Glycocalyx modulates the motility and proliferative response of vascular endothelium to fluid shear stress

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Yao Y, Rabodzey A, Dewey CF Jr. Glycocalyx modulates the motility and proliferative response of vascular endothelium to fluid shear stress. Am J Physiol Heart Circ Physiol 293: H1023–H1030, 2007. First published April 27, 2007; doi:10.1152/ajpheart.00162.2007.—Flow-induced mechanotransduction in vascular endothelial cells has been studied over the years with a major focus on putative connections between disturbed flow and atherosclerosis. Recent studies have brought in a new perspective that the glycocalyx, a structure decorating the luminal surface of vascular endothelium, may play an important role in the mechanotransduction. This study reports that modifying the amount of glycocalyx affects both short-term and long-term shear responses significantly. It is well established that after 24 h of laminar flow, endothelial cells align in the direction of flow and their proliferation is suppressed. We report here that by removing the glycocalyx by using the specific enzyme heparinase III, endothelial cells no longer align under flow after 24 h and they proliferate as if there were no flow present. In addition, confluent endothelial cells respond rapidly to flow by decreasing their migration speed by 40% and increasing the amount of vascular endothelial cadherin in the cell-cell junctions. These responses are not observed in the cells treated with heparinase III. Heparan sulfate proteoglycans (a major component of the glycocalyx) redistribute after 24 h of flow application from a uniform surface profile to a distinct peripheral pattern with most molecules detected above cell-cell junctions. We conclude that the presence of the glycocalyx is necessary for the endothelial cells to respond to fluid shear, and the glycocalyx itself is modulated by the flow. The redistribution of the glycocalyx also appears to serve as a cell-adaptive mechanism by reducing the shear gradients that the cell surface experiences.

flow; mechanotransduction

THE ENDOTHELIUM PLAYS A NUMBER of important roles in the vascular system, including mediation of leukocyte adhesion and inflammatory responses, the regulation of vascular permeability, and wound healing. Dysfunction of endothelial cells may lead to various pathological conditions, e.g., the early development of atherosclerosis (31). Endothelial cells are constantly exposed to hemodynamic forces, which in turn have a profound impact on cell morphological and functional responses (9, 15, 22). Mechanotransduction, the transformation of mechanical forces into a biochemical response, has been studied over the previous years. Endothelial cell mechanosensors that were previously proposed include integrins, G proteins, intercellular junction proteins, and ion channels (18). Recent investigation of the glycocalyx has led us into a new perspective that this “bushlike” structure (47) on the apical membrane may play an important role in mechanotransduction (12, 19, 21, 33, 37–39, 46).

The glycocalyx is composed of various proteoglycans, glycosaminoglycans (GAGs), glycoproteins, and associated plasma proteins (28). It serves as an interface between the blood flow and the endothelial cells, thus providing a structure that senses the fluid shear stress exerted by the extracellular flow, and transmits it to the intracellular structure. Florian et al. (19) reported that removal of heparan sulfate GAGs can completely stop nitric oxide production in cultured endothelial cells in response to shear. Mochizuki et al. (25) also reported that removal of hyaluronic acid GAGs can inhibit 80% of nitric oxide production in response to shear. In addition, Ainslie et al. (1) have shown that removal of chondroitin sulfate GAGs in the glycocalyx greatly affects the contraction response to increase in shear in smooth muscle cells. Together, this evidence led to the idea that maintaining the stability of the integral structure of the glycocalyx is important in mechanotransduction (38). Thi et al. (39) reported that the disruption of the glycocalyx can affect the actin cytoskeleton reorganization and focal adhesion localization after cells were exposed to 5 h of laminar shear and proposed a bumper-car model for the role of the glycocalyx. More recently, Moon et al. examined the role of cell-surface heparan sulfate proteoglycans in endothelial cell migration and proposed that heparan sulfate proteoglycans may be involved in sensing the direction of the flow (26). Recent in vivo work (42) has revealed a correlation between the glycocalyx dimension and atherosclerosis development, where after 6 wk of atherogenic diet, glycocalyx size was significantly decreased in the mouse carotid artery; at the same time, evidence of enhanced atherosclerotic deposits was observed in these mice.

