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Increase in transglutaminase 2 expression is associated with NF-κB activation in breast cancer tissues

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TABLE OF CONTENTS

1. ABSTRACT

Activation of NF-κB is reported in breast cancers. NF-κB inhibition in breast cancer cell lines results in an increase in apoptosis. However, the reason for continuous activation of this transcription factor in breast cancer is currently unclear. Interestingly, elevated transglutaminase 2 (TGase 2) expression is additionally observed in breast cancer. Recent studies showed that doxorubicin-resistant cells contain a higher level of TGase 2, compared to doxorubicin-sensitive cells. Moreover, increasing the level of TGase 2 in breast cancer cells by transient transfection leads to decreased expression of the inhibitory subunit of NF-κB (I-κBα), and up regulation of NF-κB activity. Our data show that TGase 2 expression is inversely correlated with the level of I-κBα in breast tumors, implying that the enzyme is responsible for the constitutive activation of NF-κB in breast tumors.

2. INTRODUCION

Transglutaminase 2 (TGase 2, E.C. 2.3.2.13) expression is observed in breast cancer cell lines (1,2). TGase 2 catalyzes a cross-link between peptide bound-glutamine and ε–lysine residues (3), and participates in the pathogenesis of diverse diseases (4). Costatini and colleagues reported increased accumulation of fibrinogen throughout the connective tissue in breast cancer, but not in non-malignant tissues. The group suggested that abundant fibrinogen present in the tumor contributes to the structural integrity of breast tumor tissues (5). Mehta *et al. (*6,7) showed that TGase 2 expression concomitantly increases with drug resistance in breast cancer. It is possible that the rise in TGase 2 contributes to extracellular matrix (ECM) integrity, leading to the inhibition of cancer cell metastasis, as well as anti-apoptosis (8). However, the mechanisms of anti-apoptosis associated with TGase 2 and ECM remain to be established.

NF-κB promotes the survival of cells and plays a role in neoplastic transformation (9). Resistance to the apoptotic effect of chemotherapeutic agents, such as taxol, doxorubicin, tamoxifen, and cisplatin, is linked to NF- κB activation in cancer cells (10). Interestingly, inhibition of NF-κB enhances the sensitivity of tumor cells to apoptosis induced by chemotherapeutic agents (11). However, continuous activation of NF-κB in breast cancer is not due to continuous stimulation of IKKs (12). Recent studies by our group demonstrate that TGase 2 is involved in activating the transcription factor, NF-κB, via I-κBα polymerization (4,13). If TGase 2 is responsible for the continuous activation of NF-κB in breast tumors, its expression should be inversely correlated with that of I-κBα, regardless of any other factors involved, including estrogen receptor (ER) or progesterone receptor (PR). In this report, we examine whether TGase 2 expression is associated with NF-κB activation without phosphorylation in various breast tumors that has been observed in breast cancer cells in the previous study (14).

3. MATERIALS AND METHODS

3.1. Human tissue samples

Human breast cancer samples were obtained from the Tissue bank of the National Cancer Center, Korea. From 2003 to 2004, patients with primary breast cancer were enrolled with informed consent using an IRB-approved protocol. Tissues (malignant and normal tissue pairs) were collected during surgery from seven patients and cut into two parts. Tissue was flash frozen over liquid nitrogen and stored at -80 ºC. The other half was fixed in buffered 10% formalin and paraffin-embedded for histological examination. Breast cancers were classified as infiltrating ductal carcinoma according to standard histopathological criteria. Tissues obtained from the most distant region of the tumor from the same patients that were macroscopically and histopathologically confirmed to be normal tissue. In the so-called 'normal tissue', there were neither cellular nor displastic changes observed. Samples #1 (ER-positive/PR-positive, stage I), #2 (ER-positive/PR-positive, stage IIA), #3 (ER-positive/PR-negative, stage I), #4 (ER-positive/PR-negative, stage I), #5 (ER-negative/PR-positive, stage I), #6 (ER-negative/PR-negative, stage I), and #7 (ER-negative/PR-negative, stage IIIA) were employed in this study.

