Original Article

Endothelial Glycocalyx Damage Coincides With Microalbuminuria in Type 1 Diabetes

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Chronic hyperglycemia underlies microvascular complications in patients with type 1 diabetes. The mechanisms leading to these vascular complications are not fully understood. Recently, we observed that acute hyperglycemia results in endothelial glycocalyx damage. To establish whether glycocalyx is associated with microvascular damage, we performed glycocalyx perturbation volume measurements in type 1 diabetic patients with microalbuminuria (DM1-MA group; $n = 7$), without microalbuminuria (DM1-NA group; $n = 7$), and in age-matched control subjects (CON; $n = 7$). Systemic glycocalyx volume was determined comparing intravascular distribution volume of a glycocalyx-permeable tracer (dextran 40) to that of a glycocalyx-impermeable tracer (labeled erythrocytes). Sublingual capillaries were visualized using orthogonal polarization spectral microscopy to estimate microvascular glycocalyx. Patients and control subjects were matched according to age and BMI. Glycocalyx volume decreased in a stepwise fashion from CON, DM1-NA, and finally DM1-MA subjects (1.5 ± 0.1, 0.8 ± 0.4, and 0.2 ± 0.1 l, respectively, $P < 0.05$). Microvascular glycocalyx in sublingual capillaries was also decreased in type 1 diabetes versus the control group (0.5 ± 0.1 vs. 0.9 ± 0.1 μm, $P < 0.05$). Plasma hyaluronan, a principal glycocalyx constituent, and hyaluronidase were increased in type 1 diabetes. In conclusion, type 1 diabetic patients are characterized by endothelial glycocalyx damage, the severity of which is increased in presence of microalbuminuria. *Diabetes* 55:1127–1132, 2006

Type 1 diabetes–associated micro- and macrovascular complications are major causes of morbidity and mortality (1,2). In this disorder, the presence of microvascular disease is strongly associated with increased cardiovascular risk, underlining the generalized nature of such vascular dysfunction (3,4). The exact pathways leading to this propensity for vascular disease have not been fully elucidated. In line with direct adverse effects of hyperglycemia itself (5), good glycemic control has been associated with decreased microvascular disease rates (6,7). Whereas the concept of generalized vascular dysfunctional state in type 1 diabetes has already been put forward >2 decades ago (8), a final common pathway for the vascular dysfunction has remained a matter of debate.

Recently, we demonstrated that the endothelial glycocalyx, an intraluminal layer consisting of glycosaminoglycans and hyaluronan, constitutes an important component to the vascular permeability barrier by preventing transvascular leakage of macromolecules (9). Conversely, hyaluronidase exposure severely decreases endothelial glycocalyx thickness with a concomitant increase in capillary wall permeability and induction of pericapillary edema (10,11). In fact, loss of glycocalyx leads to a wide spectrum of vascular abnormalities in experimental models, including increased vascular permeability, adhesion of mononuclear cells, and platelets to the endothelial surface and attenuated nitric oxide availability (12). Restoration of the glycocalyx is associated with reversal of these derangements (11,13). Collectively, these data suppose a potential role for the glycocalyx in determining the protective properties of the vessel wall. Recently, we validated a technique to assess the volume of the endothelial glycocalyx in humans. Using this technique, we found that acute hyperglycemia resulted in a profound perturbation of the glycocalyx, which closely coincided with vascular dysfunction and coagulation activation (14).

In the present study, we set out to evaluate whether glycocalyx loss is also present in patients with long-standing type 1 diabetes and whether more severe glycocalyx perturbation coincides with the presence of microalbuminuria. To substantiate this, we determined systemic glycocalyx volume as well as microvascular glycocalyx thickness in type 1 diabetic patients with and without microalbuminuria as well as in matched normoglycemic healthy control subjects.

RESEARCH DESIGN AND METHODS

We enrolled nonsmoking male type 1 diabetic patients either with (DM1-MA group) or without (DM1-NA group) microalbuminuria, defined as albumin-to-creatinin ratio >2.5 mg/mmol in a morning urine sample. Of note, both the DM1-MA and DM1-NA patients had been diagnosed with type 1 diabetes for at least 15 years, with comparable mean duration of diabetes in both groups. All patients were C-peptide negative, used multiple injections of insulin per day, and had HbA_{1c} (A1C) levels between 7.0 and 9.0% during the 6 months preceding the study. The presence of macrovascular disease, defined as electrocardiogram abnormalities, abnormal ankle-brachial index, or a history

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OPS, orthogonal polarization spectral.

of cardiac, cerebral, or peripheral vascular events were exclusion criteria for the study. Sex- and age-matched, normoglycemic healthy control subjects (CON; $n = 7$) were also included. All subjects gave written informed consent, and approval was obtained from the internal review board of the Academic Medical Center. The study was carried out in accordance with the principles of the Declaration of Helsinki.

