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ORIGINAL ARTICLE

TG2 protects neuroblastoma cells against DNA-damage-induced stress, suppresses p53 activation

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Abstract Tissue transglutaminase (TG2) is a multifunctional member of the transglutaminase (TGase) family (E.C.2.3.2.13), which catalyzes in a calcium-dependent reaction the formation of covalent bonds between the γ-carboxamide groups of peptide-bound glutamine residues and various primary amines. Here, we investigated the role of TG2 in a response of the neuroblastoma SH-SY5Y cells to topoisomerase II inhibitor etoposide, known to trigger DNA-damage cell response. We found an early and transient ($\sim$2 h) increase of the TG2 protein in SH-SY5Y cells treated with etoposide, along with the increase of phosphorylated and total levels of the p53 protein. Next, we showed that SH-SY5Y cells, which overexpress wild-type TG2 were significantly protected against etoposide-induced cell death. The TG2 protective effect was associated only with the transamidation active form of TG2, because overexpression the wild-type TG2, but not its transamidation inactive C277S form, resulted in a pronounced suppression of caspase-3 activity as well as p53 phosphorylation during the etoposide-induced stress. In addition, exacerbation of cell death with a significant increase in caspase-3 and p53 activation was observed in SH/anti-TG2 cells, in which expression of the endogenous TG2 protein has been greatly reduced by the antisense cDNA construct. Though the cell signaling and molecular mechanisms of the TG2-driven suppression of the cell death machinery remain to be investigated, our findings strongly suggest that TG2 plays an active role in the response of neuroblastoma cells to DNA-damage-induced stress by exerting a strong protective effect, likely by the suppression of p53 activation and p53-driven cell signaling events.

Keywords TG2 · p53 · Etoposide · Apoptosis · DNA damage · Retinoic acid

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Introduction

TG2 is the most ubiquitously expressed member of the transglutaminase family of proteins (TGases) (Lorand and Graham 2003; Mehta 2005). TG2 is a predominant TGase in cells of the mammalian nervous system (Kim et al. 1999) and in cells of the neuronal lineage, such as neuroblastoma (Piacentini et al. 1992; Liu et al. 2007). Intriguingly, increases in TG2 expression have been described in cells subjected to pathophysiological conditions (for example: Facchiano et al. 2001; Campisi et al. 2003; Ientile et al. 2007; Filiano et al. 2008; Curro et al. 2009), yet a physiological significance of these increases remains unclear. Nevertheless, an emerging consensus suggests that TG2 plays a multifunctional role in the promotion of the cell death and in cell survival (Piacentini et al. 1992; Melino et al. 1994; Piacentini et al. 1996, 2002; Tucholski and Johnson 2002; Liu et al. 2007; Filiano et al. 2008). For example, in primary cerebellar neurons or human neuroblastoma SH-SY5Y cells exposed to oxygen and glucose depravation (OGD), the increase in TG2 protein levels has been shown to play a strong protective role against OGD-induced stress (Filiano et al. 2008). In contrast, in other cell models, or even in the same SH-SY5Y, cells but exposed to other stress conditions, the increased TG2 protein levels lead to the potentiation of apoptotic cell death via the Ca^{2+}-induced and transglutaminase-dependent mechanism (Melino et al. 1994; Facchiano et al. 2001; Tucholski and

Johnson 2002). The TG2 expression has been described to be tightly regulated and increases in various cell types in response to retinoic acid (RA), epidermal growth factor (EGF), tumor necrosis factor α (TNF-α), transforming growth factor β (TGF-β), DNA demethylation (for recent reviews Lesort et al. 2000b; Lorand and Graham 2003; Ientile et al. 2007), or inhibition of histone deacetylation (HDACi) (Buommino et al. 2000; Liu et al. 2007). In a similar tight fashion, Ca^{2+}-induced transamidation activity levels of TG2 have been demonstrated to be developmentally regulated and most likely kept at a dormant state in cells under physiological condition (Achyuthan and Greenberg 1987; Lesort et al. 2000b; Bailey and Johnson 2004). The mechanism that has been proposed to inhibit in situ transamidation TG activity of TG2 involves GTP binding by TG2 (Achyuthan and Greenberg 1987; Iismaa et al. 1997). Through GTP binding and hydrolysis cycling, TG2 has been shown to function as a signal transducing G protein, coupling to cell surface receptors (Nakaoka et al. 1994; Murthy et al. 1999). In addition to TG2, other TGases such as TG3, TG5 and TG4 have also been shown to bind GTP, but a physiological significance of this process for these other TGases is unclear (Mehta 2005). Recently, other enzymatic activities have been postulated to be associated with the TG2 protein, such as protein disulphide isomerase activity (Hasegawa et al. 2003) or kinase activity (Mishra and Murphy 2006), though their physiological importance has yet to be determined.

