Imaging the Endothelial Glycocalyx In Vitro by Rapid Freezing/Freeze Substitution Transmission Electron Microscopy

Eno E. Ebong, Frank P. Macaluso, David C. Spray, John M. Tarbell

Objectives—Recent publications questioned the validity of endothelial cell (EC) culture studies of glycocalyx (GCX) function because of findings that GCX in vitro may be substantially thinner than GCX in vivo. The assessment of thickness differences is complicated by GCX collapse during dehydration for traditional electron microscopy. We measured in vitro GCX thickness using rapid freezing/freeze substitution (RF/FS) transmission electron microscopy (TEM), taking advantage of the high spatial resolution provided by TEM and the capability to stably preserve the GCX in its hydrated configuration by RF/FS.

Methods and Results—Bovine aortic EC (BAEC) and rat fat pad EC were subjected to conventional or RF/FS-TEM. Conventionally preserved BAEC GCX was ≈0.040 μm in thickness. RF/FS-TEM revealed impressively thick BAEC GCX of ≈11 μm and rat fat pad EC GCX of ≈5 μm. RF/FS-TEM also discerned GCX structure and thickness variations due to heparinase III enzyme treatment and extracellular protein removal, respectively. Immunoconfocal studies confirmed that the in vitro GCX is several micrometers thick and is composed of extensive and well-integrated heparan sulfate, hyaluronic acid, and protein layers.

Conclusion—New observations by RF/FS-TEM reveal substantial GCX layers on cultured EC, supporting their continued use for fundamental studies of GCX and its function in the vasculature. (Arterioscler Thromb Vasc Biol. 2011;31:1908-1915.)

Key Words: endothelium ■ confocal microscopy ■ glycocalyx ■ rapid freezing/freeze substitution ■ transmission electron microscopy

The endothelial cell (EC) glycocalyx (GCX) senses the force (shear stress) of flowing blood and transmits it throughout the cell to sites where force is transduced into biochemical responses (mechanotransduction). Much of the evidence to support a role for the GCX and the mechanisms of its involvement in mechanotransduction comes from experiments in which cultured EC have been treated with enzymes to selectively degrade specific components of the GCX before assessment of cell function. Such experiments have shown that the GCX mediates the shear-induced production of nitric oxide (NO),1,2 a central process in cardiovascular control, and that the NO-dependent shear-induced increases in endothelial layer hydraulic conductivity also rely on the GCX.3 Related experiments have also revealed that the characteristic remodeling of the EC cytoskeleton and intercellular junctions in response to shear stress is GCX-dependent,4 as are EC elongation and alignment in the direction of shear.5

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The validity of using cell culture experiments to study EC GCX involvement in mechanotransduction and other physiological and pathophysiological processes has recently been questioned because of findings that, on average, the thickness of the GCX in vitro6–13 may be drastically less than GCX thickness in vivo7,8,12,14–20 depending on the method of GCX assessment (Table). A major difficulty limiting the demonstration of the extent of an in vitro EC GCX has been a result of the decreased water content caused by the process of alcohol dehydration that follows fixation in preparation for electron microscopic visualization. Although immunofluorescence confocal microscopy studies in which dehydration artifacts were suppressed revealed a GCX of substantial thickness on cultured EC,4,6,9 spatial resolution of confocal microscopy is limited to several tenths of a micrometer, and glare from fluorescent labels further affects the resolution.

We report on the application of rapid freezing/freeze substitution (RF/FS) transmission electron microscopy (TEM) to assess GCX thickness and structure, taking advantage of the high spatial resolution provided by TEM while avoiding the dehydration artifacts of conventional processing that usually accompany TEM. We tested the hypothesis that RF/FS-TEM GCX thickness is signifi-
cantly greater than conventional TEM GCX thickness using TEM examination of bovine aortic EC (BAEC) fixed both conventionally and by quick freezing at liquid nitrogen temperatures, as performed and reported by Dalen et al.\textsuperscript{21} and von Schack et al.\textsuperscript{22} In quick-frozen cells, the GCX was preserved in vitrified water (noncrystalline glassy ice)\textsuperscript{23} and the vitrified water was subsequently exchanged for acetone through a process called freeze substitution, permitting the GCX and other hydrophilic cellular structures to remain configured as if alive.

