Sphingosine-1-phosphate protects endothelial glycocalyx by inhibiting syndecan-1 shedding

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Zeng Y, Adamson RH, Curry FE, Tarbell JM. Sphingosine-1-phosphate protects endothelial glycocalyx by inhibiting syndecan-1 shedding. Am J Physiol Heart Circ Physiol 306: H363–H372, 2014. First published November 27, 2013; doi:10.1152/ajpheart.00687.2013.—Endothelial cells (ECs) are covered by a surface glycocalyx layer that forms part of the barrier and mechanosensing functions of the blood-tissue interface. Removal of albumin in bathing media induces collapse or shedding of the glycocalyx. The electrostatic interaction between arginine residues on albumin, and negatively charged glycosaminoglycans (GAGs) in the glycocalyx have been hypothesized to stabilize the glycocalyx structure. Because albumin is one of the primary carriers of the phospholipid sphingosine-1-phosphate (S1P), we evaluated the alternate hypothesis that S1P, acting via S1P1 receptors, plays the primary role in stabilizing the endothelial glycocalyx. Using confocal microscopy on rat fat-pad ECs, we demonstrated that heparan sulfate (HS), chondroitin sulfate (CS), and ectodomain of syndecan-1 were shed from the endothelial cell surface after removal of plasma proteins but were retained in the presence of S1P at concentrations of >100 nM. S1P1 receptor antagonist abolished the protection of the glycocalyx by S1P and plasma proteins. S1P reduced GAGs released after removal of plasma protein. The mechanism of protection from loss of glycocalyx components by S1P-dependent pathways was shown to be suppression of metalloproteinase (MMP) activity. General inhibition of MMPs protected against loss of CS and syndecan-1. Specific inhibition of MMP-9 and MMP-13 protected against CS loss. We conclude that S1P plays a critical role in protecting the glycocalyx via S1P1 and inhibits the protease activity-dependent shedding of CS, HS, and the syndecan-1 ectodomain. Our results provide new insight into the role for S1P in protecting the glycocalyx and maintaining vascular homeostasis.

endothelial cells; glycocalyx; S1P; MMP; albumin

MAMMALIAN CELLS ARE COVERED by a surface glycocalyx layer that serves many cellular functions, including forming part of the blood-to-tissue permeability barrier (18, 20, 34), mechanotransduction (31), and limiting access of inflammatory cells to the endothelial surface (19). The stability of the glycocalyx on endothelial cells (ECs) is sensitive to physiological and pathophysiological changes. Conditions that have been shown to modify the glycocalyx include exposure to disturbed flow in large vessels (4), exposure to enzyme degradation (4, 12, 15), including activation of matrix metalloproteinases (MMPs) (19), and removal of plasma components, particularly albumin (21). The cellular mechanisms resulting in glycocalyx modification have been assumed to involve quite different mechanisms. For example, in contrast to the action of degrading enzymes that remove glycocalyx component at specific enzyme binding sites, the action of albumin has been understood in terms of albumin binding to glycosaminoglycan (GAG) side chains to form a quasi-ordered structure (29). The albumin binding appeared to be regulated by electrostatic interaction between positively charged arginine groups and negative groups in GAGs. Although such electrostatic interaction may contribute to stability, here, we describe a role of albumin, acting as a carrier of the plasma phospholipid sphingosine-1-phosphate (S1P) to regulate the stability of the glycocalyx. Specifically, we test the hypothesis that it is S1P and not albumin that modulates the endothelial glycocalyx. Furthermore, our investigations demonstrate a previously unrecognized action of sphingosine-1-phosphate in vascular endothelium to regulate MMP activity. Our results suggest a mechanism whereby the concentration of available S1P regulates the stability of the glycocalyx through modulation of MMPs.

