Protective Role of Tissue Transglutaminase in the Cell Death Induced by TNF-α in SH-SY5Y Neuroblastoma Cells

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Tissue transglutaminase (tTGase) regulates various biological processes, including extracellular matrix organization, cellular differentiation, and apoptosis. Here we report the protective role of tTGase in the cell death that is induced by the tumor necrosis factor α (TNF-α) and ceramide, a product of the TNF-α signaling pathway, in human neuroblastoma SH-SY5Y cells. Treatment with retinoic acid (RA) induced the differentiation of the neuroblastoma cells with the formation of extended neurites. Immunostaining and Western blot analysis showed the tTGase expression by RA treatment. TNF-α or C₂ ceramide, a cell permeable ceramide analog, induced cell death in normal cells, but cell death was largely inhibited by the RA treatment. The inhibition of tTGase by the tTGase inhibitors, monodansylcadaverine and cystamine, eliminated the protective role of RA-treatment in the cell death that is caused by TNF-α or C₂-ceramide. In addition, the co-treatment of TNF-α and cycloheximide decreased the protein level of tTGase and cell viability in the RA-treated cells, supporting the role of tTGase in the protection of cell death. DNA fragmentation was also induced by the co-treatment of TNF-α and cycloheximide. These results suggest that tTGase expressed by RA treatment plays an important role in the protection of cell death caused by TNF-α and ceramide.

Keywords: Cell death, C_2-Ceramide, Retinoic acid, Tissue transglutaminase, Tumor necrosis factor α

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Introduction

Tissue transglutaminase (tTGase) is a calcium-dependent enzyme that catalyzes the formation of amide bonds between the γ-carboxyl groups of glutamine residues and polypeptide-bound lysine residues or primary polyamine (Greenberg *et al.*, 1991). Recently, tTGase has attracted much attention, since there has been accumulating evidence suggesting the important roles of tTGase in various biological events. These events include the induction of apoptosis, cell adhesion and morphology change, neurodegenerative diseases, cell differentiation, cell growth, and oocyte maturation (Melino and Piacentini, 1998; Chen and Mehta, 1999; Antonyak *et al.*, 2001; Im, 2001; Kim *et al.*, 2001). It is reported that tTGase is involved in the stabilization of extracellular matrix by binding to extracellular matrix proteins (Chen and Mehta, 1999; Akimov *et al.*, 2000). There have been reports suggesting the important role of tTGase in neurodegenerative diseases, including Huntingtons disease, even though there is a report on the contradictory role of tTGase in the formation of Huntington aggregates (Chun *et al.*, 2001). Also, tTGase functions as a GTP-binding protein ($G_{αh}$), which mediates the activation of phospholipase C δ1 by the $α_1$-adrenergic receptor (Iismaa *et al.*, 2000).

It is reported that tTGase plays an important role in apoptosis by cross-linking various proteins, including core histones, glutathione S-transferase P1-1, retinoblastoma protein, troponin, and β-tubulin (Melino and Piacentini, 1998; Piredda *et al.*, 1999). In SK-N-BE neuroblastoma cells, the overexpression of tTGase increased apoptosis in response to staurosporine and retinoic acid (RA) (Piredda *et al.*, 1999). Induction of tTGase expression by RA treatment elevated apoptosis by apoptotic stimuli in U937 and vascular smooth muscle cells (Oliverio *et al.*, 1999; Ou *et al.*, 2000). Recently, however, there has been growing evidence to support the role

of tTGase in cell survival rather than apoptosis (Antonyak *et al.*, 2001; Boehm *et al.*, 2002). It has been reported that apoptosis is inhibited by the RA-induced differentiation and expression of exogenous tTGase in HL 60 cells (Antonyak *et al.*, 2001). By treatment with dexamethasone, the cell viability was much lower in thymocytes that were isolated from tTGase knockout mice than those from wild-type mice (Nanda *et al.*, 2001). Therefore, it is necessary to elucidate the exact role of tTGase in apoptotic signaling.

