Glycocalyx Regulates the Intravascular Hemostasis

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In recent years, close attention has been paid to the role of glycocalyx, a structural component of the vascular endothelium that is made up of glycoproteins, proteoglycans, sugar chains, etc. Glycocalyx has been shown to have diverse functions, including suppression of blood coagulation within the vascular lumen, as well as regulation of platelet/neutrophil adhesion to the vascular lumen, regulation of the vascular permeability, sensing of mechanical stimuli such as shear stress, etc. For technical and other reasons, few advances have been made in research on glycocalyx, which has quite delicate and fragile features. However, with the recent progress in testing methods, research on glycocalyx has entered a new phase. Now, glycocalyx is known to play important roles not only in the pathogenesis of acute diseases (e.g., sepsis and post-ischemic reperfusion disorders), but also in the pathogenesis of chronic diseases such as diabetes mellitus and atherosclerosis. In this paper, we present the latest findings on the structure and functions of glycocalyx and their relationships to illnesses, etc.

Key words: glycocalyx, syndecan, glycosaminoglycan, heparan sulfate, antithrombin

Structure and functions of glycocalyx

It is now well known that the surface of the vascular endothelium contains a structure called “glycocalyx,” and that this structure regulates thrombus formation and inflammatory reactions¹⁾. Glycocalyx is composed of a membrane-binding domain (core proteins) such as proteoglycan and glycoprotein conjugated with sugar chains and plasma protein, as well as sugar chains (hyaluronan, etc.) that is not directly bound to the cell membrane²⁾ (Figure-1). Glycocalyx is a rather fragile structure that is visualized like dense whiskers under an electron microscope. It is synthesized in the vascular endothelial cells, expressed on the endothelial cell surface, and eventually released into the blood. It repeats cycles of these metabolic changes. Glycocalyx is present not only on the endothelial cell surface, but also in the spaces between neighboring endothelial cells

and on the side of the endothelial cells close to the basal membrane. The spaces between endothelial cells are called “endothelial clefts (ETC),” and as described later, glycocalyx present at this site plays an important role in the regulation of vascular permeability³⁾.

1. Proteoglycan

Several types of proteoglycans are known, including syndecan, glypican and biglycan. Syndecan is a representative type of proteoglycan, composed of the transmembrane core protein and sugar chain called “glycosaminoglycan (GAG),” which binds to the extracellular domain of the core protein. Heparan sulfate and chondroitin sulfate are representative GAGs, with the former accounting for 50% to 90% of all GAGs. Factors controlling the coagulation cascade, such as antithrombin, activated protein C, and tissue factor pathway inhibitor (TFPI), bind to heparan sulfate, suggesting that

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Figure-1 Glycocalyx, a structural component of the vascular endothelium

The vascular endothelial surface contains a structural component called “glycocalyx,” which is composed of membrane-bound domains, such as proteoglycans and adhesion molecules (glycoproteins, etc.) and non-membrane bound domains, such as hyaluronan. Of these elements, syndecan (a representative proteoglycan) is composed of a transmembrane core protein and the sugar chain called “glycosaminoglycan” (heparan sulfate, chondroitin sulfate, etc.). Antithrombin, growth factor, cytokines, etc., bind to heparan sulfate, and the signals arising from each binding are transmitted to the endothelial cells via the endoplasmic domain of the core protein.

AT: antithrombin, Alb: albumin

heparan sulfate contributes to maintenance of the anticoagulant activity in the vascular lumen⁴⁾. Furthermore, rather interestingly, glycocalyx additionally functions as a receptor (mechanosensor) of physical stimuli, such as shear stress and mechanical stretch, and is involved in the regulation of stress fiber formation and adhesion of cells to the extracellular matrix (e.g., collagen and fibronectin). There is also a report that glycocalyx senses the physical stimuli and induces the release of nitric oxide (NO) from the vascular endothelium, thereby modulating the vascular resistance³⁾. Of the components of sugar chains, heparan sulfate additionally serves as a ligand for various growth factors such as transforming growth factor- β (TGF- β) and vascular endothelial growth factor (VEGF), and is thus involved not only in blood vessel regeneration, but also in cell differentiation and tissue morphogenesis⁵⁾.

Four subtypes of syndecan are known (syndecan 1 through 4). In the extracellular domain of the transmembrane structure, 3 to 9 molecules of heparan sulfate or chondroitin sulfate are bound to each syndecan. In regard to the cytoplasmic domain of the transmembrane structure, intracellular

transmission of various stimuli are known to take place through phosphorylation of this domain, its binding to protein kinase C or its activation. This means that syndecan functions also as a cytokine receptor, mediating the regulation of endothelial cell functions by the heparan sulfate-bound cytokines. Ishiguro *et al.* found that syndecan-4-knockout mice were more susceptible to endotoxin shock and had a higher mortality rate than wild-type mice, stating that a possible reason for this difference is the compromised inflammation control function (induced by the inability of syndecan-4 binding to TGF- β) in the syndecan-4-knockout mice as compared to the wild-type mice⁶⁾. In other words, decrease or lack of syndecan-4 leads to attenuation of the effect of TGF- β in suppressing excessive formation of inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF α) under the condition of sepsis.