S1P is a sphingolipid in plasma (0.3–0.5 μM) that plays a critical role in the cardiovascular and immune systems (22). Serum albumin and high-density lipoprotein (HDL) carry ~90% of the S1P, and both elicit the release of S1P from RBCs (3). The S1P receptor most abundantly expressed on ECs is S1P1, which contributes to maintain vascular barrier integrity (37). Although the action of S1P as an important modulator of cell-cell adhesion between endothelial cells is an area of active investigation, there have been no previous reports directly identifying a role of S1P to protect the glycocalyx via the S1P1 receptor.

The glycocalyx is composed of a variety of macromolecules, including glycoproteins bearing acidic oligosaccharides and terminal sialic acids, and proteoglycans (PGs) with GAG side chains. In the vasculature, the most prominent GAGs on the surface of ECs are heparan sulfate (HS), accounting for >50% of the total GAG pool, the rest being comprised of chondroitin sulfate (CS), and hyaluronic acid. HS and CS, both sulphated GAG (sGAG), are covalently linked to PGs, whereas hyaluronic acid is a nonsulphated GAG that is not covalently bound to a core protein. Glypican-1 and syndecan-1 are two major HSPGs on ECs. Glypican-1 is the only member of the glypican family expressed on ECs, which is exclusively substituted with HS near the COOH terminus close to the cell membrane (32). Syndecan-1 core protein contains five potential GAG attachment sites, three near its NH2-terminal ectodomain and two adjacent to the transmembrane domain near its COOH terminus. Only CS is found near the COOH terminus of syndecan-1. HS is found solely near the NH2 terminus, whereas some CS also resides. In the present investigation, we have directly investigated the shedding of HS and CS by S1P-dependent mechanisms.

Shedding of glycocalyx components including PGs and GAGs has been shown in experimental models of vascular...
barrier disruption, including TNF-α-induced inflammation (13), hemorrhagic shock (16), endotoxemia (14), and hyperglycemia (38). Moreover, loss of endothelial glycocalyx is associated with clinical conditions following surgery (30), trauma (24), and ischemia reperfusion (25). Therefore, understanding the mechanisms leading to glycocalyx shedding should aid in developing new methods to protect the endothelium from these processes.

MATERIALS AND METHODS

Cell Culture

Rat fat-pad ECs (RFPECs) were cultured at 37°C with 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin. Cells between passages 22 and 30 were then seeded onto glass-bottom dishes (MatTek) until confluence (2 to 3 days), unless indicated otherwise (33, 35, 36).

Preparation of S1P₁ Receptor Antagonist W146 and S1P

W146 is a S1P₁ receptor selective antagonist (11, 27). A stock solution of 5 mM W146 (Avanti Polar Lipids) was prepared using the solution of 1 ml of deionized water with 0.1 ml of 1 M Na₂CO₃ and 0.1 ml of 20% (wt/vol) (2-hydroxypropyl)-β-cyclodextrin (Sigma).

S1P (Avanti Polar Lipids.) was dissolved in methanol:water (95:5) at 45–65°C. The methanol:water solution was removed using a stream of dry nitrogen, evaporated at 50°C, and finally resuspended in 0.4% essential fatty acid-free bovine serum albumin (A6003, Sigma) to obtain a stock solution of 125 μM S1P.

In the present study, the final concentration of 10 μM W146 or 1 μM S1P was prepared in DMEM with 5% FBS and 0.5% BSA (p-DMEM) or DMEM without FBS and BSA (f-DMEM).

Effects of Plasma Protein on sGAG, and Measurement of S1P Concentration

The effects of plasma protein on sGAG (HS and CS) were studied by treating cells with different concentrations of FBS and BSA (A7284, Sigma) for 2 h. Then, cells were fixed and stained for HS and CS. The effects of plasma protein on CS were further determined by replacing the 5% FBS and 0.5% BSA with 5% FBS alone, 0.5% BSA alone, or 0.5% essential fatty acid-free BSA (A6003, Sigma).

