Fluorescein Permeability of the Blood–Brain Barrier Is Enhanced in Juvenile- but Not Young Adult-Onset Type 1 Diabetes in Rats

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Clinicall, neurological disorders, such as cognitive impairments and dementia, have been reported as diabetic complications, which are remarkable, especially in children with diabetes. The blood–brain barrier (BBB) is a physiologically dynamic regulatory barrier that maintains the consistency of the fluid microenvironment composition of the brain. However, the differences in BBB conditions between children and adults and the contribution of the BBB to the severity of cognitive impairments remain unclear. We generated adult-onset diabetes mellitus (DM) and juvenile-onset diabetes mellitus (JDM) diabetic rat models and investigated BBB functions in these models during the early stages of type 1 diabetes. We performed a BBB permeability assay using sodium fluorescein, a small-molecule fluorescent dye, to evaluate endothelial transport from the blood to the central nervous system. One week after diabetes onset, BBB permeability increased in the hippocampus and striatum of JDM rats, but no changes were observed in the frontal cortex and hypothalamus of JDM rats or for any region of DM rats. The double staining of tight junction proteins and astrocytes revealed no changes in the hippocampus and striatum of JDM rats. These results suggested that the observed increase in BBB permeability during early-stage diabetes onset in JDM rats, which did not depend on the expression of the interendothelial tight junction protein, claudin-5, may affect stylized neural development and cognitive function.

Key words juvenile-onset type 1 diabetes; blood–brain barrier permeability; fluorescein

INTRODUCTION

Diabetes mellitus is a disease associated with metabolic dysregulation, most notably abnormal glucose metabolism, accompanied by characteristic long-term complications, such as retinopathy, nephropathy, neuropathy, memory impairments, and dementia. Diabetes is a risk factor for cognitive and learning and memory impairments.1,2 Epidemiological studies have revealed that patients who developed type 1 diabetes before the age of 7 years have lower cognitive function scores than those who developed the disease between the ages of 7 and 17 years, at a time when they have had diabetes for more than 10 years.3 Moreover, children who develop type 1 diabetes at a very early ages were observed to present decreased attention, processing speeds,4 and more severe cognitive performance impairments5,6 than patients who developed type 1 diabetes at an older age. However, the relationship between the age of type 1 diabetes onset and the severity of cognitive impairments remains unknown.

The blood–brain barrier (BBB) is a crucial system for maintaining brain homeostasis and limiting the penetration of molecules, ions, toxins, and pathogens into the brain.7–10 Many of the BBB properties are manifested by endothelial cells that are connected by high-electrical-resistance tight junctions and contain few transcytotic vesicles, limiting the paracellular and transcellular flow of molecules from the blood into the brain.10 Structural and functional changes that affect the BBB might represent the pathophysiological basis for the development of several diseases.10,11 The ratio of albumin in the cerebrospinal fluid to the serum, which serves as an indicator of BBB disruption, was increased in Alzheimer’s disease patients compared with healthy controls.11 The knock-out of platelet-derived growth factor receptor (PDGFR), an important receptor necessary for the mobilization of pericytes around endothelial cells, results in increased vascular leakiness, neurodegeneration, and cognitive impairment in mice.12 High-glucose-induced oxidative stress also causes brain tissue damage, resulting in increased BBB permeability and associated with cerebral dysfunction. BBB permeability significantly increased in streptozotocin (STZ)-induced type 1 diabetic model rats,13–15 and the mechanism underlying this phenomenon was suggested to be the increased production of reactive oxygen species (ROS) via advanced glycation end-products (AGEs)/receptor for AGEs (RAGE) signaling and increased nuclear factor-κB (NF-κB) expression.16 Hyperglycemia may lead to the development of dementia by increasing the influx of glucose or glucose-related toxic substances in the brain due to increased BBB permeability.