2. Hyaluronan

Hyaluronan is a core protein-non-binding type of macromolecular polymer entangled with the GAGs. It has high water-retaining capability and is considered to contribute to the retention of plasma

Figure-2 Expression of glycocalyx under normal and abnormal conditions

The glycocalyx covering the vascular endothelial cell surface is also present in the endothelial clefts (ETC), i.e., the spaces between neighboring endothelial cells. The rather fragile glycocalyx is very vulnerable to detachment upon exposure to various inflammatory stimuli or environmental changes, resulting in exposure of the adhesion molecules on the endothelial surface and easier adhesion of blood cells to the endothelium. Furthermore, abnormal structure/function of glycocalyx in the ETC also causes increased vascular permeability.

proteins such as albumin and fibrinogen. According to the mechanisms proposed to date, albumin is retained in the plasma because (1) the hyaluronan-bound albumin in the ETC is negatively charged and repels the negatively charged albumin in the plasma, or (2) the sulfate group constituting the GAGs is also negatively charged and albumin cannot pass through the ETC. However, if the GAGs become detached following endothelial cell damage, it is plausible to imagine that the physical spaces will be enlarged and the electrical resistance will disappear, allowing albumin to permeate easily across the blood vessel into the extravascular space. This mechanism has been studied in an experiment using vascular endothelial cells of the glomeruli. According to that experiment, the vascular endothelial cells of glomeruli have a fenestrated structure (50 to 100 nm in diameter) with their surfaces covered by negatively charged GAGs, i.e., a structure that makes it difficult for albumin to escape into the urine. However, in patients with diabetes mellitus or acute kidney injury (AKI), hyperglycemia or oxidative stress reduces the expression of the GAGs, leading to leakage of albumin into the urine. Thus, if this phenomenon is utilized, it may become possible to assess the severity of diabetic nephropathy and AKI through measuring the urinary microalbumin level and calculating the

urinary albumin/creatinine ratio (ACR)⁷⁾.

3. Glycoprotein

Like proteoglycans, glycoproteins also bind directly to the cell membrane to constitute the glycocalyx. Adhesion molecules such as selectin and integrin are representative glycoproteins. The expressions of these molecules vary depending on the disease condition, and they regulate adhesion of leukocytes and platelets to the vascular endothelium. In this connection, it is rather interesting that the adhesion molecules, which are shorter than proteoglycans, are physiologically covered with proteoglycans, and do not contact with blood cells. However, if stimulation by inflammation or other pathological conditions causes detachment of the proteoglycans, and the blood cells can bind directly to the exposed adhesion molecules, and thereby to endothelial cells (Figure-2). In other words, leukocytes release proteases and reactive oxygen species that cause detachment of proteoglycans and create an environment suitable for their smooth landing, followed by adherence to the endothelium and subsequent inflammatory responses.

Glycocalyx morphology and observation

Glycocalyx is a rather unstable and fragile

Figure-3 Syndecan-1 expression under normal and abnormal conditions

The vascular wall is normally lined completely by a single layer of endothelial cells without any intercellular spaces. The vascular endothelium incubated under static conditions emits no fluorescence when subjected to fluorescence staining for syndecan-1 or -4, indicating the absence of syndecan expression (left). However, if the endothelial cells are injured by the addition of a small amount of histone to the culture, spaces are formed between the cells and a green chromatic response of syndecan-1 is visualized between the cells. If syndecan-1 in the culture were to be quantified, elevation of its concentration (which is under the limit of detection in the absence of stimulation), can be confirmed.

structure, its morphology and size varying depending on the method of observation used⁸⁾. For example, earlier observations by transmission electron microscopy showed that glycocalyx was several tens of nm in thickness, whereas observations using a different fixation method revealed a thickness of this structure of 5 μm or more⁹⁾. In any event, only fixed specimens can be observed under an electron microscope, and observation of glycocalyx in its natural state is not possible. As a method for estimating the glycocalyx thickness in a more physiological setting, an attempt was made to measure the distance between erythrocytes and endothelial cells under a biomicroscope. The glycocalyx thickness measured by this method was 0.4 to 0.5 μm , smaller than anticipated, suggesting that this result could be related to the limitation of diffractive spatial resolution in light microscopy. Subsequently, attempts at the use of more sophisticated observation methods have been made, such as by confocal laser scanning and dual photon laser scanning. Observation by the latter technique revealed a glycocalyx thickness in the mouse carotid artery of 4.5 μm ¹⁰⁾. Thus, the thickness of the glycocalyx layer appears to vary considerably depending on species differences, thickness of the artery, vein examined, etc. What makes research on glycocalyx rather difficult is the fact that the status of glycocalyx expression differs substantially between the static condition and blood flow