The S1P₁ receptor antagonist W146 was used to explore the role of S1P in retaining CS. Cells were treated with f-DMEM or p-DMEM in the presence or absence of W146 for the indicated time. Staining for CS was performed after cell fixation.

Fig. 1. Plasma protein protects the endothelial glycocalyx: the potential role of S1P. Immunofluorescence images (left) and coverage analysis (right) of heparan sulfate (HS; A) and chondroitin sulfate (CS; B) in the presence of p-DMEM (DMEM with 5% FBS and 0.5% BSA), DMEM with 2% FBS and 0.2% BSA, and f-DMEM (DMEM without FBS or BSA) for 2 h. C: the S1P₁ receptor antagonist W146 abolished the protection of the glycocalyx (HS and CS) by plasma protein. Note that a 10-min pretreatment with f-DMEM containing W146, which has no effect on coverage, is necessary for the reduction of CS in the p-DMEM after 2 h. Without the pretreatment, the decrease of CS coverage takes longer. For example, we observed a similar level of CS coverage in p-DMEM with W146 after 12 h. Scale bar: 20 μm. Significant difference (n = 3): *P < 0.05; **P < 0.01.
S1P concentration in solutions was measured using an ELISA kit per manufacturer’s instructions (Echelon Bioscience, K-1900) modified to match serum:saline content in standards and samples. The S1P concentrations in BSA solutions (6%) and in FBS (10%) were determined, and the levels were reported by scaling the results to the BSA (0.5%) and the FBS (5%) concentrations most commonly used in our experiments.

Effects of S1P and S1P1 Receptor Antagonist W146 on sGAG

RFPECs were treated with 1 μM S1P alone or simultaneously with 10 μM W146 in the f-DMEM or the p-DMEM for 2 h. Then, cells were fixed and stained for HS and CS. The dose-response relationships between S1P and retention of HS and CS were investigated.

sGAG Quantitation

After treatment with p-DMEM, 1 μM S1P, and f-DMEM for 2 h, the sGAG released into the culture media was determined by using a Blyscan sGAG assay (Biocolor). In brief, after incubation for 2 h with 2 ml of p-DMEM, f-DMEM, or S1P in f-DMEM, the media in T-25 flasks were collected and centrifuged at 10,000 g for 10 min to remove the cell debris. The sGAG concentrations in the culture media were determined based on the specific binding of 1,9-dimethyl methylene blue to sGAGs and quantified against a calibration curve constructed from the diluted chondroitin-4-sulfate standards.

Activation of S1P1

The activation of S1P1 on RFPECs after treatment with p-DMEM, 1 μM S1P, and f-DMEM for 2 h was detected by fluorescent staining of phosphorylated-S1P1 at threonine 236 (Abcam).

Effects of Plasma Protein on PGs

To further detect whether the depletion of plasma protein affects the shedding of PGs, syndecan-1 was detected using a rabbit polyclonal antibody H-174 (Santa Cruz) or goat polyclonal antibody N-18 (Santa Cruz), and glypican-1 was detected using a rabbit polyclonal antibody H-95 (Santa Cruz). Shedding of the syndecan-1 ectodomain in f-DMEM in the presence of S1P for 2 h was studied. The syndecan ectodomain (N-18) in media was detected using Western blotting.

Protease Inhibition

A potent broad-spectrum hydroxamic acid inhibitor of MMPs, GM6001 (EMD Millipore), and its structural analog (negative control) GM6001 NC (EMD Millipore) were added at a concentration of 10 μM to the media to prevent protease-mediated shedding of PGs.

**Significant difference (P < 0.01; n = 3).
μM into the f-DMEM, and cells were treated for 2 h to inhibit the protease activity. Cells were fixed and stained for CS and syndecan-1 ectodomain.

*Specific inhibitors of MMP-9 and MMP-13.* MMP inhibitor I (400 nM; EMD Millipore) and/or 1 μM MMP-13 inhibitor (EMD Millipore) were used to determine their effects on the coverage of CS.

