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ORIGINAL ARTICLE

Depletion of cathepsin D by transglutaminase 2 through protein cross-linking promotes cell survival

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Abstract Transglutaminase 2 (TGase 2) promotes nuclear factor-κB (NF-κB) activity through depletion of the inhibitory subunit of NF-κB (I-κBα) via protein cross-linking, leading to resolution of inflammation. Increased expression of TGase 2 contributes to inflammatory disease pathogenesis via constitutive NF-κB activation. Conversely, TGase 2 inhibition often reverses inflammation in animal models. The role of TGase 2 in apoptosis remains less clear, as both pro- and anti-apoptotic functions of TGase 2 have been demonstrated under different experimental conditions. Apoptosis is intact in a TGase 2 knock out mouse (TGase2$^{-/-}$), which is phenotypically normal. However, upon exposure to tumor necrosis factor (TNF)-α-induced apoptotic stress, mouse embryonic fibroblasts (MEFs) from TGase2$^{-/-}$ mice were more sensitive to cell death than MEFs from wild-type (TGase 2$^{+/+}$) mice. In the current study, to explore the role of TGase 2 in apoptosis, TGase 2-binding proteins were identified by LC/MS. TGase 2 was found to associate with cathepsin D (CTSD). Binding of TGase 2 to CTSD resulted in the depletion of CTSD via cross-linking in vitro as well as in MEFs, leading to decreased levels of apoptosis. Furthermore, cytoplasmic CTSD levels were higher in MEFs from TGase 2$^{-/-}$ mice than in those from TGase 2$^{+/+}$ mice, as were caspase 3 activation and poly (ADP-ribose) polymerase (PARP) processes. These results suggest that TGase 2, while not previously implicated as a major regulatory factor in apoptosis, may regulate the balance between cell survival and cell death through the modulation of CTSD levels.

Keywords Transglutaminase 2 · Apoptosis · Cathepsin D

Introduction

Transglutaminase 2 (TGase 2; E.C. 2.3.2.13, protein-glutamine γ-glutamyltransferase) belongs to a family of Ca^{2+}-dependent enzymes that catalyze N^{ε}-(γ-L-glutamyl)-L-lysine isopeptide bond formation between peptide-bound lysine and glutamine residues (Folk 1980).

Over the past few decades, TGase 2 has been shown to be required for such processes as tissue regeneration, wound healing, matrix formation, and apoptosis (Kim 2006). However, the role of TGase 2 in normal developmental and physiological processes is the subject of debate, particularly since animal models of TGase 2 gene ablation appear to have no phenotypic abnormalities (Iismaa et al. 2009).

TGase 2 has recently been shown to activate nuclear factor-κB (NF-κB) through the depletion of the inhibitory subunit of NF-κB (I-κBα) via protein cross-linking, which extends the period of NF-κB activity during inflammation (Lee et al. 2004; Kim et al. 2006; Park et al. 2006). NF-κB activity was dramatically increased by TGase 2 expression during inflammation, forming the basis for the proposed 'ouroboros theory' (Park et al. 2011; reviewed in Kim 2011), in which the protein modification of the suppressor

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I-κBα by TGase 2 and its subsequent depletion results in the amplification of NF-κB signaling pathways during inflammation.

TGase 2 is induced during apoptosis and is associated with apoptotic processes in numerous experimental models (Fesus et al. 1987; Piacentini et al. 1991; Facchiano et al. 2001; Mastroberardino et al. 2002; Ruan et al. 2007). On the other hand, increased TGase 2 expression has been shown to correlate with cell survival under conditions of apoptotic stress (Jang et al. 2010; Boehm et al. 2002; Kim et al. 2006, 2009; Yamaguchi and Wang 2006; Park et al. 2009). There is also evidence that the inhibition of TGase2 expression prevents efficient phagocytosis of apoptotic bodies (Rossin et al. 2011; Szondy et al. 2003), which suggests that rather than having a direct role in apoptosis itself, TGase 2 may be involved in the clearing of dead cells and cellular debris (Szondy et al. 2003). Thus, TGase 2 exhibits both anti- and pro-apoptotic activity under different experimental conditions, and the precise role of TGase 2 in apoptosis has yet to be fully characterized.

