Intravenous TAT-GDNF Is Protective After Focal Cerebral Ischemia in Mice

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Background and Purpose—Delivery of therapeutic proteins into tissues and across the blood-brain barrier is severely limited by their size and biochemical properties. The 11-amino acid human immunodeficiency virus TAT protein transduction domain is able to cross cell membranes and the blood-brain barrier, even when coupled with larger peptides. The present studies were done to evaluate whether TAT–glial line-derived neurotrophic factor (GDNF) fusion protein is protective in focal cerebral ischemia.

Methods—Anesthetized male C57BL/6j mice were submitted to intraluminal thread occlusion of the middle cerebral artery. Reperfusion was initiated 30 minutes later by thread retraction. Laser Doppler flow was monitored during the experiments. TAT-GDNF, TAT-GFP (0.6 nmol each), or vehicle was intravenously applied over 10 minutes immediately after reperfusion. After 3 days (30 minutes of ischemia), animals were reanesthetized and decapitated. Brain injury was evaluated by histochemical stainings.

Results—Immunocytochemical experiments confirmed the presence of TAT-GDNF protein in the brains of fusion protein–treated nonischemic control animals 3 to 4 hours after TAT fusion protein delivery. TAT-GDNF significantly reduced the number of caspase-3–immunoreactive and DNA-fragmented cells and increased the number of viable neurons in the striatum, where disseminated tissue injury was observed, compared with TAT-GFP– or vehicle-treated animals.

Conclusions—Our results demonstrate that TAT fusion proteins are powerful tools for the treatment of focal ischemia when delivered both before and after an ischemic insult. This approach may be of clinical interest because such fusion proteins can be intravenously applied and reach the ischemic brain regions. This approach may therefore offer new perspectives for future strategies in stroke therapy. (Stroke. 2003;34:1304-1310.)

Key Words: brain-derived neurotrophic factor ■ fusion protein ■ glial cell line-derived neurotrophic factor ■ ischemia ■ protein transport ■ stroke

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gial cell line-derived neurotrophic factor (GDNF), a member of the transforming growth factor-β superfamily, is a potent neurotrophic factor that promotes the survival and morphological differentiation of dopaminergic neurons1–4 and motor neurons.5,6 The protective effects of externally applied GDNF are well established in different models of cerebral ischemia.7–10 In some of these approaches, GDNF has been delivered by adeno viral vectors9,10; in others, GDNF has been applied topically11 or by intracerebral microinjection.12 Viral gene therapy is a promising method to deliver proteins but is not suited for acute stroke therapy9 because the delivery of genes into brain tissues and expression of therapeutic proteins are time-consuming, and the systemic application of genes via viral vector system may cause immunological side effects.

Moreover, cerebral protein synthesis is severely compromised in injured areas after focal ischemia,13,14 which further complicates the transduction of brain tissue. Topical application or intracerebral microinjection of GDNF protein is also not a favorable method for acute stroke therapy because of the risk of inducing intracerebral hemorrhage as a consequence of the surgical procedure.

Another disadvantage of these methods is that a putative protective factor (eg, GDNF) is not delivered directly at its site of action. Thus, none of these methods is suitable for acute stroke therapy. The common problem in these methods is that the blood-brain barrier (BBB), which is composed of specialized endothelial cells with tight junctions, prevents the passage of large molecules into the brain parenchyma.15,16 Like other neurotrophic factors, the GDNF molecule is a protein that does not readily pass the BBB. The poor penetration rate across the BBB of other lower-molecular-weight neurotrophic factors such as NGF (molecular weight, 26 kDa as a dimer) has been demonstrated using biotinylation by studies and with enzyme immunoassays.15,17

We have recently demonstrated that systemic delivery of another neuroprotective protein, Bcl-XL, does not lead to protection after focal cerebral ischemia.18 Thus, a primary
obstacle to clinical use of GDNF or other trophic factors is the lack of an efficient, noninvasive method for delivery of the respective protein across the BBB in biologically relevant amounts.

