Targeting the vessel wall in cardiovascular prevention
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Summary and perspectives
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SUMMARY

In chapter 1, we reviewed the cumulating evidence underlying our hypothesis that the endothelial glycocalyx protects the vessel wall, whereas damage increases vascular vulnerability. Chapter 2 introduced techniques to estimate glycocalyx dimension in humans. We applied these methods in chapter 4 to 6, in which we studied the effect of different atherogenic stimuli, i.e. hypercholesterolemia, inflammation and diabetes mellitus, on glycocalyx dimension in humans. All of these were indeed accompanied by a reduction in glycocalyx volume. Sulodexide, etanercept and rosuvastatin, respectively, partially restored this damage. Next, we studied the effect of chronic degradation of the glycocalyx component hyaluronan by hyaluronidase infusion on renal protein loss and atherosclerosis progression in mice (chapter 7). The results of this study are equivocal and illustrate the complexity of the system, but also point to a role for the glycocalyx in renal barrier function.

PERSPECTIVES

Measurements of endothelial glycocalyx are hampered by methodological difficulties

Although the glycocalyx is a sizeable compartment of approximately 1.5 L it is often unknown, denied or simply forgotten. One of the reasons is that direct visualization of the glycocalyx is difficult. Initial electron microscopic images greatly underestimated glycocalyx thickness (1) and estimates of glycocalyx size still vary from 20 nm to 3 μm. Moreover, data that did show the presence of glycocalyx were often put aside as artifacts. However, in the past decades several methods were developed to estimate glycocalyx volume and data accumulated to show that the endothelial glycocalyx indeed exists (2). Next to the methods described in chapter 2, Duling et al. developed a fluorescent dye exclusion technique suitable for small microvessels and Damiano et al. developed microparticle image velocimetry (μ-PIV) to determine the hydrodynamically relevant endothelial glycocalyx layer (3, 4). Both estimated the glycocalyx to be approximately 0.5 μm thick. Two-photon microscopy can also provide additional information on the endothelial glycocalyx (5). However, these techniques all require intravital microscopy and are thus unsuitable for use in humans.

OPS allows estimations of endothelial glycocalyx thickness in large scale human studies

In chapter 2, we showed that OPS enables the estimation of local, sublingual glycocalyx thickness in humans. The current analysis is based on the measurement of the change in erythrocyte column diameter before and following the passage of leukocytes. Leukocytes are more rigid than erythrocytes and compress the glycocalyx in small capillaries. Erythrocytes conform to the available space. Therefore, the change in erythrocyte diameter divided by two reflects glycocalyx thickness in capillaries whose anatomical diameter is equal to or smaller
than that of a leukocyte. Currently, Hans Vink is developing a new, leukocyte independent, semi-automatic method to analyze the images to curb some of the difficulties and limitations of the described analysis. First of all, looking for leukocytes in hours of film is labor intensive and limits the number of possible measurements. Second, as leukocytes are not really visible in OPS imaging, they could be confused with plasma gaps. Finally, leukocytes differ in size. Analysis of the passage of small lymphocytes could therefore underestimate glycocalyx thickness. Further standardization and automation of the procedure will optimize the measurements and enable the use in large clinical studies.

**Systemic glycocalyx measurement has important limitations**

The second technique is based on the findings, that the endothelial glycocalyx limits access to large plasma molecules and erythrocytes, but allows the passage of small dextrans. The systemic glycocalyx volume is estimated by subtracting circulating plasma volume from the distribution volume of dextran 40. This technique is more invasive and laborious than the first. Moreover, it is often criticized, mainly because of uncertainties whether dextran distribution volume indeed reflects the total intravascular volume. Others critics think that the systemic measurement treats the glycocalyx as uniformly distributed, amorphous 'green slime on the bottom of the sea' and does not do right to the local differences in the vessel wall and its related structures throughout the body. Even though some of the criticism on systemic measurements is understandable, the technique has been extremely helpful to obtain the first indication of the state of the glyocalyx in humans.

**Estimated glycocalyx thickness and volume add up**

To comprehend, and obviously not to proof, the relation between the two measurement, it is helpful to make a back-of-the-envelop calculation of the expected glycocalyx volume. The majority of the endothelial surface area is located in the microcirculation. These capillaries have an estimated length of 111,000 kilometers (60,000-100,000 miles equivalent to 96,000-126,000 kilometers) and a diameter of about 5-8 μm (6). Thus, the radius of these capillaries is circa 3.25 μm. Suppose that we make a long line of all these round capillaries and that these are all covered by a 0.7 μm thick glycocalyx, as estimated in OPS measurements. Calculating the expected glycocalyx volume \[\pi x (3.25 \mu m)^2 - \pi / (3.25 -0.7 \mu m)^2 x 111,000 \text{ km}\] renders a volume of 1.4 L, similar to our measurements.

