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Study on the Sliding Friction of Endothelial Cells Cultured on Hydrogel and the Role of Glycocalyx on Friction Reduction

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Abstract

In this study, we investigated the sliding friction of human umbilical vein endothelial cell (HUVEC) monolayer cultured on poly(sodium *p*-styrene sulfonate) (PNaSS) gel, intending to elucidate the role of the glycocalyx on the surface of endothelial cell (EC) in friction reduction. Three sets of HUVEC monolayers were investigated: 1) as-cultured HUVEC monolayer, 2) HUVEC monolayer treated by transforming growth factor β_1 (TGF-β_1), which increased glycocalyx by 148%, 3) HUVEC monolayer treated by heparinase I, which reduced glycocalyx by 57%, both were compared with that of the as prepared one. When being slid on flat glass surface, the frictional stress of HUVEC monolayer decreased in the order of heparinase I-treated > as-cultured > TGF-β_1-treated samples. The results suggested that glycocalyx may play a role in reducing the friction of endothelial cell monolayer.

Keywords: Friction, Endothelial cell, Glycocalyx, Gel scaffold, Lubrication

1. Introduction

It is interesting to observe that red blood cells (8 μm) deform and smoothly squeeze through capillaries with a substantially smaller diameter (5~6 μm) than their own. In this process, friction should occur between soft endothelial cells (ECs) (Young's modulus: 0.2~8 kPa)[1,2] which cover on the inner surface of blood vessel as a monolayer, and the deformable red blood cells (RBCs) (~ 0.1Pa) [3]. ECs are shielded from direct exposure to flowing blood by a layer of macromolecules known as the glycocalyx [4-10], which has an evenly distributed, discrete, bush-like structure of thickness 0.5~3 μm, greatly exceeds that of the ECs (~0.2 μm) themselves [8]. The glycocalyx is a negatively charged layer composed of glycoproteins and proteoglycans [9,10] and performs a wide variety of critical functions in the fluid environment, such as the mechano-transduction of shear stress [5,11], exclusion of red blood cells from the EC monolayer [12-15], modulation of leukocyte attachment and rolling [16-18], and inhibition of platelet adhesion [19]. However, to our knowledge, the tribological role of the glycocalyx is unclear.

Apparently, to ensure that RBCs smoothly squeeze through the capillaries, the surface friction between the EC monolayer and the RBCs should be low. In fact, the frictional shear stress exerted on the wall of blood vessel is ~2 Pa [20] under the static pressure of ~3 kPa for capillary [21].

Low sliding friction has also been observed between other biological soft tissues. For example, the cartilages of animal joints have a friction coefficient in the range of 0.001~0.03 [22], which is remarkably low even for hydrodynamically lubricated journal bearings. These fascinating tribological properties of biological systems arise due to the soft and wet nature of the tissues.

The friction between solids obeys Amonton's law (1699), $F = \mu W$, which says that the frictional force F is linearly proportional to load W, and does not depend on apparent contact area A of two solid surfaces and sliding velocity v. A hydrogel consists of a cross-linked polymer network that is solvated with water, and it therefore has a soft and wet

nature identical to that of living tissue. From this viewpoint, the frictional behaviors of various kinds of hydrogels have been extensively studied using a tribometer and rheometer and by the surface force balance technique [23-30]. We have reported that the gel friction does not simply obey this Amonton's law, and the frictional behaviors of hydrogels are very rich and complex. For example, using a tribometer and rheometer, it has been found that hydrogel friction depends on the chemical structure of the gel, the surface properties of the sliding substrates, and the measurement conditions such as normal force, sliding velocity, sample size, and sample thickness. The friction coefficient of gels, μ changes across a wide range and exhibits very low values ($\mu \approx 10^{-3} - 10^{-4}$) that are substantially lower than those between two solid materials. Furthermore, the presence of non-adhesive dangling chains on the gel surface dramatically reduces the friction, and the brush-like gels exhibit a friction coefficient as low as 10^{-4} [23].

In this study, we investigate the tribological role of the glycocalyx on the surface of ECs in a fluid environment. Glycocalyx can be considered as a hydrogel layer with negative charges, because it is a negatively charged macromolecules composed of glycoproteins and proteoglycans [11,12] whose polymer volume fraction has been theoretically estimated to be 1% ~ 2% [31,32]. We assume that the glycocalyx is crucial for the surface friction of the EC monolayer. To verify this hypothesis, we first established a system to measure the sliding friction of a living human umbilical vein endothelial cell (HUVEC) monolayer cultivated on soft and wet hydrogel surface against a flat glass substrate immersed in protein-free Humedia-EB2 medium (HEM). The frictional behavior of three sets of HUVEC monolayer were investigated: 1) as-cultured HUVEC monolayer, 2) transforming growth factor β_1 (TGF-β_1)-treated HUVEC monolayer, which increased glycocalyx by 148%, 3) heparinase I-treated HUVEC monolayer, which reduced glycocalyx by 57%, both were compared with that of the as prepared one.

Most *in vitro* studies on anchorage-dependent mammalian cells such as ECs are performed

using cells cultured on hard and dry substrates such as cover glasses and polystyrene plates. However, when ECs cultured on a hard substrate are slid against another hard surface under normal stress, they are easily destroyed due to the stress concentration. Therefore, it is not possible to study the tribological properties of EC monolayers cultured on hard and dry scaffolds. The cultivation of EC monolayers on soft and wet hydrogels, which have a three-dimensional network structure and viscoelasticity similar to that of the macromolecular-based extracellular matrix (ECM) in biological tissues, is an optimum strategy for overcoming this problem. In this study, we cultured a continuous EC monolayer on poly(sodium *p*-styrene sulfonate) (PNaSS) gel [33, 34] that contains *c.a.* 93wt% water and has a Young's modulus of 59.4 kPa. This study is helpful for understanding the low friction mechanism observed in vascular systems and for designing artificial blood vessels with low friction surfaces.

2. **Materials and methods**

2.1 Materials

N, N′-methylenebis-(acrylamide) (MBAA; Tokyo Kasei Kogyo, Tokyo, Japan), as a cross-linking agent, was purified by recrystallization from ethanol. Sodium *p*-styrene sulfonate (NaSS; Tokyo Kasei Kogyo, Tokyo, Japan), as a monomer, and 2-oxoglutaric acid (Wako Pure Chemicals, Osaka, Japan), as an initiator, were used as purchased.

2.2 Hydrogel preparation

Sheet-shaped PNaSS gel was synthesized by radical polymerization of an aqueous solution of 1 M monomer (NaSS), 10 mol% cross-linker (MBAA), and 0.1 mol% initiator (2-oxoglutaric acid) contained in the reaction cells that were formed by two parallel glass plates separated by a silicone spacer of 1.5-mm thickness for 6 h (the wavelength of UV light is 365 nm). After gelation, the gels were removed from the glass cells and immersed in a large volume of ion-exchanged water for 1 week. During this period, the water was changed 2 times every day

in order to remove residual chemicals.