To overcome these problems, acute stroke therapy needs another approach to deliver therapeutic proteins into the brain. To this end, Frankel and Pabo,19 Green and Loewenstein,20 and Schwarze et al21 showed that the application of fusion proteins linked to so-called transduction domains enables them to cross cell membranes and the BBB. Schwarze et al19 showed that intraportal injection of the 120-kDa β-galactosidase protein, fused to the protein transduction domain derived from the human immunodeficiency virus (HIV) TAT protein, results in delivery of a biologically active fusion protein in various kinds of tissues, including the mouse brain.

To evaluate the beneficial effects of this TAT transduction system, GDNF was cloned into the pTAT-HA vector to produce TAT-GDNF protein in vitro. We then evaluated whether administration of a TAT-GDNF fusion protein may prevent brain injury after transient focal ischemia by examining the effects of TAT-GDNF and TAT-GFP as a control. Both TAT fusion proteins were administered intravenously either before or after the ischemic insults induced by middle cerebral artery (MCA) thread occlusion. In this study, 2 different durations of ischemia, 30 or 120 minutes, were chosen. At these time points, disseminated neuronal injury and brain infarction, respectively, are known to occur.13,14

Materials and Methods

Experimental Groups

All experimental procedures were carried out with governmental approval according to local and National Institutes of Health guidelines for the care and use of laboratory animals. Adult male C57BL/6j mice weighing 21 to 25 g were assigned to the following experiments and groups: I, intravenous delivery of (a) 0.6 nmol TAT-GDNF dissolved in 0.1 mL of 0.1 mol/L phosphate-buffered saline (PBS), (b) 0.6 nmol TAT-GFP dissolved in 0.1 mL of 0.1 mol/L PBS or, (c) no fusion protein 1 hour before focal ischemia as induced by 120 minutes of intraluminal thread occlusion followed by 24 hours of reperfusion; II, intravenous delivery of (a) 0.6 nmol TAT-GDNF dissolved in 0.1 mL of 0.1 mol/L PBS immediately after a 120-minute episode of intraluminal thread occlusion followed by 24 hours of reperfusion; III, intravenous delivery of (a) 0.6 nmol TAT-GDNF dissolved in 0.1 mL of 0.1 mol/L PBS, (b) 0.6 nmol TAT-GFP dissolved in 0.1 mL of 0.1 mol/L PBS, or (c) no fusion protein immediately after a 30-minute episode of thread occlusion followed by 72 hours of reperfusion (n = 4 to 6 animals per group). Additional animals received intravenous infusions of 0.6 nmol TAT-GDNF or TAT-GFP but were not submitted to focal ischemia. These animals were killed 3 or 4 hours after infusion. Brains were used for the immunocytochemical analysis of TAT-GDNF or TAT-GFP and Western blot analysis of TAT-GDNF transduction (n = 3 per group).

Expression and Purification of Recombinant TAT Fusion Proteins

The GDNF coding sequence was polymerase chain reaction–amplified from rat whole-brain cDNA by use of primers 5′-attatatcagctgcaagcagaatcgca-3′ and 5′-ttattacctgagtc-agatacatcaccgc-3′ (94°C for 3 minutes, 94°C for 20 seconds, 55°C for 30 seconds, and 72°C for 45 seconds for 35 cycles; 72°C for 7 minutes). Purified polymerase chain reaction fragments were cloned into the NcoI/XhoI sites of the pTAT-HA vector (kindly provided by Dr Steven F. Dowdy, San Diego, Calif). TAT-GFP vectors were also kindly provided by Dr Steven F. Dowdy. All expression cassettes include a sequence encoding 6 consecutive histidine residues and a hemagglutinin domain. Fusion proteins were purified as described.22 Briefly, TAT-GDNF and TAT-GFP were expressed in Escherichia coli strain BL21 (DE3) (Qiagen). Salt was removed by gel filtration on Sephadex G-25 (Amersham Pharmacia Biotech). Identity of proteins was confirmed by Western blotting. Anti-hemagglutinin antibodies were purchased from BabCO.