**Not only size, but also composition matters**

Although chapter 4 to 7 largely focus on glycocalyx volume, not only size matters. Composition of glycocalyx is likely to be essential to its function. The glycocalyx consist of glucosaminoglycans, such as the heparan sulphate, chondroitin/dermatan sulphate, covalently attached to proteoglycans. These glucosaminoglycans vary in size and in sulphation pattern, both influencing the 3D conformation of these molecules (7). The transmembrane syndecans
and the membrane-bound glypicans are the two major proteoglycans on the luminal surface of endothelial cells. Hyaluronan is weaved into the glycocalyx and attached to hyaluronan receptors. These carbohydrate structures are complex to analyze. Unlike the fields of genomics and proteomics, rapid, high-throughput assessment is still in the developmental stage. Recent developments in glycomics, including advanced mass spectrometry and lectin microarray (8), will help elucidate differences in glycocalyx composition and the relation with its function. Futures studies will show whether glycocalyx degradation products in the peripheral blood or urine can serve as a biomarker of cardiovascular disease.

The endothelial glycocalyx is a dynamic structure

An additional level in the complexity of the glycocalyx arises from its dynamic nature. The interactions between glycosaminoglycans and proteins are highly dependent on the conditions of their local microenvironment, such as pH. Furthermore, endothelial cells adapt to changes in the microenvironment by actively regulating the content and properties of the glycocalyx by continuous metabolic turnover. Moreover, many factors are likely to be attached to the vessel wall via the glycocalyx. Enzymes, such as lipoprotein lipase and extracellular superoxide dismutase, and growth factors bind to heparan sulphate (9). All these factors influence glycocalyx function and can fluctuate rapidly.

Technological developments enable us to address the complexity and clinical implications of the endothelial glycocalyx

Novel and improved tools such as OPS imaging, local models in humans (e.g. using fore arm blood flow), better high-throughput detection of breakdown products, analysis of sulphation patterns of glycosaminoglycans, flow systems with cultured endothelial cells and specific knockout mice are filling our glycobiology toolkit. This will enable future research in the field of atherosclerosis, but also in other fields of medicine. Processes, influenced by the glycocalyx, such as cell adhesion and permeability are essential in many diseases. I will shortly discuss four areas, which are of special interest. The potential impact of the glycocalyx is obviously not limited to these four areas. For instance, a role of the glycocalyx in angiogenesis and cancer is also under investigation. Geerte van Sluis is currently looking into the effect of glycocalyx degradation on tumor metastasis in an experimental model.

1. Does the endothelial glycocalyx protect against atherosclerosis?

We hypothesized that an intact endothelial glycocalyx contributes to endothelial barrier function and protects against the development of cardiovascular disease. Indeed, we showed in chapter 4 to 6 that cardiovascular risk factors such as type 2 diabetes mellitus, inflammation and hypercholesterolemia are all accompanied by disturbances of the glycocalyx. Although, we did not provide direct proof of this concept in chapter 7, it is clear from previous studies that the endothelial glycocalyx prevents many pro-atherogenic events. As discussed in
chapter 1, the glycocalyx prevents cell adhesion to the vessel wall. Besides that, it functions as molecular sieve for plasma proteins and, therefore, the origin of the oncotic forces that control transcapillary fluid exchange. Thirdly, through its core proteins the endothelial glycocalyx transmits flow-mediated shear stress to the actin cytoskeleton and is thereby involved in the initiation of intracellular signaling. The signaling cascades results in production of NO and reorganization of the cytoskeleton (10-12). Finally, disruption of the glycocalyx activates coagulation (13). New approaches in animal studies, for instance conditionally knocking out synthesis or sulphation of glycosaminoglycans, will clarify the function and composition of glycocalyx and can be used to test novel compounds aimed at restoration. Future studies in larger patient cohorts will elucidate whether OPS imaging and measurement of glycocalyx degradation products, such glycosaminoglycan patterns in urine, can discern patients at high risk of cardiovascular disease.

2. Does glycocalyx contribute to (vascular complication in) type 2 diabetes?
Patients with diabetes mellitus are predisposed to vascular complications. Our hypothesis is that the reduction in glycocalyx volume in type 1 and 2 diabetes patients contributes to the development of vascular disease. Max Nieuwdorp was the first to measure glycocalyx volume in humans and showed that glycocalyx dimension is reduced during acute hyperglycemia and in type 1 diabetic patients (14, 15). Subsequently, we turned to type 2 diabetes mellitus. First, we tested the in vitro effects of sulodexide, a mixture of heparin and dermatan sulphate, on hyperglycemia induced glycocalyx dysfunction in chapter 3. We show that hyperglycemia increases the permeability of cultured endothelial cells for albumin and that sulodexide reverses this. Increased staining of glycosaminoglycans on the endothelial surface upon sulodexide is a sign of restoration of the glycocalyx. However, it must be noted that endothelial cells were not cultured under flow, which is likely to affect glycocalyx properties (16). Mirella Gouverneur and Hans Mooij are currently setting up culture methods under flow.