Human neuroblastoma SH-SY5Y cells (Biedler and Spengler 1976) are a neuroblastic subclone (N-type) of SK-N-SH cells (Ross et al. 1983). Noteworthy, SK-N-SH cells are one of the most commonly used cellular models of neuroblastoma tumors (Ross and Spengler 2007). The attribute of SH-SY5Y cells of undergoing neuronal differentiation in response to treatment with RA (Pahlman et al. 1984) or with RA and BDNF (Encinas et al. 1999), made them extensively used to study differentiation of neuronal crest cells to neuronal phenotype or to study the response of neuronal linage cells to apoptotic as well as prosurvival cell signaling events. For example, SH-SY5Y cells have been used to study the regulation and cellular targets of a p53-dependent pathway in response to irradiation (Kaghad et al. 1997; Tweddle et al. 2001). When irradiated, SH-SY5Y cells undergo cell cycle arrest at the G1 phase (Tweddle et al. 2001) via a cell signaling mechanism that is regulated by their wild-type p53 protein (Kaghad et al. 1997) and p53-dependent transcriptional downstream targets: $p21^{WAF1}$ and MDM2 (Tweddle et al. 2001). Intriguingly, SH-SY5Y treatment with RA leads to a reduction in p53 expression (Sidell and Koeffler 1988; Davidoff et al. 1992) and resistance to p53-dependent apoptosis (Thiele et al. 1985; Ronca et al. 1999; Lavoie et al. 2005). This RA-induced defect in p53 pathway has been proposed to be responsible for a dangerous "differentiation loop" that neuroblastoma tumor cells can use in vivo to survive, while escaping irradiation and chemotherapy (Reynolds et al. 1991; Tweddle et al. 2001). The importance of suppression of p53-dependent cell death during anti-neuroblastoma therapy (Keshelava et al. 2001; Barbieri et al. 2006) has been also underscored by the fact that overwhelming majority of de novo neuroblastoma tumors are wild type at the p53 locus (Maris et al. 2007).

In the light of all these findings, the current study was undertaken to determine the role of TG2 in a possible suppression of p53 activation and p53-dependent apoptosis, such as observed in RA-differentiated SH-SY5Y cells. We found that the TG2 protein levels were upregulated in naive SH-SY5Y cells subjected to the etoposide treatment, along with the increase in phosphorylated and total p53 levels. Second, overexpression of wild-type TG2 resulted in a pronounced suppression of caspase-3, as well as Ser15 phosphorylation of p53. On the other hand, suppression of the endogenous TG2 resulted in a significant exacerbation of caspase-3 activity and p53 phosphorylation. We concluded that even though the cell signaling or molecular mechanisms that may be regulated by TG2 are yet to be described, our findings suggest that TG2 plays an active role in the response of neuroblastoma cells to DNA-damage cell response by suppressing p53 activation and p53-induced apoptotic cell death.

Materials and methods

Cell culture and treatments

In the study, we used naive SH-SY5Y cells, and the following SH-SY5Y cell lines (Tucholski et al. 2001) (Fig. 2): (1) overexpressing wild-type human TG2 (SH/TG2); (2) overexpressing the transamidation inactive TG2 mutant (SH/C277S); (3) stably transfected with the antisense TG2 cDNA construct (SH/anti-TG2); or (4) with the empty vector (SH/pc). The cells were cultured in RPMI 1640 medium supplemented with 20 mM glutamine, 5% Fetal Clone II, and 10% horse serum, and 100 μg/ml G418 (naive SH-SY5Y without G418). In all experiments all cell lines were split at a cell density of 1×10^6 cells per a 60-mm dish 48 h before the treatment and cultured in 1% Fetal Clone II, and 4% horse serum media as described previously (Tucholski and Johnson 2002). At the day of the experiment, cells were transferred to RPMI serum-free media for 2 h and subsequently treated with 30 μM etoposide for an indicated period of time. Cell viability was determined based on morphological criteria described

previously (Xiang et al. 1996). Briefly, the total number of healthy attached cells was determined in the same 60-mm dish at the time 0 of the treatment and after 12 h of the treatment with 30 μM etoposide. Viable cells were judged as fully attached opaque cells with the smooth appearance.

