The Glycocalyx of the Human Umbilical Vein Endothelial Cell
An Impressive Structure Ex Vivo but Not in Culture

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Abstract—Potter and Damiano recently assessed the hydrodynamic dimensions of the endothelial glycocalyx in vivo (mouse cremaster muscle venules) and in vitro (human umbilical vein and bovine aorta endothelium cultured in perfused microchannels) using fluorescent microparticle image velocimetry (Circ Res. 2008;102:770–776). Great discrepancy was observed, the glycocalyx presenting a zone of interaction extending ≈0.52 μm into the vessel lumen in vivo, but only 0.02 to 0.03 μm from cultured cells. In an accompanying editorial, Barakat cautioned that the difference in hydrodynamic interaction did not allow one to conclude that the cultured cells totally lack a physical cell surface layer capable of mechanotransduction (Circ Res. 2008;102:747–748). To stabilize the glycocalyx for electron microscopic investigation, we perfusion-fixed 6 human umbilical veins and confluent and nonconfluent cultures (5 each) of human umbilical vein endothelial cells (HUVECs) with lanthanum/glutaraldehyde solution. Ex vivo, the thickness of glycocalyx of umbilical vein endothelium averaged 878 nm. HUVECs in vitro presented a glycocalyx with a dense-zone thickness of only 29.4 nm, plus sparse filaments reaching out on average to 118 nm, there being no difference between the nonconfluent and confluent cells. Immunohistology demonstrated the presence of heparan sulfates and syndecan-1, main constituents of the glycocalyx, both ex vivo and in vitro. These results support the observed discrepancy between glycocalyx thickness in vivo and in vitro, now for one and the same type of human cell. The presence of heparan sulfates and syndecan-1 also on cultured cells may explain why mechanotransduction phenomena can be observed even with a nonmature glycocalyx. (Circ Res. 2009;104:00-00.)

Key Words: endothelial cells • glycocalyx • heparan sulfate • syndecan • umbilical vein

Potter and Damiano have very recently assessed the hydrodynamic dimensions of the endothelial glycocalyx in an in vivo animal model (mouse cremaster muscle venules) and of endothelial cells grown in culture in perfused microchannels by means of fluorescent microparticle image velocimetry.1 A great discrepancy was observed between the 2 settings, the glycocalyx presenting a zone of interaction extending ≈0.52 μm into the vessel lumen in vivo, but only 0.02 to 0.03 μm from cultured cells. As pointed out in an accompanying editorial by Barakat, this very intriguing difference in apparent thickness of the glycocalyx does not allow one to conclude that the cultured cells totally lack a cell surface layer.2 In fact, an appeal is made for some form of direct visualization of the glycocalyx. The studies of Potter and Damiano also do not address the issue of whether there are differences in the molecular thickness or composition of the glycocalyx between in vivo and in vitro endothelial cells.3 Based on their results, Potter and Damiano caution against inferences made from in vitro studies in the areas of microvascular permeability, inflammation, mechanotransduction, and atherosclerosis, if results from such studies depend on the integrity of the endothelial cell surface chemistry.2 However, whether the glycocalyx of human endothelial cells might show similar discrepancy between the in vivo and in vitro setting remained to be shown.

A healthy vascular endothelium is coated by a variety of transmembrane- and membrane-attached molecules such as syndecans and glypicans, both carrying heparan sulfate and chondroitin sulfate side chains, which together constitute the scaffold of the endothelial glycocalyx.3,4 The glycocalyx is also made up of many functionally important molecular components such as cell adhesion molecules, eg, integrins and selectins,3 inflammatory regulators and adsorbed components, eg, of the coagulation system.5,6 Interaction with plasma colloids conveys mechanosensitivity to the endothelium, contributing to the regulation of vascular tone.7 Initially, the endothelial glycocalyx was believed to be an insignificant structure with a thickness of only a few tens of
over the past years, animal studies have suggested the glycocalyx to be much thicker and, furthermore, to play a major role in maintaining the vascular barrier function. As the interface between blood and tissue, it also prevents firm leukocyte and platelet adhesion, because, together with bound plasma proteins and solubilized glycosaminoglycans, the glycocalyx forms the endothelial surface layer with an estimated thickness ranging from 0.4 to 4 μm. This far exceeds the extension of any membrane-attached adhesion molecules, which is typically <10 nm.

