Overexpression of Tissue Transglutaminase Leads to Constitutive Activation of Nuclear Factor-κB in Cancer Cells: Delineation of a Novel Pathway

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Abstract
The transcription factor nuclear factor-κB (NF-κB) plays an important role in regulating cell growth, apoptosis, and metastatic functions. Constitutive activation of NF-κB has been observed in various cancers; however, molecular mechanisms resulting in such activation remain elusive. Based on our previous results showing that drug-resistant and metastatic cancer cells have high levels of tissue transglutaminase (TG2) expression and that this expression can confer chemoresistance to certain types of cancer cells, we hypothesized that TG2 contributes to constitutive activation of NF-κB. Numerous lines of evidence showed that overexpression of TG2 is linked with constitutive activation of NF-κB. Tumor cells with overexpression of TG2 exhibited increased levels of constitutively active NF-κB. Activation of TG2 led to activation of NF-κB; conversely, inhibition of TG2 activity inhibited activation of NF-κB. Similarly, ectopic expression of TG2 caused activation of NF-κB, and inhibition of expression of TG2 by small interfering RNA abolished the activation of NF-κB. Our results further indicated that constitutive NF-κB reporter activity in pancreatic cancer cells is not affected by dominant-negative IκBα. Additionally, coimmunoprecipitation and confocal microscopy showed that IκBα is physically associated with TG2. Lastly, immunohistochemical analysis of pancreatic ductal carcinoma samples obtained from 61 patients further supported a strong correlation between TG2 expression and NF-κB activation/overexpression (P = 0.0098, Fisher’s exact test). We conclude that TG2 induces constitutive activation of NF-κB in tumor cells via a novel pathway that is most likely independent of IκBα kinase. Therefore, TG2 may be an attractive alternate target for inhibiting constitutive NF-κB activation and rendering cancer cells sensitive to anticancer therapies. (Cancer Res 2006; 66(17): 8788-95)

Introduction
Tissue transglutaminase (TG2; EC 2.3.2.13) is an important member of the transglutaminase family of enzymes that catalyze Ca2+–dependent post-translational modification of proteins by inserting irreversible ε(γ-glutamyl)lysine cross-links between sub-

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doi:10.1158/0008-5472.CAN-06-1457
tumors and tumor cell lines, we hypothesized that TG2 plays a role in the constitutive activation of NF-κB. In the present study, we found evidence that high expression of TG2 in various types of cancer cells is associated with constitutive activation of NF-κB. We showed that inhibition of TG2 activity by synthetic inhibitors or small interfering RNA (siRNA) inhibits the constitutive activation of NF-κB. Moreover, we observed a direct association between TG2 and the IκBα/p65/p50 complex and cross-linked forms of IκBα in TG2-expressing cells. Immunohistochemical analysis of tumor samples obtained from 61 patients with pancreatic ductal adenocarcinoma revealed a strong correlation between TG2 expression and NF-κB activation. Overall, our results suggest that expression of TG2 results in constitutive activation of NF-κB via a novel IκB kinase (IKK)–independent pathway and that TG2 therefore can serve as a good alternative target for inhibiting NF-κB activation and sensitizing cancer cells to anticancer therapies.

**Materials and Methods**

**Cell lines and patient samples.** High TG2-expressing (MDA-MB-231/cl.16) and low TG2-expressing (MDA-MB-231/cl.9) breast cancer sublines were established from the parental MDA-MB-231 cell line as described previously (12). Drug-sensitive (MCF-7/WT) and drug-resistant (MCF-7/DOX) breast cancer cell lines were also established as described previously (27). Primary (WM35; radial growth phase) and metastatic (A375; vertical growth phase) melanoma cell lines were provided by Dr. Suhendan Ekmekcioglu (The University of Texas M.D. Anderson Cancer Center) and well-differentiated (BxPC3) and metastatic (Panc28) pancreatic adenocarcinoma cell lines were provided by Dr. Shrikanth A. Reddy (The University of Texas M.D. Anderson Cancer Center). All of the cell lines were maintained in the log phase of cell growth by culturing in RPMI 1640 or DMEM/F-12 of Texas M.D. Anderson Cancer Center. All of the cell lines were established from the parental MDA-MB-231 cell line as described previously (30) and were provided by Dr. Suhendan Ekmekcioglu (The University of Texas M.D. Anderson Cancer Center).

