Reversal of Drug Resistance in Breast Cancer Cells by Transglutaminase 2 Inhibition and Nuclear Factor-κB Inactivation

Dae-Seok Kim,[1] Sung-Soo Park,[1] Byung-Ho Nam,[2] In-Hoo Kim,[1] and Soo-Youl Kim[1]

[1]Molecular Oncology Branch, Division of Basic Sciences, Research Institute and [2]Cancer Biostatistics Branch, Center for Clinical Trials, National Cancer Center, Goyang, Kyonggi-do, Republic of Korea

Abstract

Induction of transglutaminase 2 (TGase 2) by epidermal growth factor (EGF) in human breast cancer cells increases their oncogenic potential and chemoresistance. The role of TGase 2 in the development of these tumor-related phenotypes remains to be elucidated, but it has been shown that expression of a dominant-negative form of TGase 2 reverses EGF-mediated chemoresistance in breast cancer cells. We examined several different breast cancer cell lines, representing both EGF receptor (EGFR)-positive and EGFR-negative breast cancers, and found that doxorubicin-resistant cells had a higher level of TGase 2 compared with doxorubicin-sensitive cells independent of the EGFR expression level. TGase 2 inhibition increased the chemosensitivity of drug-resistant cells, concomitant with a decrease in nuclear factor-κB (NF-κB) activity. Increasing the level of TGase 2 in drug-sensitive cells by transient transfection reduced the level of inhibitory subunit α of NF-κB (IκBα) and increased NF-κB activity in these cells. Inhibition of TGase 2 in drug-resistant cells by RNA interference increased the levels of IκBα, and this correlated with a shift in the accumulation of NF-κB from the nucleus to the cytosol. We recently showed that TGase 2 activated NF-κB through polymerization and depletion of free IκBα during inflammation. Therefore, increased expression of TGase 2 and subsequent activation of NF-κB may contribute to drug resistance in breast cancer cells independently of EGF signaling. (Cancer Res 2006; 66(22): 10936-43)

Introduction

Increased expression of transglutaminase 2 (TGase 2; EC 2.3.2.13) is associated with drug resistance in breast cancer (1–3). Although the mechanism is as yet undefined, it seems that TGase 2 inhibition may completely reverse the drug-resistant phenotype of certain breast cancer cell lines (3). TGase 2 is a calcium-dependent enzyme that catalyzes the formation of covalent bonds between free amine groups in one protein and protein-bound glutamines of another, creating highly cross-linked protein complexes (4). TGase 2 is ubiquitously expressed and has multiple normal physiologic functions through the targeting of specific substrates, such as blood clotting (5), wound healing (6), cell adhesion (7), apoptosis (8), and barrier formation (9). TGase 2 has also been associated with certain pathologic conditions [i.e., inflammatory diseases, such as encephalomyelitis (10), inflammatory myopathies (11), and celiac disease (12), as well as various types of cancer (1–3, 13–17)]. Long-term studies of patients with celiac disease have led to a model for disease progression, in which antigen modification by TGase 2 creates a novel epitope, which then can trigger an immune reaction in genetically predisposed individuals (18). Despite the fact that many investigators for decades have examined TGase 2 expression in cancerous tissues, the role of TGase 2 in the pathogenesis of cancer remains to be elucidated.

Mehta et al. showed that increased levels of TGase 2 are associated with drug resistance in breast cancer cells (1, 2). This was also true of invasive and metastatic breast cancer cells (2). Antonyak et al. showed that TGase 2 inhibition using a dominant-negative form of TGase 2 abolished epidermal growth factor (EGF)-mediated protection from doxorubicin-induced apoptosis in several breast cancer cell lines (3). TGase 2 may protect cells from apoptosis by interacting with the retinoblastoma protein (19) or by promoting interactions between cell surface integrins (20). It has also been proposed that the antiapoptotic effect of increased TGase 2 expression may involve modification and down-regulation of caspase-3 (21).

In the current study, we explored the role of TGase 2 in the drug-resistant phenotype of several breast cancer cell lines. We previously showed that TGase 2 activated nuclear factor-κB (NF-κB) via an IκB kinase–independent pathway involving IκBα polymerization by TGase 2 (ref. 22, reviewed in ref. 23). NF-κB promotes the survival of cells following DNA damage and plays a role in neoplastic transformation by inhibiting p53 gene expression (24). Interestingly, resistance to the apoptotic effect of the chemotherapeutic agents Taxol, doxorubicin, tamoxifen, and cisplatin has been linked to NF-κB activation in cancer cells (25). It has also been shown that inhibition of NF-κB enhances the sensitivity of tumor cells to apoptosis induced by chemotherapeutic agents (26). TGase 2 expression can be induced by EGF (3, 27). Therefore, in the current study, we examined several different breast cancer cell lines, representing EGF receptor (EGFR)-positive and EGFR-negative breast cancers, to determine the role of TGase 2–mediated NF-κB activation in drug resistance.

Materials and Methods

Cell culture and treatment conditions. The human breast cancer cell lines MCF-7 (EGFR negative, doxorubicin sensitive), MDA-468 (EGFR positive, doxorubicin sensitive), and MDA-231 (EGFR positive, doxorubicin resistant) were obtained from the American Type Culture Collection. MCF-7/DOX (EGFR negative, doxorubicin resistant) was obtained from Dr. Kenneth H. Cowan (University of Nebraska Medical Center, Omaha, NE; ref. 28). Cells were grown in RPMI 1640 (Lifetech Co.) supplemented with 10% fetal bovine serum (Lifetech), 1 mmol/L sodium pyruvate (Lifetech),

Requests for reprints: Soo-Youl Kim, Molecular Oncology Branch, Division of Basic Sciences, National Cancer Center, Research Institute, 809 Madu-dong, Ilsandong-gu, Goyang, Kyonggi-do 410-351, Republic of Korea. Phone: 82-31-920-2043; Fax: 82-31-920-2006; E-mail: kimsooyoul@gmail.com.