**Generic MMP Activity Assay**

After treatment with p-DMEM, f-DMEM, and S1P in f-DMEM for 2 h, the generic MMP activities in those cell culture media were measured using the Sensolyte 390 generic MMP activity kit (AnaSpec).

**Immunofluorescence Staining**

**Staining of HS and CS.** As previously described (35), RFPEC monolayers were fixed with 2% paraformaldehyde/0.1% glutaraldehyde, blocked with 2% goat serum (GS; Invitrogen), stained with HS mouse monoclonal antibody (1:100; 10E4 epitope, AMS Biotechnology) and biotinylated Griffonia (Bandeiraea) Simplicifolia Lectin II (GSL II; 100 μg/cm² in ×1 DPBS without calcium and magnesium; Vector Labs), and then visualized with Alexa Fluor 488 goat anti-mouse antibody (1:300; Molecular Probes) and Alexa Fluor 488 anti-Biotin (1:100 in 1 DPBS, Jackson ImmunoResearch Lab), respectively.

**Staining of phosphorylated-S1P.** Cells were fixed with 2% paraformaldehyde/0.1% glutaraldehyde, blocked with 2% GS/0.2% Triton X-100 for 30 min, stained with rabbit anti-S1P1 (1:100; phospho T236, Abcam), and finally visualized with Alexa Fluor 488 goat anti-rabbit antibody (1:300; Molecular Probes) for 30 min at room temperature.

**Staining of glypican-1 and syndecan-1.** Cells were fixed with 2% paraformaldehyde for 30 min on ice, washed three times, blocked for 30 min in blocking solution (2% GS for H-174 and H-95; 3% BSA for N-18) on ice, then incubated with primary antibody (1:50) for 1 h on ice followed by three washes, and finally visualized with Alexa Fluor 488 goat anti-rabbit antibody (1:500) for H-174 and H-95, and Alexa Fluor 488 donkey anti-goat antibody for N-18 (1:300; Molecular Probes) for 1 h at room temperature.

Antibodies were diluted in blocking buffer, unless indicated otherwise. Finally, cells were mounted in Aqua-Poly/Mount coverslipping medium (Polysciences). Negative controls were carried out by omitting primary antibodies or binding proteins. All images shown in the present paper had the background subtracted, unless indicated otherwise.

**Confocal Microscopy and Quantification Analysis**

All samples were imaged with a Zeiss LSM 510 laser-scanning confocal microscope (Confocal Microscopy Laboratory, The City College of New York) using a Plan-Apochromat ×63/1.4 Oil DIC objective, and the image stacks were analyzed with ImageJ software (version 1.46; NIH), as previously described (35). In brief, the maximum-intensity Z-projection of the green Z-series stack (Alexa Fluor 488 channel) was created to show all the staining in a given field of view (FOV). The FOV was ~133.6 × 133.6 μm² (×63). All information equal to or higher than background was selected as a region of interest (ROI). The percent coverage was determined by calculating the ratio of the area of the ROI and the FOV. The mean fluorescence intensity of phosphorylated-S1P₁ in the FOV was determined. Data are expressed as the means of the percent change ± SD of the mean fluorescence intensity compared with that in p-DMEM conditions.

**Western Blotting**

The shedding of syndecan-1 ectodomain into the culture media was analyzed by Western blotting. In brief, after incubation for 2 h with 15 ml of p-DMEM and f-DMEM, the media were collected from T-75 flask. Cell debris was removed by centrifugation. The protein in media was concentrated using the Amicon Ultra-15 Centrifugal filter device (Millipore). Protein concentration was determined by a Protein Determination Kit (Cayman). Thirty micrograms of protein were size fractionated using SDS-PAGE and electrotransferred onto PVDF.
membrane (Bio-Rad) and hybridized with polyclonal antibodies (1:200; N-18) recognizing syndecan-1 ectodomain. Detection was carried out using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Millipore). The apparent molecular masses of the proteins were estimated with prestained molecular mass markers (Bio-Rad).