3.2. Isolation of cytoplasm

Cytoplasmic cellular fractions were prepared using a CelLytic^{TM} NuCLEAR^{TM} Extraction Kit (Sigma, St. Louis, MO). Briefly, 100 mg of breast tissue was homogenized using a glass tissue homogenizer in 1 ml of cellular lysis buffer containing dithiothreitol (DTT) and protease inhibitors, and incubated on ice for 15 min. After lysis, samples were centrifuged at 11,000 x g for 20 min. We collected the supernatant containing cytoplasmic fractions. Protein concentration was determined with the Coomassie Plus protein assay^{TM} reagent (Pierce, Rockford, NY).

3.3. Western Blotting

Protein samples (cytosolic fractions prepared as described above) were applied to the wells of 4-12% gradient SDS gel, electrophoresed in Tricine buffer (Invitrogen, Carlsbad, CA), and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) using a semidry blotting apparatus (Hoefer SemiPhor, San Francisco, CA). Membranes were blocked in Tris-buffered saline containing 0.5% Tween 20 (TBS-T) and 5% milk for 1 h at room temperature, washed three times for 20 min each in TBS-T, and incubated with the required primary antibody overnight at 4^{o}C. The primary antibodies, anti-isopeptide (GGEL) (anti-mouse, 814-MAM, CovalAb, Lyon, France), anti-I-κBα (Cell Signaling Technologies, Danvers, MA), anti-phospho-I-κBα (Ser32) (Cell Signaling Technologies, Danvers, MA), monoclonal anti-TGase 2 (clone CUB 7402, NeoMarkers, Fremont, CA), anti-β-actin (Abcam, Cambridge, MA), monoclonal anti-isopeptide (GGEL) (CovalAb, Villeurbanne, France), anti-BCl_2 (Santa Cruz, Santa Cruz, CA), and anti-BAX (Santa Cruz, Santa Cruz, CA), were employed. Next, membranes were washed three times for 20 min each in TBS-T, and incubated with HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA) in TBS-T containing 1% BSA for 1 h at room temperature. Primary and secondary antibody concentrations were 5 and 0.1 μg/ml, respectively. Proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, NY). β-actin was monitored as an internal control for protein expression.

3.4. Positive control for 814-MAM antibody recognition of GGEL linkages

In vitro cross-linked fibrinogen was used as a positive control. Purified fibrinogen (2 μg) was incubated with either 10^{-4} U or 10^{-3} U of guinea pig liver TGase 2 for 1 h at 37ºC in 20 μl of Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$. After incubation, one half of the sample (10 μl) was analyzed by Western blotting for GGEL cross-links and the other half (10 μl) was analyzed by SDS-PAGE and Coomassie Blue staining for protein bands. BSA (2 μg) was used as a negative control.

3.5. TGase activity assay

We employed a modified TGase activity assay measuring the incorporation of (1,4-^{14}C)putrescine into succinylated casein (3). Samples were added to a reaction mixture (0.5 ml) containing 0.1 M Tris–acetate, pH 7.5, 1% succinylated casein, 1 mM EDTA, 10 mM $CaCl_2$, 0.5% lubrol PX, 5 mM DTT, 0.15 M NaCl, and 0.5 mCi (1,4-^{14}C)putrescine (Dupont-New England Nuclear; 118 Ci/mol). Following incubation at 37°C for 1 h, the reaction was terminated by addition of 4.5 ml cold 7.5% TCA (4°C). TCA-insoluble precipitates were collected on a GF/A glass fiber filter, washed with cold 5% TCA, dried, and counted in a scintillation counter.

