Journal of Neuroscience Research 85:3457–3464 (2007)

Glial Cell Line-Derived Neurotrophic Factor Protects Astrocytes From Staurosporine- and Ischemia-Induced Apoptosis

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Glial cell line-derived neurotrophic factor (GDNF) promotes the survival and functions of neurons. It has been shown to be a promising candidate in the treatment of ischemia and other neurodegenerative diseases. We transfected mouse astrocytes in primary cultures with a human GDNF gene and found that their conditioned medium could not only support the growth and survival of cultured dopaminergic neurons but also protect astrocytes from staurosporine- and ischemia-induced apoptosis. This indicated that these transfected astrocytes could release GDNF. A similar protective effect on astrocytes against apoptosis was evident when recombinant human GDNF was used. Moreover, GDNF reduced caspase-3 activity but not that of caspase-1 in cultured astrocytes after ischemia treatment. Thus, GDNF protects astrocytes from apoptosis by inhibiting the activation of caspase-3.

Key words: GDNF; astrocytes; lipofection; apoptosis; caspase

Glial cell line-derived neurotrophic factor (GDNF) belongs to the GDNF family of neurotrophic factors, promoting the survival and functions of various neuronal populations in the peripheral and central nervous systems (Airaksinen and Saarma, 2002). GDNF was originally isolated because of its ability to promote the survival and differentiation of dopaminergic (DA) neurons in primary cultures of embryonic ventral midbrain (Lin et al., 1993). The effects of GDNF on DA neurons in midbrain cultures are potent and relatively specific, including enhancing DA cell population, promoting dopamine uptake, changing both cell size and neurite length, improving survival, and reducing apoptosis (Airaksinen and Saarma, 2002). GDNF also has neurotrophic effects on motor neurons (Zhao et al., 2004; Parsadanian et al., 2006), sensory neurons (Buj-Bello et al., 1995; Trupp et al., 1995), noradrenergic neurons (Arenas et al., 1995), and basal forebrain cholinergic cells (Yan and Matheson, 1995; Williams et al., 1996). The neurotrophic and protective effects of GDNF on neuronal cells make GDNF a promising therapeutic candidate for gene and cell therapy for treatments of Parkinson's disease (PD; Burton et al., 2003), amyotrophic lateral sclerosis (ALS; Manabe et al., 2003), and other neurodegenerative diseases (Alberch et al., 2002).

Previously, we demonstrated that the GDNF gene could be delivered into astrocytes at a high efficiency rate via the technique of multiple liposome-mediated transfection, or multilipofection (Wu et al., 2000). Transfected astrocytes were then shown to be able to secrete GDNF effectively (Wu et al., 2000). Recently, other research groups have demonstrated that overexpression of GDNF in transgenic astrocytes promotes

Contract grant sponsor: Natural Science Foundation of China; Contract grant number: 30270426; Contract grant number: 30470543; Contract grant number: 30670644; Contract grant sponsor: Beijing National Science Foundation; Contract grant number: 703026; Contract grant number: 7051004; Contract grant sponsor: North American Medical Association Foundation, Hong Kong.

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Received 13 November 2006; Revised 26 February 2007; Accepted 1 March 2007

Published online 11 May 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21345

survival of neurons (Cunningham and Su, 2002; Zhao et al., 2004; Parsadanian et al., 2006). Thus, transgenic astrocytes were identified as feasible carriers for delivering specific gene products to the brain by means of transplantation. Interestingly, there is a lack of investigation on whether GDNF, in addition to protecting neurons, also protects astrocytes from injury.

We have shown that astrocytes in primary culture would undergo apoptosis after ischemic insult (Yu et al., 2001, 2002, 2003; Chen et al., 2005a). In this study, we confirmed that the GDNF secreted from our transgenic astrocytes (Wu et al., 2000) is functional in the aspect of promoting DA neuronal growth. We further investigated the effect of this GDNF in protecting astrocytes from apoptosis.

MATERIALS AND METHODS

Primary Culture of Cerebral Cortical Astrocytes

Cerebral cortical astrocytes were prepared from newborn ICR mice as previously described (Yu et al., 2001, 2002, 2003; Chen et al., 2005a,b). The resulting cell suspension was seeded in 35-mm Falcon tissue culture dishes (Becton Dickinson Labware) at a density of 4×10^5 viable cells per dish. All cultures were incubated in a 95% humidified incubator (Precision Scientific Inc.) at 37°C with 5% CO_2. The culture medium was changed at 3–4-day intervals with Dulbecco's modified essential medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) for the first 2 weeks and 7% (v/v) FCS thereafter. The purity of astrocyte culture was over 95% as determined by glial fibrillary acidic protein (GFAP) staining.

