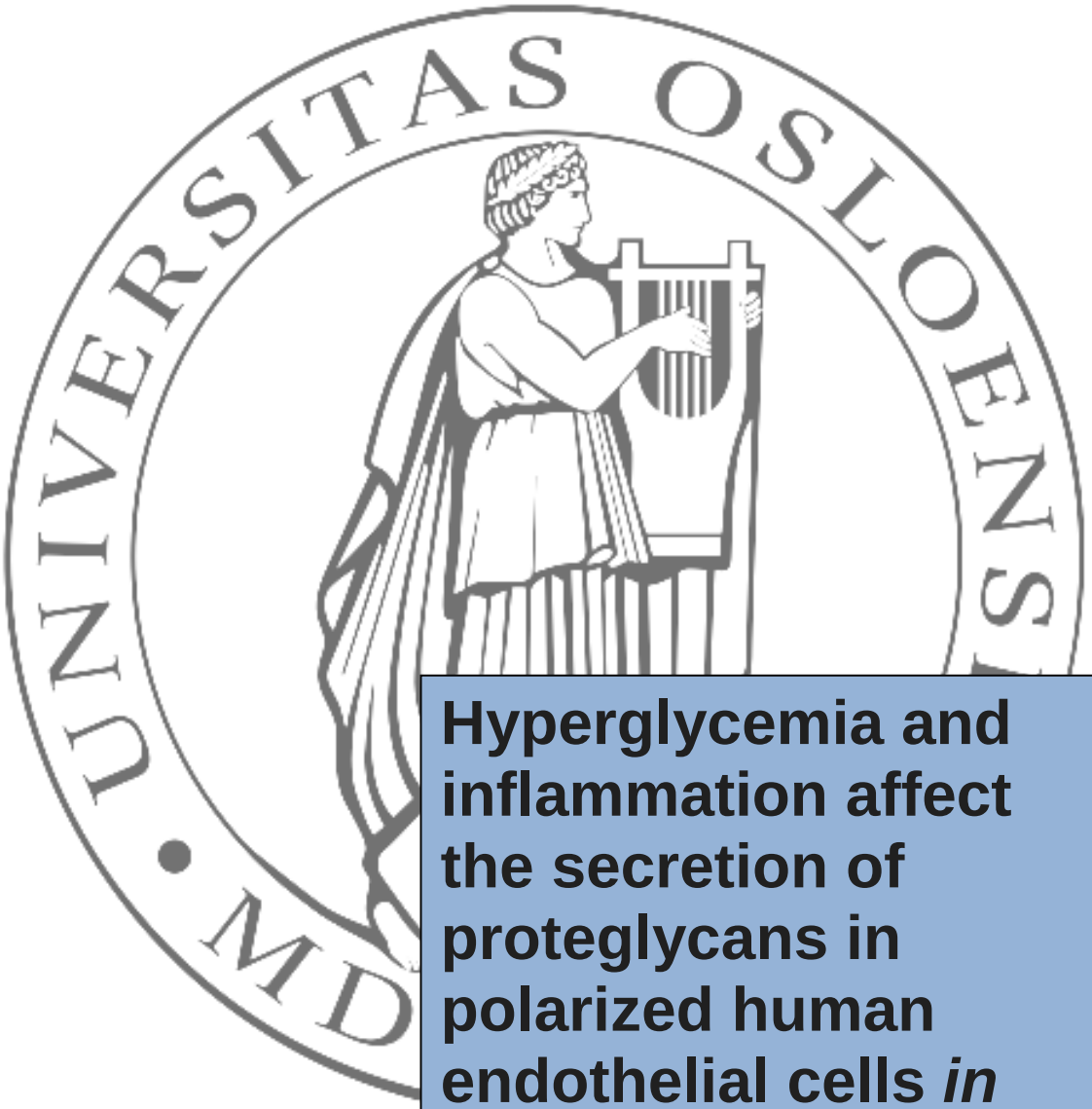

Hyperglycemia and inflammation affect the secretion of proteglycans in polarized human endothelial cells *in vitro*

Master Thesis by

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June 2011

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Siv Hilde Fjeldstad

Summary

Background and aims: Diabetes mellitus (DM) is a chronic disease characterized by hyperglycemia. Even though the general life expectancy in people with diabetes has increased, the disease still causes harmful secondary complications. The endothelium is the prime organ to be exposed to hyperglycemia, leading to endothelial dysfunction and inflammation. Both in the luminal endothelial vessel wall and in the underlying basement membrane, the proteoglycans (PGs) have important functions. In diabetes, alterations in both expression and structure of the PGs are observed, and several studies indicate that these changes are linked to DM complications. For both treatment and prevention, it is important to obtain a better understanding of the underlying mechanisms for such changes. The aims of this work were to study the secretion of PGs from polarized human umbilical vein endothelial cells (HUVEC) cultured under hyperglycemic and inflammatory conditions, as both are characteristic for the diabetic condition.

Methods: Primary HUVEC were cultured *in vitro* as a model system of the endothelium, and exposed to hyperglycemia and the inflammatory mediator interleukin 1β (IL-1β), to mimic the diabetic environment in an experimental system. Quantitative effects on total *de novo* PG synthesis and the secretion of the individual PGs perlecan, decorin, collagen XVIII and biglycan were investigated. We also studied the effect of hyperglycemia and IL-1β on endothelial permeability in HUVEC.

Results: The results obtained show that HUVEC synthesized large amounts of PGs and that the PGs were mainly secretory products. We also found that the extracellular matrix PGs perlecan, collagen XVIII and decorin were mainly secreted to the basolateral medium. Biglycan was an exception with an almost 50/50 secretion ratio between apical and basolateral media. The secretion of the different PGs was affected by hyperglycemia and IL-1β, both in total secretion and secretion pattern. Hyperglycemia and IL-1β also affected the endothelial permeability.

Conclusions: The PGs studied in this thesis was affected by the *in vitro* diabetic environment, and the altered expression may be of importance in DM complications. Further studies are needed to draw any concrete conclusions in the pathological role of specific PGs.

Sammendrag

Bakgrunn og formål: Diabetes mellitus er en type kroniske sykdommer kjennetegnet ved hyperglykemi. Forventet levealder hos personer med diabetes er økt, men sykdommen forårsaker alvorlige sekundære komplikasjoner. Endotelcellelaget i blodårene er de første cellene i kroppen som utsettes for hyperglykemi, noe som fører til endotelial dysfunksjon og inflammasjon. Proteoglykaner finnes i både endotelcellenes luminale plasmamembran og i den underliggende basalmembranen. Forandringer i proteoglykanenes uttrykkelse og struktur er observert ved diabetes, og flere studier knytter forandringer i proteoglykanene til utviklingen av diabeteskomplikasjoner. Med tanke på både forebygging og behandling, er det viktig å oppnå en bedre forståelse av de underliggende mekanismene for slike forandringer. Formålet med denne studien var å vurdere sekresjonen av proteoglykaner fra humane endotelceller fra venen i navlesnor, dyrket under hyperglykemiske og inflammatoriske forhold, som begge er karakteristisk ved diabetes.

Metode: Primære endotelceller fra venen i navlesnor ble dyrket *in vitro* som et modellsystem for endotelcellelaget *in vivo*. Cellene ble stimulert med hyperglykemi og interleukin-1β, som mål på diabetiske forhold. Kvantitative effekter av den totale *de novo* proteoglykan syntesen, og sekresjonen av de spesifikke proteoglykanene perlecan, decorin, collagen XVIII og biglycan ble undersøkt. Vi undersøkte også om hyperglykemi og IL-1β påvirket endotelial permeabilitet.

Resulater: Resultatene fra denne studien viste at humane endotelceller fra vene i navlestreng syntetisertre store mengder proteoglykaner, og at proteoglykanene hovedsakelig var sekretoriske molekyler. Vi fant også at proteoglykanene perlecan, decorin og collagen XVIII hovedsakelig ble sekretert til det basolaterale mediet. Sekresjonen av biglycan var et unntak med en tilnærmet lik sekresjon i det apikale og det basolaterale mediet. Både total sekresjonen av de ulike proteoglykanene og sekresjonsmønsteret ble påvirket av hyperglykemi og IL-1β. Den endoteliale permeabiliteten ble også påvirket av denne stimuleringen.

Konklusjon: Proteoglykanene undersøkt i denne studien ble påvirket av diabetiske stimuli *in vitro*, og disse forandringen kan være viktig i utviklingen av i diabeteskomplikasjoner. Flere studier trengs for å kunne trekke konkrete konklusjoner om spesifikke proteoglykaners rolle i denne patogenesen.

Table of contents

Abbreviations

AGE	Advanced glycation end products
BM	Basement membrane
BMI	Body mass index
cpm	Counts per minute
CS	Chondroitin sulfate
CVD	Cardiovascular disease
DAG	Diacylglycerol
DM	Diabetes Mellitus
DS	Dermatan sulfate
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	N-acetylgalactosamine
GBM	Glomerular basement membrane
GFAT	Glutamin:fructose-6 phosphate amidotransferase
GlcA	Glucuronic acid
GlcN	N-glucosamine

GlcNAc	N-acetyl glucosamine
HA	Hyaluronan
HAEC	Human aortic endothelial cell
HbA_{1c}	Glycosylated hemoglobin
HS	Heparan sulfate
HUVEC	Human umbilical vein endothelial cells
IdoA	Iduronic acid
IGF	Insulin-like growth factor
IL-1β	Interleukin 1-β
KS	Keratan sulfate
LDL	Low density lipoprotein
LRRs	Leucine-rich repeats
MDCK	Madin-Darby Canine Kidney
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor κB
NO	Nitric oxide
PG	Proteoglycan
PKC	Protein kinase C
ROS	Reactive oxygen species
SLRPs	Small leucine-rich preoteoglycans
SMC	Smooth muscle cell
STZ	Streptozotocin
TGF	Transforming growth factor
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-α
UDP-GalNAc	Uridine diphosphate N-acetyl galactosamine

UDP-GlcNAc	Uridine diphosphate N-acetyl glucosamine
VEGF	Vascular endothelial growth factor
Xyl	Xylose

1 Introduction

Diabetes mellitus (DM) is a group of chronic diseases characterized by hyperglycemia. The endothelium is lining up the interior of the vessel walls, and is vulnerable to changes in the blood composition such as hyperglycemia. This may lead to endothelial dysfunction that contributes to the development of atherosclerosis and long-term complications seen in diabetes. Proteoglycans (PGs) are major constituents of the extracellular matrix (ECM), surrounding the cells and tissues in the body. Both in the luminal endothelial vessel wall and in the underlying basal membrane, the PGs have important functions. In diabetes, alterations in both expression and structure of the PGs are observed, and several studies indicate that these changes are linked to DM complications [1, 2]. To study the implications of the PGs in diabetes in an experimental *in vitro* system, cultured endothelial cells can be exposed to hyperglycemia and inflammation, characteristics of the *in vivo* diabetic environment.

1.1 Diabetes mellitus

DM is a group of chronic diseases characterized by hyperglycemia due to lack of production and/or response to the hormone insulin [3]. In healthy individuals the normal blood glucose level varies between 4-7 mmol/l. In the diabetic state, blood glucose is increased, and the diagnostic level is fasting blood glucose $\geq$ 7 mmol/l and/or blood glucose level $\geq$ 11.1 mmol/l two hours after 75 g oral glucose load. Randomly (not fasting) blood glucose $\geq$ 11.1 mmol/l in combination with defined symptoms is also used as a diagnostic criteria [4]. Maintenance of blood glucose in a normal physiological range is the most important goal of treatment, achieved by medication, physical activity and diet. The age at onset of the disease, race, body mass index (BMI) and symptoms are used to classify the type of diabetes, which is necessary for the right treatment and follow up.

Type 1 DM is a multifactorial disease with a strong genetic component [5]. The disease is characterized by cellular mediated autoimmune destruction of pancreatic islet beta cells leading to loss of insulin production. The onset of the disease is usually at childhood, but can occur at all ages. The ongoing process that gradually destroys the beta cells in the pancreas results in clinical manifestations. Unnatural high urination volume followed by intense thirst, weight loss and fatigue are the most common symptoms [3]. The pathogenesis of type 2 DM

is characterized by insulin-resistance or reduced insulin sensitivity in insulin-dependent organs like the liver, skeletal muscle and adipose tissue. The pancreas increases the production of insulin leading to exhausting of the beta cells and decline in insulin production, resulting in hyperglycemia [6]. Several mutations that make individuals of type 2 DM more susceptible to this beta cell destruction have been found, proving that genetics is an important risk factor [7] together with obesity and sedentary lifestyle [8]. Early intervention can delay the disease and its complications many years, resulting in better life quality [9] and reduction of the economic burden of the disease [10].

There are also other types of diabetes besides type 1 and type 2 DM. When type 1 DM occurs in adulthood, the destroying process of the beta cells is very slow and can be mistaken as a type 2 DM. This is called LADA (Late onset Autoimmune Diabetes of Adulthood). Patients with LADA usually have lower C-peptide plasma concentrations and positive immunologic test like in type 1 DM [11]. MODY (Maturity-Onset Diabetes of the Young) is the most common form of monogenic mutation diabetes, even though the occurrence is rare. MODY is congenital and can often be mistaken as a type 1 or type 2 DM. Gestational DM occurs under pregnancy and can have different origins: 1) a type 1 that can debut in the pregnancy, 2) a type 2 that is plead during pregnancy, 3) patients that develop diabetes during pregnancy (the real gestational DM). WHO also emphasize that patients with impaired glucose tolerance (IGT) > 7.8 mmol/l should be classified and treated as a gestational DM [4]. If not treated, the increased blood glucose can have severe consequences for the fetus.

The global prevalence of DM is increasing: the total number of people with diabetes was 171 millions in year 2000 and is assumed to be 366 millions in 2030 [12]. The prevalence of diabetes in the American population is 24 millions, most of whom have type 2 DM which accounts for 90-95% and is more common in overweight people over the age of 45. By including the occurrence of IGT, associated with high risk of developing type 2 diabetes, the total prevalence is 74 millions [13]. In Norway the estimated total prevalence of DM is between 90.000 and 120.000, but European studies indicate that just as many may have undiagnosed diabetes. The number of people with type 1 DM in Norway is estimated to be 25.000 [14]. A recent study claims that if present trends continues, the incidence of new cases of type 1 DM in European children younger than 5 years will double from year 2005-2020

[15]. The type 2 DM prevalence among children and young adults is also increasing. This is due to the higher rate of obesity in this part of the population [16].

1.2 The endothelium

The endothelium is specialized simple squamous epithelial cells (ECs) lining the vascular system, forming an interface or barrier between circulating blood in the lumen and the rest of the vessel wall. In an adult, the endothelium consists of approximately 1×10^{13} cells, lining the interior from the heart chambers to the smallest capillaries [17]. Seen as a whole, the ECs make up an organ of approximately 1 kg in weight [18]. In order to find a link between hyperglycemia and DM complications, the ECs are obvious choices for investigations, because of their anatomical location in the vessel wall.

1.2.1 Vessel wall structure and function

Blood vessels vary in vessel wall thickness and functions. *Arteries* carry blood from the heart to the organs and are thick walled and highly elastic to bear the high pressure blood flow pumped out of the heart. A large luminal radius makes the arteries offer little resistance to the blood flow, but they become progressively smaller and branch out to reach every organ of the body. When a small artery reaches an organ to supply, it branches into numerous *arterioles*. The arterioles are highly muscular, well-innervated and have a smaller radius. The smooth muscle cell (SMC) layer can adjust the arteriolar luminal radius through vasodilatation or vasoconstriction, in response to physical or chemical changes. To reach every cell in the organ, the arterioles branch extensively forming the thin walled *capillaries* with large total cross-sectional areas, optimal for effective substance exchange. Nutrients and O_2 is delivered to the cells in exchange with waste products and CO_2 from the cells, and are then returned to the heart by the *veins*. Veins are thin walled and highly distensible vessels with a large radius, that in addition to returning the blood to the heart, also serves as a blood reservoir [19].

The walls of arteries and veins are composed of three layers called tunics: tunica intima, tunica media and tunica adventitia. Tunica intima is the innermost layer of the vessel and consists of the endothelium, the basal membrane (BM) and the subendothelial layer composed of loose connective tissues. The middle layer, tunica media, consist primarily of SMCs and a

variable degree of elastin and reticular fibers. All extracellular components of the tunica media are produced by the SMCs. The outermost layer of connective tissue, called tunica adventitia, varies in thickness and is composed of collagen tissue and elastic fibers. The PGs are present in all three layers in various extent from tissue to tissue. The capillaries are different from arteries and veins as they only consist of a single layer of ECs and their BM, this characteristic difference is illustrated by **figure 1.1** [20].

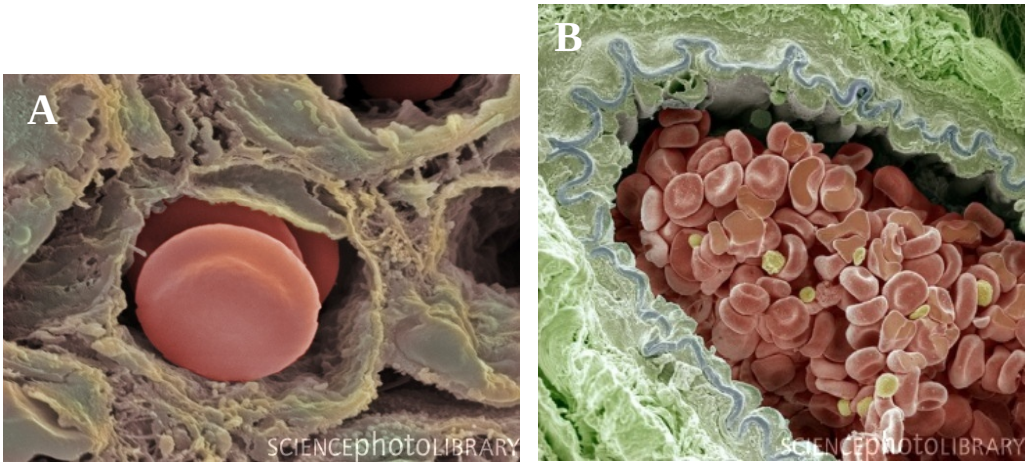

Figure 1.1A and B: Capillary and artery with erythrocytes illustrating the luminal differences. Capillaries have a minor luminal diameter with only one erythrocyte (red colored cell) passing through the vessels at a time (A). The vessel walls of capillaries are thin in contrast to the artery vessel wall, which is thick with a layer of both SMCs and connective tissue. The arteries have bigger diameter, with many erythrocytes and other plasma proteins passing through (B). *http://www.sciencephoto.com/images/download_lo_res.html?id=802120033, http://www.sciencephoto.com/images/imagePopUpDetails.html?pop=1&id=670047755&pviewid=&country=67&search=artery+AND+(vessel+AND+wall)&matchtype=FUZZY*

1.2.2 Endothelial functions

The endothelial cells regulate numerous local blood vessel functions, like vascular tone, coagulation, inflammation and proliferation through the release of different mediators [18], as illustrated in **figure 1.2**.

Local chemical changes affect the ECs which lead to the release of mediators that regulates arteriolar luminal diameter. Nitric oxide (NO) is of the most studied vasoactive mediators released from the endothelial cells, and induces vasodilatation by inhibiting the entry of Ca^{2+} into SMCs and leading to relaxation of the muscles. The endothelium may also promote vascular constriction through the release of vasoconstrictors like endothelin-1. ECs also secrete substances that promote coagulation, like von Willebrands factor. This coagulation factor binds and stabilizes the factor VIII and is a cofactor for platelet binding to exposed extracellular matrix (ECM) in injured vessel walls [21]. In the case of inflammation, ECs produce inflammatory mediators required for migration of leucocytes to injured tissues [17]. The endothelium also stimulates proliferation of SMCs and new vessel growth, through the release of vascular endothelial growth factor (VEGF) [22].

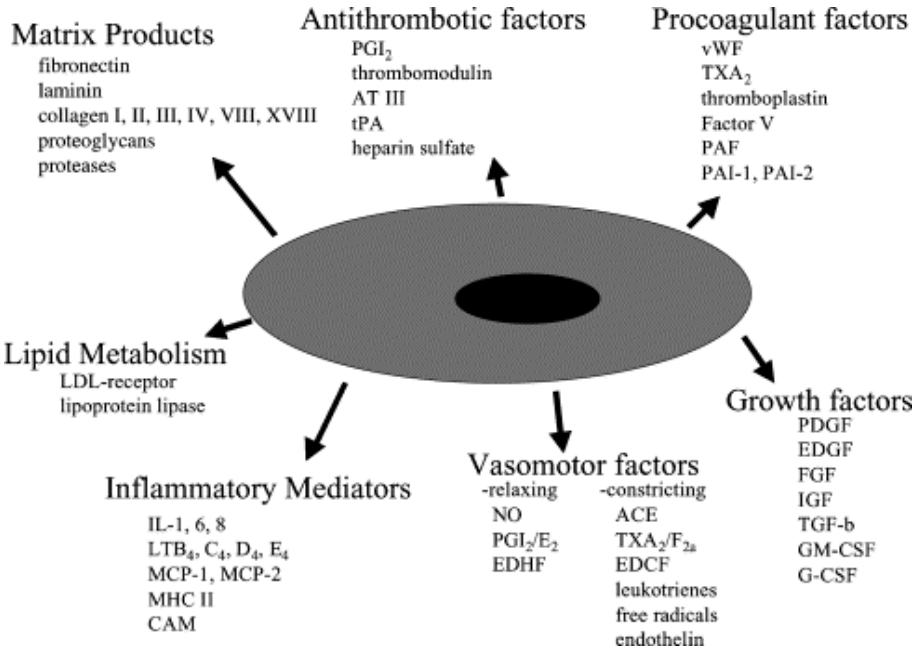

Figure1.2: Known secretory/expression products of endothelial cells. The endothelial cells produce a vast majority of molecules involved in inflammatory reactions, vasomotor function, lipid metabolism, coagulation, matrix growth and matrix components. *From: B.E Sumpio et al. Int J Biochem Cell Biol. 2002 Dec;34(12):1508-12[16].*

The endothelium is semipermeable and controls the passage of small and large molecules depending on the function of the organ. Intercellular pores exchanges water soluble molecules, and can vary in pore size from organ to organ. In the renal glomerulus, ECs are specialized to filtrate the blood and exchange deposits to the urine, maintaining the liquid balance and acid-base balance within a physiological range. In the capillaries the ECs are tightly sealed, and molecules require specialized transport mechanisms to pass the cell layer. The capillaries in the brain make up the blood-brain barrier; this protects the brain against harmful variations in blood components, because of their tight connected EC layer. The endothelium is dependent upon the BM to maintain its normal functions [23]. The BM are composed of various ECM molecules like PGs, different types of collagens, laminin and nidogen [24]. The ECs produce and secrete many of the ECM components, and are thus able to regulate the BM and its functions. The most prominent PGs in the BM are the heparan sulfate (HS)-PGs, and their functions are based on their capacity to interact with other ECM molecules, growth factors, cytokines and cellular receptors. This leads to effects on migration, proliferation and differentiation depending on the tissue in which they are localized [24]. On the luminal side, the endothelium is covered by the glycocalyx, a layer of PGs and glycoproteins. The glycocalyx is fragile and responsible for constant fluid resistance and interacts with plasma proteins. Disruptions in the glycocalyx are observed under pathologic conditions like sepsis, cardiovascular events and in diabetes [25-27].