In the following chapter 4, we measured glycocalyx volume, albumin permeability and the effect of sulodexide in 20 type 2 diabetic patients. As expected, more albumin permeates the vessel wall in diabetics and glycocalyx volume is smaller than in healthy controls. Additional analysis of glycocalyx breakdown products in plasma and urine as well as analysis of retinal angiogram to estimate vascular permeability, glycocalyx thickness and penetration of albumin into the glycocalyx layer will hopefully corroborate our initial results.

The mechanisms underlying insulin resistance, a hallmark of type 2 diabetes, are not well understood at the moment. About half of the anatomic volume in muscle capillaries is occupied by the glycocalyx. While under normal conditions this layer excludes circulating blood, recent intravital microscopic studies show that this exclusion is reduced by insulin. Bart Eskens is
currently testing the hypothesis in animal models, that insulin stimulates its own delivery to muscle cells and that this mechanism is critical for glucose uptake, by increasing glycocalyx accessibility for flowing plasma in capillaries.

3. Can restoration of the glycocalyx improve sepsis treatment?
Sepsis is a clinical syndrome that complicates severe infection. It is characterized by systemic inflammation and widespread tissue injury as well as a high mortality, mostly due to cardiovascular collapse and multiple organ dysfunction. Chapter 5 supports the evidence that glycocalyx significantly changes under inflammatory conditions, which may facilitate leukocyte rolling, adhesion and extravasation (17, 18). Cytokine-mediated activation of proteases secreted by the endothelium or leukocytes may locally reduce glycocalyx volume, specifically enabling leukocyte recruitment to inflamed tissue. Moreover, glycocalyx fragments, such as low molecular weight hyaluronan, act as proinflammatory signaling molecules (19). Indeed, glycosaminoglycan levels in plasma are increased in septic shock patients (20). This could represent a critical early step in the inflammatory response.

Besides extravasation of leukocytes, sepsis is characterized by vascular leakage as well as microcirculatory dysfunction (21, 22). Patients often have large fluid requirements and edema, which can partly be explained by increased microvascular permeability (23). Orthogonal polarization spectral imaging in humans and intravital videomicroscopy in animal models of sepsis have demonstrated impaired microcirculatory flow velocity, increased heterogeneity of regional perfusion, and low density of perfused capillaries (24, 25). Similar impairments in capillary perfusion were observed in a hamster model treated with hyaluronidase (25). This suggests that maintaining adequate glycocalyx function may prevent leukocyte accumulation, excessive leakage and capillary dysfunction in septic patients. OPS imaging could greatly contribute to research in this area as it is non-invasive and could both show glycocalyx thickness as well as capillary flow velocity and closure.

4. What is the role of the glycocalyx layer in renal barrier function?
Finally, in chapter 7 we showed that disruption of the glycocalyx by chronic hyaluronidase treatment doubled urine protein excretion. Under physiologic conditions, glomeruli produce about 180 liters of primary urine per day with a minimal loss of proteins (26). This filtrate must pass many layers, i.e. fenestrated endothelial cell with their glycocalyx, the glomerular basement membrane and podocytes. One of the major functions of the glomerulus is to allow the filtration of small solutes and water, while restricting the passage of larger molecules charge selectivity. Protein loss in urine can be interpreted a sign vascular dysfunction of is associated with a higher risk of cardiovascular disease (27). Increased clearance of large proteins is likely due to an enhanced number of larger pores and/or partial loss of the charge barrier. Recent electron microscopic studies confirm the presence of the endothelial glycocalyx as well as
an increase in albumin flux upon enzyme treatment (28, 29). Recently, cationic colloidal iron staining of human biopsy specimens confirmed the presence of endothelial glycocalyx on the surface of peritubular capillary endothelial cells of normal kidney, but not in rejected kidney transplant (30). This staining might enable further research in the role of the glycocalyx in the human kidney and its state in various diseases. Other unresolved issues are the specific contributions of the endothelial glycocalyx to the overall hydraulic resistance and macromolecule selectivity.

Therapeutic interventions can restore glycocalyx volume
Chapters 4 to 7 confirm previous in vitro and intravital microscopy studies that show that glycocalyx volume and function can be restored. So far, therapies only partially repair volume. This leaves room for smarter interventions, than simply supplying a random choice of glycocalyx components or treating conventional cardiovascular risk factors. Other options include for instance promoting glycocalyx synthesis or influencing sulphation. The status of endothelial glycocalyx research is still in its infancy and we have only scratched the surface in determining its structure and function. Hopefully, with the help of our glycobiology toolkit, the role of the glycocalyx in various disease processes will be firmly established and new interventions will be developed.

REFERENCES


