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Original Article

Glycocalyx Degradation in Retinal and Choroidal Capillary Endothelium in Rats with Diabetes and Hypertension

Fumiaki Kumase[a]*, Yuki Morizane[a], Satoshi Mohri[b], Ippei Takasu[a], Aiji Ohtsuka[c], and Hiroshi Ohtsuki[a]

Departments of [a]Ophthalmology and [c]Human Morphology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700–8558, Japan,
[b]Department of Physiology, Kawasaki Medical School, Kurashiki, Okayama 701–0192, Japan

Endothelial glycocalyx (GCX) has been reported as a protective factor for vascular endothelial cells (VEC) in diabetes and hypertension. However, the involvement of GCX impairment in ocular vasculopathy remains unclear. We evaluated the changes in the GCX thicknesses of the retinal and choroidal capillaries in rats with diabetes and hypertension by cationic colloidal iron staining using a transmission electron microscope. In the control group, the mean (standard error of the mean) thicknesses of retinal and choroidal GCX were 60.2 (1.5) nm and 84.3 (3.1) nm, respectively. The diabetic rats showed a significant decrease of GCX thickness in the retina, but not in the choroid, compared to controls (28.3 (0.3) nm, $p < 0.01$ and 77.8 (1.4) nm, respectively). In the hypertensive rats, both retinal and choroidal GCX were significantly decreased compared to the control values (10.9 (0.4) nm and 13.2 (1.0) nm, respectively, both $p < 0.01$). Moreover, we could visualize the adhesion of leukocytes and platelets on the luminal surface of VEC, at the site where the GCX was markedly degraded. These findings suggest that the GCX prevents adhesion of leukocytes and platelets to the VEC surface, and this impairment may lead to ocular vasculopathy in diabetes and hypertension.

Key words: glycocalyx, retina, choroid, diabetes, hypertension

In diabetes and hypertension, hyperglycemia and high blood pressure *per se* are potent pro-inflammatory and pro-oxidant stimuli and play a key role in the pathogenesis of ocular vasculopathy [1]. One major inflammatory reaction in the microvessels is leukocyte and platelet adhesion to vascular endothelial cells (VEC) [2]. In the process of leukocyte-VEC adhesion, it is well known that the bindings of adhesion molecules such as integrin, selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are important [3]. In regard to oxidative stress, it has been reported that hyperglycemia, advanced glycation end-product (AGE) and high blood pressure degrade VEC and thereby decrease their nitric oxide (NO) production, leading to an increase of oxidative stress [4–6]. Recently, it has been reported that the endothelial glycocalyx (GCX) plays an important role in the process of both leukocyte-VEC adhesion and decreased NO release through the degradation of VEC [7–9].

The GCX is a layer covering the intravascular lumen and consists of a negatively charged mesh of proteoglycans, glycosaminoglycans, glycoproteins and glycolipids derived from VECs [10]. The GCX has been shown to protect VEC and to orchestrate vascular homeostasis through the following actions: 1)

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*Corresponding author. Phone:+81–86–235–7297; Fax:+81–86–222–5059
E-mail:5an6m4@bma.biglobe.ne.jp (F. Kumase)

modulation of transendothelial permeability, 2) regulation of leukocyte and platelet adhesion to VEC and 3) mechanosensing of shear stress, thereby mediating shear-induced release of NO by VEC [7, 11, 12]. In the heart, brain and kidney, it has been reported that diabetes and hypertension degrade endothelial GCX and lead to an increase in vascular permeability, leukocyte-VEC adhesion and oxidative stress [4, 8, 13–16]. However, it is uncertain whether diabetes and hypertension affect the GCX of ocular microvessels, especially the retinal and choroidal capillaries.

Here, we hypothesized that the degradation of GCX plays an important role in the pathological process of diabetic and hypertensive retino-choroidal vasculopathy. To investigate this, we visualized the retinal and choroidal capillary GCX in diabetic and hypertensive rats and determined their thickness quantitatively.