1.2.3 Inflammation and endothelial dysfunction

Inflammation is characterized by translocation of leucocytes including monocytes, neutrophils and T-lymphocytes to the injured tissue where they migrate through the endothelium. The HS-PGs located at the luminal surface of the ECs, further described in chapter 1.4, have several functions in this process [28]. They may bind and release proinflammatory cytokines, like interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) which activate ECs and cause upregulation of vascular cell adhesion molecule (VCAM) required for capture of flowing leukocytes. The activated ECs also secrete interleukins, which activate integrins on leukocytes, required for tight binding to inter-cellular cell adhesion molecules (ICAM) 1 and 2, that leads to migration through the ECs [17]. This is in general a protective function, but the endogenous defenses of the vascular endothelium can be negatively affected by factors

like hypertension, hypercholesterolemia and hyperglycemia [29]. This disruption in the normal functions of the endothelium cause an imbalance of vascular tone and predisposes to vasoconstriction, changes in expression of adhesion molecules, secretion of proinflammatory molecules, alterations in the SMC proliferation and apoptosis of individual ECs, all resulting in endothelial dysfunction and increased permeability [18, 30]. Prolonged hyperglycemia or repeated hyperglycemic episodes may contribute in the initiating and developing endothelial dysfunction; an early indicator in micro- and macroangiopathy that is associated with diabetes.

1.3 Diabetic complications

The general life expectancy in people with diabetes has increased rapidly during the last 30 years. This is due to better understanding of the disease, regular follow ups and better medication. But the disease still causes harmful secondary complications. Both micro- and macrovascular vessels are affected by the diabetic state [3, 31]. Microangiopathy can lead to retinopathy, nephropathy and neuropathy, and diabetes is the lading cause of blindness, renal failure and lower limb amputations [32]. Macroangiopathy, primary atherosclerosis in the coronary, carotid and peripheral arteries increase the risk of myocardial infarction, stroke and lower limb amputations [32, 33]. It is now established that hyperglycemia is not merely a measure of insulin-resistance and diabetes, but that hyperglycemia itself contributes to the development of insulin resistance, diabetes and diabetic vascular complications [34, 35]. The extent of diabetic tissue damage is also determined by genetic determinants of individual susceptibility and by the presence of independent accelerating factors as dyslipidemia and hypertension [36], as illustrated in **figure 1.3.**

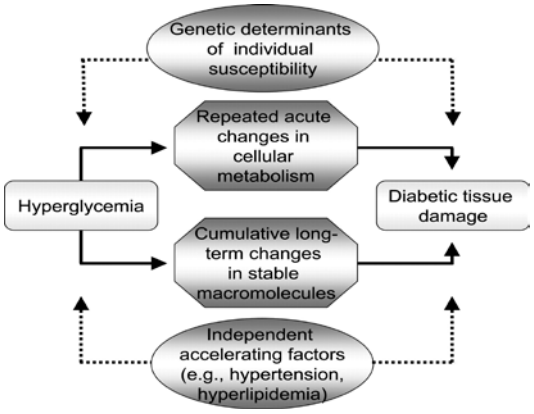

Figure 1.3: General feature of hyperglycemia-induced tissue damage. Diabetic tissue damage is determined by genetics, hypertension and hyperlipidemia together with hyperglycemia. *Taken from Brownlee M. Diabetes Vol 54, June 2005[37]*

1.3.1 Diabetic macrovascular disease

The impact of diabetes on cardiovascular disease (CVD) is so well documented that diabetes is now considered a major independent CVD risk factor [38, 39]. CVD is a progressive and multifactorial disease involving both lipid metabolism, inflammation, fibrosis and endothelial dysfunction [40]. The initiating event of atherosclerotic lesions is the formation of so called fatty streaks, recruitment of macrophages and accumulation of low density lipoprotein (LDL)-derived cholesterol in the large arteries. Monocytes and LDL migrate through the endothelial cell layer, where monocytes transform to macrophages and LDL undergoes oxidative modifications. The macrophages take up oxidated LDLs and change into foam cells, a hallmark of both early and late atherosclerotic lesions, with further recruitment of monocytes from the blood. The transition from fatty streaks to atherosclerotic lesions is characterized by the migration of SMCs into the sub-endothelial space. Here they proliferate and take up modified lipoproteins, contributing to foam cell formation and synthesize increased amounts of ECM proteins that lead to the development of lipid and ECM filled plaques. The plaques may rupture and if thrombosis occurs, heart attacks or stroke may follow [38]. Scientists struggle to find the initiating and missing link between diabetes and accelerating CVD. The retention hypothesis, one of the major theories, was first outlined by Williams and Tabas in 1995 [41] and is based on the theory that atherogenic (apo-B containing) lipoproteins is interacting with ECM molecules, particularly PGs, in the subendothelial space. This interaction leads to increased susceptibility for lipoprotein modification which can trigger an inflammatory response with alterations in SMC phenotype and function, and infiltration of inflammatory cells including macrophages [1]. Alterations in the ECM formation are observed under hyperglycemic environment *in vitro* and in *in vivo*, but the mechanisms remain unclear. The production of advanced glycated end products (AGEs), described in chapter 1.3.2, may also contribute to the changes in ECM, leading to reduced elasticity of the vessels and promoting increased binding of LDL in the intima [42].

1.3.2 Diabetic microvascular disease

It is not clearly understood how hyperglycemia leads to micro- and macrovascular complications. But the tissue-damaging effects of hyperglycemia are more severe in some cell types like capillary endothelial cells, mesangial cells and Schwann cells. This could be because these cells are not able to regulate the glucose uptake, leading to increased internal glucose concentrations in hyperglycemia. Evidence suggests that four main pathways (**figure 1.4**) are linked to the generation of diabetic complications:

- Increased polyol pathway flux
- Increased hexosamine pathway flux
- Activation of protein kinase C (PKC) isoforms
- Increased advanced glycation end products (AGE) formation

The intracellular hyperglycemia promotes high flux through the glycolysis, tricarboxylic citric acid cycle and electron transport chain, resulting in overload in the latter. This overload leads to increased superoxide production, and the reactive oxygen species (ROS) perform negative feedback control on the enzyme GADPH (Glyceraldehyde 3-phosphate dehydrogenase), inhibiting the glycolysis at glycerol-3-phosphate. This negative feedback will stop a further increase in ROS, by reducing the flux trough the already overloaded electron transport chain. Additionally, inhibition of GADPH will accumulate the glycolytic intermediates as shown in **figure 1.4,** upstream of the enzyme. As this figure illustrates, the intermediates are shunted into the four pathways, hypothesized to cause hyperglycemic damage [37]. It is likely that interaction between the different damaging factors that could lead to the diabetic complications, and that the activities of the different pathways differ from tissue to tissue.

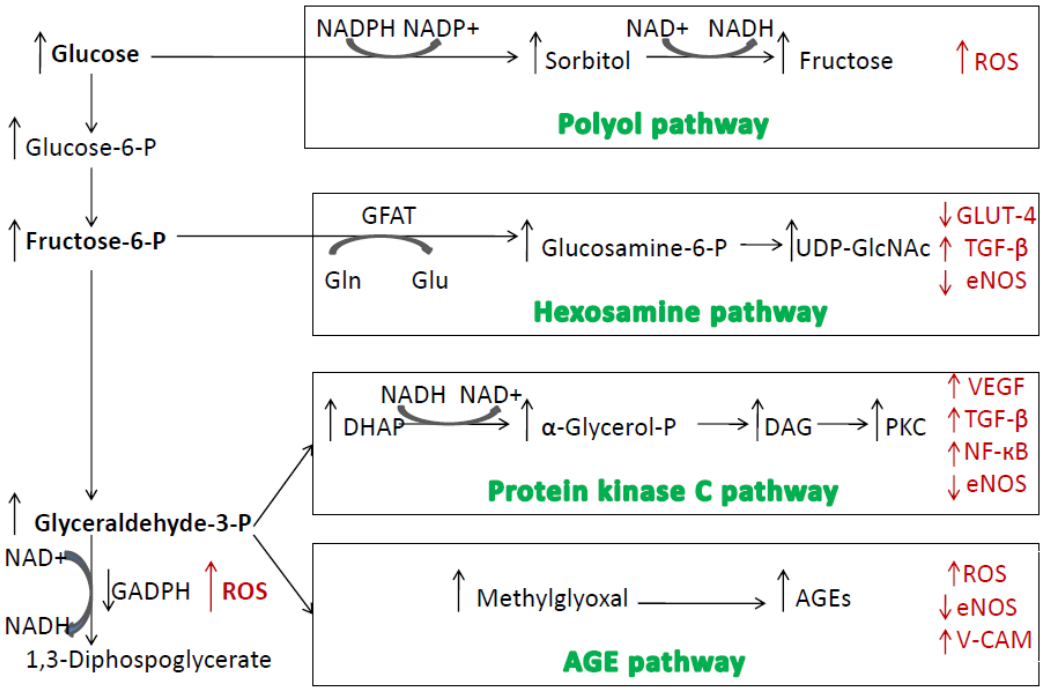

Figure 1.4: The four main proposed pathways of hyperglycemic damage. The flux through the polyol pathway, hexosamine pathway, protein kinase C pathway and AGE pathway are increased in the hyperglycemic environment seen in diabetes. The increased flux through these pathways is generated by increased ROS production in the electron transport chain and following inhibition of the enzyme glyceraldehyde 3-phosphate dehydrogenase (GADPH). ROS production is hypothesized as a common link between hyperglycemia and diabetic vascular complications. Modified from: Brownlee M. Nature Vol 414 (6865): 813-820 2001 [43].

Increased polyol pathway

The polyol pathway was the first metabolic pathway hypothesized to lead to DM complications [44]. In this pathway glucose is converted to sorbitol by the enzyme aldose reductase and its reduced cofactor nicotinamide adenine dinucleotide phosphate (NADPH). Sorbitol is then oxidized to fructose by the enzyme sorbitol dehydrogenase, with nicotinamide adenine dinucleotide (NAD+) converted to the reduced nicotinamide adenine dinucleotide (NADH). Aldose reductase is found in tissues like nerve, retina, lens, glomerulus and vascular cells [45], and has a low affinity (high K_m) for glucose. A small percentage of total glucose utilization is therefore metabolized through this pathway under normal physiological glucose concentrations [37]. However, the hyperglycemic environment leads to hyperglycemic intracellular concentrations in insulin-independent tissues, and an increase in the polyol pathway. The pathway can possibly promote ROS in different steps: 1) the pathway is consuming NADPH that is required to regenerate the endogenous antioxidant reduced

glutathione, an important scavenger of ROS. 2) The consumption of NAD+ leads to increase in the NADH/NAD+ ratio and less accessible NAD+ for the enzyme GADPH. This results in enhanced accessible substrates to the above mentioned pathways and the polyol pathway it self. Studies of inhibition of the polyol pathway *in vivo* have yielded inconsistent results. In a five-year study in diabetic dogs, aldose reductase inhibition prevented the diabetes-induced nerve conduction velocity seen in controls, but failed to prevent retinopathy and nephropathy [46].

In human lens the polyol pathway is of importance in the formation of sugar cataract. In the lens, sorbitol is produced faster than the following conversion to fructose. This accumulation leads to increased intralenticular osmotic pressure, causing the lens to swell and become opaque [47]. There are numerous publications describing oxidative stress damage to the lens fibers in diabetics, but there is no evidence that these free radicals initiate the process of cataract formation but rather accelerate and aggravate the development [48].

Increased hexosamine pathway flux

In the hexosamine pathway fructose-6 phosphate is converted to glucosamine-6 phosphate by the enzyme glutamine: fructose-6 phosphate amidotransferase (GFAT). The glucosamine-6 phosphate is then transferred to uridine diphosphate N-acetyl glucosamine (UDP-GlcNAc). This molecule serves as a substrate for two following reactions, as shown in **figure 1.5**:

1. **Proteoglycan synthesis**

UDP-GlcNAc may reversibly epimerize to uridine diphosphate N-acetyl galactosamine (UDP-GalNAc), and both components serves as sugar donors for glycosaminoclycan (GAG) synthesis. These sugar-donors are fundamental for the cellular biosynthesis of GAGs. Consequently, diabetic changes in the hexosamine pathway are likely to affect the biosynthesis of PGs.

2. ***O*-linked glycosylation of proteins**

O-linked glycosylation is the process of addition of one GlcNAc molecule on serine or threonine residues of intracellular proteins. This is a dynamic and reversible process that normally leads to functional changes in target proteins. When the intracellular glucose level is increased due to hyperglycemia, the production and availability of UDP-GlcNAc is increased

and affects modifications of proteins important in the DM complications [49]. Increased glycosylation of proteins in the Insulin receptor substrate - Protein kinase B - Glucose transporter 4 (IRS1-PKB-GLUT4) signaling pathway, results in reduced expression of GLUT 4 (in muscle and adipose tissue) and also increases the secretion of resistin from adipose tissue [49]. This promotes insulin resistance, and can be a link to the progression of type 2 DM. Reduced function of endothelial nitric oxide synthase (eNOS) following reduced NO and abnormal blood flow are also relevant examples of diabetic complications, caused by *O*-GlcNAcylation in the PKB signaling pathway [50]. Inhibition of the rate-limiting enzyme GFAT leads to a decrease in hyperglycemia-induced transcription of transforming growth factor (TGF) α and β in porcine glomerular mesangial cells, indicating that the pathway promote fibrosis [51], important in the pathogenesis of DM complications.

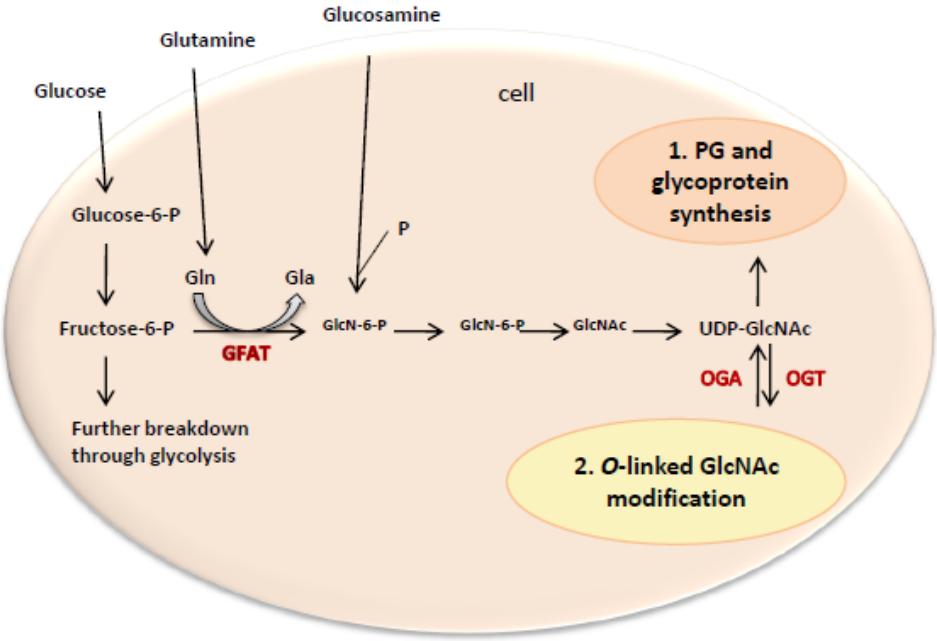

Figure 1.5: The hexosamine pathway. The hexosamine pathway is supplied with fructose-6-phosphate from the glycolysis, or from glucosamine. In a hyperglycemic environment an increased flux through the glycolysis will increase the flux of fructose 6-phosphate into the hexosamine pathway and the concentration of UDP-GlcNAc will increase. UDP-GlcNAc is a substrate for PG synthesis in the Golgi, as well as *O*-glycosylation, a post translational modification of proteins in cytosol. The increased UDP-GlcNAc concentration may lead to changes in protein modifications. (Modified from "Effects of hyperglycemic and inflammatory conditions on proteoglycan synthesis in cultured human endothelial cells", Master thesis in clinical nutrition by Trine M. Reine)

Activation of protein kinase C isoforms

De novo synthesis of diacylglycerol (DAG) is increased in a hyperglycemic environment, where the main substrate is glyceraldehyde 3-phosphate [52]. The increased DAG concentration activates nine of eleven isoforms of PKC [43], which in turn leads to multiple adverse effects. These pathogenic consequences include dysregulation of the vascular permeability and neovascularization through the expression of VEGF. Decreased eNOS activity leads to dysregulation of blood flow [53], and increased activity of nuclear factor κB (NF-κB) result in proinflammatory gene expression in ECs [54]. Increased activity of TGF-β is followed by increased production of collagen and fibronectin, leading to capillary occlusion in the mesangium [55]. The clinical relevance of a possible protective role of PKC-inhibition is inconclusive, and treatment with specific PKC-β-inhibitors may cause toxicity, since PKC activation is involved in many vital cellular functions [52].

Increased advanced glycation end products formation

Glyceraldehyde 3-phosphate can be converted to metylglyoxal in addition to DAG. Metylglyoxal is an AGE product which reacts non-enzymatically with amino groups in proteins, lipids and nucleic acids to form AGEs [56]. The production of AGEs is accumulated in a hyperglycemic environment [42] and the production starts with reducing sugars forming Schiff-bases when attached to amino groups on proteins. Schiff -bases are rapidly transformed into Amadori products, which undergo different irreversible reactions, like oxidation, and leads to the formation of AGEs. AGEs can damage cells by three general mechanisms: 1) intracellular modification of proteins leading to altered functions, 2) AGE modified ECM components interacting abnormally with other matrix components and with integrins on cell surfaces, 3) plasma proteins modified by AGE bind to AGE receptors activate pleiotropic transcription factors and cause multiple pathological changes in gene expression [57]. The sugar substrates that initiates the reaction are mainly glucose extracellulary and glucose 6-phosphate, fructose or glyceraldehyde 3-phosphate intracellulary [42]. Serum level of AGEs is increased in subjects with diabetes, positively correlated to their plasma glucose level, and is associated with degree of nephropathy and retinopathy [58, 59]. HbA_{1c}, glycosylated hemoglobin, is commonly used as a clinical marker and represents the blood glucose regulation the last 2-3 months [60]. Medication, physical activity level and diet need to be evaluated if the HbA_{1c} increases over 7.0 %, which is the set goal in diabetes treatment to prevent complications.

1.4 Proteoglycans

The ECM is a gel-like material holding the cells together and providing a porous pathway for the diffusion of nutrients and oxygen to individual cells. Collagens, elastin, fibronectin and laminin are the main constituents in the ECM together with the PGs. The ECM is found close to almost all cell types in the organism, only circulating blood cells are designed to survive and function without attachment to the ECM [19]. DM is a disease where alterations in the ECM are observed [61], and here focus will be on one major matrix component, the PGs. A variety of structural compositions leads the PGs to have essential functions for normal developmental processes as well as in response to injury and disease, like inflammation and diabetes [62].

1.4.1 Structure and synthesis

PGs are macromolecules consisting of a protein core with one or more covalently linked unbranched GAG chains. The GAGs are polymers composed of repeating disaccharides, one hexosamine and one hexuronic acid. The hexosamine is either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc). The hexuronic acid is D-glucuronic acid (GlcA) or its epimer L-iduronic acid (IdoA). Two major types of GAGs are HS and chondroitin sulfate (CS) as illustrated in **figure 1.6**. Other classes of GAGs are heparin, dermatan sulfate (DS), keratan sulfate (KS) and hyaluronan (HA). In contrast to the other GAG chains, the repeating disaccharides in KS are comprised of the hexosamines GlcNAc and galactose (Gal), in place of a hexuronic acid. HA exists only as a free GAG chain without attachment to a protein core [62].

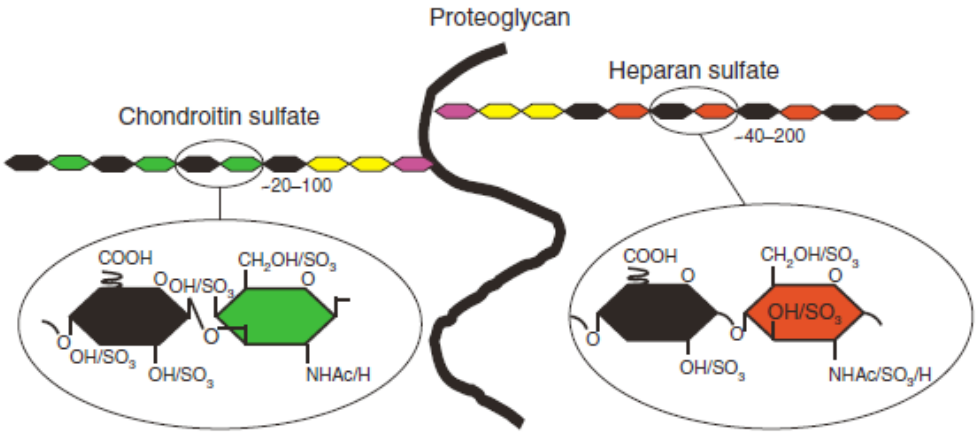

Figure 1.6: Proteoglycan with CS and HS GAG chains. GAGs are *O*-linked to serine residues in proteins through a linkage region composed of Xyl (pink) – Gal (yellow) – Gal (yellow) – GlcA (black). The two major GAGs, CS and HS, consists of repeating disaccharides: GlcA (black) + GalNAc (green) and GlcA (black) + GlcNAc (red) respectively. From Zhang L. Prog Mol Biol Transl Sci Vol 93 2010 [63].

All mammalian cells produce PGs and can secrete them into the ECM, insert them into the plasma membrane, or store them in secretory granules [64]. The protein cores of the PGs are synthesized like other proteins, by transcription in the cell nucleus and translation in the endoplasmatic reticulum. The protein is then transferred to the Golgi, where the GAG synthesis is located. The substrates for GAG synthesis, amino sugars and acidic sugars, are activated in the cytosol as UDP-sugars. They are transferred to the Golgi and added to the protein core through the actions of specific glycosyltransferases. It has been proposed that the initiating process of the GAG chain synthesis, the formation of the tetrasaccharide linker region, may start earlier in the pre-Golgi compartment. This tetrasaccharide linkage region is conserved for HS/heparin and CS/DS GAGs and composed of Xylose (Xyl), Gal, Gal and GlcA. The enzyme UDP-Xyl Transferase initiates the GAG synthesis by *O*-linking Xyl to a serine residue at the protein core. Two molecules of Gal are then added by the enzymes UDP-Gal Transferase I and II. UDP GlcA Transferase finally add a molecule of GlcA, adding the last constituent of the tetrasaccharide linker region [65]. Whether the next monosaccharide added is GlcNAc or GalNAc will determine if HS/heparin or CS/DS is synthesized, as illustrated by **figure 1.7**.

