Silencing of TGase 2 sensitizes breast cancer cells to apoptosis by regulation of survival factors

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1. ABSTRACT

The cross-linking enzyme, Transglutaminase 2 (TGase 2), contributes to physiological homeostasis and plays a role in cell death and survival. We previously showed that down-regulation of TGase 2 by cystamine or synthetic peptide R2 promotes apoptosis in drug-resistant cancer cells by restoring the level of I-κBα, leading to inactivation of NF-κB. To better define the action of TGase 2, its expression was blocked by small interfering RNA. This interference rendered, the doxorubicin-resistant breast cancer cells, highly susceptible to doxorubicin-induced apoptosis. This susceptibility, was associated with decreased levels of the cell-survival factors BCl$_2$ and BCL$_{XL}$ whereas the level of BAX remained un-changed. Together, the findings support the view that TGase 2 leads to drug-resistance by up-regulating the level of survival factors via NF-κB activation.

2. INTRODUCTION

Drug-resistance frequently occurs in response to the use of alkaloids and antibiotics, including anthracyclines such as doxorubicin and daunomycin, vinca alkaloids, actinomycin D, etoposide and paclitaxel, likely due to NF-κB activation (1-3). NF-κB activation enhances resistance and suppresses apoptosis due to chemotherapeutic agents and for this reason this activation might be important in the development of drug resistence in cancers (2-4). The activation of NF-κB occurs in response to many factors including Transglutaminase 2 (TGase 2) (5). TGase 2 cross-links protein bound-glutamine and -lysine, and leads to the formation of covalent iso-peptide bonds (6). A unqiue feature of TGase 2 is that activation of the downstream transcription factor(s) such as NF-κB, does not require ATP but rather, calcium. Expression of TGase 2 is elevated in drug-resistant cancer cell lines (7,8).

Interestingly, this drug-resistance to chemotherapeutic drugs can be counteracted by the inhibition TGase 2. Here we tested the hypothesis that this inhibiton might be mediated by virtue of changes in expression of factors that have an anti-apoptotic effect (7).

3. MATERIALS AND METHODS

3.1. Antibodies and reagents

The anti-I-κB antibody was from Cell Signaling Technologies (Beverly, MA, USA), the anti-TGase 2 antibody from NeoMarkers (clone CUB 7402; Fremont, USA), and anti-β-actin from Abcam (Headquarters: Cambridge, UK). The anti-Bax, anti-Bcl$_2$ and anti-Bcl$_{xL}$ antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Lipofectamine 2000 and Lipofectamine RNAiMAX transfection regents from Invitrogen (Carlsbad, CA, USA), Lipofectamine RNAiMAX with Stealth Negative control from invitrogen (Carlsbad, CA, USA), and propidium iodide solution and RNAase A from Sigma (St Louis, MO, USA).

3.2. Cell culture and sub-cellular preparation

The human breast cancer cell lines included MDA-468 (doxorubicin sensitive) and MDA-231 (doxorubicin resistant). These cell lines were obtained from the American Type Culture Collection. Cells were grown in RPMI (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 1mM sodium pyruvate (Gibco-BRL, Grand Island, NY, USA), and 100 U/ml penicillin-streptomycin (Gibco-BRL, Grand Island, NY, USA), and maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cytoplasmic cellular fractions were prepared using a CelLytic^{TM} NuCLEAR^{TM} Extraction Kit (Sigma, St Louis, MO, USA).

3.3. Transient transfection

For the secreted alkaline phosphatase (SEAP) reporter assay, transient transfection of a reporter plasmid carrying the NF-κB promoter (pNF-κB-SEAP; BD Bioscience, Mountain View, CA, USA) and pGAL plasmid were co-transfected using 3 μl of Lipofectamine 2000 (Invitrogen), according to the manufacturer's instruction. The data represent the average and standard deviation (SD) of three independent experiments.

3.4. NF-κB activity assay

NF-κB activity was measured using the SEAP reporter system 3 (BD Bioscience, Mountain View, CA, USA). TGase 2-specific siRNA-transfected cells were co-transfected with pNF-κB-SEAP and pGAL, then subjected to 24 and 48 h post-transfection. Then, the culture media were collected for an SEAP assay, and the cells were harvested for a β-galactosidase assay. The SEAP assay was performed according to the manufacturer (BD Bioscience, Mountain View, CA, USA) instruction. The data represent the average and SD of three independent experiments.