Materials and Methods

Animals. All animal experiments adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Male Wistar Kyoto rats (WKY) at 15 weeks of life and male spontaneous hypertensive rats (SHR) at 16 weeks of life (both from Charles River, Tokyo, Japan) were used for the experiments. Animals were fed standard laboratory food and were allowed free access to water in an air-conditioned room with a 12-hour light/12-hour dark cycle. WKY ($n = 5$) were made diabetic by an intraperitoneal injection of streptozocin (60 mg/kg; Sigma, St. Louis, MO, USA) (STZ-WKY) [17]. Forty-eight hours after the injection, the blood glucose level was monitored. A blood glucose level of more than 250 mg/dl was considered to represent hyperglycemia. In the control group ($n = 5$), citrate buffer solution was injected as a vehicle. In both groups, the visualization of glycocalyx, as stated below, was carried out on the 7th day after injection. In all rats, blood pressure was measured 5 times with tail-cuff plethysmography (Softron, Tokyo, Japan).

Visualization of glycocalyx by cationic colloidal iron staining. We visualized the glycocalyx of retinal and choroidal capillary vessels by cationic colloidal iron staining as reported previously [18, 19]. Briefly, under ether inhalation anesthesia, the chest cavity was opened and blood was drawn from the right ventricle. The rats were then perfused via the left ventricle with Ringer's lactate solution mixed with heparin. Next, the rats were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and then perfused with cationic colloidal iron [18, 19]. The eyes were enucleated and postfixed with 1% osmium tetroxide (Merck, Darmstadt, Germany) at 4℃ overnight. The eyes were then embedded in Epon 812 (Oken Shoji, Tokyo, Japan) and polymerized. Finally, we cut ultra-thin sections and observed the specimens under a transmission electron microscope (H-7100S; Hitachi, Tokyo, Japan).

Quantification of the thickness of the retinal and choroidal endothelial glycocalyx. According to the method of Lindner JR *et al.*, we used the transmission electron micrograph ($\times$ 30,000) and measured the distance from the luminal side of the VEC membrane to the luminal tip of the cationic colloidal iron deposit as the thickness of endothelial GCX [20]. The number of measuring points was at least 5 per vessel (WKY: $n = 39$ retinal and 16 choroidal vessels; STZ-WKY: $n = 29$ retinal and 18 choroidal vessels; and SHR: $n = 33$ retinal and 7 choroidal vessels).

Statistical analysis. The results are expressed as the mean $\pm$ standard error of the mean (SEM). Data were analyzed by one-way ANOVA with Scheffe's test. A p value of less than 0.05 was considered statistically significant.

Results

Table 1 shows the body weight, blood glucose and blood pressure of WKY, STZ-WKY and SHR at 16 weeks after birth. The blood glucose level of STZ-WKY (437 ± 47 mg/dL) was significantly higher than that of WKY (154 ± 10 mg/dL, $p < 0.01$) and SHR (145 ± 12 mg/dL, $p < 0.01$). The systolic and diastolic blood pressures of SHR (194 ± 1 mmHg, 153 ± 3 mmHg, respectively) were significantly higher than those of WKY (116 ± 3 mmHg, 83 ± 2 mmHg, respectively, both $p < 0.01$) and STZ-WKY (114 ± 2 mmHg, 82 ± 1 mmHg, respectively, both $p < 0.01$).

We successfully visualized both the retinal and choroidal capillary glycocalyx by staining with cationic colloidal iron (Fig. 1 and 2). In the control group

Table 1 Table 1 shows the physiological parameters of WKT, STZ-WKY and SHR at 16 weeks after birth. Mean ± SEM. One-way ANOVA with Scheffe's test. *: $p < 0.01$.

