Protective Effects of Neurotrophic Factors on Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL)-mediated Apoptosis of Murine Adrenal Chromaffin Cell Line tsAM5D*1

Tomiyasu Murata, Masaru Tsuboi, Kiyomi Hikita, and Norio Kaneda
From the Department of Analytical Neurobiology, Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468-8503, Japan

We previously established the murine adrenal chromaffin cell line tsAM5D, which was immortalized with the temperature-sensitive simian virus 40 large T-antigen. tsAM5D cells have the capacity to differentiate into neuron-like cells in response to neurotrophic factors when the culture temperature is shifted from 33 to 39 °C. In this model system, the temperature shift in the absence of neurotrophic factors led to cell death. Hoechst staining analysis revealed that typical apoptotic nuclei appeared in a time-dependent manner after the temperature shift. Upon shifting to 39 °C, the degradation of T-antigen was accompanied by the transcriptional activation of p53 protein. Among the p53 target genes, death receptor 5 (DR5), which is the receptor for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), showed the highest level of induction. Interestingly, TRAIL-neutralizing antibody protected tsAM5D cells from the temperature shift-induced apoptotic cell death by blocking the activation of caspase-8 and -3, indicating the involvement of TRAIL-mediated death signaling in the temperature shift-induced apoptosis. Glial cell line-derived neurotrophic factor (GDNF) inhibited the TRAIL-mediated activation of caspase-8 in tsAM5D cells exposed to 39 °C and cooperated with basic fibroblast growth factor (bFGF) plus ciliary neurotrophic factor (CNTF) to induce the most efficient cell survival. The combination of bFGF plus CNTF cooperated with GDNF to up-regulate the expression of neuronal cell markers in tsAM5D cells. In tsAM5D cells, the temperature shift from 33 to 39 °C induced the degradation of T-antigen and apoptosis. These results are discussed with respect to the intracellular mechanisms underlying the protective function of neurotrophic factors against TRAIL-mediated death signaling.

The temperature-sensitive tsA58 mutant of the simian virus 40 large T-antigen (tsSV40T)2 (1) has been utilized for development of mammalian cell lines displaying differentiated characteristics. Cells immortalized with the tsSV40T oncogene proliferate under the permissive temperature of 33 °C, and they differentiate at the nonpermissive temperature of 39 °C (2, 3). An advantage of tsSV40T is the ability to study the differentiation function of the cell without the interference of the transforming oncogene at the nonpermissive temperature. In fact, several tsSV40T-immortalized neuronal and non-neuronal cell lines have been shown to retain their capacity to differentiate even when the immortalizing oncogene has been inactivated (4–11). Therefore, the tsSV40T-transformed cell line can be useful for studying the regulatory mechanism by which target cells differentiate in response to extracellular stimuli.

Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of the catecholamines dopamine, norepinephrine, and epinephrine (12, 13). Tyrosine hydroxylase is selectively expressed in the central and peripheral catecholaminergic neurons and in the adrenal medullary chromaffin cells. Previously, we demonstrated that an 11-kb DNA fragment of the human tyrosine hydroxylase gene, consisting of 2.5 kb of the 5′-upstream region, the entire exon-intron portion, and 0.5 kb of the 3′-flanking region was sufficient to confer tissue-specific and high level expression of tyrosine hydroxylase in transgenic mice (14). On the basis of this experiment, we generated transgenic mice expressing the tsSV40T gene under the control of the tyrosine hydroxylase promoter to provide an ideal source for the establishment of catecholaminergic cell lines. We succeeded in establishing a conditionally immortalized adrenal chromaffin cell line, tsAM5D, from an adrenal tumor of an adult tyrosine hydroxylase-tsSV40T transgenic mouse (15). tsAM5D cells conditionally grow at the permissive temperature of 33 °C and exhibit the dopaminergic phenotype. In addition, tsAM5D cells proliferate at the permissive temperature in response to basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF). At the nonpermissive temperature of 39 °C, bFGF and CNTF act synergistically to cause tsAM5D cells to differentiate into neuron-like cells. In the presence of bFGF plus CNTF (bFGF/CNTF), the temperature shift is capable of up-regulating the expression of neuronal cell markers, including neuron-specific enolase, growth-associated protein 40 large T-antigen; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; P3K, phosphatidylinositol 3-kinase; DISC, death-inducing signaling complex; Z. benzylxoxycarbonyl; fmk, fluoromethyl ketone; PBS, phos- phate-buffered saline; RT, reverse transcription; TNF, tumor necrosis factor; MTS, tetrazolium, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium.
protein-43, microtubule-associated protein 2, and neurofilament, thereby indicating induction of the neuronal phenotype by the temperature shift. Thus, tsAM5D cells should be a good tool to allow a detailed study of the mechanisms regulating neuronal differentiation.