3.5. TGase 2 gene silencing by small interfering RNA

A small interfering RNA (siRNA) duplex targeting human TGase 2, 5′-AAGAGCGAGAUGAUCUGGAAC-3′ (Invitrogen) was introduced into the cells using

Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instruction. 48 h after transfection, the cells were harvested, and a cytosolic fraction was prepared in order to analyze the level of TGase 2 and I-κBα by Western blotting. Cells incubated with Lipofectamine RNAiMAX and Stealth Negative control (Invitrogen) were employed as the negative control. The data represent the average and SD of three independent experiments.

3.6. Western blotting

Western blotting has performed followed by the previously established method. The primary antibodies used in these studies were anti-I-κBα, anti-TGase 2, anti-β-actin, anti-Bax and anti- Bcl$_2$.

3.7. TGase activity assay

A modified TGase activity assay was used, which measured the incorporation of (1,4-^{14}C)putrescine into succinylated casein.[6]

3.8. Apoptosis assay

For the cell cycle analysis, TGase 2 siRNA-transfected cells were seeded and exposed to doxorubicin for 48 h. Then, they were collected, washed with PBS, and fixed with 100% ethanol at 4°C for 24 h. Fixed cells were washed three times and incubated for 30 min with propidium iodide solution (Sigma, St Louis, MO, USA) containing RNAase A (Sigma, St Louis, MO, USA). Cells were then subjected to cell cycle analysis in order to determine the DNA content by flow cytometry (FacsCalibur, Becton-Dickinson). The data are provided as percent hypoploidy (i.e. the percentage of cells with a sub-G1 DNA content), which reflects the percentages of apoptotic cells with fragmented genomic DNA. The analysis of annexin V binding was carried out using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions. Cells were collected, washed twice with cold PBS, and centrifuged at 1500 rpm for 5 min. Cells were then resuspended in 1x binding buffer at a concentration of $1X10^6$ cells per ml, 100 μl of the solution was transferred to a 5 ml culture tube, and 5 μl of annexin V-FITC and 5 μl of PI were added. The cells were gently vortexed and incubated for 15 min at room temperature in the dark. Finally, 400 μl of 1x binding buffer was added to each tube, and the samples were analyzed by FACScan flow cytometry. For each sample, 10,000 ungated events were acquired. PI (-)/annexin (+) cells represent the early apoptotic populations

4. RESULT AND DISCUSSION

4.1. TGase 2 expression and enzyme activity in two breast cancer cell lines

Previously, we reported that the expression of TGase 2 is elevated in drug-resistant and not drug sensitive breast cancer cell lines (7). The level of TGase 2 is up to 20-fold higher in the drug-resistant MDA231 cell line as compared with the drug-sensitive MDA468 cell line (Figure 1A). Also, the activity of the TGase 2 enzyme is up to 22-fold higher in the drug-resistant MDA231 cell line as compared with the drug-sensitive MDA468 cell line (Figure 1B).
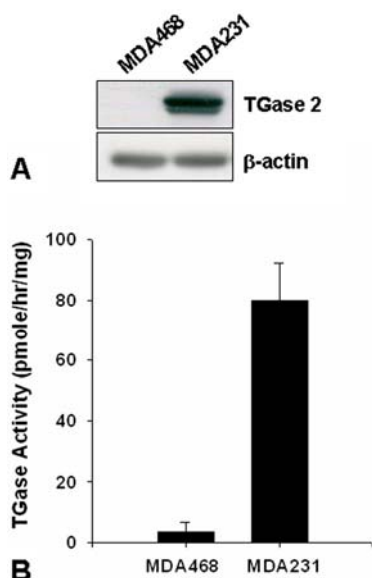
Figure 1. *In vitro* TGase 2 activity and levels of TGase 2 expression in MDA468 and MDA231 human breast cancer cells. A. Western blot of TGase 2 in MDA468 and MDA231 human breast cancer cells. β-actin was used as a loading control. B. Quantitative analysis of *in vivo* TGase 2 activity in cytosolic extract from MDA468 and MDA231 human breast cancer cells.