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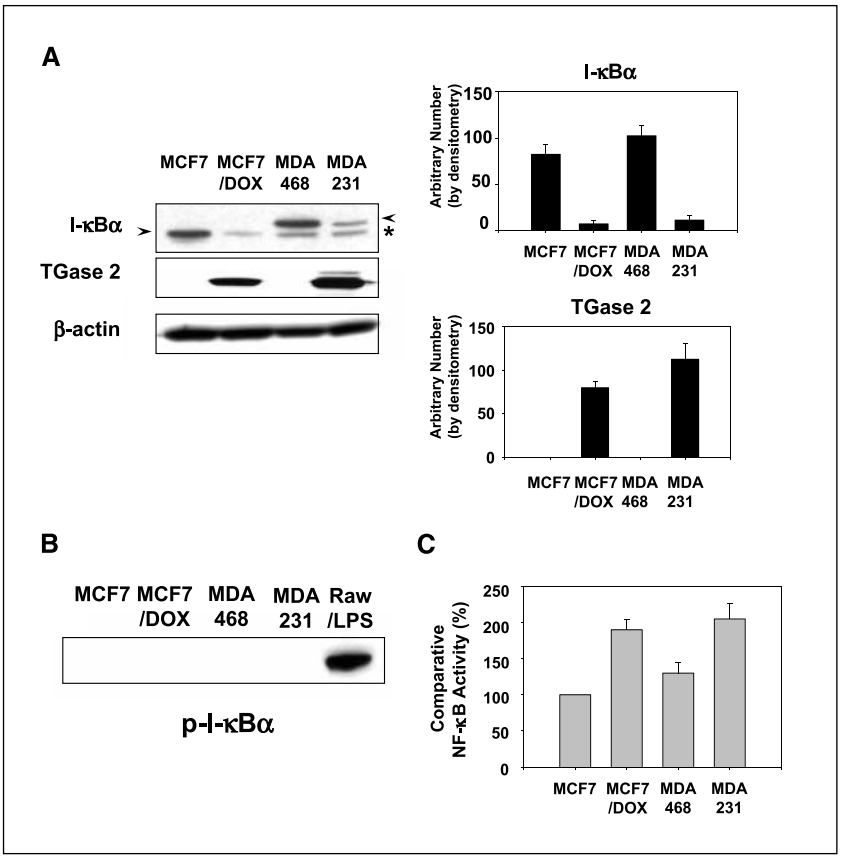

Figure 1. Western blot analysis of the levels of IκBα and TGase 2 in breast cancer cell lines. *A,* Western blot of TGase 2 and IκBα in MDA-468, MDA-231, MCF-7, and MCF-7/DOX cells. β-Actin was used as a loading control. *Arrowhead,* IκBα protein. *Asterisk,* possible degradation products (30). *B,* Western blot using anti-phosphorylated IκBα (phosphorylated Ser^{32}; *p-I-κBα*). Cytosolic cellular extract from LPS-treated raw cells was used as the positive control. *C,* NF-κB reporter assay in breast cancer cells. Values were normalized to the level of β-galactosidase activity, and the normalized value from MCF-7 cells was set as 100% activity. *Columns,* mean of three independent experiments; *bars,* SD. ANOVA and a post hoc test were done for pairwise comparisons using the Tukey procedure controlling for the overall type I error (Table 1).

and 100 units/mL penicillin-streptomycin (Lifetech) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. All four cell lines were analyzed in the TGase activity assay. To determine the effect of TGase 2 inhibition on chemosensitivity, MDA-231 and MCF-7/DOX cells were incubated with a combination of 0.1 to 1 mmol/L cystamine (CTM; Sigma Co.) and 5 μmol/L (for MDA-231) or 90 μmol/L (for MCF-7/DOX) doxorubicin for 24 to 48 hours.

Isolation of cytoplasmic and nuclear proteins. Cytoplasmic cellular fractions were prepared using a CelLytic NuCLEAR Extraction kit (Sigma). Briefly, cells were harvested, resuspended in cellular lysis buffer containing DTT and protease inhibitors, and incubated on ice for 15 minutes. After lysis, a solution of Igepal CA-630 (0.6%) was added and the samples were vortexed vigorously for 10 seconds and then centrifuged at 11,000 × *g* for 30 seconds. Supernatants were collected as the cytoplasmic fraction;

Table 1. ANOVA for the levels of IκBα and TGase 2 in breast cancer cell lines

Group	Mean	SD	Overall test*	Post hoc†
Fig. 1*A*. IκBα				
MDA-231	11.67	3.51	<0.0001	C
MDA-468	102.33	7.51		A
MCF-7	82.33	7.51		B
MCF-7/DOX	7.33	2.52		C
Fig. 1*A*. TGase 2				
MDA-231	83.67	10.60	<0.0001	B
MDA-468	0.00	0.00		C
MCF-7DOX	115.33	19.50		A
MCF-7	0.00	0.00		C
Fig. 1*C*. NF-κB activity				
MDA-231	205.00	15.00	<0.0001	A
MDA-468	130.00	10.00		B
MCF-7/DOX	190.00	10.00		A
MCF-7	100.00	1.00		C

*ANOVA.
†Tukey procedure: same alphabets indicate no significant difference and different letters indicate significant difference adjusting for the overall family-wise error (FWE) rate of 0.05.