Immunoblotting

The total levels of all studied proteins and the level of phosphorylated forms of p53 were evaluated by preparing cell extracts and quantitative immunoblotting as described previously (Tucholski and Johnson 2002, 2003). Briefly, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in sodium dodecyl sulfate (SDS) lysis buffer (2% SDS, 5 mM EGTA, 5 mM EDTA, 10% glycerol, 0.25 M Tris–HCl, pH 6.8) with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin, leupeptin, pepstatin A) and with phosphatase inhibitors (1 mM sodium orthovanadate, and 1 μM okadaic acid) as described previously (Tucholski and Johnson 2003). Cell lysates were sonicated on ice, clarified, and protein concentration was measured using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). Next, lysates (25 μg) were resolved in 10% SDS-polyacrylamide gels, transferred to nitrocellulose and probed with following antibodies: anti-TG2 (TG100) (NeoMarkers, Fremont, CA, USA), anti-Ser15-phospho p53, anti-Ser20-phospho p53, or anti-p53 (Cell Signaling, Danvers, MA, USA), anti-PARP (BD Biosciences, San Jose, CA, USA), anti-actin (Millipore, Billerica, MA, USA). Next day, after incubating with horseradish peroxidase-conjugated secondary antibodies, immunoblots were developed using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA) and quantified using a Bio Rad imaging densitometer (BioRad, Hercules, CA, USA).

[α-^{32}P] GTP photolabeling

Photoaffinity labeling of TG2 was performed as described previously (Antonyak et al. 2001; Tucholski et al. 2006). Cell lysates (50 μg) were incubated with 10 μCi of [α-^{32}P]GTP (Amersham Biosciences, Piscataway, NJ) in 50 mM Tris–HCl, pH 7.4, 2 mM EGTA, 1 mM dithiothreitol, 20% (v/v) glycerol, 100 mM NaCl, and 500 μM App(NH)p (5′-adenylylimidodiphosphate lithium) (Sigma–Aldrich, St. Louis, MO, USA) for 15 min at room temperature. Next, the samples were irradiated with UV light (254 nm) for 15 min on ice, mixed with 6× Laemmli sample buffer, boiled for 5 min and separated in 10% SDS–polyacrylamide gels. The gels were dried and exposed to a phosphoscreen overnight in the PhosphoImager cassette (Molecular Dynamics, Sunnyvale, CA). The images were acquired using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In vitro transamidation activity assay

Cell extracts were collected at time 0 of the etoposide treatment and in vitro TG activity was measured using a [^{3}H]-putrescine assay according to the previously described protocol (Tucholski et al. 1999). Finally, [^{3}H]-putrescine incorporation was determined by liquid scintillation counting. After measuring protein concentration in used cell extracts, TG activity was calculated as nanomoles of putrescine incorporated per milligram of protein per hour incubation (nmol/mg/h).

Caspase-3 activity assay

Caspase-3 activity was measured using a fluorometric assay as described previously (Tucholski and Johnson 2002). Briefly, after the treatment, the cells were rinsed once with PBS and harvested in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% NP40, 0.1 mM PMSF, and protease inhibitors). The cell lysates (20 μg) were incubated for 1 h at 37°C, in 200 μl of reaction buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM DTT), containing a caspase-3 substrate (Ac-DEVD-AMC) (Alexis Biochemicals, San Diego, CA, USA) at a concentration of 25 ng/μl. Fluorescence was measured using a fluorescence plate reader (Bio-Tek, Winooski, VT, USA). Data are presented as arbitrary caspase-3 units.

Statistics

Data were analyzed using ANOVA, and values were considered significantly different when $P < 0.05$. Results were expressed as mean ± SD.

Results

TG2 is upregulated in response of neuroblastoma cells to treatment with etoposide

To investigate the possible role of TG2 in DNA-damage-induced stress, naive SH-SY5Y cells were treated with an epipodophyllotoxin etoposide, a topoisomerase II inhibitor that is known to produce DNA breaks and induce DNA-damage cell stress (Burden and Osheroff 1998; McClendon and Osheroff 2007). Etoposide is used in anti-neuroblastoma therapy (Matthay et al. 1999), as well as for the treatment of other human malignancies, such as small cell lung cancers, leukemias, lymphomas and germ-line

malignancies (Burden and Osheroff 1998). Cells were cultured in 5% serum media for 2 days, transferred to serum-free media for 2 h and subsequently treated for indicated time with 30 μM etoposide; the concentration that has been previously demonstrated to trigger a rapid induction of p53-dependent apoptosis in SH-SY5Y cells (Rodriguez-Lopez et al. 2001). Levels of TG2, p53, Ser15- and Ser20-posphorylated p53 were determined by western blotting (Fig. 1). The phosphorylated p53 protein, which was undetectable in untreated cells, increased significantly as early as 10 min into the etoposide treatment. Throughout the treatment of SH-SY5Y cells with etoposide, a pronounce accumulation of the p53 protein was observed as previously reported by other researchers (Rodriguez-Lopez et al. 2001; Cui et al. 2002). We also discovered that the TG2 protein, which was barely detectable in unstimulated SH-SY5Y cells, was rapidly and transiently accumulated in the etoposide-treated cells. The significant accumulation of TG2 was apparent as early as 10 min into the treatment, and reached a plateau level between 1 and 2 h into the treatment (Fig. 1). At the next investigated time-point (3 h) of the etoposide treatment, the TG2 level was lower than the plateau level. Subsequently, after 3 h, the TG2 level returned to that of the control or untreated cells (data not shown). The mechanism of increase or subsequent decrease of TG2 remains to be investigated. However, the observed decrease in the TG2 protein coincided with an ongoing proteolysis of the PARP protein, the known caspase-3 substrate. Given that TG2 has been demonstrated to be a caspase-3 substrate (Fabbi et al. 1999), TG2 may be likely proteolized within this later time frame by activated caspase-3 (Tucholski, unpublished observation).