4.2. NF-κB activity is decreased by TGase 2 gene silencing

To test whether TGase 2 gene silencing affects NF-κB activity in the drug-resistant MDA231 cell line, Western blotting and an NF-κB reporter assay were peformed. This silencing led to decreased level of TGase 2 expression (about 7-fold) and TGase activity (5-fold decreased)(Figure 2A and 2B). The Western blot analysis of I-κBα showed a dramatic, dose-dependent increase of I-κBα by TGase 2 siRNA treatment (Figure 2A). Transfection with Stealth Negative control RNA, or the non-transfected MDA231 cells did not affect the levels of TGase 2 or I-κBα (data not shown). We examined whether TGase 2 gene silencing suppresses NF-κB activity in drug-resistant breast cancer cells. The reporter gene activity of the NF-κB promoter in the MDA231 cells was decreased by about 3-fold by treatment with 200 nM of TGase 2 siRNA (Figure 2C). TGase 2 gene silencing reversed the NF-κB activity via restoration of the level of I-κBα. These data concord with the previous finding that cystamine and R2 peptide reverses NF-κB activity via restoration of the I-κBα level in MCF7/Dox cells (7).

4.3. TGase 2 gene silencing reduces the level of nuclear NF-κB in the drug-resistant MDA231 cell line

To test whether TGase 2 gene silencing reduces nuclear translocation of NF-kB, immunocytochemical staining of p65 was performed after TGase 2 siRNA treatment for 48 hr (Figure 3). This treatment inhibited TGase 2 expression in MDA231 cell line (Figure 3A). Treatment of MDA231 cells with Stealth Negative control

RNA, led to peripheral nuclear staining of p65 whereas cells transfected with TGase 2-targeted siRNA showed very little nuclear staining of p65 and showed mostly staining in the cytoplasm (Figure 3B).

4.4. TGase 2 gene silencing increases sensitivity to doxorubicin-induced apoptosis in MDA231 cells

To determine whether TGase 2 gene silencing promotes chemosensitivity in the drug-resistant MDA231 cell line, we performed a cell viability MTT assay usingt doxorubicin (Figure 4). We first determined the IC_{50} value for the doxorubicin in drug-resistant MDA231 cell line. The cells were treated with various concentrations of doxorubicin, and the cell viability was determined by MTT assay after 48 hr of treatment. Treatment with 5 and 10 μM doxorubicin led to apoptosis in ~50% of the drug-resistant MDA231 cells had (Figure 4A). Ablation of TGase 2 expression did not affect the tendency to apoptosis (Figure 4B). These data are consistent with reports in TGase 2 knock-out mice, that do not show any change in apoptotic progress (27,28). To test whether TGase 2 gene silencing affects cell survivability after treatment with doxorubicine, MDA231 cells were treated with 5 or 10 μM of doxorubicin together with TGase 2 siRNA (Figure 4C). The cell viability 48 hr after treatment, showed that drug sensitivity was significantly increased, by about 2-fold (Figure 4C). The number of apoptotic cells was quantitated by flow cytometry using propidium iodide (PI) staining after transfection of cells with TGase 2 siRNA (Figure 5). The PI-stained cells were concentrated on the sub-G_1 cellular DNA fraction. Treatment of doxorubicin and transfection with TGase 2 siRNA resulted in an approximately 1.5-fold increase in the number of apoptotic DNA in the sub-G_1 fraction compared with the cells treated with the negative control siRNA (Figure 5A). A series of experiments suggested that TGase 2 siRNA sensitized the doxorubicin-resistant MDA231 breast cancer cells to the doxorubicin. The early-apoptotic rate was assessed after treatment of cells with 10 μM doxorubicin and transfection with TGase 2 siRNA by double-staining with Annexin V-FITC and PI using flow cytometry . As shown in Figures 5B and 5C, the early apoptosis rate was increased by the TGase 2 siRNA treatment, compared with control cells transfected with control siRNAs.

4.5. TGase 2 gene silencing reduces the levels of survival factors BCl_2 and BCl_{XL}

To investigate the mechanism by which TGase 2 gene silencing increases sensitivity to drug-induced apoptosis, a series of Western blotting against apoptosis factors was performed with or without TGase 2 siRNA treatment in MDA231 cells (Figure 6). The results showed that the expression of Bcl_2 and Bcl_{XL} was down-regulated dose-dependently by TGase 2 gene silencing, whereas the expression level of Bax remain un-changed.