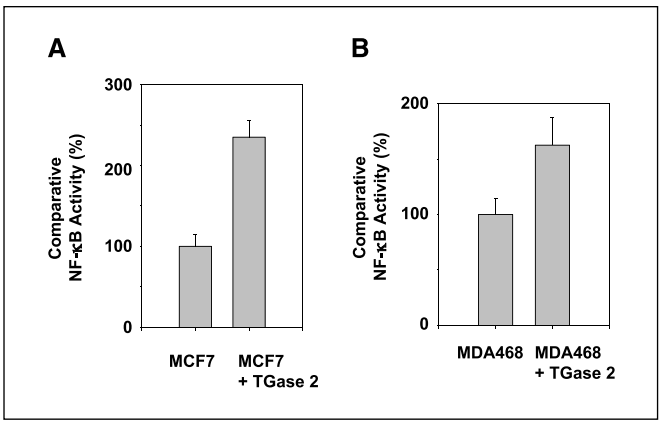

Figure 2. Effect of TGase 2 expression on NF-κB activation in breast cancer cell lines. *A* and *B,* human TGase 2, or empty vector as the control, was transiently transfected into MCF-7 and MDA-468 cells, and NF-κB activity was measured using the SEAP reporter assay. *Columns,* mean of three independent experiments; *bars,* SD. Student's *t* test resulted in probability <0.005 between groups.

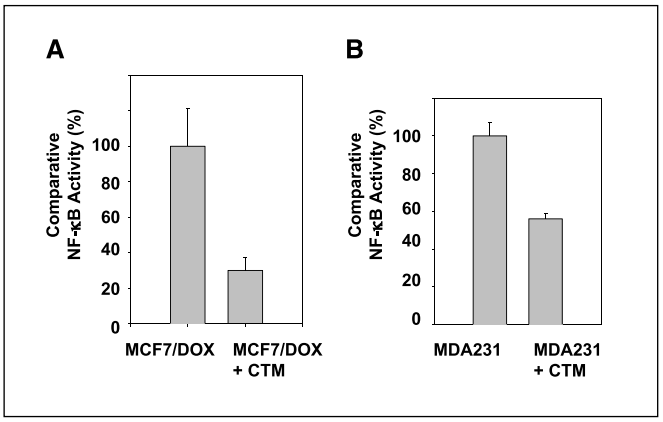

Figure 3. Effect of TGase 2 expression on NF-κB activation in MCF-7 and MDA-468 cells. *A* and *B,* MDA-231 and MCF-7/DOX cells were incubated in the presence or absence of CTM (1 mmol/L) for 24 hours. After incubation, culture medium was collected and a NF-κB assay using SEAP reporter was done. *Columns,* mean of three independent experiments; *bars,* SD. Student's *t* test resulted in probability <0.005 between groups.

pellets were resuspended in nuclear lysis buffer with vigorous vortex for 20 minutes at 4°C and then centrifuged at 14,000 × *g* for 5 minutes. The supernatant from this step was collected as the nuclear fraction. Protein concentration was determined using the Coomassie Plus Protein Assay Reagent (Pierce).

Transient transfections. Transient transfection of pcDNA3.0 containing the full-length human TGase 2 cDNA was carried out using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded at a density of 4×10^5 per well in a six-well plate. When cells reached 40% to 50% confluence, they were washed with 2 mL Opti-MEM (Invitrogen) and incubated with a DNA-LipofectAMINE mixture (1 μg DNA and 3 μL LipofectAMINE reagent) for 6 hours in a humidified 5% CO_2 environment. After 6 hours of incubation, the transfection medium was replaced with fresh culture medium, and cells were allowed to incubate for an additional 48 hours. For the secreted alkaline phosphatase (SEAP) reporter assay, a reporter plasmid carrying the NF-κB promoter (pNF-κB-SEAP; 0.5 μg; BD Biosciences Clontech) and the TGase 2 expression plasmid (0.5 μg) were cotransfected using 3 μL LipofectAMINE 2000. Transfection of pGAL plasmid (0.3 μg) was used as a control.

Cytotoxicity [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test is a colorimetric assay that measures the ability of viable cells to reduce a soluble tetrazolium salt, MTT (Sigma), to an insoluble purple formazan precipitate. MCF-7/DOX and MDA-231 cells were plated in 24-well plates at a density of 10^5 per well. When cells reached 50% confluence, they were exposed to various concentrations of CTM (0.1-1 mmol/L) alone or together with doxorubicin (90 μmol/L for MCF-7/DOX cells and 5 μmol/L for MDA-231 cells). Following treatment, cells were incubated in fresh medium containing 0.5 mg/mL MTT and incubated for an additional 4 hours. The culture medium in each well was collected, and formazan crystals were solubilized in 500 μL DMSO. The absorbance at 540 nm of each sample was determined using a microplate reader and compared with a blank prepared from cell-free medium. Cell survival was expressed as a fraction of untreated controls. Data represent the average and SD of three independent experiments.