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**Immunohistochemistry.** The levels of TG2 and NF-κB expression in patient tumor samples were evaluated using an indirect immunoperoxidase procedure (ABC-Elite, Vector Laboratories, Burlingame, CA). In brief, antigen retrieval was done by treatment of tissue sections in a steamer for 30 minutes. Antibodies against TG2 (NeoMarkers, Fremont, CA) and the p65 subunit of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 0.5 and 10 μg/mL, respectively, at 4°C for 16 hours. Secondary antibody incubation was done at room temperature for 1 hour. Nuclear staining was done using Mayer’s hematoxylin as a counterstain. Immunohistochemical staining results were evaluated independently by laboratory personnel and a pathologist. TG2 expression was categorized as low (negative or weak cytoplasmic staining in tumor cells) or high (diffuse moderate to strong cytoplasmic staining in tumor cells). The staining results for the p65 subunit of NF-κB were categorized as negative (negative or strong cytoplasmic staining in <10% of the tumor cells) or positive (strong cytoplasmic staining in >10% of the tumor cells). Statistical analysis was done using the Fisher’s exact test.

**TG2 enzymatic activity.** The level of TG2 enzymatic activity was determined using Ca²⁺–dependent incorporation of [³H]putrescine into dimethylcasein as described previously (30). The activity was expressed as nanomoles of putrescine incorporated per hour per milligram of total cell protein.

TG2 activity in intact cells was examined by preincubating cells (at 80% confluence) with 1 mmol/L 5-biotinamido-pentylamine (BPA; Pierce Biotechnology, Rockford, IL) overnight at 37°C in RPMI 1640 containing 2% FCS. To induce activation of endogenous TG2, cells were treated with calcium ionophore A23187 (2 μmol/L for 4 hours). After the incubation, cells were washed and lysed using sonication in 500 μL Tris-HCl [20 mmol/L (pH 7.4) containing 1 mmol/L EDTA, 150 mmol/L NaCl, 14 mmol/L 2-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride]. Equal amounts of cell lysates (30 μg protein) were fractionated using SDS-PAGE.

**Figure 1.** Expression of TG2 correlates with constitutive activation of NF-κB in tumor cells. A, EMSA was done with the nuclear extracts prepared from noninvasive (MDA-MB-231/cl.9) and invasive (MDA-MB-231/cl.16) breast cancer cell lines, drug-sensitive (MCF-7/WT) and drug-resistant (MCF-7/DOX) breast cancer cell lines, early-stage (WM35) and late-stage (A375) malignant melanoma cell lines, and well-differentiated (BxPC3) and highly aggressive (Panc28) pancreatic cancer cell lines. B, Western blot analysis of the TG2 expression in the eight cancer cell lines. C, comparison of TG2 activity and constitutive NF-κB activation in the eight cancer cell lines. D, supershift analysis of NF-κB using EMSA with the nuclear extracts prepared from Panc28 cells. Nuclear extracts were incubated with an anti-p65 antibody, an anti-p50 antibody, or nonradioactive (cold) or mutant NF-κB oligonucleotides and then examined for DNA binding. Representative experiments repeated at least three with similar results.
on an 8% gel and electrophoretically transferred onto a nitrocellulose membrane. The membrane was probed with horseradish peroxidase (HRP)-conjugated streptavidin (Amersham, Piscataway, NJ) and with an enhanced chemiluminescence (ECL) reagent (Amerham).

Immunoblotting and immunoprecipitation. Cell lysate proteins (30 μg) were separated using SDS-PAGE on a 7.5% gel and electrophoretically transferred onto a nitrocellulose membrane. The membrane was probed with either an anti-TG2, anti-p65 (Santa Cruz Biotechnology), anti-p-ERK (Santa Cruz Biotechnology), or anti-Bcl-xL antibody. Antibody-antibody reactions were detected using an ECL detection system. All of the membranes were stripped and reprobed with an anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:4000 to ensure even loading of proteins in different lanes.

