Targeting the vessel wall in cardiovascular prevention
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Chapter 3

Sulphated glycosaminoglycans restore glycocalyx barrier properties of cultured endothelial cells in hyperglycemia

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ABSTRACT

Introduction: Microalbuminuria is a clinical parameter of vascular dysfunction in patients with diabetes. The endothelial glycocalyx plays a role in regulation of vascular permeability. Therapeutic interventions using a mixture of glycosaminoglycans (GAGs) containing 80% heparin and 20% dermatan sulphate (sulodexide) have been shown to improve vascular barrier function by reducing microalbuminuria. Therefore we hypothesized that exogenous GAGs attenuate hyperglycemia-induced increases in endothelial permeability for albumin by restoring barrier properties of the endothelial glycocalyx.

Methods: Human umbilical vein endothelial cells (HUVECs) were cultured on semi-permeable inserts and exposed to normo- (5 mM) or hyperglycemia (25 mM) for 4 days, last 24 h in presence of the GAG mixture. Endothelial permeability was assessed by determining FITC-labeled albumin transfer over the monolayer (3 hours). Additionally, the glycocalyx glucosamine sugar residues on the endothelial cells were visualized with LEA-lectin staining.

Results: Albumin permeability of endothelial cells under hyperglycemia was increased to 122 ± 8% (p < 0.01) compared to normoglycemia. Changes in albumin permeability under hyperglycemia normalized to normoglycemic control condition was -4 ± 3% (p < 0.05) in the presence of 0.06μg/mL sulodexide. Additionally, LEA-lectin revealed a 28 ± 1% (p < 0.05) increase in glucosamine staining in hyperglycemic cells in the presence of the GAG mix.

Conclusion: GAG supplementation reverses the increased trans-endothelial albumin leakage under hyperglycemic conditions by restoring the barrier properties of the endothelial glycocalyx layer in vitro.

Keywords: Diabetes mellitus, HUVEC, endothelial cell, permeability, sulodexide

Abbreviations: DM, diabetes mellitus; GAG, glycosaminoglycans; HUVEC, human umbilical vein endothelial cells
INTRODUCTION

Patients with diabetes mellitus (DM) are characterized by both microvascular complications, comprising neuropathy, nephropathy and retinopathy, as well as macrovascular complications, mainly myocardial infarction and cerebrovascular events. Whereas traditional risk factors for atherosclerosis contribute to the increased propensity towards vascular damage, correlation studies have suggested that in diabetes the vessel wall appears to be more vulnerable towards these risk factors as compared to non-diabetics (1). Whereas the exact cause for this increased vulnerability has been a matter for intensive research, it is clear that somehow the impact of hyperglycemia on the vessel wall lining is involved in this process (2).

The endothelial glycocalyx, a layer of proteoglycans covering the endothelium, forms a barrier against atherosclerotic stimuli and regulates vascular permeability (3). This barrier is easily weakened by high levels of circulating glucose (4). In fact, progressive glycocalyx perturbation is associated with microalbuminuria in patient with type 1 diabetes (5). Moreover, therapeutic interventions using a mixture of glycosaminoglycans (GAGs) containing 80% heparin and 20% dermatan sulphate (sulodexide) have been shown to decrease microalbuminuria in both type 1 and type 2 diabetic patients (6-8). The mechanism responsible for this beneficial effect, however, remains to be established. Since GAGs are essential constituents of the endothelial glycocalyx and diabetic microalbuminuria is characterized by severe perturbation of the glycocalyx in diabetic patients, it is tempting to speculate that glycocalyx restoration may be the missing link between sulodexide and attenuation of microalbuminuria. We hypothesize that these exogenously administered GAGs function as new construction tools for the restoration of the damaged endothelial glycocalyx and therefore attenuate hyperglycemia-induced increases in endothelial permeability by restoring barrier properties of the glycocalyx.