Laser Doppler Flowmetry

During the experiments, blood flow was measured by laser Doppler flowmetry (LDF) using a flexible 0.5-mm fiberoptic probe (Perimed), which was attached with tissue adhesive to the intact skull overlying the MCA territory (2 mm posterior and 6 mm lateral from the bregma). LDF changes were monitored during ischemia and up to 30 minutes after the onset of reperfusion.

Induction of Ischemia

Animals were anesthetized with 1% halothane (30% O2; remainder, N2O). Rectal temperature was maintained between 36.5°C and 37°C using a feedback-controlled heating system. The tail vein was cannulated with a PE10 catheter for intravenous administration of TAT-GDNF or TAT-GFP solutions. After this, focal ischemia was induced using an intraluminal filament technique.21 A midline neck incision was made, and the left common and external carotid arteries were isolated and ligated. A microvascular clip (FE691, Aesculap) was temporarily placed on the internal carotid artery. An 8-0 nylon monofilament (Ethilon, Ethicon) coated with silicon resin (Xantopren, Bayer Dental) was introduced through a small incision into the common carotid artery and advanced 9 mm distal to the carotid bifurcation for occlusion of the MCA. Thread size (190 to 200 μm) was matched to body weight to ensure reproducible vascular occlusion. After ischemia, reperfusion was initiated by withdrawal of the thread. Arterial blood gases were measured at 45 minutes after onset of ischemia. After surgery and LDF recordings were finished, anesthesia was discontinued, and animals were placed in their home cages.

Neurological Deficit Scores

Neurological deficits were monitored 24 hours after vascular occlusion using the following score: 0 = normal function; 1 = flexion of torso and the contralateral forelimb on lifting of the animal by the tail; 2 = circling to the contralateral side but normal posture at rest; 3 = reclamation to the contralateral side at rest, and 4 = absence of spontaneous motor activity.23

Triphenyltetrazolium Chloride Staining

Animals were reanesthetized with halothane and decapitated. Brains were dissected into 5 equidistant slices and immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (TTX).21 The border between infarcted and noninfarcted tissue was outlined using an image analysis system, and the area of infarction was measured by subtracting the area of the nonlesioned ipsilateral hemisphere from that of the contralateral side. The volume of infarction was calculated by integration of the lesion areas.

Western Blot Analysis of GDNF Expression

Brains were dissected, complemented with lysis buffer, homogenized, and centrifuged, and supernatants were used for SDS-PAGE. Equal amounts of protein were diluted in 6× sample buffer, boiled, and loaded onto 14% polyacrylamide gels. Proteins were transferred onto polyvinyl difluoride membranes. Membranes were dried overnight, incubated in blocking solution, and immersed with polyclonal rabbit anti-GDNF (sc-328, Santa Cruz) or polyclonal rabbit anti-HA antibody (Sigma), diluted 1:200 in 0.1% Tween 20 in 0.1 mol/L...
Tris-buffered saline. Membranes were rinsed, incubated in peroxidase-coupled, goat anti-rabbit secondary antibody, diluted 1:2500 in 0.1% Tween 20 in 0.1 mol/L Tris-buffered saline, washed, immersed in ECL solution, and exposed to ECL-Hyperfilm (Amerham).

**Immunocytochemical Analysis of Tissue GFP or GDNF Content and Caspase-3 Activation**

For analysis of tissue GFP or GDNF content, brains were perfusion fixed in 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4). Brains were post-fixed for 4 hours in 4% paraformaldehyde in 0.1 mol/L PBS and immersed in 30% sucrose in 0.1 mol/L PBS at 4°C overnight. Sagittal 18-μm sections were cut on a cryostat. For analysis of activated caspase-3, brains were frozen on dry ice and cut into 16-μm coronal cryostat sections, which were immersion fixed in 4% paraformaldehyde in 0.1 mol/L PBS for 8 minutes at 4°C.