The tumor necrosis factor (TNF), produced by activated macrophages, binds to TNF receptors and triggers a series of intracellular events that result in the activation of transcription factors, such as the nuclear factors κB and c-Jun (Chen and Goeddel, 2002). TNF receptors also activate apoptotic signaling, including the TNF-receptor associated death domain, Fas-associated death domain, and caspases (Ashkenazi and Dixit, 1998; Declercq *et al.*, 1998; Kimura *et al.*, 1999). TNF activates the sphingomyelinase signaling pathway, which leads to a generation of ceramide that induces cell death through caspase 3 or the inhibition of the Akt kinase (Cuvillier *et al.*, 1996; Yoshimura *et al.*, 1998; Zhou *et al.*, 1998). Although TNF signaling in cell death has been extensively researched as a model of receptor-mediated cellular signaling, the role of tTGase in TNF-α signaling is not understood.

In this report, we present a new function of tTGase in the cell death that is induced by TNF-α in SH-SY5Y human neuroblastoma cells. RA treatment inhibited cell death caused by TNF-α and ceramide, and the inhibitory effects were eliminated by tTGase inhibitors. The protective role of tTGase was also shown by the inhibition of tTGase synthesis. Therefore, it is likely that tTGase plays a key role in the protection from cell death that is induced by apoptotic signals in SH-SY5Y cells.

Materials and Methods

Chemicals and reagents   TNF-α, C_2-ceramide (N-acetyl-D-sphingosine), all trans-retinoic acids, monodansylcadaverine, cystamine, apo-transferrin, and cycloheximide were purchased from the Sigma-Aldrich Co. (St. Louis, USA). The monoclonal antibodies against NF-κB p65, Bcl-2, and Erk1/2 were obtained from Santa Cruz (Santa Cruz, USA). Tissue transglutaminase II mAb (CUB7402) was obtained from NeoMarker (Fremont, USA). Fetal bovine serum, bovine serum albumin, penicillin/streptomycin solution, and Dulbecco's modified Eagle's medium (DMEM) were from Gibco-BRL (Gaithersburg, USA).

Cell culture   The SH-SY5Y human embryonic neuroblastoma cells were obtained from the Korea Cell Line Bank. They were maintained in DMEM containing 25 mM HEPES, pH 7.4, 10% fetal bovine serum (v/v), 100 units/ml penicillin, and 100 μg/ml streptomycin (growth medium) at 37ºC in a humidified CO_2-controlled (5%) incubator. To verify the effects of agonists in the differentiated SH-SY5Y cells, the cells were differentiated with 10

μM of RA for 3 d in growth medium, and then the subconfluent cultures were washed twice with DMEM that was supplemented with 5 μg/ml apo-transferrin, 1 mg/ml bovine serum albumin, 25 mM HEPES, pH 7.4, 100 μg/ml streptomycin, and 100 units/ml penicillin (serum-free medium). They were serum-starved for 18 h in all of the experiments.

Western blot analysis of tTGase   To investigate the tTGase expression, total cell lysates were prepared with a modified radioimmunoprecipitation buffer (50 mM HEPES, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 0.5% NP-40, 4 mM Na_3VO_4, 200 mM NaF, 1 mM EDTA, 0.1 mM phenylmethanesufonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin). The protein extracts (10-20 μg per lane) were separated by 10% sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis and transferred into a nitrocellulose membrane by using the wet-transferring system (Bio-Rad Laboratories, Hercules, USA). The membrane was blocked and incubated with an anti-mouse tTGase monoclonal antibody (1 : 1000, v/v). Horseradish peroxidase-conjugated anti-mouse IgG was used as a secondary antibody. The protein band of tTGase was then detected by the ECL™ system (Amersham Biosciences, Uppsala, Sweden).