All experiments were performed after an overnight fast in a quiet and air-conditioned room (temperature 22–24°C). Participants were asked to refrain from heavy physical exercise in the 24 h before the study visit. Patients using ACE inhibitors or angiotensin II antagonists were asked to stop this medication at least 5 days before the study visit. Patients with type 1 diabetes were asked to check their fasting blood glucose level (target levels 7.0–12.0 mmol/l), since injection of insulin was not allowed from 8 h prior until the end of the experiment. Based on 24-h urine microalbuminuria measurements in the 6 months preceding the study, patients were selected as normalbuminuric or microalbuminuric. At the beginning of the study, morning urine samples were collected to verify albumin-to-creatinin ratios. Blood pressure was measured three times, and the last two measurements were used to calculate systolic and diastolic blood pressure. During the study, two cannulas were inserted in the antecubital veins of both forearms for the collection of blood and infusion of Dextran 40 and labeled autologous erythrocytes, respectively.

Laboratory methods. After centrifugation, aliquots were snap frozen and stored at −80°C. Hematocrit (Hsys) was measured after centrifugation of heparinized blood at 10,000 rpm for 5 min (Hettich, Tuttlingen, Germany). Total cholesterol, HDL cholesterol, and triglycerides were measured by standard enzymatic methods (Roche Diagnostics, Basel, Switzerland). LDL cholesterol was calculated using the Friedewald formula. Alanine aminotransferase and aspartate aminotransferase were measured by pyridoxalphosphate activation assay (Roche Diagnostics). Creatinin was measured by Jaffé kinetic colorimetric test (Roche Diagnostics) on Modular P800 (Roche Diagnostics). A1C was measured by high-performance liquid chromatography (Reagens Bio-Rad Laboratories, Veenendaal, the Netherlands) on a Variant II (Bio-Rad Laboratories). Quantitative total plasma hyaluronan levels were measured by enzyme-linked immunosorbent assay (Echelon Biosciences, Salt Lake City, UT). Plasma hyaluronidase levels were determined with a previously described assay (intersample coefficient of variation <20%) (15). Urinary creatinin and albumin content was determined according to the Jaffé method on the P800 and by immunoturbidimetric assay, respectively (Roche Diagnostics).

Estimation of systemic glycocalyx volume. The glycocalyx allows limited access to plasma macromolecules and erythrocytes (16,17). Hence, systemic glycocalyx volume can be estimated by comparing circulating blood volume with the intravascular distribution volume of a glycocalyx-permeable tracer such as neutral dextran 40 (molecular weight 40 kDa). We recently showed that systemic glycocalyx volume can be reproducibly measured (intermeasurement coefficient of variation 15.2 ± 9.8%) and has a relatively large magnitude in healthy male volunteers (14). In short, the intravascular distribution volume of labeled, autologous erythrocytes was used to quantify circulating blood volume (18). Blood was drawn and centrifuged at 1,330 rpm for 5 min. Subsequently, 250 mg/ml of sodium fluorescein was added to the erythrocyte fraction for 5 min. After washing, labeled erythrocytes were resuspended in saline to the initial volume and reinfused. Subsequently, blood was drawn at 4, 5, 6, and 7 min after infusion. The fraction of labeled erythrocytes compared with total erythrocyte pool was used to estimate circulating erythrocyte volume. Preinjection unlabeled erythrocytes ($t = -1$) served as negative controls. Labeled erythrocytes were measured using a FACScan (FACSCalibur; Becton Dickinson, Mountain View, CA), during which at least 100,000 cells were counted to measure the circulating fraction of labeled erythrocytes. Circulating plasma volume was calculated from circulating erythrocyte volume (Vrbc) and large vessel hematocrit (Hsys) by the following formula: circulating plasma volume = ([1 − Hsys] × Vrbc)/Hsys (18).