	WKY	STZ-WKY	SHR
Body weight [g]	359 ± 3	342 ± 12	367 ± 4
Blood glucose [mg/dL]	154 ± 10	437 ± 47*	145 ± 12
Systolic blood pressure [mmHg]	116 ± 3	114 ± 2	194 ± 1*
Diastolic blood pressure [mmHg]	83 ± 2	82 ± 1	153 ± 3*

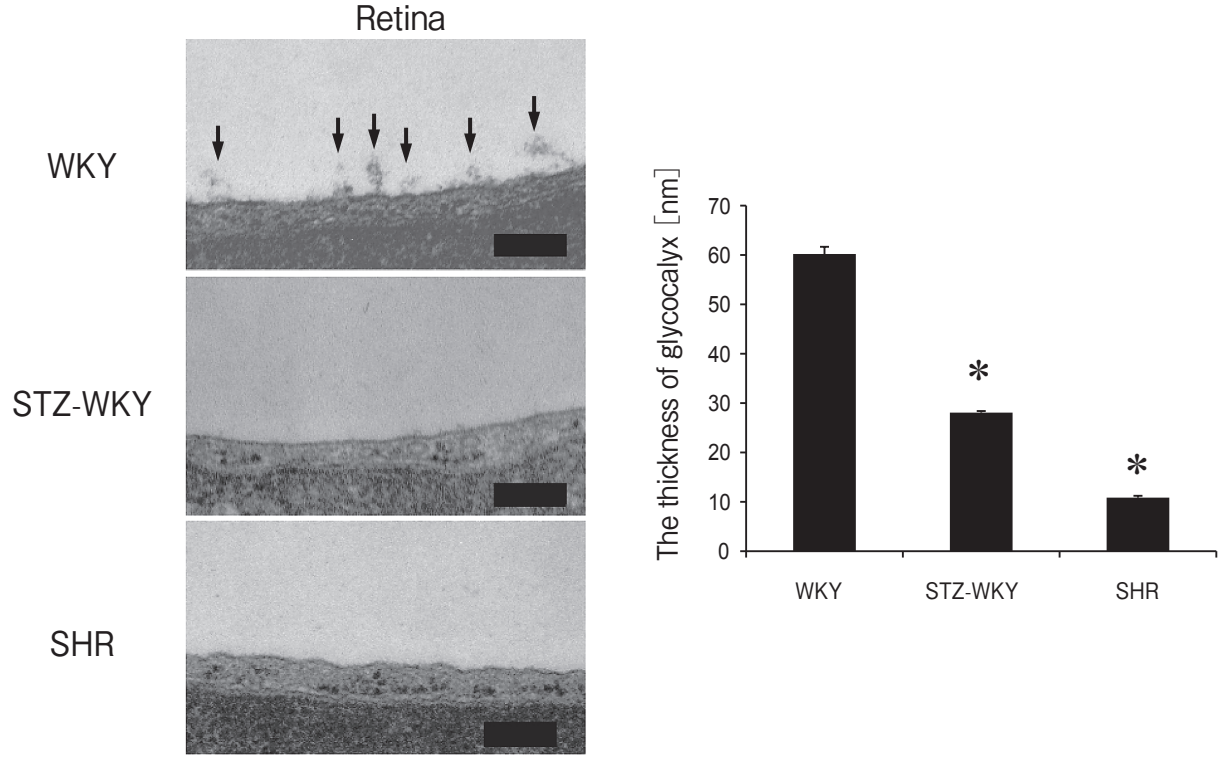

Fig. 1 The thickness of the retinal glycocalyx was decreased in both diabetic and hypertensive rats. Left, Electron micrographs of the retinal microvessels of WKY, STZ-WKY and SHR. The glycocalyx was visualized by cationic colloidal iron particles (arrows). Bar = 200 nm; Right, Thickness of the retinal glycocalyx of WKY, STZ-WKY and SHR. *: $p < 0.01$.

(WKY), the glycocalyx was situated evenly at the luminal side of all retinal and choroidal capillary vessels. Their thicknesses measured 60.2 ± 1.5 nm in the retina and 84.3 ± 3.1 nm in the choroid. In STZ-WKY, the thickness of the retinal capillary glycocalyx was decreased significantly (28.1 ± 0.3 nm, $p < 0.01$, Fig. 1). The thickness of the choroidal capillary glycocalyx in STZ-WKY did not change significantly when compared with that of WKY (77.8 ± 1.4 nm, Fig. 2). In the SHR group, the thicknesses of both the retinal and choroidal capillary glycocalyx decreased significantly (retinal capillary glycocalyx: 10.9 ± 0.4 nm, $p < 0.01$, Fig. 1; choroidal capillary glycocalyx: 13.2 ± 1.0 nm, $p < 0.01$, Fig. 2).