Immunostaining of tTGase   The cells were cultured on round coverslips for 2 d and incubated with 10 μM of RA in a growth medium for 3 d. The cells were then serum-starved for 18 h and treated with agonists. Next, the cells were fixed with 3.7% formaldehyde in Dulbeccos phosphate-buffered saline (0.9 mM $CaCl_2$, 2.7 mM KCl, 1.2 mM KH_2PO_4, 0.49 mM $MgCl_2$, 138 mM NaCl, 8.1 mM Na_2HPO_4) and permeabilized with 0.2% triton X-100 in Dulbeccos phosphate-buffered saline for 30 min at room temperature. After incubating with a blocking solution (20 mM Tris, pH 7.6, 138 mM NaCl, 0.1% triton X-100, 2% bovine serum albumin) for 30 min, the samples were incubated with a monoclonal tTGase antibody in the blocking solution (1 : 100, v/v) for 2 h at room temperature. They were further incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (1 : 200, v/v) for 1 h. The stained samples were mounted on slide glasses with GEL/MOUNT™ (Biomeda Corp., Foster City, USA) and observed by a confocal microscope with 488 nm of Ar laser and 515 nm of a longpass emission filter (Carl Zeiss 410, Jena, Germany).

Cell viability assay   Cell viability was determined by counting the number of attached cells on a culture plate after treating the cells with agonists. The cells of 4×10^4 were plated onto a 48-well culture plate, grown for 2 d, and then incubated with 10 μM of RA for 3 d. After serum-starvation for 18 h, the cells were treated with various agonists for the desired time. After treatment, each well was photographed at least 5 times by a digital camera. The attached cell numbers were counted from three randomly-selected transmitted images. Cell viability (%) was determined and the cell numbers of the treated cells were compared to those of the control.

DNA fragmentation assay   Genomic DNA fragmentation was determined by the modified procedures of Zhou *et al.* (1998), and Lee and Lim (2001). Briefly, the cells were grown in a 100 mm culture dish for 2 d, harvested, and suspended in a lysis buffer (10 mM NaCl, 1 mM EDTA, 1% SDS, and 0.2% Tris-HCl, pH 8.5).

Next, 0.1 mg/ml of proteinase K was added into the cell lysates and the cell lysates were incubated at 60ºC for 2 h. After adding 0.3 M sodium acetate, the cell lysates were placed on ice for 30 min and centrifuged at $20,000 \times g$ for 15 min. The supernatants were transferred into new tubes, precipitated with ethanol without salt, and centrifuged at 14,000 rpm for 30 min. The DNA pellet was washed with 70% ethanol, dissolved in 100 µl of distilled water, and then incubated with 0.2 mg/ml of RNase A at 55ºC for 1 h. To analyze the DNA fragmentation, 10-20 µg of DNA was loaded into 1.5% agarose gel containing ethidium bromide. The DNA band was visualized under UV light.

Results

Induction of tTGase by RA in SH-SY5Y cells In order to investigate the protective role of tTGase in cell death that is induced by TNF-α, the SH-SY5Y cells were initially treated with RA for 3 d, because RA effectively induces the tTGase expression via nuclear receptors in various cell types (Singh *et al.*, 1998; Antonyak *et al.*, 2001; Tucholski *et al.*, 2001). By the incubation with RA, the SH-SY5Y cells were differentiated into neuron-like cells with extended neurites (Fig. 1A). We then studied whether RA-induced differentiation could induce the tTGase expression in the neuroblastoma cells by immunostaining and Western blotting. When the cells were labeled with the monoclonal anti-tTGase antibody and observed by a confocal microscope, the amount of tTGase was largely elevated by the RA treatment, particularly in the cytosolic region (Fig. 1B). This was consistent with a previous report (Lesort *et al.*, 1998). The tTGase expression by RA was also shown by Western blotting analysis using the monoclonal antibody (Fig. 1C). As expected from the confocal microscopic results, a remarked increase of tTGase was observed in the cells that were treated with RA when compared to the control cells. However, the RA treatment caused no significant change in the protein levels that were related to cell proliferation or anti-apoptotic function, such as Bcl-2, Erk1/2, and NF-κB p65. The treatment with RA, therefore, resulted in the tTGase expression with the formation of extended neurites in the SH-SY5Y neuroblastoma cells.