Mouse embryonic fibroblasts (MEFs) that lack TGase 2 (TGase $2^{-/-}$) were more sensitive than TGase $2^{+/+}$ MEFs to apoptosis induced by tumor necrosis factor (TNF)-α and cycloheximide (CHX). One possibility is that, under these conditions, activation of NF-κB by TGase 2 results in the increased expression of pro-survival factors that inhibit cell death, such as cellular inhibitor of apoptosis (cIAP) (Gyrd-Hansen and Meier 2010; Lee et al. 2004). Alternatively, TGase 2 may catalyze protein crosslinking and the depletion of pro-apoptotic molecules (i.e., caspase 3) (Jang et al. 2010; Yamaguchi and Wang 2006). In the current study, to gain a better understanding of the pro-survival functions of TGase 2, we identified TGase 2-binding proteins using LC/MS techniques.

Materials and methods

Reagents

The anti-TGase 2 antibody was raised from rabbit using a C-terminal fragment of human TGase 2 (427-690) as the antigen. The anti-cathepsin D antibody was purchased from Santa Cruz (Santa Cruz, CA, USA), and the anti-β-actin antibody was purchased from Abcam (Headquarters, Cambridge, UK). Cathepsin D (CTSD) was purchased from Sigma (10 ng, 250 U/mg protein, Sigma).

Preparation of immortalized MEFs

MEFs were prepared from individual embryonic day 13.5 (E13.5) embryos using standard protocols. After removal of the head and internal organs, the remaining tissue was washed in phosphate-buffered saline (PBS) twice, and then incubated with 100 µl of typsin/EDTA for 30 min. The tissue was dissociated to near-homogeneity in complete medium and transferred to a 150 mm dish. MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 0.1 mM beta-mercaptoethanol and passaged every 2–3 days. To obtain native immortalized MEFs, primary MEFs were maintained over 10 passages. All experiments were performed with immortalized MEFs after 10 passages. MEF genotypes were confirmed by PCR using genomic DNA from the embryonic yolk sac and from the MEFs.

Analysis of apoptosis induced by TNF-α and CHX by fluorescence-activated cell sorting (FACS)

TGase $2^{+/+}$ and TGase $2^{-/-}$ MEFs were seeded at a density of 1×10^6 cells/ml and exposed to CHX (10 µg/ml) alone or in combination with TNF-α (25 ng/ml) for 1 and 5 h. Cells were collected after the indicated time point, washed with PBS, and then fixed with 100% ethanol at 4°C for 24 h. Fixed cells were washed three times and then incubated for 30 min with a solution of propidium iodide (PI) (Sigma-Aldrich, St Louis, MO, USA) containing RNAase A (Sigma-Aldrich). The cells were then subjected to cell cycle analysis by flow cytometry to determine DNA content using a FacsCalibur system (BD Biosciences, Mountain View, CA, USA). The results were presented as the percentage of cells with sub-G1 DNA content (referred to as percent hyperploidy), which reflects apoptotic cells with fragmented genomic DNA. Analysis of annexin V binding was carried out using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences), according to the manufacturer's instructions. Briefly, cells were collected, washed twice with cold PBS, and then subjected to centrifugation at 1,500 rpm for 5 min. The cell pellet was resuspended in 1× binding buffer at a concentration of 1×10^6 cells per ml and then 100 µl of the cell suspension was transferred to a 5 ml culture tube, to which 5 µl of annexin V-FITC and 5 µl of PI were added. The cells were gently vortexed and then incubated for 15 min at room temperature in the dark. Finally, 400 µl of 1× binding buffer was added to each tube and the samples were analyzed by flow cytometry. For each sample, 10,000 ungated events were acquired; PI(−)/annexin(+) cells were taken as the early apoptotic population.

Cell fractionation and isolation of cytoplasmic fractions

To facilitate the identification of TGase 2-binding proteins, the MDA-MB-231 breast cancer-derived cell line was used because the cells express a higher level of TGase 2 than MEFs. Cytoplasmic fractions of MDA-MB-231 cells were

prepared using a CelLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich). Briefly, cells were harvested and resuspended in cellular lysis buffer [which included dithiolthreitol (DTT) and protease inhibitors] and maintained on ice for 15 min. After incubation on ice, a 10% IGEPAL CA-630 solution (30 U per 500 µl of lysate) was added to a final concentration of 0.6% (6 µl per 100 µl of cell lysate). The samples were vortexed vigorously for 10 s followed by centrifugation at 11,000 rpm for 30 s. The resulting supernatant was recovered as the cytosolic fraction Fig. 1.