Here we investigated the role of the glycocalyx in both endothelial cell short-term and long-term responses by using heparinase III to cleave heparan sulfate GAGs on the cell surface. It is well recognized that cultured endothelial cells align in the direction of flow after being exposed to ~24–48 h of laminar flow (15, 30) and that the endothelial proliferation is highly suppressed during flow application (23). As observed in our experiments, heparinase-treated cells did not align in the flow direction and they proliferated as if there were no flow present. On the other hand, control endothelial cells quickly responded to the onset of laminar flow by decreasing their migration speed by 40% in the first hour (27) and increasing the amount of vascular endothelial (VE)-cadherin in cell-cell junctions (Rabodzey A, Yao Y, Luscinskas WF, Shaw S, Address for reprint requests and other correspondence: Y. Yao, 3-237, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139-4307 (e-mail: yuyao@mit.edu).

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Dewey CF Jr, unpublished observations), yet these two phenomena were not found in our heparinase-degraded cells. Our results suggest that heparan sulfate proteoglycans, or the glycocalyx in general, plays an important role in mechanotransduction.

**MATERIALS AND METHODS**

**Cell culture and heparinase treatment.** Primary bovine aortic endothelial cells (BAEC-77, passages 5–15) and human umbilical vein endothelial cells (HUVEC, passages 1–3) were a gift of Guillermo García-Cardeña (Department of Pathology, Brigham and Women’s Hospital, Boston, MA). All cell-culture reagents were purchased from Sigma unless otherwise specified. BAEC were cultured in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin. HUVEC were cultured in Medium 199 supplemented with 20% fetal calf serum, 1% L-glutamine, 1% penicillin-streptomycin, and 100 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA). Before seeding, cell culture flasks and glass slides were coated with 0.2% gelatin. Cell cultures were kept in a humidified incubator maintained at 37°C and supplied with 5% CO₂ and 95% air.

Heparinase III used to cleave heparan sulfate GAGs in glycocalyx was a gift from David Berry (Department of Bioengineering, Massachusetts Institute of Technology). To degrade the cell-surface glycocalyx, cells were washed twice in serum-free medium and were incubated in a 60 mM/ml heparinase III solution for 30 min.

**Immunofluorescence and microscopy.** Heparan sulfate antibody FITC (US Biological, Swampscott, MA) was used to assess heparan sulfate removal by heparinase III, following the staining procedure described by Thi et al. (39). Both control and shear-exposed cells were labeled by CellTrace Red-Orange AM (Invitrogen) to detect cell nucleus or with VE-cadherin antibody (BD Transduction Laboratories) to detect cell-cell junctions. Briefly, cells were incubated with CellTrace for 30 min, then chilled on ice and blocked with 2% goat serum and 2% horse serum for 30 min, followed by rinsing three times in ice-cold PBS. Then cells were treated with heparan sulfate antibody (FITC conjugated) for 1 h on ice. After that, cells were washed in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature and were mounted on microscope slides for imaging. For VE-cadherin staining, human cells were treated with mouse anti-cadherin-5 MAb primary antibody for 1 h, washed three times, and treated with Cy3-conjugated goat anti-mouse IgG secondary antibody for another 30 min. Images were acquired by using a cooled charge-coupled device camera (Apogee Instruments) on a Nikon Eclipse TE2000 microscope with a ×63 water-immersion objective and MaxIm DL software (Apogee Instruments).