5. DISCUSSION

In this study, increased expression of the survival factors BCl_2 and BCl_{XL} was observed in drug-resistant breast cancer cell lines. Furthermore, the expression level of these factors was decreased by TGase 2 gene silencing
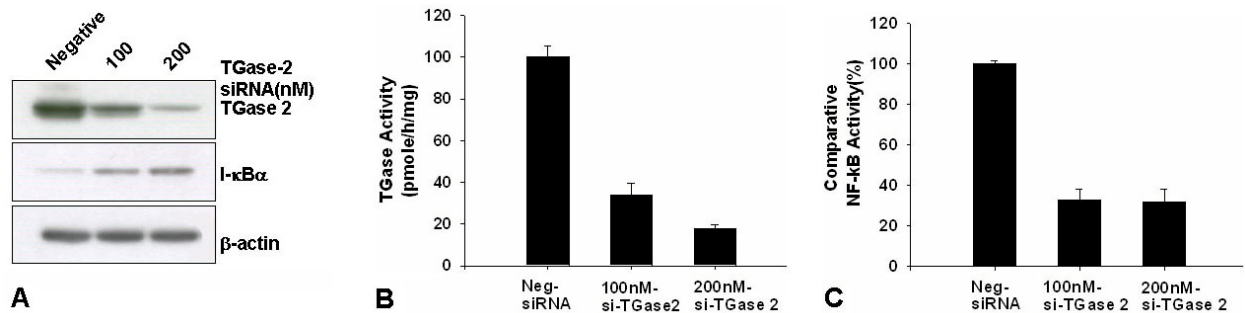
Figure 2. I-κBα levels increased by TGase 2 gene silencing. A. MDA231 cells were transfected with TGase 2-specific siRNA (100nM and 200nM) or control siRNA. 48 h post-transfection, the cells were analyzed by western blotting for expression of TGase 2 (upper panel) and I-κBα(middle panel). The actin levels were also examined to confirm that similar amounts of cytoplasmic cellular fractions were used in the assay (lower panel). B. Cytosolic TGase 2 activity was decreased in the MDA231 cells transfected with TGase 2-specific siRNA. C. NF-κB activity decreased by TGase 2 gene silencing. The MDA231 cells were transfected with TGase 2-specific siRNA (100nM and 200nM) or control siRNA. 48 h post-transfection, the relative NF-κB activity was determined by SEAP assay, as described in "Materials and methods."
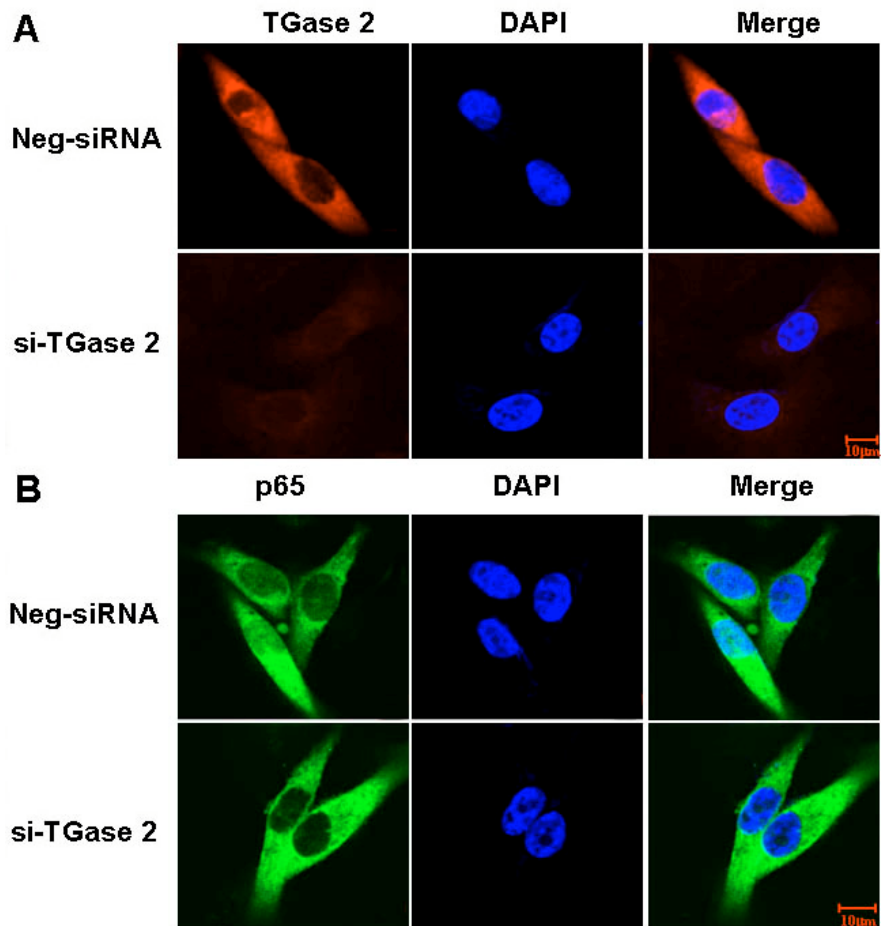