Fig. 3 shows representative electron microscopic images of leukocyte and platelet adhesion to the choroidal VECs in STZ-WKY. The glycocalyx decreased markedly in the adhesion area and its proximity.

Discussion

To our knowledge, the present study is the first to

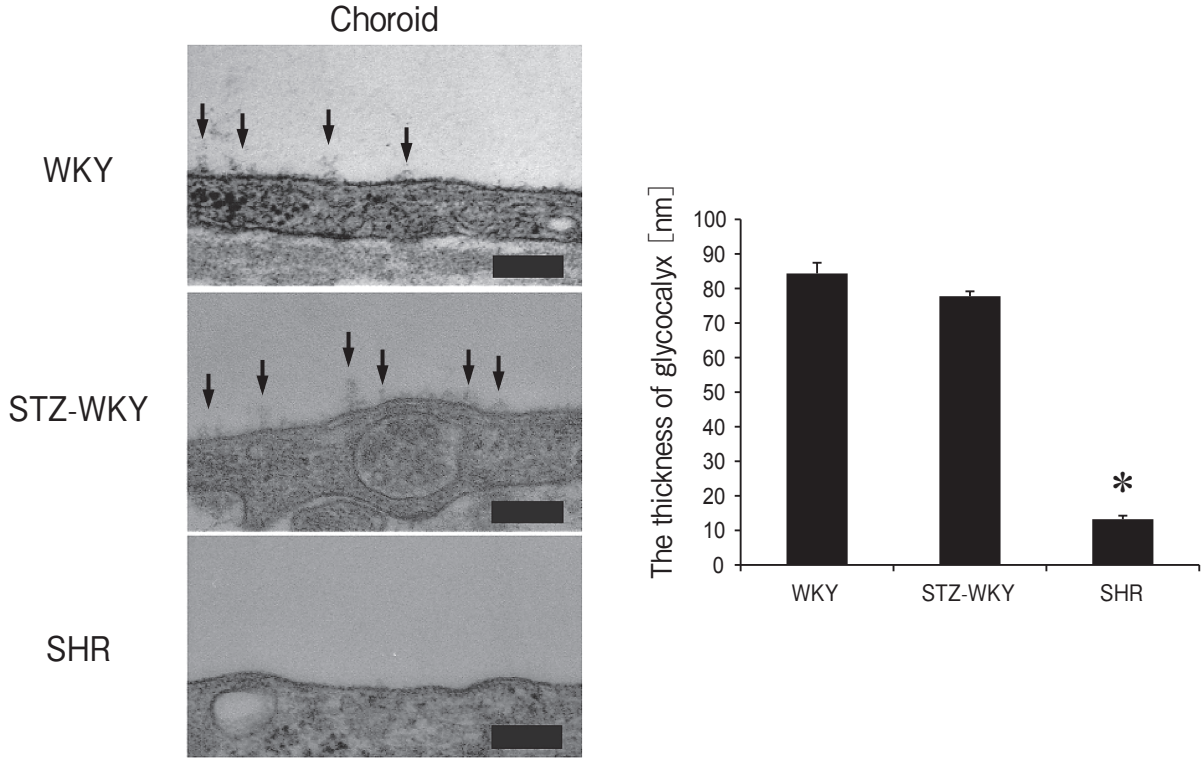

Fig. 2 The thickness of the choroidal glycocalyx was decreased in hypertensive rats but not diabetic rats.
Left, Electron micrographs of the choroidal microvessels of WKY, STZ-WKY and SHR. The glycocalyx was visualized by cationic colloidal iron particles (arrows). Bar = 200 nm; Right, Thickness of the choroidal glycocalyx of WKY, STZ-WKY and SHR. *: $p < 0.01$.

demonstrate that chronic hypertension degrades both retinal and choroidal GCX in rats. We also showed the degradation of retinal GCX, but not choroidal GCX, in early diabetic rats. In hyperglycemia, it has been reported that both AGE and hyperglycemia itself cause systemic inflammation by producing proinflammatory cytokines such as tumor necrosis factor-a (TNF-a) [9, 21]. Henry *et al.* and Mulivor *et al.* proposed that TNF-α activates leukocytes and VEC, and these cells produce reactive oxygen species (ROS) and proteases that degrade the GCX directly. In hypertension, Suematsu *et al.* reported that chronic hypertension induces oxidative stress responses and decreases the production of ROS scavengers, resulting in the degradation of the GCX [6]. Although the mechanism by which hyperglycemic and hypertensive conditions degrade the GCX in the retina and choroid remains unclear, our results showed the effect of these conditions on the homeostasis of retinal and choroidal GCX.