Chondroitin and dermatan sulfate

CS/DS-GAGs are composed of repeating disaccharides of GalNAc and GlcA or IdoA. The enzyme β GalNAc Transferase I will add GalNAc to the tetrasaccharide, committing to CS/DS synthesis [66]. Once this residue is in place, chain elongation and modification proceeds through the action of many different transferases and epimerases. The enzymes GlcA Transferase and GalNAc Transferase add GlcA and GalNAc respectively, and determine the length of the chain. In DS the enzyme C5-epimerase epimerize GlcA to IdoA, the major difference between CS- and DS-GAGs, illustrated in **figure 1.7**. The GAG chains are both sulfated by sulfotransferases, adding sulfate to OH-groups at C6 and C4 on GalNAc in both CS and DS. DS differ from CS in its content of IdoA and that an additional sulfation can occur at C2 in IdoA [64]. The synthesis and modification of CS/DS is illustrated in **figure 1.8**. CS/DS-GAGs can often occur as hybrid structures with both repeating disaccharides in one GAG chain [67].

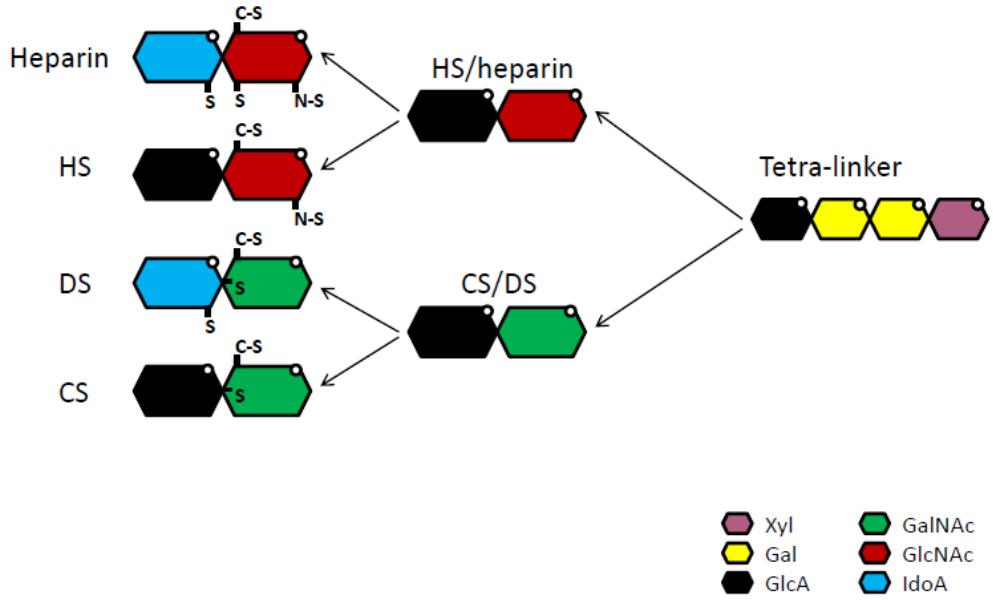

Figure 1.7: Synthesis of HS/heparin and CS/DS GAGs. The synthesis of CS/DS and HS/heparin GAGs are initiated by a tetra-linker region. Further addition of GalNAc + GlcA commits to CS/DS synthesis and GlcNAc + GlcA to HS/heparin. Modification of the GAG chains leads to structural differences between HS and heparin and CS and DS. The characteristics of heparin are based on its degree of sulfation, and the GlcNAc can be sulfated in several positions. To obtain this sulfation pattern *N*-sulfation occurs on C2 by the enzyme *N*-deacetylase/*N*-sulfatase (NDST) that in turn increase the probability of further modifications; GlcA epimerization to IdoA with possible sulfation on C2 and GlcNAc *O*-sulfation in position C6 and C3, the latter is the main characteristic of heparin. In HS, GlcNAc is sulfated to a lesser extent than in heparin. Both GlcA and IdoA can occur in HS, but as the figure illustrates the main hexuronic acid in heparin is GlcA. DS differs from CS by its GlcA epimerization to IdoA, which can be further sulfated at C2. The main characteristics of the different GAGs are not absolute, but occur in varying extents along the GAG chain. (Self-produced, Fjeldstad)

Heparan sulfate and heparin

HS is one of the more complex carbohydrate structures, and its synthesis and modification are dependent upon many different enzymes, as shown in **figure 1.8**. Addition of GlcNAc to the tetralinker region is the first step and relies upon the enzymes EXTL (exostosin-like) 3 or EXTL2, and commits to HS/heparin synthesis [66]. The GAG chains are extended by alternating addition of GlcNAc and GlcA residues forming repeating disaccharides of (GlcA-β1,4-GlcNAc-α1,4)$_n$ catalyzed by the enzymes EXT1/EXT2 [63]. While the chain is polymerized it is also modified by a series of reactions in a sequential mode. This includes *N*-deacetylation/*N*-sulfation of GlcNAc units, GlcA epimerization to IdoA followed by *O*-sulfation at C2 of IdoA. *O*-sulfation also occurs at C6 of GlcNAc residues and more rarely at C3, the latter only present in heparin [68, 69].

The enzymes responsible for the elongation and modification are present in different tissue specific isoforms [70]. Heparin is only synthesized in mast cells and contains >70 % IdoA residues and >80 % *N*-deacetylated and *N*-sulfated glucosamines. HS, is in contrast, less epimerized, and has lower density of and *N*- and *O*-sulfation and in addition higher degree of *N*-acetylation [62]. Highly modified clusters are often separated by less modified segments in one GAG chain and define a specific pattern, characteristic to different HS-PGs. This GAG chain sulfation pattern provide different site specific binding sites and cause different protein binding capacities of heparin and HS GAG chains [63].

Hyaluronan

HA is also composed of repeating disaccharides of GlcNAc and GlcA linkaged by β1,3 or β1,4. In contrast to HS and heparin, HA is non-sulfated. The molecule is of high molecular weight and with no protein core covalently attached [62]. HA is a structural component in the ECM, but can also be stored intracellulary and is expressed in most tissues. The synthesis of HA is localized to the cell surface, and not in the Golgi as for the other GAGs.

Keratan sulfate

In KS, the GAG synthesis is initiated as *N*-linked or *O*-linked oligosaccharides and extended by addition of GlcNAc and Gal [66], in contrast to the other GAG chains. KS is found predominantly in cornea and cartilage [71].

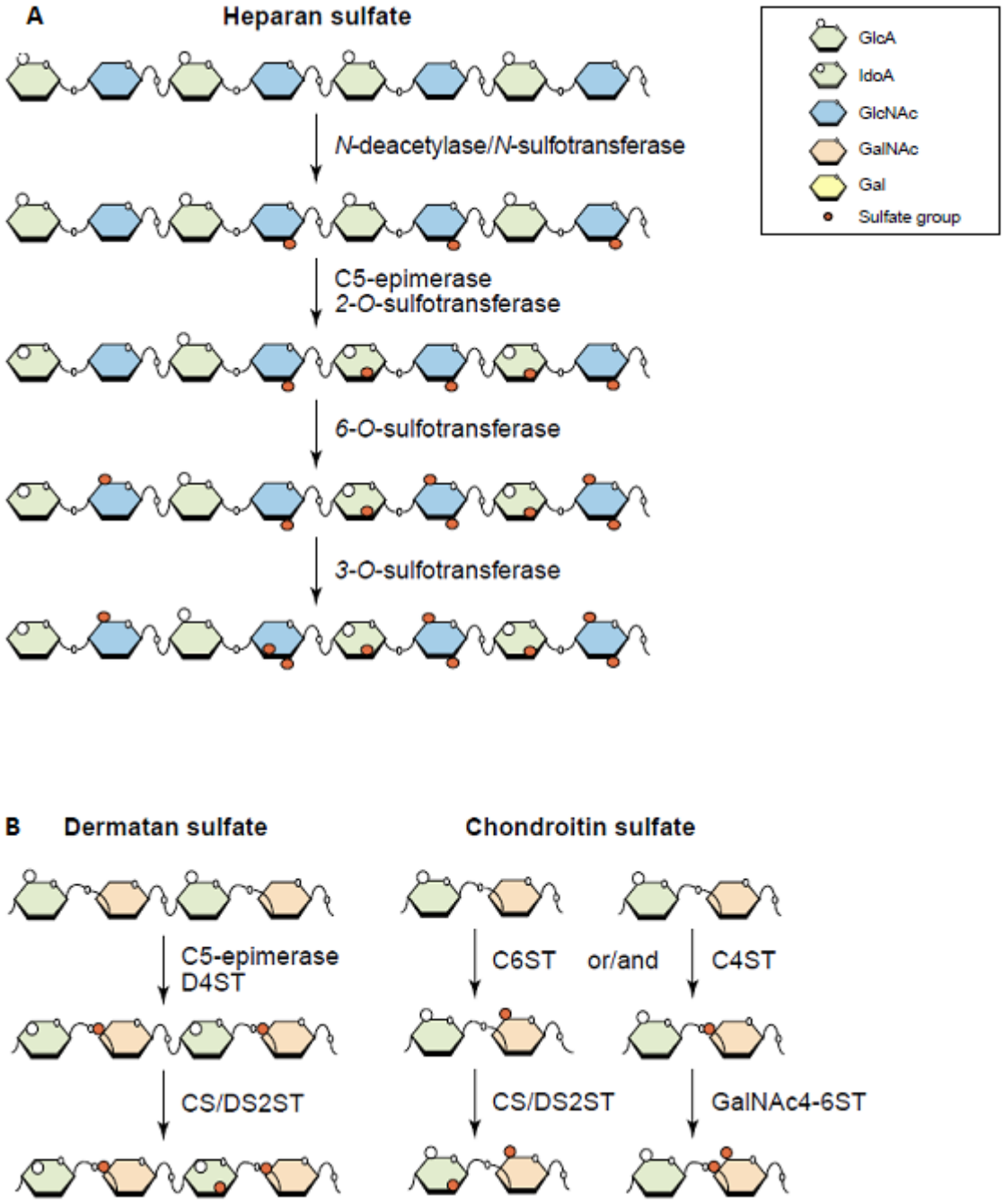

Figure1.8: Modification of the disaccharide unit of HS/heparin and CS/DS. HS/heparin synthesis is first modified by four isoenzymes, GlcNAc *N*-deacetylase/*N*-sulfatase (NDST), which removes the acetyl-group on C2 at GlcNAc, and substitutes it with a *N*-linked sulfate group. The C5 GlcA-epimerase epimerize GlcA to IdoA, by flipping the carboxyl group at C5. Further *O*-sulfation may occur of C2 on IdoA, and of C6 and C3 of GlcNAc (A). In DS, the enzyme C5-epimerase epimerize GlcA to IdoA, followed by *O*-sulfation on C2 of IdoA and at C4 at GalNAc by the enzyme 4-*O*-sulfotransferase (D4ST). In CS, sulfation may occur on C4 and C6 of GlcNAc. GlcA may also be sulfated at C2, but in a lesser extent than in DS (B) [71].

When large GAG chains are attached to PG protein cores, they often dominate the physical, chemical and biological properties of the PGs [72]. The acidic sugars and the modification by sulfate groups results in PGs becoming highly negatively charged. This gives the PGs biological properties like the ability to bind water, attraction of cations and interactions with proteins like cytokines and growth factors, and protecting them against proteolysis and promoting storage in ECM.

After synthesis, the PGs are transported to the cell membrane, to the pericellular compartment or stored in intracellular granules, depending on their biological functions and the type of cell where they are expressed.

1.4.2 Structure and function of proteoglycans

In PGs, anywhere between one to over one hundred GAG chains can be assembled on one core protein [64]. The protein cores ranges from 10 to >500 kDa in size [72] and contains biologically active domains [73]. Also, PGs can carry GAG chains of more than one type [74] and have *N*-linked or *O*-linked glycans in addition [75]. The major divergence in PG structural composition leads to a broad variety of functions including cell-cell and cell-matrix interactions, growth factor sequestration, chemokine and cytokine activation, microbial recognition, tissue morphogenesis during embryonic development, and cell migration and proliferation [62].

The PGs are classified in different ways, often after their type of GAG chain(s) attached, like CS/DS-PGs, HS-PGs or KS-PGs. The PGs can also be divided into groups according to localization, which leads to three major classes of PGs: 1) cell surface PGs, 2) ECM PGs and 3) intracellular PGs, as illustrated in **figure 1.9** [62]. In this thesis the main focus will concern the ECM associated PGs decorin and biglycan, two PGs mainly localized to loose connective tissue and perlecan and collagen XVIII, important constituents of the BMs.

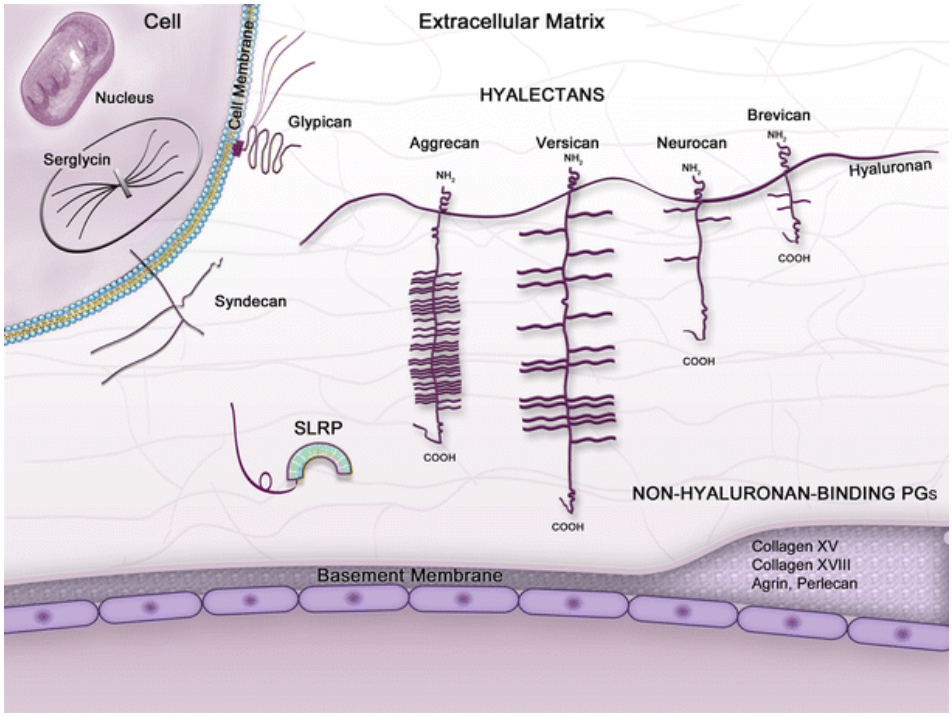

Figure 1.9: Localization of the PGs. The PGs are found intracellulary (serglycin), attached to the cell surface (syndecan, glypican) and in the ECM. In the ECM the PGs are further divided into hyalectans (e.g. aggrecan) bound to hyaluronan. The non-hyalyronan-binding PGs (e.g. collagen XVIII, perlecan) are found in the BM and the small leucine rich PGs (e.g. decorin, biglycan) are found in loose connective tissue. From: Schaefer L. Cell Tissue Res. 2010 Jan;339(1):237-46 [62].

1.4.3 Cell surface proteoglycans

The syndecans and glypicans are two major cell surface associated HS-PGs [62]. Syndecans belong to the membrane-spanning family, together with e.g. betaglycan and CD44. There are four known mammals syndecans, all usually substituted with 1-5 HS chains, although CS/DS chains may also be seen [76]. Their distribution is quite tissue specific, syndecan-1 is mostly expressed in epithelial cells, syndecan-2 mainly in mesenchymal cells, syndecan-3 is occurred mostly in neuronal tissue while syndecan-4 is more widely expressed [77]. The syndecans comprise of an ectodomain, a transmembrane domain and a short cytoplasmic domain. The HS-GAG chains are attached to the ectodomain, and are capable of binding chemokines, cytokines, growth factors and proteases, leading to cellular signaling that may regulate cell proliferation, differentiation, adhesion and migration [76]. In the case of binding to ECM molecules, the functions of syndecans are coordinated directly or indirectly with integrins, receptors with essential roles in cell-cell and cell-matrix adhesion.

These cell surface PGs, mucins and glycoproteins comprise the glycocalyx, localized on the apical side of endothelial cells in the vessel walls and other polarized cells. It is believed that this glycocalyx can be related to the pathogenesis in vascular permeability, inflammation, atherosclerosis and diabetes [26]. Matrix metalloproteinases (MMPs) are important in the ectodomain shedding, then referred to as sheddases. The shedding of PGs are proteolytic cleavages, usually near the plasma membrane, which release the ectodomains that in turn becomes soluble and can function as paracrine or autocrine effectors [78]. Increased syndecan-1 serum level is found in patients with diabetes and microalbuminuria and it has been hypothesized that syndecan-1 play a role in the pathogenesis of diabetic nephropathy [79].

Glypicans are members of the glycosyl phosphatidylinositol-linked PGs, and there are six members in the glypican family. Glypican-1 is ubiquitously expressed, whereas glypican-6 is found mainly in the heart, kidney, liver, ovaries and intestine. Glypican-3, -4, and -5 are in contrast mainly located to the CNS. Most of the *in vivo* evidence published so far indicates that the main function of membrane-attached glypicans is to regulate the signaling of Wnts, Hedgehogs, fibroblast growth factors (FGFs), bone morphogenetic proteins, Slit and insulin-like growth factor (IGF) [80]. Both syndecans and glypicans influence tumor development as their expression is abnormal in various tumor cells, but this topic is not covered here [81].

1.4.4 Extracellular proteoglycans

The ECM PGs can be divided into small leucine-rich PGs (SLRPs), HA- and lectin-binding PGs (hyalectans) and non-HA binding PGs.

Small leucine-rich proteoglycans

The SLRPs are structurally characterized by a protein core of leucine rich-repeats (LRRs) flanked by conserved cysteine clusters in both the C- and N-terminal domains [2]. They have a relatively small protein core of 36-42 kDa, covalently linked at serine residue(s) to at least one CS-, KS- or DS-GAG side chain [82]. The SLRPs are classified into five distinct groups as illustrated in **table 1.1**. The classification is based on chromosomal organization, cystein-rich regions, and conservation and homology at the protein and genome level [62]. Decorin and biglycan are both class I SLRPs, and these PGs are thought to play a role in the formation

of fibrosis and inflammatory reactions [2], relevant in the DM pathogenesis. Both decorin and biglycan are found upregulated in fibrosis and atherosclerosis [83].

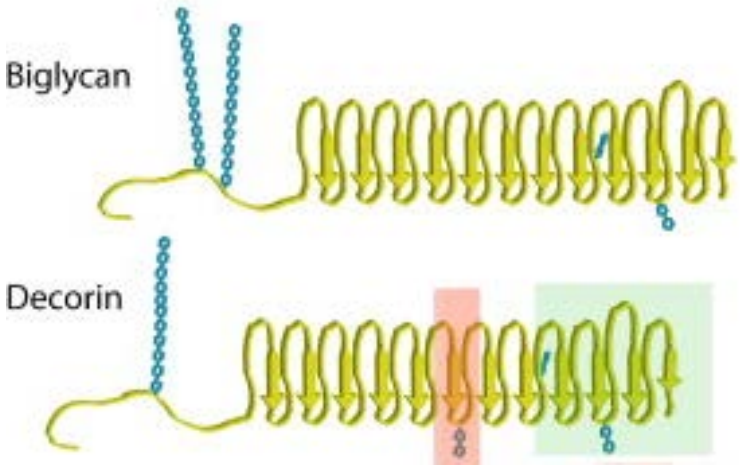

Figure 1.10: Structural composition of decorin and biglycan. Decorin and biglycan are composed of twelve Leucine-rich repeats LRR, illustrated as yellow loops in the figure. In the C-terminal, biglycan contain two *N*-glycosylated oligosaccharides while decorin has three. *O*-linked GAG chain(s) are found in the N-terminal end, one in decorin and two in biglycan. From: Kalamajski S. and Oldberg Matrix Biology 29:248-253 2010 [83].

Decorin is one of the simplest of all PGs, consisting of a core protein with one GAG chain attached to a serine residue in the N-terminal domain, as illustrated in **figure 1.10** [84]. The intact PG is around 100 kDa [85], and the core protein around 36-40 kDa [72, 85, 86]. There are three *N*-glycosylated oligosaccharides attached to the C-terminal asparagines number 211, 262 and 303, thought to aid in folding [87] and secretion of the protein [75]. The core protein consist of twelve LRRs, with conserved cys-rich N- and C-terminal domains [85]. C-terminal "ear-repeats" are distinctive features of the SLRP family, describing how the longest repeat (LRR-XI) make up a disulfide bond with LRR-XII through conserved cystein-residues [88]. The propeptide of decorin is composed of 14 amino acids and regulates the attachment of the GAG chain, with deletion leading to shorter GAG chain [89]. Decorin is a CS-PG in bone and cartilage, and a DS-PG in other tissue [84]. Decorin is a structural important compound of the ECM, with high affinity of binding to different collagens and elastic fiber components [83]. In the cornea, decorin is necessary for tight packed collagen fibrils, leading to transparency. Disruption in PG synthesis in the cornea can lead to blindness in humans, because of altered organization of the collagen fibrils [90]. Decorin deficient mice show skin fragility and dysregulation of lateral fibril growth [91]. Other studies on decorin knock out mice results in

weak tendons, lower airway resistance, slow wound healing and angiogenesis [83]. From its pericellular localization, decorin also interacts with cytokines, growth factors and receptors and can influence many different cellular functions. Through the binding to IGF-receptor, decorin affect cell survival of endothelial cells [92]. By binding to epidermal growth factor (EGF) receptor on tumor cells, it is hypothesized that decorin may play an important role in the inhibition of tumor growth [93]. Decorin also binds to Met, the receptor for hepatocyte growth factor, and thus inhibits migration and growth [94]. Decorin is with few exceptions not expressed in tumor cells, but the mechanism is still unclear and can be altered by transcription, post-transcriptional and post-translational modifications. Clinical relevance of decorin as a prognostic marker in oncology is therefore studied, but the results are as yet inconclusive [81]. Decorin and biglycan can both bind and immobilize the proinflammatory cytokine TNF-α [95] and the profibrotic TGF-β [96]. Decorin is expressed only to a limited extent in the kidneys, but increase in expression is observed in DM nephropathy supporting the theory of its possible anti-fibrotic functions [97]. The normal distribution of class I SLRPs in the kidneys was mainly found in the glomeruli [98]. Both decorin and biglycan were also found in the glomerular mesangial matrix to a limited extent, while biglycan was also found in endothelial cells and occasionally in epithelial cells in glomeruli. Decorin was found in all layers of arterial blood vessels in peritubuli and peritubular matrix. In tunica adventitia of preglomerular arterioles, biglycan was found in the endothelium and SMCs. The SLRPs are not one of the established BM components, and these findings may therefore suggest possible new aspects of decorin functions.

