The influence of hyperglycemia on neutrophil extracellular trap formation and endothelial glycocalyx damage in a mouse model of type 2 diabetes

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Running Head: NETs formation in diabetic mice

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ABSTRACT

Objectives: Hyperglycemia induces vascular dysfunction that is thought to be initiated by neutrophils. Neutrophil activation produces endothelial injury by pathways that include NETosis, a type of specific cell-death. In this study, we investigated the effects of hyperglycemia on neutrophil activation, cell death, NETosis, and endothelial glycocalyx damage using a mouse diabetes model.

Methods: We used db/db mice as a type 2 diabetes model, and C57BL/6 mice were the controls. At 5, 8, and 12 weeks of age, the proportion of CD11b+ granulocytes, neutrophil extracellular traps (NETs)-forming granulocytes, damaged and nonviable granulocytes were analyzed. In addition, serum levels of high mobility group box-1, histone H3, and glycocalyx components that included syndecan-1 and hyaluronan were measured.

Results: In diabetic mice, we observed an increased proportion of CD11b+ granulocytes. The proportion of NETs-forming granulocytes increased from the early stages of the experiments. The proportions of damaged and nonviable granulocytes increased over time. In the 12-week old diabetes mice, serum histone H3 levels increased. Circulating levels of syndecan-1 and hyaluronan decreased over time and were lower in diabetes mice.

Conclusion: Neutrophil activation and cell death induce endothelial glycocalyx damage, and NETs formation also participates in the mechanisms of vascular injury in type 2 diabetes.

Keywords:
Diabetes mellitus, Hyperglycemia, Neutrophil Extracellular Traps, Vascular Injuries
Glycocalyx

List of abbreviations
NETs = neutrophil extracellular traps
HMGB1 = high mobility group box-1
MPO = myeloperoxidase
7-AAD = 7-Amino-Actinomycin D
FITC = fluorescein isothiocyanate
AGEs = advanced glycation end products
ELISA = Enzyme-Linked Immuno Sorbent Assay
Introduction

Vascular disorders caused by persistent hyperglycemia lead to the diabetic complications that include neuropathy, retinopathy, kidney damage, and arteriosclerosis.\(^1\) The mechanisms underlying vascular injury in diabetes are complex, and one hypothesized pathway is that vascular injury is initiated by endothelial damage following the activation of neutrophils, particularly NETs formation. Previous studies have described the interaction between activated neutrophils and endothelial cells with hyperglycemia.\(^2\),\(^3\)

Activated neutrophils express adhesion molecules on their surface, such as integrins and selectins, and attach to the vascular endothelial cells,\(^4\) and also change their cell membrane composition and express phosphatidylserine.\(^5\) Subsequently, the activated neutrophils undergo cell-death and release their cytotoxic components to induce inflammation. The neutrophil cell death has unique characteristics that includes the release of web-like DNA decorated with antimicrobial proteins such as histones, elastases, and MPO, namely NETs.\(^6\),\(^7\) NETs are also known to induce potent proinflammatory and procoagulant reactions in the surrounding area.\(^8\) Although the inflammatory response induced by neutrophil activation and cell death is an important inherent host defense mechanism, the excessive response can also damage the host. As a result, neutrophil activation and cell death caused by hyperglycemia are considered to be a critical event for the development of diabetic vascular disorders and complications.\(^3\)

As previously noted, NETosis is a specific type of neutrophil cell death characterized by NETs release.\(^9\) The estimated role of NETs is primarily pathogen-killing, but they are also cytotoxic to the host.\(^10\) During the course of NETosis, arginine residues of histones should be citrullinated, loosens histone-DNA binding, and cause chromatin to become filamentous.\(^11\) Histones that are released have a cytotoxic effect on the vascular
endothelium, and previous reports note that NETs play a major role in the development of vascular injury.\textsuperscript{3,12}