The pH and ionic strength of the solution in the gel were adjusted to 7.4 and *c.a.* 0.15 M, respectively, by immersing the gel in HEPES (Sigma) buffer solution (HEPES, 5×10^{-3} M; $NaHCO_3$, 1.55×10^{-2} M; NaCl, 0.14 M; pH, 7.4). The above gel was denoted as HEPES buffer-equilibrated gel.

2.3 Cell culture

After sterilization by autoclaving (120˚C, 20 min), the HEPES buffer-equilibrated gel disks of radius 7.5 mm and thickness *ca.* 3 mm were used for HUVEC (KURSBO Biomed. Bus.). A HUVEC suspension (2.26×10^4 cells/cm^2 in 20% FBS Medium-199) was directly seeded on the gel surfaces that not been modified by any cell-adhesive proteins or peptides before cell culture. The HUVEC-loaded samples were cultured at 37°C in a humidified atmosphere of 5% CO_2 until the cells proliferated to a monolayer (144 h). The details were described in a previous paper [34].

2.4 Hydrogel characterization

The Young's modulus (*E*) of the PNaSS gel was measured by compressive stress-strain measurements using a tensile-compressive tester (Tensilon RTC-1310A, Orientec Co.). From the stress-strain curve, *E* was determined by the average slope in a strain ratio range of 0 to 0.1. *E* of the PNaSS gel was 59.4 kPa and 60.2 kPa after equilibration in protein-free and 20% FBS-containing HEM, respectively, which did not obviously change because the ionic strength (*c.a.* 0.15 M) of the two media was identical. The details were described elsewhere [35].

2.5 Glycocalyx modulation and immunofluorescence.

Heparan sulfate proteoglycans (HSPGs) which consist of a core protein and heparan sulfate-type glycosaminoglycan, are main proteoglycans of glycocalyx [7, 8] To increase the amount of HSPGs on the HUVEC surface, the HUVEC monolayers were incubated for 48 h in 20% FBS-containing HEM with 20 ng/ml TGF-β_1 [36, 37]. On the other hand, to disrupt HSPGs, HUVEC monolayers were incubated for 4 h in protein-free HEM containing 1.45

U/ml heparinase I (Sigma) [17, 38]. Detection of HSPGs was performed as follows [39]: HUVECs were chilled briefly on ice and rinsed three times with ice cold 1×PBS. Then the HUVECs were incubated on ice with primary heparan sulfate antibody (US Biological, Swampscott, MA), 1:200, for 1 h, followed by ice-cold 1×PBS rinses. Secondary antibody Alexa 488 (Molecular Probes) was used at 1:200 for 1 h. After rinsing three times with ice cold 1×PBS, immunostained samples were examined on an Olympus FluoView FV500 Laser Scanning Confocal Microscope (Olympus, BX 50). The fluorescence intensity of the TGF-β_1- or heparinase I-treated HUVEC monolayers relative to that of the as-cultured HUVEC monolayers corresponds to the change in the amount of HSPGs on the HUVEC surface.

2.6 Friction measurement

All frictional tests were performed using a rheometer (Advanced Rheometric Expansion System, "ARES," Rheometric Scientific Inc.). A schematic representation of the friction measurement of the EC monolayers is shown in Fig. 1. Disk-shaped gel of radius 7.5 mm with a HUVEC monolayer on one surface was used as the test sample. The bare surface of the gel was glued to the upper surface of a coaxial disk platen by using cyanoacrylate, an instant adhesive agent (Toagosei Co., Ltd.). A square, flat glass substrate (30 mm × 30 mm) or disk-shaped PNaSS gel of radius 12.5 mm with the same HUVEC monolayer on one surface was used as the opposing substrate.

Three sets of cell-glass experiments were performed: 1) as-cultured HUVEC monolayers vs. glass, 2) TGF-β_1-treated HUVEC monolayer vs. glass, 3) heparinase I-treated HUVEC monolayer vs. glass. In addition, three sets of cell-cell experiments were performed: 1) as-cultured HUVEC monolayers vs. as-cultured HUVEC monolayers 2) TGF-β_1-treated HUVEC monolayer vs. TGF-β_1-treated HUVEC monolayer, 3) heparinase I-treated HUVEC monolayer vs. heparinase I-treated HUVEC monolayer.

The opposing substrates were glued to the bottom of the container with an inner radius of 60 mm, which served as the lower platen parallel to the upper platen. The test sample and

opposing substrate were submerged in a container containing protein-free HEM (37°C), and the two surfaces were compressed against each other under a normal pressure (P) of 3.3 kPa. This pressure is the minimum value to obtain stable and reproducible friction stress of this measurement system, which is similar to the pressure exerted on the capillaries (*c.a.* 3 kPa) [21] but much higher than the pressure acts to compress the glycocalyx *in vivo* [11]. After 10 min of static loading, the opposing substrate was rotated at an angular velocity (ω) of 10^{-3} to 10^{-2} rad/s, corresponding to a maximum sliding velocity ($v = \omega R$) of 7.5×10^{-6} to 7.5×10^{-5} m/s at the edge of the disk-shaped gel. The torque ($T(\omega)$) was detected when the opposing sliding substrate rotated with an angular velocity, ω, at a strain-constant mode. Since the sliding velocity changes with the distance from the center of the axis, the obtained torque, $T(\omega)$, is an integrated value all over the plate [26].

Our previous study has shown that the frictional stress corresponding to the sliding velocity of ωR was, $\sigma(\omega R) = [(3+\alpha)T(\omega)]/(2+\alpha)\pi R^3$, supposing that the torque has a power relation with ω as $T(\omega) \approx \omega^{\alpha}$. Usually, α lies between 0 and 1. Therefore, we use $\sigma(\omega R) = [4T(\omega)]/3\pi R^3$ to calculate the frictional force by setting, $\alpha = 1$. This gives a good approximation [26]. The details of the friction measurement were described in a previous paper [42].

3. **Results and discussion**

3.1 Establishment of measurement method

We previously reported that when two like-charged flat surfaces (negatively charged hydrogel vs. flat glass or two negatively charged hydrogels) are slid against each other in aqueous solution, the osmotic repulsion of the dissociated counter-ions of the charged surfaces sustains a lubricating water layer at the interface and leads to lubrication even under pressures up to the order of sub-MPa[23-28]. The EC surface is negatively charged because the glycocalyx layer has acidic glycosaminoglycans side chains, that contain many carboxyl and sulfate groups and

contribute to its negative charge and hydration [10]. On the other hand, the SiOH groups on the surface of glass dissociate with an increase in the pH and are negatively charged at pH = 7.4 [43]. Thus, we used flat glass (surface roughness: R_a=2 nm, contact angle to water: 22°) as the opposing substrate, expecting an osmotic repulsion of the counter-ions of the interface between the HUVEC monolayer and the flat glass substrate, which will prevent non-specific adhesion of glycocalyx to the substrate.