Transfection of Human GDNF in Astrocytes

The gene encoding a human GDNF (hGDNF) was cloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). Astrocytes were transfected through the use of an optimized multilipofection method, with 1.5 mg hGDNF/pcDNA3 and 3 ml Lipofectin (1 mg/ml; GibcoBRL, Life Technologies, NY), which would yield maximal transfection efficiency as previously described (Wu et al., 2000). In this study, young cultured astrocytes (2 days old) were used instead of cultures more than 3 weeks old. After each lipofection, cells were allowed to recover for 12 hr in fresh DMEM containing 10% (v/v) FCS. In total, lipofection was repeatedly performed four times in some cultures.

After multitransfection, culture medium tentatively containing GDNF was collected as GDNF-conditioned medium (GDNF-CM). Similarly, vector-only (transfected with 1.5 μg pcDNA3 DNA)-conditioned medium (vector-CM) and medium from nontransfected cells (UT-CM) were also collected.

Primary Culture of Embryonic Mesencephalic DA Neurons

Midbrain neuronal primary cultures were prepared from E15 rat mesencephalon as previously described (Shimoda et al., 1992), but with minor modifications. Briefly, the fetal brainstem was isolated free of meninges, and the mesencephalic dopaminergic region (MDR) was dissociated in Ca^{2+}- and Mg^{2+}-free Hank's medium (GibcoBRL, Life Technologies) containing 0.25% trypsin at room temperature for 10 min with constant shaking. FCS was added to the dissociation mixture to a final concentration of 10% (v/v), and centrifugation of this mixture was carried out for 5 min at 190*g*. After resuspension of the cell pellet with serum-free neuronal medium, the suspension was filtered through a 70-μm sterile Mesh Nylon filter (Spectrum Laboratories Inc.). Neuronal medium was similar to DMEM except that the concentration of glucose increased from 7.5 to 30 mM and the neuronal medium was without glutamine. Finally, glutamine was added freshly to a concentration of 2 mM. The filtered cells were subsequently seeded in a poly-D-lysine (Sigma Chemical Co., St. Louis, MO)-coated 24-well plate (Nunc) at a density of 7.5×10^4 cells/cm^2 in 500 μl 10% FCS-neuronal medium. Cells were incubated at 37°C in 5% CO_2 for 30 min, and medium was replaced by neuronal medium containing freshly added 10% FCS.

Immunostaining of Embryonic Mesencephalic DA Neurons

Neuronal medium (400 μl) mixed with 100 μl GDNF-CM (or vector-CM, or UT-CM) was used to replace the media in the wells, in which the DA neurons were grown. Cells in regular growth medium without any treatment served as negative controls. After culturing for 7 days, the mesencephalic DA neurons were washed twice with PBS and then fixed with 4% paraformaldehyde at room temperature for 20 min. They were then neutralized with 0.1 M glycine and permeabilized with 0.1% Triton X-100. Cells were subsequently incubated with monoclonal antityrosine hydroxylase (TH) antibody (1:5 dilution; Boehringer, Mannheim, Germany) at room temperature for 1 hr, followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (1:20 dilution; Boehringer). Immunoreactivity for TH was visualized with a confocal microscope (Bio-Rad, Hercules, CA). The TH-positive cells were determined by counting the number of TH-positive neurons in five random fields under a fluorescent microscope (Axiophot; Zeiss).

Ischemia Model

Ischemia was induced in cultured astrocytes by using an anaerobic chamber (model 1029; Forma Scientific Inc.), according to previously described procedures (Lau and Yu, 2001; Yu et al., 2001, 2002, 2003; Chen et al., 2005a,b). After ischemic treatment, normoxic DMEM (2 ml) was added to the cultures, which were then kept at 37°C in 5% CO_2 for various periods, followed by terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end labeling (TUNEL) assay. Control cultures in normoxic DMEM were incubated in parallel, but in a normoxic CO_2 incubator.

GDNF Treatment to Astrocytes Prior to Staurosporine Treatment

Cultured astrocytes (4–5 weeks old) were incubated in 10 μl GDNF-CM (mixed with 990 μl 10% FCS-DMEM), 1 ml GDNF-CM, 1 ml vector-CM, or recombinant human GDNF (rhGDNF; 100 ng in 1 ml 10% FCS-DMEM) for

3 hr at 37°C in 5% CO_2. Thereafter, cells were treated with 1 μM staurosporine (STS; Sigma) for 24 hr to induce apoptosis. To stain apoptotic nuclei, Hoechst 33342 (Sigma) was added into the culture medium at a final concentration of 1 μg/ml for 10 min before STS treatment ended. Cells were examined under a fluorescent microscope (Axiophot). Similarly, cultured astrocytes (4–5 weeks old) were incubated in 1 ml GDNF-CM, rhGDNF (100 ng in 1 ml 10% FCS-DMEM), 1 ml vector-CM, or 1 ml UT-CM for 6 hr before STS treatment. Untreated astrocyte cultures served as controls.