The vascular endothelial injury caused by neutrophil activation can be evaluated by the damage of glycocalyx.\textsuperscript{13} The glycocalyx is a gel-like component that covers the vascular endothelial surface and is mainly composed of proteoglycans that include syndecan-1, glycoprotein, and hyaluronan, sugar chains that do not bind directly to the cell membrane. The glycocalyx provides important functions that include anticoagulation, control of platelet and neutrophil adhesion to the vessel wall, and regulating vascular endothelial permeability.\textsuperscript{14} Accordingly, the glycocalyx protects blood vessels and contributes to vascular patency, however, since this component is quite fragile, it is easily damaged by various stimuli that can release components into the circulation following injury. As a result, circulating levels of glycocalyx components such as syndecan-1 and hyaluronan are used to evaluate vascular injury.\textsuperscript{15,16}

In diabetes, neutrophil activation and cell death are considered to be key factors producing endothelial injury, and previous studies report that NETs formation can be stimulated in hyperglycemia.\textsuperscript{17} However, most studies were performed using isolated neutrophils and observed acute responses to high glucose levels.\textsuperscript{18} At present, few studies evaluated the NETs-formation along with the progression of the hyperglycemia \textit{in vivo}. In the present study, we focused on the effect of high-glucose level on NETs formation and subsequent glycocalyx damage in a prolonged hyperglycemic state in a model of type 2 diabetes.

\textbf{Materials and methods}

\textit{Animal models}
The study was performed employing 4-week-old BKS.Cg-Dock7m+/+Leprdb/J male mice (db/db, as diabetes model) and C57BL/6J mice (BL/6, as normal control) (n= 5 or 6 in each group at each time point), which were purchased from Charles River (Tokyo, Japan). Animal procedures were performed in accordance with the guidelines for animal experimentation of the Japanese association for laboratory animal science. To acclimate to the laboratory conditions, mice were housed individually under a 12h:12h light-dark cycle at 22–25°C and 40–60% humidity for one week. Mice were fed CE-2 (CLEA Japan, Tokyo, Japan) and allowed access to water ad libitum throughout the study.

**Body weight, blood cells, and fluorescent staining**

At 5, 8, and 12 weeks of age, the bodyweight of each mice was measured, and then mice (n= 5 or 6 per time point in each group) were anesthetized and whole blood was collected. We added 14 ml of ACK lysing buffer (0.15 M NH4Cl, 1.0 mM KHCO3, 0.1 mM EDTA) to 1.0 mL of whole blood and mixed, then incubated for 5 min at room temperature to deplete red blood cells, followed by centrifugation at 2,000 rpm for 5 min at room temperature. The supernatant was carefully removed and pellets were rinsed with 5.0 mL of cold RPMI 1640 medium (Gibco, NY, USA) and mixed gently, and followed by centrifugation at 2,000 rpm for 5 min at 4 ºC, and supernatant was removed. Blood cells were suspended in 500 μL of RPMI 1640 medium. The number of blood cells was counted under the microscope, 5×10^5 cells per tube were dispensed into test tube. To assess NETs formation, blood cells were stained with the following antibodies: BV421-conjugated anti-Ly-6G antibody (clone 1A8, BD PharMingen, San Diego, CA) at 1:1000 dilution, APC-Cy7-conjugated anti-CD11b antibody (clone M1/70, Biolegend, San Diego, CA) at 1:1000 dilution, anti-histone H3 (citrulline R2 + R8 + R17) antibody (rabbit IgG, clone ab5103, Abcam, Cambridge, UK) at 1:300 dilution, and FITC-conjugated anti-myeloperoxidase
antibody (clone 2D4, Abcam) at 1:50 dilution were used. Antibodies were determined in reference to the previous report.\textsuperscript{19} Incubated for 30 min on ice in the dark. After incubation, centrifuged at 2,000 rpm for 5 min at 4 °C and aspirate supernatant. Washed with stain buffer (FBS, BD Pharminogen) and added BV510-conjugated Donkey anti-rabbit IgG (clone Poly 4064, Biolegend) at 1:60 dilution. Incubated for 30 min on ice in the dark and wash with stain buffer, filter the cell suspension on the cell-strainer cap of a 5-mL round-bottom tube (Corning, NY, USA). To assess the phosphatidylserine expression and DNA exposure, firstly, blood cells were stained with BV421-conjugated anti-Ly-6G antibody (clone 1A8, BD PharMingen) at 1:1000 dilution and APC-Cy7-conjugated anti-CD11b antibody (clone M1/70, Biolegend) as described above. After that, FITC Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend) was used according to manufacturer’s instructions.