Biglycan carries two GAG chains near the N-terminus and two *N*-linked oligosaccharides in the C-terminus, as shown in **figure 1.10** [99]. Released from the ECM or macrophages, biglycan can act as an endogenous ligand of Toll-like receptors (TLR) 2 and 4 and play important role in immunity and inflammatory responses [100]. Under normal circumstances biglycan is confined to the ECM, but secreted from the ECM in stress situations by mechanisms not defined [82]. Downstream of TLRs, biglycan increase activation of NF-κB, a proinflammatory transcription factor. Lack of biglycan in mice causes osteoporosis and spontaneous aortic dissection (in male animals only) [62], the latter indicating an important function in the artery vessel wall. Biglycan is also co-localized with atherogenic lipoproteins within atherosclerotic plaques, supporting the hypothesis that biglycan may contribute to the pathogenesis of atherosclerosis by trapping lipoproteins in the artery wall [101].

Hyaluronan- and lectin-binding proteoglycans (hyalectans)

Versican, neurocan and aggrecan are members of the hyalectans, which are composed of a tri-domain structure; central domain with 3-100 GAGs attached, C-terminal domain capable of binding to lectins and a N-terminal domain capable of binding to HA. Aggrecan was one of the first observed PGs, named after its ability to form "aggregates" with HA. Aggrecan has highly anionic functions, as it contains approximately 100 CS-GAGs and approximately 60 KS chains. This leads to highly osmotic environment, necessary to retain water in tissues [102]. Aggrecan is found in cartilage and tendons, where high compressive load caused by water binding is necessary. The KS chains attached to the aggrecan protein core have been shown to bind with high affinity for collagen [102].

Non-hyaluronan binding proteoglycans

Non-HA binding PGs, like perlecan, collagen XVIII and agrin, are all found in BMs, flexible thin (40-120 nm) layers of specialized ECM that underlie all endothelial and epithelial layers, and surround many other cells including Schwann cells and muscle cells. The BM is composed of several ECM molecules together with PGs, such as different types of collagens, laminins and nidogens [24]. Agrin, perlecan and collagen XVIII are HS-PGs and the negative charge of their highly sulfated GAG chains are of importance in basal membranes such as the glomerular basement membrane (GBM), where they are part of the filtration barrier.

Perlecan has a 400-467 kDa protein core with three to five HS-GAGs and additionally numerous *O*-linked oligosaccharides, resulting in a molecular size of ~800 kDa [103, 104]. Perlecan is mainly localized in the BM in vascular tissue [105], and is found in the mesangial matrix, GBM, tubular basement membrane and in Bowman's capsule in the kidneys [106, 107]. During development perlecan is pivotal in the GBM, and perlcan -/-mice die *in utero* or shortly after birth with disruptions of the BM [62]. Both the protein core and the GAG chains of perlecan are able to bind to different ECM molecules and growth factors like FGF2, VEGF and platelet derived growth factor [24, 103]. Perlecan is believed to participate actively in the negative electrostatic charge barrier in the GBM together with agrin and collagen XVIII [108]. It remains unclear whether the HS-PGs are the most prominent compounds in the GBM filtration [109]. However, it is certain that they have functions of importance [110].

Collagen XVIII is a HS-PG with the protein core of 187 kDa [62]. The core is consistent of three domains characterized by a central triple helical collagenous domain flanked by N- and C-terminal non-collagenous domains. The HS chains are attached to the N-terminal and the non-collagenous sequences in the central domain [103]. The main localization of the PG is in the BM throughout the body [111]. The C-terminal part of the core protein, endostatin, can be cleaved and have potential biological functions as an inhibitor of angiogenesis in tumor growth and endothelial cell proliferation [112]. Angiogenesis is the development of new blood vessels from preexisting ones, and is a common feature in severe DM retinopathy [22]. The collagen XVIII/endostatin also has essential functions in development and maintenance of visual function [111].

1.4.5 Intracellular proteoglycans

Serglycin is the most prominent intracellular PG, found in endothelial cells, neutrophils, mast cell, platelets, cytotoxic T-cells and macrophages. This PG is present in storage granules, important for storing enzymes, growth factors and other compounds. In ECs, the PG is shown to be secreted, together with chemokines like growth-related oncogene α (GRO-α/CXCL1) [113]. The GAG chains attached to serglycin core protein differ between the different cell types. CS-GAGs are apparent in neutrophils, T-lymphocytes and platelets. In connective tissue mast cells, serglycin is composed of heparin GAGs, a powerful blood coagulant, and in macrophages and monocytes the serglycin has both CS- and HS-GAG chains [114].

Table 1.1: Overview of PGs based on their localization and common features.

PG group			PG	GAG sort/no	Protein core kDa
Intracellular			Serglycin	Heparin/CS 10-15	10-19
Cell surface	GPI-coupled		Glypican 1-6	HS 1-3	~60
	Integral		Syndecan 1-6	HS/CS 1-3	31-45
ECM	SLRPs	Class I	Decorin	CS/DS 1	36
			Biglycan	CS/DS 2	38
		Class II	Fibromodulin	KS 4	42
			Lumican	KS 1	38
		Class III	Osteoglycin	KS 2-3	35
			Epiphycan	CS/DS 2-3	36
		Class IV	Chondroadherin	KS	36
			Nyctalponin	?	50
		Class V	Podocan	0-1	70
	Hyalectans		Versican	CS 12-15	265
			Aggrecan	CS/KS ~ 100	208-220
			Brevican	CS 0-4	96
			Neurocan	CS 1-2	145
	Non-hyalectans		Perlecan	HS/CS 3-5	400-467
			Collagen XVIII	HS 3	187
			Agrin	HS 3	212

1.4.6 Degradation of proteoglycans

Enzymes responsible for ECM and PG degradation and remodeling include MMPs, serine proteases, endoglucuronidases and sulfatases. Inhibitors such as serine protease tissue inhibitors (TIMPs) and plasminogen activator inhibitor (PAI-1) are important for the regulation of these processes [61]. For further degradation the PGs are transported to the lysosomes where proteases, sulfatases and glycosidases degrades both protein and GAGs to their constituent units. Mutation in some of these enzymes may lead to lysosomal storage diseases, such as mucopolysaccharidoses (type I-VII) e.g. Hunters disease with impaired degradation of DS and HS caused by iduronide-2-sulfate sulfatase mutation.

Heparanase is an endoglucuronidase that specifically cleaves the glycobond between GlcA and GlcNAc in HS-GAGs, whose gene expression is regulated by inflammatory cytokines and hypoxia. The fragmentation results in 5-7 kDa pieces of HS which may contain biological active functions [115]. Increase in heparanase levels are observed in urine and blood samples

in type 2 diabetic patients [116]. It is hypothesized that increased heparanase production in endothelium and macrophages can be related to the reduced content of HS-PGs observed in atherosclerosis [117]. Another posttranslational modification can be obtained by the plasma-membrane-bound endosulfatases, by removing specific sulfate groups of extracellular HS-GAG chains [28]. The reduced degree of sulfation either promotes or inhibits binding of different molecules, and can affect the PGs functions.

1.5 Extracellular matrix and diabetic complications

Early in the course of diabetes, endothelial dysfunction leads to vasoconstriction and increased permeability of the blood vessels. Further, accumulation of ECM caused by increased synthesis or reduced degradation, leads to fibrosis, a hallmark of diabetic complications [61]. One organ affected by these pathologic changes is the kidneys, clinically observed by thickening of the GBM and glomerulosclerosis. ECM alterations in the larger arteries are linked to the accelerated process of atherosclerosis seen in diabetes, and the ECM alterations are thus of great importance in both micro- and macrovascular complications.

2 Aims

2.1 Study rationale

Diabetes is characterized by hyperglycemia and chronic inflammation. Even though the general life expectancy in people with diabetes has increased, the disease still causes harmful and serious secondary complications. For both treatment and prevention, it is important to obtain a better understanding of the underlying mechanisms.

The endothelium is the prime organ to be exposed to hyperglycemia, and hyperglycemia is now considered a primary causal factor in the development of DM complications. The PGs are related to the endothelium and changes in their structure and/or distribution are observed in atherosclerosis, retinopathy, nephropathy and neuropathy, the most common DM complications. Studies have shown that exposing human umbilical vein endothelial cells (HUVEC) to IL-1β and hyperglycemia alter the endothelial PG expression and secretion [113].

In order to elaborate on this observation, primary HUVEC were cultured *in vitro* and exposed to hyperglycemia and inflammatory conditions, characteristics of a diabetic environment, to determine secretion of PGs from such endothelial cells. Quantitative effects on total *de novo* PG synthesis and the individual ECM PGs perlecan, decorin, collagen XVIII and biglycan were investigated. We also studied the effect of hyperglycemic and inflammatory conditions on endothelial permeability in HUVEC, using FITC-labeled albumin as target molecule.

2.2 Hypothesis

Specific hypotheses of this thesis:

a) ECM-type PGs are predominantly secreted to the basolateral side.

b) The secretion is affected by hyperglycemic and inflammatory conditions.

c) The cellular permeability is affected by hyperglycemic and inflammatory conditions.

3 Methods

3.1 Human umbilical vein endothelial cells (HUVEC)

The HUVEC are primary vein ECs from human umbilical cord, obtained from the Woman and Child clinic, Oslo University hospital. Ethical approval for the use of umbilical cords was obtained from the Regional Committees for Medical and Health Research Ethics, through our clinical collaborators and was approved by the mothers in advance.

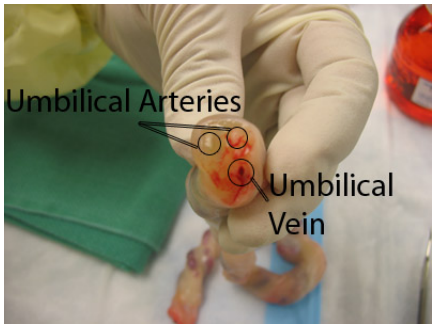

Figure 3.1: An Umbilical cord with two arteries and one vein. The vein supply oxygen and nutrient rich blood from the placenta to the fetus. http://homepages.cae.wisc.edu/~bme300/umbilical_f07/

3.1.1 Isolation of HUVEC from umbilical cord

HUVEC were isolated enzymatically from infant umbilical cords as earlier described [118]. The umbilical cords were from normal pregnancies and stored under sterile conditions and established as primary cell cultures.

Procedure:

1. The umbilical cords were stored in a sterile cord buffer at 4°C, for a maximum of 24 hours before isolation.

2. The vein was rinsed with PBS and damaged areas were removed.

3. Collagenase was infused into the vein and the cord was incubated in PBS for 10 min at 37°C. After incubation, the collagenase solution containing the ECs was flushed by the cord by perfusion of PBS. If needed, an additional 10 mL of PBS was added to detach the cells.

4. The solution containing the cells was finally collected in a centrifuge tube, and centrifuged at 1500 rpm for 10 min. The cell pellet was then resuspended in 15 mL medium and cultured in T75 cell flasks at 37°C and 5.0 % CO_2.

Equipment and solutions:

EQUIPMENT	ADDITIONAL EQUIPMENT NEEDED	SOLUTIONS
2 metal cannulas	2 single use syringes 50 mL	1X PBS autoclaved
4 compresses	1 single use syringe 15 mL	Cord buffer
Aluminium foil	2 centrifuge tubes 50 mL	0.2 % collagenase
4 plastic straps	1 cell flask 75 cm^2	Complete medium
2 plastic tubes (2-3 cm)	Sterile glows	
3 glass vials	Water bath, 37°C	
A sterile tray to work on	1 sterile surgical blade	

All equipment was autoclaved.

3.1.2 Culturing HUVEC

HUVEC were established at 37°C and 5.0 % CO_2 in plastic bottles in complete MCDB-131 medium (Sigma-Aldrich) with 7 % fetal calf serum (FCS) changed every second day. The cells were grown in 5 ml medium in 25 cm^2 bottles, or in 15 ml in 75 cm^2 bottles. FCS was heat inactivated at 60ºC for 30 min.

3.1.3 Polarized HUVEC

To achieve polarization, cells were cultured on semipermeable filters, a useful *in vitro* model system with relevance to the *in vivo* situation.

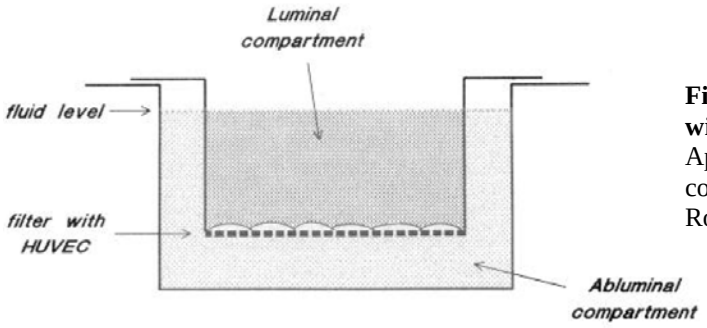

Figure 3.2: Schematic illustration of one well with filter inserts for culturing polarized cells. Apical (luminal) and basolateral (abluminal) compartments are filled with medium. Taken from Rosengren S et. Al, J. Appl Physiol. 1991[119]

Costar Transwell clear polyester membrane inserts (Corning Life Sciences) with 0.4 µm pore size, were preincubated with MCDB medium for 1-16 hours, coating the membrane with plasma proteins. Both 12- and 6-well inserts were used having a growth area of 1.12 cm^2 and 4.67 cm^2 respectively. The fluid volume was dependent on well size and proportional to the surface area, resulting in an even liquid level for the inner and other compartment.

Solutions:

Basis MCDB medium

M-8537 (MCDB-131) powder and 1.18 g $NaHCO_3$ was dissolved in 850 mL. PH was adjusted to 7.3 with 1 M HCl or NaOH while stirring. Finally, water was added to a total of 1000 mL. The medium was sterile filtrated before use.

Complete MCDB medium

Basis MCDB medium was added antibiotics and growth factors as listed below, to obtain the complete MCDB medium.

COMPONENTS	VOLUME	FINAL CONCENTRATION
MCDB-131 medium	Powder for 500 mL	
$NaHCO_3$	0.59 g	1.0 mM
mQ-H_2O	500 mL	
FGF 10 µg/ml	50 µL	1 ng/mL
Hydrocortisone 10 mg/ml	50 µL	1 µl/mL
EGF 10 µg/ml	500 µL	10 ng/mL
Gentamicine 50 mg/ml	500 µL	50 µg/ml
Fungizone 250 µg/ml	500 µL	250 ng/mL
FCS (heat inactivated)	37.5 mL	7 %

3.1.4 Splitting of HUVEC

1. The cells were split when 90 % confluent. They were usually split in a 1:3 ratio.

2. All solutions were brought to room temperature before use.

3. The conditioned medium was removed. The cells were then washed with 4 mL PBS prior to trypsination, to remove divalent cations and proteins that inhibit trypsin action. 4 mL trypsin/EDTA was added to depolymerize the adhesion proteins in cell-cell and cell-matrix interactions.

4. After approximately 5 min the endothelial cells detached from the flask and acquired round shapes, observed visually by microscope. Trypsin was then inactivated by adding equal amount of medium containing FCS. The cell solution was transferred to a centrifuge tube and spinned down at 1300 rpm for 3 min.

5. The supernatant was removed and the cell pellet was resuspended in new medium and seeded in culturing flasks or in filter wells.

6. The cells were incubated at 37°C and 5.0 % CO_2.

3.2 General Experimental Outline

The cells were established on conventional plastic and passaged from one to four times. To obtain polarized cells, HUVEC were cultured on semipermeable filters. This was, to be able to analyze the apical and basolateral compartments respectively. The cells were routinely grown to 100 % confluence before used in experiments, observed visually by microscope as a tight monolayer.

Both polarized HUVEC and cells cultured in conventional cell culture flasks were stimulated with 0.5 ng/mL IL-1ß, together with medium with either low glucose (LG, 5 mM) or high glucose (HG, 25 mM) in 24 hours. LG alone was used as a control. The stimulations were performed in MCDB-medium with FCS reduced to 2 %.

Total amount of newly synthesized PGs was determined by ^{35}S-Sulfate labeling. When labeling with ^{35}S-Sulfate, sulfate free RPMI 1640-medium was used, added only 2 % FCS and 5 mM L-glutamine. The medium was not added growth factors or antibiotics.

3.3 Labeling with ^{35}S-sulfate

Total amount of newly synthesized PGs was determined by ^{35}S-sulfate labeling. The radioactive isotope was added to the sulfate-free medium in cell cultures and the cells were incubated for 24 hours in 0.1 milli-Curie ^{35}S-sulfate/mL medium. GAG chains are highly sulfated compared to other macromolecules in the cells. By using sulfate-free medium without sulfate containing antibiotics, added the radioactive sulfate, radioactive sulfate will be incorporated into the GAGs of newly synthesized PGs, and can thus be easily detected.

After labeling, the medium and cell fraction were harvested as described below. To remove free ^{35}S-sulfate and ^{35}S incorporated in smaller molecules, the samples were purified by Sephadex G50-fine gel filtration.

3.4 Harvesting

The medium was harvested as one unit from cell flasks of same stimulations, or divided as apical and basolateral medium from the polarized cells in wells, and collected in centrifuge tubes. The ECs were washed with PBS prior to 15 min incubation with 0.5 mL RIPA- or guanidine buffer. A cell scrape was used to detach the cells and the lysate was transferred to a centrifuge tube. Both medium and cell lysate was kept on ice. The medium was spinned down, 1500 rpm for 3 min to remove cell debris, then analyzed immediately or frozen before further analysis.

RIPA buffer, 100 mL

COMPONENTS	VOLUME	FINAL CONCENTRATION
1 M Tris-HCl pH 7.4	5 mL	0.5 M
2 M NaCl	7,5 mL	0.15 M
Triton X-100/NP-40	1.0 mL	1 %
SDS	0.1 mL	0.1 %
Na-deoxycholate	0,5 mL	0.5 %
EDTA	37.6 mg	1 mM
Sodium pyrophosphate ($Na_4PO_20_7$)	446 mg	10 mM

Protease inhibitor was added fresh to the buffer, 1 tablet per 10 mL.

Guanidine/Trition X-100 harvesting buffer for ^{35}S-labeled cells

COMPONENTS	VOLUME	FINAL CONCENTRATION
Guanidine HCl	229.27 g	4 M
Triton X-100	12 mL	2 %
M Acetatebuffer pH 6,5	Up to 600 mL	0.1M

Guanidine HCl was added to the Acetatebuffer and the volume adjusted to 600 mL. Then the solution was rinsed while stirring with charcoal over night, and then filtered twice before Trition X-100 was added to a final concentration of 2 % and pH adjusted to 6.0.

3.5 Gel filtration- Sephadex G-50 fine

Proteins can be separated according to molecular size by filtration trough a porous matrix gel. The pores of the gels are of different sizes. Small molecules elute trough both small and larger pores, while larger molecules are confined to the less numerous large pores or outside the gel particles. In this way larger molecules will travel a shorter way than the small ones, and can thus be eluted at an earlier stage. Sephadex G50-fine separate proteins of 0.5-10 kDa, and is a

suitable method for exclusion of both smaller molecules containing ^{35}S-Sulfate and free unincorporated ^{35}S-Sulfate

Procedure:

1. Sephadex G-50 fine powder was swollen over night in a 0.05 M NaCl Tris-HCl buffer with pH 8,0.

2. Columns were made of 10 mL pipettes with glass wool in the bottom filled with 4 mL Sephadex G-50 fine gel.

3. 1 mL sample was added to the columns.

4. The first elute of 1 mL volume was discarded.

5. By adding 1.5 mL buffer, the PGs were eluted and collected. Free ^{35}S-Sulfate was retained in the column and was discarded.

6. Aliquots of this elute (2x50 µL) was counted for radioactivity using a scintillation counter.

Solutions:

Sephadex G50-fine solution

COMPONENTS	VOLUME
Sephadex G50-fine powder	10 teaspoons
0.05M NaCl in 0.05M Tris-HCl, pH 8.0	500 mL

The solution was swollen over night.

3.6 Protein quantitation

BC (bicinchoninic acid) Assay was used to quantify protein by spectrometric measurement of a color produced by a reaction between proteins and copper (Cu^{2+}). The copper-protein complex can be measured by optical absorbance at 562 nm. The absorbance is directly proportional to protein concentration, and can be calculated with a reference curve obtained for a protein standard.

Procedure:

1. A standard curve was made from dilutions from a BSA stock solution of known concentration.

2. 25 µL of the standards and samples were added in duplicates to a 96-well microtiter plate. Then 200 µL AB mix was added to all of the wells. The AB mix was made according to manufacturer's protocol.

3. Incubated for 30 min at 37ºC.

4. The absorbance at 562 nm was measured with the Titertec multiscan PLUS spectrophotometer.

5. A standard curve was created from the measured absorbance (Y-axis) and known protein concentrations (X-axis).

6. The protein concentration of the samples was calculated from the standard curve.

3.7 Scintillation counting

The amount of radio-labeled molecules in a metabolically labeled sample can be estimated using a liquid scintillation counter, which measures radiation from β-emitting nuclides such as ^{35}S. The kinetic energy of the β-particles is absorbed by solvent molecules, and the energy of these excited molecules is emitted as UV-light. The samples were placed in small transparent vials loaded into a liquid scintillation counter, where light flashes emitted per minute are counted. In this way, each beta emission results in a pulse of light, and the amount of light emitted is correlated to the amount of radioactivity of the sample. Each sample was counted for three minutes, and measured as counts per minute (cpm).

3.8 DEAE – anion-exchange chromatography

DEAE (Diethylaminoethyl) Sephacel, was used to separate macromolecules by charge. Because DEAE is a weak anion-exchanger, it binds highly negatively charged molecules, like PGs, by ionic bonds. By changing the salt concentration of the buffer, the PGs can be separated from other macromolecules according to charge. This was done by washing the gel with 0.15/0.3/ 2.0 M NaCl in 0.05 M Tris-HCl pH 8. Low salt will elute slightly negatively charged molecules, high salt will elute highly negatively charged molecules, like PGs.

Procedure:

1. DEAE Sephacel was centrifuged and the ethanol supernatant was discarded. The resulting gel was resuspended in an equal amount of 0.05 M Tris Start-buffer.
2. 500 µL of the DEAE-solution/gel was applied to polyprep columns.
3. The gels were washed with 3 mL Start-buffer.
4. The samples were added to the columns, and the negatively charged PGs will bind to the DEAE.
5. 3 mL Start buffer was added.
6. By adding 3 mL 0.3 M Wash-buffer, slightly negative charged molecules were eluted.
7. The highly negatively charged PGs were eluted with 1500 µL Eluting-buffer.