To accurately measure the surface friction of the HUVEC monolayer by using a rheometer (Fig. 1), we need to identify suitable testing conditions under which most HUVECs remain adhered to the gel surface in the process of friction test. During this process, when the HUVEC monolayer was compressed against the opposing substrate (flat glass or identical HUVEC-monolayers) and then the opposing substrate was subsequently rotated, a shearing stress was applied to the HUVEC monolayer. Therefore, the HUVECs were easily deformed, and they were even detached from the gel surface when the testing time is long. Fig. 2A shows the percentage of HUVEC that remained adhered to the gel surface after the friction test conducted at a sliding velocity of 7.5×10^{-6} m/s against the glass substrate. Comparing to that before friction test (Figure 2B), the density of HUVEC gradually decreased with testing time. Before 800 s, more than 80% HUVECs remain adhered to the gel surface (Fig. 2A, C). In contrast, the percentage of HUVEC was only 60% and 40%, at a testing time of 1200 s and 1800 s, respectively, due to high frictional stress (*i.e.*, frictional force per unit area) between HUVEC monolayer and flat glass. As shown in Fig.2, morphology of the most of HUVECs changed from polygonal shape (Fig. 2B) to fusiform shape (Fig. 2C), and to concentric circles (Fig.2D) after friction test, indicating that the HUVECs were deformed by the frictional shear stress.

To confirm if the frictional stress of the HUVEC monolayer could be measured by the current method as shown in Fig. 1, we first measured 3 sets of samples for a short testing time of 100s, within which almost 100% of cells remain adhered on the gel surface: a) HUVEC monolayer

vs. glass, b) bare PNaSS hydrogel vs. glass, c) ECM layer of HUVEC vs. glass. Here, the ECM layer of HUVEC is obtained by exfoliated the HUVEC monolayer from the PNaSS hydrogel scaffold by using cellular scraper, so that only ECM beneath the HUVEC remained on the surface. All the samples were equilibrated in HEM before test. As shown in Fig. 3, during sliding, the shear stresses of HUVEC monolayer and bare PNaSS hydrogel rapidly increased initially, reaching a peak of *c.a.* 300 Pa at a few seconds, then gradually decreased with the sliding time, and became constant after several tens of seconds. On the other hand, the shear stresses of ECM sample did obviously show a peak at initial sliding, and the shear stress was much higher than the other two samples, showing an unstable behavior. The frictional stresses of the three samples increased in the order of HUVEC monolayer < bare PNaSS hydrogel < ECM sample. These results indicate that for a short testing time, the frictional stress of the HUVEC monolayer can be accurately measured by the current method.

Based on the above results, the frictional stress, where a constant value with testing time was reached and at the same time 100% HUVECs remained adhered on the gel surface, was adopted as dynamic frictional stress (σ_{dyn}). In addition, the peak value of the frictional stress was adopted as the static friction stress (σ_{stat}) that is required to start the sliding between the HUVEC monolayer and the flat glass substrate. The ratio of σ_{stat} (σ_{dyn}) to the normal stress applied, corresponding to the static friction coefficient μ_{stat} (dynamic friction coefficient μ_{dyn}), was estimated in the following experiment.

3.2 Effect of glycocalyx on EC monolayer friction

3.2.1 Glycocalyx modulation

The glycocalyx consists of negatively charged glycoproteins and proteoglycans [7, 8]. Of proteoglycans, the main component is heparan sulfate proteoglycans (HSPGs) (50~90wt%), which consist of a core protein and heparan sulfate-type glycosaminoglycan, containing

numerous negatively charged sulfate groups and binds several plasma proteins [7]. The amount of glycocalyx on the EC surface can be enhanced using TGF-β_1, a cytokine that stimulate heparan sulfate proteoglycans (HSPGs), the main proteoglycan of glycocalyx synthesis [37, 38]. It was reported that TGF-β_1 treatment can increase the HSPG content on the EC surface by more than 50%. Furthermore, the glycosaminoglycan chains of a kind of HSPG synthesized by the ECs, *i.e.*, bioglycan, were elongated by the TGF-β_1 treatment [38].

On the other hand, the HSPGs on the EC surfaces can be disrupted by heparinase I, an enzyme that cleaves glycosaminoglycan side-chains from HSPGs [18, 39, 40]. It was demonstrated that heparinase treatment reduced the fluorescence intensity of fluorescence-labeled wheat germ agglutinin by 27-51% [41] and the fluorescence intensity associated with the heparan sulfate antibody by 45.9% [40]. Furthermore, the thickness and negative charge of the glycocalyx were reduced by heparinase disruption [44].

In this study, we intended to modulate the amount of the glycocalyx on HUVECs by treating the as-prepared HUVEC monolayers with TGF-β_1 and heparinase I. To verify the extent of change in the HSPGs after treatment, the as-cultured, TGF-β_1-treated, and heparinase I-treated HUVEC monolayers were subjected to immunostaining analysis. Fig. 4A shows the fluorescence intensity ratios of HUVEC monolayers treated with TGF-β_1 or heparinase I to that of the as-cultured HUVEC monolayer. Morphology of HUVEC monolayer treated by heparinase I and TGF-β_1 are shown in Fig.4C and Fig.4D, respectively. Pictures of immunostained HUVEC monolayers examined by a Laser scanning confocal microscope are shown in Fig.4E, Fig.4F, and Fig.4G for untreated, heparinase I treated, and TGF-β_1 treated samples, respectively. The fluorescence intensity clearly decreased to 57.0% due to heparinase I treatment and increased to 148% due to TGF-β_1 treatment. The results demonstrated that the amount of HSPGs in the glycocalyx substantially increased and decreased after TGF-β_1 and heparinase I treatment, respectively.

3.2.2 Frictional behavior of EC monolayer

Fig. 4B shows the typical time profiles of the frictional shear stresses of the as-cultured, TGF-β_1-treated, and heparinase I-treated HUVEC monolayers slid against the flat glass substrate at a sliding velocity of 7.5×10^{-6} m/s. The three samples showed similar time profiles, and the frictional stress changed remarkably with glycocalyx treatment. The static (σ_{stat}) and dynamic (σ_{dyn}) frictional stress of HUVEC monolayer decreased in the following order: heparinase I-treated > as-cultured > TGF-β_1-treated samples. The static (σ_{stat}, μ_{stat}) and dynamic (σ_{dyn}, μ_{dyn}) frictional stress and frictional coefficient for the samples with various amount of glycocalyx are summarized in Fig. 5.

To judge if cells underwent apoptosis or not after friction test, we cultivated the cells remained adhered on the hydrogel scaffolds after friction test by placing the samples in culture dish and incubated at 37°C in a humidified atmosphere of 5% CO_2. We have found that the ECs remained adhered and spread on the gel surface after 48 h, indicating that ECs did not undergo apoptosis, because if ECs underwent apoptosis, they would become round and detach from the hydrogel scaffolds.