3.6. Statistical Analysis

The TGase activity in control and cancer tissues was compared using Student's t-test in Figure 1. Western blottings of I-κBα, TGase 2, BCl_2, and BAX were compared using Student's t-test in Figs 2 and 3. A p-value less than 0.05 was considered as being statistically significant. Correlalation coefficiency was calculated between level of I-κBα and TGase 2 activity in breast

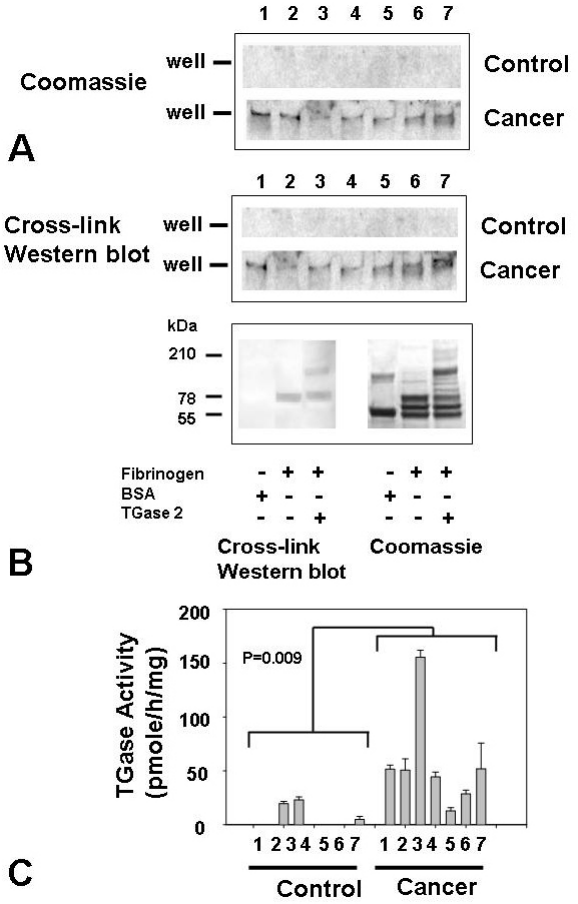

Figure 1. Increase in high molecular weight (HMW) protein expression and TGase 2 activity in breast cancer tissues. (a) HMW proteins were significantly increased in cancer tissues. The cytosolic fractions of breast tumors were separated by 10% SDS-PAGE, and stained with Coomassie brilliant blue. HMW proteins that could not be separated from the stacking gel remained in the well, which appear to contain TGase-mediated cross-links detected by GGEL antibody. (b) Positive controls for (a). Lane 1 contained 2 μg of BSA as a negative a control. Lane 2 contained 2 μg of fibrinogen incubated in the absence of TGase 2. Lane 3 contains fibrinogen incubated with 10^{-3} U of guinea pig TGase 2. (c) Cytosolic TGase activity was elevated in breast tumors, compared to the control. Data represent the means ±SD of three independent experiments; Student's t-test resulted in less than 0.009 between groups.

cancer cases by use of Microsoft Excel program. The value less than –0.5 was considered as being statistical significance.

4. RESULTS

4.1. Increase in TGase and high molecular weight protein levels in breast tumors

Previously, others and we reported elevated TGase 2 expression and activity in drug-resistant cells (14). Moreover, immunoblotting using an iso-peptide specific antibody revealed an abundance of high molecular weight (HMW) proteins in doxorubicin-resistant breast cancer cell lines (MCF-7/DOX and MDA231), compared to drug-sensitive cell lines (data not shown). The TGase 2 cross-link is responsible for increasing HMW protein expression in drug-resistant breast cancer cells (14). Notably, HMW protein levels were dramatically reduced after treatment with a TGase inhibitor (data not shown). Therefore, upregulation of HMW protein expression is closely correlated with breast cancer. In our experiments, HMW protein levels were enhanced, together with TGase 2 activity (Figure 1C) in breast cancers concomitantly with increase of cross-link (Figure 1A). Specifically, TGase activity was increased up to 100-fold, compared to the control. *In vitro* cross-linked fibrinogen was used as a positive control (Figure 1B).