Statistical Analysis

Data are presented as means ± SD obtained from at least three independent experiments, unless indicated otherwise. The images for coverage calculations were obtained from at least 12 maximum-intensity Z-projection images from three independent experiments. Statistical analysis was performed by one-way ANOVA with either the least significant difference (LSD) test or Tamhane’s T2 test (depending on Levene’s statistic for homogeneity of variance), using the SPSS 20.0 software package. Differences in means were considered significant if $P < 0.05$.

RESULTS

Plasma Protein Protects the Endothelial Glycocalyx

The effects of plasma protein on HS and CS are shown in Fig. 1, A and B. Similar to our laboratory’s previous study (35), the initial coverage of HS and CS on RFPECs were 83.3 ± 6.2 and 86.6 ± 2.7%, respectively, in the presence of p-DMEM for 2 h. The coverage of HS and CS were significantly decreased by 22.6 and 53.1%, respectively, in the presence of f-DMEM for 2 h. The large decreases in the coverage of HS and CS suggest that the structural stability of the glycocalyx depends strongly on the presence of plasma protein. Furthermore, the reductions of sGAG coverage were dependent on the concentration of plasma protein. In the presence of 2% FBS and 0.2% BSA, the coverage of CS was significantly decreased by 21.1%, whereas that of HS was not changed. Furthermore, replacing the 5% FBS and 0.5% BSA with 5% FBS alone, 0.5% BSA alone, or 0.5% essential fatty acid-free BSA greatly diminished the effect of the media in protecting CS (Fig. 2). The fatty acid-free BSA in DMEM was not distinguishable from DMEM alone in reducing the protection of CS. It appears that both 5% FBS and 0.5% BSA contribute significantly to the protective effect of p-DMEM. Because CS was reduced much more than HS in protein-free media, most of the subsequent data focuses on CS, not HS.

Although changes in coverage are emphasized throughout the study for CS, it is noted that the thickness of the small amount of CS that remained after treatment with f-DMEM was slightly diminished (from 1.14 ± 0.24 to 0.92 ± 0.59 μm; $P < 0.01$).

Measurement of S1P- and S1P-Dependent Phosphorylation

To examine the role of S1P1 further, we measured S1P concentrations in our basic media. FBS (5%) contributed 100 ± 11 nM and BSA (A7284, 0.5%) contributed 87 ± 22 nM to our basic media. Fatty acid-free BSA (A6003, 0.5%) contributed a lower amount (56 ± 14 nM). The result that the total S1P in 5% FBS plus 0.5% BSA is 187 nM and protects the glycocalyx, whereas 0.5% BSA alone with 87 nM S1P fails to protect the glycocalyx suggests a steep relationship between S1P concentration and its protective effect between 100 and 200 nM. S1P ligation of S1P1 is associated with threonine 236 phosphorylation (17). Therefore, we measured the phosphorylation of S1P1 (threonine 236) in different media as shown in Fig. 3. S1P1 is phosphorylated on exposure to either p-DMEM or 1 μM S1P, whereas the phosphorylation of S1P1 was suppressed by f-DMEM, suggesting that S1P1 is activated by S1P in p-DMEM media.

S1P Plays a Dominant Role in Protecting the Glycocalyx via the S1P1 Receptor

Figure 1C shows that the selective antagonist (W146) to the most prominent S1P receptor in endothelial cells (S1P1) significantly decreased the coverage of CS by 86.6% in the presence of p-DMEM. The action of W146 to reduce CS coverage in the presence of p-DMEM, compared with p-DMEM as control conformed to the hypothesis that S1P in p-DMEM plays a dominant role in protecting the glycocalyx. Note that, when W146 was added directly into the f-DMEM, it did not reduce CS after 2 h. For the reduction of CS in the p-DMEM after 2 h, a 10-min pretreatment with f-DMEM containing W146 is necessary. Without the pretreatment, the decrease of CS coverage takes longer. For example, we observed a similar level of CS coverage in p-DMEM with W146 after 12 h (data not shown).

