Reduction of Ischemic Brain Injury by Topical Application of Glial Cell Line–Derived Neurotrophic Factor After Permanent Middle Cerebral Artery Occlusion in Rats

H. Kitagawa, MS; T. Hayashi, MD; Y. Mitsumoto, PhD; N. Koga, MS; Y. Itoyama, MD, PhD; K. Abe, MD, PhD

Background and Purpose—Glial cell line–derived neurotrophic factor (GDNF) plays important roles in the survival and recovery of some mature neurons under pathological conditions. However, the effect of GDNF in ameliorating ischemic brain injury has not been well documented. Therefore, we investigated a possible effect of GDNF on the changes of infarct size, brain edema, DNA fragmentation, and immunoreactivities for caspases after permanent middle cerebral artery occlusion (MCAO) in rats.

Methods—For the estimation of ischemic brain injury, we calculated the infarct size of MCA region and also measured the brain water content as edema formation at 24 hours after the MCAO. Terminal deoxynucleotidyl transferase–mediated dUTP-biotin in situ nick labeling (TUNEL) staining was performed for the detection of DNA fragmentation. Immunoreactivities for caspase-1 (ICE), caspase-2 (Nedd-2), and caspase-3 (CPP32) were stained.

Results—Both infarct size and brain edema after permanent MCAO were significantly reduced by topical application of GDNF (48% and 30% decreases, P < 0.01). TUNEL staining and immunoreactivities for caspases were markedly induced at 12 hours after permanent MCAO in the vehicle-treated animals. However, the spatial distribution of those immunohistochemically positive cells was dissociative in each caspase. Induction of TUNEL staining and immunoreactivities for caspases-1 and -3 was greatly reduced with GDNF treatment, whereas the reduction of caspase-2 staining was only minimum.

Conclusions—These data suggest that the reduction of infarct size and brain edema by GDNF was greatly associated with the reduction of DNA fragmentation and apoptotic signals predominantly through caspases-1 and -3 cascades. (Stroke. 1998;29:1417-1422.)

Key Words: caspases ■ cerebral ischemia ■ GDNF ■ middle cerebral artery occlusion ■ rats

Glial cell line–derived neurotrophic factor, a member of the TGF-β superfamily,1 plays important roles not only for the differentiation of neurons during normal development but also for the survival and recovery of many populations of mature neurons. It has been reported that GDNF has protective effects on various injuries for central and peripheral nervous systems in vitro and in vivo.2–6 A part of ischemic injury is associated with apoptosis detected by TUNEL staining.7 Our group recently demonstrated that topical application of GDNF significantly ameliorated brain edema formation in reperfused rat brain in association with the reduction of TUNEL staining.8 Another report showed that ventricular and intraparenchymal administration of GDNF diminished the volume of cortical infarction after transient MCA ligation with the inhibition of nitric oxide release.9 However, a possible protective effect of GDNF on permanent focal cerebral ischemia and the exact mechanism of ameliorative effect of GDNF on brain ischemic injury are not fully understood.

Members of ICE-like cysteine protease are related to mammalian apoptosis and inflammation,9–15 and have recently been designated as “caspases.”16 Among them, caspase-1 (ICE), caspase-2 (Nedd-2/Ich-1), and caspase-3 (CPP32) have been thought to play an important role in ischemic neuronal injury. Expression or upregulation of mRNAs for caspases has been reported in some ischemic injury models.17–19 Inhibition of caspase family proteases reduced ischemic and excitotoxic neuronal damage,20 and expression of a dominant negative mutant of ICE or a mutant ICE inhibitory protein in transgenic mice prevented or attenuated ischemic brain injury.21,22 Therefore, the progression of ischemic neuronal injuries may be greatly associated with activation of these caspases under an apoptotic process.

There has been much evidence to suggest a relationship of these caspases to transient or reperfused ischemic brain injury.17,19,20,22 However, it has been uncertain whether...
caspases are also induced or involved in the neuronal death of permanent MCAO, which may be related more to necrotic than apoptotic processes as compared with transient MCAO. Therefore, we investigated the possible protective effect of GDNF on the infarct area and brain edema in association with modification of DNA fragmentation and immunoreactivities for caspases after permanent MCAO in rats.