TUNEL and Hoechst Double Labeling

Apoptotic profiles were analyzed by a nonisotopic TUNEL technique based on the nucleosomal fragments generated by apoptosis-induced endonuclease cleavage (Gavrieli et al., 1992). An In Situ Cell Death Detection Kit (Boehringer) was used to perform the TUNEL experiment after ischemic treatment of astrocyte cultures as described previously (Yu et al., 2001). Hoechst 33342 (1 μg/ml) was used in parallel to confirm apoptotic nuclei.

Measurement of Caspase Activity

After ischemic treatment, astrocyte cultures were allowed to recover in normoxia for 4 hr (postischemia incubation) and were subsequently washed twice with 2 ml chilled PBS. After addition of 200 μl PBS, the cells were collected with a cell scraper and subjected to centrifugation at 450*g* for 5 min at 4°C. The caspase activity in astrocytes was measured by a CaspACE Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

Western Blot Analysis

Soluble crude protein samples were prepared by centrifugation of the collected CM at 15,000*g* for 5 min at 4°C. Protein samples from ischemia-treated astrocytes were prepared by an ice-cold phosphate-buffered saline (PBS) wash, followed by cell lysis in 200 μl lysis buffer containing 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml leupeptin, and 0.5 g/ml pepstatin. After being chilled on ice for 20 min, the cell lysate was centrifuged at 13,000*g* for 10 min at 4°C. The subsequent supernatant from cell lysis and the supernatant (20 μl) from each CM were resolved on a 12.5% reducing SDS-polyacrylamide gel. Western blot analysis was performed as previously described (Wu et al., 2000; Jiang et al., 2002; Yu et al., 2003). GDNF was detected by using an anti-GDNF antibody conjugated with horseradish peroxidase (HRP; 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA). The Western blot signals were visualized with an electrochemiluminescent (ECL) detection system (Amersham Pharmacia Biotechnology).

Statistical Analysis

All data in this paper were computed to give values of mean ± SEM from at least three individual experiments and analyzed by ANOVA. Statistical significance was defined as $P < 0.05$.

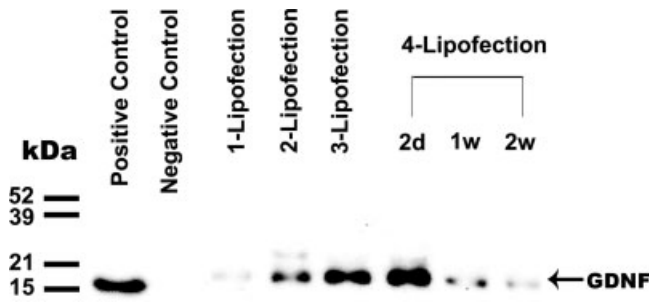

Fig. 1. GDNF levels in culture medium after 1-, 2-, 3-, and 4-lipofections. GDNF-CM and vector-CM (negative control) from transfected astrocytes were analyzed by Western blot to detect the presence of GDNF 48 hr after 1-, 2-, 3-, and 4-lipofections. A prestained protein standard was used as the molecular weight marker, and the rhGDNF protein (2 μg/well) was loaded as the positive control.

RESULTS

GDNF Expression in Astrocytes After Multilipofection

An hGDNF gene was transfected into 2-day-old cultured astrocytes via multiple liposome-mediated transfection—or multilipofection—and CM was analyzed by Western blot 48 hr after multilipofection. The parameters for multilipofection into astrocytes were optimized for maximal transfection efficiency without causing toxicity or cell disturbance, as previously described (Wu et al., 2000). The hGDNF protein (~20 kDa) could be detected in CM from GDNF-transfected astrocyte cultures with 1-lipofection (Fig. 1). Other primary cultures of astrocytes were then subjected to increasing times of lipofections—twice (2-), thrice (3-), and quartic (4-)—resulting in increasing levels of hGDNF in the CM of the astrocytes. The level of hGDNF in CM stably expressed by the astrocytes peaked at 4-lipofection for at least 2 days, after which the GDNF protein levels started to decrease in the CM but remained at a detectable level 2 weeks after the last transfection.

The amount of GDNF in CM increased progressively with the number of lipofections (from 1- to 4-; Table I). Levels of GDNF in CM of cultures subjected to 1-, 2-, 3-, and 4-lipofections exceeded that in the UT-CM by ~90%, ~576%, ~1,099%, and ~1,285%, respectively. Furthermore, compared with 1-lipofection, 2-, 3-, and 4-lipofections increased GDNF levels in the CM by ~255%, ~530%, and ~628%, respectively.