Preparation of immobilized TGase 2 and purification of TGase 2-binding proteins

To purify TGase 2-binding proteins, affinity chromatography was carried out using UltraLink Biosupport Medium (UBM). Purified human recombinant TGase 2 (2.0 mg) was conjugated to azlactone-containing UBM beads (20 mg) via amide bonding, according to the manufacturer's instruction (Pierce; Rockford, IL, USA). TGase 2-conjugated UBM beads were incubated with cytosolic

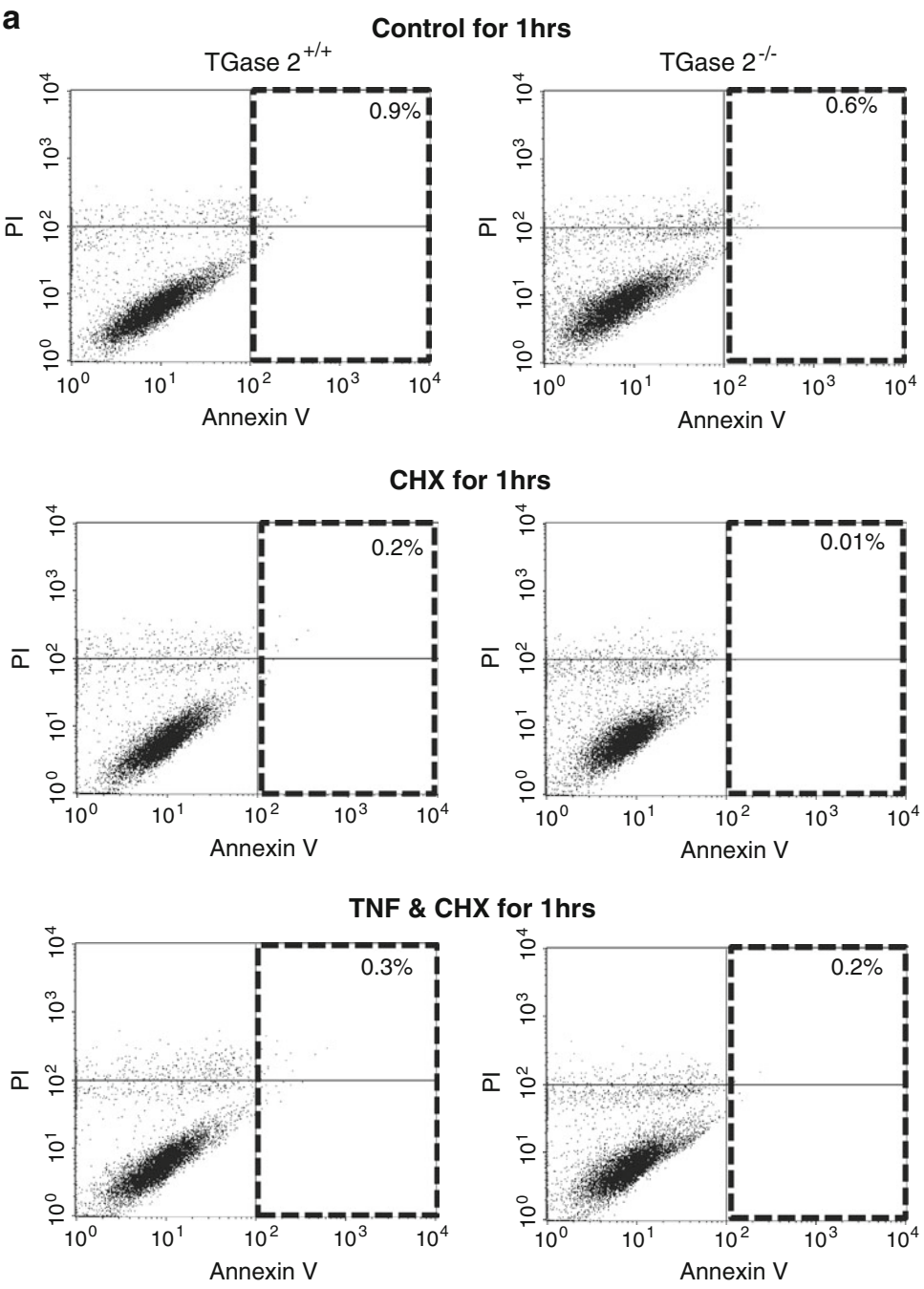

Fig. 1 MEFs from TGase2$^{-/-}$ mice are more susceptible to TNF-α–induced apoptosis. TGase 2$^{+/+}$ and TGase2$^{-/-}$ MEFs were seeded and exposed to CHX (10 µg/ml) alone or in combination with TNF-α (25 ng/ml) for 1 h (**a**) and 5 h (**b**). After treatment, cells were co-stained with Annexin V-FITC and PI and then analyzed by flow cytometry. The number of cells in early apoptosis was increased in TGase2$^{-/-}$ MEFs after treatment with TNF-α and CHX for 5 h. **a** Annexin-V expression in TGase 2$^{+/+}$ and TGase2$^{-/-}$ MEFs after 1 h, showing no difference in apoptosis. **b** After treatment for 5 h, 66.6% of TGase2$^{-/-}$ MEFs were in early apoptosis, versus 25.3% of TGase 2$^{+/+}$ MEFs