Further Validation of the Protection of Glycocalyx by Exogenous S1P via S1P1 Receptor

The effects of S1P and W146 on both HS and CS were further examined as shown in Fig. 4. Exogenous S1P (1 μM) significantly inhibited the shedding of HS from RFPECs in f-DMEM (Fig. 4A). When cells were treated with W146 in the presence of S1P, the effect of S1P on HS with f-DMEM was significantly attenuated (Fig. 4B). Similar to its effect on HS, the presence of S1P in f-DMEM retained the same level of CS on RFPECs as p-DMEM alone (Fig. 4C). Control experiments demonstrated that the addition of S1P when p-DMEM was present did not change coverage of HS or CS. W146 abolished the protective role of S1P in the f-DMEM-induced CS loss (Fig. 4D). Figure 4D also shows that, when W146 was added directly into the f-DMEM, it did not reduce CS. In summary, both p-DMEM and 1 μM S1P protected the glycocalyx, and W146 abolished the protective role of S1P.

Fig. 5. The sGAG levels in media. The sGAG (HS and CS) released into media in the presence of p-DMEM and f-DMEM with or without S1P for 2 h was determined using a Blyscan sGAG assay. p-DMEM and f-DMEM plus S1P protect against sGAG release into the media. *Significant difference ($P < 0.05$; $n = 6$).
The concentration dependence of the protective effect of exogenous S1P was tested. Whereas 0.2 μM protected CS as well as 1 μM (Fig. 4F), the effectiveness of 0.1 μM was significantly diminished (Fig. 4, E and F). Because S1P has been shown to compromise endothelial barriers at higher concentrations, we also tested 10 μM S1P. At this concentration, coverage was diminished.

**SIP Inhibits the f-DMEM-Induced sGAG Release**

To investigate the mechanisms leading to reduced coverage of the glycocalyx, the sGAG released into the media was determined by the Blyscan assay (Fig. 5). After a 2-h treatment, the base level of sGAG released into the p-DMEM was 1.6 ± 0.4 μg/ml; the sGAG released into the f-DMEM significantly increased by 42.9% (P < 0.05). However, the sGAG released into the f-DMEM in the presence of S1P decreased to the base levels.

**Glypican-1 is not Shed: SIP Inhibits Shedding of Syndecan-1**

The role of the core proteins carrying the HS and CS chains was further investigated using labels for the core proteins as shown in Fig. 6A. In p-DMEM, the coverage of H-95, which labeled the glypican-1 ectodomain near its NH2 terminus, was 44.4 ± 11.5% (Fig. 6B). When cells were treated with f-DMEM for 2 h, the coverage of H-95-labeled glypican-1 did not change significantly. For syndecan-1, the coverage of H-174, which labeled syndecan-1 from the transmembrane domain to the ectodomain, on f-DMEM-treated cells was not different than that on p-DMEM-treated cells (55.5 ± 8.4%) (Fig. 6C). However, the coverage of N-18, which labeled the...
NH2-terminal ectodomain of syndecan-1, was 54.8 ± 9.8% in p-DMEM and significantly decreased by 56.9% after f-DMEM treatment (Fig. 6D). Using Western blotting, a strong band for N-18 (ectodomain of syndecan-1) in the f-DMEM media compared with the p-DMEM was also observed (Fig. 6E). All of this indicates that the ectodomain is shed, not the transmembrane domain. The decrease of syndecan-1 ectodomain could be abolished by adding S1P (Fig. 6D).