NF-κB activity assay. NF-κB activity was measured using the SEAP Reporter System 3. Cells were transfected with pNF-κB-SEAP, and then 24 and 48 hours after transfection, the cell culture medium was collected for a SEAP assay and the cells were harvested for a β-galactosidase assay. The expression vector pcDNA3.0 was used as a negative control. To normalize for expression levels in the different cell lines, cells were cotransfected with pGAL (1 μg) and the various expression plasmids and SEAP activity was normalized to β-galactosidase activity. The SEAP assay was done according to the manufacturer's instructions (BD Biosciences Clontech). Briefly, culture medium (25 μL) was mixed with 25 μL of dilution buffer in a 96-well flat-bottomed microtiter plate and incubated at 65°C for 30 minutes. The plate was chilled on ice for 2 minutes, and then 97 μL of assay buffer

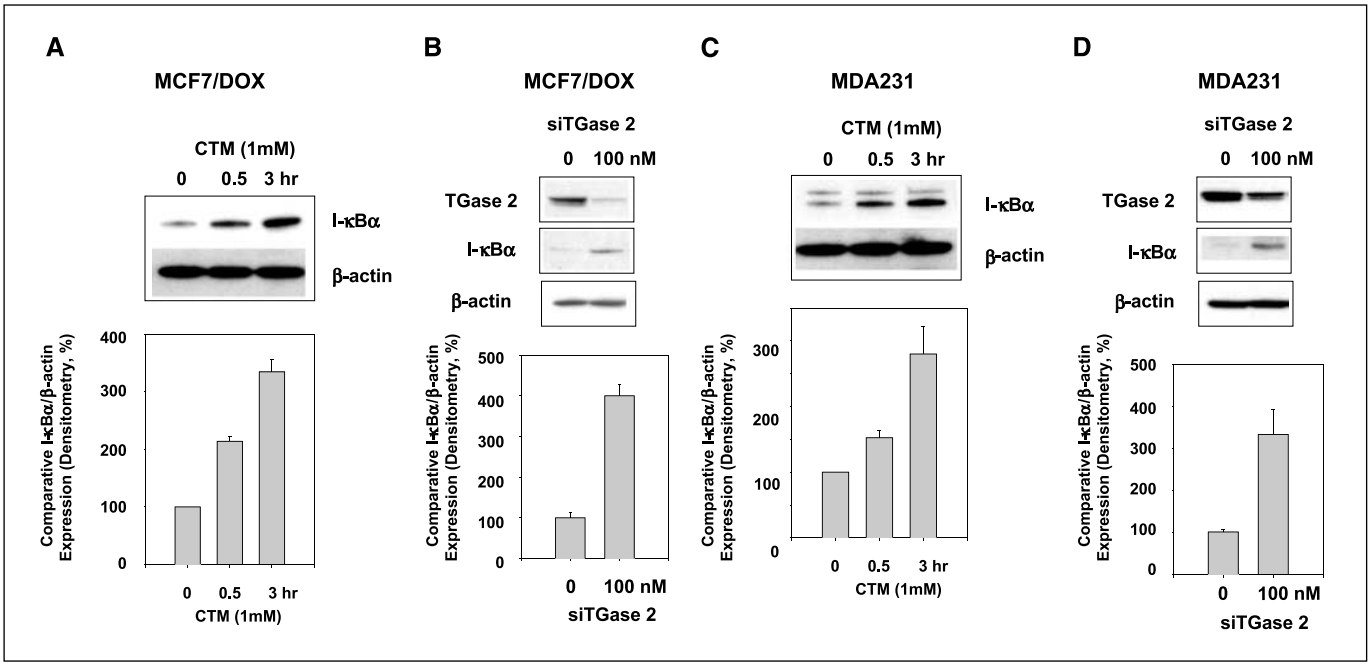

Figure 4. Effect of TGase 2 inhibition on cytosolic IκBα in MCF-7/DOX and MDA-231 cells. *A* and *C,* cells were incubated with 1 mmol/L CTM for 30 minutes or 3 hours. After treatment, cytosolic cellular fractions were examined by Western blot using anti-IκBα antibodies. *Bottom,* Western blots were quantitated using densitometry, and the results are presented in graph form. β-Actin was used as a protein loading control. *B* and *D,* MCF-7/DOX and MDA-231 cells were transfected with TGase 2–specific siRNA (*siTGase 2*), and the level of cytosolic IκBα was analyzed as in (*A*) and (*C*). *Columns,* mean of three independent experiments; *bars,* SD. Student's *t* test was included in Table 2. ANOVA and a post hoc test were also done for pairwise comparisons using the Tukey procedure controlling for the overall type I error (Table 2).

Table 2. ANOVA for effect of TGase 2 inhibition on cytosolic IκBα in MCF-7/DOX and MDA-231 cells

Fig. 4*A*

Group (MCF-7/DOX)	Mean	SD	Overall test*	Post hoc †
IκBα_0 h	100.00	1.00	<0.0001	C
IκBα_0.5 h	214.00	6.00		B
IκBα_3 h	334.67	15.01		A

Fig. 4*B*

Group (MCF-7/DOX)	Mean	SD	Pr > |t| ‡
IκBα_siTG2_0	99.67	10.02	<0.0001
IκBα_siTG2_100nM	401.33	20.13	

Fig. 4*C*

Group (MDA-231)	Mean	SD	Overall test*	Post hoc †
IκBα_0 h	100.00	2.00	<0.0001	C
IκBα_0.5 h	153.00	7.55		B
IκBα_3 h	275.00	31.22		A

Fig. 4*D*

Group (MDA-231)	Mean	SD	Pr > |t| ‡
IκBα_siTG2_0	101.33	4.73	0.0104
IκBα_siTG2_100nM	332.33	42.50	

*ANOVA.
†Tukey procedure: same alphabets indicate no significant difference and different letters indicate significant difference adjusting for the overall FWE rate of 0.05.
‡Student's *t* test.

were added to each well. Plates were incubated at room temperature for 5 minutes, and then 3 μL of 1 mmol/L MUP fluorescent substrate were added and the plates were incubated for an additional 60 minutes in the dark at room temperature. The fluorescence of each sample was measured using a 96-well fluorescent plate reader (FluoroScan II, Lab Systems) equipped with a 360-nm wavelength excitation filter and a 460-nm wavelength emission filter. The promoter activity under each set of transfection conditions was expressed as a fold increase in fluorescence with respect to control vector–transfected cells. Data represent the average and SD of three independent experiments.