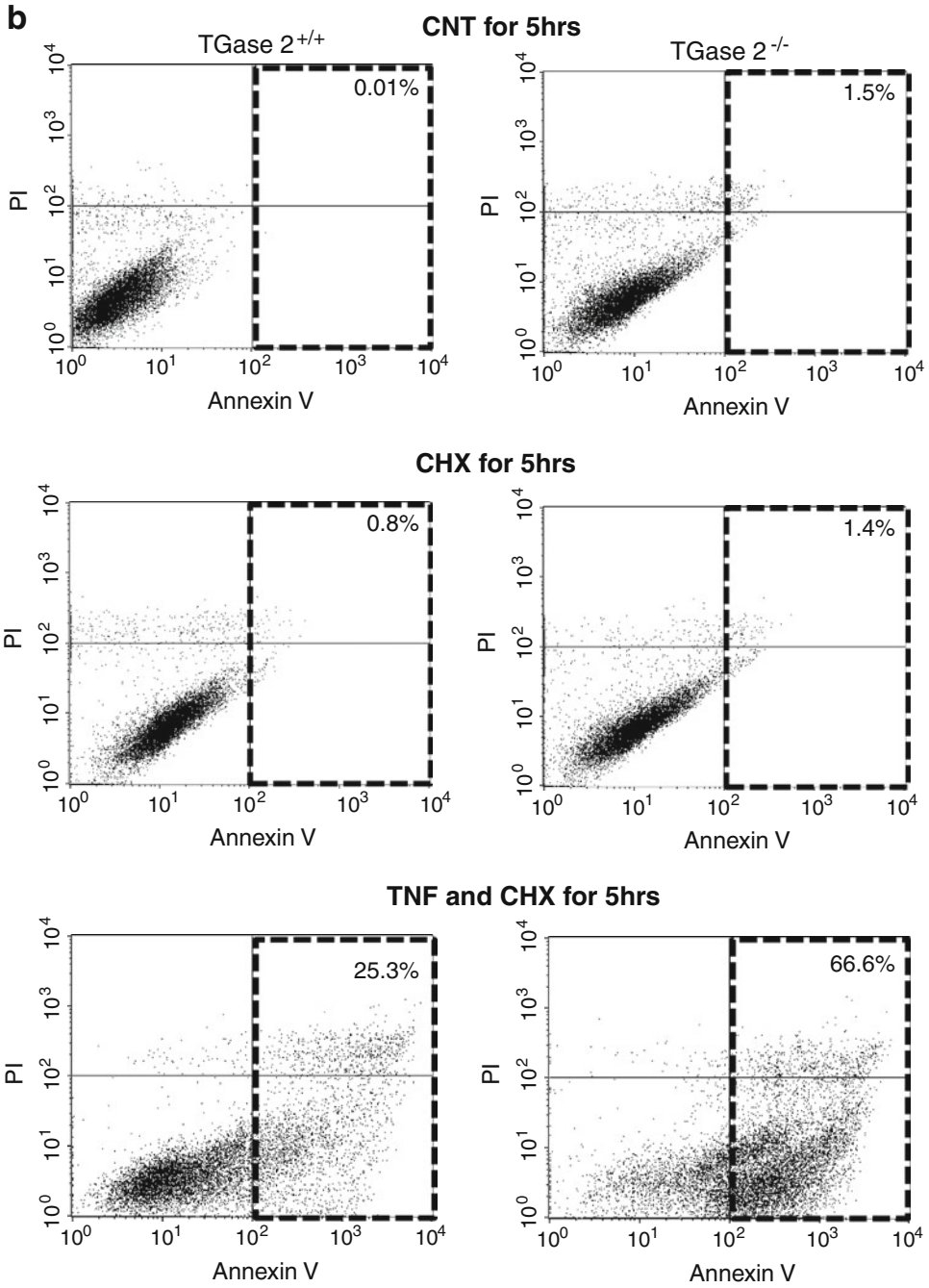

Fig. 1 continued

fractions prepared from MDM-MB-231 cells at room temperature for 1 h. The incubated mixture was centrifuged at 3,000×*g* for 5 min at 4°C and then the beads were washed three times with PBS. Proteins bound to the TGase 2-conjugated UBM beads were eluted using IgG elution buffer (Pierce) and concentrated using a centrifugal filter device (Ultracel YM-3; Millipore, Billerica, MA, USA). Concentrated binding proteins were separated by 4–20% NuPage gel electrophoresis (Invitrogen, Carlsbad, CA, USA) and visualized by Coomassie staining. Gel fragments were then subjected to LC/MS analysis (Fig. 2a).

LC-MS/MS and database search

