White Matter Damage and the Effect of Matrix Metalloproteinases in Type 2 Diabetic Mice After Stroke

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Background and Purpose—Diabetes mellitus leads to a higher risk of ischemic stroke and worse outcome compared to the general population. However, there have been few studies on white matter (WM) damage after stroke in diabetes mellitus. We therefore investigated WM damage after stroke in mice with diabetes mellitus.

Methods—BKS.Cg-m<sup>+/+</sup>Lepr<sup>db/db</sup> type 2 diabetes mellitus mice and db<sup>+</sup> non-diabetes mellitus mice were subjected to middle cerebral artery occlusion. Functional outcome, immunostaining, zymography, Western blot, and polymerase chain reaction were used.

Results—After stroke, mice with diabetes mellitus exhibited significantly increased lesion volume and brain hemorrhagic and neurological deficits compared to mice without diabetes mellitus. Bielshowsky silver, luxol fast blue, amyloid precursor protein, and NG2 expression were significantly decreased, indicating WM damage, and matrix metalloproteinase (MMP)-9 activity was significantly increased in the ischemic brain of mice with diabetes mellitus. Subanalysis of similar lesions in mice with and without diabetes mellitus demonstrated mice with diabetes mellitus had significantly increased WM damage than in mice without diabetes mellitus (<i>P</i> < 0.05). To investigate the mechanism underlying diabetes mellitus-induced WM damage, oxygen–glucose deprivation-stressed premature oligodendrocyte and primary cortical neuron cultures were used. High glucose increased MMP-2, MMP-9, cleaved caspase-3 levels, and apoptosis, as well as decreased cell survival and dendrite outgrowth in cultured primary cortical neuron. High glucose increased MMP-9, cleaved caspase-3 level, and apoptosis, and decreased cell proliferation and cell survival in cultured oligodendrocytes. Inhibition of MMP by GM6001 treatment significantly decreased high glucose-induced cell death and apoptosis in cultured primary cortical neuron and oligodendrocytes but did not alter dendrite outgrowth in primary cortical neuron.

Conclusions—Mice with diabetes mellitus have increased brain hemorrhage and show more severely injured WM than mice without diabetes mellitus after stroke. MMP-9 upregulated in mice with diabetes mellitus may exacerbate WM damage after stroke in mice with diabetes mellitus. (Stroke. 2011;42:445-452.)

Key Words: diabetes □ mice □ stroke □ white matter

Diabetes mellitus (DM) is a major health problem leading to a higher risk of ischemic stroke and worse outcome compared to that of the general population. Diabetic subjects are more prone to more and earlier white matter (WM) high-intensity lesions. We seek to elucidate the changes and mechanisms underlying the adverse effects of diabetes on the impaired WM after stroke.

Matrix metalloproteinases (MMP) degrade extracellular matrix proteins and are implicated in blood–brain barrier (BBB) breakdown and neuronal injury early after stroke. Disruption of the BBB leads to WM lesions. MMP promote BBB disruption, glial cell activation, and WM lesions after chronic cerebral hypoperfusion. Ischemic degradation of myelin basic protein is significantly reduced in the WM in MMP-9 knockout mice. Increased circulating MMP-9 is associated with a high prevalence of large WM hyperintensities in brain ischemia patients. MMP-9 protein level and activity are augmented in isolated cerebrovessels of the Goto-Kakizaki rats with diabetes. Whether DM exacerbates WM damage after stroke and whether MMP contribute to this WM damage in mice with DM have not been investigated.

Materials and Methods

Animal Middle Cerebral Artery Occlusion Model and Experimental Groups
Adult male BKS.Cg-m<sup>+/+</sup>Lepr<sup>db/db</sup> mice with DM and control db<sup>+</sup> mice without DM (age, 2–3 months) were purchased from Jackson Laboratory (Wilmington, MA). Right temporal (60 minutes) middle cerebral artery occlusion (MCAO) was induced using the filament model as previously described. Mice with MCAO

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werefemal24 hours after MCAO for immunostaining (n=11 per group)and for zymography, Western blot, and real-time polymerase chain reaction (PCR) assays (n=4 per group).

Blood Glucose Measurement
Blood glucose was measured before and 24 hours after MCAO by using test strips for glucose (Polymer Technology System, Indianapolis, IN).

Functional Test
A battery of behavioral tests (modified neurological severity score) and foot-fault tests were performed at 1 day after MCAO by an investigator who was blinded to the experimental groups.

Histological and Immunohistochemical Assessment
The brains were fixed in 4% paraformaldehyde. Seven coronal sections of tissue were processed and stained with hematoxylin and eosin for calculation of volume of cerebral infarction and presented as a percentage of the lesion compared with the contralateral hemisphere.