For investigating the effect of glycocalyx on EC monolayer frictional behavior, surface friction behavior between two HUVEC monolayers was further studied. The results are also shown in Fig. 5. The static frictional stress (σ_{stat}) did not change obviously with the amount of the glycocalyx, and all the 3 samples showed a similar value. On the other hand, the dynamic frictional stress (σ_{dyn}) decreased in the following order: heparinase I-treated > as-cultured > TGF-β_1-treated samples, which is in a similar tendency with that between HUVEC and glass.

3.2.3 Sliding velocity effect

HUVECs were easily detached from the gel surface at a high sliding velocity. The time that

more than 80% HUVECs remained adhered to the gel surface was denoted as $T_{80\%}$. Fig. 6 (white bar) shows $T_{80\%}$ as a function of the sliding velocity for the HUVEC slid on glass. Compared to the $T_{80\%}$ of the as-cultured HUVEC monolayer, that of the TGF-β_1-treated monolayers did not change obviously, while that of the heparinase I-treated monolayers obviously decreased, especially when the sliding velocity was greater than 2.37×10^{-5} m/s. The $T_{80\%}$ of all three HUVEC samples rapidly decreased to less than 100 s when the sliding velocity was increased to 7.5×10^{-5} m/s. When the sliding velocity was increased to 7.5×10^{-4} m/s, the HUVECs were detached from the gel surface in a few seconds before the frictional stress became constant (data no shown).

Fig. 6 (oblique bar) shows $T_{80\%}$ as a function of the sliding velocity for the HUVEC slid on HUVEC. In the case of the as-cultured and TGF-β_1-treated HUVEC monolayers, at a sliding velocity of 7.5×10^{-6} m/s, the $T_{80\%}$ of HUVEC monolayer vs. HUVEC monolayer was larger than that of HUVEC monolayer vs. glass; in contrast, when the sliding velocity was increased to 2.37×10^{-5} m/s, the $T_{80\%}$ of HUVEC monolayer vs. HUVEC monolayer was less than that of HUVEC monolayer vs. glass. In the case of the heparinase I-treated HUVEC monolayers, the $T_{80\%}$ values of HUVEC monolayer vs. HUVEC monolayer at 7.5×10^{-6} m/s and 2.37×10^{-5} m/s were obviously less than those of HUVEC monolayer vs. glass. Furthermore, when the sliding velocity was increased to 7.5×10^{-5} m/s, the $T_{80\%}$ values of HUVEC monolayer vs.HUVEC monolayer and HUVEC monolayer vs. glass were identical, regardless of TGF-β_1 or heparinase I treatment. The results suggested that the glycocalyx brushes on the as-cultured and TGF-β_1-treated HUVEC monolayers easily interpenetrated mutually at static loading (3.3 kPa, 10 min), although this condition changed with changes in the sliding velocity.

The lower $T_{80\%}$ of the heparinase I-treated sample compared to other samples is associated with two effects: one is its higher frictional stress as shown in Fig. 4B, and the other is the reduced strength of EC adhesion on the gel scaffold. Li *et al.* reported that heparinase-treated ECs were more easily exfoliated than the as-cultured ECs from a flat glass substrate under the same shear

stress, because HSPG disruption with heparinase decreases the size of the prominent focal adhesion that is correlated to the adhesion strength of the ECs [39].

3.3 Discussions

3.3.1 Hydrogel lubrication model

The glycocalyx can be considered as a hydrogel layer with negative charges, whose polymer volume fraction has been estimated as 1% ~ 2%, that is, the water volume fraction of glycocalyx could be estimated as 98% ~ 99%. Here we try to apply the hydrogel friction model to discuss the frictional behavior of ECs. In order to favor the readers who stand at the point for biomaterials science, we shortly describe the hydrogel friction due to viscose drag here, that is, hydrogel lubrication model. When a gel sliding on a solid substrate with a liquid trapped at the gel-substrate interface, the dynamic frictional stress by the viscose drag of the liquid is [26],

$$\sigma_{dyn} = \frac{\eta v}{h + \sqrt{K_{gel}}} \qquad (1)$$

Here η is the viscosity of liquid, v the sliding velocity, h the thickness of the trapped liquid, K_{gel} the permeability of the gel. K_{gel} is related to the correlation length ξ of the gel as $\xi = \sqrt{K_{gel}}$, which decreases with the increase in the network cross-linking density. For a conventional 3-dimentionally cross-linked gel, ξ is in the order of 1-10 nm. Equation 1 indicates that the shear flow can penetrate into the gel with a thickness of the correlation length, or the equivalent non-slippery boundary of a gel surface is located at a depth of ξ from the gel surface. On the other hand, the thickness of the trapped liquid h is from two possible effects: 1) The electric double layer (EDL) formation at the like-charged interface. When two like-charged surfaces approach each other, which is also the case of our system, an osmotic repulsion exists between the interfaces to form an EDL. This EDL is strongly dependent on the ionic strength or the Debye length. At the physiological condition, the Debye length is in the order of 1nm; 2) Drainage liquid at interface. When two surfaces approach each other under compression in a liquid, a finite drainage time is required before the surfaces getting contact. So liquid is, transiently, trapped at the interface and serves as lubrication layer.

The drainage layer thickness *h* is related to the drainage time τ, the surface area *A*, and the compression pressure *P* as $h = \sqrt{3\eta A / 4P\tau}$ [45].

3.3.2 Discussion of RBC friction in vivo using hydrogel lubrication model

In the *in vivo* system, the shear stress exerted on the wall of the capillary was found about 2 Pa [20]. If we assume that this dynamic shear stress is contributed by the viscose drag of the liquid entrapped at the RBC- EC interface with an average thickness *h* and in the glycocalyx matrix with a permeability K_{gel} on the ECs surface, the apparent lubricating thickness $h + \sqrt{K_{gel}}$ is in the order of 1μm, estimated from Eq. 1 using the viscosity of the plasma $\eta = 10^{-3}$Pas and $\nu = 10^{-3}$m/s for RBC flow [46]. This value is much larger than the Debye length (1nm) of the physiological environment, indicating that the EDL surrounding the EC monolayer is so thin that plays an insignificant role in friction reduction. Furthermore, the drainage layer thickness for RBC is estimated as 80 nm using the size of RBC 8μm, drainage time 1 s (for a single RBC in capillary it takes 0.5 s for the water to escape), and pressure 10 Pa [11]. This estimation suggests that the dominant origin of lubrication is not due to the formation of EDL or the drainage layer, but due to the permeability of the glycocalyx matrix to liquid, that is, $h + \sqrt{K_{gel}} \approx \sqrt{K_{gel}}$. The apparent lubricating thickness of 1μm is much larger than the correlation length ξ of conventional 3-dimentionally cross-linked gels, and it is in the same order of brush-like glycocalyx layer thickness. The result suggests that the one-end anchored glycoclayx brushes will both sustain the load and enhance the effective lubricating layer thickness owing to their easy deformation, and therefore reduce the shear resistance dramatically. Similar dramatic friction reduction effect by drafting the polymers on gel surfaces has been observed in synthetic gel systems[47,48].