Following SDS-PAGE, protein bands of interest were excised from the gel. Gel pieces were destained with 50% acetonitrile in 0.1 M ammonium bicarbonate and then dried in a SpeedVac evaporator. Dried gel pieces were

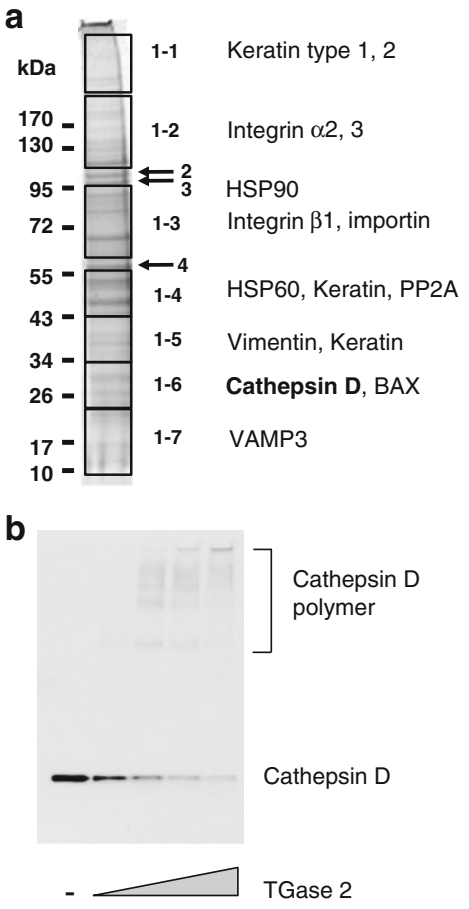

Fig. 2 Identification of TGase 2-binding proteins by LS/MS. TGase 2-binding proteins were isolated from cytoplasmic fractions of MDA-MB-231 cells and then subjected to LC/MS. **a** Purified binding proteins were separated by SDS-PAGE. Following in-gel digestion, proteins were analyzed by LC/MS (see Supplementary Table 1 for identified protein masses). Major TGase 2-binding proteins are indicated. **b** TGase 2 depleted CTSD via protein cross-linking in a dose-dependent manner in vitro