### Table. Selected Publications of GCX Thickness Measurements

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<td>0.03</td>
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<td>RFPEC</td>
<td>2.00**</td>
<td>Thi et al. (2004)\textsuperscript{4}</td>
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<td>HABP-FITC and fluorescent anti-HS</td>
<td>BLMVEC</td>
<td>2.00 to 3.00</td>
<td>Stevens et al. (2007)\textsuperscript{9}</td>
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</table>

Glut indicates glutaraldehyde; PFA, paraformaldehyde; RR, ruthenium red; IM, intravital microscopy; TPLSM, two-photon laser scanning microscopy; CLSM, confocal laser scanning microscopy; WGA, wheat germ agglutinin; SDF, sidestream dark field; FITC, fluorescein isothiocyanate; HABP, hyaluronic acid binding protein; AF555, Alexa Fluor 555; AF488, Alexa Fluor 488; \(\mu\)-PIV, microparticle image velocimetry; BLMVEC, bovine lung microvascular ECs; HAEC, human aortic ECs; CiGEnC, conditionally immortalized human glomerular endothelial cells; MCMVEC, murine cardiac microvascular ECs; HDMVEC, human dermal microvascular ECs; BLMVEC, bovine luteal microvascular ECs.

\textsuperscript{*}5.1\(\pm\)0.1 \(\mu\text{m}\) diameter.
\textsuperscript{†}45\(\pm\)20 \(\mu\text{m}\) diameter.
\textsuperscript{‡}148\(\pm\)5 \(\mu\text{m}\) diameter.
\textsuperscript{§}90 \(\mu\text{m}\) diameter.

\textsuperscript{¶}Cells were plated in \(\sim\)110 to 150 \(\mu\text{m}\) diameter collagen channels.
\textsuperscript{‖}Cells were plated in \(\sim\)88 to 109 \(\mu\text{m}\) diameter collagen channels.

\textsuperscript{**}GCX thickness was not reported in the publication but was obtained in reference to the scale bar and relative to the cell dimensions.
We also assessed the utility of RF/FS-TEM for detecting differences in the GCX depending on cell type and on changes in environmental conditions. BAEC macrovascular GCX was compared with rat fat pad EC (RFPEC) microvascular GCX after both cell types were processed by RF/FS-TEM. BAEC and RFPEC were chosen for several reasons. BAEC are the most widely used, and therefore the most important, in vitro model for studies of fluid shear stress effects on EC. We have published GCX studies with BAEC, and much of what is known about EC response to shear stress was established in BAEC. We have also used RFPEC for studies of the role of the GCX in EC remodeling to shear stress. In addition to comparing 2 different cell types, some EC were cultured in the presence of the heparanase III at a concentration that selectively removes ~50% of the heparan sulfate (HS) component of the GCX, and others were cultured in the absence of serum protein and bovine serum albumin (BSA) because Adamson and Clough reported that insufficient protein in the endothelial environment leads to GCX collapse. We found that RF/FS-TEM enabled in vitro stabilization of the GCX (including its HS component) in its hydrated and protein-rich configuration and observation of a surface layer of significant thickness that has never before been reported. This method clearly discerned thickness differences between the GCX of cells of different origin and a dramatically altered GCX structure or reduced thickness due to changing the culture environment.

Taken together, our findings suggest that EC cultures are appropriate for fundamental studies of GCX function in the vasculature. RF/FS-TEM fills a critical gap in the current state of the art in GCX research.

Methods

Cell Culture

BAEC and RFPEC were cultured as previously described. Briefly, BAEC were harvested from bovine thoracic aortas or obtained from VEC Technologies (Rensselaer, NY). Whenever possible, experiments were conducted using both sets of BAEC and little difference in cell morphology and function were noted. RFPEC were cloned from cells isolated from capillaries of rat epididymal fat pads. reported that insufficient protein in the endothelial environment leads to GCX collapse. We found that RF/FS-TEM enabled in vitro stabilization of the GCX (including its HS component) in its hydrated and protein-rich configuration and observation of a surface layer of significant thickness that has never before been reported. This method clearly discerned thickness differences between the GCX of cells of different origin and a dramatically altered GCX structure or reduced thickness due to changing the culture environment.