The above discussion on the friction of RBC is in agreement with the direct observation of the RBC-EC gap by Han et al [49]. Han et al have reported when RBCs flowing through hamster

cremaster muscle capillary, RBCs change their morphology and squeeze through the capillary in the center, there is clearly identified gap between the membrane of flowing RBCs and ECs in the capillary. The dimension of RBC-EC gap is in the order of 1μm. It has been found that the RBC-EC gap is cell free and only contains plasma (and probably the glycocalyx layer), so the gap is also called a plasma layer. This plasma layer was assumed to serve as lubricating layer, contributing for RBCs smoothly flow through on EC surface. So the results discussed based on the gel friction model seems to be in agreement with this assumption.

3.3.3 Discussion of ECs gel friction in vitro using hydrogel lubrication model

The frictional stress observed in this work was in the order of 10~100 Pa even for the TGF-β_1-treated sample, which is considerably higher than the shear stress exerted on the wall of the blood vessels of the *in vivo* system that has a value of 2 Pa [20]. The high frictional stress cannot be understood by the viscose drag mechanism. In fact, if we apply Equation 1, the apparent lubricating layer thicknesses were estimated as $\sqrt{K_{gel}}$ =0.058 nm, 0.25 nm, 0.022 nm, for as-cultured, TGF-β_1-treated, and heparinase I-treated HUVEC monolayer slide against flat glass, respectively (using $\eta = 10^{-3}$Pas, sliding velocity in this friction test $v = 7.5\times10^{-6}$m/s, and average σ_{dyn} = 130 Pa, 30 Pa, and 340 Pa for as-cultured, TGF-β_1-treated, and heparinase I-treated samples, respectively). These apparent lubricating layer thicknesses have no any physical meanings, indicating that it is not proper to consider that the friction is predominantly originated from viscose drag. Direct contact between the glass substrate and the glycoxalyx or the cell surface should occur, and accordingly the dominant contribution to the frictional shear stress is from this direct contact. The large difference observed in this study and *in vivo* might be associated with the following three reasons:

(i) Friction was tested under a normal pressure, 3.3 kPa, which is 10 to100 times higher than the pressure exerted on the EC by the red cell membrane *in vivo* [11]. Under such a high

pressure, the glycocalyx layer was highly compressed, and this will lead two effects that increase the friction: 1) An increase in the contact of solid component (glycocalyx) with the opposing surface; 2) Decrease of the permeability to liquid.

(ii) Poor growth of the glycocalyx layer on the HUVECs cultured *in vitro*. Recent studies have found glycocalyx deficiency when the ECs are cultured *in vitro*[50, 51]. Damiano E R. et al showed that the glycocalyx thickness of *in vitro* cultured human umbilical vein was 0.03 μm under standard cell culture conditions, *c.a.* 17 times shorter than that of microvessels *in vivo* (0.52 μm) [50]. Although our study indicated that the amount of glycocalyx on HUVECs cultured on the negatively charged synthetic hydrogels is obviously higher than that cultured on polystyrene culture dish[34], it is not clear if the former is as much as that *in vivo*. As indicated by Damiano E R [50], a deficiency in glycocalyx for ECs cultured under standard cell culture conditions likewise casts doubt on the applicability of using these EC cells to many of the vascular function and pathology studies.

(iii) The hard nature of the opposing sliding substrate (flat glass) is not favorable for sustaining a large amount of water as lubrication layer at the interface. The drainage layer thickness is theoretically estimated as 100 nm using the lateral dimensions of platen, 7.5 mm, the pressure 3 kPa, and the overall measurement time 2000 s, including the preload time. This thickness probably is too thin to maintain a continuous lubricating layer at the interface, considering the possible mis-alignment between the two parallel platens of the apparatus, and some part of the sample would be in direct contact with the hard glass surface[52].

When the HUVEC monolayers are treated with TGF-β_1, HSPG synthesis is stimulated and the amount of HSPGs on the surface of the HUVEC monolayer increases [37, 38]. This results in an increase in the amount of glycocalyx, either by increase in the number or in the length of the brushes. This enhancement in glycocalyx will favor the water holding ability of the glycocalyx layer against the normal compression and therefore reduce the friction. On the other hand, when the HUVEC monolayers are treated with heparinase I, HSPGs are disrupted, *i.e.*, the

amount of HSPGs on the HUVEC monolayers decreases [18, 40]. This leads to a decrease in the glycocalyx of the HUVEC monolayers [42], and the water holding ability decreases. In addition, after been treated by heparinase I for 4 h in serum free culture medium, the cell-cell contact became looser and cell became a little smaller than before HUVEC treatment. Cell shape and membrane characteristics could also influence sliding properties.

Finally, we discuss the friction behavior between HUVEC monolayers. When two HUVEC monolayers are compressed against each other and a shearing stress is subsequently applied, the overlapping of the two soft HUVEC monolayers will significantly influence the frictional behavior. Mutual interpenetration of the brush-like glycocalyx between the opposing HUVEC monolayers might occur because the normal pressure is high. This mutual interpenetration will increase the resistance against shear motion, and the depth of interpenetration should increase with the contact time. This effect may explain why the static friction (σ_{stat}) between the HUVEC monolayers did not obviously change with the amount of glycocalyx: during compression, the glycocalyx brushes on the surface of the as-cultured and TGF-β_1-treated HUVEC monolayers might interpenetrate mutually to give a high resistance.

4. **Conclusions**

In this study, we established a direct experimental method for investigating the surface sliding friction of EC monolayers cultured on a soft, PNaSS gel scaffold by using a rheometer, and we studied the role of the glycocalyx on the surface friction of ECs. Using a rheometer to determine the frictional behavior of the EC monolayer has several advantages such as the possibility of measuring a small (several mm) living EC monolayer cultured on a flat plate, the high sensitivity in detecting extremely small frictional torque, and the possibility of performing the measurement in the culture medium.

The frictional stress on a smooth flat glass surface was found to decrease when the HUVEC monolayers were treated with TGF-β_1, which stimulates the synthesis of HSPGs on the

HUVEC surface, while it increased when the HUVEC monolayers were treated with heparinase I, which disrupts HSPGs synthesis. Comparing with the as cultured and TGF-β_1-treated HUVECs, heparinase I-treated HUVECs are easily detached from the cell culture scaffold, because of high friction and weak focal adhesion. So under the present experimental condition, an enhancement in the amount of glycocalyx will increase the osmotic pressure of the glycocalyx layer which sustains the high pressure applied and retain more liquid in the glycocalyx gel layer, and favors the low friction. The study suggests that the glycocalyx play an important role in reducing the surface friction of ECs.