Inhibitory effect of RA treatment on the cell death by TNF-α and C₂-ceramide Next, we investigated the effect of the tTGase expression on the cell death induced by TNF-α. The SH-SY5Y cells were differentiated with RA for 3 d. Cell death was determined by a microscopic observation of the cell shrinkage and detachment from the culture plates. In the undifferentiated control cells, the cells began to shrink and detach from the culture plates at 3 h after incubation with TNF-α. Within 24 h, a large number of cells were detached from the culture plates (Fig. 2A). However, TNF-α had no effect on the cells that were differentiated with RA. Reportedly, TNF-α activates the sphingomyelinase-signaling pathway, leading to a generation of ceramide that induces cell

death through the activation of caspase 3 or inhibition of Akt kinase (Cuvillier *et al.*, 1996; Yoshimura *et al.*, 1998; Zhou *et al.*, 1998). Therefore, we also investigated the effect of cell differentiation on the cell death that is induced by C₂-ceramide, a cell-permeable ceramide analogue. As shown in Fig. 2A, a large number of cells were detached after being shrunk within 24 h by C₂-ceramide in undifferentiated cells, but no cells were affected by C₂-ceramide in the RA-treated cells. Considering the tTGase expression by the RA treatment, these results indicate the possible role of tTGase in the prevention of cell death that is caused by TNF-α and C₂-ceramide.

Cell viability was also investigated by determining the percentage of normal (un-shrunken) cells that was attached on the culture plates after incubation by TNF-α or C₂-ceramide for 24 h. As shown in Fig. 2B, TNF-α and C₂-ceramide significantly decreased the cell viabilities by 47 and 18% of the control level, respectively, in undifferentiated cells. In contrast, the RA treatment enhanced cell viability when compared to the undifferentiated cells. Also, TNF-α and C₂-ceramide had no significant effect on the viability of the cells
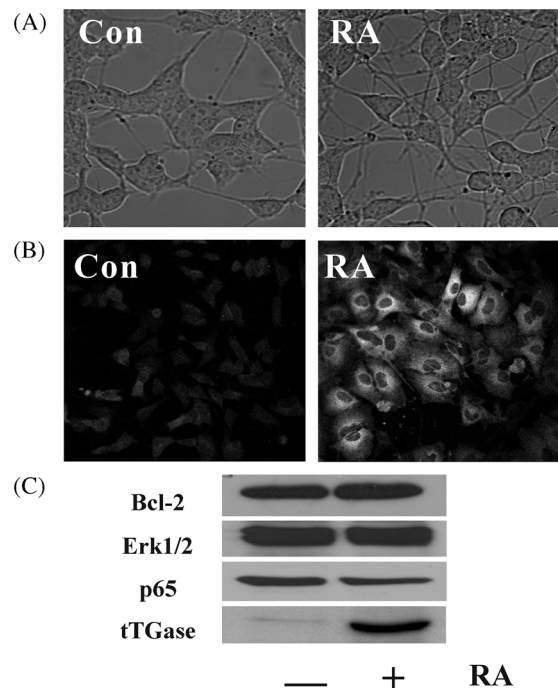

Fig. 1. Changes of cell morphology (A), tTGase expression (B), and protein expression (C) by RA-treatment in SH-SY5Y cells. The cells were treated with vehicle (Con) and 10 µM of RA for 3 d in growth medium. (A) Morphology of neuroblastoma cells incubated with control (Con) or retinoic acid (RA). The bar is 10 mm. (B) The cells were stained with monoclonal tTGase antibody (CUB 7402) and observed by a confocal microscope as described in Materials and Methods. (C) The proteins were extracted from the cells that were treated with vehicle or RA, and separated with 10% SDS-PAGE. Then, Bcl-2, Erk1/2, NF-κB (p65), and tTGase were detected by Western blotting analysis as described in Materials and Methods.
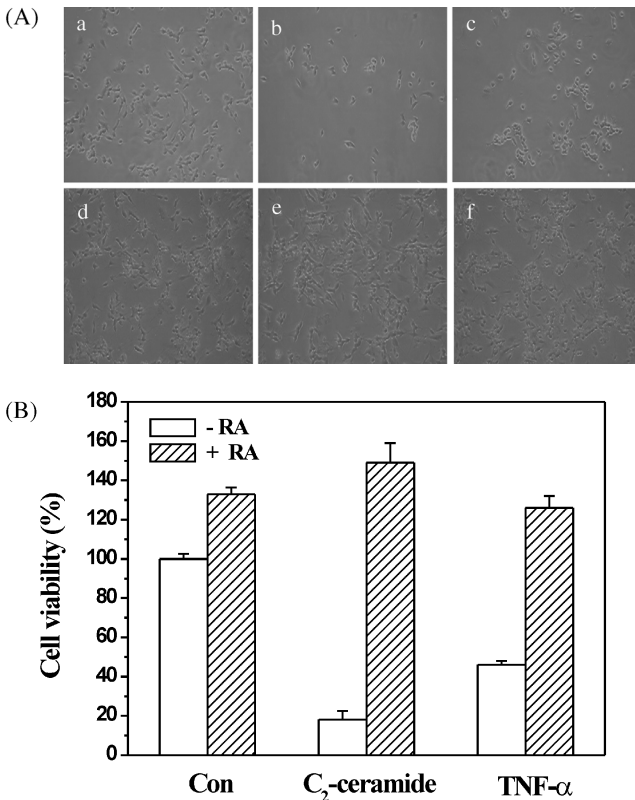
(A)