4.2. Depletion of free I-κBα correlates with increased TGase 2 expression in breast tumors

The I-κBα level was particularly high in the control group (normal pair) of samples (Figure 2). However, I-κBα in cancer tissues was significantly decreased (Figure 2). We examined whether the decrease in I-κBα was associated with breast cancers. Most breast cancer cases displayed NF-κB activation, regardless of the estrogen receptor or PR level (method section). Interestingly, the I-κBα level was decreased about 2- to 10-fold in the cancer group, and correlated inversely with TGase 2 expression in breast tumors. Cancer patients #1~3 displayed relatively higher TGase expression, and corresponding relatively lower I-κBα level on Western blots, compared to the other patients (Figure 2). Moreover, phosphorylated I-κBα was not detected in breast tumors (data not shown), supporting the theory that increased TGase 2 expression leads to the loss of free I-κBα. Correlation coefficiency was calculated between level of I-κBα and TGase activity in cancer patients. The result was –0.59, which represents inverse correlation between two groups supported by meaningful p value in each group of I-κBα level and TGase activity.

4.3. Increased NF-κB activity in breast tumors

Electrophoretic mobility shift assay (EMSA) reactions were performed with nuclear extractions from controls or tumors using a double stranded consensus oligonucleotide for NF-κB end-labeled with (^{32}P)ATP (Figure 3). Gel-shift showed about 3-fold increase of NF-κB in breast tumors. This result complies with the decrease of I-κBα in Figure 2. Positive control was employed using breast cancer cell line (MDA 231) treated with TNF-α (50 ng/ml) for 30 min.

4.4. Decrease in the BCl_2/BAX ratio in breast tumors

The balance between the anti-apoptotic gene, *bcl-2*, and the pro-apoptotic gene, *bax*, is a good indicator of apoptosis (15). An increase in the Bcl-2/Bax ratio in breast cancer tissues (up to 4-fold; Figure 4) was observed, compared to the control group. Interestingly, NF-κB activation triggers Bcl-2 expression (16), resulting in decreased cell death.

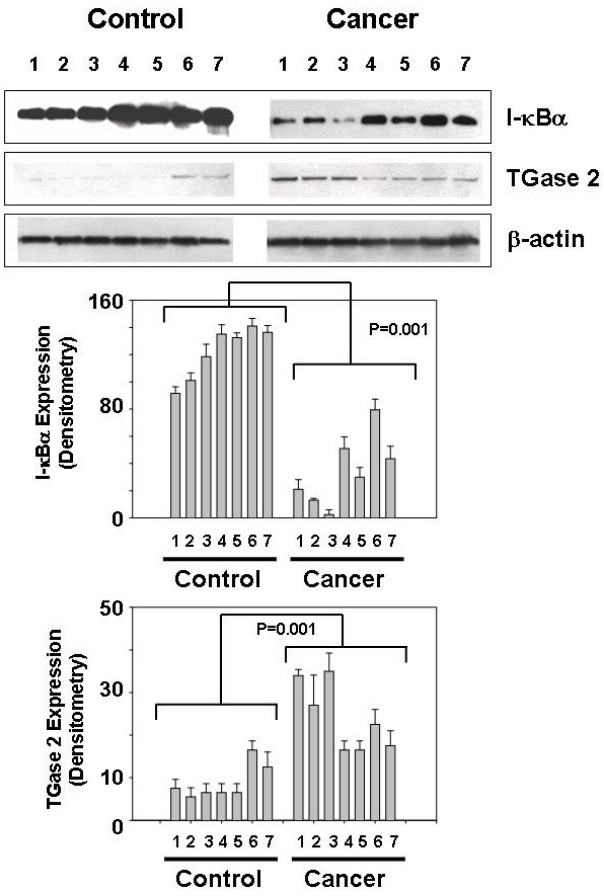

Figure 2. Inverse correlation of TGase 2 and I-κBα expression in breast tumors. Western blot analyses revealed a dramatic decrease in the I-κBα level, and a corresponding increase in TGase 2 in breast cancer tissues. β-actin was employed as the internal control. Data represent the means ±SD of three independent experiments; Student's t-test resulted in less than 0.001 between groups both in I-κBα and TGase 2.