Effect of GDNF on Growth and Morphology of DA Neurons

We used the CM from the transfected astrocytes to incubate DA neurons in culture to confirm the biological and functional activity of this released GNDF protein in CM. Figure 2b shows the DA neurons cultured in vector-CM. They were TH-positive cells visualized by monoclonal anti-TH antibody and FITC-conjugated anti-mouse antibody labeling. When DA neurons were

TABLE I. Levels of GDNF in the Culture Medium After Multilipofection of Astrocytes in Primary Cultures*

	Untransfected control	Time of lipofection				Time after 4-lipofection	
		1-	2-	3-	4-	1 week	2 weeks
GDNF level (ADU)	98.6 ± 17.08	187.6 ± 12.61	677.2 ± 84.47[a]	1182.6 ± 107.71[a]	1365.4 ± 80.07[a]	224.2 ± 25.97[b]	183 ± 17.33[b]

*ADU, arbitrary densitometric unit; 1, lipofection one time; 2, lipofection two times; 3, lipofection three times; 4-, lipofection four times; data from five different experiments and expressed as mean ± SEM.
[a]Significantly different from GDNF level after 1-lipofection.
[b]Significantly different from GDNF level 48 hr after 4-lipofection(ANOVA; $P < 0.05$).

grown in the presence of GDNF-CM from astrocytes with 4-lipofections of GDNF gene for 7 days, their cell bodies became larger (Fig. 2c) and exhibited much longer neurites (Fig. 2a) than the DA neurons grown in the CM from astrocytes with 4-lipofections with vector only (vector-CM; Fig. 2b). The neurotrophic effect of GDNF-CM on DA neurons is best demonstrated by the TH-positive cells, which refer to the average number of TH positive neurons counted in five random fields per well under a fluorescence microscope (Table II). The TH-positive cells for DA neurons grown in UT-CM and vector-CM were 162 ± 74 and 160 ± 77, respectively, whereas the TH-positive cells for DA neurons grown in GDNF-CM were 320 ± 66 (Table II). The results demonstrated that GDNF released from GDNF-transfected astrocytes processed the neurotrophic effects. The apparent data may have been significant if the experiment had been repeated several times or if more random fields were counted.

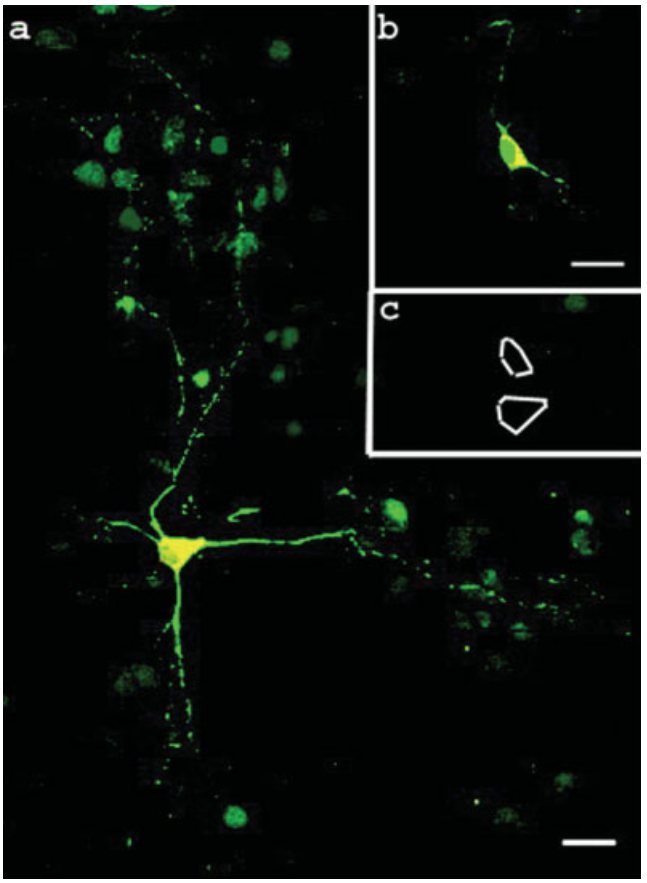

Fig. 2. Effect of GDNF on the morphology of TH-positive DA neurons. DA neuron cultures were incubated in GDNF-CM (**a**) or in vector-CM (**b**) for 7 days, followed by immunostaining for tyrosine hydroxylase (TH). The appearance of DA neuron is shown in a and b, whereas **c** indicates the outline of the soma of the TH-positive neurons in a (the lower outline) and b (the upper outline) for easy comparison of their soma size. Scale bars = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Effect of GDNF on STS-induced Apoptosis in Astrocytes

We previously used STS to induce apoptosis in astrocytes (Yu et al., 2001). After treatment with 1 μM STS for 24 hr, the culture disintegrated (Fig. 3a), and many astrocytes underwent apoptosis with condensed nucleus as revealed by Hoechst staining (Fig. 3f). Pretreatment of astrocytes with the rhGDNF (Fig. 3c,h) or 1 ml GDNF-CM (Fig. 3e,j) for 3 hr prior to STS treatment exerted some protection by reducing the extent of apoptosis. However, pretreatment with 1 ml vector-CM (Fig. 3b,g) or 10 μl GDNF-CM (Fig. 3d,i) resulted in an amount of condensed nuclei similar to that observed in cultures receiving no pretreatment (Fig. 3a,f). Table III summarizes the protective effect of GDNF on astrocytes against STS-induced apoptosis. The extent of apoptosis was measured by calculating the percentage of cells with condensed nucleus vs. the total number of cell count. Astrocytes pretreated with GDNF exhibited significantly ($P < 0.05$) fewer apoptotic cells than cells receiving no pretreatment prior to STS treatment.

