cell expression of extracellular matrix genes after arterial injury.* Am J Pathol, 1994. **144**(6): p. 1348-56.
170. Kojima, T., A. Takagi, M. Maeda, T. Segawa, A. Shimizu, K. Yamamoto, T. Matsushita, and H. Saito, *Plasma levels of syndecan-4 (ryudocan) are elevated in patients with acute myocardial infarction.* Thromb Haemost, 2001. **85**(5): p. 793-9.
171. Yeaman, C. and A.C. Rapraeger, *Membrane-anchored proteoglycans of mouse macrophages: P388D1 cells express a syndecan-4-like heparan sulfate proteoglycan and a distinct chondroitin sulfate form.* J Cell Physiol, 1993. **157**(2): p. 413-25.
172. Wang, W., C.A. Haller, J. Wen, P. Wang, and E.L. Chaikof, *Decoupled syndecan 1 mRNA and protein expression is differentially regulated by angiotensin II in macrophages.* J Cell Physiol, 2008. **214**(3): p. 750-6.
173. Kolset, S.O., L. Kjellen, R. Seljelid, and U. Lindahl, *Changes in glycosaminoglycan biosynthesis during differentiation in vitro of human monocytes.* Biochem J, 1983. **210**(3): p. 661-7.
174. Levene, C.I., R. Kapoor, and G. Heale, *The effect of hypoxia on the synthesis of collagen and glycosaminoglycans by cultured pig aortic endothelium.* Atherosclerosis, 1982. **44**(3): p. 327-37.
175. Christner, J.E. and J.R. Baker, *A competitive assay of lipoprotein: proteoglycan interaction using a 96-well microtitration plate.* Anal Biochem, 1990. **184**(2): p. 388-94.
176. Srinivasan, S.R., J.H. Xu, P. Vijayagopal, B. Radhakrishnamurthy, and G.S. Berenson, *Low-density lipoprotein binding affinity of arterial chondroitin sulfate proteoglycan variants modulates cholesteryl ester accumulation in macrophages.* Biochim Biophys Acta, 1995. **1272**(1): p. 61-7.
177. Oorni, K. and P.T. Kovanen, *Enhanced extracellular lipid accumulation in acidic environments.* Curr Opin Lipidol, 2006. **17**(5): p. 534-40.
178. Murata, K., A. Murata, and K. Yoshida, *Heparan sulfate isomers in cerebral arteries of Japanese women with aging and with atherosclerosis--heparitinase and high-performance liquid chromatography determinations.* Atherosclerosis, 1997. **132**(1): p. 9-17.
179. Beavan, L.A., M. Davies, J.R. Couchman, M.A. Williams, and R.M. Mason, *In vivo turnover of the basement membrane and other heparan sulfate proteoglycans of rat glomerulus.* Arch Biochem Biophys, 1989. **269**(2): p. 576-85.
180. Iozzo, R.V., *Turnover of heparan sulfate proteoglycan in human colon carcinoma cells. A quantitative biochemical and autoradiographic study.* J Biol Chem, 1987. **262**(4): p. 1888-900.
181. Moon, J.J., M. Matsumoto, S. Patel, L. Lee, J.L. Guan, and S. Li, *Role of cell surface heparan sulfate proteoglycans in endothelial cell migration and mechanotransduction.* J Cell Physiol, 2005. **203**(1): p. 166-76.
182. Parish, C.R., C. Freeman, and M.D. Hulett, *Heparanase: a key enzyme involved in cell invasion.* Biochim Biophys Acta, 2001. **1471**(3): p. M99-108.
183. Takahashi, H., H. Matsumoto, A. Smirkin, T. Itai, Y. Nishimura, and J. Tanaka, *Involvement of heparanase in migration of microglial cells.* Biochim Biophys Acta, 2008. **1780**(4): p. 709-15.
184. Fukai, T., R.J. Folz, U. Landmesser, and D.G. Harrison, *Extracellular superoxide dismutase and cardiovascular disease.* Cardiovasc Res, 2002. **55**(2): p. 239-49.
185. Landmesser, U., R. Merten, S. Spiekermann, K. Buttner, H. Drexler, and B. Hornig, *Vascular extracellular superoxide dismutase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation.* Circulation, 2000. **101**(19): p. 2264-70.