For immunostaining, a series of 6-µm-thick sections were cut from standard paraffin blocks (bregma, −1 mm to +1 mm). Antibody against NG2 (oligodendrocyte progenitor cell marker, 1:100; Chemicon, CA) and amyloid precursor protein (dilution 1:50; Cell Signaling Technology) were used. Bielschowsky silver immunostaining was used to demonstrate axons, and luxol fast blue staining was used to demonstrate myelin. Control experiments consisted of staining brain coronal tissue sections as outlined, but nonimmune serum was substituted for the primary antibody. The immunostaining analysis was performed by an investigator blinded to the experimental groups.

MMP Zymography
Cerebral tissue was homogenized in lysis buffer, including protease inhibitors, and MMP zymography was performed as previously described. Gelatinolytic activity was demonstrated as clear zones or bands at the appropriate molecular weights.

Real-Time PCR
Brain tissues or cells were harvested and total RNA was isolated following a standard protocol. Quantitative PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems) using 3-stage program parameters. Each sample was tested in triplicate, and analysis of relative gene expression data using the 2−ΔΔCt method. The following primers were used: MMP-2: forward, CGA GGA ATG AGT ACT GGG TCT ATT; reverse, ACT CCA GTT AAA GGC ATG AGT ACT GGG TCT ATT; MMP-9: forward, ATC TCT TCT AGA GAC TGG GAA GGA G; reverse, AAG CTG ATT GAC TAA AGT AGC TGG A; and GAPDH: forward, AGA ACA TCA TCC CTG CAT CC; reverse, CAC ATT GGG GGT AGG AAC AC.

Western Blot
Western blot was performed as previously described. Specific proteins were visualized using a SuperSignal West Pico chemiluminescence kit (Pierce). Anti-MMP-2 (1:500; Santa Cruz Biotechno-

Primary Cultured Neuron Culture and Quantification of Dendrite Outgrowth in Primary Cultured Neurons
Primary cultured neurons (PCN) were obtained from pregnant Wistar rat embryos 17 days old and cultured with Neuralbasal-A medium (GIBCO) containing 2% B27 medium supplement (GIBCO); 1-hour oxygen-glucose deprivation was induced. The PCN cultures were then divided into (n=6 per group): (1) control (25 mmol/L glucose); (2) high glucose (HG; 37.5 mmol/L glucose); (3) HG supplemented with 10 mmol/L DMSO (0.4 µM/L); and (4) HG+GM6001 (10 µmol/L) for 24 hours. Western blot, real-time PCR, lactate dehydrogenase (LDH) assays, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining were performed, respectively.

To measure PCN dendrite outgrowth, TUJ1 immunofluorescent staining (1:1000; Covance) was performed. TUJ1-positive cells were captured at 40× magnification with a digital camera. The average length of TUJ1-positive neuronal dendrite outgrowth was presented.

Premature Oligodendrocyte Cell Line Culture
An immortalized mouse premature oligodendrocyte cell line (N20.1; generously provided by Dr Anthony Campagnoni, University of California at Los Angeles) was used. N20.1 cells were cultured in DMEM/F12 media with 10% fetal bovine serum and 100 µ/mL G418. Oxygen-glucose deprivation was induced and cells were...
treated with: (1) control; (2) HG; (3) HG+DMSO; and (4) HG+GM6001 (10 μmol/L) for 24 hours. Western blot, real-time PCR, LDH, MTS assays, and TUNEL staining were performed, respectively.

**LDH Assay**
The CytoTox 96 non-radioactive cytotoxicity assay kit (Promega) was used following standard protocol. Secreted (media) and total (media and cells) LDH levels were measured. Data are presented as percentage of LDH level in the media to total LDH.

**MTS Assay**
Using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), 5000 cells were plated and 20 μL MTS was added to each well and incubated after treatment. Absorbance was recorded at 490 nm.

**Figure 2.** Diabetes mellitus (DM) increased white matter (WM) and axon damage in the ischemic brain compared to mice without DM. Immunostaining of luxol fast blue (dark blue; A), NG2 (brown; B), Bielschowsky silver (black; C), and amyloid precursor protein (brown; D) and quantification data in the ischemic boundary zone in control mice with and without DM (n=11 per group). Scale bar in A–D=100 μm.

**Figure 3.** Subanalysis in similar lesion animals. Quantification data in the ischemic boundary zone for luxol fast blue (A), NG2 (B), and Bielschowsky silver (C) staining in similar lesion of mice with and without diabetes mellitus (n=5 per group).
TUNEL Staining
TUNEL for measuring apoptosis was performed using a commercial kit (S7100; ApopTag kit; Chemicon). TUNEL-positive cells were counted. The percentage of TUNEL-positive cell in total DAPI-positive nuclear cells was presented.