Materials and Methods

Permanent MCAO

Adult male Wistar rats (body weight, 250 to 280 g) were anesthetized with an intraperitoneal injection of pentobarbital (10 mg/250 g rat), and a burr hole with a diameter of 2 mm was carefully made in the skull using an electric dental drill to avoid traumatic brain injury. The location of the burr hole was 3 mm dorsal and 4 mm lateral to the skull using an electric dental drill to avoid traumatic brain injury. Dura mater was preserved at this time. The animals were anesthetized with an intraperitoneal injection of pentobarbital (10 mg/250 g rat), and a burr hole with a diameter of 2 mm was carefully made in the skull. The Spongel was buried in the skull bone. The surface of the skull cortex of the ischemic area; therefore, it was thought that this was the major route of applied GDNF to reach its biological target. Sham control animals were treated in the same way without MCAO. The above operation were performed in a sterile fashion. Blood samples (90 L) were collected before or at 8 hours after MCAO from ventral tail artery for measurement of PO2, PCO2, and pH (blood gas analyzer model ABL330, Radiometer). Blood pressure was also measured by blood pressure monitor (OMEGAWAVE).

Estimation of Infarct Area and Brain Edema After Permanent MCAO

To examine a possible effect of GDNF on infarct size after permanent MCAO, the rat forebrains were removed and divided into 6 coronal (2 mm) sections at 24 hours of occlusion with vehicle (n=7) or GDNF (n=9) treatment. The coronal sections were stained with saline containing 2% 2,3,5-triphenyltetrazolium chloride at 37°C for 30 minutes, after which sections were fixed in 10% neutralized formalin, according to a technique reported previously.24 The infarct area of each section was measured NIH Image software, version 1.62, and then infarct areas on each slice were summed and multiplied by slice thickness to give the infarct volume.25 In this experiment, regional CBF of left frontoparietal cortex region was measured before, immediately after (0), or at 8 or 24 hours after MCAO through the Burr hole using a laser blood flowmeter (Flo-C1, Omegawave).

For the measurement of brain edema, rats were decapitated under a deep anesthesia at 24 hours of permanent MCAO in vehicle- (n=5) or GDNF-treated (n=7) groups, and cerebral cortices of the occluded MCA territory were dissected. Water contents were then measured by the dry-weight method we reported previously.23 Sham control cerebral cortices (n=6) were also dissected immediately after the sham operation without MCAO. Statistical analyses were performed using Student’s t tests.

Detection of DNA Fragmentation and Immunohistochemical Study

For histochuneral stainings for DNA fragmentation and caspases, the rat forebrains were removed and quickly frozen at 12 hours of occlusion with vehicle (n=4) or GDNF (n=4). Coronal sections at the caudate and dorsal hippocampal levels were cut on a cryostat at −18°C to a 10 mm thickness and collected on glass slides coated with poly-l-lysine. Sham control sections were also obtained. Histochuneral staining for TUNEL was performed with a kit (TACS TdT in situ apoptosis detection kit No. #80-4625-00, ZYMED). After a detection of double-strand breaks in genomic DNA with 2,3′-diaminobenzened trihydrochloride (0.5 mg/mL in 50 mmol/L Tris-HCl buffer, pH 7.4), the sections were counterstained with methyl green according to the protocol in the kit.

Immunostaining for caspases was performed by the avidin-biotin-peroxidase method (ABC) using a kit (PK-6102, Vector Laboratories). The fresh-frozen sections were fixed for 10 minutes in ice-cold acetone and air-dried. Then the sections were rinsed three times in PBS (pH 7.4). After blocking with 10% normal rabbit serum for 2 hours, the slides were incubated for 16 hours at 4°C with a first antibody: a goat polyclonal antibody against caspase-1 (ICE p20, M-19), caspase-2 (Nedd-2, N-19), or caspase-3 (CPP32 p20, L-18) (Santa Cruz Biotechnology Inc, catalog No. sc-1225, sc1218, or sc-1217, respectively), diluted in PBS (1:200) containing 10% normal rabbit serum and 0.3% Triton X-100. Some sections were treated simultaneously without the first antibody. Specificity of the caspase antibodies has been described elsewhere.26,29 Endogenous peroxidase was blocked for 20 minutes with PBS containing 0.3% H2O2 and 10% methanol. The sections were then washed and incubated for 3 hours with the second antibody (1:200), biotinylated rabbit anti-goat IgG (p50232, ZYMED Laboratories Inc), in the buffer, followed by incubation for 30 minutes with avidin-biotin-horseradish peroxidase complex. Staining was developed with 2,3′-diaminobenzene tetrahydrochloride (0.5 mg/mL in 50 mmol/L Tris-HCl buffer, pH 7.4), and counterstained with methyl green.