Taken together, our findings suggest that EC cultures are appropriate for fundamental studies of GCX function in the vasculature. RF/FS-TEM fills a critical gap in the current state of the art in GCX research.

Conventional and RF/FS-TEM

EC GCX was labeled and examined by conventional TEM as previously described. Briefly, EC were fixed for 1 hour in 2.0% paraformaldehyde and 2.5% glutaraldehyde containing 0.075% ruthenium red, 75 mmol/L lysine, and 0.1 mol/L cacodylate, pH 7.2, and then fixed in the same solution with lysine removed. The ruthenium red component of the fixative solution binds to the GCX glycosaminoglycans. Following a rinse with 0.1 mol/L cacodylate alone, cells were postfixed with 1% osmium tetroxide to enhance contrast between cellular components, dehydrated through a graded series of ethanol solutions, and embedded in LX112 resin

For most of our GCX studies, RF/FS was used to preserve cell samples. The purpose of using this method was to limit GCX dehydration, damage, collapse, and loss of ultrastructure. For RF/FS-TEM, cells were quick frozen in a Life Cell CF 100 slam freezer by impacting the cell monolayer against a polished sapphire surface cooled to liquid nitrogen temperature. Frozen cells were transferred to a FS7500 Freeze Substitution Unit (RMC Inc, Tucson, AZ), where they were freeze substituted in acetone containing 1% osmium tetroxide, thereby stabilizing the GCX while enhancing contrast to visualize of the GCX. They were stored at ~90°C for 3 days and then slowly raised to room temperature over 2 to 3 days, rinsed in acetone, and embedded in LX112 resin.

Conventional and RF/FS preservation experiments were repeated 3 times per cell type and condition examined. From each experiment, 2 or 3 samples were cut perpendicular to the growth plane into multiple 80-nm sections using a Reichert UCT ultramicrotome. Sections were stained with uranyl acetate followed by lead citrate using a Leica EMStainer. Sections were observed at 80 kV using a JEOL 1200EX transmission electron microscope. Among the sections that were observed, we discarded sections in which the EC monolayer was not intact, the EC surface GCX was damaged or discontinuous, or freeze damage of the EC was observed. The occurrence of GCX loss and freeze damage, inherent but undesirable effects of handling the fragile GCX layer and sometimes cryopreserving the cells below the critical rate of rapid freezing, limited the number of sample sections (n) to 1 to 3 per experiment, which is a total of at least 3 sections (n) per data set. Images of these sections were recorded on film. The negatives were scanned to create high-resolution digital images. The contrast of all images was adjusted using Adobe Photoshop, and some images were grouped together to create panoramas using the Photoshop automated photomerge feature. Images were later assessed for GCX presence, organization, and thickness. GCX thickness data sets were averaged, expressed as mean thickness ± SEM, and analyzed using a t test, with P < 0.05 indicating the statistical significance of the thickness of RF/FS-TEM-processed BAEC GCX compared with the thickness of GCX on 1 of the following: BAEC processed by conventional TEM, RFPEC processed by RF/FS-TEM, BAEC cultured in the presence of heparinase, or BAEC culture in the absence of protein.