Statistical Analysis
Independent samples \( t \) test was used for testing lesion volume, hemorrhage rate, functional outcome, immunostaining, real-time PCR, Western blot, and zymography assays in the brain tissues. One-way ANOVA and least-significant difference analysis after a post hoc test were performed to assess data of LDH, MTS, TUNEL, and neurite outgrowth in vitro. All data are presented as mean±SE.

Results
Mice with DM had significantly increased blood glucose (before MCAO, 427.0±41.1; after MCAO, 478.6±35.8 mg/dL) levels compared to control mice without DM (before MCAO, 130.6±16.1; after MCAO, 139.2±9.7 mg/dL; \( P<0.05 \)), respectively.

Mice with DM exhibit increased lesion volume and brain hemorrhage and have worse neurological outcome after stroke (Figure 1). Figure 1 shows that the lesion volume, mortality rate, and brain hemorrhage (Figure 1B) were significantly increased after stroke in mice with DM (mortality, 38%; brain hemorrhage, 46.2%) compared to control mice without DM (mortality, 8%; brain hemorrhage, 6%; \( P<0.05 \)). Neurological functional recovery was significantly attenuated in mice with DM on modified neurological severity score and foot-fault tests at 1 day after stroke compared to mice without DM subjected to MCAO (Figure 1B; \( P<0.05 \)).

Mice with DM exhibit increased WM and axonal damage in the ischemic brain (Figure 2). Bielschowsky silver is a marker for axons and luxol fast blue shows myelin. Amyloid precursor protein is a sensitive marker of axonal damage and WM lesions. NG2 is an oligodendrocyte progenitor cell marker. Figure 2 shows that luxol fast blue (Figure 2A), NG2 (Figure 2B), and Bielschowsky silver (Figure 2C) expression were significantly decreased in the ischemic boundary zone.
in mice with DM and MCAO compared to control mice without DM and MCAO. Amyloid precursor protein (Figure 2D) expression was significantly increased in the ischemic boundary zone in the mice with DM and MCAO compared with control animals without DM and MCAO.

To exclude the possibility that worsening WM damage in mice with DM may be attributed to the increased lesion volume after stroke, subanalysis was performed on similar lesion volumes in wild-type (13.36% ± 1.4) and mice with DM (13.71% ± 2.98, n=5 per group; P<0.05) and stroke. WM damage identified by luxol fast blue (Figure 3A), NG2 (Figure 3B), and Bielschowsky silver (Figure 3C) in mice with DM was significantly increased compared to similar lesion of mice without DM, indicating that worsening of WM damage in mice with DM compared to mice without DM is independent of lesion volume.

Mice with DM exhibit increased MMP-2 and MMP-9 expression in the ischemic brain (Figure 4). DM significantly increased MMP-2 and MMP-9 genes (Figure 4A) and increased MMP-9 protein (Figure 4B) expression in the ischemic brain compared to control mice without DM. Zymography data showed that MMP-9 activity was significantly increased in the ischemic brain in mice with DM compared to mice without DM (Figure 4C).

HG increases cell death, decreases neurite outgrowth, and regulates MMP-2 and MMP-9 expression in cultured PCN. HG significantly increased PCN cell death measured by LDH assay (Figure 5A; P<0.05). HG also significantly increased MMP-2 and MMP-9 genes (Figure 5A) and protein expression, as well as increased cleaved capase-3 level (Figure 5B) and apoptosis measured by TUNEL staining (Figure 6A) in cultured PCN (P<0.05). Figure 6C shows that HG significantly decreased dendrite outgrowth in cultured PCN compared to normal glucose control (P<0.05). The MMP inhibitor, GM6001, significantly increased PCN survival (Figure 5A) and decreased apoptosis (Figure 6A), but it did not attenuate HG-induced impairment of dendrite outgrowth in cultured PCN compared to HG control (Figure 6C). The data
suggest that MMP contributes to, but is not the sole mediator of, neuronal cell death.

HG increases oligodendrocyte cell death and increases MMP-9 expression in cultured oligodendrocytes. Oligodendrocytes are the myelin-producing cells of the central nervous system. HG significantly increased oligodendrocyte cell death (Figure 5C), apoptosis (Figure 6B), and decreased oligodendrocyte proliferation (Figure 5C), respectively. HG increased MMP-9 gene (Figure 5C) and protein expression (Figure 5B) compared to normal glucose control. GM6001 treatment significantly attenuated HG-induced oligodendrocyte cell death (Figure 5C; \( P < 0.05 \)) and apoptosis (Figure 6B), but it did not attenuate HG-induced decreased oligodendrocyte proliferation (Figure 5C).