To immunoprecipitate the protein of interest, 500 μg cell lysate [in 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40] was precleared by incubation with 50 μl TruBlot anti-mouse or anti-rabbit IgG-coated beads (eBioscience, San Diego, CA). The primary antibody (5 μg) was then added to the precleared cell lysate and incubated for 1 hour at 4°C. Immune complexes were recovered by incubation with the TruBlot anti-mouse or anti-rabbit IgG-coated beads overnight at 4°C. At the end of the incubation period, the pellets were washed four times in ice-cold TBS (50 mmol/L, pH 8.0) and resuspended in 1× loading buffer (50 μL). The immunoprecipitates were analyzed using SDS-PAGE followed by immunoblotting and autoradiography.

NF-κB activation. To assess NF-κB activation, electrophoretic mobility shift assay (EMSA) was done as described previously (31). Briefly, nuclear extracts prepared from cells (1 × 10⁶/mL) were incubated with a 3²P end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg protein with 16 fmol DNA) from the HIV long terminal repeat, 5’TGTGTTACAAGG-CAGTTCGCTGGGACCTTTCCAGGGAGGCGTGG-3’ (boldface indicates NF-κB-binding sites), for 30 minutes at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5’TGTGTTACAACCCTTCCAGGGAGGCGTGG-3’, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined using competition with the unlabeled and mutated oligonucleotides. For supershift assays, nuclear extracts were incubated with antibodies against either the p50 or the p65 subunit of NF-κB for 30 minutes at 37°C before the complex was analyzed using EMSA.

NF-κB-dependent reporter gene expression assay. NF-κB-dependent reporter gene transcription was analyzed using secretory alkaline phosphatase (SEAP) assay as described previously (31). Briefly, Panc28 and MCF-7 cells (5 × 10⁶/well in a six-well plate) were transiently transfected by the calcium phosphate method in a 1 mL medium containing 0.5 μg NF-κB promoter DNA linked to the heat-stable SEAP gene or with the control plasmid pcMV-FLAG1 DNA. Transfected cells were either left untreated or treated with 1 μmol/L A23187 (Sigma-Aldrich), 1 mmol/L BPA, or 1 mmol/L tumor necrosis factor-α (TNF-α; Genentech, South San Francisco, CA). Twenty-four hours after treatment, the conditioned medium was removed and assayed for SEAP activity according to the manufacturer’s instructions (Clontech Laboratories, Mountain View, CA) using an automated microplate reader (Perkin-Elmer, Wellesley, MA). In some experiments, cells were cotransfected with dominant-negative construct of IκBα (DN-IκBα) along with NF-κB reporter gene.

To inhibit endogenous TG2 expression, we designed two siRNA duplexes to target the coding sequence of human TG2 mRNA and were synthesized by Qiagen Sciences (Germantown, MD). The target sequence is 5’-AAGGCGCTTTGTTTCCACTAAAG-3’ for TG2 siRNA1 and is 5’-AAGGGC-GAACCCACCTGAAACA-3’ for siRNA2. Scrambled siRNA, in which the sequence was scrambled but the nucleotide composition was the same as that in the siRNA1, was used in parallel as a control. For transfections, cells at 70% confluence in six-well plates were incubated with 5 μL volume of TG2-specific or scrambled (control) siRNA containing 30 μL RNAiFect reagent (Qiagen Sciences) in 2 mL total volume made with serum-containing RPMI 1640. After 48 hours of transfection, cells were washed and collected to measure NF-κB activation using EMSA or TG2 expression using immunoblotting and enzymatic activity.