Several studies have indirectly assessed the dimensions of the endothelial glycocalyx via intravital microscopy or using fluorescent antibodies. Visualizations with electron microscopy after fixation have succeeded in isolated rat and guinea pig hearts and in vivo in the mouse. Corresponding copy after fixation have succeeded in isolated rat and guinea pig hearts and in vivo in the mouse.

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Figure 1. A through C, The human endothelial glycocalyx stained in umbilical veins in situ, showed at different magnifications. Caveolae and invaginations at the endothelial surface are also filled with glycocalyx. D, Umbilical vein, not perfusion- but merely immersion-fixed. Shed heparan sulfate is homogeneously mixed with plasma and surrounding red blood cells, bordering the endothelium. EG indicates endothelial glycocalyx; VL, vascular lumen; RBC, red blood cell; arrowheads denote the endothelial plasmalemma.

Materials and Methods

Before the study, we recruited mothers expecting elective cesarean birth of a child and who assented to donating 10 cm of umbilical cord post partum. Cords were cut and, within 1 minute of birth, veins of 6 were cannulated and flushed at room temperature in the course of 1 minute with 20 mL of a freshly prepared solution composed of 2% glutaraldehyde, 2% sucrose, 0.1 mol/L sodium cacodylate buffer (pH 7.3), and 2% lanthanum nitrate. The veins were diced and 3 to 4 pieces of ~1 mm each were immersed in the fixation solution for 2 hours at 20°C. The pieces then remained overnight in solution, without glutaraldehyde, before being washed in alkaline (0.03 mol/L NaOH) saccharose (2%) solution. Following contrast enhancement with a solution containing 2% osmium tetroxide and 2% lanthanum nitrate, embedding in araldite, and microtomic sectioning, electron microscopy of the glycocalyx was performed as previously described. This ex vivo fixation enabled us to visualize the structure and thickness of the endothelial glycocalyx of a large human vessel after only a very brief cessation of blood flow. For comparison, a section of each umbilical cord was not perfused but just immersed in the fixation solution. The thickness of the glycocalyx was determined as described by Vogel et al in each electron microscopic picture and then averaged for all slices of a given umbilical vein (6 to 9 each), or endothelial cell culture (see below).

Primary HUVECs were isolated enzymatically from umbilical veins other than those above according to standard procedures. The cells were propagated in standard plates on type IV collagen under quiescent conditions using human endothelial cell–specific growth medium C-22010 (PromoCell, Heidelberg, Germany) supplemented with 10% FCS. After 3 passages, cells were seeded into 10 perfusable chambers (IBIDI µ-Slide I, Munich, Germany) coated with type IV collagen and grown in the same medium as above at 37°C with 5% CO2. Confluence was reached after ~10 days under quiescent growth conditions. The IBIDI slides allow for linear perfusion in a rectangular flow geometry, microscopic examination, and the flexible bottom membrane to be cut by microtome together

Results

An endothelial glycocalyx was visualized ex vivo in the umbilical veins (Figure 1) and on cultured HUVECs in vitro (Figure 2) using perfusion fixation with a solution containing lanthanum ions and glutaraldehyde. Interestingly, we found major differences in its thickness, both among umbilical cords and as compared to the cultured cells at either stage of maturity. Also the density differed, with umbilical vein glycocalyx appearing homogeneous, whereas that of the
HUVECs had a dense inner zone and a further-reaching, rarified zone of fibrils (Figure 2).

As can be seen in Figure 1, the glycocalyx ex vivo was an impressively delicate structure. It reached a thickness of 355±133, 623±254, 792±177, 910±530, 1079±437, and 1,210±616 nm, respectively, in the 6 cords examined, the average extent being 878±612 nm (mean±SD, n=6). Cultured HUVECs, in contrast, presented only a rudimentary glycocalyx with an average thickness of 29.4±5.8 nm for the dense zone and of 117.9±39.1 nm for the outer zone (mean±SD, n=10 cultures). The quantitative data on thickness revealed no statistically significant difference between nonconfluent (dense zone, 26.8±5.5 nm; outer filaments, 119±33 nm) and confluent cultures (dense zone, 32.0±4.9 nm; outer filaments, 126±42 nm).

No glycocalyx was electron microscopically visible in umbilical veins that had not been perfusion-fixed, but merely immersion-fixed (Figure 1D). Similarly, the umbilical arteries, which we were unable to cannulate rapidly, were seemingly devoid of any appreciable glycocalyx (result not shown).