Dextran 40 is used as a probe to estimate total intravascular volume, which includes the glycocalyx compartment (14,16,17,19). A bolus of 10 ml dextran 1 (Promiten; NPBI International, Emmercompascuum, the Netherlands) was injected to attenuate the risk for anaphylactic reactions. At least 1 h later, 100 ml dextran 40 (Rheomacrodex; NPBI International) was intravenously injected, followed by repeated blood sampling at 5, 7, 10, 15, 20, and 30 min. Dextran 40 concentration was calculated by measuring the increase in glucose concentration in the postinfusion samples after hydrolyzation of dextran 40 glucose polymers (19). Glucose concentration per time point was assessed in duplicate using the hexokinase method (Gluco-quant, Hitachi 917; Hitachi). To determine the initial intravascular distribution volume of dextran 40, the concentration of dextran 40 at the time of injection was estimated by exponential fitting of the measured dextran 40 concentrations.

Visualization of the capillary endothelial glycocalyx. To determine the thickness of the endothelial glycocalyx in individual capillary blood vessels, we used orthogonal polarization spectral (OPS) imaging of the sublingual microcirculation (Cytoscan; Cytometrics, Philadelphia, PA), which has been extensively validated (20,21). Preceding the systemic glycocalyx volume measurement, images were obtained with a times-five objective (on screen magnification ×650). A total of five representative sublingual capillaries per person ($n = 21$; 7 healthy control and 14 type 1 diabetic subjects) were identified for microvascular glycocalyx analysis. As previously reported, the change in capillary red cell column width following capillary leukocyte passage can be used to provide an estimate of the anatomic capillary diameter (i.e., glycocalyx compressed; Dcap_anat), whereas the red cell column width before leukocyte passage reflects the functionally perfused capillary diameter (Dcap_func) (22). Subsequently, subtracting functional capillary diameter from anatomic capillary diameter ([Dcap_anat − Dcap_func]/2) provides an estimate of individual capillary glycocalyx thickness. Measurements and analysis of the images were performed with Image Pro Plus (Mediacybernetrics, Silver Spring, MD) by the same person, who was unaware of the clinical details of the participants.

Statistical analysis. All results are expressed as means ± SE except those listed in Table 1 (means ± SD). Differences between groups were tested by Kruskall-Wallis test. Mann-Whitney U test (two tailed) was used for comparison of vascular volume compartment determinants and unpaired Student's t test (two tailed) for all other parameters. Statistical differences were first calculated for all type 1 diabetic patients versus control subjects and then separately for DM1-NA versus DM1-MA subjects. Correlation coefficients between systemic glycocalyx volume and microvascular glycocalyx thickness and biochemical parameters were calculated with the Spearman's rank correlation test (two tailed). A probability value of <0.05 was considered significant.

RESULTS

Baseline parameters. Clinical characteristics of subjects are listed in Table 1. Compared with healthy control subjects, type 1 diabetic patients had higher fasting plasma glucose and A1C levels (Table 1). Between DM1-NA and DM1-MA patients, albumin-to-creatinin ratio and plasma creatinin were significantly different (Table 1).

Systemic glycocalyx volume. All procedures during the test day were well tolerated, and no adverse events occurred during systemic glycocalyx volume measurements. Systemic glycocalyx volumes were significantly decreased in type 1 diabetic patients compared with control subjects (CON: 1.5 ± 0.1 vs. type 1 diabetes: 0.5 ± 0.1 l, $P < 0.01$, Fig. 1*A*). Markedly, the reduction in systemic glycocalyx volume was significantly higher in DM1-MA compared with DM1-NA subjects (0.2 ± 0.1 and 0.8 ± 0.4 l, respectively, $P < 0.05$, Fig. 1*A*). Changes in dextran 40 distribution volumes were predominantly responsible for the decreased systemic glycocalyx volume in type 1 diabetes (CON: 4.5 ± 0.7 vs. DM1-NA: 3.7 ± 0.9 and DM1-MA: 3.4 ± 0.6 l), as evidenced by the changed dextran 40 clearance curves (Fig. 1*B*). Circulating plasma volumes were not significantly different (CON: 3.0 ± 0.4 vs. DM1-NA: 2.9 ± 0.4 and DM1-MA: 3.2 ± 0.5 l, NS). Hematocrit values were comparable between groups and did not change during dextran 40 infusion (data not shown).