Immunofluorescence Microscopy

Previously described immunocytochemistry methods were used as the basis for the studies we conducted to confirm cell surface localization of the GCX, with slight modifications. In brief, HS, hyaluronic acid (HA), and BSA GCX components were labeled on 2% paraformaldehyde/0.1% glutaraldehyde fixed EC. HS and HA were labeled with anti-HS (US Biologicals) and HA binding protein (Associates of Cape Cod), respectively. BSA was labeled with anti-BSA (Invitrogen Molecular Probes). Immunofluorescence was visualized with a Zeiss LSM 510 Duo confocal microscope, using a ×60 objective at a zoom of 2. z-stack images (planar sections collected 0.33 μm apart), three-dimensional reconstructions, and cross-sectional cuts were performed using Zeiss LSM software. Using the cross-section images, HS, HA, and BSA thicknesses were measured per individual EC in the region overlying the center of the cell nucleus, but only when the EC surface GCX was continuous in that cell. Maximum HS, HA, and BSA thicknesses in each cross-section were also measured, as well as HS, HA, and BSA thicknesses at cell-cell junctions. Each GCX thickness data set was generated from at least 3 cells or cell-cell junctions (n), averaged, expressed as mean thickness ± SEM, and analyzed using ANOVA.

Results and Discussion

In conventional TEM studies in which EC have been subjected to alcohol dehydration, leading to the collapse of
normally hydrated structures, GCX thickness has been found to be on the order of tens of nanometers.\textsuperscript{7,10} We repeated these conventional TEM experiments to compare the data to the results of our RF/FS-TEM studies. When BAEC were preserved by paraformaldehyde/glutaraldehyde fixation, dehydrated with alcohol, stained with ruthenium red, treated with a postfixative containing osmium tetroxide contrast agent, and examined by TEM, the endothelial monolayer was found to be intact (Figure 1A). All intercellular structures could be distinguished, and the structures were found to be well preserved (Figure 1C). A ruthenium red– and osmium tetroxide–positive extracellular layer, 41.68 ± 3.49 nm thick, was observed on the apical surface of the cells (Figure 1C and 3), with a few GCX strands extending 150 to 200 nm from the cell surface (Figure 1C). Similar results were reported by Luft,\textsuperscript{15} who performed the first TEM study of ruthenium red–stained GCX on explanted vascular tissue that underwent alcohol dehydration. Our conventional TEM findings also corroborate results of the in vitro TEM study of dehydrated, ruthenium red–stained BAEC GCX reported by Ueda et al\textsuperscript{10} and the recent TEM study by Chappell et al,\textsuperscript{7} who used lanthanum staining in place of ruthenium red to view GCX on dehydrated BAEC and human umbilical vein EC (HUVEC). Lanthanum binds to negatively charged glycoprotein\textsuperscript{27} in a fashion similar to ruthenium red, but lanthanum also stabilizes the GCX.\textsuperscript{28} Therefore, it is reasonable to expect lanthanum-labeled GCX to be thicker than ruthenium red–stained GCX. The fact that a thicker GCX was not reported by Chappell et al,\textsuperscript{7} even with the use of lanthanum, is difficult to explain but may be due to the sensitivity of EC in vitro to cell culture and electron microscopy methods.