**Blood glucose and triglyceride**

The serum samples collected from each mouse were stored at -80°C condition until the test were performed. A biochemical analyzer FUJI DRI-CHEM 7000 (Fujifilm Corp., Tokyo, Japan) were used to measure blood triglyceride and casual blood glucose following manufacturer’s instruction.

**Flow cytometric analysis**

Stained cells were analyzed by flow cytometry using a FACS Melody (BD Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed by using the FACS Chorus software (BD Biosciences). White blood cells were identified by Forward Scatter (FSC) and Side Scatter (SSC) properties. The proportion of Ly-6G\(^+\)CD11b\(^+\) cells (activated granulocyte), Ly-6G\(^+\)CD11b\(^+\)MPO\(^+\)H3Cit\(^+\) cells (NETs-forming granulocyte), Ly-
6G^+CD11b^+annexin V^+7-AAD^- cells (damaged granulocyte) and Ly-6G^+CD11b^+annexin V^+7-AAD^+ cells (dead granulocyte) in white blood cells were calculated.

**ELISA assay**

Enzyme-linked immunosorbent assays (ELISA) were performed using the commercially available kits to analyze serum levels of histone H3 (Shino-test, Tokyo, Japan), HMGB1 (Diaclone, Besancon, France), syndecan-1 (Shino-test) and hyaluronan (R&D systems, Minneapolis, USA). Each assay was performed according to the manufacturer’s instructions.

**Statistical analysis**

Results were expressed as mean ± standard error of the mean (SEM). Data were analyzed using SPSS 20.0 J (IBM Japan, Tokyo, Japan). Student’s t-test was used to identify significant differences between the groups. A P-value of less than 0.05 was considered significant.

**Results**

Body weight, blood glucose, and triglyceride level

During the study period, body weight was kept higher in db/db mice compared to BL/6 mice (Fig. 1). The blood glucose levels in BL/6 mouse were approximately 380 mg/dL, and it was stable throughout the study period. While the blood glucose levels in db/db mice were twice as much as that of BL/6 at 5 weeks-old and continued to increase with age and reached to over 1000 mg/dL (Fig. 2A). The triglyceride levels did not change over time during the study period in both mice, and the triglyceride levels in db/db mice were significantly higher than those in controls at 5, 8, and 12 weeks of age (Fig. 2B).
Neutrophil activation and NETs-formation

In the flow cytometry analysis, the gating strategies in this study are shown in Fig. 3. The populations of activated (CD11b+) granulocyte was not different between db/db mice and BL/6 mice at 5 weeks old. While the populations of activated granulocyte increased over time and were significantly higher at 8 and 12 weeks-old in db/db mice than those in BL/6 mice (Fig. 4A). As for NETs-forming cells, the population in db/db mice kept significantly higher than that in BL/6 mice from 5 weeks-old till the end of the study (Fig. 4B). The population of damaged (annexin V+) granulocyte in db/db mice increased over time and was significantly higher than that in BL/6 mice at 12 weeks-old of age (Fig. 4C). The population of dead (7-AAD+) granulocyte in db/db mice was significantly higher at 8 and 12 weeks old (Fig. 4D).

Changes of HMGB1 and histone H3

Serum levels of histone H3 were low in both animals and not exceeded 0.2 ng/mL until 8 weeks-old. There serum histone H3 levels significantly increased at 12 weeks only in db/db mouse (Fig. 5A). There were no significant differences in serum HMGB1 levels between db/db and BL/6 mice throughout the study period (Fig. 5B).