Confocal microscopy. To determine colocalization of TG2 and p65, Panc28 cells were grown on glass coverslips and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Fixed cells were then incubated with the primary antibodies overnight, washed with PBS, and incubated again with the secondary antibodies conjugated with either Alexa 546 (red) or Alexa 488 (green; Molecular Probes, Eugene, OR). The DNA dye Topro-3 (Molecular Probes) was used to costain the nuclei (blue). Cells treated with the secondary antibodies alone were used as controls. Confocal scanning analysis of the cells was done with a Zeiss (Thornwood, NY) laser scanning confocal microscope or with an Olympus (Levville, NY) Fluoview 300 confocal microscope in accordance with established method using sequential laser excitation to minimize the fluorescent emission bleed-through. Each section was examined for the presence of each stain at two excitations (546 nm and 488 or 633 nm as indicated in the text), and the data were compared pixel by pixel. Each image represented 2-sections at the same cellular level and magnification; a three-dimensional reconstructed image was used to visualize the whole sample. Merging red and green showed colocalization of two proteins giving yellow color.

TG2 adenosine generation. An adenosine containing a TG2 cDNA construct was provided by Dr. Ugra Singh (Texas A&M University System Health Science Center College of Medicine, Temple, TX; ref. 32). Briefly, TG2 cDNA cloned into a pcDNA3.1 vector was first subcloned into a psuttle vector and then in a BD adenoX adenoaviral vector (BD Biosciences, Palo Alto, CA). HEK293 cells were transfected with a recombinant adenoaviral plasmid for packaging of adenoaviral particles. The adenosine was purified on a C18 gradient and used at 25 multiplicities of infection. Cells infected with the lacZ adenosine were used controls.

Statistical analysis. Unless otherwise stated, all experiments were conducted independently thrice and the data reported are as mean ± SD.

**Figure 2.** Activation and overexpression of TG2 lead to activation of NF-κB in tumor cells. A, level of constitutive and inducible (calcium ionophore A23187-mediated) activation of endogenous TG2 was determined according to BPA conjugation with cellular proteins as described in Materials and Methods. B, EMSA showing NF-κB activation in BxPc3 and Panc28 cells in response to treatment with A23187 (24 hours at a concentration of 1 or 2 nmol/L). MCF-7/WT cells that lacked TG2 expression failed to show any constitutive or A23187-induced NF-κB activation. C, TG2 overexpression causes NF-κB activation. EMSA (top) and Western blot (bottom) showing constitutively activated NF-κB and TG2 levels in untreated (UT), TG2-infected (Ad.TG2), and vector alone–infected (Ad) BxPc3 cells. Representative experiments repeated at least twice to thrice with similar results.
TG2 Expression Leads to Constitutive NF-κB Activation

Results

TG2 expression correlates with constitutive NF-κB activation. As the first step to determining the significance of increased TG2 expression in constitutive activation of NF-κB, we compared the TG2 expression and constitutive NF-κB activation in the cell lines MDA-MB-231/cl.19, MDA-MB-231/cl.16, MCF-7/WT, MCF-7/Dox, WM35, A375, BxPC3, and Panc28. Using EMSA, we observed high constitutive NF-κB activation in MDA-MB-231/cl.16, MCF-7/Dox, A375, and Panc28 cells (Fig. 1A). Western blot analysis revealed that the cell lines with high constitutive activation of NF-κB also had high basal levels of TG2 protein expression (Fig. 1B).

The increased expression of TG2 in various cancer cell lines correlated well with a parallel increase in the enzymatic activity and activation of NF-κB (Fig. 1C). We observed a similar correlation between TG2 expression and constitutive NF-κB activation in several MiaPaca-2 pancreatic cancer cell line-derived sublines (data not shown). These results clearly suggested that there is a direct correlation between TG2 expression and NF-κB activation in various cancer cell lines.

Figure 1D shows the specificity of a NF-κB band visualized using EMSA. Incubation of the nuclear extracts from Panc28 cells with antibodies against the p50 and p65 subunits of NF-κB before EMSA caused a shift in the band to a higher molecular weight. Similarly, the addition of an excess cold unlabeled NF-κB oligonucleotide (100-fold) resulted in a complete disappearance of the band, whereas the addition of mutated NF-κB oligonucleotide did not affect the binding.