Immunohistochemical staining of cultured HUVECs and the umbilical veins after fixation with formaldehyde revealed the presence of both heparan sulfate (Figure 3) and syndecan-1 (Figure 4) on the endothelial surface. Clearly, the extent of the heparan sulfate–positive layer in the immersion-fixed material was quite thin. Furthermore, the nonperfused umbilical arteries contained a mass of heparan sulfate–positive material in the lumen (Figure 3 and 4). This suggests spontaneous shedding of the endothelial glycocalyx during immersion fixation. The syndecan-1–positive layer likewise did not extend appreciably from the surface into the lumen or away from the cultured cells. Noteworthy, however, is the nonuniform intensity of staining among individual cells of a given culture (Figure 4).

**Discussion**

The images of the human endothelial glycocalyx provided in this study using electron microscopy and immunohistology on ex vivo–perfused umbilical veins and in vitro–cultured HUVECs fully corroborate the results of Potter and Damiano. This is true both for the qualitative difference between the ex vivo and the in vitro cells, as well as for the quantitative dimensions of the glycocalyx in the 2 distinct settings.

Although it did not allow for a strictly in vivo examination, as performed by Potter and Damiano on cremaster muscle venules of the mouse, the human umbilical vein was chosen, because its ready access facilitates rapid onset of perfusion with a lanthanum/glutaraldehyde-containing fixative after birth. Another reason for choosing the umbilical vein simply lies in the fact that this allows for a direct comparison of the same endothelial cell type in vitro and in situ. The unavoidable interruption of blood flow in the experiments was less than 1 minute, typical of an ex vivo fixation protocol. The speed of fixation and stabilization of the heparan sulfate-- and
chondroitin sulfate–containing structures by the polyvalent lanthanum ion seem paramount in preserving the very labile structure of the glycocalyx for electron microscopic investigation. This is documented here by the dramatic difference between perfusion-fixed and immersion-fixed umbilical vessels, where the latter did not exhibit a glycocalyx. Similarly, immunohistological staining of heparan sulfate and syndecan-1 moieties, although positive for the endothelial lining of both umbilical veins and umbilical arteries, did not reveal a glycocalyx of measurable thickness. A structure of 0.5 to 2 μm thickness, as noted in electron microscopy, should have been visible. However, the tissue for these investigations had also been subjected to slow fixation by immersion.

Controversy exists in the literature concerning the thickness of the glycocalyx. The uncertainty arises in part from (1) differences in the technique applied to estimate the dimension (eg, indirect measurement of exclusion zones for blood cells as opposed to fluorescent tracers; calculations based on flow resistance measurements and optical vessel width; ex vivo determinations after fixation by various approaches, etc), (2) the question of whether the glycocalyx alone (as in crystalloid-perfused systems) or the endothelial surface layer (as in vivo) is being addressed; and (3) the possibility that dimensions vary for different vascular beds, different organs, different species, and different flow rates. For instance, capillaries seem to have a glycocalyx of 0.2 to 0.5 μm thickness, whereas large vessels have been proposed to have a glycocalyx of up to 2 to 4 μm. Our results on the umbilical vein (a large vessel with low shear stress) lie between these 2 extremes and are in line with a recent study using intravital microscopy to visualize the human glycocalyx, reporting a thickness of 0.58 ± 0.16 μm in the sublingual microvasculature. The umbilical cords in our study varied with respect to the dimension of the glycocalyx from 0.35 to 1.21 μm, suggesting that the expression of the endothelial glycocalyx may be not only dependent on the vessel type but also an individual trait. However, handling of the cords was not completely standardizable at this stage of the investigation, so there may be experimental causes behind the different thicknesses.

Obviously, isolation and preparation of endothelial cells from the human umbilical vein for culture seem to deteriorate the fragile glycocalyx. We do not know how long the glycocalyx needs to reconstruct itself or whether it is at all able to do so in vitro. However, HUVECs cultured to confluence for 12 days showed no significant difference in the dimension of the endothelial glycocalyx compared to nonconfluent HUVECs after 6 days. Heparan sulfate, a negatively charged constituent of the glycocalyx needed to bind lanthanum and to stabilize the structure during fixation, was undoubtedly present on HUVECs in amounts at least as great as on umbilical vessels. Thus, failure to react with the fixative cannot be held responsible for the observed rudimentary glycocalyx of HUVECs. Even though inadequate reorganization could be a reason for the thin glycocalyx layer on HUVECs, ongoing proteolytic shedding in the nonphysiological, promitotic conditions pertaining to endothelial cultures probably accounts for the poorly developed glycocalyx. The differing intensity of syndecan-1 staining of individual cells in culture indicates that there may also be variations in expression of constituents.