It was reported that in some of tsSV40T-immortalized cell lines, the shift up to the nonpermissive temperature not only induces growth arrest but also causes cell death (16–22). The temperature shift-induced cell death of tsSV40T-immortalized cells has been demonstrated to be due to apoptosis, based on the observation of nuclear fragmentation or DNA fragmentation. However, the molecular basis for the apoptosis of tsSV40T-immortalized cells is not yet understood. During the course of our study to characterize tsAM5D cells, we found that these cells dramatically underwent cell death by the temperature shift from 33 to 39 °C in the absence of bFGF/CNTF. In this study, we examined the mechanism of cell death triggered by this temperature shift. Here we report that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated the cell death induced by the temperature shift in a p53-dependent fashion. Furthermore, by using this model system, we demonstrated the protective effects of neurotrophic factors against this TRAIL-mediated apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following materials from the indicated sources were used in this study: trypsin solution, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and G5 supplement from Invitrogen; BioCoat type IV collagen-coated 24- and 96-well plates and mouse type IV collagen from BD Biosciences; mouse anti-SV40T-antigen antibody (clone polyclonal antibody 101), rat anti-mouse TRAIL antibody from Pharmingen (San Jose, CA); goat anti-mouse Fas ligand antibody, caspase-8 inhibitor (Z-IE TD-fmk), caspase-3 inhibitor (Z-DEVD-fmk), and general caspase inhibitor (Z-VAD-fmk) from R & D Systems, Inc. (Minneapolis, MN); Hoechst 33342 dye from Sigma; LY294002 and PD98059 from Calbiochem-Novabiochem; goat anti-mouse TNF-α antibody, rat glial cell line-derived neurotrophic factor (GDNF), human bFGF, and rat CNTF from Genzyme Techne (Minneapolis, MN); Vectashield mounting medium from Vector Laboratories (Burlingame, CA); CellTiter 96 AQueous One Solution reagent, pRL-TK vector, TransFast reagent, dual-luciferase assay system, and pGEM-T Easy cloning vector from Promega (Madison, WI); pp53-TA-Luc vector, pTA-Luc vector, and A poAlert caspase fluorescence assay kit from Clontech; ISOGEN from Nippon Gene (Toyama, Japan); TaqMan reverse transcription reagent from PerkinElmer Life Sciences; QuantiTect SYBR Green RT-PCR kit from Qiagen (Valencia, CA); BCA protein assay reagent kit and 3,3′,5,5′-dithiobis(sulfosuccinimidylpropionate) from Pierce; goat anti-mouse death receptor 5 (DR5) antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); peroxidase-conjugated donkey anti-mouse IgG antibody and goat anti-goat IgG antibody from Jackson ImmunoResearch (West Grove, PA); hamster anti-mouse DR5 antibody from eBioscience (San Diego, CA); protein assay kit from Bio-Rad; protein G-Sepharose 4B and ECL Western blotting detection reagents from Amersham Biosciences; and Alexa Fluor 568-conjugated donkey anti-goat IgG antibody from Molecular Probes (Eugene, OR).

**Cell Culture**—tsAM5D cells were seeded into type IV collagen-coated dishes and cultured in DMEM supplemented with G5 supplement (insulin, transferrin, selenite, biotin, hydrocortisone, bFGF, and epidermal growth factor), 10% heat-inactivated FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin under a humidified atmosphere of 5% CO2 and 95% air at 33 °C (15).

**Measurement of Cell Viability**—To examine the effects of culture temperature on cell viability, we plated tsAM5D cells (4 × 10⁴ cells/well for the permissive temperature; 8 × 10⁴ cells/well for the nonpermissive temperature) on type IV collagen-coated 96-well dishes and maintained them in the defined growth medium at 33 °C. After 24 h (day 0), the cells were washed twice with DMEM containing 10% FBS and then cultured at 33 or 39 °C in 150 μl/well of the serum-containing medium. To examine the involvement of death receptor signaling and the caspase cascade in the cell death, we preincubated cells with neutralizing antibodies (10 μg/ml), control IgG (10 μg/ml), or caspase inhibitors (50 μM) for 2 h prior to temperature shift. The neutralizing antibodies used were rat anti-mouse TRAIL antibody, goat anti-mouse Fas ligand antibody, and goat anti-mouse TNF-α antibody. The caspase inhibitors used were caspase-8 inhibitor (Z-IE TD-fmk), caspase-3 inhibitor (Z-DEVD-fmk), and general caspase inhibitor (Z-VAD-fmk). To examine the effect of neurotrophic factors on the temperature shift-induced cell death, we added 50 ng/ml of various neurotrophic factors at day 0. At the time points indicated in the figures, the number of viable cells was assessed by adding 30 μl/well of CellTiter 96 AQueous One Solution reagent containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS tetrazolium). After 2 h of incubation at 33 or 39 °C, the absorbance at 490 nm was measured with a microtiter plate reader (Bio-Rad). Background absorbance because of the medium was subtracted from the data.

**Quantification of Apoptosis**—After the cells had been treated on 4-well chamber slides previously coated with mouse type IV collagen, they were fixed with 4% paraformaldehyde in PBS for 30 min, washed once with PBS, and then stained for 15 min with Hoechst 33342 dye. After being washed with PBS, the coverslips were mounted using Vectashield, and the cells were then observed under a Zeiss fluorescence microscope (Carl Zeiss Microl Imaging, Inc., Jena, Germany). Apoptotic cells were defined by condensed and/or fragmented chromatin in the blue fluorescence-emitting nuclei. At least 250 cells from randomly selected fields were counted in each experiment.

**Morphological Analysis**—Cells were plated on type IV collagen-coated 35-mm dishes at an initial density of 80% confluence and maintained in the defined growth medium at 33 °C for 24 h after plating. After the cells had been washed with 10% FBS-containing medium, the culture medium was switched to serum-containing medium supplemented or not with 50 ng/ml of various neurotrophic factors and maintained at 39 °C for the times indicated in the figures; and the cultures were then photographed under a phase-contrast microscope (model TE300, Nikon, Tokyo, Japan).
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Assay of Transcriptional Activity of p53—Cells were plated on type IV collagen-coated 24-well dishes to ~80% confluence and maintained in defined growth medium at 33 °C. After 12 h, either pp53-TA-Luc vector (0.5 μg/well) or an equivalent molar amount of pTA-Luc vector (negative control) was used along with pRL-TK vector (0.125 μg/well) to co-transfect the cells. We used a synthetic cationic lipid component, TransFast reagent, according to the manufacturer’s instructions, for the transfection. The pRL-TK vector containing the Renilla luciferase gene under the control of the minimum promoter from herpes simplex virus thymidine kinase was used as an internal control to correct for transfection efficiency and differences in cell number. After the transfection, the transfected cells were cultivated in the defined growth medium at 33 °C. After 18 h (time 0), the cells were cultured at 33 or 39 °C for the desired periods. At the end of the culture period, the transfected cells were lysed, and the luciferase activity in each cell lysate was then measured by conducting the dual-luciferase assay according to the manufacturer’s instructions. The reporter gene activity was expressed as the firefly luciferase activity of pp53-TA-Luc vector divided by the Renilla luciferase activity of the pRL-TK vector. The luciferase activity of the pTA-Luc vector was subtracted from that of pp53-TA-Luc vector.