**Shear apparatus.** A parallel-plate flow chamber was used to expose cultured endothelial cell monolayers to laminar fluid shear stress. The chamber is composed of two acrylic plates (developed in our laboratory) separated by a piece of silicon gasket (Allied Biomedical), and the flow channel is created by removal of a 10 mm × 150 mm rectangular section from the gasket. A coverslip with a confluent endothelial cell monolayer is placed in a rectangular recess on the bottom plate, and the central region of the monolayer is exposed to flow. The flow chamber is maintained in a customized hood setup on the microscope stage to maintain 37°C and 5% CO₂. Laminar flow is generated by a peristaltic pump (Barnant) that is connected to a damper to eliminate pulsations. At a flow rate of 80 ml/s, cells are subjected to a shear stress of 15 dyn/cm².

**Cell-migration speed and alignment measurement.** Cell speeds were determined by time-lapse video microscopy. Bright-field images of a confluent endothelial cell monolayer were taken every 5 min. About 35 cells were randomly chosen in each experiment, and their nucleus positions in each image were tracked over time by using Image J software (National Institutes of Health). The crawling speed of each cell was thus calculated based on the nucleus trajectories, and the mean speed was calculated based on the statistics of 35 cells. Each experiment was conducted at least three times.

Cell alignment was quantified by measuring the angle of the longest cell-body direction with respect to flow direction. The axis of the cell elongation was manually identified by using Image J for 100 cells in each experiment, and its relative angle to flow direction was then calculated and grouped into the corresponding category, ranging from 0° to 90° for each 10° increment.

**RESULTS**

Heparan sulfate GAG removal by heparinase III. To evaluate the effectiveness of heparan sulfate GAG removal by heparinase III, we compared the fluorescent intensity of cellsurface heparan sulfate GAGs between control BAEC and enzyme-degraded BAEC. As shown in Fig. 1, heparinase treatment caused a 60.1 ± 5.4% decrease in the intensity of heparan sulfate antibody stained on the cell surface. This distinct immunofluorescent result confirmed the effectiveness of the enzyme treatment because a much lower level of heparan sulfate GAGs were observed after degradation. We strictly followed the immunostaining protocol provided by Thi et al. (39), where three-dimensional reconstruction of confocal fluorescent images has shown heparan sulfate proteoglycan staining specifically on cell apical surface. All results are reported here as the means ± SD (n = 3) unless otherwise noted.

**Long-term flow response of endothelial cells.** Long-term laminar flow application affects endothelial cell morphology in various ways. Control endothelial cells in a monolayer under static conditions display a polygonal shape with no preferred orientation. Previous results have shown that when endothelial cells were exposed to laminar shear stress for ~24–48 h, cells elongate and align in the direction of the flow (16, 30). We subjected both untreated BAEC and heparinase-treated BAEC to a shear stress of 15 dyn/cm². The untreated BAEC elongated and aligned in the direction of the flow as expected; however, heparinase-treated BAEC did not align under flow (Fig. 2A). Cell alignment was quantified by measuring the angle of the major axis of cell elongation with respect to the flow direction. Under static culture conditions, cell elongation in both control and treated BAEC monolayers distributed randomly in all possible angles from 0° to 90° (Fig. 2C), whereas long-term flow exposure caused only control cells aligning in the direction of flow (with most of the cells elongating in the axis <20° degrees with respect to flow). Similarly, heparinase III treatment also blocked the alignment of HUVEC in response to shear (data not shown). Another key flow response of endothelial cells is that shear stress causes a dose-related reduction of endothelial cell proliferation rate (23). We found that glycocalyx-degraded endothelial cell proliferation was not suppressed by flow and that those cells tended to proliferate at the same rate as they would under static conditions. Figure 2B compares the BAEC proliferation rate under the following four conditions: 1) control BAEC no flow, 2) control BAEC with flow, 3) degraded BAEC no flow, and 4) degraded BAEC with flow. The results corroborated that heparinase treatment alone did not significantly affect endothelial cell proliferation under static conditions; when subjected to flow, the degraded cells proliferated at the same rate as if there were no flow present. Considering the fact that two distinct features of control endothelial cell flow response were not observed on degraded cells,
these results suggest that the glycocalyx plays a pivotal role in mechanotransduction of applied shear.