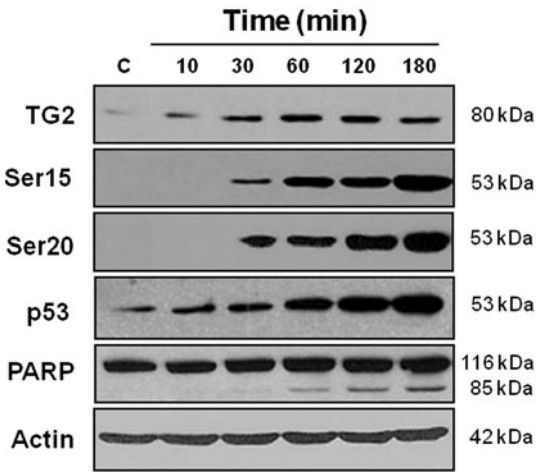

Fig. 1 The TG2 protein is increased transiently in response to the etoposide treatment in SH-SY5Y cells. Naive SH-SY5Y cells were cultured in 5% serum media for 2 days, transferred to serum-free media for 2 h, and subsequently treated for up to 3 h with 30 μM etoposide. The western blotting analysis of collected cell lysates shows an increase of TG2, p53 total and its Ser15- and Ser20-phosphorylated forms, and total and proteolized PARP protein, and actin as loading control for each sample

Increase in TG2 expression is protective, whereas suppression of TG2 expression exacerbates etoposide-induced cell death

Given the findings that the TG2 level was increased in naive SH-SY5Y cells in response to the etoposide treatment, we investigated the consequences of changes in TG2 expression on the etoposide-induced cell death. In the study, we used SH-SY5Y cell lines generated in our laboratory (Fig. 2) (Tucholski et al. 1999, 2001), which have been used extensively in studies of the TG2's role in the biology of SH-SY5Y cells, particularly during neuronal differentiation (Tucholski et al. 2001; Singh et al. 2003; Tucholski and Johnson 2003; Joshi et al. 2006) or in response to stress (Lesort et al. 2000a; Tucholski and Johnson 2002; Robitaille et al. 2004; Filiano et al. 2008).

The same amount (25 μg) of collected cell lysates from SH/TG2 and SH/C277S or SH/anti-TG2 were separated and western blotted along with the lysate of control SH/pc cells (Fig. 1a, a top panel). As demonstrated in Fig. 1a, SH-SY5Y cell lines express the TG2 protein at dramatically different level. SH/TG2 cells constitutively express significantly elevated level of the wild-type TG2, whereas SH/C277S cells express at the similar level the TG2 mutant without its transamidation activity (C277S) (Lee et al. 1993; Tucholski et al. 1999) (a short exposure blot, ~5 s). In SH/anti-TG2 cells, the endogenous TG2 protein expression has been significantly decreased with the antisense cDNA TG2 construct (anti-TG2) (Tucholski et al. 2001) (a long exposure blot, ~15 min). The control SH/pc cells, transfected the empty vector, expressed TG2 comparable level to that in naive SH-SY5Y (Tucholski et al. 1999, 2001). Next, we showed that overexpressed wild-type TG2 and C277S-TG2 proteins bind GTP by using the [α-^{32}P]GTP photolabeling assay (Fig. 1a, a bottom panel). Unfortunately, we were unable to detect any GTP-bound TG2 protein in either SH/pc or SH/anti-TG2, likely, given rather low levels of TG2 protein in both cell lines. In the next step, we confirmed that TG2 protein expressed in SH/TG2 cells was an active TGase enzyme by measuring in vitro transamidation activity in cell lysates as described previously (Tucholski and Johnson 2002). As indicated in Fig. 2b, only in SH/TG2 cells there was a significant increase in in vitro transamidation activity when compared to SH/pc. In contrast, we were unable to detect any significant changes, namely, a decrease in transglutaminase activity in SH/anti-TG2 when compared to control SH/pc cells, most likely given the already very low transglutaminase activity in control cells (Fig. 2b).