Solutions:

Start-buffer

COMPONENTS	VOLUME	FINAL CONCENTRATION
1 M Tris	10 mL	0.5 M
2 M NaCl	15 mL	0.15 M
mQ-H_20	Up to 200 mL	

pH was adjusted with 1M HCl to 8.0

Wash-buffer

COMPONENTS	VOLUME	FINAL CONCENTRATION
1 M Tris	10 mL	0.5 M
2 M NaCl	30 mL	0.3 M
mQ-H_20	Up to 200 mL	

pH was adjusted with 1M HCl to 8.0

Eluting-buffer

COMPONENTS	VOLUME	FINAL CONCENTRATION
1 M Tris	5 mL	0.05 M
2 M NaCl	11,688 mL	2.0 M
mQ-H_20	Up to 100 mL	

pH was adjusted with HCl to 8.0

3.9 Glycosaminoglycan depolymerization

3.9.1 Chondroitinase ABC (cABC)

Chondroitinase ABC is an enzyme that depolymerizes CS/DS GAG chains. It is specific for cleavage between beta-1,4-galactosaminic bonds between N-acetylgalactosamine (GalNAc) and either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) and will produce a mixture of oligo-, tetra- and disaccharides.

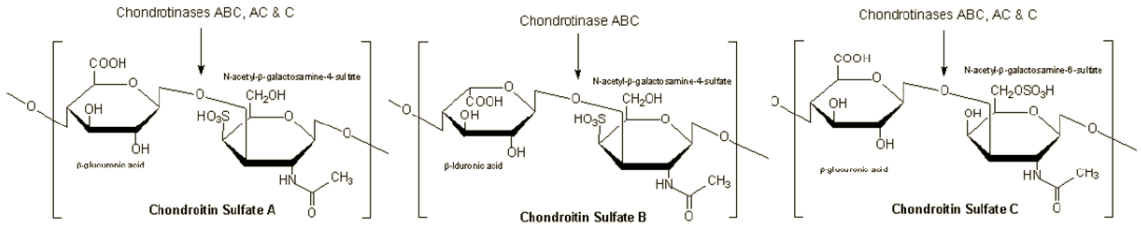

Figure 4.3: Chondroitinase ABC. The enzymes Chondroitinase A, -B and –C cleaves the β-1,4-galactosaminic bonds between GalNAc and either GlcA or IdoA. http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/carbohydrate-analysis/carbohydrate-analysis-ii.html#Chondroitin Sulfate.

Procedure:

1. 0.01 U of cABC enzyme was added to each sample of 10 µL.
2. 10x cABC-buffer was added until 1x. 1 µL 10x buffer was added to each sample of 10 µL.
3. Samples were incubated over night at 37ºC.

Solutions:

10X c-ABC buffer: 0.5 M Tris HCl pH 8.0, 0.5 M sodium acetate, 10 mL

COMPONENTS	VOLUME	FINAL CONCENTRATION
1 M Tris pH 8.0	5 mL	0.5 M
NaAc*3H_20 (sodium acetate)	0.68 g	0.5 M
mQ-H_20	Until 10 mL	

Stored at 4ºC.

3.9.2 HNO_2 – Cleavage of N-sulfated GAGs

Nitrous acid (HNO_2) is prepared by mixing nitrite salts with acid, and cleave the glycosidic bonds of N-sulfated GlcN residues at room temperature and pH 1.5 to yield a mixture of saccharides depending on N-sulfation pattern. Only HS and heparin will be polymerized by

this treatment because of their characteristic N-sulfation. Nitrous acid is degraded and unstable. The solution must therefore be used within 30 min after preparation.

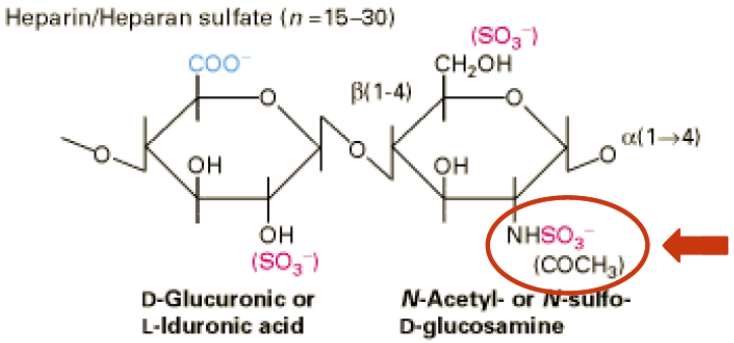

Figure 4.4: Illustration of a heparin/HS disaccharide unit and the location of nitrous cleavage. N-sulfation is unique in GlcNAc and constitute to a characteristic feature of heparin/HS. This is the target location of nitrous cleavage, marked with red arrow, used to distinguish CS/DS-PGs form HS/heparin. http://courses.cm.utexas.edu

Procedure:

1. Equal amounts of HNO_2 and sample were mixed and incubated for 10 min in room temperature.

2. The reaction was stopped by adding 2 M Na_2CO_3 in 1/10 of the sample volume. Then the pH was increased to 8.5, measured with pH-paper.

Solutions:

2 M Na_2CO_3

COMPONENTS	VOLUME	FINAL CONCENTRATION
Na_2CO_3	2.12 g	2 M
mQ-H_20	Up to 10 mL	

Stored at 4ºC.

0.5 M H_2SO_4

COMPONENTS	VOLUME	FINAL CONCENTRATION
H_2SO_4	1.33 mL	0.5 M
mQ-H_20	Up to 50 mL	

Stored at 4ºC.

0.5 M $Ba(NO_2)_2$

COMPONENTS	VOLUME	FINAL CONCENTRATION
$Ba(NO_2)_2$	0.62 g	0.5 M
mQ-H_20	Up to 5 mL	

Freshly made.

HNO_2 pH 1.5

COMPONENTS	VOLUME
0.5 M H_2SO_4	0.5 mL
0.5 M $Ba(NO_2)_2$	0.5 mL

Freshly made, mixed and kept on ice. Centrifuged for 5 min. The supernatant is the HNO_2 solution. Chemical reaction: $H_2SO_4 + Ba(NO_2)_2 \longrightarrow 2HNO_2 + BaSO_4$.

3.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a procedure that separates proteins according to their molecular weight on a polyacrylamide gel. The gel contains acrylamide and N,N-methylenebisacrylamide (Bis) in a 3-dimentional network. The pore size, organized by this polymerization, is determined by the amount of acrylamide and the number of crosslinks between the two substances, and separates proteins by size. Sodium dodecyl sulfate (SDS) is added to the samples in the loading buffer prior to application on the gel. The SDS denaturate the proteins, and give them a near uniform negative charge along the length of the polypeptide chain. Migration through the gel can then be assumed to be directly related to the size of the proteins and not the charge.

The samples from DEAE were concentrated on vivaspin columns, with molecular mass cut-off 30 kDa (GE Healthcare), and the high salt solution was discarded. The volume was adjusted to 30 µl, by addition of start buffer or by condensation on the vacuum centrifuge.

Glycerol was added to the loading buffer to make the sample sink in the wells and maintain the sample sunk until start of electrophoresis. To enable visualization bromphenol blue was used. The dye is a small anion, migrating through the gel and monitoring the separation progress. 2-mercaptoethanol was used to reduce disulfide bonds in the proteins.

Protein standards were used to assess the migration distance of differently sized molecules.

Procedure:

1. 4X loading buffer were added to the samples to a final concentration of 1X and the samples were incubated for 15 min in room temperature.

2. Precast gels (Criteron^{TM} Precast Gel, 4-20 % Tris-HCl) were submerged in running buffer in the electrophoresis apparatus.

3. Protein standards were applied along with the samples in separate wells, in order to determine the size of the proteins.

4. The apparatus was attached to an electrical supply, 110 V for approximately 2 hours, until the bromphenol blue reached the bottom of the gel.

5. The gel was immediately incubated in transfer buffer to prevent diffusion of proteins before further analysis by western blotting. Gels containing radiolabeled samples were fixed, treated with amplifier, dried and subjected to autoradiography.

Solutions:

4X loading buffer

COMPONENTS	VOLUME	FINAL CONCENTRATION
0.5 M Tris buffer pH 6,8	1.0 mL	0.05 M
Glycerol	4.0 mL	40 %
SDS	0.8 g	0.08 %
Bromphenol Blue	8.0 mg	8 %
2-mercaptoethanol	1.0 mL	10 %
mQ-H20	Up to 10.0 mL	

10X running buffer

COMPONENTS	VOLUME
Tris base	29.0 g
Glycin	144.0 g
SDS	10.0 g
mQ-H20	Up to 1000 mL

1X running buffer

COMPONENTS	VOLUME	FINAL CONCENTRATION
10X Running buffer	100 mL	1X
mQ-H_20	900 mL	

3.10.2 Drying of SDS-PAGE gel and autoradiography

The gel containing radiolabeled samples was dried after treatment with fixing solution and amplifier. Fixing solution was used to prevent diffusion of proteins from the gel, and amplifier was used to enhance the radioactive signal. The gel was soaked in both solutions for 15 minutes each. Then the gel was transferred to a soaked filter paper, covered with plastic foil and placed on gel drier. The lid was tightly closed with suction and heated to 70ºC for 2 hours. The gel and its radioactive material were dried onto the filter paper, and the filter paper was placed on a film in a hyper cassette in -20ºC or -70ºC before development.

3.11 Western blotting

Western blotting allows individual proteins in a mixture to be identified and analyzed. Proteins separated by SDS-PAGE are transferred onto a membrane, followed by immunological detection of individual proteins. The method is therefore also called immunoblotting. The transfer is dependent upon electricity, generated by a voltage (V) that makes the proteins migrate to the membrane following a current (I) depending on the resistance (R) generated by the materials placed between the electrodes. The process is following Ohm's law: V = I x R, and the dependence upon electricity causes the process to also be referred to as electro-blotting. The blotting system consists of: a cathode, a gel holder, fiber pad, filter paper, gel, polyvinylidene fluoridine (PVDF) membrane, filter paper, fiber pad, gel holder and an anode. Together with a transfer buffer to maintain the conductivity and pH of the system during transfer, the proteins migrate from the cathode to the anode. Heat production decreases resistance of the transfer buffer, and can cause the transfer buffer to loose its buffering capacity. A cooling agent is used and changed if needed during the blotting. When the proteins have reached the membrane, they are immobilized, making them accessible for specific antibodies and enables quantitative detection. There are different types of membranes, PVDF blotting membrane was used because of high mechanical strength and chemical stability as recommended for western blotting. There are both wet- and dry transfer options. Wet transfer was performed, as recommended for large proteins.

A typical experimental system utilizes two layers of antibody in the detection process. The primary antibody is directed against the target antigen: here the PG protein cores. The

secondary antibody is specific for the primary antibody and is here conjugated to Horseradish Peroxidase (HRP), which forms a color precipitate and the light signal is captured on film.

Procedure:

1. The gel was washed with transfer buffer in 15 min to remove SDS-remnants.

2. The PVDF membrane was activated by soaking in methanol in 15 sec, mQ-H_20 in 2 min and transfer buffer in 10 min on agitator.

3. Filter-paper was soaked in cold transfer buffer.

4. According to the instructions of the distributor; filter pad, filter paper, gel and membrane were added in a sandwich. The tight packed sandwich was placed in a cassette and placed in the blotting thank.

5. The tank was filled with cold transfer buffer together with a cooling agent and a stir bar. The tank was placed on a magnetic stirrer.

6. The apparatus was attached to an electrical supply, 0.7 A for 2 hours.

3.11.2 Blocking the membrane

Before the membrane was incubated with the primary antibody, a blocking buffer was used to prevent non-specific background binding of the antibody. Non-fat milk concentrate in TBST was used as blocking-buffer for 1 hour on agitator at room temperature.

3.11.3 Incubation with primary antibody

The primary antibody was diluted in TBST and the membrane incubated on an agitator at 4ºC over night.

	PRIMARY ANTIBODY	TBST	RATIO
Perlecan	20 µL	10 mL	1:500
Collagen XVIII	25 µL	5 mL	1:200
Decorin	2.5 µL	10 mL	1:4000
Biglycan	2 µg	10 mL	0.2 µg/mL

3.11.4 Incubation with secondary antibody

Unbound antibodies were removed by washing 6x5 min in TBST. The secondary HRP-linked antibody was diluted in TBST, and incubated for 1 hour in room temperature. Unbound secondary antibodies were removed by washing 6x5 min in TBST.

	SECONDARY ANTIBODY	TBST	RATIO
Perlecan (rabbit)	0.5 µL	25 mL	1:50 000
Collagen XVIII (rabbit)	5 µL	25 mL	1:5000
Decorin (goat)	10 µL	10 mL	1:1000
Biglycan (goat)	10 µL	10 mL	1:1000

3.11.5 Development

ECL western blotting (GE Healthcare) is a light emitting non-radioactive method for detection of immobilized specific antigens, directly or indirectly with Horseradish Peroxidase (HRP) labeled antibodies. Luminol in the ECL-solution is converted by HRP to a light emitting substance.

The membrane was added ECL-solution (regular or plus). After 5 min with ECL-plus or 1 min with ECL, the membrane was transferred to a hypercassette and developed using Hyperfilm^{TM} MP and Hyperprocessor Amersham Pharmacia biotech developer.

3.11.6 Stripping and reprobing

To be able to probe the membrane with a different antibody, stripping and re-probing was performed. The membrane was then washed:

a. 5 min in mQ-H_20
b. 5 min in blocking buffer
c. 5 min in mQ-H_20
d. 15 min in 2M NaOH
e. 5 min in mQ-H_20
f. 15 min in TBST
g. 1 hour in blocking buffer
h. 15 min in TBST

Before incubation in primary and secondary antibodies as described.

3.11.7 Visualization of proteins in gel

Immediately after the electroblotting, the gel was incubated with 0.1 % (w/v) Coomassie blue R-250 in 45 % methanol 10 % glacial acidic acid for 30 minutes before 1 hour destaining in water. Coomassie blue binds proteins, and this dye is frequently used to visualize proteins and determine if the transfer process from the gel to the membrane was complete.

Solutions:

10X Transfer buffer pH 7.4, 1000 mL

COMPONENTS	VOLUME
Tris-HCl	39.4 g
Glycine	143.0 g
mQ-H20	Up to 1000 mL

1X Transfer buffer

COMPONENTS	VOLUME	FINAL CONCENTRATION
Methanol	100 mL	10 %
10x Transfer buffer	100 mL	1X
mQ-H_20	700 mL	

pH adjusted with 1M NaOH.

10X TBS pH 7.5, 1000 ml

COMPONENTS	VOLUME
Tris base	24.22 g
NaCl	80.15 g
1 M HCl	38 mL
mQ-H20	Up to 1000 mL

pH adjusted with 10 M HCl. Stored at 4ºC.

TBST

COMPONENTS	VOLUME	FINAL CONCENTRATION
10X TBS	200 mL	1X
Tween 20	2 mL	1 %
mQ-H_20	Up to 2000 mL	

Blocking buffer

COMPONENTS	VOLUME	FINAL CONCENTRATION
TBST	100 mL	1 % Tween 20
Dried milk	5 g	5 %

3.12 Albumin permeability assay

FITC (Fluorescein isothiocyanate Conjugated)-albumin (Sigma-Aldrich) was used to determine cellular confluence of polarized HUVEC. When a confluent cell layer is achieved, intercellular pores determine the degree of passage by water soluble molecules. The passage of albumin will be lower at confluence compared to non-confluent cell layer. Cells were cultured on Costar Transwell clear polyester membrane inserts with 0.4 µm pore size and growth area of 1.12 cm^2 (Corning Life Sciences) in 12-well plates. FITC-albumin was applied to the apical medium and both apical and basolateral medium were collected after the incubation time. The passage of FITC over the cell layer was assessed by measurement of fluorescence on each side of the cell layer.

The FITC looses fluorescence (bleaches) when exposed to light at 495 nm, and at pH < 7,0 with or without light exposure. The analysis was therefore done with black eppendorph tubes which are impenetrable to light, and without light in the LAF-bench.

Procedure:

1. A standard curve was made by known concentrations of FITC-albumin.

2. Cells were grown in 7 % FCS MCDB medium to confluence on Transwell inserts.

3. 3.5 µg/µL FITC-albumin in 7 % FCS medium was added to the apical medium and incubated for 45 min.

4. Medium from both apical and basolateral compartment was collected, and diluted to a final concentration of 0.07 µg/µL.

5. The fluorescence was measured at wavelength 495 nm for excitation max and 518 nm for emission max, measured in 96-well microtiter plate with Spectramax Gemini.

6. To test the influence of high glucose and IL-1β on cellular permeability, cells were stimulated 24 hours prior to FITC-application. The concentrations used were the same as prior to western blotting.

3.13 Quantitative analysis

The software Quantity One (Bio Rad), was used to analyze the intensity of the bands obtained after western immunoblotting.

4 Results

Both endothelial dysfunction and changes in ECM are important in the pathogenesis of diabetes. To study the mechanisms behind diabetic complications, HUVECs were used as a model system for the ubiquitous endothelial cells. Hyperglycemia and proinflammatory cytokine IL-1β, both landmarks of the pathogenesis of diabetes, were used as agents to mimic the diabetic state. The widespread and diverse PGs, molecules of the ECM, are related to the endothelium and changes in their structure and/or distribution are observed in atherosclerosis, retinopathy, nephropathy and neuropathy, the most common DM complications. In order to study the implication of PGs in diabetes in an experimental system HUVEC were exposed to hyperglycemia and inflammatory conditions, and secretion and structure of PGs expressed by endothelial cells were investigated.

Both polarized HUVEC and cells cultured in conventional cell culture flasks were stimulated with 0.5 ng/mL IL-1β, high glucose (25 mM) or both for 24 hours. Cells cultured in low glucose (5 mM) were used as a control. The stimulations were performed in MCDB-medium with FCS reduced to 2 %.

4.1 The effect of hyperglycemia and IL-1β on the total proteoglycan synthesis and secretion

A pilot study was performed to investigate whether the PG synthesis was affected by hyperglycemia and/or the inflammatory agent IL-1β. Biosynthetic labeling with ^{35}S-sulfate was performed to study *de novo* synthesis of sulfated macromolecules like PGs. The amount of newly synthesized PGs was determined after harvesting medium and cell fractions and subjecting the fractions to gel chromatography.

The *de novo* ^{35}S-PG synthesis was increased after all stimulations compared to control (**figure 4.1**). The cpm values show that the PGs are mainly secreted and not stored intracellularly.

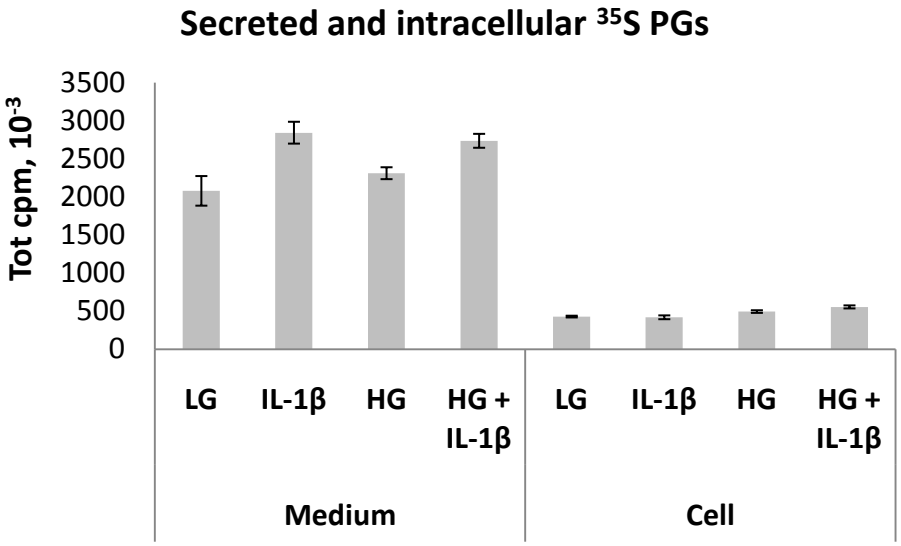

Figure 4.1: Cell-associated and secreted ^{35}S-PGs. HUVEC were cultured and labeled with ^{35}S-Sulfate for 24 hours, in medium with low glucose (control), IL-1β, high glucose (HG) or IL-1β and HG together. ^{35}S-PGs were assessed by scintillation counting after G-50 fine gel filtration.

Medium samples were subjected to either cABC digestion or HNO_2 treatment and separated by SDS-PAGE. The secreted ^{35}S-labeled macromolecules were clearly separated into three parts of ~100, ~220 and > ~250 kDa size (**figure 4.2**). As described in chapter 3.8, cABC treatment depolymerizes CS/DS-GAGs, and HNO_2 degrade heparin and HS-GAGs. These experiments revealed that treatment with HNO_2 completely degraded the macromolecules of > ~250 kDa, indicating that these ^{35}S-macromolecule fractions were composed of HS-PGs (**figure 4.2, lane 3, 6, 9 and 12**). In contrast, the two fractions of ~100 and ~220 kDa were not affected by the HNO_2 treatment, but were completely degraded by cABC treatment (**figure 4.2, lane 2, 5, 8 and 11**). This indicates that these bands of ^{35}S-macromolecules were also PGs, though substituted with CS/DS-GAGs. Neither this pattern, nor the size of the PG fractions, were affected by stimulations by IL-1β (lane 4-6), high glucose (lane 7-9) or high glucose and IL-1β together (lane 10-12).

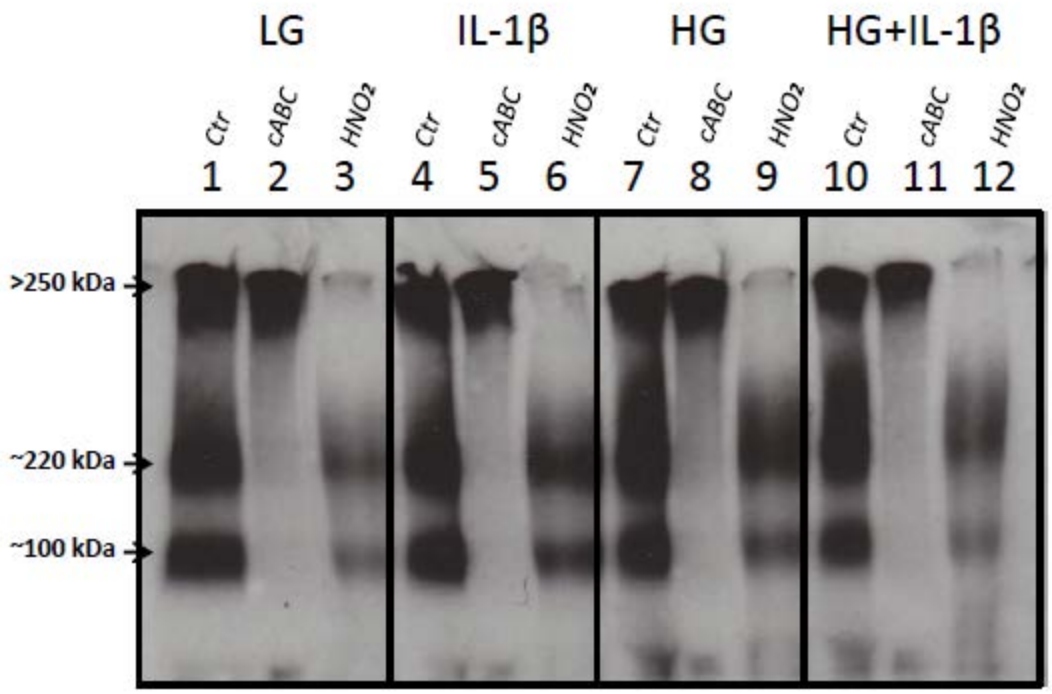

Figure 4.2: SDS-PAGE of secreted ^{35}S-PGs, treated with cABC and HNO_2. HUVEC were cultured and labeled with 35S-Sulfate for 24 hours, in low glucose (control), IL-1β, high glucose (HG) or IL-1β and HG together. Samples were subjected to gel chromatography followed by cABC treatment to degrade CS/DS, HNO_2 to degrade HS or no treatment (Ctr). The samples were finally analyzed by SDS-PAGE and autoradiography. The migration positions of molecular mass markers (in kDa) are shown at the left with arrows.