Microvascular glycocalyx volume. Glycocalyx thickness in sublingual capillaries was reduced in type 1 diabetic patients compared with control subjects (CON: 0.9 ± 0.1 vs. type 1 diabetes: 0.5 ± 0.1 μm, $P < 0.01$), with a nonsignificant difference between DM1-NA and DM1-MA subjects (0.5 ± 0.1 and 0.4 ± 0.1 μm, respectively, Fig. 2*A*). The reduced glycocalyx thickness in type 1 diabetic patients was accompanied by a modest reduction in anatomic capillary diameter compared with control subjects (CON: 6.8 ± 0.2 vs. DM1-NA: 5.7 ± 0.1 and DM1-MA: 5.0 ± 0.2 μm, $P < 0.05$). In addition, a close correlation was found between systemic glycocalyx volume and sublingual glycocalyx thickness in all subjects ($r = 0.73$, $P < 0.01$, Fig. 2*B*).

TABLE 1
Clinical characteristics of the study subjects

	CON group	DM1-NA group	DM1-MA group
n	7	7	7
Duration of diabetes (years)	—	29 ± 13	32 ± 7
Daily insulin use (IU)	—	50 ± 18	48 ± 17
Age (years)	52 ± 11	47 ± 12	51 ± 10
BMI (kg/m^2)	24 ± 4	23 ± 2	24 ± 3
Smoking (yes/no)	0/7	0/7	0/7
Systolic blood pressure (mmHg)	134 ± 18	137 ± 10	150 ± 17
Diastolic blood pressure (mmHg)	74 ± 10	77 ± 7	80 ± 9
Heart rate (bpm)	64 ± 7	64 ± 10	70 ± 10
Fasting plasma glucose (mmol/l)	5.1 ± 0.4	9.1 ± 2.4*	9.3 ± 2.2*
Total cholesterol (mmol/l)	4.8 ± 0.8	4.8 ± 1.0	4.8 ± 0.8
LDL cholesterol (mmol/l)	3.0 ± 0.6	2.9 ± 0.9	2.6 ± 0.8
HDL cholesterol (mmol/l)	1.5 ± 0.3	1.6 ± 0.4	1.9 ± 0.4
Triglycerides (mmol/l)	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
Albumin-to-creatinine ratio (mg/mmol)	0.4 ± 0.1	0.8 ± 0.7	30 ± 27*†
Plasma creatinine (μmol/l)	69 ± 6	67 ± 6	86 ± 1*†
Aspartate aminotransferase (U/l)	21.9 ± 5.9	23.9 ± 4.6	24.0 ± 5.0
Alanine aminotransferase (U/l)	22.0 ± 11.6	22.6 ± 8.1	19.3 ± 5.1
A1C (%)	5.2 ± 0.3	7.7 ± 0.6*	8.3 ± 1.0*
High-sensitivity C-reactive protein (mg/l)	1.3 ± 1.1	1.6 ± 1.2	2.8 ± 1.6

Data are means ± SD. *$P < 0.05$ control vs. diabetic subjects. †$P < 0.05$ DM1-NA vs. DM1-MA subjects.

Systemic biochemical markers of glycocalyx perturbation. Plasma levels of hyaluronan were increased in type 1 diabetes (DM1: 118 ± 9 vs. CON: 65 ± 8 ng/ml, $P < 0.01$), with a further increase in type 1 diabetes with microalbuminuria (DM1-MA: 136 ± 29 vs. DM1-NA: 100 ± 17 ng/ml, $P < 0.05$, Fig. 3*A*). Plasma hyaluronidase levels were increased in type 1 diabetic patients (type 1 diabetes: 236 ± 8 vs. CON: 170 ± 19 units/ml, $P < 0.01$), with a trend toward higher values in type 1 diabetes with microalbuminuria (DM1-MA: 240 ± 13 and DM1-NA: 232 ± 10 units/ml, Fig. 3*B*). Plasma hyaluronan and hyaluronidase ($r = -0.75$ and -0.66, respectively, $P < 0.01$), plasma creatinin ($r = -0.58$, $P < 0.05$), and albumin-to-creatinin ratio ($r = -0.54$, $P < 0.05$) were inversely correlated with systemic glycocalyx volume. Systemic glycocalyx volume showed no correlation with fasting plasma glucose ($r = -0.31$, NS), but there was a trend for A1C ($r = -0.41$, $P = 0.06$).