Although type 1 diabetic animal models generated by STZ administration have been widely used, most studies have not focused on juvenile-onset diabetes mellitus (JDM), which may correspond with childhood-onset type 1 diabetes. We previously reported working memory and spatial learning deficits in JDM rats without associated motor deficits, as revealed by the Y-maze and Morris water maze tests.17,18 During the early stages of type 1 diabetes, we also observed impairments in hippocampal long-term depression at the Schaffer collateral-CA1 pyramidal synapses in JDM but not in young adult-onset diabetes mellitus (DM) rats, which was attributed to NR2B-containing N-methyl-D-aspartate receptors (NMDARs).
and protein kinase A (PKA) activity. By contrast, long-term potentiation in the hippocampus was impaired in DM but not in JDM rats.\textsuperscript{18,19} These results suggested differences in electrophysiological properties between JDM and DM rats, which might cause the behavioral deficiencies observed in JDM rats. The aim of this study was to investigate changes in cerebral vascular permeability in JDM and DM rats 1 week after diabetes induction.

**MATERIALS AND METHODS**

**Animals** All experiments were approved by the Institutional Animal Care and Use Committee of Tokyo University of Science. Male and female Wistar/ST rats were housed under 12-h–12-h light–dark conditions and received food and water ad libitum. Animals were classified as diabetic when blood glucose levels were measured at >300 mg/dL. Blood glucose levels were measured at the time of plasma collection. Diabetes mellitus was induced by the one-time STZ administration (85 mg/kg, intraperitoneally (i.p.)) to 17-d-old rats (JDM) and 10-week-old rats (DM), as previously described.\textsuperscript{17–19} To investigate region-specific changes in BBB permeability, rat brains were dissected: frontal cortex, hippocampus, hypothalamus, and striatum.

**Fluorescein Extravasation** Sodium fluorescein (NaFL) permeability experiments were performed as described previously with minor modification.\textsuperscript{20} Rats were anesthetized with pentobarbital (40 mg/kg, i.p.), and NaFL (40 mg/kg, intravenously (i.v.)) was infused via the tail vein and allowed to circulate for 30 min. To inhibit NaFL discharge, probenecid (200 mg/kg, i.p.), a multidrug resistance-associated protein 2 inhibitor\textsuperscript{21} was administered 45 min before anesthesia, and heparin (100 U/mL, i.p.) was injected 15 min before opening the chest. Rats were perfused transcardially with ice-cold phosphate-buffered saline (PBS) for 10 min. The brain was removed and divided into each brain region. Brain tissues were mechanically homogenized in 7.5% (w/v) trichloroacetate and neutralized by the addition of 5 NaOH to the supernatant prior to the fluorometric determination of NaFL concentrations (excitation 485 nm, emission 535 nm) by Envision (PerkinElmer Japan Co., Ltd., Yokohama, Japan). Plasma samples were collected from the perfusion circuit, diluted in 7.5% (w/v) trichloroacetate, and measured using the same methods used to measure tissue concentrations. The content of fluorescein (ng) in plasma and brain tissue samples was calculated from the calibration curve. To evaluate NaFL extravasation, the ratios of tissue NaFL concentrations to plasma NaFL concentrations (µL/g) was calculated by dividing the fluorescein concentration in plasma (ng/µL) by the fluorescein concentration in brain tissue (ng/g).

**Immunohistochemistry** Rats were anesthetized with pentobarbital, opened, and transcardially perfused with ice-cold PBS for 10 min, after which the brain was immediately removed and flash frozen with liquid nitrogen. The brain was cut into 20-µm-thick coronal sections using a cryostat (Leica Microsystems GmbH), and slices were collected on slides (hippocampus: Bregma −3.00 to −4.16 mm; striatum: Bregma −0.40 to +0.48 mm, according to Paxinos and Franklin\textsuperscript{22}). The sections were dehydrated, defatted, and fixed with 100% ethanol and acetone. For double staining, the same section was preincubated for 90 min in 5% normal donkey serum in PBS containing 0.3% Triton X-100 and then incubated in primary antibodies (mouse anti-claudin-5, Thermo Fischer Scientific K.K. (Tokyo, Japan); and rabbit anti-glial fibrillary acidic protein (GFAP), Sigma-Aldrich Co., LLC. (Tokyo, Japan)) at 4°C for 1 d. After three washes in PBS, secondary antibodies (donkey anti-mouse immunoglobulin G (IgG)-Alexa Fluor\textsuperscript{488}, 488, diluted 1:100 and donkey anti-rabbit IgG-Alexa Fluor\textsuperscript{555}, 555, diluted 1:100) were incubated for 1 h at room temperature. Slices were observed with a confocal laser microscope LSM 5 Exciter (excitation wavelength 488 or 543 nm). Four images (130 × 130 µm) were taken per region, and the average of the positive area and co-localized area was calculated as the mean value using ImageJ (NIH).