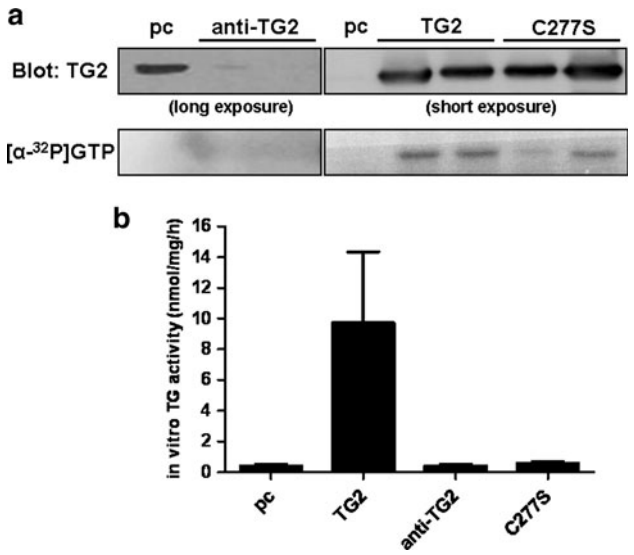

Fig. 2 TG2 expression and its enzymatic activities in different SH-SY5Y cell lines. Human neuroblastoma SH-SY5Y cell lines used in the study that express either wild-type human TG2 (TG2) or TG2 without transamidation activity (C277S), or stably transfected with empty pcDNA vector (*pc*) or anti-sense cDNA TG2 construct (anti-TG2). **a** *Top panel* the representative TG2 immunoblot showing the TG2 protein level (ran in duplicates) in pc, anti-TG2 cells at higher exposure time and the TG2 protein level in pc, TG2 and C277S cells at lower exposure time. The TG2 immunoreactivity was barely detectable in anti-TG2 cells. *Bottom panel* representative autoradiograph showing photolabeling of the TG2 protein by [α-^{32}P]GTP in pc, anti-TG2, TG2 and C277S cells. Cells lysates (50 μg) were photolabeled with [α-^{32}P]GTP prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Only TG2 and C277S cells show photolabeling of TG2 protein with [α-^{32}P]GTP. **b** A representative graph of an in vitro TG activity in pc, TG2, anti-TG2 and C277S cells, cultured in regular RPMI media. Cell lysates from all cells were used in an in vitro putrescine incorporation assay with labeled [^{3}H]-putrescine and dimethylcasein as a putrescine donor. In vitro TG activity was calculated is presented as nanomoles of putrescine incorporated per milligram of protein per hour (nmol/mg/h) (mean ± SD)

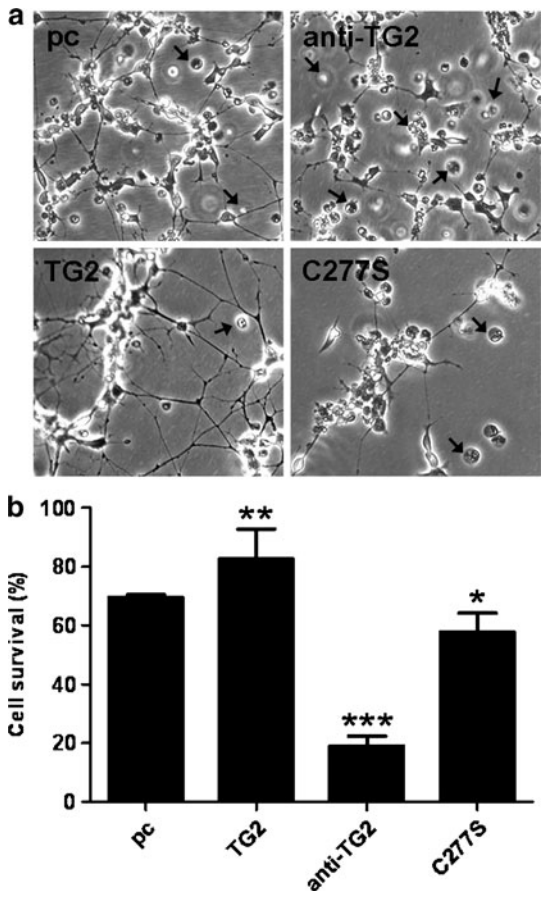

Fig. 3 Contrasting changes in cell morphology of the different cell lines in response to the etoposide treatment. **a** Representative phase-contrast photomicrographs showing changes in morphology of the different cell lines after 12 h treatment with etoposide (30 μM). The treatment of TG2 cells with etoposide resulted in a predominantly differentiated cell morphology with extensive network of neurites. In contrast, the same treatment of anti-TG2 cells produced a significant amount of detached and floating attached cells. SH/C277S cells represented mixture of "differentiated" and detached floating cells. **b** Cell survival was quantitatively assessed by counting healthy cells in the same field at time 0 and 12 h after with 30 μM etoposide. A quantification of number of viable attached cells after 12 h treatment. A percentage of attached viable cells was significantly different when compared to control pc cell (*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$). The most striking effect was demonstrated for etoposide-treated SH/anti-TG2 cells