The sections were examined by light microscope, and the stained cells in 0.25 mm2 of 3 random MCA areas were counted, summed, and categorized into 4 grades in the following manner: no staining or a small (2 to 50), moderate (50 to 200), or large (200 to 500 or more) number of stained cells into −, +, 2+, and 3+, respectively. Distribution and the staining grades of positive cells are schematically illustrated in Figure 4, comparing vehicle- and GDNF-treated groups.

Results

There was no significant difference in physiological parameters between the vehicle- and GDNF-treated groups before (mean blood pressure, 85.7±8.6 and 87.8±9.2 mm Hg, respectively; PO2, 135.0±18.4 and 128.0±24.0 mm Hg; PCO2, 43.1±3.7 and

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**Selected Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
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<tr>
<td>CPP32</td>
<td>cysteine protease p32</td>
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<tr>
<td>GDNF</td>
<td>glial cell line–derived neurotrophic factor</td>
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<tr>
<td>ICE</td>
<td>interleukin-1β-converting enzyme</td>
</tr>
<tr>
<td>Ich-1</td>
<td>ICE and ced-3 homolog-1</td>
</tr>
<tr>
<td>MCA</td>
<td>middle cerebral artery</td>
</tr>
<tr>
<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
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<tr>
<td>Nedd-2</td>
<td>neuronal precursor cell–expressed developmentally downregulated gene 2</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase–mediated dUTP-biotin in situ nick end labeling</td>
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44.6±2.1 mm Hg; pH, 7.46±0.03 and 7.45±0.02) or 8 hours after (mean blood pressure, 97.4±14.0 mm Hg, 97.8±11.7 mm Hg; PO\textsubscript{2}, 144.3±41.7 and 140.8±19.4 mm Hg; PCO\textsubscript{2}, 36.7±2.5 and 34.7±2.7 mm Hg; pH, 7.48±0.02 and 7.52±0.04) MCAO.

While infarct volume in the brain sections of the sham control group was not measured, those of the vehicle-treated or GDNF-treated group at 24 hours after permanent MCAO were 343.3±112.4 mm\textsuperscript{2} (mean±SD, n=7) and 176.5±119.8 mm\textsuperscript{2} (n=9; P<0.01 versus the vehicle-treated group), respectively (Figure 1A). Infarct areas of 4 coronal sections (4, 6, 8, and 10 mm caudal from frontal pole) from the GDNF-treated group were also significantly smaller than those of vehicle group (Figure 1B). Sham control cortices showed 80.6±0.4% (mean±SD, n=6) of water content, while those of vehicle- or GDNF-treated tissues at 24 hours after permanent MCAO showed 86.5±0.6% (n=5; P<0.001 versus the sham control group) and 84.7±1.4% (n=7; P=0.01 versus the vehicle-treated group), respectively (Figure 1C). Regional CBF of both vehicle- and GDNF-treated groups was reduced to less than 50% that of the control group immediately after MCAO and persisted to 24 hours (Figure 1D). There was no significant difference between the 2 groups (Figure 1D).