condition in the blood vessel¹¹⁾. For example, in our observation of cultured endothelial cells using fluorescent antibodies, we could not identify syndecan, or GADs such as heparan sulfate, on the cell surface under static culture conditions. However, when the endothelial spaces were dilated by the addition of injurious factors (such as histone) to the cultured endothelial cells, the expression of syndecan-1 and -4 was unmasked, suggesting that syndecan is expressed in the ETC even under static conditions and plays a role in intercellular adhesion (Figure-3). In view of these results, it seems difficult to estimate the status of the glycocalyx in the living body solely on the basis of the results of *in vitro* studies. This important point should be borne in mind when interpreting the results of research on this novel structure.

Glycocalyx as a biomarker

The fragile structure “glycocalyx” is vulnerable to detachment and release into the blood upon exposure to various stimuli. Making use of this feature, attempts have recently been made to utilize glycocalyx in pathophysiological analysis and diagnosis through measuring each element of glycocalyx circulating in the blood. For example, the results of blood glycocalyx measurements in patients with chronic diseases (diabetes mellitus, atherosclerosis, etc.), trauma, sepsis, post-ischemic reperfusion

disorders, etc., have been reported, and the usefulness of glycocalyx as a biomarker is being recognized^{12) 13)}. In regard to the mechanism of release of glycocalyx into the blood, it has been reported that in patients with sepsis, cells of the granulocyte family, such as neutrophils, release proteases (elastase, etc.) and reactive oxygen species to degrade the core protein, and that plasma cells release heparanase and matrix metalloproteinase that separate the GAGs¹⁴⁾. It has also been reported that hyperglycemia promotes the degradation of glycocalyx and that the glycocalyx layer is thinner in diabetic patients¹⁵⁾. Furthermore, it has been reported that in patients with post-ischemic reperfusion injury, degradation of glycocalyx is stimulated by the change in the blood pH. Because glycocalyx in the vascular endothelium plays important roles, such as in anticoagulation, fibrinolysis, and blood cell adhesion, and also regulates protein retention and vascular permeability, it is expected that estimation of endothelial dysfunction may be enabled by quantification of these elements of glycocalyx in the blood¹⁶⁾. As stated above, 4 forms of syndecan are known to exist. Of these 4 forms, syndecan-4 is widely known as an antithrombin-binding molecule. The distribution of each subtype of syndecan is unique. For example, syndecan-1, -2 and -4 are expressed between vascular endothelial cells and on the side of the vascular endothelial cells closer to the basal membrane. Johansson *et al.* evaluated syndecan-1 as a marker for predicting the prognosis in trauma patients, expecting that its level would rise following endothelial injury or high-dose catecholamine treatment¹⁷⁾. They found the plasma syndecan-1 served as an independent predictor of death, although the odds ratio was only 1.01 (95% confidence interval [CI]: 1.00–1.02), leaving open the question about the usefulness of this substance as a prognostic marker. Ostrowski *et al.*, on the other hand, conducted syndecan-1 measurement and thrombelastography (TEG) in 184 patients with severe sepsis, and reported that the data reflected overcoagulation in the presence of sepsis¹⁸⁾. In another study conducted by Thorevska *et al.*, the ACR was measured in 104 severely sick patients admitted to the intensive care unit (ICU), and increase of ACR was noted in 69% of the patients¹⁹⁾. They stated that ACR was a useful

predictor of in-hospital death, comparable in reliability to the Acute Physiology and Chronic Health Evaluation II score (APACHE II score) or Sequential Organ Failure Assessment score (SOFA score).

Syndecan and antithrombin

Antithrombin, a physiological anticoagulant, has long been known to possess not only anticoagulant activity, but also anti-inflammatory activity²⁰⁾. Regarding the mechanism of its anti-inflammatory activity, it has been considered that the binding of antithrombin to the heparan sulfate of syndecan-4 stimulates the production of prostacyclin (PGI₂) in the vascular endothelial cells, and that the PGI₂ formed thus suppresses neutrophil/platelet adhesion to vascular endothelial cells and other inflammatory reactions. Heparin is known to attenuate the anti-inflammatory activity of antithrombin, because concomitant use of heparin inhibits the binding of antithrombin to heparan sulfate, i.e. to syndecan-4, because of competitive binding²¹⁾.

Close attention has also been paid to the syndecan-4-protective activity of antithrombin. Matriptase is a trypsin-like protease belonging to the type II transmembrane serine protease family and is known to decompose substrates such as extracellular matrix, cell adhesion molecules, growth factor-like protein, and syndecan. It has been reported that antithrombin bound to syndecan-4 on the endothelial cell surface controls the activity of matriptase and thereby suppresses the decomposition and the release of syndecan²²⁾.

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Conflicts of Interest

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