Brain sections were washed 3 times for 5 minutes in 0.1 mol/L PBS and immerses for 30 minutes in 0.1 mol/L PBS containing 0.3% triton (PBS-T) and 10% normal goat serum (NGS). After 3 further rinses in 0.1 mol/L PBS, sections were incubated overnight at 4°C with PBS-T containing 2% NGS and polyclonal rabbit antibody against GFP (8367-2, BD Biosciences, Clontech, diluted 1:10), polyclonal rabbit antibody against GDNF (sc-328, Santa Cruz, diluted 1:200), monoclonal mouse anti-neuronal specific nuclear protein (NeuN) antibody (Chemicon, Temecula, diluted 1:250), or p20 fragment of caspase-3 (CM-1 antibody, Idun Pharmaceuticals Inc, diluted 1:500). The next day, sections were washed 3 times for 5 minutes in 0.1 mol/L PBS and incubated for 2 hours with 0.1 mol/L PBS in 10% NGS containing anti-rabbit Cy3 or anti-mouse FITC antibody, diluted 1:250. After 3 further rinses in PBS, sections were counterstained with DAPI, coverslipped with Mowiol, and evaluated under epifluorescence using appropriate filters.

**Terminal Transferase Biotinylated-dUTP Nick End Labeling**

For detection of DNA fragmentation, sections were fixed for 20 minutes with 4% paraformaldehyde in 0.1 mol/L PBS, rinsed 3 times for 5 minutes in PBS, and washed in 70% ethanol for 5 minutes. After another 3 rinses in PBS, sections were incubated for 5 minutes at 37°C in PBS containing proteinase K at a concentration of 2 μg/mL. After 3 further rinses in PBS, sections were immerses for 30 minutes in terminal deoxynucleotidyl transferase (TDT) buffer containing 25 mmol/L NaCl, 500 mmol/L cacodylic acid, 1 mmol/L CoCl₂, 0.05% bovine serum albumin, and 0.5 mmol/L diithiothreitol before incubation over 90 minutes at 37°C with a TDT mix consisting of TDT buffer, 12.5 mg/mL TDT (Boehringer-Roche Mannheim), and 25 mg/mL biotinylated dUTP (Boehringer-Roche Mannheim). The reaction was terminated by transferring the sections to 2× sodium chloride/sodium citrate for 30 minutes at room temperature. Sections were then rinsed 3 times for 5 minutes in PBS and blocked for 20 minutes in PBS-T containing 10% NGS, 1% gelatin, and 1% bovine serum albumin. Sections were then incubated overnight at 4°C with PBS-T containing 10% NGS, 1% bovine serum albumin, and streptavidin-FITC, diluted 1:50. The next day, sections were washed 3 times for 5 minutes in PBS, counterstained with DAPI, and coverslipped with Mowiol.

Other brain sections were used for cresyl violet staining according to a standard histological protocol. Terminal transferase biotinylated-dUTP nick end labeling (TUNEL) and cresyl violet sections were analyzed by counting the number of DNA-fragmented cells and viable neurons in a total of 6 regions of interest throughout the striatum. Mean values were calculated for these regions of interest.

**Statistical Analysis**

Brain sections were examined by 2 independent and blinded investigators. All values are given as mean±SD. Differences between groups were compared by using 1-way analysis of variance, followed by Scheffé’s tests. Values of P<0.05 were considered to indicate statistical significance.

**Results**

**TAT-GDNF–Mediated Tissue Transduction**

Western blot analysis reproducibly revealed a robust GDNF transduction in TAT-GDNF–treated animals. No protein transduction was detectable in control animals (Figure 1).

In TAT-GFP–treated control animals, the intrinsic level of GDNF immunoreactivity was below the level of detection both 3 and 4 hours after intravenous delivery. On the other hand, strong GDNF staining was noted around blood vessels 3 hours after TAT-GDNF infusion. After 4 hours, GDNF staining was detected in all brain regions intracellularly inside the brain parenchyma (Figure 2A and 2B).

After 4 hours, the TAT-GFP–treated control animals showed no detectable vital GFP fluorescence (without GFP immunoreactivity). GFP staining was detected in all brain regions intracellularly inside the brain parenchyma (with anti-GFP antibody) (Figure 2C).