(B)

Fig. 2. Effect of RA-treatment on the viability of SH-SY5Y cells treated with C_2 ceramide and TNF-α. (A) The cells were incubated with vehicle (a, b, c) or 10 μM RA (d, e, f) for 3 d, then serum-starved for 18 h. The cells were treated with the control (a, d), 20 mM C_2-ceramide (b, e), and 10 ng/ml TNF-α (c, f) for 24 h. The transmitted images were then taken by a digital camera. (B) Three transmitted images were randomly selected from each treatment, and the attached cell numbers were counted as described in Materials and Methods. Cell viability (%) was determined by comparing the cell numbers of the control with those of the treated cells. Data are means ± S.E. from three separate experiments.

that were differentiated with RA. These results suggest that the RA treatment caused cellular resistance against the cell death that is induced by TNF-α and C_2-ceramide in SH-SY5Y cells.

Role of tTGase in protection against cell death by TNF-α or C_2-ceramide Our previous results showed that the RA treatment induced the tTGase expression and protected it from the cell death that is induced by TNF-α and C_2-ceramide. Thus, we investigated whether tTGase was responsible for the protection against cell death by TNF-α and C_2-ceramide in RA-treated cells. The tTGase role was studied by preincubation with tTGase inhibitors, monodansylcadaverine, and cystamine (Ou *et al.*, 2000; Kim *et al.*, 2001). As shown in Fig. 3, monodansylcadaverine and cystamine largely reversed the protection from cell death in response to TNF-α and C_2-ceramide. However, the inhibitors alone had no
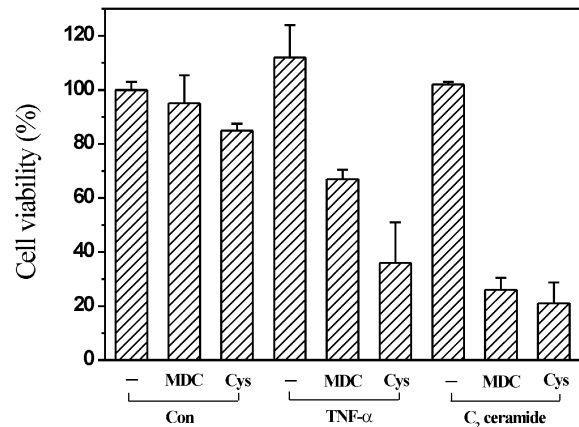

Fig. 3. Effect of tTGase inhibitors on the viability of SH-SY5Y cells. The cells were incubated with 10 mM RA for 3 d and serum-starved for 18 h. The cells were then preincubated with vehicle (−), 20 μM monodansylcadaverine (MDC), or cystamine (Cys), and treated with the control (Con), TNF-α, and C_2-ceramide with for 18 h. Cell viability was determined as described in the legend of Fig. 2. Data are means ± S.E. from three separate experiments.

significant effect on the cell viability. These results suggest that the cell protection that was caused by the RA treatment contributed to the tTGase expression.