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Figure captions

Figure 1　Schematic representation of the experimental setup for friction measurement of EC monolayers cultured on PNaSS hydrogel. (1) Disk-shaped gel (radius R = 7.5 mm, thickness H = 3 mm), (2) HUVEC monolayer cultured on the gel, (3) protein-free HEM, (4) opposing substrate (glass plate or HUVEC monolayer cultured on PNaSS gel; H = 3 mm). Normal stress: 3.3 kPa; temperature: 37 °C.

Figure 2　Information regarding HUVECs on the gel surface after the friction test. (A) Ratio of the number of HUVECs after and before the HUVEC monolayers were slid against the glass substrate for different times at a sliding velocity of 7.5×10^{-6}m/s. The average number was determined by counting the number of HUVECs after sliding for different times. The number of HUVECs was counted using micrographs ((B) and (C)) that were taken over a region 5~7.5 mm from the center of the disk-shaped samples; 4 micrographs were taken per sample. The error ranges are standard derivation over 3~5 samples. Normal stress: 3.3 kPa. (D) A representative phase-contrast micrograph of a TGF-β_1-treated HUVEC monolayer after it was slid against an identical HUVEC monolayer for 1200 s at a sliding velocity of 7.5×10^{-6} m/s. Concentric circles were clearly observed. The image is that of a disk-shaped sample attached to the upper plate of the rheometer.

Figure 3　Time profiles of the frictional stress for 3 sets of samples: a) HUVEC monolayer vs. glass, b) bare PNaSS hydrogel vs. glass, c) ECM of HUVEC vs. glass. Sliding velocity: 7.5×10^{-6} m/s.

Figure 4　Analysis of cell surface HSPGs in the HUVEC glycocalyx (A) and time profiles of frictional stress (B) of the corresponding HUVEC monolayers. (A) Fluorescence intensity ratio

of HUVECs monolayers treated with heparinase I or TGF-β_1 to the as-cultured HUVEC monolayer; 5 micrographs were taken per sample. The error ranges are standard derivation over 3~4 samples. (B) Time profiles of frictional stress of as-cultured, heparinase I-treated, and TGF-β_1-treated HUVEC monolayers. Sliding velocity: 7.5×10^{-6} m/s. Normal stress: 3.3 kPa. Morphology of HUVEC monolayer treated by heparinase I and TGF-β_1 are shown in C and D, respectively. Pictures of immunostained HUVEC monolayers examined on a laser scanning confocal Microscope are shown in E, F, and G for untreated, heparinase I treated, and TGF-β_1 treated samples, respectively. Scale bar in E, F, G:200μm.

Figure 5 Static (σ_{stat}) and dynamic (σ_{dyn}) frictional stress of HUVEC monolayer vs. glass, HUVEC vs HUVEC monolayers, for as-cultured, heparinase I-treated, and TGF-β_1-treated samples. The right vertical axis is the corresponding results of static (μ_{stat}) and dynamic (μ_{dyn}) frictional coefficient that is defined as the ratio of the frictional force to the normal load applied. Sliding velocity: 7.5×10^{-6} m/s. Normal stress: 3.3 kPa.

Figure 6 Time for which more than 80% HUVECs remained adhered to the PNaSS gel surfaces after the friction test for HUVEC monolayers sliding against a glass substrate ($T_{80\%}$ of HUVECs-glass) and those sliding against identical HUVEC monolayers ($T_{80\%}$ of HUVECs-HUVECs) for as-cultured, heparinase I-treated, and TGF-β_1-treated HUVEC monolayers. White bar: HUVECs-glass, bar with oblique lines: HUVECs-HUVECs. The average time was determined by counting the number of HUVECs after sliding different for times. The number of HUVECs was counted using micrographs that were taken over a region 5~7.5 mm from the center of the disk-shaped samples; 4 micrographs were taken per sample. The error ranges are standard derivation over 3~5 samples. Normal stress: 3.3 kPa.

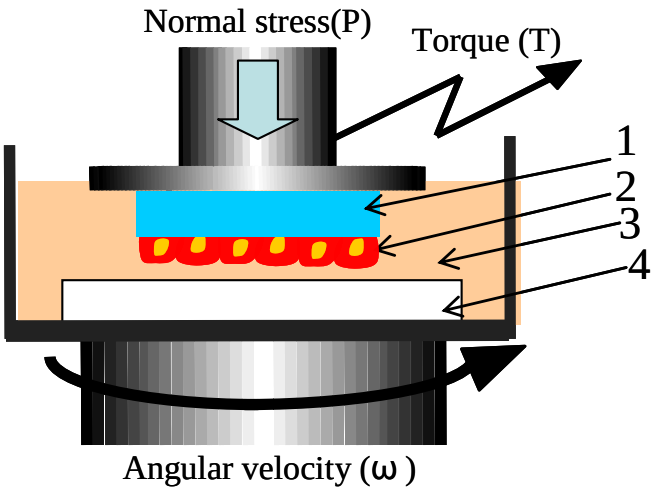


Fig. 1

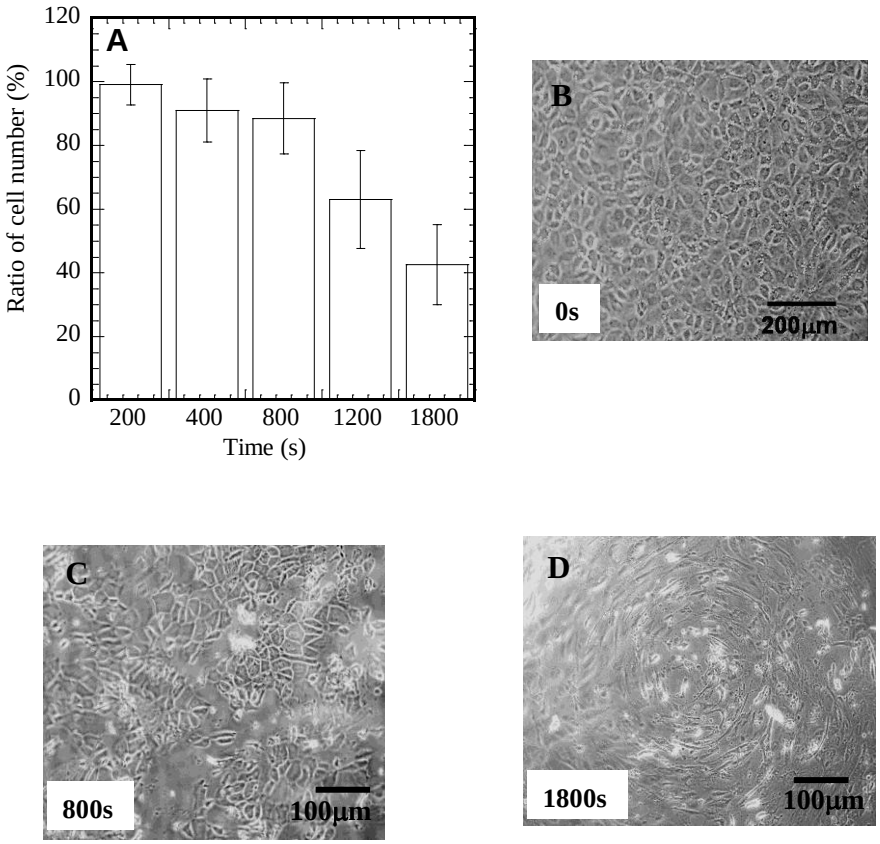

A
Ratio of cell number (%)
120
100
80
60
40
20
0
200
400
800
1200
1800
Time (s)
B
0s
200μm
C
800s
100μm
D
1800s
100μm