Changes of syndecan-1 and hyaluronan

Serum levels of syndecan-1 were gradually declined along with the time, and the levels were lower at 5 and 12 weeks in db/db mice compared with those in BL/6 mice (Fig. 5C). Similarly, hyaluronan levels decreased with time, and the levels were lower in db/db mice throughout the study period (Fig. 5D).
Discussion

Type 2 diabetes is one of the most common lifestyle-related diseases worldwide. The number of patients with type 2 diabetes is increasing, and one in 11 adults are estimated to be diabetic. Type 2 diabetes is caused by various factors that include genetic background, excessive caloric intake, lack of exercise, and aging. If not appropriately treated, diabetes causes multiorgan injury due to vascular dysfunction. Therefore, it is important to alter the progression from vascular injury to the irreversible organ damage in the management of type 2 diabetes.

In the present study, we evaluated age-dependent granulocyte changes of activation, injury, and cell death, including NETs-formation and endothelial glycocalyx damage in a type 2 diabetes mouse model. We found age-dependent increases of activated, damaged, and dead granulocytes based on flow cytometry analysis. In addition, the incidence of NETosis was also increased. Our findings suggest that hyperglycemia facilitates granulocyte attachment and damage to the vascular endothelium, and shifts the endothelial surface to a proinflammatory and procoagulant state, and NETs formation further accelerates the vascular changes noted.

In this study, the neutrophil activation was assessed by adhesion molecule CD11b expression, also called integrin αM chain, a critical adhesion molecule expressed on inflammatory cells that include granulocytes and monocytes. Delgado-Rizo et al. reported that increased CD11b expression on neutrophils occurred in diabetic microangiopathy in patients with type 2 diabetes, and increases in CD11b expression levels represent neutrophil activation and intensified adhesion capability. The relationship between the blood glucose level and release of NETs was also reported in in vitro studies with increases in NETs production in isolated neutrophils from db/db mice. NETs release is also reported to be dependent on exogenous glucose and glycolysis. Therefore, we
hypothesized that prolonged hyperglycemia in vivo promotes neutrophil activation and NETosis, and those phenomena were confirmed in our animal model.

Histone H3 and HMGB1, major damage-associated molecular patterns that are released by cell death and NETosis, are known to damage the vascular endothelium. Previously, histone measurements in serum have been challenging due to the presence of interfering substances. Recently, a new modified ELISA following specific preparation techniques has made it possible. Using this method, the present study confirmed that circulating histone H3 levels increased in the diabetic mouse at 12 weeks, which probably contributes to the vascular endothelial damage due to persistent hyperglycemia. On the other hand, HMGB1 did not increase over time, and no significant difference was observed between diabetes and control mice. HMGB1 is known as a ‘death mediator’ in sepsis, and Wang et al. reported elevated HMGB1 and death in the endotoxin-induced sepsis model. The reason for the unexpected result in this study is unknown, but sustained hyperglycemia may not trigger the increase of HMGB1.

The endothelial glycocalyx has also been reported to be decreased in type 2 diabetes. Hyperglycemia-induced atherosclerosis a complex inflammatory process produced by multiple factors that include endothelial injury, smooth muscle cell proliferation, and foam cell infiltration. Glycocalyx injury and loss is a critical part of this response, however, the mechanisms are not well described. In this study, serum syndecan-1 and hyaluronan levels decreased in both types of mice along with age, and a recent report has also shown similar decreases with age in humans suggesting these changes might be the results of aging. Interestingly, serum syndecan-1 and hyaluronan levels were significantly lower in diabetic mice compared to controls, and the decreases seemed to be in parallel with the activation, damage, cell death, and NETosis. These time-dependent reductions in glycocalyx expression were presumably the result of persistent endothelial injury evoked
by the hyperglycemia-stimulated neutrophils. In fact, previous reports have reported that the thickness of the glycocalyx layer was reduced due to persistent hyperglycemia. However, it is suggested that serum syndecan and hyaluronan levels are elevated in diabetics, and they are the independent markers of cardiovascular risk. For the discrepancy between our results and other observations, additional studies are necessary.