Activation and overexpression of TG2 results in activation of NF-κB. Next, we sought to determine the effect of the catalytic function of TG2 (protein cross-linking activity) on activation of NF-κB. For this purpose, we used calcium ionophore A23187 to induce an increase in the level of free cytosolic Ca2+, which is needed to activate the TG2 cross-linking functions. We treated low TG2-expressing BxPC3 cells, high TG2-expressing Panc28 cells, and TG2-negative MCF-7 cells with A23187 (Fig. 2A) and monitored in situ activation of TG2 by determining the level of covalent conjugation of the substrate inhibitor BPA into cellular proteins. The results shown in Fig. 2A indicated a complete lack of BPA labeling in MCF-7 cells in the presence or absence of A23187. In BxPC3 cells, BPA labeling of cellular proteins became evident but only after treatment with A23187. Panc28 cells, on the other hand, showed some basal TG2 activity (as evidenced by BPA labeling of the cellular proteins) that became more prominent in response to treatment with A23187 (Fig. 2A).

We then examined the effect of A23187-induced TG2 activation on NF-κB activation. The results shown in Fig. 2B clearly showed a strong correlation between in situ TG2 activity and NF-κB activation. Treatment of BxPC3 cells with A23187 resulted in strong activation of NF-κB that reached the level of untreated Panc28 cells (Fig. 2B). In Panc28 cells that showed some basal TG2 activity (Fig. 2A) and high constitutive NF-κB activation (Fig. 2B), both could be further induced by treatment with A23187. On the other hand, MCF-7 cells that lacked basal TG2 expression and activation in response to treatment with A23187 failed to show an increase in NF-κB activation even after treatment with A23187. Time-course and dose-response studies using BxPC3 cells revealed that treatment with 1 μmol/L A23187 for 24 hours is optimal for NF-κB activation (data not shown). Next, we sought to determine the effect of increased TG2 expression on NF-κB activation by reconstituting TG2 levels in low TG2-expressing BxPC3 cells. We tested cells infected with an adenoviral construct containing a full-length TG2 cDNA or empty vector alone for TG2 levels and NF-κB activation (Fig. 2C). Indeed, increased TG2 protein expression in BxPC3 cells was associated with increased constitutive NF-κB activation. Vector alone–infected cells showed no increase in TG2.

from a representative experiment. The paired t test was used for comparing samples of unequal variance and statistical significance was based on two-sided P < 0.05.
protein expression or NF-κB activation. These results suggested that increased expression of TG2 and its transamidation activity were essential for NF-κB activation.

**Inhibition of TG2 activity inhibits activation of NF-κB.**

Because the activation of TG2 by treatment with A23187 induced activation of NF-κB, in another experiment, we sought to determine the effect of two TG2-specific inhibitors, monodansylcadaverine and BPA, on NF-κB activation. Panc28 cells treated with BPA showed ~70% inhibition of constitutive activation of NF-κB (Fig. 3A). Moreover, preincubation of Panc28 cells with BPA failed to further augment A23187-induced activation of NF-κB. Similarly, treatment of Panc28 cells with monodansylcadaverine (50 μmol/L for 24 hours) resulted in ~60% inhibition of NF-κB activation and blocked A23187-induced activation (Fig. 3A, lanes 5 and 6).

Using EMSA, we showed that BPA and monodansylcadaverine could effectively block DNA binding of NF-κB protein to its consensus sequence. However, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting a role for an additional regulatory step (24). To determine the effect of BPA on NF-κB-dependent target gene expression, we transiently transfected Panc28 and MCF-7 cells with the NF-κB SEAP reporter construct. The results shown in Fig. 3B showed that transient transfection of the SEAP reporter in MCF-7/WT cells resulted in its expression (14-fold) only in response to treatment with TNF-α that could be effectively inhibited by DN-IκBα but not by BPA. No basal or A23187-induced activity of SEAP in the NF-κB SEAP reporter was seen in MCF-7/WT cells. In contrast, untreated Panc28 cells showed a substantial increase (13-fold) in SEAP expression that was significantly inhibited (P < 0.001) by BPA. SEAP expression did not increase further in response to treatment with A23187 (1 mmol/L for 24 hours) and was not inhibited by DN-IκBα.