Figure 3. Effect of TGase 2 gene silencing on NF-κB localization. Immunocytochemical staining using anti-TGase 2 (A) and anti-p65 antibody (B) in MDA231 cells. DAPI staining was employed as a nuclear counter stain. The scale bar represents 10 μm.

while the level of BAX remained constant. NF-κB-mediated induction of BCl₂ and BClₓₗ is involved in cancer

cell survival (9,10). Therefore our finding implies that gene silencing of TGase 2 reduces cell survival by decreasing
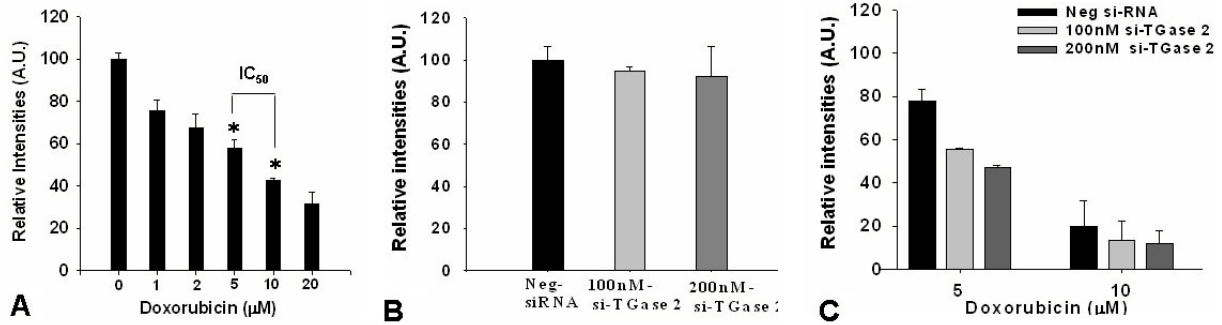
Figure 4. Decreased resistance to doxorubicin of MDA231 cells by TGase 2 gene silencing. A. The MDA231 cells were treated with various concentrations of doxorubicin, and cell viability was determined by MTT assay after 48 h treatment. B. The MDA231 cells were transfected with TGase 2-specific siRNA (100 nM and 200 nM) or control siRNA. After 48 h incubation, the relative cell numbers were determined by MTT assay, as described in Materials and methods. C. After siRNA treatment for 48 h, the cells were exposed to doxorubicin (5 and 10 μM) for another 48 h. After incubation, the relative cell numbers were determined by MTT assay.
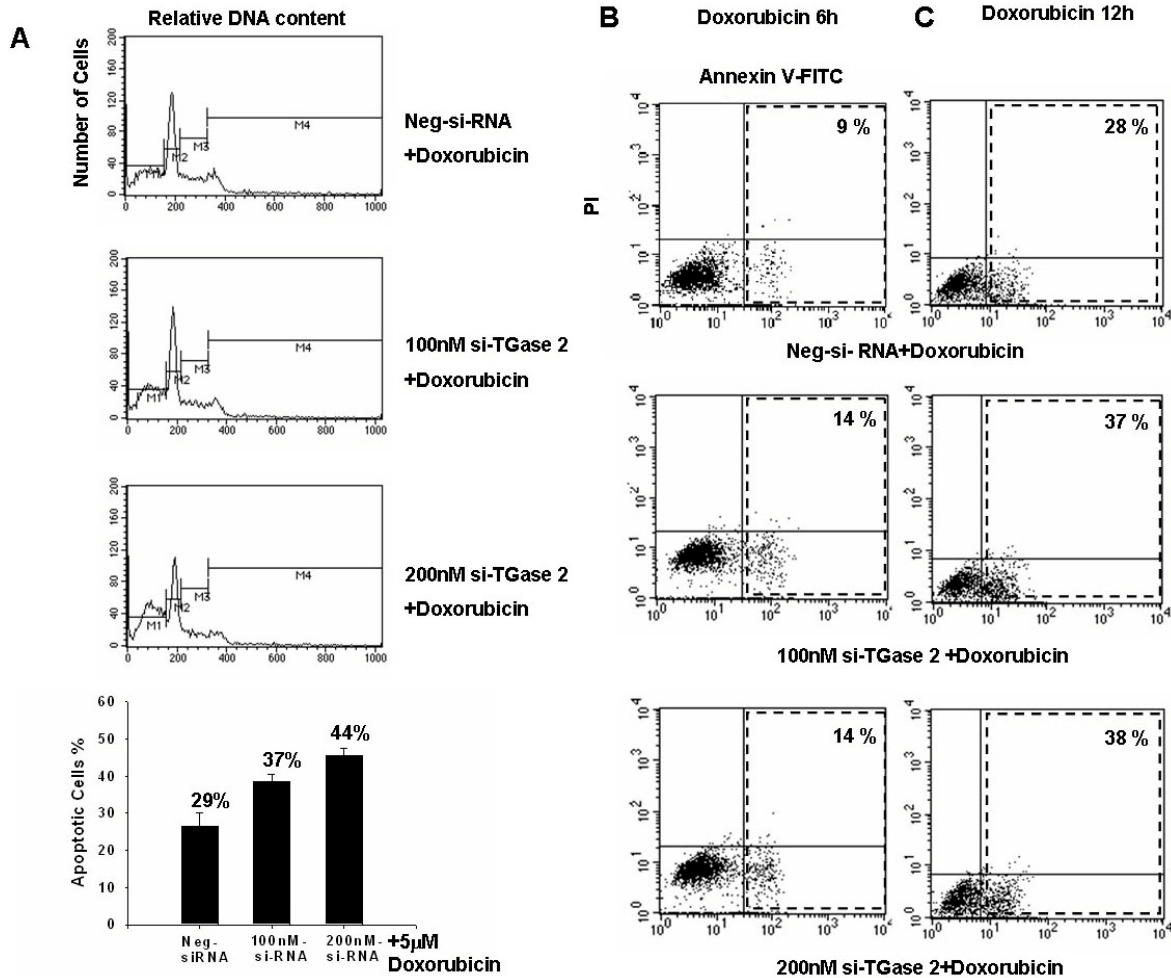