MATERIALS AND METHODS

Chemicals
M199 media, L-glutamine, antibiotic-antimycotic and trypsin were obtained from Gibco-BRL, PBS pH 7.4 from Fresenius Kabi and Fetal Bovine Serum (FBS) from Biowhittaker. The following chemicals were obtained from Sigma; heparin, endothelial cell growth supplement (ECGS), sodium chlorate and 4-methylumbelliferone. D(+)glucose was obtained from Merck. Fibronectin was a kind gift from Central Laboratory for Blood transfusion (CLB), Amsterdam, The Netherlands. Semi-permeable inserts 12 wells 3 μm pores were purchased from Greiner. Sulodexide® was kindly provided by Alfa Wassermann.
Cell cultures

HUVEC cells were isolated from human umbilical cords from the department of obstetrics of the AMC in Amsterdam. Briefly, umbilical veins were canulated and rinsed with PBS before applying trypsin solution. The trypsin solution was incubated for 37 °C to detach the endothelial cells from the venous vessel wall. The trysin solution was collected and the vein was rinsed with PBS. The cell solution was centrifuged for 10 min at 1100 rpm, supernatant was removed and the cell pellet was resuspended in 5 mL M199 medium. The cells were grown on 10 μg/mL fibronectin-coated cell culture flasks in M199 media supplemented with 20% heat-inactivated fetal bovine serum, 50 μg/mL heparin and 12.5 μg/mL endothelial cell growth supplement, 0.2 mmol/L L-glutamine and 100 U/mL penicillin-G, 100 U/mL Streptomycin sulphate, 25 μg/mL amphotericin-B at 37°C in 5% CO₂.

Glycocalyx inhibitors

Sodium sulphate inhibits sulphate donor PAPS and decreases sulphation of glycosaminoglycans and 4-methylumbelliferone acts as substrate analogue in hyaluronan synthesis, and to lesser extent heparan and keratan sulphate (9, 10). Cells were incubated for 24 hours in the presence of inhibitors at concentration 50 mM sodium chlorate and 200 μM 4-methylumbelliferone and endothelial permeability was assessed.

Permeability assay

Human umbilical vein endothelial cells (HUVECs) were cultured on semi-permeable inserts and exposed to normo- (5 mM) or hyperglycemia (25 mM) for 5 days, the last 24 hours in the presence of the GAG mixture at concentrations ranging up to 60 μg/mL. Endothelial permeability was assessed by determining FITC-labeled albumin transfer over the monolayer. Top compartment with HUVEC cells was incubated with 400 μg/mL FITC-labeled albumin in 1% BSA/RPMI 1640 media without phenol red. After 3 hours the media from the bottom well was removed and FITC-albumin content was measured by fluorescent spectrometry in Fluostar.

Lectin staining

After 5 day incubation, HUVEC cells were washed two times with ice cold RPMI 1640 and fixed in 4% paraformaldehyde for 30 min at room temperature. The cells were rinsed three times in lectin buffer (0.1% BSA in HBSS) and incubated with 67 μg/mL FITC-LEA, which is directed against glucosamine residues and 69 μg/mL TRITC-BSI, which is directed against galactosamine residues and 10 μg/mL HOECHST, which stains cell nuclei, for 30 min at room temperature. After 30 min the cells are washed with lectin buffer and imaged with fluorescence microscope.

Statistical analysis

For statistical analysis, two-way unpaired t tests were used. A value of p < 0.05 was considered statistically significant. Values are means ± SE.
RESULTS

The isolated HUVECs were cultured for 5 days under normoglycemic (5 mM) or hyperglycemic (25 mM) conditions on a semi-permeable membrane. After 5 days, the formation of the monolayer was assessed by measuring endothelial transfer of FITC-labeled albumin over the layer. Data are presented as percentage change in permeability.