Rapid flow response of endothelial cells. Endothelial cells quickly respond to shear stress as soon as the flow starts. Within a few seconds of flow application, intracellular free calcium concentration increases significantly (35). The motility of BAEC in a confluent monolayer slows down by 40% during the first couple of hours of flow application, whereas at the same time the actin-filament turnover rate increases and the amount of polymerized actin decreases, resulting in the acceleration of actin-filament remodeling in individual cells (27). The accelerated actin-filament remodeling should have enabled the cells to migrate faster, and the suppression of cell movement may be attributed to the strengthening of cell-cell junctional forces (Rabodzey A, Yao Y, Luscinskas WF, Shaw S, Dewey CF Jr, unpublished observations). We measured the migration speed of endothelial cells in a confluent monolayer and found that the normal cells respond to flow with a 40% drop in the first couple of hours as previously reported (27), whereas the migration speed of glycocalyx-degraded cells after flow onset maintained the same level as static condition (Fig. 3A). Cell-migration speed curves as long as 24 h for both cases are shown in Fig. 3B, indicating that the initial motility drop for control cells does not extend over hours in the later time course under flow. In addition, HUVEC in a confluent monolayer exhibit a similar response: control cells experience motility drop under shear initiation, whereas heparinase-degraded cell motility is unaffected by the flow initiation (data not shown). We measured the migration speed of endothelial cells in a confluent monolayer and found that the normal cells respond to flow with a 40% drop in the first couple of hours as previously reported (27), whereas the migration speed of glycocalyx-degraded cells after flow onset maintained the same level as static condition (Fig. 3A). Cell-migration speed curves as long as 24 h for both cases are shown in Fig. 3B, indicating that the initial motility drop for control cells does not extend over hours in the later time course under flow. In addition, HUVEC in a confluent monolayer exhibit a similar response: control cells experience motility drop under shear initiation, whereas heparinase-degraded cell motility is unaffected by the flow initiation (data not shown).

Pretreatment of heparinase III on a confluent endothelial cell monolayer, however, slightly reduces the motility of endothelial cells in static cultures by ~20%. Degraded cell crawling speeds gradually recover over a period of ~6 h (Fig. 3B), which is of the same time scale required for the glycocalyx layer to be regrown (personal communication with Dr. R. Sasishekaran, Massachusetts Institute of Technology). Because cell migration is a complex process including both mechanical driving forces and biochemical reactions, it is plausible (though not fully understood) that the role of glycocalyx as a transport facility for biochemical molecules such as growth factors (2, 6) can explain this slight motility change of the degraded cells under static conditions.

Fig. 1. Heparinase III (HepIII) treatment (60 mU/ml for 30 min; A) removes cell-surface heparan sulfate proteoglycans (HSPG) by 60.1 ± 5.4% (n = 3). Representative immunostaining of HSPG on confluent monolayers of control bovine aortic endothelial cells (BAEC; B) and heparinase-degraded BAEC (C) are shown.

Redistribution of heparan sulfate proteoglycan on cell surface after flow application. After 24 h of laminar flow application, we examined the heparan sulfate proteoglycan distribution to check whether the state of cell-surface glycocalyx itself may be altered by flow. As shown in Fig. 4, endothelial cells without flow application exhibit a uniform distribution of glycocalyx across the cell surface. After 24 h of flow application, a distinct peripheral staining pattern was observed. A double staining of CellTrace in red and glycocalyx antibody in green on BAEC after flow reveals that the glycocalyx is most prominent in the junctional regions away from the nucleus. This was further confirmed by observing the colocalization of the junctional protein VE-cadherin (red) and glycocalyx (green) in HUVEC (HUVEC was used in this case because of the availability of the VE-cadherin antibody).