Quantitative Real Time RT-PCR—Cells were plated on type IV collagen-coated 35-mm dishes at an initial density of 80% confluence and maintained in the defined growth medium at 33 °C for 24 h after plating. After the cells had been washed with 10% FBS-containing medium, the culture medium was switched to serum-containing medium supplemented or not with 50 ng/ml of various neurotrophic factors, and the cells were then maintained at 33 or 39 °C for the desired periods. Total RNA was isolated from the cultured cells by using Isogen according to the manufacturer’s instructions and then treated with RNase-free DNase. In a 100-μl reaction, the purified total RNA (2 μg) was reverse-transscribed at 48 °C in the presence of oligo(dT) primers by using TaqMan reverse transcription reagent kit. Supernatants containing 50 μg/ml of various neurotrophic factors, and the cells were then maintained at 33 or 39 °C for the desired periods. The following thermal profile was used: PCR amplification, 1 cycle at 94 °C for 15 min and then 45 cycles at 94 °C for 15 s, 57–59 °C for 30 s, and 72 °C for 1 min. For the quantitative determination of the mRNA level of target genes, the accumulation of PCR products was measured directly by monitoring fluorescence intensity with an ABI PRISM 7700 sequence detector (PerkinElmer Life Sciences). Amplified DNA fragments were cloned into the pGEM-T Easy cloning vector and then sequenced with an automated fluorescence DNA sequencer (ABI PRISM 310-2 Genetic Analyzer, PerkinElmer Life Sciences) to confirm the identity of the cloned inserts. The housekeeping gene β-actin was also amplified to normalize the cDNA content of each sample, because the expression level of β-actin mRNA did not change under any culture conditions. The cycle threshold (Ct) values for target genes and β-actin were measured, and the change in mRNA level of target genes was calculated by using the comparative Ct method (User Bulletin 2, Applied Biosystems).

Assay of Caspase Activity—The activities of caspases-8 and caspase-3 were measured by using an ApoAlert caspase fluorescence assay kit according to the manufacturer’s instructions. Cells were cultured on type IV collagen-coated 24-well dishes under the appropriate conditions, washed with ice-cold PBS, and lysed in caspase lysis buffer for 15 min on ice. To study the effects of LY294002 and PD98059 on caspase activity, we preincubated cells with either inhibitor 1 h prior to treating them with neurotrophic factors at 39 °C. After centrifugation at 15,000 × g for 15 min at 4 °C, the protein concentration of supernatants was determined with a micro-BCA protein assay reagent kit. Supernatants containing 50 μg of protein were incubated with 50 μM IETD-AFC (caspase-8 substrate) or DEVD-AFC (caspase-3 substrate) for 1 h. Caspase activity was determined by fluorometric detection of the hydrolyzed products with a microplate spectrofluorometer (Bio-Rad). Excitation at 400 nm and emission at 505 nm were used for caspase-3 and caspase-8 analyses.

Immunoblot Analysis—Cells were cultured on type IV collagen-coated 60-mm dishes under the appropriate conditions. For the analysis of tsSV40T, cells were lysed in lysis buffer consisting of 125 mM Tris–HCl, pH 6.8, containing 4% SDS, 100 mM NaCl, 1 mM EDTA, and 15% glycerol. For the analysis of DR5, cells were lysed in a different lysis buffer (20 mM Tris–HCl, pH 7.6, containing 0.5% Nonidet P-40, 150 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture). After centrifugation at 15,000 × g for 15 min at 4 °C, the protein concentration of supernatant was determined with BCA protein assay reagent kit. The supernatant (100 μg of protein) was boiled in Laemmli sample buffer containing 5% 2-mercaptoethanol. The proteins were resolved by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes.
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After blocking for 1 h with TBS-T (20 mM Tris-HCl, pH 7.6, containing 100 mM NaCl and 0.1% Tween 20) supplemented with 3% skim milk, the membranes were incubated with mouse anti-SV40T-antigen antibody (1:100) or goat anti-mouse DR5 antibody (1:100) in TBS-T containing 1% skim milk and then with peroxidase-conjugated donkey anti-mouse IgG antibody or anti-goat IgG antibody in the same buffer. Bound antibody was visualized by using the ECL system. The same blots were stripped and reprobed with anti-β-actin antibody, as β-actin was used as a loading control.

**TRAIL Receptor DR5 Cross-linking and Immunoprecipitation**—Cells were treated with 2 mM 3,3′-dithiobis(sulfosuccinimidylpropionate), a cleavable cross-linker, in PBS for 20 min at 4 °C. After the reaction had been stopped with 10 mM ammonium propionate, a cleavable cross-linker, in PBS for 20 min at 4 °C.

**Immunoprecipitation**—The immunocomplexes were washed five times with lysis buffer and booted in Laemmli sample buffer containing 5% 2-mercaptoethanol. The proteins were resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. To rule out the possibility that the bands were nonspecific bands derived from the cell lysate proteins and immune IgG used for immunoprecipitation, we treated the beads with either cell lysate alone or anti-DR5 antibody alone in lysis buffer. These beads were then mixed and boiled in Laemmli sample buffer containing 5% 2-mercaptoethanol, and the samples were loaded as an immunoprecipitation negative control. By using the goat anti-mouse DR5 antibody and peroxidase-conjugated donkey anti-goat IgG antibody, we performed the immunoblotting of DR5 as mentioned above.

**Immunocytochemical Analysis**—After the cells had been cultured on 2-well chamber slides coated previously with mouse type IV collagen, they were fixed with 4% paraformaldehyde in PBS for 30 min. After washing three times with PBS, the cells were blocked with Block Ace for 1 h and then incubated with goat anti-mouse DR5 antibody (1:50) in Block Ace solution at 4 °C overnight. After incubation with the primary antibody, the cells were washed three times with PBS and incubated for 1 h with Alexa Fluor 568-conjugated donkey anti-goat IgG antibody (1:200) in Block Ace solution. Subsequently, they were washed with PBS, and coverslips were mounted using Vectashield, and the cells were analyzed by Zeiss LSM510 META confocal laser-scanning microscopy (Carl Zeiss MicroImaging, Inc.). For quantification of the fluorescence signals, images of labeled cells were acquired by using all the same settings below saturation at 512 × 512 pixels. The z-series images were collected at 0.4-μm steps, and a maximum transparency projection in Zeiss-LSM Image Browser version 3.5 was used for producing figures. Immunofluorescence analysis for each experiment was performed 3–5 times, and the most representative results were displayed.

**Statistical Analysis**—The significance of differences was estimated by using Student’s t test. A p value of less than 0.05 was considered significant.