Figure 1. Endothelial albumin permeability in presence of inhibitors
Albumin permeability is 4-fold increase in the presence of glycocalyx synthesis inhibitors 4-methylumbelliferone and sodium chlorate in cultured HUVECs

Permeability of the endothelial monolayer after glycocalyx inhibitors: Choice of tracer
To determine the barrier function of the glycocalyx in albumin permeability, we incubated HUVECs grown under normoglycemic conditions for the last 24 hours with the glycocalyx inhibitors sodium chlorate and 4-methylumbelliferone. Fluorescent spectrometry showed a 4-fold increase of albumin permeability in the presence of glycocalyx inhibitors compared to baseline conditions after 3 hours of incubation with FITC-albumin. As the inhibitors prevent the degree of synthesis of GAGs these findings demonstrate the importance of the glycocalyx barrier function in albumin permeability.

Permeability of the monolayer after normo- and hyperglycemia
The HUVECs were cultured for 5 days under hyperglycemic (25 mM) conditions to investigate whether or not this condition would affect endothelial permeability. Figure 2 shows the permeability of the HUVECs grown under normo- and hyperglycemia. The albumin transfer rate of the HUVECs grown under normoglycemic conditions was set to 100%. Hyperglycemia increased permeability of albumin by 22% (p < 0.01), indicating a impairment of the barrier.

Permeability of the monolayer after supplementation of the compound sulodexide
In an attempt to restore the hyperglycemia induced increase in permeability, increasing amounts of sulodexide were added to the model to determine the optimal dose, which
could restore endothelial barrier properties. The cultured cells were incubated in the last 24 hours with sulodexide at a dose of 0.06, 0.6 and 6 μg/mL. The dose-range in Figure 3 shows that the lowest concentration sulodexide of 0.06 μg/mL has the most beneficial effect on the restoration of hyperglycemic-induced increase in permeability. GAG incubation restored permeability in hyperglycemic cells. Changes in albumin permeability normalized to normoglycemic control condition was 122 ± 8% when no sulodexide was present and -4 ± 3%; (p < 0.05) in the presence of 0.06 μg/mL sulodexide.

Figure 2. Endothelial albumin permeability under normo- and hyperglycemia
High glucose condition increases the endothelial albumin permeability by 22% (p < 0.01)

Lectin-staining of the endothelial cells after compound supplementation in normo- and hyperglycemic condition
FITC-LEA lectin staining was used to determine the heparan sulphate/hyaluronan content of the glycocalyx layer of the HUVECs. Supplementation of 0.06 μg/mL sulodexide increases
glycocalyx staining in hyperglycemic cells by 28 ± 1% (p < 0.05), indicating partial restoration of the glycocalyx perturbation by compound supplementation. Staining of the cells with TRITC-BSI, a lectin directed against galactosamine residues showed no difference between the normo and hyperglycemic cultured cells in the presence of sulodexide.

**DISCUSSION**

In the present study, we show that hyperglycemic conditions increase albumin permeability through a cultured monolayer of HUVECs, which can be restored by incubation with a mixture of 80% heparin and 20% dermatan sulphate (sulodexide). Endothelial permeability for albumin after incubation with sulodexide decreased to values lower than baseline albumin leakage. Increased staining of the HUVECs with a GAG specific glucosamine-binding lectin after incubation with sulodexide supported the restoration by showing increased incorporation of glucosamine containing GAGs on the endothelial surface. These findings implicate that the glycocalyx has an important barrier function for negatively charged proteins. According to these results, amelioration of the vascular function through restoration of glycocalyx damage caused by hyperglycemia could be a promising new strategy in the prevention of cardiovascular disease in patients with diabetes.