DISCUSSION

In the present study, we show that systemic glycocalyx volume is markedly reduced in patients with long-standing type 1 diabetes compared with normoglycemic control subjects. In fact, the magnitude of systemic glycocalyx reduction was largest in type 1 diabetic patients with microalbuminuria. Using OPS imaging of the sublingual microcirculation, we were also able to confirm the reduction of glycocalyx volume in type 1 diabetic patients at the level of the microcirculation. The reduction of systemic glycocalyx volume was correlated with increased levels of circulating hyaluronan and its degrading enzyme hyaluronidase. The close relation between glycocalyx perturbation and microvascular complications in type 1 diabetes warrants further studies to assess whether loss of glycocalyx may actually be a causal factor for vascular complications in type 1 diabetes.

Systemic and microvascular glycocalyx reduction in patients with type 1 diabetes. Previously, we have validated the estimation of glycocalyx volume by comparison of erythrocyte and dextran 40 distribution volumes in individual vessels and in vivo (14,16,17). The size of systemic glycocalyx volume in healthy control subjects is in line with its predicted dimensions, based on a thickness between 0.5 and 3.0 μm (23,24) and an estimated endothelial surface area between 1,000 and 7,000 m^2 (25). Microvascular OPS imaging of glycocalyx thickness in sublingual capillaries further substantiated our systemic findings. In analogy with our previous findings of glycocalyx perturbation during acute hyperglycemia in healthy individuals (14), a marked reduction in systemic glycocalyx volume was observed in type 1 diabetic patients. In fact, overall systemic glycocalyx volume was reduced to 40–50% of the volume observed in matched normoglycemic control subjects, with an even more profound reduction in type 1 diabetic patients characterized by microalbuminuria. In addition, we also found a trend between A1C levels and glycocalyx volume, whereas no correlation was found for fasting plasma glucose levels. Therefore, the impact of (long-term) glucose regulation on glycocalyx volume will have to be addressed in a larger cohort of type 1 diabetic patients.

Interestingly, the reductions in dextran 40 intravascular distribution volumes in type 1 diabetic patients are not accompanied by concomitant increases in circulating blood volume. The latter implies that the lost glycocalyx volume is apparently compensated for by a reduction in total vascular volume. Since the majority of the glycocalyx volume is located within the microvasculature, these changes should become apparent at the level of the capillaries. Indeed, we observed a significant reduction in anatomic capillary dimensions in type 1 diabetes. The mechanism by which glycocalyx loss leads to a decrease in capillary diameter needs further study. One of the mechanisms could relate to the formation of pericapillary edema, which has been shown to cause a reduction of capillary diameters following glycocalyx degradation upon hyaluronidase infusion in rats (10). In analogy, reductions in capillary volume have been consistently reported in