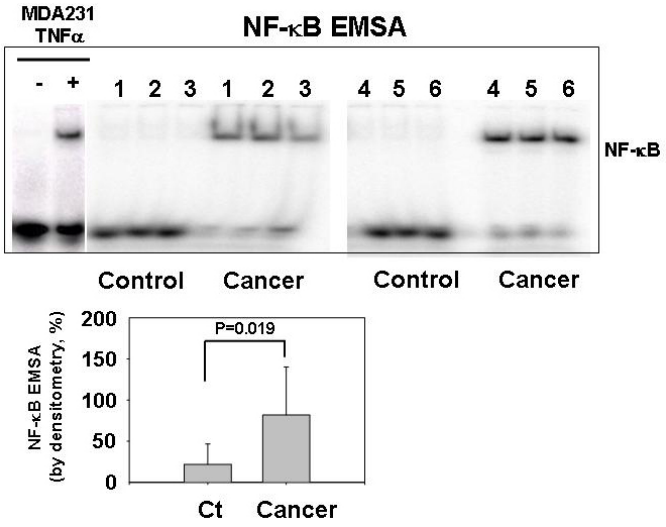

Figure 3. NF-κB activation in breast tumors. EMSA was performed with nuclear fractions of breast tumors and controls. Binding reactions were performed with nuclear extractions from controls or cancer tumors using a double stranded consensus oligonucleotide for NF-κB end-labeled with (^{32}P)ATP. A gel-shift assay showed about 3-fold increase of NF-κB in breast tumors. Positive control was employed using breast cancer cell line (MDA 231) treated with 50 ng/ml TNF-α for 5 hr. Data represent the means ±SD of three independent experiments; Student's t-test resulted in 0.019 between groups.

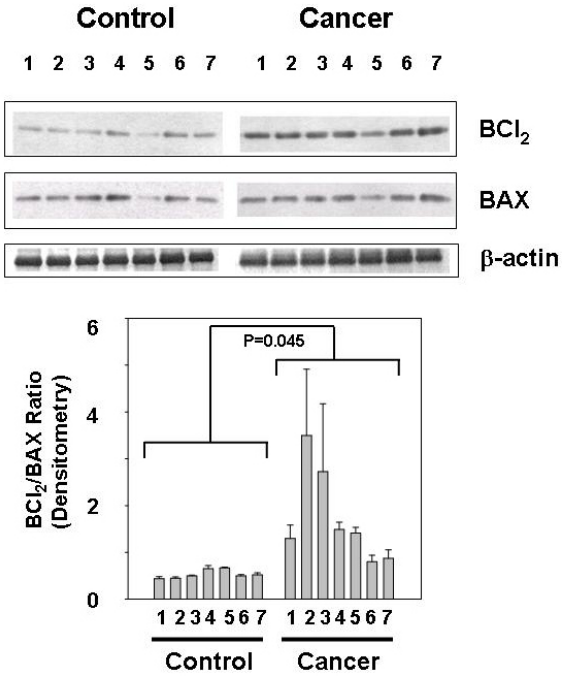

Figure 4. Bcl-2 and Bax expression in breast tumors. Western blot analyses disclosed an increase in Bcl-2 in breast tumors. The high Bcl-2 and Bax ratio indicates prevalence of Bcl-2 in breast cancer. Bcl-2 expression was induced by NF-κB activation. Data represent the means ±SD of three independent experiments; Student's t-test resulted in 0.045 between groups.