In summary, these data indicate that HUVEC synthesize large amounts of PGs and that the PGs are mainly secreted. High glucose and IL-1β increase the PG synthesis and secretion. In the cell fraction, a minor increase in the ^{35}S-PG level could be observed after exposure to hyperglycemia with or without IL-1β.

To investigate these results in more detail, we next wanted to analyze the secretion pattern of specific PGs.

4.2 Effect of hyperglycemia and IL-1β on proteoglycan secretion in polarized HUVEC

Pilot studies were performed on HUVEC cultured on conventional plastic. However, a more *in vivo* like approach would be to culture the cells on semipermeable filters, allowing them to polarize. By analysis of the apical and basolateral medium respectively, differences in the sorting of secreted PGs could be investigated.

The protein concentrations of the cell lysates after the various stimulations were determined, and the amount of medium analyzed was adjusted according to the protein concentrations.

The HUVEC were cultured with medium with IL-1β with or without high glucose and high glucose alone. The secretion of different specific PGs was studied using western blotting. The gels were incubated with coomassie blue, which determined that the proteins were completely transferred from the gel to the membrane. The HS-PGs perlecan and collagen XVIII, and the CS/DS-PGs decorin and biglycan, were detected and analyzed. These PGs were chosen because of their location in the BM and loose connective tissue and because of possible changes during diabetic complications. Perlecan and collagen XVIII are important constituents of the BM, important in both glomerular filtration and atherosclerosis because of their HS-GAG chains and their high binding capacity [1, 108]. The SLRPs decorin and biglycan are constituents of loose connective tissue, and are thought to play a role in the fibrosis and inflammatory reactions [2].

The blots were quantified and changes in PG expression and secretion was determined. The results obtained revealed biological differences between different HUVEC donors, giving rise to variations between the different experiments using cells originating from different umbilical cords.

4.2.1 Perlecan

Perlecan is a basement membrane HS-PG with a molecular size of ~800 kDa. The perlecan secretion in polarized HUVEC was assessed by western blotting. **Figure 4.3A** shows one of three perlecan western blots. The intensity of the protein bands of all three blots were quantified and presented in **figure 4.3B-D.** The total secretion of perlecan was increased after all stimulations; IL-1β resulted in a 68 ± 37 % increase, hyperglycemia 80 ± 55 % and

hyperglycemia together with IL-1β increased the perlecan secretion by 94 ± 62 % (**figure 4.3B**). The total increase in secretion can be related to increased levels of both apical and basolateral secretion, where the most prominent increase was observed to the apical side (**figure 4.3C and D**). Hyperglycemia led to the most prominent increase apically, whereas IL-1β alone or together with high glucose, gave the most prominent increase basolaterally. However, at the basal level (LG) the basolateral secretion of perlecan by far exceeded the apical secretion (**figure 4.3A and 4.4**)

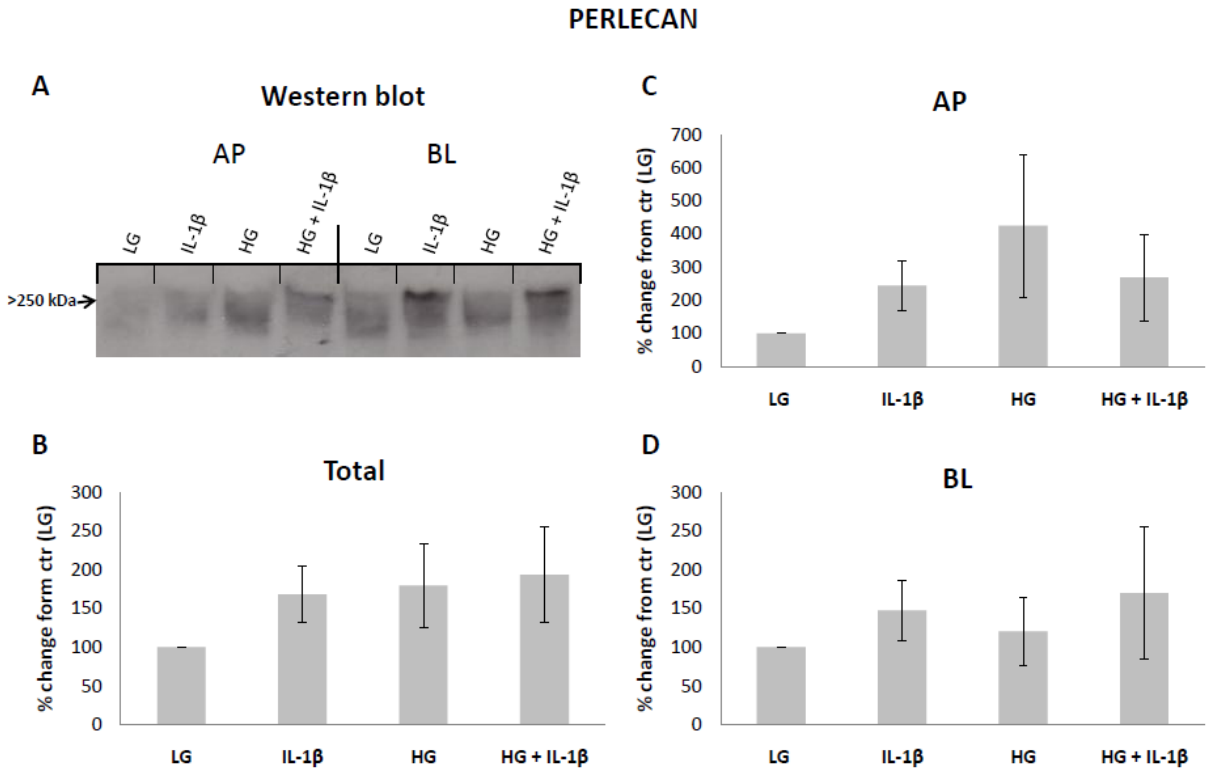

Figure 4.3A-D: Western blot of secreted perlecan in apical (AP) and basolateral (BL) medium from HUVEC exposed to IL-1β, high glucose (HG) and HG and IL-1β for 24 hours (A). The figures B-D are based on quantitation of western blots on material from three different individuals and are presented as % of control (low glucose, LG) with error bars. B: Total secretion, C: Apical secretion, D: Basolateral secretion.

In the control, 77 ± 8.3 % of the perlecan was secreted to the basolateral side. This is illustrated by **figure 4.4**, along with changes in this pattern caused by the various stimulations. An increase in the ratio between the apical and the basolateral secretion was seen for all stimulations. Hyperglycemia alone gave the most prominent change, observed as an almost 50/50 ratio, with a percentage secretion of 48 ± 9.4 % apically and 52 ± 9.4 % basolaterally.

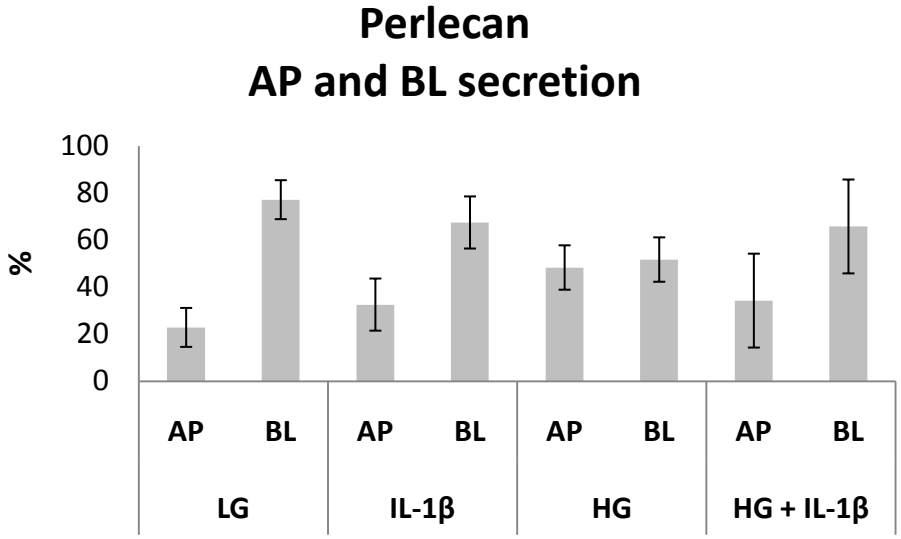

Figure 4.4: Apical (AP) and basolateral (BL) secretion of perlecan after stimulation with IL-1β, high glucose (HG) and both HG and IL-1β for 24 hours. The data are based on quantitation of three experiments and presented as mean with error bars. The values are given as % secretion to the AP or BL side, with the total secretion set to 100 %.

4.2.2 Collagen XVIII

Collagen XVIII is a 300 kDa HS-PG mainly located to the BM. The collagen XVIII secretion in polarized HUVEC was assessed by western blotting. **Figure 4.5A** shows one of two collagen XVIII western blots. The intensity of the protein bands of both blots were quantified and presented in **figure 4.5B-D**. **Figure 4.5B** shows the total secretion of collagen XVIII, which was slightly increased after stimulation with IL-1β (10 ± 3.5 %) and hyperglycemia (19 ± 24.3 %). However, hyperglycemia and IL-1β together reduced the secretion by 34 ± 34.1 %. This reduction could be caused by a major reduction in the basolateral secretion (59 ± 8.7 %) after this stimulation (**figure 4.5D**). On the other hand, the increase in total secretion after IL-1β and high glucose stimulation was related to an increase in apical secretion (**figure 4.5C**), while the basolateral secretion was somewhat reduced (**figure 4.5D**).

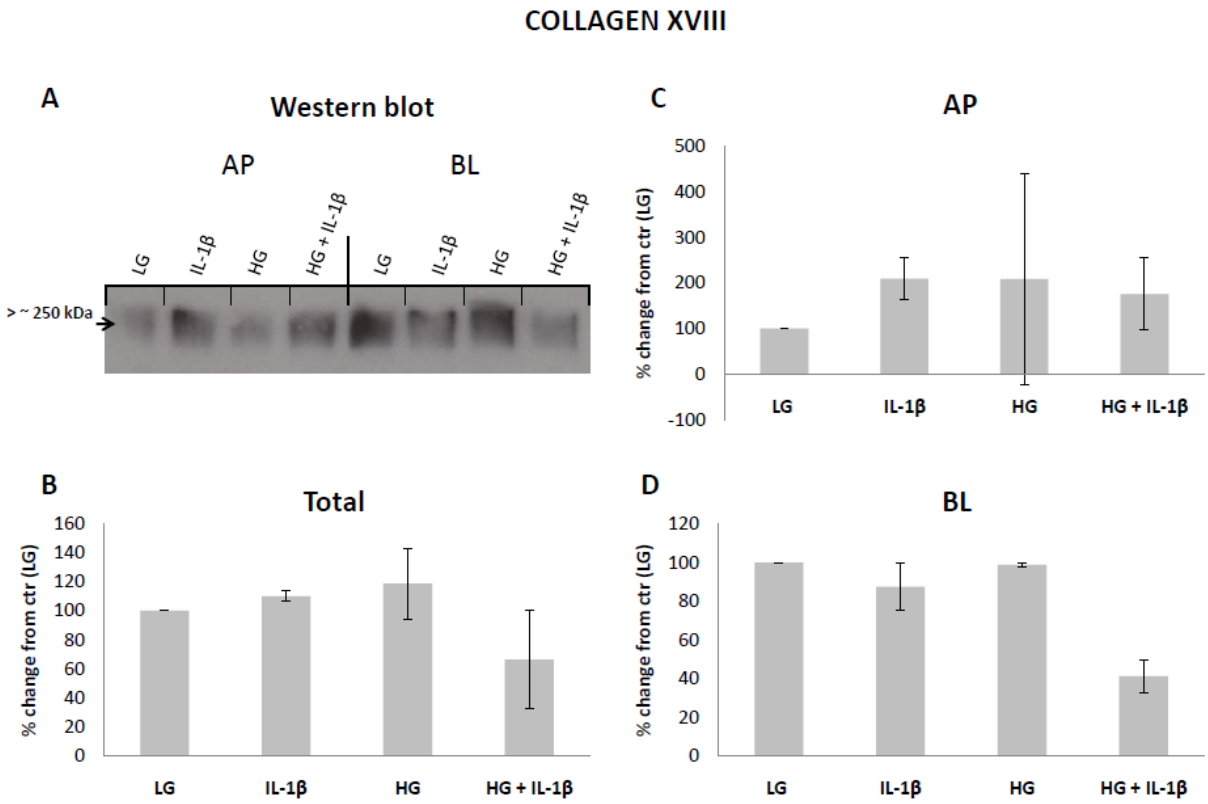

Figure 4.5A-D: Western blot of secreted collagen XVIII in apical (AP) and basolateral (BL) medium from HUVEC exposed to IL-1β, high glucose (HG) and HG and IL-1β for 24 hours (A). The figures B-D are based on quantitation of western blots on material from two different individuals and are presented as % of control (low glucose, LG) with error bars. B: Total secretion, C: Apical secretion, D: Basolateral secretion.

Collagen XVIII was mainly secreted to the basolateral medium as shown in **figure 4.6**. As seen from this figure, all stimulations increased the ratio, i.e. by increasing the apical secretion and decreasing the basolateral secretion. At baseline the basolateral secretion was 81 ± 10.9 %, altered to 57 ± 25 % after stimulation with both hyperglycemia and IL-1β. The apical secretion was altered from 19 ± 10.9 % at baseline to 43 ± 25 % after stimulation with both hyperglycemia and IL-1β.

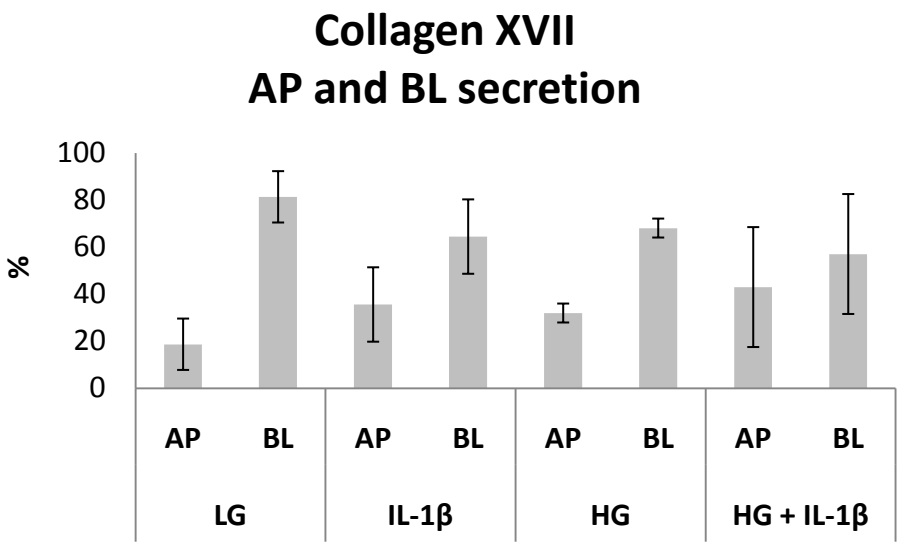

Figure 4.6: Apical (AP) and basolateral (BL) secretion of collagen XVIII after stimulation with IL-1β, high glucose (HG) and both HG and IL-1β for 24 hours. The data are based on quantification of two experiments and presented as mean with error bars. The values are given as % secretion to the AP or BL side, with the total secretion set to 100 %.

4.2.3 Decorin

Decorin is an ECM PG with a core protein of 39 kDa and one CS- or DS-GAG chain attached near the N-terminal. The intact PG is around 100 kDa in molecular weight. The decorin secretion from polarized HUVEC was assessed by western blotting. **Figure 4.7A** shows one of three decorin western blots, and the intensity of the protein bands of all blots were quantified and shown in **figure 4.5B-D**. The total secretion of decorin was increased by 50 ± 70 % after stimulation with IL-1β. Hyperglycemia with or without IL-1β decreased the total secretion of decorin by respectively 18 ± 31 % and 15 ± 25 % (**figure 4.7B**). The total increase in secretion by IL-1β can be related to the basolateral secretion, and possibly by the apical secretion as well (**figure 4.7C and D**). The variations in the response in apical secretion between HUVEC from different donors were too large to show a clear trend.

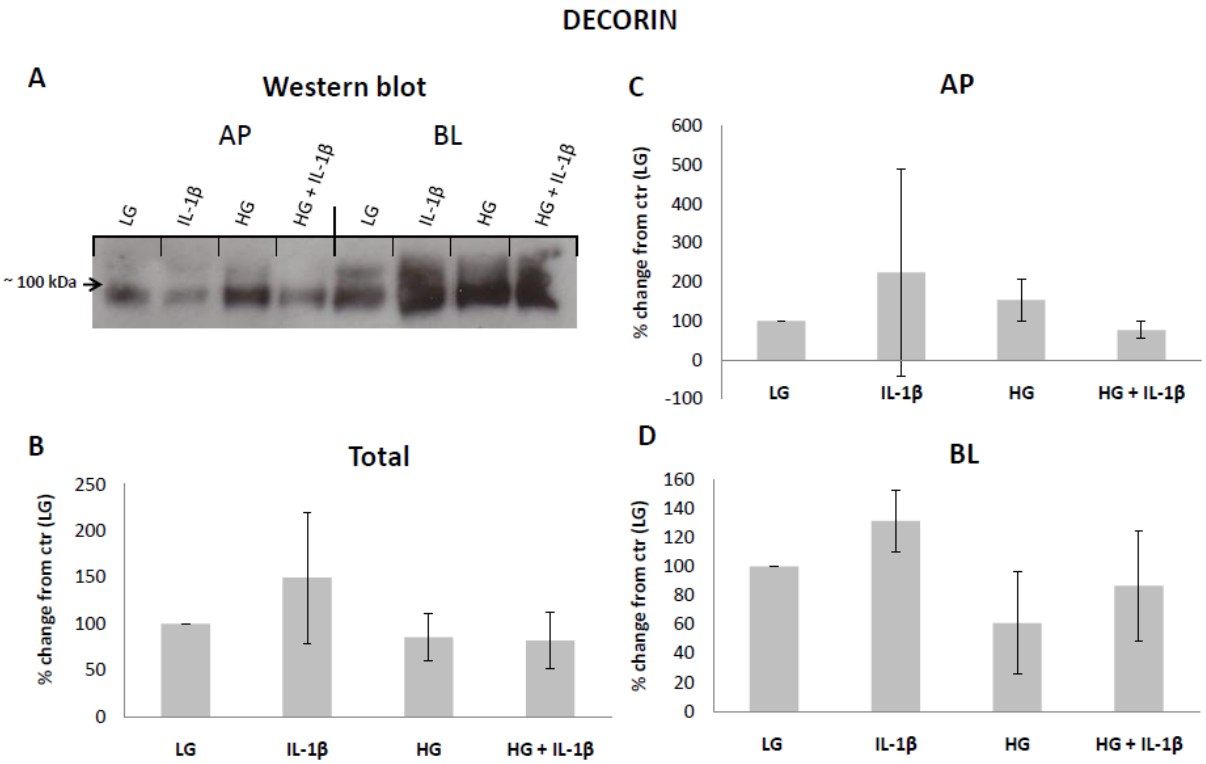

Figure 4.7A-D: Western blot of secreted decorin in apical (AP) and basolateral (BL) medium from HUVEC exposed to IL-1β, high glucose (HG) and HG and IL-1β for 24 hours (A). The figures B-D are based on quantiation of western blots on material from three different individuals and are presented as % of control (low glucose, LG) with error bars. B: Total secretion, C: Apical secretion, D: Basolateral secretion.

Figure 4.8 show that decorin is mainly secreted to the basolateral medium at baseline (67 ± 14.2 %). There were only small differences observed after stimulations with IL-1β alone or together with high glucose. In contrast, hyperglycemia alone altered the secretion ratio substantially i.e. 57 ± 17 % to the apical side and 43 ± 17 % to the basolateral side.

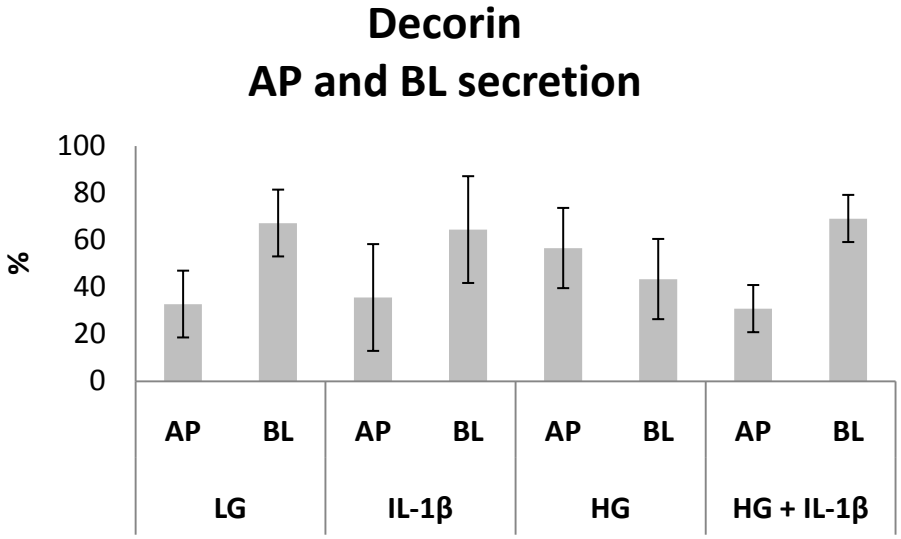

Figure 4.8: Apical (AP) and basolateral (BL) secretion of decorin after stimulation with IL-1β, high glucose (HG) and both HG and IL-1β for 24 hours. The data are based on quantitation of three experiments and presented as mean with error bars. The values are given as % secretion to the AP or BL side, with the total secretion set to 100 %.