re-swollen with 30 µl of 25 mM sodium bicarbonate, pH 8.8, containing 50 ng of trypsin (Promega, Madison, WI, USA) at 37°C overnight. Samples were desalted using Zip-Tips C18 (Millipore) and dissolved in 10 µl of 2% acetonitrile in 0.1% formic acid. Analysis was performed using a LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific) at the Proteomics Core of the National Cancer Center, Korea. MS was carried out using positive mode ESI. A syringe pump was used to introduce the calibration solution for automatic tuning and calibration of the LTQ. Digested samples were separated by liquid chromatography and then infused into the ionization source. The spray voltage was set at +1.1 kV, the temperature of the capillary was set at 200°C, the capillary voltage was set at +20 V, and the tube lens voltage was set at +100 V. The auxiliary gas was set to zero. Full scans were performed using a linear ion trap in the 150–2,000 m/z range. MS/MS was carried out by changing the relative collision energy and monitoring the intensities of the fragmented ions. Data were collected and analyzed using Sequest software (Thermo Fisher Scientific), which was programmed to search the uniprot_sprot database, assuming tryptic digestion. Fragment ion mass tolerance was set at 1.00 Da and parent ion tolerance was set at 1.2 Da. Oxidation of methionine was specified as a modification variable (Supplementary Table 1).

Polymerization of CTSD by TGase 2 in vitro

Purified guinea pig liver TGase 2 (0, 1, 2, 4, 8 mU; Sigma Aldrich) was incubated with human CTSD (10 ng, 250 U/mg protein; Sigma Aldrich) at 37°C for 30 min in reaction buffer [100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 mM $CaCl_2$, and 5 mM DTT]. The reaction was stopped with sample buffer and the reaction mixture was subjected to SDS-PAGE followed by immunoblot analysis using an antiCTSD antibody (Fig. 2b).

Results

Significant differences in TNF-α-induced apoptosis in TGase $2^{+/+}$ and TGase$2^{-/-}$ MEFs.

To determine the role of TGase 2 in apoptosis, we established naturally immortalized MEFs from TGase $2^{+/+}$ and TGase$2^{-/-}$ mice. Both sets of MEFs behaved similarly under normal conditions. However, in response to TNF-α-induced apoptosis, significant differences between TGase $2^{+/+}$ and TGase$2^{-/-}$ MEFs emerged. MEFs were seeded and exposed to CHX (10 µg/ml) alone or in combination with TNF-α (25 ng/ml) for 5 h. After treatment, cells were co-stained with Annexin V-FITC and PI and then analyzed by flow cytometry to determine cell cycle distribution. Cells in early apoptosis were increased in the population of TGase $2^{-/-}$ MEFs after treatment with TNF-α and CHX for 1 and 5 h. As shown in Fig. 1a and b, early apoptosis was significantly higher in TGase$2^{-/-}$ MEFs at 5 h compared to TGase $2^{+/+}$ MEFs.

TGase 2-binding proteins comprised a variety of structural and apoptotic molecules.

An important approach to understanding the role of TGase 2 in apoptosis is the identification of TGase 2-binding proteins. TGase 2-binding proteins were purified from cytosolic fractions of MDA-MB-231 cells and then subjected to LC/MS (Fig. 2a). A detailed list of the proteins identified is presented in Supplementary Table 1. CTSD and BAX were among the TGase 2-binding proteins identified by LC/MS. Previously, Kim et al (2009), investigating the mechanism of increased sensitivity to drug-induced apoptosis by TGase 2 silencing, showed though a

series of immunoblot experiments that apoptotic factors were differentially regulated by TGase 2 siRNA treatment in MDA231 cells (Kim et al. 2009). The expression of Bcl-2 was downregulated by TGase 2 gene silencing, whereas the expression of Bax appeared to be unchanged. BCL-2 is induced by NF-κB activation (Turco et al. 2004), while BAX expression is induced by p53 (Miyashita et al. 1994). Thus, the regulation of BAX by TGase 2 may be indirect and not through depletion via protein cross-linking. Therefore, we selected CTSD for further analysis (Fig. 2b).

Efficient depletion of CTSD by TGase 2-mediated protein cross-linking.