To confirm the possible tTGase role in cell protection, the tTGase protein level, cell viability, and DNA fragmentation were investigated after co-treatment of the RA-treated cells with TNF-α and cycloheximide (Fig. 4). Cycloheximide inhibits protein synthesis and induces cell death in many cell types (Setkov *et al.*, 1992; Suzuki *et al.*, 1992; Tang *et al.*, 1999). The tTGase protein level was investigated by Western blotting after incubation with TNF-α and cycloheximide. There was no change in the tTGase protein level until 3 h. However, the tTGase protein level was largely decreased by the co-treatment of TNF-α and cycloheximide for 18 h with no change by TNF-α or cycloheximide alone (Fig. 4A). We then investigated the effect of cycloheximide on the cell viability by TNF-α. The co-treatment of TNF-α and cycloheximide decreased cell viability to about 40% of the control level in the RA-differentiated neuroblastoma cells (Fig. 4B). However, cycloheximide alone had no significant effect on the tTGase level and cell viability (data not shown). In addition, DNA fragmentation was observed within 48 h by the co-treatment of TNF-α and cycloheximide, but not by TNF-α alone (Fig. 4C). These results suggest that the tTGase expression by RA is responsible for the protection from apoptotic cell death that is induced by TNF-α.

Discussion

In this report, we demonstrated the protective role of tTGase in the death signaling that is induced by TNF-α signaling in

(A)

3h

18h

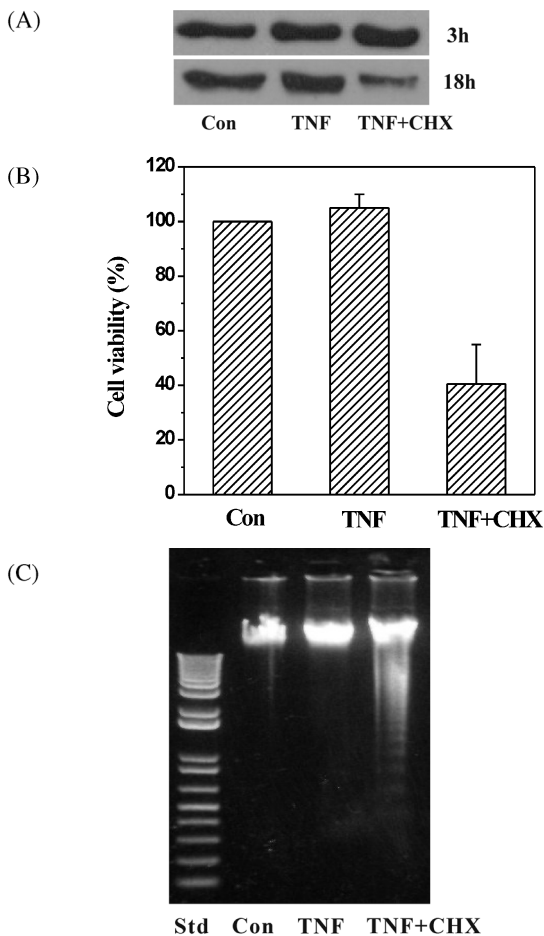Con   TNF   TNF+CHX

(B)

(C)

Std Con TNF TNF+CHX

Fig. 4. Effect of cycloheximide on tTGase expression (A), cell viability (B), and DNA fragmentation (C) by TNF-α in SH-SY5Y cells. The cells were incubated with 10 mM RA for 3 d and serum-starved for 18 h. The cells were then treated with the control (Con), TNF-α alone (TNF), or TNF-α and 10 μg/ml cycloheximide (TNF + CHX) for 18 h. (A) The proteins were extracted at 3 h and 18 h after treatment, and tTGase was detected by a Western blotting analysis. (B) Cell viability assay. Cell viability was determined as described in Materials and Methods. Data are means ± S.E. from three separate experiments. (C) DNA fragmentation assay. DNA was extracted, separated by 1.5% agarose gel containing ethidium bromide, and then visualized under UV light as described in Materials and Methods. Std, DNA 1 kb marker.

SH-SY5Y cells. RA treatment induced the tTGase expression, which was verified by Western blotting and confocal microscopic observation. The protective role of tTGase was shown by three different approaches. First, TNF-α-induced cell death was largely inhibited by RA treatment. Similar results were also obtained by ceramide, which is generated by the sphingomyelin signaling of TNF-α (Cuvillier et al., 1996; Xu et al., 1998). Secondly, the protective role of the RA treatment in the cell death that is induced by TNF-α or ceramide was significantly eliminated by the tTGase inhibitors, monodansylcadaverine and cystamine. In addition,

cell viability was decreased when the tTGase protein level was reduced by co-treatment of TNF-α and cycloheximide.