GCX visualized by RF/FS-TEM was 100-fold thicker than the conventional TEM measurements. After EC were rapidly slam frozen and freeze substituted in acetone containing osmium tetroxide to avoid dehydration and collapse of hydrated cellular structures, when viewed by TEM the cell monolayer exhibited no damage (Figure 1B). Membrane and individual organelles could be easily identified because of differences in affinity for osmium tetroxide (Figure 1B and 1D). Cell structures were evenly distributed and classical in appearance, demonstrating successful preservation of cell morphology by slam freezing. Attached to the cell membrane and extending into the luminal and abluminal extracellular space, we could observe osmium tetroxide–positive material, which was taken to be GCX. Morphologically, RF/FS-preserved BAEC luminal GCX had the appearance of a fibril mesh, unlike the shrub-like appearance of conventionally preserved in vivo GCX structures (eg, van den Berg et al\textsuperscript{29}), a result of using RF/FS cryofixation in place of conventional chemical fixation.\textsuperscript{30} Ameye et al showed that chemical fixation of the cuticles of the pedicellaria primordial in the sea urchin \textit{Paracentrotus lividus} and of the tube foot disc in the sea star \textit{Asterias rubens} results in the preservation of a proteoglycan coat consisting of an accumulation (shrub) of very electron-dense material, whereas rapid cryofixation and freeze substitution preserves a proteoglycan coat made up of a loose meshwork of delicate fibrils.\textsuperscript{30} In a similar fashion, the type of fixation used may explain the difference between the periodic structure of the GCX that has drawn much attention in previous studies (eg, van den Berg et al\textsuperscript{29}) and the mesh structure of the GCX that we observed in our investigation. We particularly noted that RF/FS-preserved BAEC GCX was less dense, was more porous, and exhibited many elongated elements compared with its conventionally preserved in vitro counterpart. The morphology and thickness of the GCX varied spatially along the cell surface. It is particularly clear in Figure 1B for BAEC that there were areas of the GCX in which elongated structures emanating from the cell surface were prominent, there were areas in which the elongated structures extended out to only about half the thickness of the GCX, and there were areas where there were no elongated structures at all. In locations where there were no elongated structures, the GCX...
layer had a distinct, more amorphous appearance that extended to the outer boundary of the GCX. The RF/FS-TEM-processed GCX was 11.35 ± 0.21 μm thick (ranging from 10.8 to 12.5 μm) on BAEC (Figure 1D and 3). This ~11-μm BAEC GCX thickness is much greater than values derived from in vitro conventional TEM studies conducted by us (Figure 1A, 1C, and 3) and others. However, the RF/FS-preserved BAEC GCX thickness that we report is comparable to the results of a recent study by Broekhuizen et al.,14 who estimated a GCX thickness of 8.9 μm in vessels greater than 90 μm in diameter in live humans using sidestream dark field imaging and a fluorescent plasma tracer that was sterically excluded by the GCX.

When we applied RF/FS-TEM to assess the GCX on RFPEC, its morphology was that of a mesh (Figure 2B), similar to the morphology of RF/FS-preserved BAEC GCX (Figure 2A), but denser and more amorphous, with fewer distinguishable elongated elements. Morphological differences between EC of different vascular origin are well documented by several previously reported electron microscopy studies (reviewed by Tse and Stan). The distinct morphology that we observed in RFPEC GCX is, at least superficially, consistent with its microvascular function in the regulation of transendothelial permeability. In addition, we also found by RF/FS-TEM that RFPEC GCX was 5.83 ± 1.13 μm thick (ranging from 3.6 to 6.7 μm) (Figure 2B and 3), which is about half the thickness of BAEC GCX, showing that RF/FS-TEM can detect thickness variations due to different cell origins. The in vitro RFPEC GCX thickness we report is consistent with the previous in vivo study by Megens et al. and ex vivo study by van den Berg et al., who imaged a 4.5-μm-thick GCX using 2-photon and confocal laser scanning microscopy, respectively. In these studies in which mouse carotid arteries were examined, the endothelium was not dehydrated before obtaining thickness measurements, similar to our studies.

It is well established that the components of the hydrated GCX include membrane-bound proteoglycans, glycosaminoglycans, and glycoproteins, along with plasma proteins that bind to this structure. We expected that the GCX would be reduced in thickness after the addition of heparinase to EC culture medium to partially remove membrane-bound HS, based on a previous report of the effects of heparinase and another GCX degrading enzyme, hyaluronidase, which cleaves membrane-bound HA. The previous studies of the effects of heparinase and hyaluronidase treatment on GCX thickness were performed in vivo and using vessels perfused with high concentrations of enzyme (50 U/mL of heparinase and 3000 to 15 000 U/mL of hyaluronidase, where U is Sigma Units) dissolved in buffer that, in some cases, contained no BSA to stabilize the GCX. We conducted our study in a different manner, by investigating the influence of 15 mU/mL of heparinase treatment (this concentration blocks mechanotransduction events1–5 and has less drastic effects on the GCX) on RF/FS processed BAEC that were cultured in vitro and under static conditions, and we did not observe changes in GCX thickness (Figure 3) but modifications in its ultrastructure (Figure 2C). Heparinase-treated RF/FS-preserved BAEC GCX was less dense and exhibited more elongated elements in the inner region closest to the plasma membrane (Figure 2C) than its untreated counterpart (Figure 2A). In our investigation, the effects of removing other physiologically and functionally relevant membrane-bound GCX components (chondroitin sulfate, HA, glypican-1, and syndecans-1, -2, and -4) were not examined. To fully identify the nature of the components that make up the GCX mesh in RF/FS-preserved EC, further study of the dose-dependent effects of heparinase and other glycosaminoglycan degradation enzymes, as well as systematic RNA inhibition of known proteoglycans, is required.