TGase 2 gene silencing by small interfering RNA. A small interfering RNA (siRNA) duplex targeting human TGase 2, 5′-AAGAGCGAGAUGAUCUG-GAAC-3′, was introduced into cells using LipofectAMINE 2000 according to the manufacturer's instructions. One day after transfection, complexes were added to the cells, they were removed, and cells were incubated in serum-free medium for an additional 72 hours. Cells were harvested, and cytosolic fractions were examined for TGase 2 and IκBα by Western blot analysis. Cells incubated with LipofectAMINE 2000 alone were used as the nontreated control. Data represent the average and SD of three independent experiments.

Western blotting. Protein samples (cytosolic fractions, prepared as described above) were applied to the wells of 4% to 12% gradient SDS gel, electrophoresed in Tricine buffer (Invitrogen), and then transferred to polyvinylidene difluoride membranes (Bio-Rad) using a semidry blotting apparatus (Hoefer SemiPhor). Membranes were blocked in TBS containing 0.5% Tween 20 (TBS-T) and 5% milk for 1 hour at room temperature and then washed twice for 20 minutes each in TBS-T. The membranes were then incubated with primary antibody overnight at 4°C. The primary antibodies used in these studies were the following: anti-IκBα (Cell Signaling Technology), phosphorylated IκBα (Ser^{32}; Cell Signaling Technology), TGase 2 (clone CUB 7402, NeoMarkers), and β-actin (Abcam). Membranes were washed twice for 20 minutes each in TBS-T and then incubated with horseradish peroxidase–conjugated secondary antibody (Bio-Rad) in TBS-T containing 1% bovine serum albumin for 1 hour at room temperature. The concentration of primary and secondary antibodies was 5 and 0.1 μg/mL, respectively. Proteins were visualized using enhanced chemiluminescence (Pierce). β-Actin was monitored as an internal control for protein levels.

Immunocytochemical analysis. The translocation of p65 was visualized by immunostaining and confocal microscopy. Cells were seeded in a two-well slide chamber (1×10^5 per well) 1 day before treatment. The next day, cells were treated with CTM (1 mmol/L) or R2 peptide (100 nmol/L; ref. 29) for 3 and 6 hours, respectively. Cells were incubated in 3.7% paraformaldehyde on ice for 15 minutes and then washed with 0.1% PBS-T. Cells were permeabilized using 0.5% PBS-T for 5 minutes, washed again in 0.1% PBS-T, and then incubated for 1 hour in 10% calf serum in PBS containing 0.5% gelatin followed by a final wash with 0.1% PBS-T. Fixed cells were incubated with mouse monoclonal anti-p65 antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature, washed with 0.1% PBS-T, and then incubated with a FITC-conjugated anti-mouse IgG (1:200 dilution; Jackson ImmunoResearch) for 1 hour at room temperature. The cells were washed with 0.1% PBS-T, incubated with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) nuclear dye for 5 minutes at room temperature, washed with 0.1% PBS-T again, and then treated with VectaShield (Vector Laboratories). NF-κB expression was visualized using a Zeiss Axiovert LSM510 microscope.

Statistical analysis. The NF-κB promoter activity in MCF-7 and MDA-468 cells with or without transient transfection of TGase 2 was compared using Student's *t* test. ANOVA was done to examine whether TGase 2 inhibition promoted chemosensitivity in breast cancer cells. After observing an overall significant difference in drug sensitivity among different conditions, we did a post hoc test for pairwise comparisons using the Tukey procedure controlling for the overall type I error. The Statistical Analysis System (SAS Institute, Inc., Cary, NC) procedures called 'PROC TTEST' and 'PROC ANOVA' were used. All the statistical tests were two sided. A $P < 0.05$ was considered as being statistically significant.

Results

NF-κB activation correlates with increased TGase 2 expression in breast cancer cell lines. We did Western blot analysis of IκBα in several different breast cancer cell lines: MDA-468, MDA-231, MCF-7, and MCF-7/DOX cells. We found that the level of free IκBα in the drug-resistant cell lines MDA-231 and MCF-7/DOX was as much as 10-fold lower than that in the drug-sensitive cell lines (Fig. 1*A*). Interestingly, the level of IκBα correlated inversely with TGase 2 protein levels in that TGase 2 was nearly undetectable in the drug-sensitive cell lines and present at significantly higher levels in MDA-231 and MCF-7/DOX cells (Fig. 1*A*). The decreased levels of IκBα implied that NF-κB was activated in the drug-resistant breast cancer cells. We examined NF-κB promoter-specific activity using the SEAP reporter assay in the cell lines to determine whether it correlated with TGase 2 levels. We found that NF-κB activity in MDA-231 and MCF-7/DOX cells was up to 2-fold higher than in the two drug-sensitive cell lines (Fig. 1*C*), and this increase in NF-κB activity correlated well with the level of TGase 2 expression. To examine the mechanism of IκBα depletion in drug-resistant cells, we did Western blot analysis using anti-phosphorylated IκBα antibodies. The phosphorylation level of IκBα was undetectable compared with that seen in the control lipopolysaccharide (LPS)-treated cells, indicating that the depletion of IκBα was not due to phosphorylation (Fig. 1*B*). We did an ANOVA test to examine whether there is an overall difference among groups and post hoc test (Tukey procedure) for pairwise comparisons. Interestingly, the increase in TGase 2 levels in doxorubicin-resistant cells was true regardless of EGFR expression levels, suggesting that TGase 2 is involved in the development of doxorubicin resistance independently of EGF signaling (Fig. 1*A* and *C*; Table 1).