**RESULTS AND DISCUSSION**

We recently succeeded in establishing a conditionally immortalized adrenal chromaffin cell line named tsAM5D (15). In serum-containing medium supplemented with bFGF/CNTF, cells proliferate at the permissive temperature of 33 °C, and they differentiate into neuron-like cells at the nonpermissive temperature of 39 °C (15). However, when cultured at 39 °C in the absence of bFGF/CNTF, the cells dramatically undergo cell death (15). One purpose of our study was to investigate the intracellular mechanism responsible for the cell death at 39 °C in the absence of neurotrophic factors. We first examined the cell viability of tsAM5D cells in medium containing serum alone at 33 and 39 °C. To assess cell viability, we used the CellTiter 96 aqueous assay kit, which is based on the metabolic conversion of MTS tetrazolium compound to a colored formazan product by living cells. The absorbance of the formazan product is directly proportional to the number of viable cells in culture. As shown in Fig. 1A, when the cells were cultured at 33 °C in the presence of 10% FBS alone, the MTS level was constant, but it dramatically decreased when the temperature was increased to 39 °C. Upon morphological analysis, a small number of floating dead cells was seen at 6 h after the temperature shift, and most of cells had detached from the culture dish by 30 h (Fig. 1B). At the permissive temperature (33 °C), the morphology of the cells did not change over a 30-h period (data not shown). To determine whether the loss of cell viability elicited by the temperature shift was because of apoptosis, we monitored the nuclear morphology of the cells by Hoechst staining. As shown in Fig. 1C, the percentage of apoptotic cells at 39 °C gradually increased in a time-dependent manner. Interestingly, when the temperature was shifted to 39 °C, the tsSV40T protein was gradually degraded and became undetectable after a 12-h incubation (Fig. 1D). At the permissive temperature, however, the expression level of tsSV40T did not change over the same period of time (data not shown). These results suggest that apoptotic cell death induced by the temperature shift depended on the expression level of tsSV40T.

The tsSV40T-immortalized cell lines TLR2 (17) and RETsAF (18) have been reported to undergo apoptosis at the nonpermissive temperature. T-antigen is thought to induce immortalization by interacting with p53 or retinoblastoma suppressor protein (23–25). In the case of RETsAF and TLR2 cells, it was demonstrated that p53 forms complexes with T-antigen at the permissive temperature and that the shift up to the nonpermissive temperature leads to the degradation of T-antigen and the liberation of an abundance of p53 from the degrading complexes. Because p53 has been shown to be capable of inducing apoptotic cell death (26–29), these data imply that apoptosis is induced at the nonpermissive temperature by the transient increase in the level of active p53. Therefore, we speculated that functional activation of p53 is involved in the apoptosis of tsAM5D cells at the nonpermissive temperature. To examine
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Whether the transcriptional function of p53 in tsAM5D cells is activated by the temperature shift, we transfected the cells with a p53-sensitive reporter vector, incubated the transfectants at 33 or 39 °C, and estimated the p53-derived luciferase activity. The luciferase activity from the p53 reporter vector did not change at 33 °C, but it increased in a time-dependent manner at 39 °C (Fig. 2A). p53 has been shown to transactivate a number of genes important for apoptosis (26, 27, 29), cell cycle control (30), and DNA repair (31). So we examined the effect of the temperature shift on the mRNA expression levels of five typical p53 target genes, i.e. DR5, p21, Mdm2, Fas, and Bax. As shown in Fig. 2B, all of these target genes were induced in a time-dependent manner after the shift to 39 °C. In contrast, their mRNA levels remained constant over a 10-h period at 33 °C (data not shown). These results suggest that the temperature shift-induced degradation of T-antigen led to the functional activation of p53 in tsAM5D cells.

In p53-mediated apoptosis, genes encoding death receptors TRAIL receptor DR5 and Fas are well established targets of p53 (32). In fact, the mRNA levels of these two death receptor genes were up-regulated in tsAM5D cells by the temperature shift (Fig. 2B). It was reported earlier that the death-inducing ligands TRAIL, Fas ligand, and TNF-α cooperatively amplified apoptosis via cross-linking of their cognate receptors (33). Considering these previous findings, we examined whether the temperature shift-induced apoptosis of tsAM5D cells involved death-inducing ligand/receptor systems such as TRAIL, Fas ligand, and TNF-α. Interestingly, the temperature shift-induced decrease in the MTS level was significantly inhibited by TRAIL-neutralizing antibody but not by Fas ligand- or TNF-α-neutralizing antibodies (Fig. 3A). In addition, the induction of apoptotic cells by the temperature shift was also inhibited by TRAIL-neutralizing antibody but not by the other antibodies (Fig. 3B). The specificity of the protective action elicited by the anti-TRAIL antibody in both MTS level and apoptotic cell number was demonstrated by the lack of effect of the isotype IgG control. These results suggest that endogenous TRAIL was activating DR5, thereby triggering the cell death process. Because the real time RT-PCR analysis showed that the mRNA level of TRAIL did not change after the temperature shift (data not shown), the TRAIL-mediated cell death caused by the temperature shift may be attributable to the up-regulated expression of DR5. In addition, because the mRNA level of the Fas ligand gradually dropped in a time-dependent manner (data not shown), the down-regulated expression of this ligand may be involved in the lack of effect of the anti-Fas ligand antibody.