**Drugs and Chemicals** Streptozotocin was purchased from Sigma-Aldrich Co., LLC. and dissolved in PBS. Sodium fluorescein, probenecid, trichloroacetate, and NaOH were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and were dissolved in distilled water or PBS. Sodium pentobarbital was purchased from Tokyo Chemical Industry (Tokyo, Japan) and was dissolved in PBS. Heparin was purchased from EA Pharma (Tokyo, Japan). All chemicals were commercial products of the highest available quality.

**Data Analysis and Statistics** All statistical analyses were performed using Excel (Microsoft, Redmond, WA, U.S.A.) with the Statcel4 add-in software (OMS, Tokyo, Japan). All data are presented as the mean ± standard error of the mean (S.E.M.). Significant differences between means were evaluated by Welch’s t-test or one-way ANOVA, followed by Bonferroni’s post hoc test for multiple comparisons. $p$-Values less than 0.05 were considered significant.

**RESULTS AND DISCUSSION**

Both males and females in the STZ-treated groups showed significant decreases in body weight and increases in blood glucose levels 1 week after STZ treatment compared with age-matched control animals (Table 1). The combined results for both males and females are as follows. The body weight for young control and JDM groups were 54.3 ± 2.7 g (n = 16) and 37.7 ± 1.9 g (n = 18), respectively (p < 0.0001), and the blood glucose levels were 110.8 ± 5.3 mg/dL (n = 16) and 516.6 ± 21.1 mg/dL (n = 18), p < 0.0001). The body weights for adult control and DM groups were 284.3 ± 24.9 g (n = 6) and 220.4 ± 18.6 g (n = 7), respectively (p < 0.0001), and the blood glucose levels were 155.2 ± 22.2 mg/dL (n = 6) and 488.1 ± 40.9 mg/dL (n = 7, p < 0.0001). Blood glucose levels did not differ significantly between males and females for either the JDM or DM groups (Table 1). Therefore, we confirmed that the diabetic model was successfully generated in both sexes of the JDM and DM groups, and all following experiments were conducted without distinction between males and females. Because the blood glucose levels between the JDM and DM groups were not significantly different, the following results cannot be attributed to differences in blood glucose levels.

We performed a BBB permeability assay using NaFL to evaluate endothelial transport from the blood to the central nervous system. As shown in Fig. 1, BBB permeability increased in the hippocampus and striatum of JDM rats but not in the frontal cortex and hypothalamus compared with the permeability of the age-matched control group 1 week after
STZ administration. Changes in BBB permeability were not observed in DM rats or either age-matched control group. NaFL is a low-molecular-weight tracer (376 Da) that can pass through the BBB more easily than higher-molecular-weight tracers. Thus, the increase in BBB permeability to NaFL may represent the earliest and the most sensitive indicator of BBB disruption. These results indicated that the BBB of the hippocampus and striatum in JDM but not DM rats became vulnerable 1 week after diabetes induction.