In the pathogenesis of diabetic and hypertensive vasculopathy, leukocyte and platelet-endothelial adhesion occurs as an inflammatory response to hyperglycemia, AGE and high blood pressure [3, 22, 23]. In this process, adhesion molecules such as integrin, selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) play a key role in binding leukocytes and platelets to the VEC [17]. Recently, the GCX has been considered an antiadhesive factor in the process due to its relatively large dimensions, which exceed the length of these adhesion molecules [9]. For example, in the heart, the thickness of the GCX has been reported as 50–500 nm, whereas ICAM-1 and P-selectin extend only 18.7 nm and 38 nm above the surface of the endothelium [9, 24]. In the control group (WKY) of the present study, we showed that the GCX thicknesses of retinal and choroidal capillaries were 60.2 $\pm$ 1.5 nm and 84.3 $\pm$ 3.1 nm, respectively. It has been reported that the number of leukocytes that adheres to

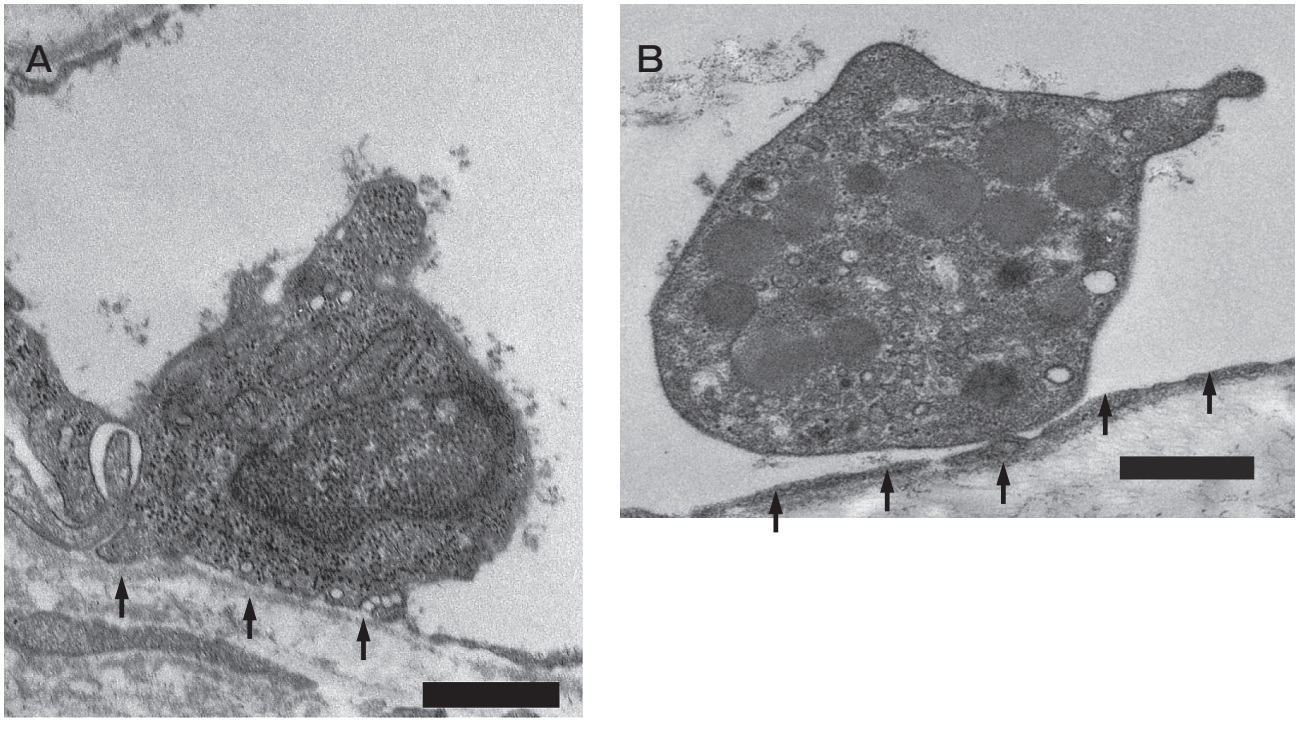

Fig. 3 Degradation of the choroidal glycocalyx at the site of leukocyte and platelet adhesion. Electron micrographs of leukocyte (**A**) and platelet (**B**) adhesion to choroidal vascular endothelial cells in STZ-WKY. A leukocyte (**A**) and a platelet (**B**) adhered to the choroidal vascular endothelial cells. In A, the glycocalyx is degraded at the site of leukocyte adhesion and its proximity (arrows). In B, the glycocalyx is totally degraded (arrows). Bar = 500 nm.

the VECs reaches a maximum level in the very early phase of diabetic retinopathy—*i. e.*, 7 days after the STZ injection [17]. To detect the change in the GCX in this period, we examined the diabetic rat choroidal capillaries on the 7th day after STZ injection and observed a degradation of the GCX at the site of leukocyte and platelet-endothelial adhesion and its proximity. These findings coincide with the theory that the GCX functions as a barrier against proinflammatory stimuli in diabetes and hypertension.

In early diabetic rats (STZ-WKY), the thickness of the retinal GCX decreased by 53% compared to that of the control group (WKY). However, Fitzgerald *et al.* reported that the thickness of the retinal GCX did not change in spontaneous diabetic rats [25, 26]. As an explanation for the discrepancy, we consider that the differences in the method of GCX staining may have played a role. After perfusion fixation, Fitzgerald *et al.