These results suggested that TG2-mediated activation of NF-κB is mediated via some novel pathway that is independent of IKK.

For a more specific approach to determine the significance of TG2 expression in constitutive activation NF-κB, we investigated the effect of TG2 siRNA on the expression of NF-κB target gene, Bcl-xL. Using Western blot analysis, we found that siRNA transfection (siRNA1 and siRNA2) of Panc28 cells resulted in 70% to 80% inhibition of TG2 activity (data not shown) and protein expression (Fig. 3C). Reprobing of membrane with an anti-Bcl-xL antibody revealed that siRNA effectively blocked Bcl-xL protein expression. Mobility shift assay showed that constitutive activation of NF-κB could be effectively blocked by knocking down TG2 expression. The siRNA-mediated down-regulation of NF-κB could not be reversed by treatment with A23187. These results showed that NF-κB activation in Panc28 cells is dependent on TG2 expression.

**TG2 is associated with NF-κB in Panc28 cells.** Immunoprecipitation of Panc28 cytoplasmic extracts with anti-IκBα antibody
Treatment of cells with A23187 further augmented the formation of antibody revealed the presence of a 66-kDa dimeric IκB we studied the effect of TG2 on IκB complex with p65/p50. complex in the cytoplasm and translocates to the nucleus in a possible mechanism by which TG2 mediates NF-κB overexpression, revealing a significant correlation between TG2 expression and NF-κB activation.

**Figure 5.** TG2 expression correlates with NF-κB activation in pancreatic tumors. A, sections from pancreatic cancer samples immunostained for the p65 subunit of NF-κB and TG2 protein expression. Representative sections with positive and negative immunostaining for TG2 and p65. Left, arrows, locations of tumor cells. The TG2 staining in the negative sample (bottom left) shows endothelial and other stromal components that constitutively express TG2. Original magnification, ×200. B, NF-κB activation/overexpression was observed in 29 (85%) of the 34 pancreatic carcinoma samples with high levels of TG2 expression. In comparison, 13 (48%) of the 27 pancreatic carcinoma samples with low levels of TG2 expression showed a lack of NF-κB activation/overexpression, revealing a significant correlation between TG2 expression and NF-κB activation in these samples (P < 0.0098).

effectively pulled down the TG2 protein in addition to the IκBα and p65 proteins, suggesting that TG2 forms a part of the complex between p65/p50 and IκBα (Fig. 4A). We confirmed this association by immunoprecipitating TG2 and showing the presence of p65 and IκBα in the immune complex (Fig. 4C). Furthermore, confocal microscopy data supported the colocalization of TG2 with p65 in the cytoplasm (Fig. 4B). The association between TG2 and p65 further increased in response to treatment with A23187, which led to increased translocation of the p65/TG2 complex in the nucleus (Fig. 4B, inset). These results clearly suggested that TG2 is closely associated with the IκBα/p65/p50 complex in the cytoplasm and translocates to the nucleus in a complex with p65/p50.

**Mechanism of TG2-mediated NF-κB activation.** To delineate the possible mechanism by which TG2 mediates NF-κB activation, we studied the effect of TG2 on IκBα. Immunoprecipitation of a cytoplasmic extract from TG2-rich Panc28 cells with an anti-IκBα antibody revealed the presence of a 66-kDa dimeric IκBα band (Fig. 4D, left) in addition to the 33-kDa monomeric IκBα band. Treatment of cells with A23187 further augmented the formation of dimeric bands and resulted in the appearance of another polymeric IκBα band. We also observed these polymeric forms of IκBα in *in vitro* labeling of IκBα with BPA. We labeled the IκBα immunoprecipitates from Panc28 cytoplasmic extracts with BPA *in vitro* in the presence of Ca²⁺ (3 mmol/L) and probed them with HRP-conjugated streptavidin in a Western blot–type assay. The results shown in Fig. 4D (right) showed the presence of high molecular weight forms (dimeric and polymeric) of IκBα, confirming that TG2 is closely associated with IκBα and that IκBα can serve as a substrate for TG2-catalyzed cross-linking reactions.