In the next step, we studied the response of all above cell lines to the etoposide treatment. Cells were cultured as previously, and treated for 12 h with 30 μM etoposide. In response to this treatment, SH/TG2 cells differentiated to a neuronal-like morphology with almost no floating cells (Fig. 3a). On the other hand, other cell lines responded to the etoposide treatment by producing neurites, but also a significant amount of detached and floating cells (Fig. 3), what was most dramatically visible for SH/anti-TG2 cells (Fig 3b). This initial result suggested a possible suppression of a cell death process in SH/TG2 cells. Therefore, in the next step, the extent of apoptotic cell death was measured by determining the active caspase-3 levels after 2 and 4 h of etoposide treatment using an ELISA assay (Fig. 4). The results demonstrate that the levels of caspase-3 activity were increased in all the investigated cells only after 4 h treatment. However, the magnitude of this increase was dramatically smaller in SH/TG2 cells than in the other cells. The most dramatic difference was seen when levels of active caspase-3 were compared between SH/TG2 cells and SH/anti-TG2 cells: while caspase-3 activity levels were significantly suppressed in the first cell line, in SH/anti-TG2 cells this activity was greatly potentiated (Fig. 4). Last, overexpression of C277S-TG2 did not have any protective effect on SH-SY5Y cells against the etoposide treatment (Figs. 3, 4).

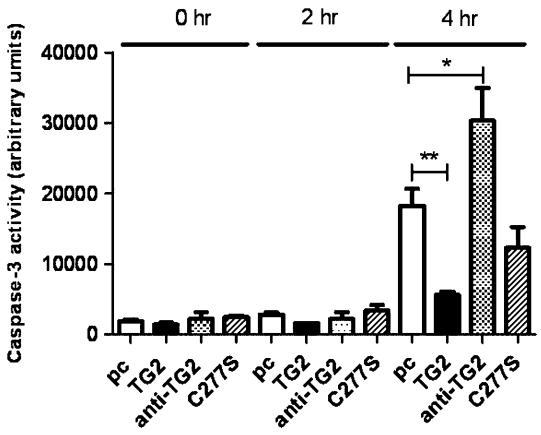

Fig. 4 Caspase-3 activation is attenuated in cells overexpressing wild-type TG2, and potentiated in cells with depleted TG2 expression. Caspase-3 was measured in collected cell lysates from pc, TG2, anti-TG2 and C277S cells after 2 or 4 h treatment with 30 μM etoposide in serum-free media. The level of caspase-3 activity was increased in all the investigated cells only after 4 h treatment. TG2 cells exhibited a significant decrease in caspase-3 activity ($n = 3$; $^{**}P < 0.001$), while anti-TG2 cells exhibited significant increase in caspase-3 activity levels ($n = 3$; $^{*}P < 0.05$) compared with SH/pc cells

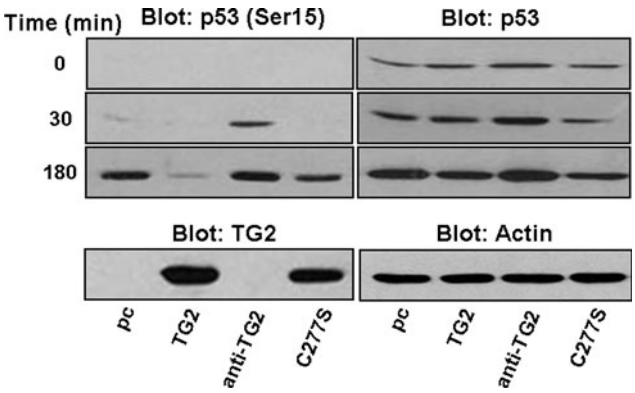

Fig. 5 TG2 expression affects the time-dependent changes in p53 phosphorylation. Representative immunoblots of total and Ser15 phosphorylated p53 levels in pc, TG2, anti-TG2, and C277S cells. All cells were cultured in 5% serum media for 2 days, transferred to serum-free media for 2 h, and subsequently treated up to 3 h with etoposide (30 μM). In TG2 cells, phosphorylation and less accumulation of p53 were significantly delayed compared to the other cells. In contrast, in anti-TG2 cells phosphorylation of p53 was significantly accelerated. *Bottom panel* shows TG2 or actin levels in cell sample from all cell lines subjected to etoposide treatment for 180 min

Increased TG2 level leads to significant suppression of p53 phosphorylation during etoposide treatment