On the basis of these results, we conclude that long-term flow application can lead to the redistribution of glycocalyx into the junction regions. We propose that the redistribution of glycocalyx may be a cell-adaptive mechanism to reduce the shear gradient experienced by the cell apical surface. The hypothesized model of glycocalyx is illustrated in Fig. 5. As reported previously (13), endothelial cells in a disturbed-flow region with a high shear gradient do not align under flow, and most of them tend to escape from that region. Even in the laminar region, the waviness of the monolayer surface due to the heterogeneity of endothelial cell morphology still results in shear-stress gradients on a subcellular scale (4). Atomic force microscopy (AFM) measurement has shown that cell-height modulation under flow was ~1.8 ± 0.5 μm (4), which is of the...
same order as the in vivo glycocalyx thickness (42, 46); at the same time, mathematical modeling has predicted the maximum subcellular shear gradient close to $10^4$ dyn/cm$^3$ (32), even higher than the shear gradients in a disturbed-flow region (4, 14). Hence, the mechanical stiffness of this glycocalyx structure may function to smooth the surface of cell monolayer under flow, thus reducing the shear gradients to which the cell body is exposed. Until now, limited data existed for in vitro glycocalyx thickness, and previously reported electron-microscopy results (41) suggest the level of glycocalyx expression on cultured endothelial cells may not be as abundant as in vivo and may also be regulated by the duration and strength of flow exposure; therefore, a careful in vitro study of glycocalyx thickness would be extremely useful.

**DISCUSSION**

In this study, we have examined the role of the glycocalyx in mechanotransduction by studying the well-characterized responses of endothelial cells to fluid shear stress. The responses of the glycocalyx-degraded cells differ significantly from control cells in several ways: 1) ~24–48 h of laminar flow application is not sufficient to induce a degraded cell’s alignment and proliferation suppression compared with normal cells, and 2) flow onset does not cause degraded cells to migrate significantly more slowly than in the static condition, unlike in untreated cells, which rapidly decrease their motility by 40% during the first few hours of flow application. In concert with several other recent studies of the endothelial...
glycocalyx (19, 37–39), we conclude that the glycocalyx plays an important role in mechanotransduction and furthermore that the removal of the glycocalyx appears to turn off the endothelial cell response to shear stress. The evidence of reorganization of glycocalyx under flow also leads us to speculate that not only does the glycocalyx transform mechanical force into biochemical signals inside the cells, but it also has an adaptive mechanism to reorganize itself so as to reduce the shear gradient to which individual endothelial cells are exposed under flow. This may also be of interest to studies such as the effect of tissue loading in the cleft region (49). Other researchers have reported that fluid shear stress could stimulate the incorporation of hyaluronan into the glycocalyx matrix (21) and that increased synthesis of sulfated GAGs was also observed under high shear-stress (40 dyn/cm²) conditions (3). Fluid shear may as well promote heparan sulfate proteoglycan synthesis, but our data at the moment are insufficient to resolve this question.

Moon et al. (26) found that disruption of heparan sulfate proteoglycans increased isolated endothelial cell migration speed. This may seem contradictory to our observation that heparinase III treatment itself slightly decreased cell migration speed by ~20% in a monolayer. However, we should notice that in the Moon et al. study, cells were detached and resuspended in heparinase solution to remove glycocalyx before they were seeded on fibronectin-coated surfaces for experiments. Therefore, the disruption of heparan sulfate proteoglycans not only occurred on cell apical surface but in the basal membrane as well. Since heparan sulfate proteoglycans are also actively involved in cell-adhesion formation, the reported increase in migration speed may be attributed to the weakening of cell adhesion, which is in concert with the data shown later in their study that decreasing cell adhesion by lowering fibronectin density enhanced endothelial cell migration. In our study, intact cell monolayers were treated with heparinase for 30 min to remove the apical-surface glycocalyx without significantly affecting the existing focal adhesions; therefore, our approach differs from that of Moon et al. and leads to different observations. As we know, the glycocalyx serves various important functions that maintain the health of endothelium, such as providing a selective permeability barrier for macromolecules (17, 42, 43, 45), forming a lubrication layer to assist red blood cell passing through capillaries (11, 34), and interacting with fibroblast growth factor in regulating proliferation (7, 8). The mechanism behind this motility change by glycocalyx degradation is still under study.