Fig. 2

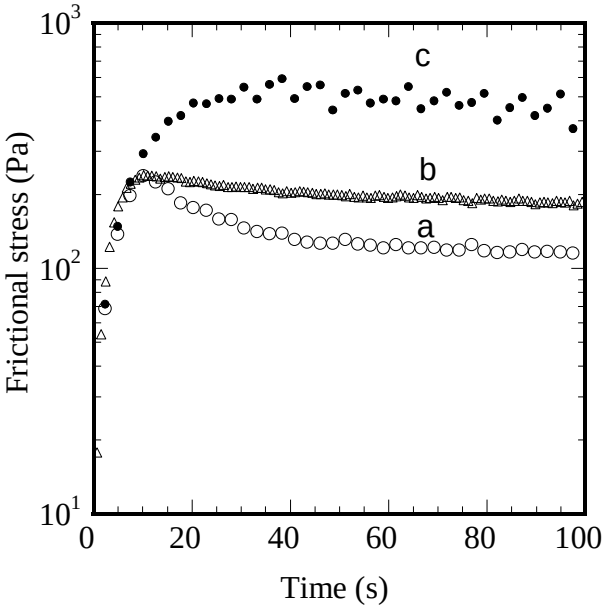
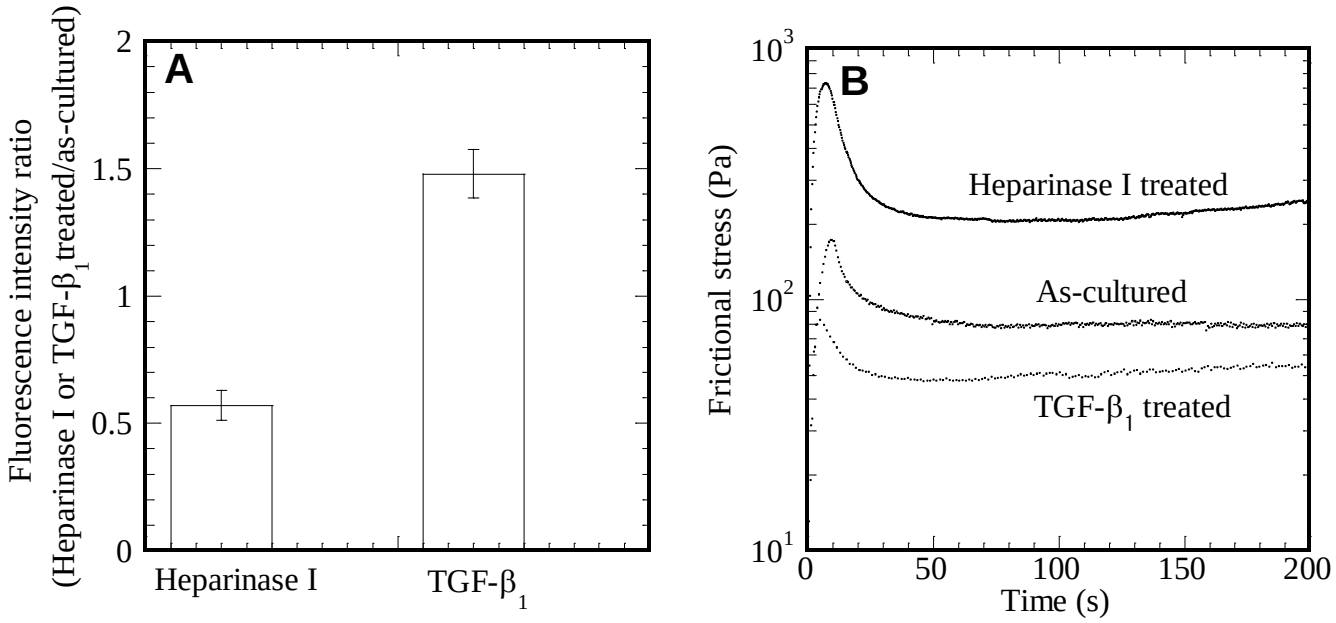
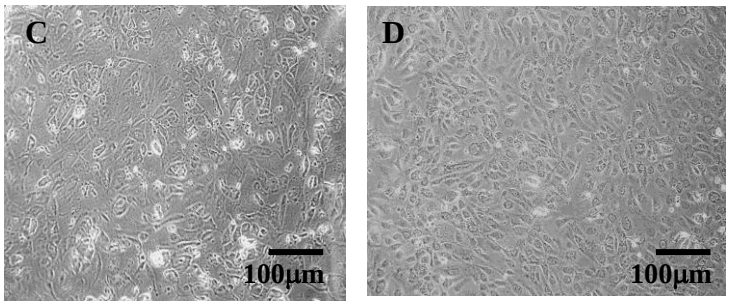


Fig. 3


A
Fluorescence intensity ratio
(Heparinase I or TGF-β1 treated/as-cultured)
2
1.5
1
0.5
0
Heparinase I
TGF-β1
B
Frictional stress (Pa)
10^3
10^2
10^1
Heparinase I treated
As-cultured
TGF-β1 treated
0
50
100
150
200
Time (s)