4.2.4 Biglycan

Biglycan carries two GAG chains near the N-terminus, and the molecular size is around 100 kDa. The biglycan secretion in polarized HUVEC was assessed by western blotting. **Figure 4.9A** shows one of two biglycan western blots, where the intensity of the protein bands of both blots were quantified and presented in **figure 4.9B-D**. The total secretion of biglycan was increased by 23 ± 32.4 % after stimulation with IL-1β together with high glucose. The two stimulations separately gave a reduction of 11 ± 63 % for IL-1β and 6 ± 0.3 % for high glucose (**figure 4.9B**). The total increase in biglycan expression after stimulation with high glucose and IL-1β together can be related to both increased apical (24 ± 70.8 %) and basolateral (15 ± 2.2 %) secretion, as shown in **figure 4.9C and D**. However, the variation in response in the apical secretion does not allow any conclusions. IL-1β alone increased the apical secretion and reduced it to the basolateral side. This is in contrast with the effect of high glucose, which increased the basolateral secretion and reduced the apical secretion.

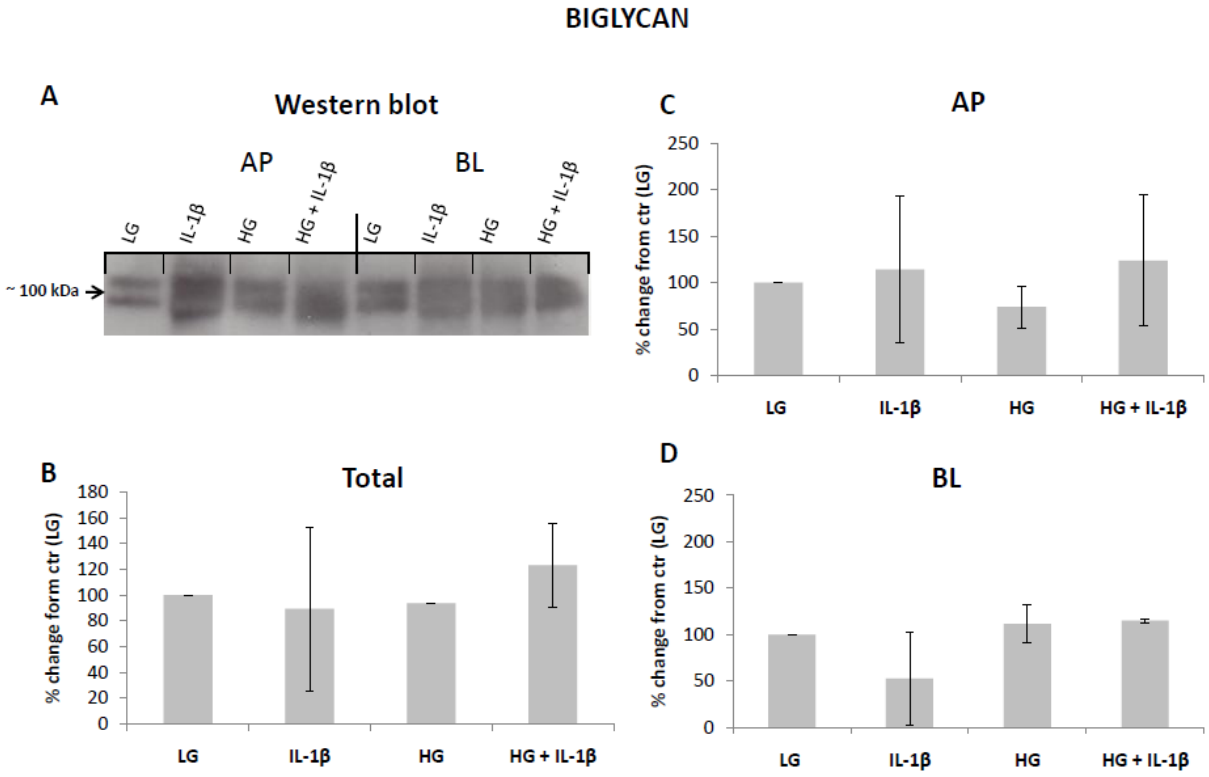

Figure 4.9: Western blot of secreted biglycan in apical (AP) and basolateral (BL) medium from HUVEC exposed to IL-1β, high glucose (HG) and HG + IL-1β for 24 hours (A). The figures B-D are based on quantitation of two experiments and presented as % of control (low glucose, LG) with error bars. B: Total secretion, C: Apical secretion, D: Basolateral secretion.

The secretion pattern of biglycan was different from the other PGs studied. At baseline 48 ± 0.4 % was recovered in the apical medium and 52 ± 0.4 % in the basolateral medium, which was almost a 50/50 ratio (**figure 4.10**). In contrast, the other PGs have mainly been secreted to the basolateral medium at baseline. IL-1β alone increased the apical to basolateral secretion ratio, while high glucose had the opposite effect, decreasing the ratio. When hyperglycemia and IL-1β were administered together, these opposite effects compensated each other, and resulted in no net effect.

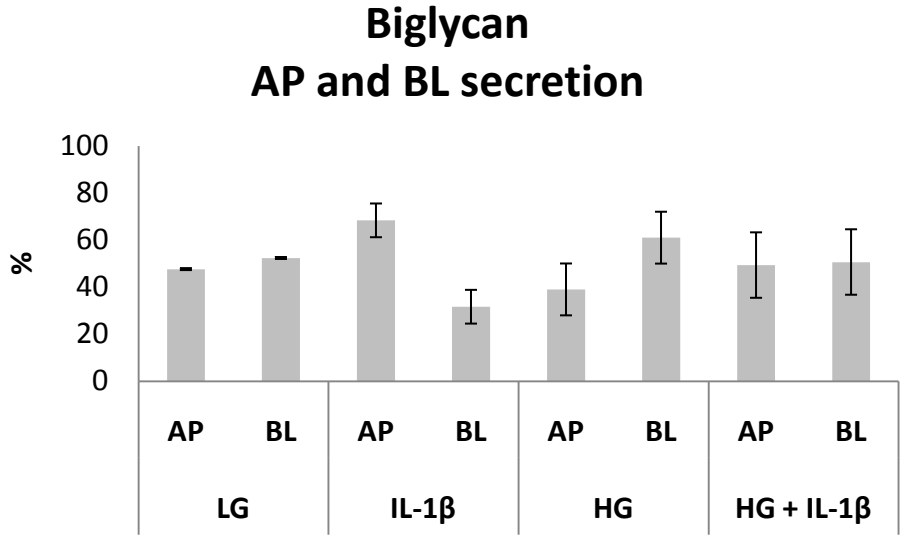

Figure 4.10: Apical (AP) and basolateral (BL) secretion of biglycan after stimulation with IL-1β, high glucose (HG) and both HG and IL-1β for 24 hours. The data are based on quantitation of three experiments and presented as mean with error bars. The values are given as % secretion to the AP or BL side, with the total secretions of all stimulations set to 100 %.

4.3 Total effect of different stimulations

The total effect of each stimulator on the expression of the different PGs was compared.

IL-1β is a proinflammatory cytokine, and increased the total secretion of perlecan (68 ± 36.8 %), decorin (50 ± 70.7 %) and collagen XVIII (10 ± 3.5 %), while it reduced the secretion of biglycan (11 ± 63.5 %), as illustrated in **figure 4.11A**. High glucose increased the total secretion of the HS-PGs perlecan (80 ± 55 %) and collagen XVIII (19 ± 24.3 %), while it reduced the total secretion of the CS/DS-PGs decorin (15 ± 25 %) and biglycan (6 ± 0.3 %) (**figure 4.11B**). High glucose and IL-1β together resulted in additive increase of perlecan by 94 ± 62 %, and biglycan levels was increased by 23 ± 32.4 %. Collagen XVIII and decorin expression, on the other hand, was reduced by 34 ± 34.1 % and 18.2 ± 31 %, as illustrated in **figure 4.11C**.

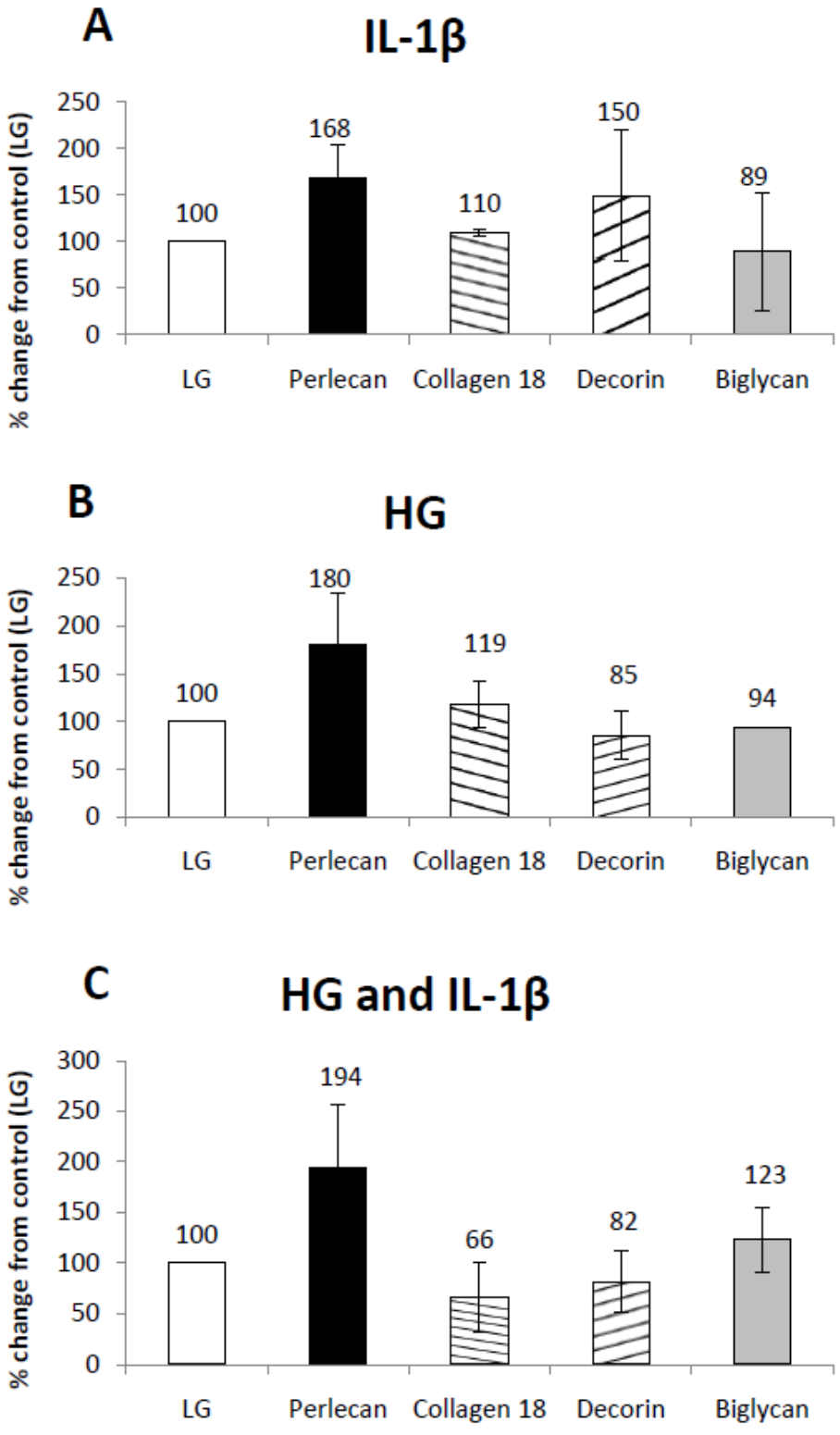

Figure 4.11: Total secretion of perlecan, collagen XVIII, decorin and biglycan in HUVEC exposed to IL-1β (0.5ng/mL), high glucose (25 mM) or both for 24 hours. The data are based on quantification of two or three western blots, and presented as % of control (low glucose, LG) with error bars.

4.4 Cellular permeability

HUVEC were cultured to confluence on semipermeable filter well inserts in MCDB-medium with 7 % FCS. Medium from both apical and basolateral compartments were discarded and new medium containing FITC-albumin (3.5 mg/mL) was applied to the apical medium. Medium without FITC was applied basolaterally and the cells were incubated in 45 minutes. The apical and basolateral media were harvested and fluorescence was measured in both medium fractions. The same experiment was performed in wells without cells. In empty wells, the fluorescent FITC-labeled albumin was found both in apical (65.6 ± 1.5 %) and basolateral medium (34.4 ± 1.5 %) (**figure 4.12A**). In wells with confluent cell layer, the apical fluorescence was increased to 77.6 ± 4.7 % and the basolateral fluorescence reduced to 22.4 ± 4.7 %, as illustrated in **figure 4.12B**. This indicated that less FITC-albumin had crossed the cell layer and that the cell layer was confluent.

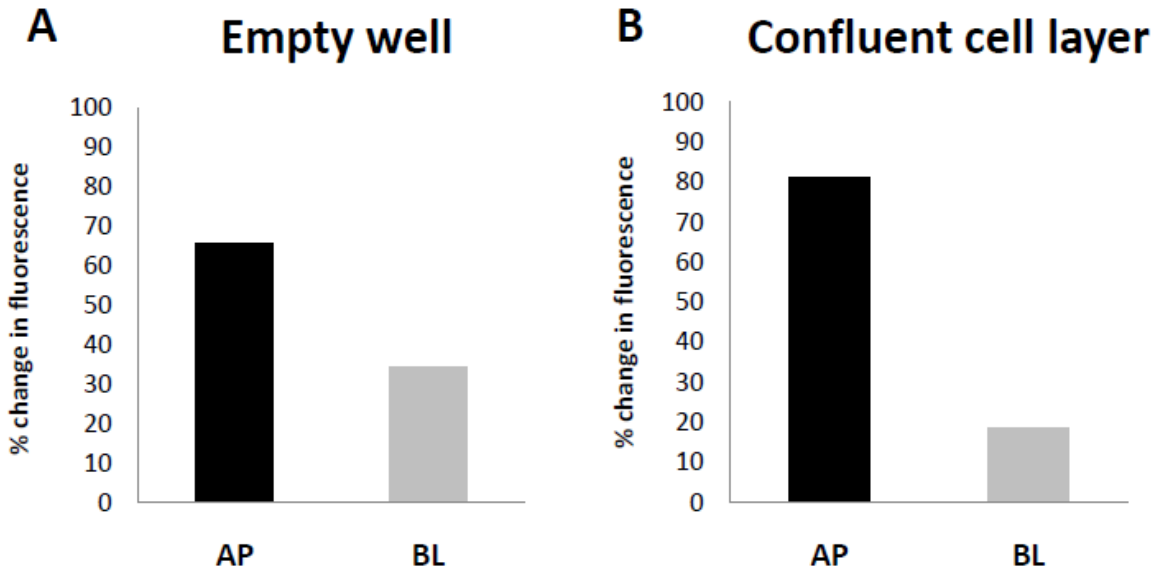

Figure 4.12: FITC-albumin permeability assay. Fluorescence of FITC-albumin measured from apical (AP) and basolateral (BL) medium from empty wells (A) and wells with confluent cell layer (B).

HUVEC were also exposed to IL-1β (0.5ng/mL), high glucose (25 mM) or both for 24 hours in MCDB-medium with 2 % FCS, after confluent cell layer was achieved. After incubation, the medium were harvested and new medium with FITC-albumin was applied to the apical

compartment and new medium without FITC was applied in the basolateral compartment. After 45 minutes incubation the medium from both fractions were harvested, and fluorescence was measured. The results are illustrated in **figure 4.13**.

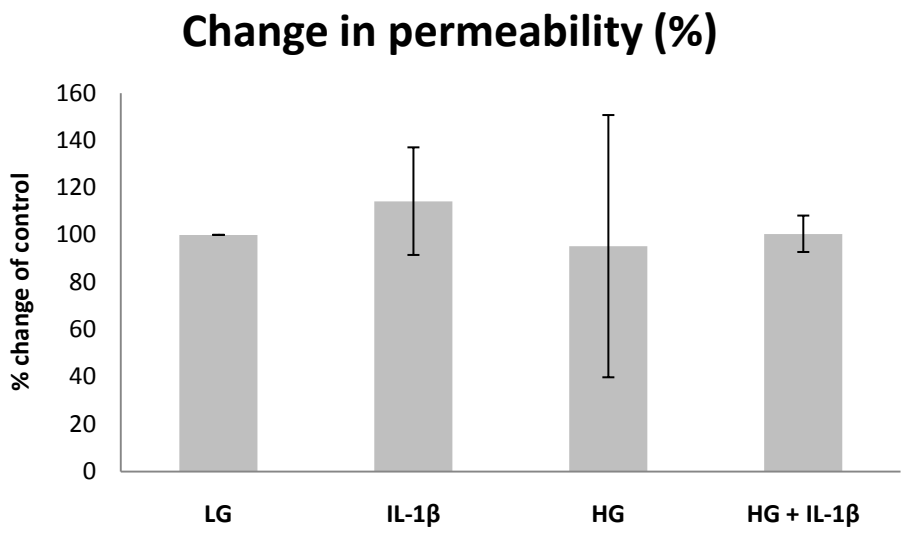

Figure 4.13: Differences in fluorescence after different stimulations. HUVEC were grown on semipermeable filter well inserts until confluence. The cells were stimulated with IL-1β, high glucose (HG) and IL-1β and HG in 24 hours. Low glucose (LG) was used as a control.

Compared to control (low glucose), stimulation with the proinflammatory agent IL-1β resulted in a 14.3 ± 22.7 % increase in permeability. After exposing cells to high glucose, the fluorescence was reduced by 5.2 ± 55.4 %. Whit high glucose and IL-1β stimulation simultaneously, the fluorescence increased by 0.5 ± 7.7 %.

5 Discussion

Long-term micro- and macrovascular complications are the principal cause of morbidity and mortality among diabetic subjects [32, 33]. Because of the high prevalence of DM, these complications amount to significant national health problems. Large clinical trials have demonstrated that normalization of the hyperglycemia can greatly reduce the incidence of DM complications [34, 35], but this is often difficult to obtain in practice. It is therefore relevant to study the mechanisms behind hyperglycemia and DM complications, which can lead to new therapeutic approaches and prevention of the disease.

The endothelium is the prime organ to be exposed to hyperglycemia, leading to endothelial dysfunction and inflammation [29]. Both in the luminal endothelial vessel wall and in the underlying BM, the PGs have important functions. In diabetes, alterations in both expression and structure of the PGs are observed, and several studies indicate that these changes are linked to DM complications [1, 2].

In order to study this altered endothelial PG expression HUVEC were cultured *in vitro* as a model system of the endothelium. To mimic the diabetic environment in an experimental system the cells were exposed to hyperglycemia and the inflammatory mediator IL-1β. Hyperglycemia and IL-1β have previously been shown to affect HUVECs and their expression of PGs [113]. By culturing HUVEC on semipermeable filters, the cells polarize and basolateral and apical secretory products can be analyzed separately. We wanted to study the secretion of different specific PGs. Immunoblotting was used to detect and quantitate the HS-PGs perlecan and collagen XVIII, and the CS/DS-PGs decorin and biglycan. These PGs were chosen because of their location and possible functions in diabetic complications. Perlecan and collagen XVIII are important constituents of the BM, important in both glomerular filtration and atherosclerosis [1, 108]. The SLRPs decorin and biglycan are constituents of loose connective tissue, and are thought to play a role in fibrosis and inflammatory reactions [2].

5.1 Major findings

In this thesis we found that HUVEC synthesize large amounts of PGs and that the PGs are mainly secretory products.

We also found that the ECM PGs perlecan, collagen XVIII and decorin were mainly secreted to the basolateral medium. Biglycan was an exception with equal secretion to the apical and basolateral medium.

The secretion of the HS-PGs perlecan and collagen XVIII were increased after stimulation with IL-1β and hyperglycemia. Perlecan secretion also increased after exposure to IL-1β along with hyperglycemia, whereas collagen XVIII secretion was decreased under this condition. As for perlecan and collagen XVIII, decorin was increased after stimulation with IL-1β, while biglycan was increased after exposure to hyperglycemia and IL-1β together. The experiments of biglycan need to be repeated because of conflicting results and considerable differences between the separate experiments.

In summary, the secretion of PGs to the basolateral and apical media are affected, although differently by hyperglycemia with or without inflammatory stimuli.

5.2 Methodological considerations

- We used primary HUVECs in our experiments. HUVEC are often used in *in vitro* experiments, making our results directly comparable with other studies using such cells. The umbilical cords are no longer needed after birth and are normally discarded. The isolation of HUVECs from these umbilical cords are therefore a non-invasive method that do not require any sacrifice or suffering from the donors.

- Human primary cells in culture closely mimic the *in vivo* state and generate more physiologically relevant data compared to endothelial cell lines. Primary ECs from different donors may have both different genotypes and phenotypes. This can result in extensive variations in response to stimuli [120]. Even tough the ECs are obtained from the same vascular tissue, phenotypic variations between different portions of the vasculature may occur. Generalization of the data obtained should therefore be done with caution. However, use of HUVEC to study endothelial cell biology will provide useful novel insight [17, 121].

- By culturing HUVEC on semipermeable filters, the cells polarize and we obtain a more *in vivo* like morphology, which is an improvement compared to cells cultured on conventional plastic. The secretory products of confluent polarized cells are separated

in the apical and the basolateral media. Alterations in the secretion pattern are potentially relevant to the *in vivo* situation and endothelial pathobiology. Using polarized HUVEC should be of interest in future studies on endothelial dysfunction and diabetes.

- The diagnostic level of DM is fasting blood glucose ≥ 7 mmol/l and/or blood glucose level ≥ 11.1 mmol/l two hours after 75 g oral glucose load. Randomly (not fasting) blood glucose ≥ 11.1 mmol/l in combination with symptoms is also used as a diagnostic criteria [4]. 25 mM glucose is a common concentration used when mimicking hyperglycemia in short-term cell culture experiments [122, 123]. This concentration is higher than 11 mM, but in humans with diabetes the blood glucose concentration is fluctuating throughout the day and it can potentially reach > 25 mM. When incubated for 24 hours the 25 mM glucose load can be reduced in the cell culture system through cellular metabolism. Also, *in vitro* experiments often require higher exposure than *in vivo* exposure, in order to show the effects experienced *in vivo* in short-term experiments.

- Western blotting has certain limitations as a quantitative method, but can be used to demonstrate changes and trends. In SDS-PAGE, small proteins may pass through the whole gel if the time is overrun. After the SDS-PAGE is run, the proteins captured in the gel may diffuse out of the gel if not western blotting is performed immediately after SDS-PAGE. It is also some disadvantages by the software Quantity One, where the background can affect the measurement of the intensity of the protein bands. All this may affect the accuracy of the data. One of the benefits of using western blotting is that the results are related to the protein level, which is the final and crucial product in the protein synthesis in contrast to e.g. mRNA determination.

- The HNO_2 reagent, used to depolymerise HS and heparin, has a low pH at 1.5 which can denature proteins. The enzyme heparitinase could be preferred as a more gentle treatment, compared with HNO_2-treatment. The two analyzing tools have approximately the same HS/heparin cleavage characteristics, but the heparitinase analysis is more expensive and time-consuming.