It is well established that binding of TRAIL to its death domain receptor leads to activation of caspase-8, with subsequent activation of caspase-3, finally resulting in apoptosis condensation and fragmentation of apoptosis relative to the total number of counted cells (250 cells/time point). All values represent the means ± S.E. of three independent experiments, in which measurements in each were made in triplicate. *, p < 0.05 compared with 33 °C treatment at the same time. B, morphological analysis. Cells were observed by phase-contrast microscopy. Original magnification, ×200. C, apoptosis assay. Apoptosis was determined by staining nuclear chromatin with Hoechst 33342. Results are expressed as the percentage of the cells showing characteristic nuclear morphological features (nuclear condensation and fragmentation) of apoptosis relative to the total number of counted cells (250 cells/time point). All values represent the means ± S.E. of three independent experiments, in which measurements in each were made in triplicate. *, p < 0.05 compared with 33 °C treatment at the same time. D, Western blotting analysis of tsSV40T. The proteins in a total cell lysate (100 μg) were separated by SDS-PAGE (12%) and subjected to immunoblotting with antibodies against SV40 large T-antigen. The same blots were stripped and reprobed with anti-β-actin antibody to ensure that equal amounts of proteins had been loaded.
So next we examined the involvement of caspase-8 and caspase-3 in the temperature shift-dependent apoptosis by measuring the enzymatic activity of these caspases. The activity of caspase-8 was activated by the temperature shift in a time-dependent manner when the enzymatic activity of caspase-8 was analyzed by measuring IETDase activity (Fig. 4A). Interestingly, the addition of TRAIL-neutralizing antibody almost completely inhibited the temperature shift-dependent increase in caspase-8 activity within 12 h after the temperature shift (Fig. 4A). These results indicate that the TRAIL ligand/receptor system mediated the temperature shift-induced activation of caspase-8. In addition, when the enzymatic activity of caspase-3 was analyzed by measuring DEVDase activity, caspase-3 was also activated by the temperature shift; and likewise, its activation was significantly blocked by the addition of the TRAIL-neutralizing antibody (Fig. 4B). These results suggest that the TRAIL-mediated activation of caspase-8 led to downstream caspase-3 activation. To confirm that the temperature shift-dependent cell death was mediated through the activation of intracellular caspases, we examined the ability of caspase inhibitors to prevent the temperature shift-induced apoptotic cell death. Caspase-8 inhibitor (Z-IETD-fmk), caspase-3 inhibitor (Z-DEVD-fmk), and a pan-caspase inhibitor (Z-VAD-fmk) significantly protected the cells from the temperature shift-induced increase in the number of apoptotic cells (Fig. 4C). Taken together, these results suggest that the temperature shift-induced apoptosis of tsAM5D cells was dependent on TRAIL-mediated activation of caspase-8 and its downstream cascade. The residual apoptotic cell death was still observed at 39 °C in the presence of TRAIL-neutralizing antibody (Fig. 3, A and B).
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In addition, as shown in Fig. 4, A and B, within 12 h after the temperature shift, when the activation of caspase-8 was completely inhibited by TRAIL-neutralizing antibody, the downstream caspase-3 activity was still observed. Furthermore, as shown in Fig. 4C, when the caspase-8 activation was inhibited by the pharmacological inhibitor Z-IETD-fmk, the apoptosis was still evident. Thus, regardless of the blockage of TRAIL-mediated caspase-8 activation, the caspase-3 activation and apoptosis partially occurred upon the temperature shift. In the apoptotic signaling pathway, mitochondrial dysfunction is thought to lead to the release of several pro-apoptotic factors, including cytochrome c, and to the subsequent activation of caspase-9 and then caspase-3. A number of genes known to be involved in mitochondrial apoptosis, including Bax, Puma, Noxa, Bid, and p53AIP1, have been identified as direct transcriptional targets regulated by p53 (26, 27, 29). In fact, upon the temperature shift, the up-regulation of Bax was correlated with the p53 activation (Fig. 2B). It has been shown that p53 triggers apoptosis by inducing mitochondrial dysfunction through a transcription-dependent mechanism (26, 27, 29). In addition to the transcriptional role of p53, transcription-independent, pro-apoptotic activities of p53 related to mitochondria have been reported (26, 28). It is therefore possible that these mitochondrial intrinsic apoptotic signals also occur upon the temperature shift-mediated p53 activation. Thus, regardless of TRAIL signaling, some other p53-mediated mitochondrial intrinsic apoptotic signals may be partially involved in the temperature shift-induced caspase-3 activation and subsequent apoptosis. The residual cell death and apoptosis still observed in the presence of TRAIL-neutralizing antibody (Fig. 3, A and B) may reflect the action of such signals.