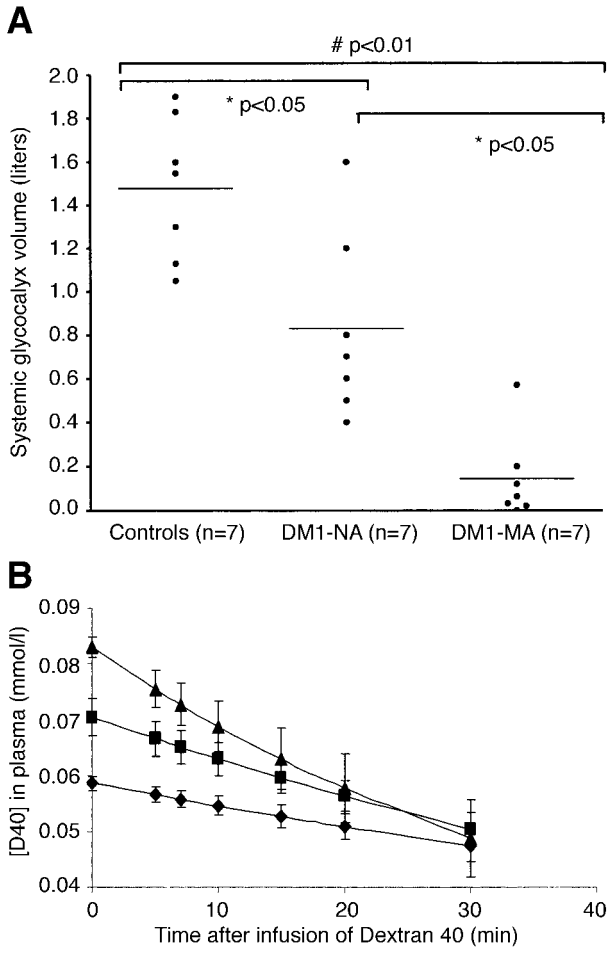

FIG. 1. ***A*: Systemic glycocalyx volumes are decreased in type 1 diabetic patients (DM1-NA and DM1-MA; groups 2 and 3) compared with matched control subjects (Controls; group 1). Between type 1 diabetic patients, reduction was highest in DM1-MA compared with DM1-NA. *P < 0.05 and #P < 0.01. *B*: Plasma dextran 40 clearance curves in matched control (♦), DM1-NA (■), and DM1-MA (▲) subjects. In type 1 diabetes, the rate of dextran 40 plasma clearance is increased compared with matched control subjects. Values on each time point are expressed as means ± SE.**

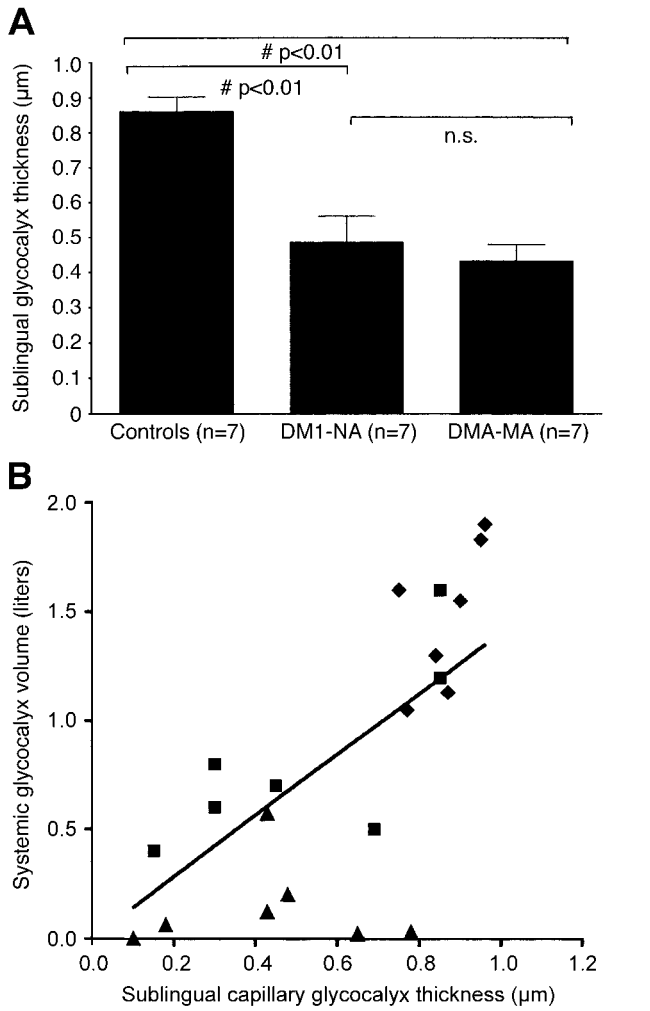

FIG. 2. ***A*: Capillary glycocalyx thickness is reduced in type 1 diabetes (DM1-NA and DM1-MA bars) compared with age-matched healthy control subjects (Controls bar). Individual capillary glycocalyx thickness is estimated from the transient widening of the erythrocyte column upon compression of the glycocalyx during capillary leukocyte passage. #P < 0.01. *B*: Relation between systemic glycocalyx volume and sublingual capillary glycocalyx thickness in matched control (♦), DM1-NA (■), and DM1-MA (▲) subjects.**

animal models of both acute and chronic hyperglycemia (26–28).

Possible mechanisms of glycocalyx perturbation. Glycocalyx thickness depends on the rate of synthesis and shedding of glycosaminoglycans. Over the last years, we and others (14,29,30) have demonstrated that hyaluronan is an important component of the endothelial glycocalyx and that hyperglycemia is associated with increased synthesis of hyaluronan both in humans and in vitro models. Moreover, recent animal studies (31–33) have shown that the endothelial glycocalyx is an important component of the glomerular barrier, the magnitude of which firmly decreases after hyaluronidase infusion. The observation of both increased hyaluronan and hyaluronidase levels in both plasma and kidney of diabetic animal models is consistent with our present finding in type 1 diabetic subjects, correlating the reduction in glycocalyx volume with enhanced shedding of these components (29,34). So far, six hyaluronidase-regulating genes have been identified in humans, of which four are functionally active, and the presence of renal hyaluronidase acitivity has long been recognized (35,36). It is tempting to speculate that genetically determined changes in hyaluronan and hyaluronidase metabolism may contribute to the sensitivity toward glycocalyx perturbation, which can be associated with the likelihood of developing vascular dysfunction in type 1 diabetes.