**Discussion**

In this study, we are the first to our knowledge to demonstrate that DM induces severe WM and axonal damage in the ischemic boundary zone compared to mice without DM after stroke. DM significantly increased MMP-9 expression and activity in the ischemic brain. HG increased MMP-9 expression and decreased neuronal and oligodendrocyte survival and neurite outgrowth in PCN and oligodendrocyte cultures. Inhibition of MMP decreased neuronal and oligodendrocyte death but did not attenuate HG-induced impairment of neurite outgrowth. Therefore, MMP may contribute to worsening of WM damage and functional deficit in DM after stroke.

**DM Increases Lesion Volume, Functional Deficit, and WM Damage After Stroke**

Stroke in diabetic patients has a specific clinical pattern and a poor prognosis for motor function.\(^2^1\) Reasons for the altered prognosis in diabetes-associated stroke are multifactorial. Hyperglycemia at the time of ischemia is likely to be important. Diabetic patients with poor glycemic control exhibit neuronal dysfunction and WM damage in brain.\(^2^2\) Hyperglycemia is associated with increased mortality and poor recovery in stroke patients and is associated with hemorrhagic transformation in patients with acute ischemic stroke treated with thrombolysis.\(^2^3\) After stroke, hyperglycemia induces frequent and extensive hemorrhagic transformation.\(^2^4\) Hyperglycemia also inhibits Schwann cell proliferation and migration and restricts regeneration of axons in adult murine dorsal root ganglia.\(^2^5\) In this study, we found that mice with DM had significantly increased blood glucose level, increased lesion volume, and an increased incidence of brain hemorrhage compared to control mice. In addition, HG decreased neuronal and oligodendrocyte cell survival and neuronal dendrite outgrowth, as well as increased cleaved caspase-3 and apoptosis in vitro. WM and axonal damage were more severe in the DM ischemic brain compared to mice without DM after stroke. WM change in stroke patients is often associated with a higher risk of death and poor neurological outcome.\(^2^6\) DM mice showed significantly worse functional outcome after stroke compared to mice with stroke but without DM. Therefore, DM increases lesion volume, brain hemorrhage, and WM damage, all of which may promote neurological deficit after stroke in the DM population. In addition, using a subanalysis with similar lesions and mice with and without DM, we also found that WM damage was significantly increased in mice with DM compared to mice without DM. Therefore, DM inducing worsening WM damage is independent of lesion volume.

**Figure 6.** Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining in cultured primary cultured neurons (PCN) and oligodendrocyte. High glucose (HG) decreased neurite outgrowth in cultured PCN. Inhibition of matrix metalloproteinase did not attenuate HG-induced neurite outgrowth damage. TUNEL staining and quantitative data in PCN (A) and oligodendrocyte (B). C, TUJ1 immunostaining and neurite outgrowth quantitative data in cultured PCN (red, TUJ1; blue, DAPI; \( n = 6 \) per group).
MMP-9 Might Contribute to the Increased Brain Hemorrhage, WM, and Neuronal Damage in Mice With DM

HG induces oxidative stress, and both upregulate MMP-9 activity. Increased MMP-9 expression and proteolytic activation promote BBB damage and brain hemorrhage after stroke.\(^2\) Damage to the BBB with leakage of serum components in the WM may induce WM damage.\(^3\) MMP change the permeability of the BBB and contribute to WM damage after chronic cerebral hypoperfusion and induce vascular cognitive impairment.\(^4,28\) In addition, MMP have the potential to disrupt myelin-associated glycoprotein-dependent axon–glia communication and to generate bioactive fragments.\(^29\) Inhibition of MMP prevented macrophage-induced axonal retraction.\(^30\) We found that mice with DM exhibit increased WM damage as well as increased MMP-9 expression and activity in the ischemic brain compared to mice without DM and MCAO mice. HG increases MMP-9 and MMP-2 expression in cultured PCN and oligodendrocytes compared to normal glucose control. Inhibition of MMP by GM6001 significantly attenuates HG-induced neuronal and oligodendrocyte cell death. In addition, MMP promote WM damage in brain hypoxic injury.\(^31\) The aberrant excessive activity of MMP, especially MMP-9, contributes directly to neuron apoptosis and brain damage.\(^32\) Treatment with MMP inhibitors in mice reduces hippocampal death and reduces neuronal apoptosis after stroke.\(^33\) Thus, MMP may contribute to the enhanced DM-induced WM and axon damage in the ischemic brain.

Conclusion

There are a number of limitations and caveats to the present study. In this study, we only measured outcome at 24 hours after transient MCAO. Additional studies are warranted under conditions of permanent focal stroke and long-term outcomes. Studies of DM under conditions of hyperglycemic control also should be performed. In addition, many factors, not only MMP, might contribute to the enhanced WM damage. Dysregulated proteinolytic within the neurovascular unit also might, at least partially, be responsible for the increased WM injury in mice with DM after stroke and this warrants investigation.

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Disclosures

None.

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