We next examined the effect of neurotrophic factors on the temperature shift-induced loss of tsAM5D cell viability (Fig. 5A). When GDNF was added to serum-containing medium, the temperature shift-dependent decrease in MTS level was significantly less than that with serum-containing medium alone (control), but the level did decrease in a time-dependent manner. The addition of either bFGF or CNTF to serum-containing medium also reduced the decrease in the MTS level, but the effectiveness of these factors was less than that of GDNF. In a further study to investigate the effects of bFGF and CNTF on the GDNF responsiveness of tsAM5D cells, we examined the effects of bFGF and CNTF on the survival of GDNF-treated cells. The combination treatment with GDNF plus bFGF (GDNF/bFGF) was more effective than GDNF alone in lessening the drop in viability; however, the loss of viability was still not completely prevented. Interestingly, the combination of GDNF plus CNTF (GDNF/CNTF) completely protected the cells from the temperature shift-induced decrease in viability as judged from the MTS level. The effects of neurotrophic factors on the temperature shift-induced increase in apoptotic cell number, as seen in Fig. 5B, reflected the data from cell viability assays (Fig. 5A). After 48 h of treatment with GDNF, bFGF, CNTF, or GDNF/bFGF, the surviving cells extended relatively short processes (Fig. 5C). When GDNF was added to serum-containing medium, the GDNF responsiveness of tsAM5D cells, we examined the effects of bFGF and CNTF on the survival of GDNF-treated cells. The combination treatment with GDNF plus bFGF (GDNF/bFGF) was more effective than GDNF alone in lessening the drop in viability; however, the loss of viability was still not completely prevented. Interestingly, the combination of GDNF plus CNTF (GDNF/CNTF) completely protected the cells from the temperature shift-induced decrease in viability as judged from the MTS level. The effects of neurotrophic factors on the temperature shift-induced increase in apoptotic cell number, as seen in Fig. 5B, reflected the data from cell viability assays (Fig. 5A). After 48 h of treatment with GDNF, bFGF, CNTF, or GDNF/bFGF, the surviving cells extended relatively short processes (Fig. 5C). However, after combination treatment with GDNF/CNTF, the cells extended long and elaborately branched processes that formed a network, indicating that these cells had undergone stable neuronal differentiation (Fig. 5C). In the GDNF/CNTF-treated cultures, we consider the terminal neuronal differentiation to have materialized because of the complete protection against the temper-
FIGURE 5. **GDNF prevents the temperature shift-dependent cell death of tsAM5D cells and cooperates with bFGF and CNTF.** The cells were seeded in type IV collagen-coated culture dishes and then incubated in defined growth medium at 33 °C for 24 h. After the medium had been changed to 10% FBS-containing medium supplemented with various neurotrophic factors (50 ng/ml) (0 time), the cells were shifted to 39 °C and incubated for the indicated periods. A control experiment was performed without neurotrophic factors. After the indicated period of culture, the cells were processed for the assays of cell viability, morphology, and apoptosis. The cell viability and apoptosis assays were performed as described in the legend of Fig. 1. All values represent the means ± S.E. of three independent experiments, in which measurements in each were made in triplicate. *, p < 0.05 compared with control at the same time. #, p < 0.05 compared with GDNF treatment at the same time. In the morphology assay, the cells were treated with various neurotrophic factors (50 ng/ml) in 10% FBS-containing medium at 39 °C for 48 h and then viewed by phase-contrast microscopy. Original magnification, ×200.
ature shift-induced apoptotic cell death. Considering the involvement of TRAIL signaling in the temperature shift-induced cell death of tsAM5D cells, it is probable that the cell protection by the combination of neurotrophic factors was based on blockage of the TRAIL-mediated apoptosis. In tsAM5D cell cultures, mitogenic growth factors (G5 supplement) are routinely added to 10% FBS-containing medium to maintain cell proliferation at 33 °C (15). When tsAM5D cells maintained in the defined growth medium were cultured at 33 °C in 10% FBS-containing medium alone, no cell proliferation was observed (15). In this study, we confirmed that the cell number was constant when cells were incubated at 33 °C for 30 h in medium supplemented with serum alone (Fig. 1A). We also previously found that the addition of neurotrophic factor (e.g. bFGF or CNTF) to serum-containing medium at 33 °C led to cell proliferation (15). In this study, we confirmed that the cell number was constant when cells were incubated at 33 °C for 30 h in medium supplemented with serum alone (Fig. 1A). We also previously found that the addition of neurotrophic factor (e.g. bFGF or CNTF) to serum-containing medium at 33 °C led to cell proliferation (15). Thus, the neurotrophic factors were considered to have the ability to support the T-antigen-mediated signal for cell proliferation. As shown in Fig. 5A, upon co-treatment with GDNF/CNTF at 39 °C, the cells slightly proliferated (~1.7-fold) during the first 24 h, but subsequently no proliferation was observed. Because T-antigen protein was degraded within 12 h after the temperature shift (Fig. 1D), it is possible that after the temperature shift, the combination of GDNF/CNTF supported the residual T-antigen-mediated mitogenic signal for cell proliferation, leading to the cell proliferation over a 24-h period. Also, it is possible that from 24 to 48 h, the disappearance of T-antigen-mediated mitogenic signal led to the cessation of cell proliferation and then triggered the synergistic differentiation-inducing effects of GDNF and CNTF.

Next, we examined the effect of neurotrophic factors on TRAIL-related signal transduction pathway in tsAM5D cells following exposure to 39 °C. The first approach was to analyze the effects of the neurotrophic factors on the temperature shift-induced activation of caspase-8 (Fig. 6A). GDNF significantly prevented the activation of caspase-8. bFGF or CNTF also prevented the activation of caspase-8, but their effectiveness was
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A

Relative DR5 mRNA level (fold increase)

Time at 39°C (hours)

B

39°C (10 h)

DR5

β-actin

C

Excess Ab (5 μg/ml)

Limiting Ab (0.5 μg/ml)

39°C (10 h)

33°C (39°C, 0h)

Control (39°C, 10 h)

GDNF (39°C, 10 h)

bFGF (39°C, 10 h)

CNTF (39°C, 10 h)

GDNF/bFGF (39°C, 10 h)

GDNF/CNTF (39°C, 10 h)
less than that of GDNF. The protective effect of GDNF was slightly enhanced by the combination with bFGF. Interestingly, co-treatment of cells with GDNF/CNTF resulted in almost complete protection against the activation of caspase-8. In addition, the order of potency of neurotrophic factors in preventing the activation of caspase-3 was the same as that in preventing activation of caspase-8 (Fig. 6B). These results imply that the protective effect of neurotrophic factors on the temperature shift-induced apoptosis of tsAM5D cells was because of suppression of TRAIL-mediated signaling and that the treatment with neurotrophic factors affected events upstream of caspase-8 activation, i.e., the formation of TRAIL and its death receptor complex for transducing the death signal.

It is well known that GDNF (37, 38), FGF (39, 40), and CNTF (41, 42) promote neuronal cell survival by activating the phosphatidylinositol 3-kinase (PI3K) pathway. So we examined whether inactivation of PI3K signaling by LY294002 would alter the protective effect of neurotrophic factors against TRAIL-mediated caspase-8/-3 activation. The cells were pretreated with LY294002 for 1 h and then incubated at 39 °C for 12 h in the presence or absence of neurotrophic factors. In the absence of neurotrophic factor as a control experiment, LY294002 did not affect the temperature shift-induced activation of caspase-8. However, LY294002 attenuated the protective effect of GDNF, bFGF, CNTF, GDNF/bFGF or GDNF/CNTF on the temperature shift-induced activation of caspase-8 (Fig. 7A). In addition, the neurotrophic factor-mediated protection against the caspase-3 activation, which is downstream event of caspase-8 activation, was also inhibited by LY294002 (Fig. 7B). These results suggest that PI3K signaling was required for preventing the TRAIL-mediated caspase-8/-3 activation by neurotrophic factors. In contrast to the PI3K inhibitor, the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor PD98059 did not alter the ability of neurotrophic factors to inhibit TRAIL-mediated activation of caspase-8/-3 (data not shown). Although LY294002 attenuated the protective effects of neurotrophic factors against the activation of caspase-8/-3, its effect was modest (Fig. 7, A and B). These results imply that other signaling mechanisms in addition to the PI3K one are likely to be involved in these protective effects.