Study limitations. Due to the relatively small sample size, we were unable to perform multivariate analysis in order to identify determinants predictive for glycocalyx damage. In fact, we chose to include a homogenous group of type 1 diabetic patients with or without microalbuminuria. The clear difference between patient categories and control subjects underscores potential clinical relevance of the observation. Secondly, the accuracy of glycocalyx volume estimates is largely determined by the accuracy of dextran 40 distribution volume estimates. Because of its small size and neutral charge, dextran 40 is also cleared from circulation. Therefore, we estimated the intravascular dextran 40 concentrations before vascular leakage or renal clearance by extrapolating dextran 40 concentrations to the time of injection. As can be appreciated from the clearance curve in Fig. 1*B*, the error of the estimated initial dextran 40 concentration is relatively small and will

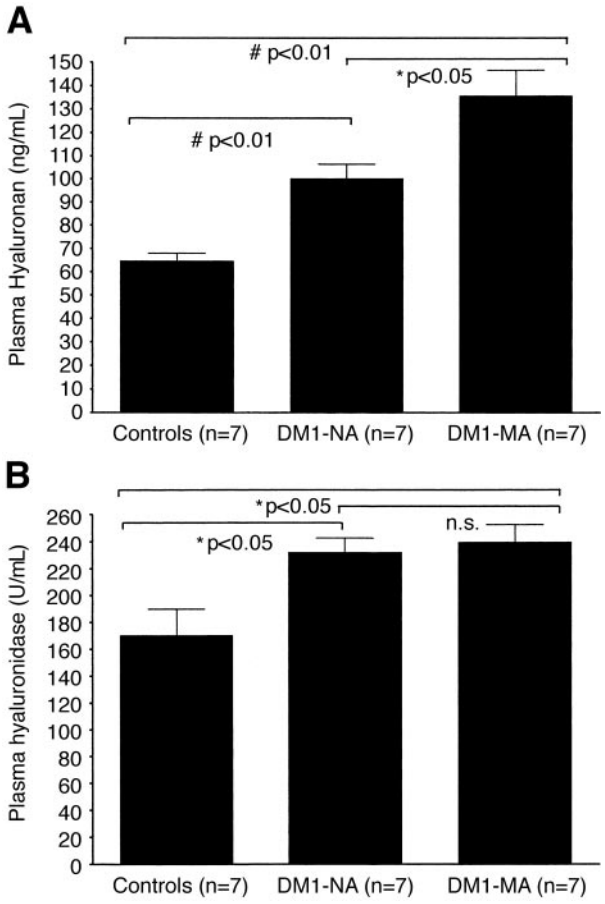

FIG. 3. *A*: Circulating hyaluronan levels increase in a stepwise manner from control to DM1-NA and DM1-MA subjects. Data are presented as means ± SE. *$P < 0.05$ and #$P < 0.01$. *B*: Plasma hyaluronidase activity is increased in type 1 diabetic patients compared with control subjects. Data are presented as means ± SE. *$P < 0.05$.

therefore have no major impact on the estimates of glycocalyx volume. Finally, whereas the microvasculature with its large endothelial surface area contains the majority of systemic glycocalyx volume, the macrovasculature determines the circulating blood volume. So, decreased glycocalyx volume with stable blood volume could also indicate selective loss of microvascular capillary volume. However, in the present study this scenario is highly unlikely. Thus, capillary density and dimension between diabetes without versus diabetes with microalbuminuria during OPS imaging were not significantly different, in spite of a significant decline in glycocalyx volume.

Clinical implications. The finding of a gradual reduction in glycocalyx volume in association with the presence of microalbuminuria in type 1 diabetic subjects emphasizes the generalized nature of glycocalyx perturbation in the development of diabetes-related microvascular disease. Further studies are needed to address whether glycocalyx perturbation indicates a poor vascular outcome and whether restoration of the glycocalyx is a valuable target to prevent vascular disease progression.

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