C
100μm
D
100μm

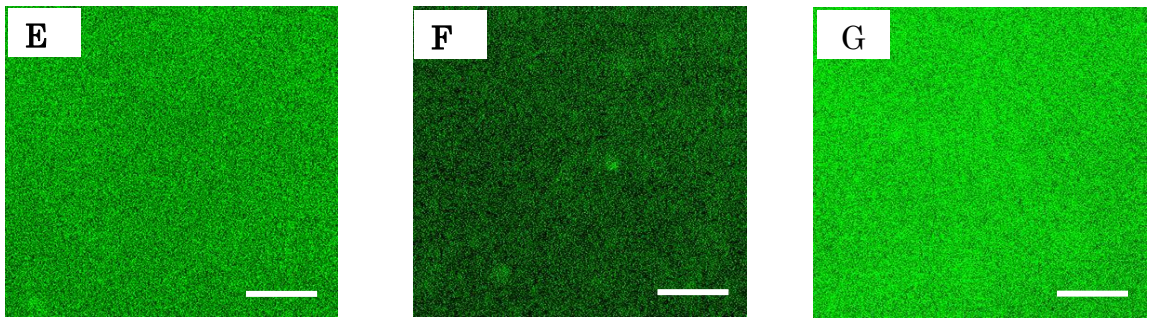

E
F
G

Fig. 4

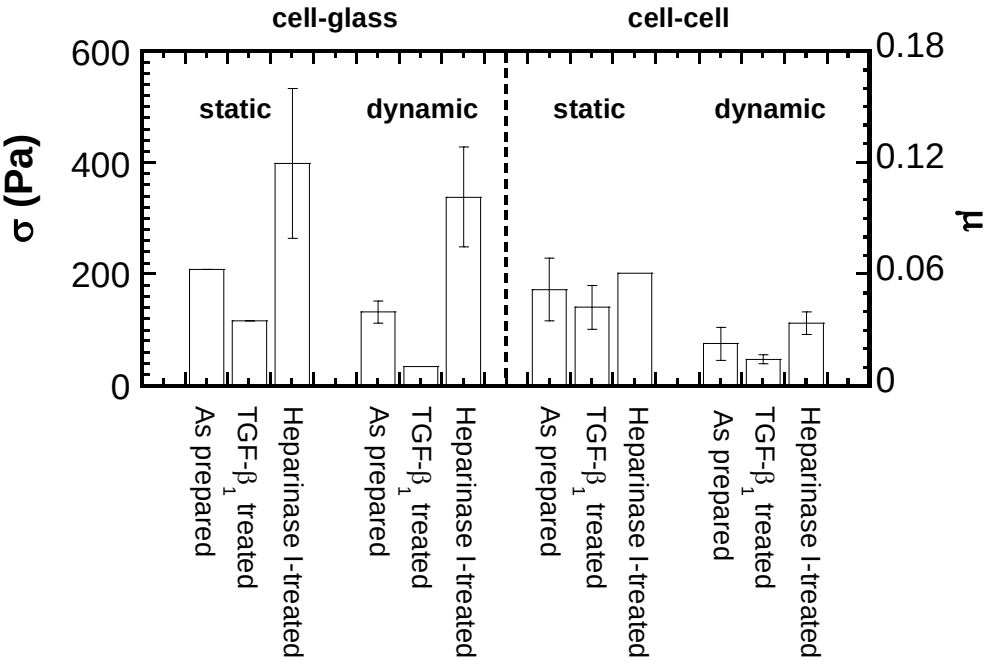

cell-glass
cell-cell
600
400
200
0
σ (Pa)
0.18
0.12
0.06
0
η
static
dynamic
static
dynamic
As prepared
TGF-β1 treated
Heparinase I-treated
As prepared
TGF-β1 treated
Heparinase I-treated
As prepared
TGF-β1 treated
Heparinase I-treated
As prepared
TGF-β1 treated
Heparinase I-treated

Fig. 5

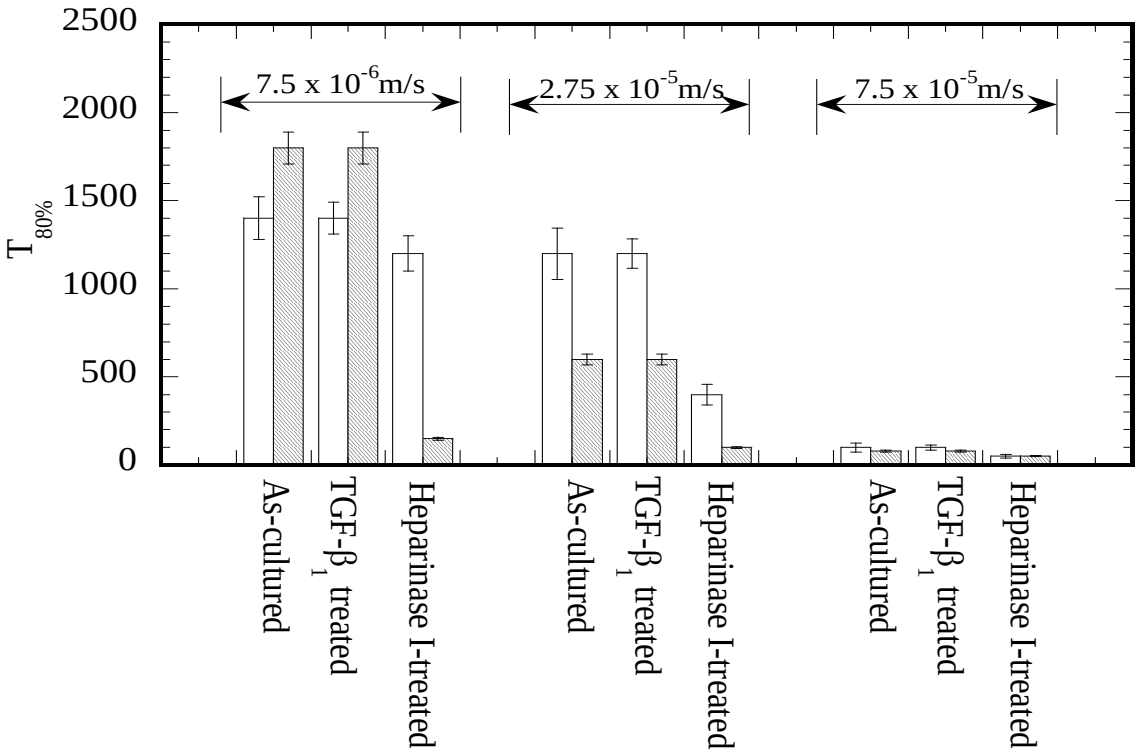

2500
2000
1500
1000
500
0
$T_{80\%}$
7.5 x 10^{-6}m/s
2.75 x 10^{-5}m/s
7.5 x 10^{-5}m/s
As-cultured
TGF-β_1 treated
Heparinase I-treated
As-cultured
TGF-β_1 treated
Heparinase I-treated
As-cultured
TGF-β_1 treated
Heparinase I-treated

Fig. 6

Table of Contents

Study on the Sliding Friction of Endothelial Cells Cultured on Hydrogel and the Role of Glycocalyx on Friction Reduction

Sliding friction of human umbilical vein endothelial cell (HUVEC) monolayer cultured on poly(sodium *p*-styrene sulfonate) (PNaSS) hydrogel was investigated, intending to elucidate the role of the glycocalyx on the surface of endothelial cell in friction reduction. Three sets of HUVEC monolayers were investigated: 1) as-cultured HUVEC monolayer, 2) HUVEC monolayer treated by transforming growth factor β_1 (TGF-β_1), which increased glycocalyx, 3) HUVEC monolayer treated by heparinase I, which reduced glycocalyx. The frictional stress of these samples against glass substrate decreased in the order of heparinase I-treated > as-cultured > TGF-β_1-treated samples, suggested that glycocalyx may play a role in reducing the friction of endothelial cells.