In the human system, TRAIL triggers apoptosis through interaction with death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) (36). As assessed by crystal structure analysis, TRAIL and its cognate death receptor DR5 have been shown to oligomerize as the earliest biochemical event necessary for death signal transduction (43, 44). Since the mouse homologue of DR5 has been identified (45), we analyzed the effect of neurotrophic factors on the oligomerization of mouse DR5 in the temperature shift-induced apoptosis. To assess the oligomerization, we employed the technique of limited antibody immunoprecipitation with a cleavable chemical cross-linker. This technique has been used to assess TRAIL and Fas receptor oligomerization (46–49) and is based on the concept that oligomerized, cross-linked proteins are more efficiently immunoprecipitated than monomers. We first examined the effects of the neurotrophic factors on the induction of DR5 mRNA by the temperature shift. As shown in Fig. 8A, the time-dependent induction of DR5 mRNA by the temperature shift was not affected by GDNF, bFGF, CNTF, or by GDNF/bFGF but was enhanced by GDNF/CNTF. In fact, after 10 h of incubation at 39 °C, the protein level of DR5 reflected the expression level of its mRNA (Fig. 8B). The DR5 protein was detected by SDS-PAGE as two bands of ~43 and ~38 kDa. Although two alternative spliced forms of human DR5 have been identified (50), the mouse homologue does not contain the 3′ and 5′ splice sites for the alternative splicing. It will be interesting to examine the reason for the appearance of two bands in the immunoblotting. Because the expression level of DR5 protein was different in each treatment, in DR5 cross-linking and immunoprecipitation experiments, we adjusted the samples to have an equal amount of DR5 by diluting the cell lysates with lysis buffer. Performance of the immunoprecipitation by using an excess amount of antibody confirmed that equal amounts of DR5 protein were present in each treatment (Fig. 8C). In contrast, a limiting amount of antibody precipitated more DR5 in the absence of neurotrophic factors after the temperature shift than before it (Fig. 8C; cf. 33 °C, 0 h and 39 °C, control), suggesting that the oligomerization of DR5 occurred after the temperature shift. Interestingly, under the limiting antibody condition, the enhanced DR5 immunoprecipitation by the temperature shift was significantly inhibited by the treatment with GDNF/CNTF but not by that with GDNF, bFGF, CNTF, or GDNF/bFGF (Fig. 8C). These results indicate that GDNF cooperatively inhibited the oligomerization of DR5 with CNTF but not with bFGF.

By using immunocytochemical analysis of nonpermeabilized cells, we next addressed whether the temperature shift could induce the DR5 oligomerization in the cell membrane (Fig. 8D). At 33 °C, DR5 was weakly expressed and distributed diffusely throughout the cell membrane surfaces, and only a low level of spot-like immunofluorescence was observed. In contrast, at 10 h after the temperature shift to 39 °C, DR5 was highly...

**FIGURE 8. Oligomerization of TRAIL receptor DR5 in tsAM5D cells exposed to 39 °C is inhibited by GDNF/CNTF treatment but not by GDNF, bFGF, CNTF, or GDNF/bFGF treatment.** Treatment of cells with neurotrophic factors (50 ng/ml) was performed as described in the legend of Fig. 5. A, expression analysis of DR5 mRNA. The expression level of DR5 mRNA was examined by the real time RT-PCR using specific primers. All values represent the means ± S.E. of three independent experiments, in which measurements in each were made in triplicate. * p < 0.05 compared with mRNA level at 0 time. #, p < 0.05 compared with control at the same time. B, Western blotting analysis of DR5 protein. The proteins in a total cell lysate (100 µg) were separated by SDS-PAGE (12%) and subjected to immunoblotting with antibody against DR5. The same blots were stripped and reprobed with anti-β-actin antibody as a loading control. C, analysis of DR5 cross-linking and immunoprecipitation. Cells were treated with the cleavable cross-linking agent 3,3′-dithiobis(succinimidylpropiionate) and lysed for immunoprecipitation using limited (0.5 µg/ml) or excess (5 µg/ml) amounts of anti-DR5 antibody. Immunoprecipitation (IP) and Western blotting were performed as described under “Experimental Procedures.” Immunoprecipitation (IP) negative control was used to rule out the possibility that the bands were nonspecific bands derived from cell lysate proteins and the heavy chain of antibody used for the immunoprecipitation as described under “Experimental Procedures.” D, immunocytochemical analysis of DR5. Cells were stained with goat anti-mouse DR5 antibody followed by Alexa Fluor 568-conjugated donkey anti-goat IgG antibody and inspected by confocal fluorescence microscopy. The images were acquired by the maximum transparency projections of z-stacks, including all planes of the specimen as described under “Experimental Procedures.” The rotation pictures of each projection image are available as supplemental material.
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Oligomerization (Fig. 6) activates caspase-8 in TRAIL signaling but not the DR5 single treatment with GDNF, bFGF, or CNTF inhibited the activated caspase-8 (34, 51, 52). In this study, we found that the temperature shift-induced oligomerization of DR5. Thus, these immunocytochemical data support our conclusion based on the results of the DR5 cross-linking and immunoprecipitation experiment. The rotation pictures of each projection image in Fig. 8D are available as Supplemental Material.

In the TRAIL signaling, following ligation of TRAIL to its receptor and oligomerization of the receptor, a death-inducing signaling complex (DISC) is formed by recruitment of both Fas-associated death domain protein and procaspase-8 to the ligand-receptor complex (34, 51, 52). The formation of DISC triggers the autoprocessing of procaspase-8, leading to activated caspase-8 (34, 51, 52). In this study, we found that the single treatment with GDNF, bFGF, or CNTF inhibited the activation of caspase-8 in TRAIL signaling but not the DR5 oligomerization (Fig. 6A and Fig. 8, C and D). These results imply that GDNF, bFGF, and CNTF individually modulate the TRAIL signaling at the level of DISC. We also found that the PI3K inhibitor LY294002 protected the expression of GDNF, bFGF, and CNTF against TRAIL-mediated caspase-8 activation in tsAM5D cells at 39 °C (Fig. 7A), suggesting that GDNF, bFGF, and CNTF exerted their inhibitory effect at the DISC level by PI3K signaling. In Fas-mediated apoptosis, PI3K signaling has been shown to suppress the potential of the Fas-DISC (53–56). Because the system of TRAIL-DISC mediates its signal through Fas-associated death domain protein and caspase-8 as key apoptosis signal transducers and because its biochemical events resemble those of Fas-DISC, the modulation by PI3K signaling of TRAIL-DISC may be intact in tsAM5D cells exposed to 39 °C. Unfortunately, we could not obtain clear results on TRAIL-DISC analysis using anti-DR5 antibody and tsAM5D cells. This failure may be ascribed to an insufficient titer of the antiserum against the mouse DISC components. Because antisera against the human DISC components are readily available, in order to examine the effects of GDNF, bFGF, and CNTF on TRAIL-DISC, it will be necessary to establish a human cell line sensitive to exogenous TRAIL and responsive to GDNF, bFGF, and CNTF. In this study, our most interesting finding is that co-treatment with GDNF/CNTF led to remarkable suppression of DR5 oligomerization, which occurs upstream of the formation of DISC. We consider the inhibitory effect of GDNF/CNTF on TRAIL-mediated activation of caspase-8 in tsAM5D cells to be a significant reflection of the suppression of DR5 oligomerization. LY294002 did not affect the inhibitory effect of GDNF/CNTF on the temperature shift-induced oligomerization of DR5 (data not shown), suggesting that PI3K signaling was not involved in the DR5 oligomerization. The results shown in Fig. 7A suggest that in the case of GDNF/CNTF treatment, PI3K signaling inhibited the temperature shift-dependent activation of caspase-8. This protective effect of PI3K signaling on caspase-8 activation may have resulted from the prevention of biochemical events at the slightly remaining TRAIL-DISC but not from the inhibition of DR5 oligomerization. These data provide the first insight into the mechanism of how GDNF and CNTF promote survival from TRAIL-mediated apoptosis. It will be interesting to investigate the molecular mechanism by which GDNF cooperates with CNTF to inhibit DR5 oligomerization.