Figure 2. RF/FS preservation, osmium tetroxide staining, and TEM of untreated BAEC GCX (A), untreated RFPEC GCX (B), BAEC GCX treated with heparinase III to degrade the heparan sulfate component of the GCX (C), and GCX of BAEC cultured in the absence of FBS and BSA (D).
When we studied the contribution of adsorbed protein to GCX thickness, by removal of FBS and BSA from EC culture medium, BAEC (Figure 2D) and RFPEC (not shown) GCX thickness was severely reduced to a level that was not visible by RF/FS-TEM (Figure 3), even at the same high magnification that permitted us to see the collapsed conventional TEM-processed GCX shown in Figure 1C. Our findings corroborate a previous report that the absence of plasma proteins collapses frog mesenteric capillary GCX. In our study, protein removal did not simply collapse the GCX but led to its complete absence. This apparent shedding of the GCX is possibly due to the fact that without protein in its microenvironment, the GCX is destabilized and strips away before or during the RF/FS process. The fact that substantial GCX was observed on EC grown in the presence of protein (Figure 2A) and no GCX could be found on EC cultured in the absence of protein (Figure 2D) provides a strong reminder of the importance of maintaining sufficient protein content in EC culture to sustain the GCX, allowing for proper assessment of GCX-mediated EC functions, such as mechanotransduction. This result of protein removal on the GCX, taken together with the effect of heparinase treatment on the GCX, suggests the effectiveness of using RF/FS-TEM to reveal GCX modifications in response to microenvironmental changes, such as protein content modification and enzymatic activity.

RF/FS-TEM has several advantages for observing hydrated, multicomponent structures such as the GCX, as outlined above. However, there are limitations. For example, although RF/FS-TEM reveals that in vitro GCX thickness extends to 5 μm for microvascular GCX and 11 μm for macrovascular GCX, no evidence is provided to suggest that these are the dimensions of the micro- and macrovascular GCX in vivo. In the future, we plan to examine in vivo GCX ultrastructure in RF/FS-preserved and TEM-visualized tissue explanted from murine blood vessels. In addition, experiments targeted at studying the mechanisms by which the GCX undergoes marked degradation at sites of platelet- or white blood cell–EC adhesion are accessible using the RF/FS-TEM approach and cultured EC incubated with platelets or white blood cells. Ideally, addressing the question of GCX thickness in vivo using the RF/FS-TEM approach would best be accomplished if EC or vascular tissue were rapidly frozen in situ under fluid flow conditions and in the presence of circulating cells. This is technically not feasible at present.

A second limitation of RF/FS-TEM is that post-RF/FS immunogold labeling techniques adversely affect cell morphology, and pre-RF/FS use of traditional immunogold or novel immuno-nanogold/silver enhancement labeling cause ice damage and subsequent loss of the GCX and other cellular structures. To overcome this constraint, immunoconfocal microscopy was used to supplement RF/FS-TEM data, revealing the localization of individual GCX components, although with limited spatial resolution. Immunoconfocal studies of the GCX uncovered abundant HS, HA, and BSA on EC in vitro. BAEC and RFPEC exhibit HS, HA, and BSA layers of nonuniform thickness, ranging from ∼1.4 μm to ∼4.3 μm (Figure 4). Before this work, similar thicknesses were reported in vitro by Barker et al and Stevens et al. Although thickness differences are not statistically significant, BAEC HA (Figure 4B) extends farther from the cell surface than BAEC HS (Figure 4A), and RFPEC HA (Figure 4E) appears to be confined to a region closer to the plasma membrane than RFPEC HS (Figure 4D). The layer of BSA seen on BAEC and RFPEC (Figure 4C and 4F) is comparable in thickness to the HS and HA layers, demonstrating that the adsorbed protein is well integrated into the structure of the GCX. Unfortunately, because immunoconfocal microscopy has limited spatial resolution and because of glare from the fluorescent labels, it would be difficult to use this technique to generate a detailed ultrastructural map of the GCX and its components. As such, we are currently developing a working immunogold labeling protocol for RF/FS-TEM.