Fig. 7. Role of MMPs and MMP activity. f-DMEM induced shedding of CS (A), and syndecan-1 ectodomain (B) was abolished by the general MMP inhibitor GM6001 after 2 h (n = 3). GM6001 NC served as negative control. C: f-DMEM-induced activation of MMPs was suppressed by S1P (2 h) (n = 4). Data shows the involvement of MMPs in CS shedding. **Significant difference (P < 0.01).

Fig. 8. The separate and concurrent effects of specific MMP inhibitors on CS. Before staining of CS, cells were treated with p-DMEM, 400 nM MMP-9 inhibitor alone, 1 μM MMP-13 inhibitor alone, and f-DMEM for 2 h (A and C), or were treated with p-DMEM, 400 nM MMP-9 inhibitor and 1 μM MMP-13 inhibitor together, and f-DMEM for 2 h (B and D). MMP-9 plus MMP-13 can account for all CS shedding. Scale bar: 20 μm. **Significant difference vs. p-DMEM control (P < 0.01; n = 3).
MMP Inhibitor Abolishes f-DMEM-Induced Losses of CS and Syndecan-1 Ectodomain

The general MMP inhibitor GM6001 and its negative control GM6001 NC were used initially to investigate the potential roles of MMPs in the losses of CS and syndecan-1 ectodomain (Fig. 7, A and B). Similar results were obtained for both CS and syndecan-1 ectodomain, and showed that GM6001 completely abolished the f-DMEM-induced shedding of CS and syndecan-1. GM6001 NC had no effect.

Specific inhibitors of MMP-9 and MMP-13 were used to determine their effects on the coverage of CS. MMP-9 inhibitor (400 nM) or 1 μM MMP-13 inhibitor partially reduced the shedding of CS (Fig. 8, A and C). The combination of 400 nM MMP-9 inhibitor and 1 μM MMP-13 completely abolished the shedding of CS (Fig. 8, B and D), suggesting that the activation of MMP-9 and MMP-13 mediate the shedding of CS.

S1P Abolishes the f-DMEM-Promoted MMP Activity

After 2 h of treatment, the MMP activities in p-DMEM, f-DMEM, and f-DMEM with S1P were measured using the Sensolyte 390 generic MMP activity kit (Fig. 7C). The MMP activity in f-DMEM was significantly increased by 116.2% compared with p-DMEM controls. The presence of S1P attenuated the MMP activity to near the level of p-DMEM, showing S1P abolished the f-DMEM-induced increase of MMP activity.

DISCUSSION

The present study reveals a new mechanism by which plasma protein affects the structural stability of the endothelial glycocalyx. Previous studies suggested that the negative charges on GAGs interacted with positively charged arginine residues on albumin to provide a stabilizing electrostatic interaction that was disrupted when protein was removed, leading to a collapse of the overall glycocalyx structure (1, 21, 35). The new mechanism is quite different, centering on S1P binding to its S1P₁ receptor to inhibit protease activity-dependent shedding of the syndecan-1 ectodomain.

In recent years, many studies have revealed a substantial glycocalyx on ECs in vitro (9, 33, 35). In the present study, we have demonstrated that 5% FBS and 0.5% BSA (p-DMEM) can maintain the structural stability of the glycocalyx on ECs. The observation that removal of the plasma proteins decreased the surface coverages of HS and CS (Fig. 1) is consistent with previous research from our laboratory showing a 27.4% reduction of HS after removing plasma protein for 5 h on RFPECs (33) and observations using high-resolution rapid freeze electron microscopy that removal of all protein from the culture media bathing bovine aortic EC led to a complete loss of glycocalyx (9). Our observations are also consistent with the well known observation in capillaries demonstrating shedding or collapse of glycocalyx visualized using electron microscopy after albumin removal from the perfusate (1). Furthermore, the average reduction of CS in f-DMEM (Figs. 1, B and C, 4C, and 7A) is much greater than the reduction of HS, suggesting that stabilizing interactions between HS and CS are minimal, as we demonstrated previously by enzymatic removal of individual GAG components (35).