Given the central role of p53 in etoposide-induced DNA-damage response (Rodriguez-Lopez et al. 2001; Cui et al. 2002), we investigated whether differences in activation of apoptotic cell death in different SH-SY5Y cell lines were dependent upon differential activation of p53. Specifically, we investigated phosphorylation of p53 on Ser15 as indicative of activation of DNA-damage response during etoposide treatment. Cell lysates were prepared from all four cell lines following the treatment with 30 μM etoposide for indicated time and the total and Ser15-phosphorylated levels of the p53 protein were determined by western blotting analysis. As in the previous experiment (Fig. 4), the most striking differences were discovered between SH/TG2 and SH/anti-TG2 cells (Fig. 5). In SH/TG2 cells, phosphorylation on Ser15 of the p53 protein was significantly delayed compared to the other cell lines. In contrast, this process was significantly exacerbated in SH/anti-TG2 cells (Fig. 5). Also, as in previous experiment, overexpression of the transamidation inactive TG2 (C277S) protein did not have any effect on levels of Ser15-phospho p53 (Fig. 5).

Discussion

In the current study, we described a new protective role of the TG2 protein in response of human neuroblastoma SH-SY5Y cells to the treatment with etoposide, a chemotherapeutic agent that inhibits topoisomerase II and by this mechanism induces the DNA-damage stress response of neuroblastoma cells. First, we found that TG2 was upregulated in naive SH-SY5Y cells early during etoposide treatment, along with total and phosphorylated levels of p53. Next, we determined the role of TG2 in the etoposide-induced cell death process by measuring the levels of active caspase-3 and the levels of total and phosphorylated form of p53 in different SH-SY5Y cell lines. We discovered that overexpression of the wild-type TG2, but not its transamidation inactive C277S mutant, resulted in a pronounced suppression of etoposide-induced cell death, illustrated by significantly lower levels of active caspase-3 and the phosphorylated form of p53, compared to the other cell lines. The opposite effect, exacerbation of etoposide-induced cell death with a significantly increased caspase-3 and p53 activation was observed in SH/anti-TG2 cells, which have been engineered to express endogenous TG2 protein at almost undetectable level (Tucholski et al. 2001). Even though cell signaling and molecular mechanisms of TG2-driven suppression of etoposide-induced cell death remain to be investigated, our findings suggest that TG2 may play an active role in etoposide-induced response of neuroblastoma cells by exerting a protective effect through suppression of p53 activation and the p53-driven signaling events. We also believe that this study sheds light on possible clinical consequences of a chronic TG2 increase in neuroblastoma tumor cells, given a central role of p53 in how neuroblastoma and other tumor cells respond to anti-tumor therapy. Although, TG2 protein levels have been

already described to increase in response to the DNA-damage related stress, we described here such an increase for the first time in cells of a neuronal lineage. Previously, for example, a TG2 level has been shown to increase dramatically in thymocytes in response in vivo γ-irradiation (Szondy et al. 1997, 2003), the process accompanied by rapid p53-dependent apoptotic-like cell death triggered by DNA damage (Clarke et al. 1993; Lowe et al. 1993). Interestingly, TG2 protein accumulation has been also observed in a number of immortalized cancer cells, such as MCF-7 human breast (Mehta 1994) or PC14 lung cancer (Han and Park 1999) or HeLa cervical cancer cells (Jeong et al. 2009) when treated with doxorubicin, another topoisomerase II inhibitor (Gewirtz 1999). These findings along with our finding suggest that TG2 may be potentially an important player in response of some cancer cells to DNA-damage-induced stress. The precise mechanism of the TG2 upregulation in current and the previous studies is unknown, although it likely may be triggered, as suggested previously (Szondy et al. 1997, 2003), through activation of positive transcriptional elements such as responsive to retinoid binding (Nagy et al. 1996), or alternatively, suppression of negative regulatory elements, such as DNA methylation (Bastien and Rochette-Egly 2004; Ai et al. 2008) or histone deacetylation (Liu et al. 2007). Interestingly, such an intricate transcriptional regulation of TG2 levels has been recently described in the Liu et al. studies where differentiation of neuroblastoma cells in the HDACi treated cells was caused by depression of recruitment of N-Myc and to the HDAC1 protein to the Sp-1 binding site in the TG2 core promoter (Liu et al. 2007).

Following DNA damage, cells activate distinctive signaling networks that mediate cell cycle checkpoints, DNA repair, and apoptosis (Sancar et al. 2004). The master regulator of the DNA-damage response is the protein kinase ATM, which in turn phosphorylates p53, and other proteins: H2AX, MDC1, Rad50, Nbs1, Chk2, and Mdm2 (Sancar et al. 2004). However, p53 is the main target of ATM, which contributes to two major cellular responses following DNA damage: cell cycle arrest and apoptosis (Prives and Hall 1999; Vousden 2000).