Effect of GDNF on Ischemia-Induced Apoptosis in Astrocytes

Astrocytes displayed apoptotic characteristics signified by Hoechst-stained condensed nucleus 3 hr after ischemic insult (Fig. 4f). With the exception of a few cells, most of the Hoechst-stained condensed nuclei were also positive to the TUNEL assay (Fig. 4f,h). Pretreating astrocytes for 6 hr with 1 ml GDNF-CM (Fig. 4d) or 100 ng of rhGDNF as positive control (Fig. 4e) prior to ischemic insult resulted in a markedly lower

TABLE II. Neurotrophic Effect of GDNF on DA Neurons as Demonstrated by TH-Positive Cells

	Treatment with conditioned medium			
	No treatment (n = 5)	UT-CM (n = 5)	Vector-CM (n = 5)	GDNF-CM (n = 5)
Mean TH^+ cells[a]	185 ± 53	162 ± 74	160 ± 77	320 ± 66[b]

[a]Mean TH^+ cells are determined by averaging the number of TH^+ neurons counted in five random fields under a fluorescent microscope.
[b]Significantly different from DA neurons with no treatment (ANOVA; $P < 0.05$). Data were expressed as mean ± SEM; n equals to number of cultures.

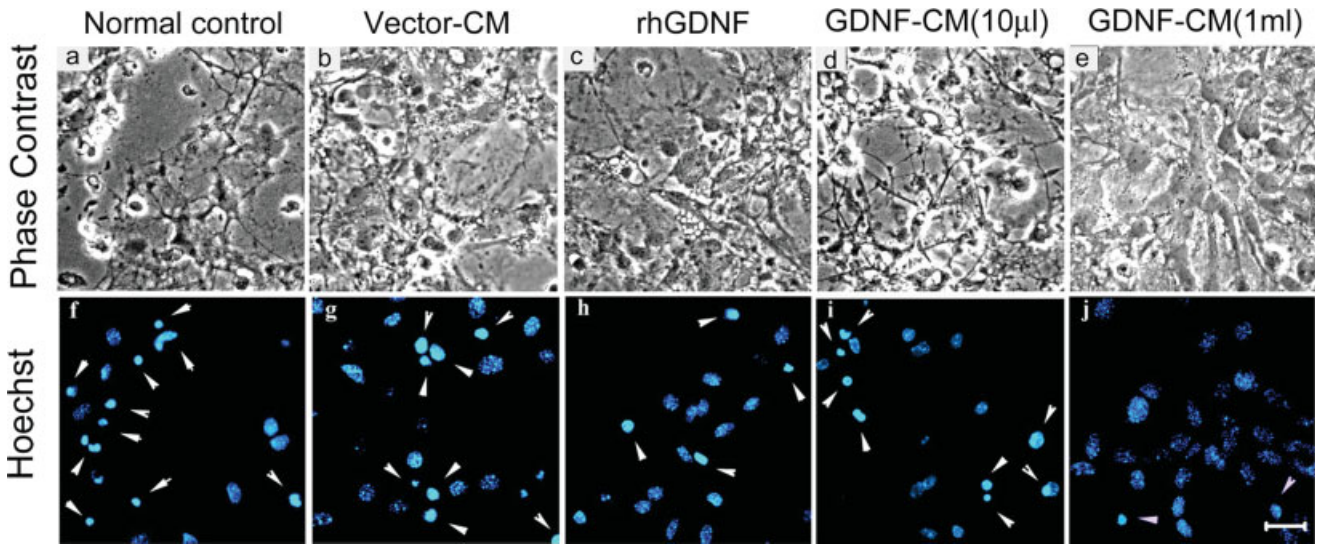

Fig. 3. Effect of GDNF on STS-induced apoptosis in cultured astrocytes. Prior to STS treatment, cultured astrocytes were pretreated with various conditioned media. The extent of apoptosis was visualized under a phase-contrast microscope (upper panels) and by Hoechst staining (lower panels). Cultures with no pretreatment (normal control, **a,f**) exhibit greater number of condensed nuclei than those pretreated with 100 ng rhGDNF (**c,h**) or 1 ml GDNF-CM (**e,j**) after exposure to STS. On the contrary, cultures pretreated with 1 ml vector-CM (**b,g**) or 10 μl GDNF-CM (**d,i**) display an extent of condensed nuclei similar to that in a. Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE III. Effect of GDNF on Hoechst-Positive Astrocytes After STS-Induced Apoptosis