To investigate whether free CTSD was depleted by TGase 2, purified CTSD and guinea pig TGase 2 were incubated in vitro and then analyzed by SDS-PAGE (Fig. 2b). CTSD was decreased upon incubation with TGase 2 in a dose-dependent manner, and this correlated with the appearance of high molecular weight cross-linked CTSD polymers. Thus, CTSD associated with TGase 2 in the absence of calcium (Fig. 2a), and the polymerization of CTSD by TGase 2 occurred in the presence of calcium (Fig. 2b).

To determine whether CTSD levels were regulated by TGase 2 in cells, we employed a model of TNF-α- and CHX-induced cell death (Fig. 3). Immortalized MEFs from TGase $2^{+/+}$ and TGase$2^{-/-}$ mice were treated with TNF-α and CHX for 5 hrs. TGase$2^{-/-}$ MEFs were more susceptible to cell death under these conditions than TGase $2^{+/+}$ MEFs (Fig. 3a), exhibiting markedly higher levels of apoptosis. Under the same conditions, CTSD levels were also markedly increased in TGase$2^{-/-}$ MEFs (Fig. 3b). The levels of CTSD were approximately tenfold higher in TGase$2^{-/-}$ MEFs compared to TGase $2^{+/+}$ MEFs (Fig. 3b, lower panel bar graph). Interestingly, CTSD was increased in TGase$2^{-/-}$ MEFs in response to CHX treatment, suggesting that TGase 2 may regulate CTSD under normal conditions as well. These results indicated that increased TGase 2 expression promotes cell survival under conditions of apoptotic stress.

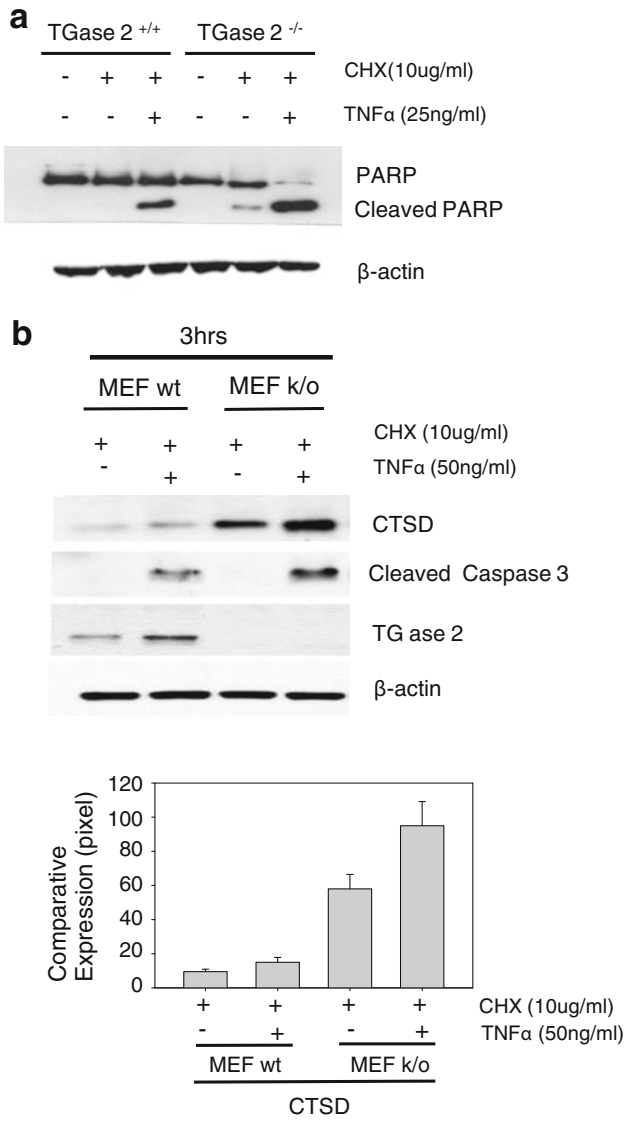

Fig. 3 Role of TGase 2 in TNF-α–induced apoptosis. TGase $2^{+/+}$ and TGase $2^{-/-}$ MEFs were seeded and exposed to CHX (10 μg/ml) alone or in combination with TNF-α (25 ng/ml) for 3 h. **a** Increased PARP processes in TGase$2^{-/-}$ MEFs in response to apoptosis. **b** CTSD expression was increased 5-fold in TGase$2^{-/-}$ MEFs compared to TGase $2^{+/+}$ MEFs in response to TNF-α and CHX treatment for 5 h. Increased susceptibility of TGase 2-null MEFs to TNF-α-induced apoptosis may involve the depletion of CTSD through protein cross-linking