Because of the small molecular weight of fluorescein, we focused on claudin-5, one of the tight junction-related molecules, and examined its expression level by immunostaining. As shown in Fig. 2, no significant differences were observed in claudin-5 expression in either the hippocampus or striatum compared with that in age-matched control rats (Figs. 2A, B, left). These results suggested that changes in the expression levels of claudin-5 do not contribute greatly to the increased BBB permeability observed in JDM rats. Next, we examined the expression levels of glial cell markers and the percentage of glial cells that colocalized with claudin-5 by performing immunostaining in the hippocampus and striatum of JDM rats (Figs. 2A, B, middle and right). The overlap between the astrocyte marker GFAP and claudin-5 tended to decrease in JDM rats; however, the ratio of claudin-5 and GFAP double-positive areas to claudin-5-positive areas remained unchanged (Fig. 2C). These results indicated that the association between claudin-5 and astrocytic end-feet might not be involved in the enhancement of BBB permeability in JDM rats. The proper- ties of the BBB are largely dependent on endothelial cells, but cells associated with the basement membrane, pericytes, glial cells, neurons, and perivascular macrophages, which are also located in the BBB and comprise the neurovascular unit, are

|                | Control (young) | JDM | Control (adult) | DM |
|----------------|----------------|-----|----------------|----|
|                | Male  | Female | Male  | Female | Male  | Female | Male  | Female |
| Body weight (g) | 57.0  | ±4.0   | 50.4  | ±3.3   | 38.6  | ±2.2   | ** 35.6 | ±3.9 |
| Blood glucose (mg/dL) | 110.2 | ±6.7   | 111.8 | ±9.7   | 515.6 | ±26.4*** | 519.0 | ±37.0*** |
| n               | 10    | 6      | 13    | 5      | 3     | 3      | 3     | 4      |

Data are presented as the mean ± S.E.M. ***p < 0.001, **p < 0.01 vs. age-matched male control, †††p < 0.001, †p < 0.01, †p < 0.05 vs. age-matched female control. Welch’s t-test.
also important for the restrictions imposed by the BBB. 7–9) Endothelial cells in the central nervous system are held together by tight junction proteins, which greatly limit the paracellular flux of molecules and ions in the blood. 23) Claudin-5 is a major cell adhesion molecule associated with tight junctions and expressed by the endothelial cells of the brain. A previous tracer assay revealed that BBB permeability against small molecules (<800 Da) increased in claudin-5-deficient mice, but permeability to larger molecules remained unaffected. 24) Claudin-5 was also reported to be expressed at the endothelial junctions as early as embryonic day 12, and tracer experiments using biotin (0.5 kDa) revealed that the BBB was functional during embryogenesis, even before astrocyte generation. 25) However, our results suggested that the expression levels of claudin-5 do not contribute greatly to the observed increase in BBB permeability in JDM rats. Astrocyte coverage, which supports endothelial cells, and tight junction proteins are important for the maintenance of BBB function. 26) The basal process end-feet of astrocytes, which represent a major glial cell type, almost completely ensheath the vascular tube, which is generated directly after birth and extends processes that contact vessels during the first postnatal week. 27) The coverage of blood vessels by astrocytic end-feet, which is characteristic of the adult brain, is not completed until 3 weeks of age in mice. 27) In this study, STZ was administered on postnatal day 17, when the cerebral blood vessels might not yet be completely covered by astrocytic end-feet, and the BBB function might not yet be mature; however, astrocytes did not appear to contribute greatly to the increased BBB permeability observed in JDM rats in our study.

These results suggested that the onset of type 1 diabetes during brain development leads to an increase in cerebrovascular permeability during the early stages of type 1 diabetes mellitus, which may contribute to the development of working memory and spatial learning deficits in JDM. 17,18) Many transportation systems and cellular junctions components are expressed in BBB cells, 10) and the expression and appearance of basement membrane proteins, pericyte coverage, and transporters also begin during embryogenesis, contributing to the formation of a primitive BBB during embryogenesis. 10) As for the permeation of small molecules into the brain parenchyma, there are various mechanisms such as not only paracellular transports but also transporters and vesicular transport, further studies remain necessary to examine these systems.

CONCLUSION

In conclusion, the permeability of glucose and other substances may be increased at an early stage of type 1 juvenile diabetes, and this may affect stylized neural development and cognitive function. Further studies are required to determine what protein would be attributed to the increased BBB permeability in JDM rats. Our findings lead us to consider the cerebrovascular protection may be important for the treatment of
cognitive decline in childhood-onset type 1 diabetes.

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Conflict of Interest The authors declare no conflict of interest.

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