Our RF/FS-TEM measurements of GCX thickness (reaching 11 μm for BAEC and 6 μm for RFPEC) are significantly greater than our confocal immunolabeling measurements (4 μm maximum for BAEC and 3 μm maximum for RFPEC). This discrepancy is best explained by comparing the effects
of RF/FS fixation for TEM with the effects of paraformaldehyde and glutaraldehyde fixation for confocal microscopy. Although the RF/FS-preserved GCX well represents the GCX of live cells,²⁷ the paraformaldehyde/glutaraldehyde-preserved GCX is distorted because of aldehyde-induced cross-linking of GCX components and subsequent pulling of the GCX inward toward the cell surface.²⁸ Notably, compared with the aldehyde preserved configuration, the conventionally studied aldehyde fixed and dehydrated GCX least resembles the live structure (Figure 5).²⁸ Our RF/FS-TEM and immunoconfocal microscopy studies, taken together, provide a new life-like perspective of the GCX as a fibril mesh with well-integrated components and substantial thickness.

The GCX thicknesses measured by RF/FS-TEM and immunoconfocal microscopy far exceed the estimated thickness that was recently reported by Potter and Damiano³ and Potter et al., who claimed that a significant GCX is not present in vitro, based on their particle tracking measurements in the near wall region. Despite the fact that these authors examined cultured EC that were not subjected to alcohol dehydration that would lead to GCX collapse, they exposed EC to other factors that might explain the altered GCX structure. Live BAEC or HUVEC were grown inside 100- to 150-μm collagen channels. Most in vitro experiments have been conducted on glass or plastic coated with fibronectin, not collagen, which may contribute to changes in GCX structure. In addition, EC-lined channels were perfused with medium supplemented with a high concentration (4%) of 70-kDa dextran, to raise the flow viscosity and minimize medium consumption. The nonconventional dextran use may have been problematic, as it has been shown to be sterically excluded from the GCX.²⁹ Rather than being incorporated into the GCX and contributing to the stabilization and enhancement of its thickness, as do other components of the medium, the dextran increases the viscosity of the core flow and may lead to deformation, compression, or shedding of the GCX. Microscopy experiments and antibody or lectin staining would be required to verify the presence of a GCX and confirm that the dextran in the medium does not alter GCX structure compared with normal medium.

Figure 4. Confocal micrographs of EC HS (red), HA (green), BSA (green), and nuclei (blue). A, BAEC HS; B, BAEC HA; C, BAEC BSA; D, RFPEC HS; E, RFPEC HA; F, RFPEC BSA. Arrowheads indicate locations of cell-to-cell junctions. Scale bars = 5 μm. G, Average ± SEM. GCX thickness (n = GCX-covered EC nuclei), maximum GCX thickness per cross-section, and junctional GCX thickness.

Figure 5. This cartoon, redrawn from Pahakis et al² on the basis of Heuser’s review,²⁸ depicts how live conditions, aldehyde fixation, and aldehyde combined with dehydration differentially affect GCX thickness and ultrastructural composition.
In summary, we have shown that compared with conventional methods, RF/FS-TEM provides high-resolution detection of the EC GCX in vitro. RF/FS-TEM allows for the maintenance of hydration needed to stabilize hydrophilic cellular structures after cell preservation. The use of RF/FS-TEM makes it possible to detect small structural alterations, as we have shown by comparing cell types and culture environment. We hope that this work clarifies much of the ambiguity surrounding the existence of EC GCX in vitro. The cell culture model continues to play an important role in fundamental studies of the relationship of the GCX and endothelial mechanotransduction, other physiological processes, and vascular disease.

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Disclosures

None.

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