In contrast to the work of Potter and Damiano, who cultivated cells in vitro in perfused cylindrical microchannels, the HUVECs in our study grew in stationary culture. This mode was adopted because most studies of HUVEC characteristics reported in the literature rely on such cultures. In any case, it did not affect the excellent qualitative and quantitative accordance with the dimensions of the glycocalyx, as assessed by microviscosimetry. Fixation of endothelial cultures by perfusion, practiced by us, might explain the similarity. In contrast to the result shown here and to those of Potter and Damiano, another group has reported HUVECs to have a glycocalyx of up to 2.5 μm thickness. However, in that particular study, a fluorescent lectin (binding to sialyl acid groups in hyaluronic acid and heparan sulfates) was allowed to diffuse onto cell cultures in quiescent medium for an extended period of time. It may well be that the rarified fibrillar structure, extending outward by up to 200 nm from our perfusion-fixed cells, extends much further in unstirred media. Another study using fluorescent lectin binding on isolated mouse arterial vessel preparations reports an extremely extensive glycocalyx, reaching out by 4.5 μm. This could well be attributable to lectin binding to long stands of hyaluronic acid, extending out beyond the denser part of the glycocalyx in stationary media. Accordingly, van den Berg et al and our group, using perfusion fixation, albeit with different fixation media, have found the dimension of the glycocalyx in various vessels of rat and guinea pig to be in the order of 0.4 μm.

HUVECs provide a model for a large, worldwide community of researchers looking at endothelial cell physiology and pathology. Many studies deal with adhesion of leukocytes and platelets to the endothelium or with permeability in HUVEC monolayers, and it is generally believed that HUVECs provide a good in vitro setup, with results being transferable not only to pathophysiological but also to physiological situations in vivo. Given the major impact of the endothelial glycocalyx on leukocyte adhesion and vascular permeability, as well as the findings of Potter and Damiano, the utility of HUVECs needs to be investigated. However, the immunohistochemical demonstration of the presence of heparan sulfate and syndecan-1 on cultured HUVECs helps to explain why these cells display evidence of mechanotransduction that is dependent on components of the endothelial glycocalyx, but not on the mature structure itself.

Because it is extremely difficult to visualize the glycocalyx in humans in vivo, mainly because of its fragility, one possible means for investigating the state of the glycocalyx and the endothelial surface layer, respectively, has been to measure plasma levels of constituent parts, eg, heparan sulfate, syndecan-1, or hyaluronan. Another approach to measure the presence and extent of the endothelial surface layer is to compare intravascular distribution volumes of glycocalyx-permeable and -impermeable tracers. The difference of the 2 values is generally presumed to represent the systemic volume of the glycocalyx. Dextran 40 and indocyanine green (via immediate binding to plasma proteins) are believed to be glycocalyx-permeable tracers; a
suitable impermeable tracer is the fluorescently labeled erythrycte. With dextran, Nieuwdorp et al reported a glycocalyx of \( \sim 1.5 \) L volume. Using indocyanine green, our group measured a total volume of the endothelial surface layer in humans of \( \sim 720 \) mL. Taking this value and a total endothelial surface area of 350 \( \text{m}^2 \), Pries and Kuebler estimated the average thickness of the endothelial surface layer to be \( \sim 2 \) \( \mu \text{m} \). In the present study, we confirm these assumptions in an intact human vessel.

This large dimension suggests that the endothelial glycocalyx/surface layer could be relevant for various vascular and circulatory diseases. For example, damage to the endothelial glycocalyx could be a trigger for macro- and microangiopathies as in diabetes- and non–diabetes-associated arteriosclerosis, sepsis, and disseminated intravascular coagulation. Recently, constituent parts of the endothelial surface layer, in particular heparan sulfates, have been shown to be involved in several aspects of cancer biology including tumor progression, angiogenesis, and metastasis. They are also believed to promote cancer growth by providing a loose matrix for migrating tumor cells and mediating adhesion of cancer cells.

For such pathological situations, with a degraded glycocalyx, HUVECs would seem to be a valid model. In accordance with Potter and Damiano, we speculate that the differences between in vitro–cultured endothelial cells and the in situ endothelium not only occur in the umbilical vein but are generally valid for all organs. Thus, as voiced in the closing remarks of the editorial by Barakat, further studies will be necessary to prove this assumption and to show whether in vitro setups hold their place as suggested (patho)physiological in vivo simulations.

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**Disclosures**

None.

**References**

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