TUNEL staining was negative in the sham control brain sections. However, heavy staining was seen in the brain sections at 12 hours after the permanent MCAO (Figure 2a, arrowheads). TUNEL-positive cells were distributed mainly in the ischemic core of the cerebral cortex and dorsal caudate of the occluded MCA area (Figure 3b, arrowheads, and Figure 4). Approximately 50% to 70% of cells were positive for TUNEL in the above-mentioned area, and the staining was essentially found in the nucleus of neuronal cells. However, TUNEL-positive cells were not evident in the inner boundary zone of the infarct (Figure 3b, zone between arrowheads and dotted line). No TUNEL-positive cells were found in other areas of the ipsilateral hemisphere or in the contralateral side. The treatment with GDNF greatly reduced the number of TUNEL-positive cells at 12 hours after permanent MCAO (Figure 2e, arrowhead).
Figure 3. Dissociative distribution of TUNEL and caspase staining in the boundary region of the infarct at 12 hours after permanent MCAO. Adjacent sections in the same vehicle-treated sample were stained with hematoxylin-eosin (a), TUNEL (b), caspase-1 (c), caspase-3 (e), and caspase-2 (f). The boundary region in these panels is shown in the schematic (d, arrow). Dotted line shown in panel d represents the boundary of the infarction. Right or left side of the dotted line in each panel is noninfarction area or infarction area, respectively. Note the presence of some TUNEL-positive cells in the ischemic core region of the infarct core (b, arrowheads) in contrast to moderate numbers of stained cells for caspase-1 (c, arrowheads) and caspase-3 (e, arrowheads) at the inner boundary zone of the infarct. Caspase-2–stained cells were found not only in the inner boundary of the infarct (f, filled arrowheads) but also outside the infarct area (f, open arrowheads). Magnification, ×50. Bars, 0.08 mm.

Immunoreactivities for caspases were not detectable in the sham control brain sections (data not shown). However, caspases-1 and -3 became markedly present at 12 hours after permanent MCAO in the neuronal cytoplasm of the cerebral cortex (Figure 2b and 2c, arrowheads), especially in the inner boundary zone of the infarct (Figure 3c and 3e, arrowheads) and caudate in the MCA territory (Figure 4). On the other hand, only a few stained cells for those caspases were found in the ischemic core region. The number of stained cells for both caspase-1 and caspase-3 was reduced in the GDNF-treated tissues (Figure 2f and 2g, arrowheads). Immunoreactivity for caspase-2 was also induced after the MCAO but was more widely distributed in the cerebral cortex (Figures 2d and 4) and caudate in the MCA territory than in the tissues treated with caspases-1 and -3 (Figure 4). The stained cells in the cortex were distributed not only in the inner boundary zone of the infarct (Figure 3f, filled arrowheads) but also outside the infarct area (Figure 3f, open arrowheads). The stained area was only slightly reduced with GDNF treatment (Figure 2h, arrowheads) than those with vehicle (Figure 2d, arrowheads). The spatial distributions and the grade of TUNEL staining and immunoreactivities for caspases-1, -2, and -3 in the brain sections are summarized in Figure 4 and the Table, respectively.

Discussion

In the present study, TUNEL–positive cells were predominantly located in the ischemic core region rather than in the ischemic penumbra (Figures 2a, 3b, and 4). Permanent MCAO reduces the regional CBF persistently, while recirculation recovers the CBF in hours. In this study, the reduction rate of regional CBF in the frontoparietal cortex after occlusion was about 50% to 55%, in accordance with a previous report, and the reduction persisted to the end of the experiment (Figure 1D). Therefore, ischemic neuronal damage in permanent MCAO is more related to the reduction of ATP level than in transient MCAO. Thus, in contrast to transient ischemia, permanent ischemia may be related more to necrotic than apoptotic cell death. Apoptotic neurons were localized primarily in the inner boundary zone of the infarct, whereas necrotic cells were mainly distributed in the ischemic core after transient focal ischemia in rats. Furthermore, DNA fragmentation, in both apoptotic and necrotic neurons in the brain section after transient MCAO, was detected by the TUNEL method, even if they could have been discriminated morphologically. Thus, in the present study, TUNEL–positive cells located in the ischemic core may be found mainly during the necrotic process. In fact, the spatial distributions between TUNEL– and caspase-positive cells were different (Figure 4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TUNEL</th>
<th>Caspase-1</th>
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Staining was performed at 12 hours after permanent MCAO, and was categorized into the following 4 grades: no staining, or a small (2-50), moderate (50-200), or large (200-500) number of stained cells into (-), (+), (2+), and (3+), respectively. Sham control group, n=2; vehicle and GDNF groups, n=4.
Caspases, which are strongly related to the process of apoptotic cell death, were induced and mainly located in the ischemic penumbra, suggesting that the expansion of neuronal cell damage in this area may be primarily by an apoptotic mechanism. Of interest is the fact that caspase-2–positive cells were more widely distributed than caspase-1– and caspase-3–positive cells (Figures 3c, 3e, 3f, and 4). While the CBF of the ischemic core region in the permanent MCAO is markedly reduced (to 10% to 13% of that of the control tissues), that of the penumbral region is only mildly reduced (about 70% to 80% of control). Therefore, caspase-2 may be activated or induced even under such a mild ischemic condition. A recent report indicated that mRNA of caspase-2 (Nedd-2, Ich-1) was transiently induced earlier than those of other caspases, and became a peak at 8 hours in rat brain after permanent MCAO. Another report demonstrated that the caspase-2 gene expression was selectively upregulated in the gerbil brain after transient global ischemia, whereas caspases-1 and -3 were not. Moreover, a cleavage of caspase-2 gene expression was selectively upregulated in the ischemic core region of the permanent MCAO. Although the cascade of caspases related to apoptotic cell death is not fully understood.