**GDNF Delivery Before and After 120 Minutes of MCA Thread Occlusion**

**LDF Measurement**

LDF measurements during and after 120 minutes of MCA occlusion are summarized in Figure 3. After thread insertion, mean LDF reproducibly declined to ~10% of preischemic control levels in all animal groups. LDF values returned to slightly below control levels within 20 minutes after thread retraction. No statistically significant differences were seen between various groups. Arterial blood gases (PO₂, PCO₂, pH) also did not differ between groups 45 minutes after onset of ischemia (~145, ~44, and ~7.3 respectively).

**TTC Stainings**

In untreated and TAT-GFP–treated control groups, reproducible brain infarcts were obtained (Figures 4 and Figure 5A). The infarct was significantly smaller in TAT-GDNF–treated animals when fusion proteins were applied before thread occlusion (Figure 5A). In animals treated with TAT-GDNF after reperfusion, there also was a trend toward an improvement of injury, although this trend did not reach statistical significance (Figure 5B).

**Neurological Deficits**

The reduction in infarct size by TAT-GDNF was associated with an improvement in neurological deficits, which was significant in those animals treated before ischemia (Figure 5B).
GDNF Delivery After 30 Minutes of MCA Thread Occlusion

**LDF Measurements**

After thread insertion, LDF values decreased to ~10% of preischemic levels. After thread retraction, LDF values even increased above preischemic control levels in these animals. Again, no differences were detected between various animal groups.

**Ischemic Injury**

In accordance with our previous findings, 30 minutes of thread occlusion resulted in disseminated neuronal injury of the striatum but not the overlying cortex 3 days after reperfusion. Notably, the number of injured cells in the striatum, as assessed by TUNEL staining, was significantly reduced in TAT-GDNF– versus TAT-GFP–treated and untreated animals (Figure 6A). On the other hand, the number of viable neurons was significantly increased in TAT-GDNF– versus TAT-GFP–treated and untreated animals, as revealed by cresyl violet staining (Figure 6B).
Activation of Caspase-3
In ischemic animals treated with the TAT-GFP and in untreated animals, a considerable number of cells immunopositive for activated caspase-3 were found in the striatum 3 days after thread occlusion (Figure 6C). The number of caspase-3-positive cells was significantly lower in animals treated with TAT-GDNF (Figure 6C).

Neurological Deficits
After 30 minutes of MCA occlusion, neurological deficits were mild in all animal groups (range of deficit score, 0 to 1). Therefore, no differences were detectable between different groups.

Discussion
The delivery of therapeutic proteins into tissues and across the BBB is severely limited by the size and biochemical properties of the respective proteins. Thus, therapeutic compounds, peptidyl mimetics, and proteins may pass cell membranes only when the molecules are small, (ie, <600 Da). Diffusion of bioactive peptides across the BBB is also restricted to short (<6 amino acids) and highly lipophilic peptides. Yet, it was discovered in 1988 independently by Frankel and Pabo and Green and Loewenstein that the HIV-TAT protein can cross cell membranes. In 1994, Fawell et al further corroborated that heterologous proteins chemically cross-linked to a 36-amino acid domain of TAT were able to transduce into cells. More recently, Schwarze et al showed that intraperitoneal injection of the 120-kDa β-galactosidase protein, fused to the protein transduction domain of the HIV TAT protein, results in delivery of biologically active protein into various tissues in mice, including the brain. They determined the distribution of TAT-FITC peptide and TAT-β-galactosidase in brain sections after intraperitoneal injection and, similar to the present study, were able to show a progressive translocation of TAT proteins into brain tissue.

Cerebral ischemia induces disruption of the BBB, allowing even large molecules such as proteins to enter the brain. However, this disruption occurs after several hours of ischemia, whereas the therapeutic time window for most neuroprotective agents is <6 hours. Thus, to be effective, such neuroprotective agents must cross a still "intact" BBB.