	No pretreatment control (n = 5)	Vector-CM (n = 5)	rhGDNF 100 ng (n = 6)	GDNF-CM 10 μl (n = 5)	GDNF-CM 1 ml (n = 6)
Percentage $Hoechst^+$ cells[a]	17.3 ± 2.8	16.7 ± 2.5	12.4 ± 1.6[b]	14.6 ± 2.7	7.1 ± 1.2[b]

[a]Percentage $Hoechst^+$ cells is determined by counting the total number of TH^+ cells vs. number of $Hoechst^+$ cells. Data are shown as mean ± SEM; n refers to the number of cultures.
[b]Statistically different from the No pretreatment control (ANOVA; $P < 0.05$).

number of Hoechst- and TUNEL-positive cells than cells pretreated with UT-CM (Fig. 4b,g,l) or vector-CM (Fig. 4c,h,m). The proportion of Hoechst- and TUNEL-positive cells 24 hr after ischemia is summarized in Table IV. The GDNF-CM or rhGDNF pretreatment led to, at least, a 2.5-fold reduction in Hoechst- and TUNEL-positive cells compared with cells receiving no GDNF pretreatment (Table IV). The significant reduction ($P < 0.05$) in apoptotic cell number under ischemia in cultures with GDNF treatment demonstrates that GDNF could protect astrocytes against ischemia-induced apoptosis.

Effect of GDNF on Caspase-3 Activity in Ischemic Astrocytes

The activities of caspase-1 and caspase-3 were determined in astrocytes with and without 3 hr ischemia and 4 hr postischemic recovery. Some of the ischemic astrocytes were also pretreated with rhGDNF. Figure 5a indicates that the caspase-1 activity in astrocytes was not affected by the treatments. In contrast, the caspase-3 activity was elevated by over 100% ($P < 0.05$) in astrocytes with a 3-hr ischemia and a 4-hr postischemia recovery (Fig. 5b). Interestingly, this caspase-3 activity was