Some limitations should be noted in this study. First, the genetic diabetic mouse was utilized as a type 2 diabetes model, and it is not possible to distinguish whether the phenomenon observed in this study is due to diabetes or due to genetic factors. For example, together with hyperglycemia, hypertriglyceridemia was recognized in this model that may also affect endothelial damage. However, blood glucose levels increased over time in diabetic mice, and the increase was parallel with the activation of neutrophil and glycocalyx damage. In contrast, the triglyceride level was consistent throughout the study period, thus the time-dependent increases of granulocyte activation, cell damage, cell death, and NETs formation are more likely to be induced by the hyperglycemia. Second, blood glucose levels differ among species, and the glucose levels of mice is much higher than humans. The glucose level was over 300 mg/dL in the control mouse, and that reached over 1000 mg/dL in diabetic mouse. In addition, the proportion of neutrophils differs between humans and mice considerably. Species-difference should be considered when the present data is applied to humans. Finally, other important pathways involved in endothelial damage such as oxidative stress, thrombosis, and formation of AGEs and activation of AGE receptors were not examined in this study. Additional studies that include other important factors should be considered.

**Perspective:**
The present study demonstrated the time-dependent increases of neutrophil activation, damage, and cell death, especially the NETs formation in a diabetic mouse model. The relationships between hyperglycemia and damage-promoting capabilities of neutrophils were also observed, and this inflammatory injury, as described is thought to lead to endothelial glycocalyx damage and vascular dysfunction in type 2 diabetes. This study provides important information for the maintenance of microcirculation in diabetes.

**Acknowledgement:**

None.
References


Figure 1. Body weight. Changes in the body weight during the study period. db/db mice at 5, 8, 12 weeks-old (black circle, solid line, n=6, 6, 5, respectively) and BL/6 mice at 5, 8, 12 weeks-old (diamonds, dashed line, n=6). Data are represented as mean ± standard error of the mean (SEM).
Figure 2. Blood glucose and triglyceride. Blood glucose and triglyceride levels are shown in the figures; (A) blood glucose, and (B) triglyceride in db/db mice at 5, 8, 12 weeks-old (black bars, n=6, 6, 5, respectively) and BL/6 mice at 5, 8, 12 weeks-old (gray bars, n=6). Data are represented as mean ± standard error of the mean (SEM).
Figure 3. Gating strategy in flow cytometry. Upper three figures represent gating strategy for CD11b+ granulocytes and NET-forming granulocyte. Single cell suspensions were first gated by forward scatter (FSC) and side scatter (SSC). CD11b+ granulocytes were selected as double-positive for CD11b and Ly-6G, quantified, and used for further analysis. NET-forming granulocyte were selected as double-positive for myeloperoxidase (MPO) and citrullinated histone H3 (H3Cit). Lower three figures represent gating strategy for damaged (phosphatidylserine-positive) granulocytes and nonviable (7-AAD-positive) granulocytes. After selection of CD11b+ granulocytes, damaged granulocytes were selected as negative for 7-AAD and positive for Annexin-V. Dead granulocytes were selected as double-positive for 7-AAD and Annexin-V. The same gating strategy was used to all analysis.
Figure 4. Neutrophil activation, NETs-forming and cell death. Each graph represented as (A) activated (CD11b\(^{+}\)) granulocytes, (B) NETs-forming granulocytes, (C) damaged (Annexin-V\(^{+}\)) granulocytes and (D) nonviable (7-AAD\(^{+}\)) granulocytes in both db/db mice at 5,8,12 weeks old (black bars, n=6,6,5, respectively) and BL/6 mice at 5,8,12 weeks old (gray bars, n=6). The vertical axis represents the proportion to the total white blood cell. Each data was represented as mean + standard error of the mean (SEM).
Figure 5. Measurement of histone H3, HMGB1, hyaluronan and syndecan-1. Each graph represented as serum levels of (A) histone H3, (B) HMGB1, (C) serum syndecan-1 and (D) hyaluronan in both db/db mice at 5, 8, 12 weeks old (black bars, n=5) and BL/6 mice at 5, 8, 12 weeks old (gray bars, n=5). Each data was represented as mean ± standard error of the mean (SEM).