Neuroblastoma tumors are capable of undergoing p53-dependent apoptotic cell death in response to DNA-damage-inducing stress (Keshelava et al. 2001; Barbieri et al. 2006), given the fact that the overwhelming majority of de novo neuroblastoma tumors are wild type at the p53 locus (Maris et al. 2007). It is believed that DNA damaging drug-induced apoptosis in SH-SY5Y cells is regulated by wild-type p53 and activated p53-dependent signaling pathway (Rodriguez-Lopez et al. 2001; Cui et al. 2002). Thus, phosphorylation of p53 on Ser15 and Ser20 are clearly indicative of activation of DNA-damage response during etoposide treatment of SH-SY5Y cells. Modulation of DNA-damage response has been suggested as a possible therapeutic target in anti-neuroblastoma treatment. For example, inhibition of Mdm2, a critical p53 ubiquitin ligase, sensitizes neuroblastoma and other cancer cells to etoposide treatment and other DNA-damage-induced chemotherapeutic agents through the process of activation of p53-dependent apoptotic pathway (Barbieri et al. 2006). Moreover, RA-treated SH-SY5Y cells have been characterized by a reduction in p53 expression (Sidell and Koeffler 1988; Davidoff et al. 1992) and resistance to p53-dependent apoptosis (Thiele et al. 1985; Ronca et al. 1999; Lavoie et al. 2005). Thus, given that TG2 is upregulated by RA (Tucholski et al. 2001) and is an essential element of RA-induced differentiation of SH-SY5Y cells, TG2 may be potentially involved in RA-induced reduction in p53 expression (Sidell and Koeffler 1988; Davidoff et al. 1992) or in resistance of SH-SY5Y cells to p53-dependent apoptosis (Thiele et al. 1985; Ronca et al. 1999). This possibility awaits further extensive investigation in future studies.

Is it possible that levels and status of p53 and TG2 proteins together predetermine neuroblastoma cell response to stress? Such a conclusion, we believe, can be drawn from the previous and our current studies. For example, in human neuroblastoma SK-N-BE(2) cells, in which the p53 protein is mutated and p53-dependent pathway is inactivated (Kaghad et al. 1997), the overexpression wild-type human TG2 has been shown to result in a drastic reduction in cell proliferation capacity, an induction of spontaneous apoptosis and in their higher susceptibility to apoptotic stimuli (Melino et al. 1994). On the other hand, overexpression of the same protein in SH-SY5Y cells, which have the active wild-type endogenous p53 protein and p53-dependent pathway, led to spontaneous differentiation of those cells and did not produce any changes in a rate of spontaneous apoptosis (Tucholski et al. 2001). The findings of the current study coincide with data presented by Datta et al. (2006), who has found that overexpression of wild-type TG2, but not transamidation defective mutant, led to a significant protection of breast cancer SKBR3 cells against doxorubicin treatment. However, our findings are in a contrast to findings by Mishra et al. (Mishra and Murphy 2006), who suggested that TG2 acts as a Ser/Thr kinase that can phosphorylate p53 protein at Ser15 residue (an ATM phosphorylation site, Prives and Hall 1999; Vousden 2000), as well as at Ser20 residue (a check point kinase Chk2 phosphorylation site, Sancar et al. 2004). This striking difference might be associated with a completely different experimental setting of both studies: all data reported by Mishra et al. are from the in vitro experiments, either with guinea pig liver TG2 or recombinant human TG2. On the other hand, our data were collected from cells treated in culture with the agent that induces p53

activation. Nonetheless, Mishra's and our study strongly point at p53 protein as a possible target regulation by TG2. There are a number of provisional targets or mechanisms of how TG2 can affect p53 activation that protects neuroblastoma cells against DNA-damage-induced stress. Some of the mechanisms have been suggested in the past, such as regulation of DNA-repair enzymes (Han and Park 1999) or a delay of cell cycle progression/inhibition of cell proliferation (Mian et al. 1995). Another hypothetical mechanism that has been suggested is regulation of Mdm2-dependent p53 ubiquitination and proteosomal degradation by TG2 (Mishra and Murphy 2006). Intriguingly, a TG2-mediated regulation of the ubiquitin–protesome degradation system has been demonstrated in the of spinobulbar muscular atrophy (SBMA) (Mandrusiak et al. 2003).

In summary, the current study demonstrates for the first time that TG2 protein is increased in human neuroblastoma SH-SY5Y cells treated with topoisomerase II inhibitor etoposide. Next, we showed that the chronic increase in wild-type TG2 protein results in a significant protection of SH-SY5Y cells against etoposide treatment, likely by suppression of the phosphorylation of p53 and of p53-induced cell death. Given the critical role of both p53 and TG2 proteins in regulation in cell stress response, we believe that further studies are urgently warranted to fully understand cell signaling and molecular mechanisms that may culminate in a TG2-driven protection against DNA-damage-induced stress.

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