* stained the retinal GCX by immersing the tissue in cationized ferritin (CF), which binds to the GCX [25]. Then they quantified the amount of GCX by counting the number of CF particles. On the other hand, we stained the GCX by perfusion of cationic colloidal iron, immediately after perfusion fixation. Because the GCX is attached to intravascular lumen, perfusion staining is more effective than immersion fixation to stain the GCX. Furthermore, the diameter of the colloidal iron particles (0.5–1.0 nm) is much smaller than that of CF particles (12.0 nm). This enables us to improve the demonstration of GCX [18, 27]. Therefore, we consider that their method may have lower sensitivity than our method and may underestimate the change in the length of GCX. In humans, it is well known that the incidence of diabetic choroidopathy is lower than that of retinopathy [28]. The result that the thickness of choroidal GCX was not decreased (*i. e.*, that the GCX was preserved) is compatible with this clinical feature of diabetic ocular vasculopathy. Although it is unclear why choroidal GCX is less vulnerable than retinal GCX, our result suggests that the lower vulnerability of choroidal GCX may explain the

decreased occurrence of choroidal retinopathy.

Hypertension is the main secondary risk factor associated with diabetic retinopathy [29, 30]. Pinto *et al.* reported that experimentally induced diabetes increases macrophage migration, ICAM-1 expression, VEGF and NF-κB p65 levels in the SHR retina [30]. In the present study, not only diabetic rats (STZ-WKY) but also hypertensive rats (SHR) showed degradation of retinal and choroidal GCX. Although we did not examine the model of diabetes associated with hypertension, our results imply that hypertension can augment the degradation of GCX in diabetes and result in more severe diabetic retinopathy than diabetes alone.

In conclusion, we showed that 1) the length of both the retinal and choroidal GCX in WKY is longer than that of adhesion molecules; 2) the retinal GCX was degraded in both diabetic and hypertensive rats and the choroidal GCX was degraded in hypertensive rats; 3) the degradation of GCX coincided with leukocyte and platelet adhesion to VECs. These findings suggest that the GCX functions as a protective factor in the pathogenesis of retino- and choroidopathy in diabetes and hypertension.

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