It was reported that tTGase is an important factor for apoptosis in various cells, including human promonocytic and vascular smooth muscle cells (Melino and Piacentini, 1998; Oliverio et al., 1999; Ou et al., 2000). The U937 promonocytic cells that were treated with RA, which showed an elevated tTGase expression, were susceptible to apoptosis. In addition, cell death significantly decreased by the inhibition of the tTGase expression or the expression of the antisense tTGase gene (Oliverio et al., 1999). The tTGase expression by the RA-treatment also elevated cell death in vascular smooth muscle cells (Ou et al., 2000). Recently, however, there have been reports that tTGase is involved in cell survival rather than cell death (Antonyak et al., 2001; Antonyak et al., 2002; Boehm et al., 2002). The differentiation of the HL60 and NIH 3T3 cells with RA, which increased the activity and expression of tTGase through phosphoinositide 3-kinase, inhibited apoptosis that is caused by N-(4-hydroxyphenyl) retinamide (Antonyak et al., 2001; Antonyak et al., 2002). The inhibition of apoptosis by the RA treatment was also explained by the transamidation of the retinoblastoma protein (Boehm et al., 2002). Our results showed the protective role of tTGase against cell death by TNF-α. Thus, it is likely that tTGase has dual functions, cell survival and death, which might be dependent on the type of cell system or extracellular signal.

TNF-α induced cell death in the RA-treated cells when cycloheximide was co-treated. By the same treatment, the tTGase protein level significantly decreased and DNA fragmentation was clearly observed within 48 h, which is a representative phenomenon of apoptosis. Reportedly, TNF rarely triggers apoptosis unless protein synthesis is blocked (Xu et al., 1998). This suggests that cellular factors (for example NF-κB and AP-1) might suppress the apoptotic stimulus that is generated by TNF (Ashkenazi and Dixit, 1998). We presented here that the RA treatment activated the tTGase expression with no significant changes in the protein levels that are related to the cell proliferation or anti-apoptotic function, such as Bcl-2, Erk1/2, and NF-κB p65. Therefore, the tTGase expression could be a prerequisite condition for protection against cell death. Also, the continuous tTGase expression might confer cellular resistance against death stimuli, such as TNF-α and C_2-ceramide.

What is the possible mechanism by which tTGase protects against cell death that is caused by TNF-α? It is well known that TNF induces cell death by apoptotic signaling, including the TNF-receptor associated death domain, Fas-associated death domain, and caspases (Ashkenazi and Dixit, 1998; Declercq et al., 1998; Kimura et al., 1999). TNF is also known to trigger anti-apoptotic signaling, resulting in the activation of transcription factors, nuclear factor kB, and c-Jun (Chen and Goeddel, 2002). TNF-α also activates the sphingomyelinase signaling pathway, leading to a generation of ceramide that induces cell death through caspase 3 or the

inhibition of the Akt kinase (Cuvillier *et al.*, 1996; Yoshimura *et al.*, 1998; Zhou *et al.*, 1998). It is also reported that TNF-α generated intracellular ROS by a phospholipase A$_2$ cascade (Woo *et al.*, 2000). In this work, we present evidence suggesting the anti-apoptotic role of tTGase in the cell signaling by TNF-α. Thus, it is possible to predict that tTGase inhibited TNF-α-stimulated cell death by two possible mechanisms, the direct inhibition of the apoptotic signaling pathway by tTGase, or the indirect inhibition of the apoptotic pathway through the activation of the anti-apoptotic pathway. However, it is necessary to elucidate the tTGase role(s) in TNF-α signaling.

In summary, tTGase has a very important function to regulate cellular fate, death, or survival, but its exact mechanisms are not yet well understood. In this report, we provided evidence to demonstrate the function of tTGase in cellular protection against death signaling that is induced by TNF-α and ceramide in neuroblastoma cells.

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