In the present study, we detected the immunoreactive caspases after permanent MCAO. However, it is uncertain whether the caspases are biologically activated after ischemia or not. Activation of caspase-2 or caspase-3 is required for apoptosis in cultured neurons. Moreover, activation of caspase-1 and caspase-3 contributes to neuronal apoptosis after traumatic brain injury in vivo. Therefore, activation of these caspases may be greatly associated with neuronal apoptotic processes. However, further study will be required for evidence of caspase activation in ischemic brain injury.

Neurotrophic factors have recently been classified into several groups (eg, neurotrophins, cytokines, the fibroblast growth factor family, and the TGF-β superfamily). GDNF, a member of the TGF-β superfamily, is thought to be the most potent among neurotrophic factors for the survival of cultured neurons. It has been also reported that GDNF has a protective effect on various injuries for central and peripheral nervous systems in vitro and in vivo. In this study, we showed that topical application of GDNF significantly ameliorated both infarction and brain edema formation of MCA region (48% and 30% decreases, respectively) after permanent MCAO. There was no difference in regional CBF between vehicle- and GDNF-treated groups (Figure 1D), suggesting that the ameliorative effect of GDNF was less involved in the improvement of CBF. Interestingly, the effect was greatly associated with the marked reduction of TUNEL staining and the moderate decrease of immunoreactivities for caspases-1 and -3 but not caspase-2. Although the infarct size became smaller after the GDNF treatment, the reduction of the numbers of TUNEL–, caspase-1–, and caspase-3–positive cells elicited by the GDNF treatment was not simply dependent on the reduction of the infarct size but was related to the decrease of the density of positive cells (Figures 2 and 4). Furthermore, the dissociative spatial distributions of

TUNEL–, caspase-1–, caspase-3–, and caspase-2–positive cells were similar between the vehicle-treated and GDNF-treated tissues (Figure 4). These data suggest that the mechanism of the ameliorative effect of GDNF on brain ischemic injury after permanent MCAO may be related not only to the reduction of necrotic cells but also to the reduction of the apoptotic process through the inhibition of the caspase-1 and caspase-3 pathway.

In conclusion, the present study first demonstrated the ameliorative effect of GDNF on ischemic brain injury induced by permanent MCAO, that was strongly associated with the reduction of apoptotic and necrotic processes. Therefore, GDNF could become a strong candidate for use as a therapeutic agent in the treatment of ischemic brain diseases in the near future.

Acknowledgment

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References

Reduction of Ischemic Injury by GDNF


Editorial Comment

In the manuscript published above, Kitagawa and his colleagues report that glial cell line–derived neurotrophic factor (GDNF) reduced infarct size and edema associated with ischemia induced by permanent middle cerebral artery occlusion in rats. Although a large number of compounds have been identified that minimize tissue injury and protect the brain from the effects of ischemia, this compound appears to have unique properties. The authors showed that the effect of GDNF was not mediated by a change in blood flow to the ischemic area. In addition, they found that the increased activity of the caspases was minimized by treatment with GDNF, providing a possible clue to the mechanism of action. These enzymes are important in mediating apoptosis and inflammation, and their activation may be an important mediator of neuronal death from ischemia. Additional work is needed to clarify the mechanism of action of GDNF further and to provide a firmer base for its consideration as a possible therapeutic agent for ischemia.

The pathogenesis of neuronal damage and death after ischemia is likely to be multifactorial; it is therefore useful to identify compounds that have multiple mechanisms of action and that may bring benefit by a variety of mechanisms. Ultimately, the combination of these agents may provide a more effective mechanism for minimizing the effects of ischemia on brain tissue.

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