NF-κB activity is regulated by TGase 2. To test whether TGase 2 expression increased NF-κB activity in drug-sensitive breast cancer cells, the NF-κB reporter assay was done in MCF-7 and MDA-468 cells following transient transfection of TGase 2 (Fig. 2*A* and *B*). NF-κB promoter activity in MCF-7 and MDA-468 cells was compared against these cells following transient transfection of TGase 2 (Fig. 2). NF-κB promoter activity significantly increased approximately 2.5- and 2-fold in MCF-7 and MDA-468 cells, respectively. In the reciprocal analysis, we examined whether TGase 2 inhibition suppressed NF-κB activity in drug-resistant breast cancer cells. MCF-7/DOX and MDA-231 cells were treated with the TGase inhibitor CTM (1 mmol/L) for 24 hours, after which the NF-κB reporter assay was done (Fig. 3*A* and *B*). We found that CTM treatment dramatically reduced NF-κB activity compared with untreated controls, resulting in approximately 2- and 3-fold decreases in MDA-231 and MCF-7/DOX cells, respectively.

Inhibition of TGase 2 increases the level of IκBα in drug-resistant breast cancer cells. To test whether TGase 2 inhibition effected NF-κB activation in the drug-resistant breast cancer cells, NF-κB activity was analyzed indirectly by Western blot analysis of IκBα following treatment of MCF-7/DOX and MDA-231 cells with 1 mmol/L CTM. Western blot analysis using anti-IκBα antibodies revealed a dramatic increase in free IκBα over time, up to 3-fold, on inhibition of TGase 2 in these cells (Fig. 4*A* and *C*). We also inhibited TGase using TGase 2–targeted siRNA and examined the effect on IκBα levels (Fig. 4*B* and *D*). MDA-231 or MCF-7/DOX was incubated with TGase 2–specific siRNA (100 nmol/L) for 72 hours. After incubation, the cytosolic cellular fractions were analyzed for changes in IκBα levels by Western blot. IκBα levels increased approximately 4- and 3-fold in MCF-7/DOX and MDA-231 cells, respectively, compared with control, nontransfected cells (Fig. 4*B* and *D*). We did an ANOVA test to examine whether there is an overall difference among groups and post hoc test (Tukey procedure) for pairwise comparisons. Interestingly TGase 2 inhibition rescued the level of cytosolic IκBα in MCF-7/DOX and MDA-231 cells (Fig. 4*A-D*; Table 2).

TGase 2 inhibition promotes apoptosis of drug-resistant breast cancer cells. To determine whether TGase 2 inhibition

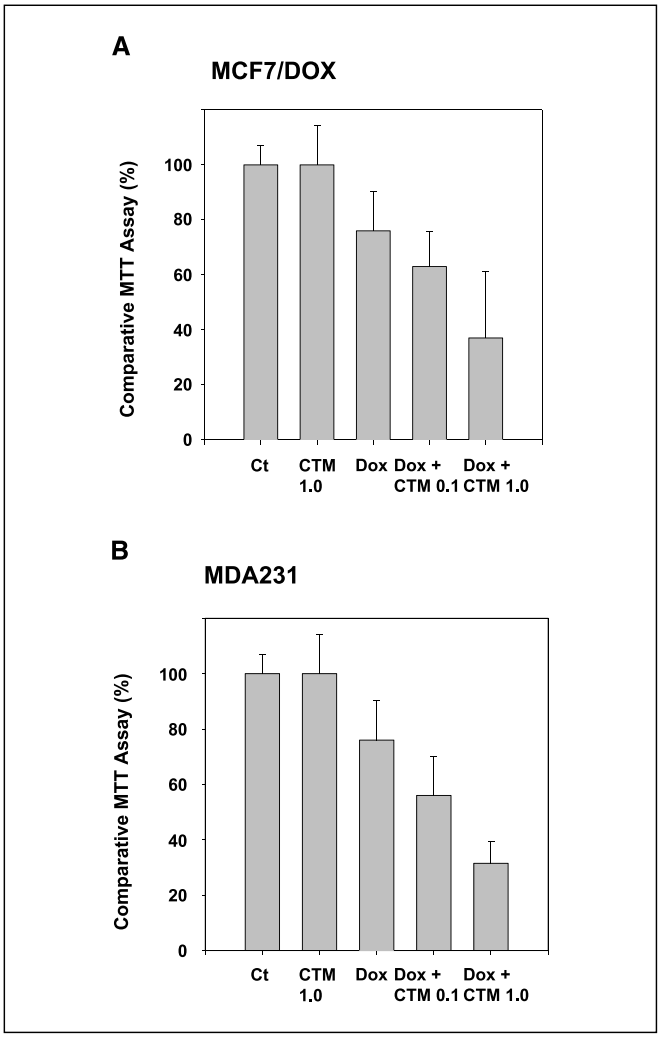

Figure 5. Effect of TGase 2 inhibition on the viability of MCF-7/DOX and MDA-231 cells treated with doxorubicin. MCF-7/DOX (*A*) and MDA-231 (*B*) cells were treated with doxorubicin (*Dox*) in the presence or absence of CTM (0.1 and 1.0 mmol/L) for 24 hours. After treatment, cell viability was measured using the MTT assay. Viability is expressed as percentage of control, DMSO-treated cells. *Columns,* mean of three independent experiments; *bars,* SD. ANOVA and a post hoc test were done for pairwise comparisons using the Tukey procedure controlling for the overall type I error (Table 3).