Several theoretical models of the glycocalyx are formed on the basis of the prediction that the Brinkman layer of flow interior to the glycocalyx is very small (11, 28, 38). Therefore, the fluid shear stress is exerted on the top surface of the glycocalyx, and the plasma membrane itself bears little shear stress (37–39, 47). One possible mechanism of mechanotransduction through glycocalyx is that the fluid shear is sensed by the core proteins in the glycocalyx structure and transmitted to the actin cytoskeleton via the transmembrane domain. Heparin sulfate GAGs are known as the most abundant GAGs on the endothelial surface, comprising ~50–90% of the total GAG
amount (20). Syndecan-1, -2, and -4, all expressed on endothelial cells, have sufficient GAG-attachment sites in the extracellular domain as well as a cytoplasmic tail linked with actin cytoskeleton (5, 24, 36, 40). Therefore, the syndecans are the most likely candidates for transmitting the fluid shear stress. Their cytoplasmic domains bind to several intracellular proteins, including ezurin, tubulin, Src kinase, cortactin, dynamin, and α-actinin (37, 40, 48), and signaling through syndecans can regulate cytoskeleton organization through their clustering, association with actin cytoskeleton, binding to cytoplasmic binding proteins, and intracellular phosphorylation (48). As a result, fluid shear stress transmitted through syndecans may be able to induce the displacement of actin filaments and start the intracellular signaling cascades (39, 47).

Thi et al. (39) describe a “bumper-car” model that proposes that the shear force exerted on the glyocalyx may be sufficient to overcome a threshold of mechanical force that may lead to the adherens-junction rupture and the formation of new stress fiber and focal adhesion. Considering the other possible biochemical pathways under flow, the role of glyocalyx under flow may well be a complicated coordination of both mechanical force and biochemical stimuli. The glyocalyx is acknowledged to be a permeability barrier for the endothelium (10, 45), and both theoretical and experimental studies have shown the important sieving property for macromolecules as represented by the permeability of the glyocalyx (44, 47). Therefore, it is logical to assume that once the flow starts, the solute influx across the glyocalyx into the cell generated by the hemodynamic forces and the resulting intracellular signaling events may be affected by altering the state of the glyocalyx. Various biochemical molecules, such as growth factors, are involved in the control of endothelial activities, such as proliferation and migration, and further studies are required to understand the role of the glyocalyx in modulating the transport of these molecules under flow. Because the glyocalyx is a complex matrix composed of a large amount of highly negatively charged GAG strands, the flow-modulated membrane diffusion and redistribution of some important signaling molecules such as adhesion or junctional proteins may also be affected by changing the state of the glyocalyx. In vitro or in vivo direct

Fig. 4. HSPG redistribution under flow application. i: BAEC no flow, with CellTracker in red and HSPG in green. ii and iii: BAEC and human umbilical vein endothelial cells (HUVEC), respectively, after 24 h of laminar flow application, showing a peripheral staining pattern of glyocalyx. HSPG is shown in green, with either CellTracker in red (ii) or vascular endothelial cadherin (VE-Cad) in red (iii). Note, HUVEC are used in iii because VE-Cad antibody works against HUVEC.

Fig. 5. Hypothesized model of glyocalyx redistribution under flow. Under static conditions, cells express a relatively uniform glyocalyx layer on apical surface. During flow application, glyocalyx components relocate from the central region to cell-cell junctions, resulting in a distinct peripheral pattern, which may help reduce the shear gradient that cells experience under flow.
measurements of glycoalyx mechanical properties would be of great help in understanding the mechanism of red blood cell passing in small capillaries as well as white blood cell interaction with the endothelium. Combined with the recent discovery of apparent linkage between glycoalyx and atherosclerosis (42), it appears that the glycoalyx occupies a pivotal role in modulating vascular mechanotransduction and vascular disease.

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