Figure 5. A. MDA231 cells were transfected with TGase 2-specific siRNA (100nM and 200nM) or control siRNA. 48 h post-transfection, cells were exposed to 5μM doxorubicin, and after 48 h, apoptotic DNA fragmentation was determined on the single-cell level by flow cytometric measurement of cellular DNA content. Percentage of apoptotic cells displaying a sub-G1 DNA content is given. The Bar graph illustrates the results of a statistical analysis of the apoptosis assay data. B. Flowcytometric analysis of TGase 2 specific siRNA combination with doxorubicin-induced apoptosis after double staining with Annexin V-FITC and PI. Early apoptosis was increased in the TGase 2 specific siRNA transfectants after treatment with 10μM doxorubicin for 6 h and 12 h
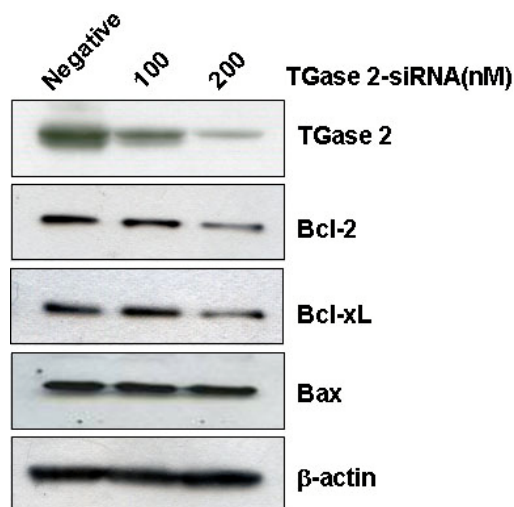
Figure 6. Suppression of Bcl-2 and Bcl-xL protein expression level by TGase 2 gene silencing in MDA231 cells. Western blot analysis revealed specific downregulation of the Bcl-2 and Bcl-xL proteins in proportion to the knock-down of TGase 2 protein, whereas the expression of the Bax proteins was not altered. The actin levels were also examined to confirm that similar amounts of cytoplasmic cellular fractions were used in the assay (lower panel).
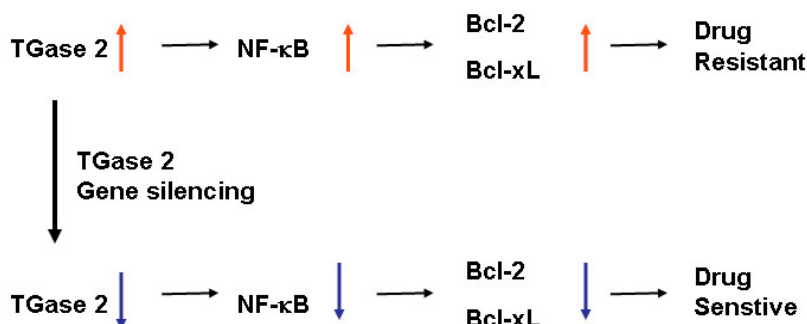