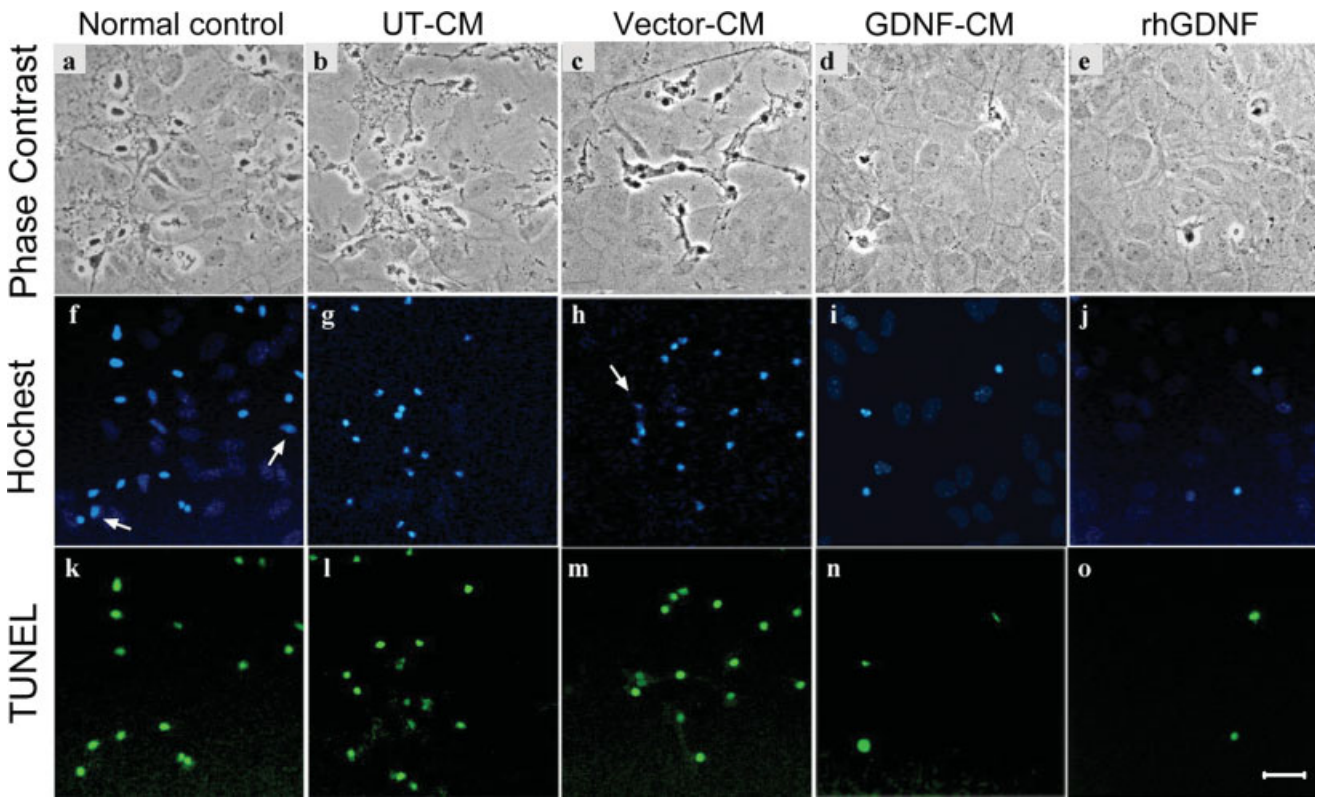

Fig. 4. **a–o:** Effect of GDNF on ischemia-induced apoptosis in cultured astrocytes. Astrocytes were pretreated with rhGDNF or GDNF-CM prior to ischemic exposure. Apoptosis was revealed by the Hoechst and TUNEL staining, and the astrocytes were observed under a phase-contrast microscope (upper panels) and fluorescent microscope (middle and lower panels). Astrocytes visualized by Hoechst stain (middle panels) are also detected by the TUNEL assay (lower panels), except for a few astrocytes (in f and h, indicated by arrows). Cultures with no pretreatment (normal control) exhibit higher number of condensed nuclei than cultures pretreated with 1 ml GDNF-CM (d,i,n) or 100 ng rhGDNF (e,j,o) after ischemic insult. In contrast, cultures pretreated with 1 ml UT-CM (b,g,l) or 1 ml Vector-CM (c,h,m) display extent of condensed nuclei similar to that in a. Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE IV. Effect of GDNF on Hoechst$^+$ or TUNEL$^+$ Astrocytes 24 Hr After 3 Hr of Ischemic Insult

	No pretreatment control (n = 5)	UT-CM (n = 5)	Vector-CM (n = 5)	GDNF-CM (n = 5)	rhGDNF (n = 6)
Percentage Hoechst$^+$ cells[a]	25.1 ± 1.0	24.2 ± 4.2	23.5 ± 1.2	10.2 ± 0.8[b]	6.3 ± 1.9[b]
Normal/Hoechst$^+$ (cell number)[c]	2,050/706	1,951/563	2,047/619	2,463/292	2,937/203
Percentage TUNEL$^+$ cells[a]	22.4 ± 1.8	21.6 ± 3.0	21.2 ± 1.4	8.4 ± 1.2[b]	5.0 ± 1.0[b]
Normal/TUNEL$^+$ (cell number)[c]	2,050/612	1,951/498	2,047/546	2,463/242	2,937/159

[a]Percentage Hoechst$^+$ or TUNEL$^+$ cells determined by counting the total number of TH$^+$ vs. the number of Hoechst$^+$ or TUNEL$^+$ cells, respectively. Data are shown as mean ± SEM; n refers to the number of cultures.
[b]Statistically different from the No protreatment control (ANOVA; $P < 0.05$).
[c]The cell numbers are counted in five random fields of each dish. The length of the field is about 132 μm.

reduced by about 50% ($P < 0.05$) in ischemic astrocytes pretreated with GDNF (Fig. 5b). This indicated that GDNF protects astrocytes from apoptosis under ischemia by reducing caspase-3 activity.

DISCUSSION

GDNF in rat functions as a homodimer with molecular weight ranging between 33 and 45 kDa (Lin et al., 1994), depending on the extent of glycosylation. The actual molecular weight of the hGDNF in CM produced by the transfected astrocytes in this study is approximately 20 kDa, indicating a consistent size range for the expressed GDNF monomer, as reported previously (Lin et al., 1993; Wu et al., 2000). In this study, we have confirmed that the hGDNF synthesized and released by transfected astrocytes in primary culture exerts biological functions, as shown in its support of the survival and growth of DA neurons in culture.