186. Khan, W.S., A.B. Adesida, and T.E. Hardingham, *Hypoxic conditions increase hypoxia-inducible transcription factor 2alpha and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients.* Arthritis Res Ther, 2007. **9**(3): p. R55.
187. Coyle, C.H., N.J. Izzo, and C.R. Chu, *Sustained hypoxia enhances chondrocyte matrix synthesis.* J Orthop Res, 2009. **27**(6): p. 793-9.

188. Pfander, D., T. Cramer, E. Schipani, and R.S. Johnson, *HIF-1alpha controls extracellular matrix synthesis by epiphyseal chondrocytes.* J Cell Sci, 2003. **116**(Pt 9): p. 1819-26.
189. Semenza, G.L., *Hypoxia-inducible factor 1: control of oxygen homeostasis in health and disease.* Pediatr Res, 2001. **49**(5): p. 614-7.
190. Li, J., N.W. Shworak, and M. Simons, *Increased responsiveness of hypoxic endothelial cells to FGF2 is mediated by HIF-1alpha-dependent regulation of enzymes involved in synthesis of heparan sulfate FGF2-binding sites.* J Cell Sci, 2002. **115**(Pt 9): p. 1951-9.
191. Shohet, R.V. and J.A. Garcia, *Keeping the engine primed: HIF factors as key regulators of cardiac metabolism and angiogenesis during ischemia.* J Mol Med, 2007. **85**(12): p. 1309-15.
192. Lofstedt, T., E. Fredlund, L. Holmquist-Mengelbier, A. Pietras, M. Ovenberger, L. Poellinger, and S. Pahlman, *Hypoxia inducible factor-2alpha in cancer.* Cell Cycle, 2007. **6**(8): p. 919-26.
193. Holmquist-Mengelbier, L., E. Fredlund, T. Lofstedt, R. Noguera, S. Navarro, H. Nilsson, A. Pietras, J. Vallon-Christersson, A. Borg, K. Gradin, L. Poellinger, and S. Pahlman, *Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype.* Cancer Cell, 2006. **10**(5): p. 413-23.
194. Wilson, P., K. Drennon, and L.R. Tannock, *Regulation of vascular proteoglycan synthesis by metabolic factors associated with diabetes.* J Investig Med, 2007. **55**(1): p. 18-25.
195. Olsson, U., G. Bondjers, and G. Camejo, *Fatty acids modulate the composition of extracellular matrix in cultured human arterial smooth muscle cells by altering the expression of genes for proteoglycan core proteins.* Diabetes, 1999. **48**(3): p. 616-22.
196. Rodriguez-Lee, M., G. Ostergren-Lunden, B. Wallin, J. Moses, G. Bondjers, and G. Camejo, *Fatty Acids Cause Alterations of Human Arterial Smooth Muscle Cell Proteoglycans That Increase the Affinity for Low-Density Lipoprotein.* Arterioscler Thromb Vasc Biol, 2005.
197. Morawski, M., M.K. Bruckner, P. Riederer, G. Bruckner, and T. Arendt, *Perineuronal nets potentially protect against oxidative stress.* Exp Neurol, 2004. **188**(2): p. 309-15.
198. Sato, H., J. Onose, H. Toyoda, T. Toida, T. Imanari, M. Sagai, N. Nishimura, and Y. Aoki, *Quantitative changes in glycosaminoglycans in the lungs of rats exposed to diesel exhaust.* Toxicology, 2001. **166**(3): p. 119-28.
199. Wu, Y., J. Wu, D.Y. Lee, A. Yee, L. Cao, Y. Zhang, C. Kiani, and B.B. Yang, *Versican protects cells from oxidative stress-induced apoptosis.* Matrix Biol, 2005. **24**(1): p. 3-13.
200. Kim, J.W., I. Tchernyshyov, G.L. Semenza, and C.V. Dang, *HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia.* Cell Metab, 2006. **3**(3): p. 177-85.
201. Saha, P., B. Modarai, J. Humphries, K. Mattock, M. Waltham, K.G. Burnand, and A. Smith, *The monocyte/macrophage as a therapeutic target in atherosclerosis.* Curr Opin Pharmacol, 2009. **9**(2): p. 109-18.