- The results in this thesis show biological natural occurring differences, and the variation in response does not always allow any conclusions. If the number of subjects

was higher, it would be relevant to test the results by further statistical analysis. Due to the low number of subjects, eventually stasticial significant values would somehow not be of great importance. Conclusions of the normal distribution of the results is also difficult to obtain because of the few donors.

5.3 General discussion

5.3.1 Total proteoglycan synthesis

We found that HUVEC synthesize PGs under normoglycemic conditions and that the PGs are mainly secretory products *in vitro*. These results were obtained by labeling with ^{35}S-sulfate, to study *de novo* synthesis of sulfated macromolecules like PGs. When separated by SDS-PAGE, three PG populations of ~100, ~220 and > ~250 kDa were detected. When HUVEC were exposed to IL-1β, high glucose and IL-1β and high glucose together, the total secretion of PGs was increased. The stimulations did not affect the expression of PGs in either size or GAG-chain assembly. This result is in accordance with our previously obtained data in the study of Meen et al. [113].

PGs have previously been known to be secretory products from vascular ECs [124]. Shick B.P et al. found that the majority of PGs are secreted and not stored intracellularly [125], consistent both with our previous results [113] and the data presented in this thesis. The normal expression and secretion pattern of specific PGs in ECs is still known only to a limited extent.

5.3.2 Secretion of specific proteoglycans from polarized HUVEC

In this thesis we found that perlecan, collagen XVIII, decorin and biglycan were secreted from HUVEC. The cell fractions have therefore not been studied in this work. By culturing the cells on semipermeable filter, the HUVEC polarize and secretion products from apical and basolateral medium can be analyzed. The HS-PGs can be found in both the apical and basolateral medium of the ECs. Apically they can be bound to the cell surface possibly as syndecans and glypicans [76], while in the basolateral medium BM and ECM HS-PGs like perlecan and collagen XVIII dominate [62]. In this study we have shown that the HS-PGs

perlecan and collagen XVIII are predominantly secreted to the basolateral side, which is consistent with their location in the BM. Decorin was also found to be secreted mainly to the basolateral side, while in contrast biglycan was found to be secreted equally to both medium fractions. Both decorin and biglycan are CS/DS-PGs and known constituents of the ECM. They would therefore be hypothesized to mainly be secreted to the basolateral medium, like the other ECM PGs. Biglycan has immunological functions that can explain the somewhat different secretion pattern. Immunologically important proteins are transported by the blood stream to the site of injury or inflammation, and the apical sorting route in the ECs is thought to be the shortest way to the blood stream. The higher apical secretion of biglycan could consequently be related to inflammation. Previous studies have demonstrated that HUVEC secrete several PG species, either with HS- or CS/DS-GAG chains [113, 123]. However, a comparison of the regulation of apical and basolateral secretion has not been the subject of these studies. Studies of cells from other species are therefore included in the discussion although direct comparable conclusions not can be undertaken. A study on apical and basolateral secretion of PGs in Madin-Darby Canine Kidney (MDCK) cells, found that more extensively sulfated PGs were secreted to the basolateral medium than to the apical medium, indicating that the degree of sulfation may be a regulatory factor in the sorting of PGs [126]. Both MDCK I and II cells have previously been found to express perlecan, where the majority could be recovered from the basolateral medium [127]. Previous findings in HUVECs have found that serglycin is predominantly secreted to the apical side, while perlecan and decorin are predominantly secreted to the basolateral side [113]. The results in this thesis are therefore consistent with previous findings in HUVEC, regarding perlecan and decorin [113].

The possible cellular mechanisms that determines the sorting of PGs, are not known. As already mentioned, degree of sulfation can be one possible regulating factor. It is also suggested that glycosylation affect the secretion pattern. Secretion of decorin is shown to be altered if the *N*-linked glycans are removed in *spodoptera frugiperda 21* (Sf21) insect cells [86] and in Chinese hamster ovary (CHO) cell lines deficient in specific glycosylation steps [75]. Structures in the core protein may also affect the sorting. A study has shown that a globular domain in the core protein of aggrecan, affect GAG modification and secretion [128]. In MDCK cells, the dependence on pH in seceretory vesicles was suggested as a possible sorting mechanism [129]. Mores studies are needed to determine regulatory mechanisms of the PG-sorting. Alterations in the secretion pattern after different stimulatory mediators can be of great relevance for pathological disorders. We have therefore further

investigated if the secretion patterns were affected after stimulation with high glucose and/or IL-1β.

5.3.3 Effect of high glucose and IL-1β on proteoglycan synthesis on polarized HUVEC

Perlecan

Perlecan is an ECM HS-PG with a molecular size of ~800 kDa. The importance of the HS-PGs in the BM has been highlighted, and changes in HS-PG biosynthesis and turnover will affect their functions. In this thesis we have shown that perlecan was increased after all stimulations. This is in contrast with previous studies, e.g. by a study that observed significant decreased amounts of perlecan core protein after stimulation with high glucose in renal parenchymal cells [130]. Another study found no change in perlecan synthesis, when human aortic endothelial cell (HAEC) line was exposed to high glucose [131]. Reduced sulfation of the secreted HS-GAGs of perlecans was found in these HAEC, which in contrast not was found in perlecan secreted from SMCs. The effect of hyperglycemia of perlecan synthesis is possibly different in different cell types. Changes in the HS-GAG composition is relevant in the BM or subendothelial space of the arteries, the main location of lipid accumulation in atherosclerosis [132]. Whether this reduction of sulfation is caused by a reduced number of GAGs, reduced length of the GAGs, alterations of HS-GAGs to CS/DS-GAG ratio or reduced amount of the HS-PGs is not known. But regardless of the reason, an altered structure of the perlecan HS-GAGs can cause disruption in protein binding capacity and other functions of the PG. One study found that hyperglycemia affect the binding of monocytes to the sub-endothelial space, and that this increase was due to reduced sulfation of the HS-PG perlecan [133]. In the kidneys, studies have shown the importance of HS-GAGs in the GBM as a negative charge barrier, and reduction of HS-GAGs leads to loss of negative charge [108]. This may be one of the mechanisms resulting in impaired glomerular filtration capacity and to kidney dysfunction, clinically manifested as microalbuminuria [134]. In streptozotocin (STZ) rats the total PG content constitute of 20 % CS/DS-GAGs and approximately 80 % HS-GAGs in kidney. This study found that total sulfation was reduced by 12 %, and that CS/DS was found to be more affected than HS [67]. This indicates that the HS-PGs may not be that important in the kidneys as hypothesized. However, differences in PG composition and

synthesis are somehow affected by a diabetic environment, but it is still need for further studies to evaluate the mechanisms involving perlcan in DM complications.

Collagen XVIII

Collagen XVIII is a ~300 kDa HS-PG mainly located to the BM. A study detected collagen XVIII as an L-selectin binding HS-PG in distal tubuli in rat kidney [135]. L-selectin is expressed on neutrophils and monocytes, leading to the hypothesis that collagen XVIII is involved in leukocyte migration into inflammatory tissue, and that collagen XVII may be upregulated in inflammatory conditions. The secretion of the PG was increased after stimulation with IL-1β in this thesis, but together with hyperglycemia the secretion was reduced. Hyperglycemia is associated with increased antiangiogenic signaling. In this thesis hyperglycemia increased the secretion of collagen XVIII. Collagen XVIII is a precursor to the antiangiogenic molecule Endostatin, cleaved and released from the intact collagen XVIII protein. In DM patients with coronary artery disease, endostatin is reported to be increased in myocardial tissue, positively correlated with blood glucose level [136]. The study did not show increased expression of the protein level of collagen XVIII, inconsistent with our results. MMPs and other ECM degrading molecules cleave collagen XVIII resulting in release of endostatin. An upregulation of one or more of these molecules could be a possible mechanism behind the increased endostatin level. The formation of coronary collateralization is an important survival benefit for patients suffering of coronary artery disease. A possible reduction in neovascularization due to increased endostatin level in coronary artery disease, may cause increased mortality risk of DM patients suffering from the disease. DM retinopathy is associated with increased neovascularization, and increased levels of endostatin is found in the vitreous significantly correlated with severity of the disease [137]. Used as a therapeutic treatment in STZ mice with DM nephropathy, endostatin prevented progression of the disease [138]. Further studies on collagen XVIII in HUVEC is needed to elaborate on hyperglycemic events and the links to angiogenesis.

Decorin

In this study the total secretion of decorin was increased after stimulation with IL-1β. In contrast, hyperglycemia with or without IL-1β decreased the total secretion of decorin. Bolton et. al showed that serum-decorin is increased in type 2 DM patients with IGT, compared with

people which are normoglucose tolerant [139]. The secretion ratio after high glucose stimulation in this thesis is altered to somewhat shifted apical secretion. This is an interesting notion related to the observed increased plasma levels of decorin in type 2 DM. Immunohistochemical analysis of adipose tissue in these obese type 2 DM patients with IGT show that decorin is predominantly localized to regions adjacent to blood vessels. The total decrease in decorin secretion after high glucose stimulation with or without IL-1β, was unexpectedly based on the knowledge that decorin is upregulated in fibrosis and atherosclerosis [83]. Decorin bind TGF-β and probably has antifibrotic properties, important in the pathogenesis of diabetes [96]. One study found that mRNA expression of decorin was increased in all stages of DM nephropathy, but the protein level was not correlated with the increase in mRNA [97]. Decorin at the protein level was only found in small amounts in the glomeruli. The authors therefore hypothesize that decorin is bound to TGF-β and that the decorin-TGF-β-complexes are eliminated in the urine. In healthy subjects, decorin was found in concentrations of <1µg/L. In comparison, a patient with membranous nephropathy had 40 µg/L of decorin in the urine. TGF- β was detected in the urine as well, but only obout 6 % of the total amount of the secreteted molecule was decorin-bound. This is interesting results that should be further elaborated before any prove of the antifibrotic functions of decorin *in vivo* could be revealed. The plasma level of decorin in the same patients were analyzed and shown to be significantly increased in patients with manifest DM nephropathy. Again, an increase in plasma deorin levels is correlating with our observations of increased apical decorin secretion after hyperglycemic exposure.

Biglycan

Biglycan is an important constituent of the vessel wall, and is hypothesized to mediate in the plaque lipoprotein retention. The amount of biglycan was previously found to be upregulated in atherosclerosis [83] and to bind to apolipoproteins in both LDL and high density lipoprotein (HDL) in the artery wall [101]. Our study found that hyperglycemia and IL-1β alone decreased the secretion of biglycan, inconsistent with the previous findings. In a type 1 DM rat model, biglycan was found to bind to apoB48 in the artery vessel wall, and the binding affinity to the lipoprotein was higher when biglycan was glycated [140]. Glycation of the protein core or agents that modify the length and/or sulfation of vascular GAG chains may be more important than the protein level in lipoprotein retention. Proof for the role of biglycan in mediating vascular lipoprotein retention is lacking, and more studies are needed to confirm

a role for biglycan in diabetes and the accelerated process of atherosclerosis. Biglycan is predominantly located in the ECM and released by stress through undefined mechanism [82]. As previously discussed, we saw a secretion pattern of biglycan to a higher extent in the apical medium in untested cells compared to the other PGs. The hypothesized release of biglycan from the ECM in stress related situations may instead be released from ECs in response to stress. Further studies are needed to examine this mechanisms, but biglycan is somehow a proinflammatory mediator through binding of TLR2 and 4, receptors on macrophages of importance in the innate immune system [100]. This initiates a robust inflammatory response which attracts additional neutrophils and macrophages to the inflammatory tissue. In turn, macrophages can synthesize biglycan by an inflammatory response, and additionally increase the inflammatory reactions. One study demonstrates that biglycan -/- mice have survival benefits in induced sepsis [100]. The reduced secretion of biglycan after stimulation with IL-1β and hyperglycemia alone in this thesis may be a protective function to avoid a possible extensive inflammatory response. The expression of pro-IL-1β mRNA is stimulated when biglycan binds to TLR2/4, and by combined signaling it also increase proinflammatory cytokine IL-1β, used in this study as a stimulator [141]. Biglycan -/- mice have lower levels of IL-1β in a model of noninfectious inflammation in the kidney [82]. This connection can be a possible mechanism behind the individual variations observed after the IL-1β stimulation, and rice a “chicken and egg” problem for discussion. Further studies on biglycan in HUVEC are needed to elaborate its role in hyperglycemia and inflammation.

5.3.4 Cellular permeability

Disruptions in the barrier integrity of the endothelium lead to increased permeability. It has previously been found that by exposing HUVEC to IL-1β the endothelial permeability was increased [142]. In this thesis IL-1β alone had the most prominent increase in cellular permeability. In a study HUVEC were exposed to whole blood of healthy subjects and added bacterial lipopolysaccharide which increased the endothelial permeability. Newly produced IL-1β in the blood increased further the permeability observed by the bacterial stimulation alone. The measured concentration of IL-1β reached 50 ng/mL in that study [143], while 0,5 ng/mL was used to stimulate the HUVECs in this thesis. This indicates that the inflammatory response possibly leads to a much higher concentration of IL-1β, and that higher increases in permeability would be found by higher stimulatory concentrations. Hyperglycemia has

previously been found to increase the endothelial permeability through reduced volume of glycocalyx [144] and increased apoptosis of ECs [16]. In this thesis the hyperglycemic stimulation in contrast resulted in reduced endothelial permeability. Hyperglycemia and IL-1β together resulted in slightly increased permeability. The mechanisms behind these observations have not been studied here. The decorin PG analyzed in this thesis is however in previous studies related to cellular signaling and apoptotic functions. Decorin is shown to inhibit apoptosis by signaling trough the IGF-receptor in normal cells [92]. This is an interesting notion related to the fact that decorin secretions were highest after IL-1β stimulation, which also gave the highest increase in endothelial permeability. A study on STZ mice with decorin deficiency, found that these mice experienced enhanced apoptosis of tubular epithelial cells in the kidneys. This was achieved through decorin binding of IGF-receptor and lack of decorin was related to overexpression of the receptor in diabetic tubular kidney cells [145]. The possible role of decorin as an antiapoptotic signaling molecule, need to be further studied before any conclusions can be made. But it is possible that decorin may have these functions in humans as well, which can be relevant for both prevention and treatment of DM complications.

6 Conclusions

In the present study we have shown that

a) The ECM PGs perlecan, collagen XVIII and decorin were predominantly secreted to the basolateral medium of polarized HUVEC. Biglycan was secreted equally to the apical and basolateral media.

b) The secretion of PGs was affected by hyperglycemic and inflammatory conditions.

c) The cellular permeability was affected hyperglycemic and inflammatory conditions.

7 Future perspectives

- The level of PGs can be affected by both synthesis and degradation. By simultaneously analyzing expression of e.g. MMPs together with the PGs, a more detailed understanding can be obtained.

- The PGs could also be modified after synthesis, both extracelluary and intracellulary. It could be of great relevance to study the PG composition and whether any alterations in the structure are observed after exposure to high glucose and IL-1β. Changes in GAG structure would benefit from analyses of enzymes such as heparanase and sulfatases.

- Elevated TGF-β levels are observed in DM and it should be analyzed whether this profibrotic molecule is a potent stimulator of endothelial PG synthesis. Further analyses could then investigate if high glucose and/or IL-1β stimulation affect the level of TGF-β in cell cultures.

- Insulin level in the plasma is increased in insulin resistant type 2 DM patients and in people with IGT that still have functional insulin production. The increased plasma levels of insulin may also affect the ECs in a diverse manner, and could be a potent stimulator of PG synthesis. The effects of insulin on polarized HUVEC should provide novel insight to this point.

- Polarized cells comprise an important experimental system that makes the *in vitro* mechanisms more relevant to the *in vivo* situation. Further studies focusing on diabetes should be in use of this cell system, to obtain data from a cell system with a morphology more relevant to the *in vivo* situation.

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Appendix I – Materials

Cells

Human Umbilical Vein Endothelial Cells (HUVEC)	Rikshospitalet, OUS

Chemicals

2-mercaptoethanol	Sigma-Aldrich
^{35}S-Sulfate as NO_2SO_4	Perkin Elmer
Antobodies, primary:	
Perlecan, rabitt	Gift from Iozzo, R. (Department of Pathology, Anatomy and Cell Biology and the Kimmel Cancer Centre, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA)
Decorin, goat	R&D systems
Biglycan, goat	Novus Biologicals / Bio Site
Collagen XVIII, rabitt	Santa Cruz Biotechnology Inc.
Antibody, secondary:	
Anti-rabitt HRP-linked	GE Healthcare
Anti-goat HRP-linked	R&D Systems
Chondroitin ABC lyase	Sigma-Aldrich
Coomassie Blue R250	BIO-RAD
Barium nitritt ($Ba(NO_2)_2$	Sigma-Aldrich
Bromphenol Blue	Bio-Rad Laboratories
Chondroitin ABC lyase	Sigma-Aldrich
Collagenase 0.2 %	Sigma-Aldrich

Complete Mini, EDTA-free protease inhibitor	Roche
Coomassie Blue R250	BIO-RAD
D-(+)-Glucose	Sigma-Aldrich
DEAE-Sephacel	GE Healthcare
ECL™ Plus Western Blotting system	GE Healthcare
ECL™ Western Blotting solutions	GE Healthcare
EDTA	Sigma-Aldrich
EGF	R&D systems
FCS	Sigma-Aldrich
FGF	R&D Systems
FITC-albumin	Sigma-Aldrich
Fungisone	GIBCO Invitrogen Corporation
Gentamicine	GIBCO Invitrogen Corporation
Glycerol	Sigma-Aldrich
Glycin	Sigma-Aldrich
Guanidine HCl	Sigma-Aldrich
Hydrocortisone	Sigma-Aldrich
IL-1β	R&D Systems
Methanol	Merck
MCDM-131 medium powder	Sigma-Aldrich
Precision Plus protein standard, All blue	BIO RAD
Precision Plus protein standard, Dual Colour	BIO-RAD
SDS	Sigma-Aldrich
Sephadex G50-fine powder	GE Healthcare
Skim milk powder	Fluka
Sodium acetate trihydrat ($NaAc*3H_2O$)	Merck

Sodium Carbonate (Na_2CO_3)	Sigma-Aldrich
Sodium Chloride (NaCl)	Merck
Sodium deoxycholate	Sigma-Aldrich
Sodium hydrogencarbonate ($NaHCO_3$)	Kebo
Sodium hydroxid (NaOH)	Merck
Sodium pyrophosphate ($Na_4PO_2O_7$)	Sigma-Aldrich
Sulphuric acid (H_2SO_4)	Merck
Trisma HCl	Sigma-Aldrich
Trisma base	Calbiochem
Triton X-100	Fluka
Trypsin / EDTA	Sigma-Aldrich
Tween 20	Merck

Equipment

Amersham Hyperfilm^{TM} ECL	GE Healthcare
Autoradiography cassette, Hypercassette^{TM}	Amersham Biosciences
Cell culture flasks (25, 75, 150 cm^2)	BD Labware
Cell culture plastic plates (6 and 12 wells)	BD Labware
Compresses, Meosoft	Tendra
Criterion Precast Gel, 4-20 % Tris-HCl, 12+2 & 18 wells	BIO-RAD
Criterion TM gel system, for SDS-PAGE and Western blotting	BIO-RAD
Eppendorf tubes (1.5 ml & 2.0 ml)	Sarstedt
Falcon pipette tubes 5, 10, 25 ml	BD Labware
Falcon tubes, 15 and 50 ml	BD Labware
Filter flask (for medium), sterile	TPP

Finnpipette	Thermo labsystems
Glass wool	Paragon
pH paper	Merck
Pipetboy	IBS Integra Biosciences
PVDF membrane, ImmobilionTM Transfer Membranes	Millipore
Scalpel, single use	Paragon
Sterile needle, Sterican	Braun
Sterile surgical glows with biogel coating	Regent
Syringe filter 0.2 μm, sterile	VWR
Syringe, single use 15/50 ml, Omnifix®	B.Braun
Whatman® filter paper	Schleicher & Schwell

Apparature

Centifuges:

Labofuge 400 R	Heareus
Centri Kubota 5930	Kubota
Centrifuge 5417C	Eppendorf
DNA speedvac, DNA 100	Savant
Film Developer	Hyperprocessor amersham pharmacia biotech developer
Incubator, Forma	Scientific Incorporation
Microscope	Leica DMIL
pH meter, pHm210	Radiometer Analytical
Spectramax, fluorescense measurer	Gemini
Titerek multiscan PLUS, spectrphometer	Eflab
Water Bath	Lauda

Appendix II - Solutions

Cord buffer

COMPONENTS	VOLUME	FINAL CONCENTRATION
Streptomycin	0.5 g in 1 mL sterile mQ-H_2O	50 mg/L
1X PBS (sterile)	1000 mL	

0.2% collagenase

COMPONENTS	VOLUME	FINAL CONCENTRATION
Collagenase	200 mg	0.2 %
1X PBS (sterile)	100 mL	

Sterile filtrated and kept at -20ºC.

15 mL 1.0 M Glucose

COMPONENTS	VOLUME	FINAL CONCENTRATION
D-(+)-Glucose	2.703 g	1.0 M
mQ-H_2O	15 mL	

The solution was sterile filtrated.

10X PBS pH 7.2-7.4

COMPONENTS	VOLUME
2.5 mM NaH_2PO_4 * $2H_2O$	3.9 g
8 mM NaH_2PO_4	14.1 g
NaCl	85.2 g
mQ-H_2O	Up to 1000 mL

pH was adjusted to 7.2-7.4 with 1 M NaOH or 1 M HCl.

1.0X PBS

COMPONENTS	VOLUME	FINAL CONCENTRATION
10X PBS	100 mL	1X
mQ-H_2O	900 mL	

1.0 M Tris buffer pH 8.0

COMPONENTS	VOLUME	FINAL CONCENTRATION
Tris base $C_4H_{11}NO_3$	121.1 mg	1.0 M
mQ-H_2O	800 mL	
HCl concentrated	42 mL	
mQ-H_2O	Up to 1000 mL	

pH was adjusted to 8,0 with HCl.

1.0 M Tris buffer pH 7.4

COMPONENTS	VOLUME	FINAL CONCENTRATION
Tris base $C_4H_{11}NO_3$	121.1 mg	1.0 M
mQ-H_2O	800 mL	
HCl concentrated	42 mL	
mQ-H_2O	Up to 1000 mL	

pH was adjusted to 7.4 with HCl.

10 M NaOH

COMPONENTS	VOLUME	FINAL CONCENTRATION
NaOH	40 g	10 M
mQ-H_2O	Up to 100 mL	

1.0 M NaOH

COMPONENTS	VOLUME	FINAL CONCENTRATION
10 M NaOH	10 mL	1.0 M
mQ-H_2O	90 mL	

10 M HCl

COMPONENTS	VOLUME	FINAL CONCENTRATION
10 M NaOH	10 mL	10 M
mQ-H_2O	90 mL	

1.0 M HCl

COMPONENTS	VOLUME	FINAL CONCENTRATION
10 M HCl	10 mL	1.0 M
mQ-H_2O	90 mL	