**TG2 expression correlates with NF-κB activation in pancreatic tumors.** To further confirm the significance of our *in vitro* observations that expression of TG2 results in constitutive activation of NF-κB, we constructed a human tissue microarray containing 61 pancreatic ductal carcinoma samples using formalin-fixed, paraffin-embedded archival tissue blocks. We evaluated the expression of TG2 and NF-κB using immunohistochemistry under identical standardized conditions for all of the samples with consecutive tissue sections cut from the array block. Examples of both positive and negative staining are shown in Fig. 5A. Quantification of the staining showed that in the 34 pancreatic carcinoma samples with high levels of TG2 expression, 29 (85%) also showed activation/overexpression of NF-κB. In contrast, of the 27 pancreatic carcinoma samples with low levels of TG2 expression, 13 (48%) did not show activation/overexpression of NF-κB (Fig. 5B). Strong TG2 immunopositivity was associated with activation/overexpression of NF-κB (P = 0.0098, Fisher’s exact test). These data further supported the notion that expression of TG2 contributes to constitutive activation of NF-κB in pancreatic cancer.

**Discussion**

Our results show that a great majority of pancreatic cancer cells and cell lines have high levels of expression of the multifunctional protein TG2. Our data provide the first evidence that TG2 forms a ternary complex with NF-κB/IκBα. By catalyzing post-translational modification (cross-linking) of IκBα, TG2 results in constitutive activation of NF-κB. The activation of TG2 by calcium ionophore A23187 and overexpression of TG2 in the low TG2-expressing BxPc3 cells strongly induced activation of NF-κB. Moreover, treatment with the TG2-specific inhibitors BPA and monodansyl-cadaverine caused strong inhibition of NF-κB activation, an observation that was further supported by the use of TG2-specific siRNA. Overall, these results suggest a strong rationale for inhibiting endogenous TG2 activity to inhibit constitutive NF-κB activation, which can improve the sensitivity of cancer cells to antitumor therapies.

We and others showed previously that, irrespective of their source or type, cancer cells selected for resistance to chemotherapeutic drugs exhibit high levels of TG2 expression (15, 27, 30, 33). Notably, down-regulation of TG2 by stable transfection with TG2-specific antisense RNA (33) or with siRNA (15) rendered the cells sensitive to chemotherapeutic drugs, suggesting that TG2 plays a role in acquisition of drug resistance. Similarly, metastatic cancer cells and cell lines have high levels of TG2 expression. Indeed, TG2 was 1 of the 11 proteins whose expression was selectively increased in metastatic lung cancer cells as determined using proteomic analysis (34). Similarly, several metastatic breast cancer cell lines have exhibited increased levels of TG2 protein expression (12, 35). Importantly, lymph node metastases obtained from patients with breast cancer showed significantly higher TG2 expression than did
primary tumors obtained from the same patients. These observations suggest that the development of drug resistance and metastatic phenotypes in cancer cells is associated with increased expression of TG2. However, a direct link among TG2 expression, drug resistance, and metastasis has not been established.

The data presented here suggest that TG2 expression contributes to the development of drug resistance and metastatic phenotypes by inducing constitutive activation of NF-κB. Many cancer cells and cell lines have constitutive NF-κB activation, which enables malignant cells to escape apoptosis (19, 20, 36, 37). In contrast, activation of NF-κB in normal cells is transient, which prevents abnormal cell growth and survival. Therefore, constitutively activated NF-κB in cancer cells may play a role in the development of drug resistance by attenuating the apoptotic response of cells to genotoxic anticancer drugs and ionizing radiation. Indeed, the expression of several genes that encode antiapoptotic proteins that either act at the mitochondrial level, such as Bcl-xL and BFL1, or directly block caspase activation, such as inhibitors of apoptosis protein 1 and 2 and X-linked inhibitor of apoptosis protein (XIAP), can be directly regulated by NF-κB (19, 20). In particular, the failure of a human lung carcinoma cell line to respond to chemotherapeutic drugs has been attributed to NF-κB-induced expression of Bcl-xL and BFL1 (38).