Lipophilic compounds may cross the BBB by diffusion, but polar compounds are excluded unless they are substrates for transporters present at the BBB. Thus, brain-derived neurotrophic factor or GDNF, as neuroprotective agents, do
not cross the intact BBB in pharmacologically significant amounts.28

Schwarze et al21 further demonstrated that the BBB remained intact in TAT–β-galactosidase–treated mice as revealed by the absence of extravasated coinjected Evan’s blue albumin complex in the brain parenchyma. Because fusion proteins might open new ways to treat neurodegenerative disorders, we were interested whether a systemic, ie, intravenous delivery of a TAT fusion protein may prevent brain injury after focal ischemia.

GDNF, a member of the transforming growth factor-β superfamily, was originally identified by its capacity to promote the survival of cultured midbrain dopaminergic neurons.29 Uregulation of GDNF mRNA in the rodent brain has been shown to occur after excitotoxicity induced by glutamate,10 kainate,31 or ischemia.3 GDNF has been shown to protect neurons against oxidative stress in cultured mesencephalic neurons and glial cells,32 against ischemia- or hypoxia-induced brain injury in neonatal rats,33 after brain injury following permanent or transient focal cerebral ischemia in rats or mice,34,35 after cortical cold injury,10 and in primate models of Parkinson’s disease.35 GDNF promotes cell survival in multiple ways; recent publications showed that GDNF upregulates antiapoptotic Bcl-2 and Bcl-X, in apoptosis-induced rat mesencephalic neurons, resulting in a reduction in caspase activation.34,36 Chao and Lee37 showed that acute GDNF infusion significantly increased glutathione peroxidase, superoxide dismutase, and catalase activity that counters the reactive oxygen radicals and induces neuroprotection. Apart from this neuroprotective property of GDNF, it regulates endogenous levels of X-linked inhibitor of apoptosis protein and neuronal apoptosis inhibitory protein in motor neurons after sciatic nerve axotomy. These results showed that X-linked inhibitor of apoptosis protein and neuronal apoptosis inhibitory protein are essential for intracellular signaling of GDNF in motor neuron survival.38 GDNF modulates the Ret receptor tyrosine kinase, which might play an important role in brain injury caused by ischemia.12

Because of its neuroprotective effects, we chose to study the effects of a TAT-GDNF fusion protein in the present study. We examined an intraluminal thread occlusion model, which is a highly reproducible model previously characterized in detail by metabolic imaging techniques.13,14 As shown earlier, short episodes of focal ischemia induced by 30 minutes of thread occlusion result in disseminated, predominantly neuronal injury of the striatum, which involves activation of apoptotic cascades but is associated with intact energy metabolism.13 On the other hand, more severe episodes of ischemia induced by thread occlusions of ≥1 hour lead to reproducible infarcts of the MCA territory.14 Under these conditions, the evolution of brain injury is predominated by a failure of the cerebral energy state.14 It is noteworthy that TAT-GDNF delivery prevented both apoptotic and necrotic injury after short (30-minute duration) and longer (120-minute duration) ischemia, ie, disseminated injury and brain infarction. This suggests that GDNF protein may particularly be suited for the treatment of stroke, where both kinds of injury occur.

In the present experiments, protection of brain tissue was seen when TAT-GDNF was administered both before and 30 minutes after onset of ischemia. This demonstrates that this protein may indeed be a powerful tool for the immediate treatment of stroke, particularly with the newly established technique of intravenous fusion protein delivery. Because the method of application (ie, intravenous infusion) is both simple and elegant, it requires no surgical interventions (such as trephination of the skull), and these proteins nevertheless reliably reach their site of action (as shown in the present experiments), fusion proteins may be useful tools providing fascinating perspectives for future research.

In summary, the present report shows that intravenous delivery of an HIV-TAT–derived peptide fused to GDNF efficaciously reduces brain injury after 30 minutes of focal ischemia induced by MCA thread occlusion in mice. Protective effects on the cellular level were observed after short ischemic episodes leading to disseminated neuronal injury and longer-lasting insults resulting in reproducible tissue infarcts. The size of the infarction was significantly reduced when TAT-GDNF was applied before a 2-hour thread occlusion. Accordingly, fusion proteins might open fascinating perspectives for future acute strategies in stroke treatment.

References
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