The structural stability of the glycocalyx depends much more strongly on the presence of S1P bound to plasma protein than to any individual GAG component. Even when there is no carrier for S1P, albumin, HDL, and LDL are all lacking in f-DMEM, and both 0.2 and 1 μM S1P can completely protect the glycocalyx against shedding in this condition (Fig. 4, A, C, and F). To demonstrate that the S1P₁ receptor is the key mediator of the S1P effect, we used a specific inhibitor of S1P₁.
(W146) to block the protective effect of protein or S1P in the media (Figs. 1 and 4) and a specific antibody against p-S1P1 to demonstrate activation of S1P1 by S1P bound to plasma protein and exogenous S1P (Fig. 3). The concentration range over which S1P maintains the glycocalyx is similar to that which maintains endothelial barrier function (7). Our recent in vivo study in mammalian microvessels has shown that S1P below 100 nM fails to maintain normal hydraulic conductivity and solute permeability in intact microvessels, whereas concentrations above 300 nM stabilized the barrier (6). In plasma, S1P concentration is between 100 and 500 nm (22). The average S1P content of our media was 100, 87, and 57 nM in 5% FBS, 0.5% BSA, and 0.5% fatty acid-free BSA, respectively, and 187 nM in p-DMEM (5% FBS plus 0.5% BSA). Taken together, these data suggest that S1P is the active component in p-DMEM that protects the glycocalyx. We also note that S1P at 10 μM caused an increase in hydraulic conductivity in rat venular microvessels (2). High levels of S1P resulted in degradation of the S1P1 receptor (23), whereas physiological levels of S1P induce activation of S1P1 (Fig. 3).

Others have shown that modification of the arginine, but not the lysine, residues on albumin led to a loss of endothelial barrier function, suggesting that the positively charged arginine residues on albumin interact with negative charges on the endothelial surface GAG side chains to stabilize and order the glycocalyx structure (21). The present results suggest the alternate interpretation of those studies that modified arginine sites reduced the binding of S1P to albumin, thus restricting its delivery to the glycocalyx and the cell surface. This is also suggested by recent studies of S1P binding to apolipoprotein M (ApoM) (5) and binding sites on albumin (28).

To evaluate mechanisms leading to loss of glycocalyx coverage, we used the Blyscan assay to demonstrate that the sGAGs released into culture media significantly increased in the presence of f-DMEM (Fig. 5). In addition, we found losses of HS (Fig. 1A), CS (Fig. 1B), and syndecan-1 ectodomain that was labeled by the N-18 antibody (Fig. 6), suggesting that the cleavage site is below the CS attachment site and that the losses of CS and HS are due to shedding of the syndecan-1 ectodomain. Glypican-1 is exclusively substituted with HS in the COOH terminus close to the cell membrane. This combined with the observation that there was no change in glypican-1 distribution (Fig. 6B) can explain why HS was reduced less than CS (Fig. 1).

Syndecan-1 shedding occurs via proteolytic cleavage close to the cell membrane. We found that inhibition of MMP activation by the zinc chelating hydroxamic acid inhibitor GM6001 attenuated the shedding of CS and syndecan-1 ectodomain in the absence of plasma protein, showing that shedding of the syndecan-1 ectodomain depends on MMP activity (Fig. 7, A and B). Specifically, both MMP-9 inhibitor and MMP-13 inhibitor reduced shedding of CS, and together completely abolished it (Fig. 8). Furthermore, S1P greatly diminished f-DMEM-induced MMP activity (Fig. 7C). Although the exact intracellular signaling pathways mediating syndecan-1 shedding from the cell surface are not yet completely understood, we suggest that Rac-1-dependent pathways may mediate the inhibition of MMP activation by S1P phosphorylation. It has been demonstrated that Akt-mediated phosphorylation of S1P1 receptor (T236) is indispensable for S1P-induced Rac activation, endothelial migration, and morphogenesis (17).