Discussion

Protein cross-linking by TGase 2 is emerging as a new paradigm of post-translational modification and signaling. Several reports have shown that TGase 2 can regulate cell death through decreased caspase 3 activation (Jang et al. 2010; Rossin et al. 2011; Yamaguchi and Wang 2006). Jang et al. demonstrated that caspase 3 is depleted via TGase 2-mediated polymerization only under conditions of hypoxia. Under normoxic conditions, TGase 2 inhibition induced apoptosis in a dose-dependent manner with no detectable caspase 3 polymerization (Jang et al. 2010). Interestingly, Jang et al. and Yamaguchi et al. both showed that only activated caspase 3 but not pro-caspase 3 was depleted in cells with high TGase 2 expression under conditions of normoxia. Upstream of caspase 3, CTSD appears to be the major factor involved in the induction of caspase 3 activity through BID-BAX-Caspase 9 (Heinrich et al. 2004). Our results suggest that decreased CTSD levels in the cytoplasm due to TGase 2-mediated cross-linking may be a key factor underlying the reduced levels of caspase 3 activity under normoxic conditions in TGase

$2^{+/+}$ MEFs. This is supported by the results of the current study, in which the increased susceptibility of TGase$2^{-/-}$ MEFs to TNF-α-induced apoptosis correlated with increased CTSD levels in the cytoplasm compared to TGase $2^{+/+}$ MEFs (Fig. 3).

The apoptotic potential of CTSD has been well documented. Overexpression of CTSD sensitizes cells to apoptosis upon serum starvation (Shibata et al. 1998) and oxidative stress (Kagedal et al. 2001). MEFs from $CTSD^{-/-}$ mice are resistant to adriamycin- and etoposide-induced apoptosis (Wu et al. 1998). Furthermore, microinjection of CTSD induces caspase-dependent apoptosis in human fibroblasts (Roberg et al. 2002). In addition to the caspase family of proteases, lysosomal proteases such as CTSD have recently emerged as important regulators of the cell death process (Salvesen 2001). Caspases and Bid are potential targets for cleavage by CTSD. Therefore, the release of CTSD from endolysosomes into the cytoplasm may be an important prerequisite for the activation of apoptotic signaling pathways. Negative regulation of CTSD by TGase 2 may be an important protection mechanism to deadly apoptotic signaling.

In summary, in further support of caspase 3 as a TGase 2 target, we demonstrated that TGase 2 depletes CTSD via protein cross-linking to promote cell survival under conditions of apoptotic stress. CTSD polymers may be degraded by calpain and/or the proteasomal system, similar to the mechanism of degradation of polymerized I-κBα (Kim et al. 2010; Park et al. 2006). The detailed mechanism of CTSD degradation remains the subject of future studies.

Acknowledgments Dr. Folk devoted his life to developing the story of TGase 2. He told me that he avoided using 'TG' because 'TG' stands for triacylglycerol in Biochemistry. His wit named the 'TG-ase' as an enzyme. Dr. Waelsch discovered TGase 2 in liver tissue (Sarkar et al. 1957) at a point when it had only been detected in blood (FXIIIa). Dr. Folk solved the enzymatic reaction of TGase 2 in detail (Folk and Cole 1965; Folk and Chung 1985). Dr. Folk gave me the scientific courage to pursue TGase expression in bacterial systems, which was ultimately a success (Kim et al. 1994), when others claimed that it was impossible to express active TGase 2 in a bacterial system (Wong et al. 1991). I am especially grateful to Dr. Folk as well as his friends and my mentors Soo-Il Chung and Peter Steinert, great leaders in the TGase field. I will never forget Dr. Folk's apron and his smile when he made mistakes in his experiments. God be with you, Jack. This work was supported by a research grant (NCC1110011-1) from the National Cancer Center in Korea and by a National Research Foundation grant funded by the Korean Government (MEST) (No. 2010-0029919).

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