In the human system, other receptors for TRAIL have been identified, including DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and soluble osteoprotegerin, all of which serve as decoy receptors to block TRAIL-mediated apoptosis (36). In the mouse system, two decoy receptors mDcTRAILR1 and mDcTRAILR2, which are quite structurally distinct from the known human decoy TRAIL receptors, have been identified so far (57). These mouse decoy receptors have been demonstrated to efficiently block the TRAIL ligand-dependent apoptosis of Jurkat cells by directly competing for the ligand. A third mouse TRAIL-binding decoy receptor is osteoprotegerin (58), but its relevance to TRAIL biology remains to be established. We examined the effects of neurotrophic factors on the expression of mouse decoy receptors in tsAM5D cells following exposure to 39 °C. The expression level of mDcTRAILR1 did not change within 10 h after the temperature shift. In addition, the treatments with GDNF, bFGF, CNTF, and GDNF/bFGF slightly induced the mRNA expression of mDcTRAILR1 to the same degree within 10 h after the temperature shift. Interestingly, GDNF/CNTF treatment remarkably enhanced the expression level of mDcTRAILR1 compared with the other treatments. Unfortunately, we could not obtain clear results on the expression of mDcTRAILR2 and osteoprotegerin due to their low expression level. When overexpressed in tsAM5D cells, mDcTRAILR1 protected against the temperature shift-induced apoptosis of the cells. These observations imply that mDcTRAILR1 can act as a decoy receptor for temperature shift-induced TRAIL signaling in tsAM5D cells. It will be interesting to investigate the role of mDcTRAILR1 in the effect of neurotrophic factors on DR5 oligomerization and TRAIL-DISC function. Previous studies have demonstrated that phosphorylation events occur within some members of the TNF receptor superfamily. For example, TNF receptor 1 (CD120a) (59–61) and DR3 (61) have been shown to be phosphorylated by ERK2. In addition, TNF receptor 2 (CD120b) is reportedly phosphorylated by casein kinase-1 (62). Furthermore, Fas has been demonstrated to be phosphorylated by a kinase associated with the membrane-proximal cytoplasmic domain of Fas (63). To our knowledge, there have been no previous reports on the phosphorylation of

3 T. Murata, M. Tsuboi, K. Hikita, and N. Kaneda, unpublished data.
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To clarify the mechanism by which neurotrophic factors block DR5 activation, it will be also interesting to investigate the phosphorylation level of DR5 in each neurotrophic factor treatment at 39 °C.

GDNF/CNTF stimulation supported both the survival and differentiation of cells (Fig. 5, A–C), even though the combined factors also increased the expression of pro-apoptotic protein DR5 (Fig. 8, A, B, and D). Thus, a paradox is created. The increased cell survival is attributable to the inhibition of DR5 oligomerization. It will be interesting to investigate the molecular mechanism responsible for this GDNF/CNTF protection against DR5 oligomerization. It is possible that GDNF/CNTF signaling directly blocks this oligomerization. It is also possible that the neuronal differentiative events elicited by GDNF/CNTF indirectly reduce the density of DR5 on the surface of the plasma membrane, thereby wholly or partly accounting for the inhibition of DR5 oligomerization.

TRAIL displays potent pro-apoptotic effects on a number of tumor cell lines (64). Also, with regard to normal tissues, the brain (65) and hepatocyte cells (66) are potentially susceptible to TRAIL exposure. In the human brain, TRAIL is not constitutively expressed, although its receptors are found in neurons, astrocytes, and oligodendrocytes (67). Recombinant TRAIL has been shown to induce apoptosis in rat cortical neurons (68) and in human brains slices (65). Recently, several studies have suggested a significant role for the TRAIL signaling pathway in neurodegenerative processes. For example, TRAIL was reported to mediate ischemia-induced apoptosis in neurons (68). In addition, TRAIL has been demonstrated to be one of the relevant contributors to β-amyloid toxicity in a human neuronal cell line (69) and to be expressed in the brain cells of patients with Alzheimer disease, which is characterized by progressive neuronal loss associated with deposits of amyloid fibrils (70). Furthermore, the TRAIL ligand/receptor system has been proposed to be involved in T-cell-mediated autoimmune diseases of the central nervous system such as multiple sclerosis (71, 72). Finally, in a murine model of human immunodeficiency virus infection of the central nervous system, TRAIL was demonstrated to mediate neuronal death in the human immunodeficiency virus-infected brain (73), and a role for TRAIL in mononuclear phagocyte-mediated neurotoxicity in human immunodeficiency virus-associated dementia was proposed (74). In this study, we demonstrated for the first time the intracellular mechanism for the protective effects of GDNF, bFGF, and CNTF against TRAIL-mediated apoptosis. Our findings suggest that these neurotrophic factors may be applied in the future as therapeutics for TRAIL-mediated neurological disorders.

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