5. DISCUSSION

TGase 2 expression is upregulated during apoptosis, and appears to play a critical role in this process (17). In some cases, TGase 2 itself may be insufficient for triggering apoptosis, but an increase in expression may sensitize cells to apoptotic stimuli (18). Interestingly, TGase 2 expression is also closely related to anti-apoptosis (19). It is possible that elevated TGase 2 expression balances the promotion of apoptosis in response to apoptotic stress and cell survival in anti-cancer drug chemical stress. Several possible pathways for the anti-apoptotic mechanism of TGase 2 are suggested, including protection against the degradation of tumor suppressor protein p110 Rb (20), inhibition of caspase 3 (2), and promotion of cell adhesion for survival signaling pathways by activation of integrins on the cell surface. Indeed, TGase 2 plays an important role in the formation of ECM (22), which may contribute to enhanced cell survival in breast tumors. Cells must suffer serious damage from toxic protein aggregates when HMW proteins may not be cleared and accumulated during growth. However, we found that the prepared cellular extracts contained nearly equal amounts of HMW proteins regardless of tumor tissues. This suggests that HMW proteins are balanced in a steady state between continuous formation and degradation. Therefore, pohysiological specific role of high molecular weight (HMW) prorteins remains to be elucidated.

Mehta *et al.* (1,2) showed that increased TGase 2 levels are associated with drug resistance in invasive and metastatic breast cancer cells. In invasive tumors, the TGase 2 antigen no longer localizes to the normal tissue boundary, but is dispersed around the tumor cells. The upregulation of TGase 2 in intraductal and invasive human breast cancer and its localization to the ECM and neovasculature indicate a role in the regulation of tumor growth and metastasis (2), possibly via an indirect mechanism.

The transcription factor, NF-κB, plays a critical role in maintaining tumor growth and metastasis (23). Factors that induce constitutive NF-κB activation have been identified in previous analyses. In our experiments, no increase in I-κBα phosphorylation was evident in breast tumors (data not shown). Thus, it is likely that continuous activation of NF-κB in tumors does not occur via the canonical pathway. However, the possibility of rapid degradation of phosphorylated I-κBα during tumor biopsy cannot be excluded. TGase 2 is capable of activating NF-κB through depletion of free I-κBα. Specifically, TGase 2 polymerizes I-κBα without kinase activation, resulting in decreased I-kBα levels. Theoretically NF-κB is activated continuously concomitant with TGase 2 expression. Moreover, TGase 2 expression is inversely correlated with that of I-κBα (Figs. 1 and 2). This remarkable finding strongly supports the role of TGase 2 as an NF-κB activator in breast tumors. Bcl-2, a cell survivor factor that acts by inhibiting apoptosis, is upregulated in breast cancers (Figure 3). The protein is induced following NF-κB activation (16). Therefore continuous activation of NF-κB may stimulate the increase in Bcl-2 in breast cancers.

To assess the clinical effects of TGase 2 expression, more breast cancer cases require investigation. While our data are not statistically significant, they are consistent with previous findings in cancer cell lines. Expression of TGase 2 and NF-κB can be induced by chemotherapeutic agents (24). Generally it has to be feedback by induction of inhibitors such as I-κBα (23). However, failure of homeostasis between TGase 2 and NF-κB may exacerbate cancer pathogenesis. While the mechanism responsible for constitutive activation of TGase 2 in breast tumors is unknown, our findings strongly indicate that combined inhibition of TGase 2 and NF-κB results in increased sensitivity to chemotherapeutic drugs, and thus represents a potentially useful therapeutic approach for breast cancer treatment.

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Abbreviations: TGase : transglutaminase; NF-κB: nuclear factor-κB; I-κBα**:** inhibitory subuniț of NF-κB

Key Words: Transglutaminase, Breast Cancer, I-kappaBalpha, NF-kappaB, Review

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