promoted chemosensitivity in breast cancer cells, we did a cell viability assay using MTT (Fig. 5*A* and *B*). The drug-resistant breast cancer cell lines MCF-7/DOX and MDA-231 were treated with doxorubicin for 48 hours in the presence or absence of CTM (0.1 and 1.0 mmol/L). Under conditions of doxorubicin treatment (5 μmol/L for MDA-231 cells and 90 μmol/L for MCF-7/DOX cells), we found that ~50% of the drug-resistant cells underwent apoptosis. Under similar conditions, there was 100% cell death in cultures of MCF-7 and MDA-468 cells (data not shown). We did an ANOVA test to examine whether there is an overall difference among groups and post hoc test (Tukey procedure) for pairwise comparisons. Interestingly TGase 2 inhibition significantly increased drug sensitivity up to 2-fold in both MCF-7/DOX and MDA-231 cells (Fig. 5*A* and *B*; Table 3).

Inhibition of TGase 2 reduces the level of nuclear NF-κB in drug-resistant beast cancer cells. Using immunocytochemistry of p65 to analyze the cellular location of NF-κB in drug-resistant cells, we found that there was an abundant level of p65 in the nuclei of MDA-231 and MCF-7/DOX cells (Fig. 6*A* and *B*). Because inhibition of TGase 2 was shown to increase the levels of IκBα in drug-resistant cells (Fig. 4*A* and *C*), we were interested in whether NF-κB was retained in the cytosol by TGase 2 inhibition. In cells treated with either CTM or the TGase 2 inhibitor peptide R2, there was a depletion of nuclear p65 and retention of the protein in the nucleus.

Discussion

In this study, we found that increased levels of TGase 2 correlated with drug resistance in breast cancer cells (Fig. 1). This observation agrees well with previously reported data (1, 2). Interestingly, the high level of TGase 2 in doxorubicin-resistant cells was independent of EGFR status (Fig. 1), suggesting that TGase 2 is involved in the development of doxorubicin resistance independently of EGF signaling. It has been shown that TGase 2 can be induced by EGF (3, 27). However, TGase 2 can also be activated or induced by a variety of chemical, physical, and biological stimuli (23). Because TGase 2 is a calcium-dependent enzyme, TGase 2 activity may also be elevated in response to factors that increase calcium uptake. We previously showed that TGase 2 activated NF-κB in microglia and neuroblastoma cells through IκBα polymerization (23). Here, we showed that introduction of TGase 2 into MCF-7 and MDA-468 cells, which are two drug-sensitive breast cancer cell lines, resulted in an increase in NF-κB activity using the SEAP reporter assay (Fig. 2). TGase 2 inhibition in drug-resistant MCF-7/DOX and MDA-231 cells resulted in a dramatic decrease in NF-κB activity (Fig. 3). These results clearly suggested that aberrant TGase 2 expression may be involved in the constitutive activation of NF-κB activation in drug-resistant breast cancer cell lines.

Reduction of TGase 2 activity in drug-resistant cells using either CTM treatment or TGase 2–targeted siRNA resulted in a dramatic increase in cytosolic levels of IκBα (Fig. 4), indicating that TGase 2 inhibition also resulted in the inactivation of NF-κB. This was supported by immunocytochemical analysis of p65, which showed that inhibition of TGase 2 resulted in the retention of NF-κB in the cytosol in drug-resistant cells. TGase 2 inhibition also increased the susceptibility of MDA-231 and MCF-7/DOX to doxorubicin (Fig. 5) possibly by a mechanism that involved down-regulation of NF-κB. We also found that IκBα phosphorylation was not increased in the drug-resistant cells, indicating that TGase 2–mediated NF-κB activation and drug resistance was mediated through IκBα polymerization (Figs. 1-5).

It has been shown that >40% of cell surface–associated integrins are associated with TGase 2 (20). Mehta et al. suggested that increased TGase 2 expression in MCF-7/DOX cells augmented the binding of integrins to extracellular matrix proteins (31). Increased expression of TGase 2 correlates with increased cell motility and adhesion (20, 32, 33), and cell adhesion induces activation of focal

Table 3. ANOVA for effect of TGase 2 inhibition on chemosensitivity in breast cancer cells

Fig. 5*B*

Group (MCF-7/DOX)	Mean	SD	Overall test*	Post hoc †	
CT	100.67	5.13	<0.0001	A	
CTM	100.33	10.02		A	
DOX1.0	75.67	10.02		B	
CTM0.1/DOX	55.67	10.02		B	
CTM1.0/DOX	31.33	5.51		C	

Fig. 5*B*

Group (MDA-231)	Mean	SD	Overall test*	Post hoc †	
CT	103.33	12.58	<0.0001	A	
CTM1.0	102.00	7.55		A	
DOX	78.33	6.11		A	B
CTM0.1/DOX	56.67	13.20		C	B
CTM1.0/DOX	32.33	10.60		C	

*ANOVA.
†Tukey procedure: same alphabets indicate no significant difference and different letters indicate significant difference adjusting for the overall FWE rate of 0.05.