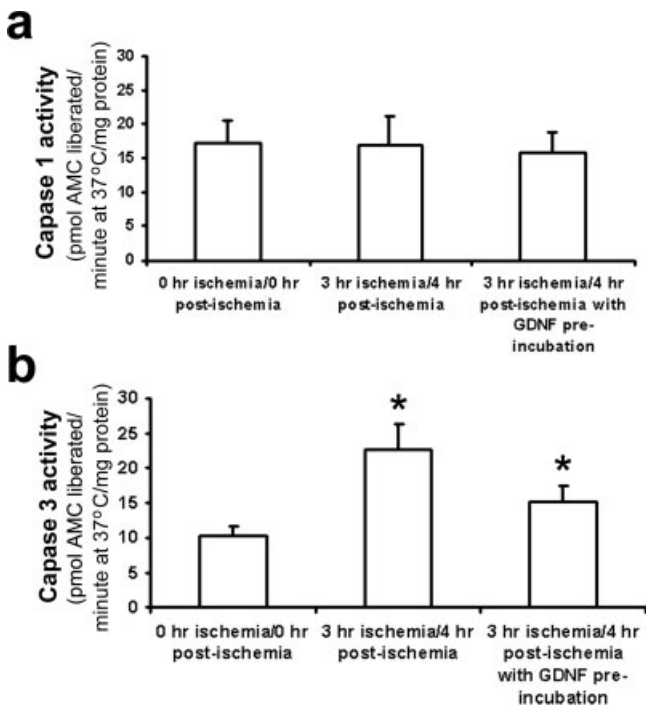

Fig. 5. Inhibitory effect of GDNF on caspase-1 (**a**) and caspase-3 (**b**) activities during postischemia recovery. Astrocytes were exposed to ischemia for 3 hr and allowed to recover in normoxic medium at 37°C for 4 hr (postischemia). GDNF preincubation was accomplished by providing the astrocytes with GDNF-CM for 6 hr prior to the ischemic exposure. After recovery for 4 hr under postischemia, the activities of caspase-1 and caspase-3 were determined in these astrocytes. All data were derived from three independent experiments, and error bars indicate SEM.

It is known that the levels of GDNF mRNA and protein could be elevated in cultured astrocytes when exposed to inflammatory cytokines (Appel et al., 1997), kainate and glutamate (Ho et al., 1995), fibroblast growth factor (Suter-Crazzolara and Unsicker, 1996), apomorphine (Li et al., 2006), and valproate (Chen et al., 2006). We have shown, in this study and in our previous study (Wu et al., 2000), transgenic GDNF expression and elevated intracellular GDNF, which is then released into the culture medium. This GDNF in CM not only supports neuronal growth but also protects astrocytes from STS- and ischemia-induced apoptosis. This also revealed that the GDNF therapeutic effects observed in ischemic brain (Harvey et al., 2005) also embrace astrocytes. Therefore, GDNF produced by astrocytes serve two purposes: an autocrine effect in protecting itself and a paracrine effect on neighboring astrocytes and neurons.

GDNF-reduced apoptosis in ischemic astrocytes was through a reduction of caspase-3 activity but not caspase-1. It is known that both the caspases are elevated in the CNS under ischemia (Kitagawa et al., 1998), and GDNF treatment would reduce the two caspases in the infarct tissues (Kitagawa et al., 1998; Harvey et al., 2005). However, in this study, the lack of effect of GDNF on caspase-1 activity in ischemic astrocytes might indicate some variants in neuronal and astrocytic response to GDNF, an issue requiring future clarification. GDNF is known to exert its protective effect through a multicomponent receptor complex composed of the tyrosine kinase product of c-Ret and the ligand-binding protein GDNF receptor (GFR; Sariola and Saarma, 2003). Ret was detected in astrocytes (Nakamura et al., 1994), but GFRα1 was once thought not to exist in astrocytes (Walker et al., 1998). Bresjanac and Antauer (2000) have reported the existence of GFRα1 in reactive astrocytes. Hauck et al. (2006) also reported GFRα1 and Ret expression in retinal Mueller glial cells. Preliminary study in our laboratory also detected the expression of both Ret and GFRα1 mRNA in primary cultures of astrocytes (unpublished data).

Although astrocytes could be chemically induced to produce GDNF, the level of GDNF from these astrocytes would not be able to achieve any therapeutic effects. In this study, we used the multilipofection method to transfect the GDNF gene to astrocytes in primary cultures. This is based on its advantages of simplicity, safety of use, biological inertness, ease of automation, and nonimmunogenicity. We have demonstrated that it is a mild and efficient nonviral gene transfer method with high specificity for gene delivery to astrocytes in primary cultures (Wu et al., 2000). One does not have to be concerned with the side effects of viral gene transfer technology, including the possibility of some viruses, to disturb the host DNA transcription and synthesis, regain their pathogenic activity, and generate an immune response. Therefore, multilipofection provides an alternative means for gene transfer in clinical gene/cell therapy before an absolutely safe virus is identified.

One of the factors determining the efficiency of transplantation is the survival of the transplanted cells. Many of these cells cannot survive long enough to exert their effects because of the microenvironment of the site of transplantation, which is usually ischemic. Our finding of GDNF protecting astrocytes further suggests that GDNF and astrocytes make a great combination for gene/cell therapy. The transfected astrocytes release GDNF into the microenvironment, and, in addition to exerting their neuroprotective effect on the host, they guard the transplanted astrocytes to survive until the mircocirculation is reestablished in the damaged tissue.

We previously demonstrated the feasibility of using multilipofection to introduce GDNF into cultured astrocytes without causing any observable disturbances to the cells themselves (Wu et al., 2000). The level of GDNF transcripts and protein in cultured astrocytes is extremely low or even undetectable, whereas high levels of GDNF protein can be detected in the CM after 4-lipofections of the GDNF gene. Recent evidence has indicated that astrocytes are involved in many pathological and physiological conditions of the CNS. The potent protective effect of GDNF on astrocytes is an important addition to its well-documented neuronal protection on DA neu-

rons, noradrenergic neurons, motor neurons, and basal forebrain cholinergic neurons.

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