Figure 7. Proposed mechanism of TGase 2 gene-silencing-induced apoptosis in drug-resistant MDA231 cells. TGase 2 activates NF-κB, causing the activation of Bcl-2 and Bcl-xL. These signals promote the drug-resistance properties of MDA231 cells. TGase 2 gene silencing increases sensitivity to drug-induced apoptosis by NF-κB inactivation, which inhibits Bcl-2 and Bcl-xL activity.

BCl$_2$ and BCl$_{XL}$ expression. Recently Mehta *et al.* suggested that TGase 2 over-expression causes constitutive activation of the FAK and PI3K/Akt system, which can result in increased levels of survival factors (11). Interestingly, Akt signaling also activates NF-κB via IKK activation, which might result in increased BCl$_2$ expression (12). This observation is in concordance with recent findings that increased TGase 2 expression induces NF-κB activation in various cell lines including microglia (5), neuroblastoma (5), human kidney (13), breast cancer (7), astrocyte (14), and melanoma (15).

Previous studies have revealed that increased expression of TGase 2 appears to prolong cell survival via suppression of apoptosis (antonyak 2004, boehm 2002, Mehta 2005) (16-18). Contrarily, many reports also found evidence of TGase 2 involvement in apoptosis; specifically, whereas its over-expression triggered cells to die, its inhibition by gene silencing rendered the cells resistant to apoptosis (19-26). However, there is the discrepancy that,

in other studies, Tgm-/- mice showed no statistically significant differences, compared with Tgm2+/+ mice, for any of the measured parameters (27-28). Indeed, the cells were triggered to die without TGase 2. The lack of TGase 2 activity in Tgm-/- mice is only associated with smaller thymuses and less TUNEL positivity, owing to decreased cell death or an increased rate of dead cell clearance (28). TGase 2 over-expression enhances the response to apoptosis signaling (29), but the specific mechanism of TGase 2 in apoptosis remains to be elucidated. TGase 2 certainly plays a role both in cell death and survival that can contribute to homeostasis (24).

Although we demonstrated that via TGase 2 over-expression, cell survivability increases concomitantly with constitutive activation of NF-κB, there are other possible explanations. TGase 2 can increase cell survivability as a G protein. According to Aeschlimann *et al.*, TGase 2 can promote cell adhesion by regulating and being regulated by phospholipase C through binding to it with the non-

transamidating GTP form of the enzyme (30-31). This pathway involves protein kinase C, Rho and focal adhesion kinase, but the mechanism is independent of the transamidation of TGase 2. The Szondy group has also shown that TGase 2, as a G protein, inhibited Fas-mediated cell death (32). As a cross-linking enzyme, TGase 2 prolongs cell survivability by polyamine modification of Rb. TGase 2 protects Rb from caspase-induced degradation in a transamidation-dependent manner (17), suggesting that transamidation of Rb by TGase 2 is necessary for its ability to inhibit apoptosis. We also demonstrated that drug-resistant cancer cells with high NF-κB activity turned into drug-sensitive cells as a result of treatment with either of the TGase inhibitors cystamine or synthetic peptide R2 (7).

It remains to be clarified whether increased cell survivability as a result of increased TGase 2 expression is dependent on transamidation or G protein; still, the reduced expression of TGase 2 using siRNA dramatically decreased the levels of the cell-survival factors BCl_2 and BCL_{XL}.

6. ACKNOWLEDGEMENTS

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