**Protective properties of the glycocalyx**

The glycocalyx serves as a first defense against pro-atherogenic stimuli and shields vascular endothelial cells from direct exposure to flowing blood by forming a highly hydrated mesh.
of negatively charged membrane-associated proteoglycans, glycosaminoglycans, glycoproteins and glycolipids on top of the endothelial lining. Experimental models have confirmed that the glycocalyx indeed exerts a wide array of anti-atherogenic effects such as inhibition of the coagulation cascade, nitric oxide production and activation of leukocytes (11). In these models, damage to the glycocalyx has been shown to play a key role in the development of atherosclerosis, characterized by increased vascular permeability and adhesiveness and a deteriorated endothelial function (12-15). Also, the charge restriction imposed by the glycocalyx may determine accessibility of selected proteins, such as the anion protein albumin. Damage to the glycocalyx breaks down this barrier and could contribute to development of diabetic micro- and macro vascular angiopathy. A review of Mehta and Malik describes evidence that supports the key contribution of the glycocalyx in maintaining the endothelial barrier function. In several animal experiments in which the glycocalyx was disrupted or the negative charge was neutralized by enzymes, photolysis or TNF, it was shown that this degradation caused an increase in endothelial permeability for macromolecules (16).

**Hyperglycemia and glycocalyx perturbation**

The increase in endothelial albumin permeability in this in vitro model supports previous studies which show acute and chronic hyperglycemia induced reduction of glycocalyx volume in humans (4, 5). Nieuwdorp et al found a reduction in glycocalyx volume in patients with type 1 DM with and without microalbuminuria compared to healthy controls. Additionally, reduced glycocalyx volume in patients with chronic hyperglycemia due to type 1 DM was associated with an increase in microvascular complications (4). In hyperglycemia, several mechanisms could be involved in the loss of glycocalyx volume. A plausible explanation might be that oxygen radicals have a direct effect on the synthesis of GAGs as hyperglycemia is a potent pro-oxidant and pro-inflammatory stimulus. However, injury to the vascular injury may cause increased shedding of GAGs, resulting in an up-regulation of GAG synthesis to compensate for increased degradation (17, 18). Additionally, high glucose condition has been shown to increase heparanase activity and heparin compounds were able to inhibit this activity (19).

GAGs are formed from amino-sugars or hexosamines to form large complexes of repeating disaccharide units. It has been suggested that activation of the hexosamine pathway might be partially responsible for the adverse effects of chronic hyperglycemia by shunting of excess intracellular glucose into the hexosamine pathway. This may contribute to increased oxidative stress which will eventually lead to B-cell dysfunction and insulin resistance (20). There is evidence that activation of the pathway influences expression of certain genes and protein function, for instance eNOS, which might interfere with a healthy functioning glycocalyx. (17)
Effect of sulodexide on endothelial cells in hyperglycemia

GAGs and albuminuria
We investigated the ability of a mixture with heparin and dermatan sulphate to restore the glycocalyx and found that hyperglycemia induced damage to the glycocalyx and increased albumin permeability could be reversed by GAG incubation. GAGs are long, unbranched, negatively charged polysaccharides present on cell surfaces and within the extracellular matrix (21). Throughout the body, they form an impermeable border on top of the endothelial lining but GAGs are also present in the kidney’s glomerulus where they have an important function in maintaining the negatively charged filtration barrier (22). Interruption of this barrier allows larger molecules like albumin to pass through the membrane and be excreted in urine. Proteinuria in patients with DM is a marker of kidney dysfunction and is also associated with an increased risk of vascular complications. A mixture of GAGs (sulodexide) is used in diabetic patients worldwide to diminish microalbuminuria. Experimental data did already suggest that supplementation of glycocalyx constituents may have the capacity to restore glycocalyx damage to some extent (6, 7). In the present study, we found that GAG supplementation reverses the increased trans-endothelial albumin leakage under hyperglycemic conditions by restoring the barrier properties of the endothelial glycocalyx layer in vitro.

Clinical implications
In conclusion, glycocalyx damage is involved in the deterioration of the vascular barrier function, which can ultimately lead to cardiovascular disease. Treatment strategies aiming to restore and protect the endothelial glycocalyx by exogenous administration of GAG components are of important value in the cause to reduce complications in DM patients. Reversal of permeability by GAG mixture may imply restoration of not only vascular permeability, but also of other glycocalyx protective effects. Further studies in patients with diabetes appear necessary to investigate the effects of sulodexide or other glycocalyx restoring strategies on the subsequent development of cardiovascular disease.
REFERENCES