Similarly, increased expression of TG2 may also contribute to the development of metastatic phenotypes by constitutively activating NF-κB. The metastatic process requires the migration of cancer cells into and out of the walls of blood vessels. Several adhesion molecules, such as ICAM-1, VCAM-1, and ELAM-1, whose expression is under the direct control of NF-κB, play a critical role in this process (39). Moreover, inducible nitric oxide synthase, the expression of which is linked to the metastatic ability of cancer cells (40), can also be regulated by NF-κB. In breast cancer cells, NF-κB has been shown to facilitate the cell motility by up-regulating the expression of the chemokine receptor CXCR (41). Conversely, another study found that inhibition of NF-κB activity by expression of a mutant IκBα inhibited liver metastasis (42). All of these observations support the involvement of NF-κB in the development of metastatic phenotypes in cancer cells. In view of these observations, researchers have developed or are developing numerous NF-κB inhibitors that target the upstream kinase IKK, but none of these molecules have proven to be specific for IKK or effective in inhibiting constitutively activated NF-κB.

Based on our earlier observations that chemoresistant and metastatic cancer cells have high levels of TG2 expression and our present observation that TG2 overexpression results in constitutive NF-κB activation, we infer that TG2 expression plays a role in conferring drug-resistant and metastatic phenotypes to cancer cells. Figure 6 summarizes the possible pathways that lead to constitutive activation of NF-κB by TG2. We speculate that TG2 catalyzes cross-linking of IκBα and destabilizes the IκBα/NF-κB complex, resulting in the release and activation of NF-κB. Indeed, previous observations by Lee et al. (43) showed that TG2 could effectively cross-link IκBα and induce its polymerization in vitro. Importantly, the polymerized form of IκBα was unable to bind to p65/p50 in their study. Alternatively, association of TG2 with p50/p65 may interfere with the binding of IκBα to NF-κB complex resulting in its constitutive activation.

The data reported here also show for the first time that TG2 is closely associated with the IκBα/p65p50 complex in the cytoplasm. The significance of this association is not clear at this time, but we are currently investigating it in our laboratory. We speculate that TG2 plays a role in destabilizing the IκBα/p65p50 complex and thus in regulating NF-κB activation. We also observed nuclear localization of TG2 in association with p65. The nuclear association of TG2 with p65 is also evident according to confocal microscopy data in A23187-treated cells (Fig. 4B). Recent reports suggest that, under certain conditions, TG2 can serve as a kinase, and its serine-threonine kinase activity can phosphorylate histones

Figure 6. Schematic representation of TG2-independent (IKK-dependent) and TG2-dependent (IKK-independent) NF-κB activation pathways. By catalyzing cross-linking of IκBα, TG2 can destabilize its association with p65:p50 complex resulting in constitutive activation of NF-κB and its translocation to the nucleus. The fate of TG2-catalyzed polymeric IκBα forms is not yet known. Alternatively, association of TG2 with p65:p50 complex could mitigate the binding of IκBα to NF-κB complex, resulting in its constitutive activation. TG2-mediated activation of NF-κB can result in constitutive expression of various antiapoptotic target genes, such as Bcl-xL and XIAP, resulting in a chemoresistance phenotype.
and p53 (44). Because p65 undergoes phosphorylation by various kinases at Ser256 (45, 46), we are also investigating the possibility that p65 serves as substrate for TG2 kinase activity.

In summary, our study provides the first evidence that expression of TG2 results in constitutive activation of NF-κB in an IKK-independent manner. We expect that inhibitors designed to target TG2 can inhibit NF-κB activity and may improve the response of cancer cells to anticancer treatment.

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8795 Cancer Res 2006; 66 (17). September 1, 2006

Acknowledgments

Received 4/21/2006; revised 6/2/2006; accepted 7/7/2006.

Grant support: NIH grants CA092115 (K. Mehta) and CA98823 and CA98823 (B. Manavathu and R. Kumar) and Specialized Programs of Research Excellence in Pancreatic Cancer at The University of Texas M.D. Anderson Cancer Center grant P20CA101936.

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