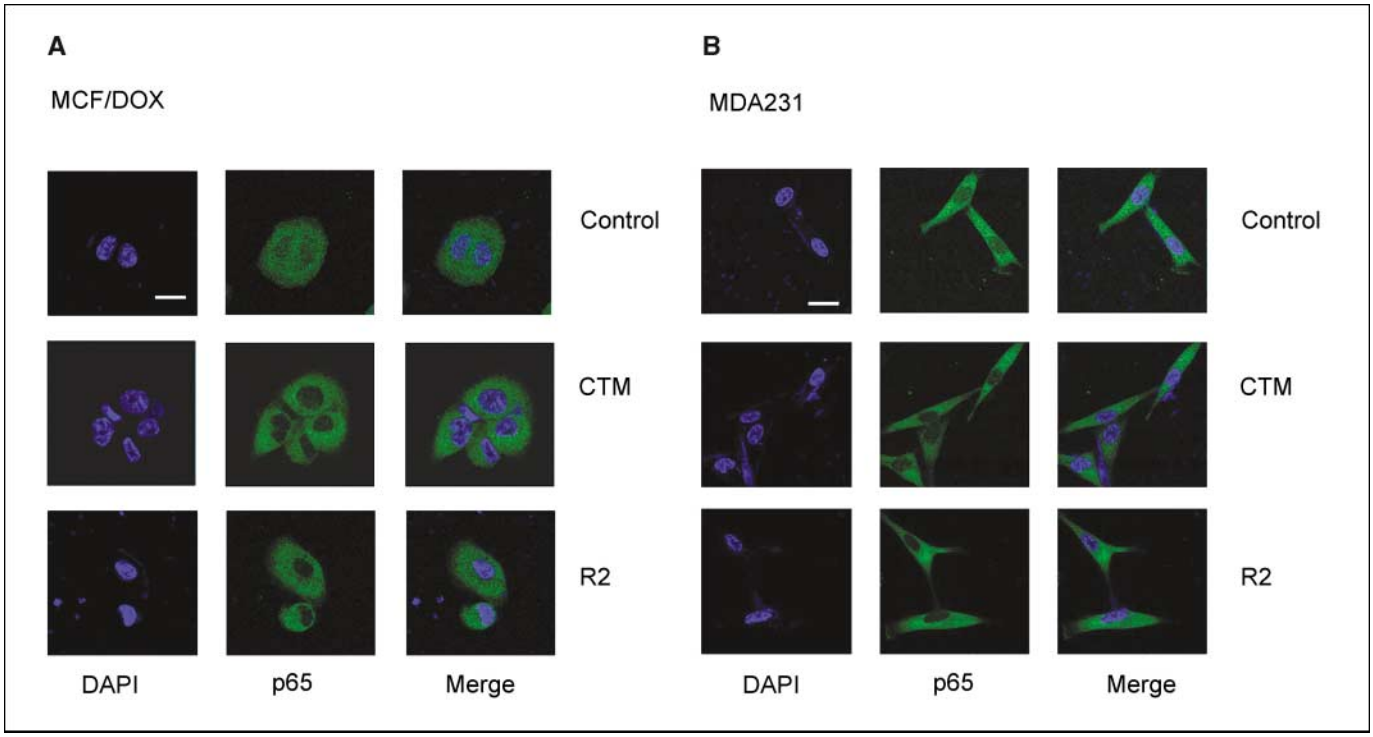

Figure 6. Effect of TGase 2 inhibition on NF-κB localization. *A* and *B*, immunocytochemical staining of NF-κB using anti-p65 antibody in MCF-7/DOX or MDA-231 cells revealed a scattered, spot-like staining pattern in the nucleus. MCF-7 or MDA-468 cells showed very little nuclear staining of p65 (data not shown). Cells were treated with CTM or R2 peptide for 3 and 6 hours, respectively. NF-κB translocated to the cytosol in both cell types under both conditions. DAPI staining was used as a nuclear counterstain. Bar, 20 μm.

adhesion kinase (FAK)-dependent signaling, which can lead to NF-κB activation (34). TGase 2 expression may be induced at least in part by FAK signaling (20); however, the role of FAK in NF-κB activation in breast cancer cells must be limited, as we observed constitutive activation of NF-κB in drug-resistant cells in the absence of IκBα phosphorylation (Fig. 1*B*).

Understanding the mechanisms of drug resistance is the key to developing new therapeutic options. Drug resistance is a complex process that involves hundreds of changes in gene expressions. To date, many types of proteins have been identified that are altered in drug-resistant cells, such as membrane proteins (35), proteins associated with glutathione transferases and detoxification (28), and proteins of the DNA repair system (36). From the perspective of transcriptional regulation, it has been shown that chemotherapy activates NF-κB, which results in resistance to apoptosis (37). The NF-κB family of transcription factors is ubiquitous in mammary carcinogenesis. NF-κB-associated pathways are linked not only to antiapoptosis in breast cancer cells but also to multiple survival pathways, including proliferation, invasion, angiogenesis, and metastasis (38). Based on these observations, inhibition of NF-κB is generally viewed as a promising new intervention in cancer therapy (39). Based on previous observations that TGase 2 was able to activate NF-κB, through depletion of free, cytosolic IκBα, we hypothesize that TGase 2 may be one of the genes involved in the induction of NF-κB activity in cancer cells (23). In the current study, we showed that TGase 2 inhibition promoted sensitivity to the chemotherapeutic drug doxorubicin and that this correlated with NF-κB inactivation, indicating that TGase 2 inhibition in breast cancer cells is an effective approach to promoting chemosensitivity. This is supported by the observation that the combined use of TGase inhibitors and chemotherapeutic agents decreased the size of glioblastoma tumors by 50% compared with treatment with chemotherapeutic agents (40). Given that expressions of TGase 2 and NF-κB can be induced by chemotherapeutic agents, this raises the possibility that TGase 2 inhibition in combination with NF-κB inhibition may result in increased sensitivity to chemotherapeutic drugs and represent a potentially useful therapeutic approach to the treatment of certain cancers (25, 41).

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