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Tissue transglutaminase: from biological glue to cell survival cues

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1. ABSTRACT

Tissue transglutaminase (TG2, EC 2.3.2.13) is a ubiquitous enzyme that catalyzes Ca^{2+}-dependent post-translational modification of proteins by inserting highly stable (epsilon-[gamma-glutamyl] lysine) isopeptide bonds or by conjugating polyamines at selected peptide-bound glutamine residues. The TG2-catalyzed cross-linked products (generally high molecular mass scaffold of proteins) are of great physiological significance; they are highly stable and resistant to mechanical, chemical and proteolytic degradation. The accumulation of isopeptide bonds can be observed in skin, hair and during blood clotting and wound healing. In addition to transamidation activity, TG2 also exhibits GTPase activity and in response to certain agonist hormones can serve as a signal transducing G protein. Although predominantly a cytosolic protein, TG2 can translocate to the nucleus with the help of importin-alpha-3 protein or to the membranes in association with integrins. Moreover, TG2 can also be secreted outside the cell (by yet unknown mechanism) where it crosslinks proteins of the extracellular matrix (ECM) and promotes cell adhesion and spreading. Another important property of TG2 is that it has high binding-affinity for the ECM component protein, fibronectin and thus can promote interaction between cell surface integrin with fibronectin. In this review, we discuss the implications of increased TG2 expression in drug-resistant and metastatic cancer cells and that how TG2 expression can contribute in the development of these phenotypes.

2. INTRODUCTION

Despite major advances in prevention, therapeutic modalities, and adjuvant therapies, cancer has continued to be a major health problem associated with high mortality rates throughout the globe. For example, according to recent estimates, 6.2 million people are dying from the disease every year with approximately 5.3 million men and 4.7 million women being diagnosed with cancer every year (1). The vast clinical experience has taught us now that long-term survival of patients is mainly dependent on the early detection and treatment of the cancer. Unfortunately, most patients are first seen when the disease is in an advanced stages, that is, when it has progressed to a more malignant phenotype as characterized by invasive and metastatic foci. These tumors are not only inoperable, they are also resistant to chemotherapeutic interventions.

An important insight that is enabling researchers to develop new treatments for cancer is that it is a disease primarily caused by defects or dysfunction of pathways that govern cell cycle regulation and homeostasis (2). Indeed, the proteins encoded by many oncogenes and tumor suppressor genes either constitute intrinsic components of the cell cycle machinery or have the ability to control cell cycle progression. A common feature of advanced cancers is that they are resistant to apoptosis (3), a genetically controlled form of cell death that plays an important role in organ development and homeostasis. It is further becoming apparent that some oncogenic mutations disrupt apoptosis, thereby leading to tumor initiation, progression, and

Table 1. Role of apoptotic proteins in drug resistance

Protein	Role in drug resistance
p53	Mutated/altered expression in many cancers. p53-deficient cells are resistant to drug-induced apoptosis
Bcl-2	Overexpressed in many tumors. Inhibits drug-induced apoptosis
Bax	Mutated or decreased expression in many tumors. Sufficient but not necessary for drug-induced apoptosis
Bak	Mutated or decreased expression in many tumors. Sufficient but not necessary for drug-induced apoptosis
Apaf-1	Mutated or silenced in melanoma and leukemia cell lines. Apaf-1-deficient cells are chemoresistant
CD-95/Fas	mutated/down regulated in lymphoid and solid tumors. Loss is associated with resistance to drug-induced apoptosis
TRAIL-R1/R2	Mutated in metastatic breast cancer. Mutation leads to suppression of death receptor-mediated apoptosis
Caspase-8	Gene silenced in neuroblastoma, which results in drug-induced apoptosis
IAPs	Over expressed in cancer. Down-regulation results in apoptosis in chemoresistant tumors
NF-κB	Constitutively activated in many tumors. Can induce resistance to drug-induced apoptosis
Akt	Often amplified in solid tumors. Induces resistance to a variety of apoptosis-inducing stimuli, including drugs
PTEN	Mutated or altered expression in many cancer. Regulates Akt activation. Loss of PTEN results in resistance to apoptosis
PI3K	Expression altered in some cancers. Inhibition enhances drug-induced apoptosis
Ras	Mutated and activated in many cancers. Inhibits drug-induced apoptosis
FLIP	Overexpressed in some cancers. Prevents apoptosis induced by some chemotherapeutic drugs

metastasis. Moreover, the realization that diverse anticancer drugs kill tumor cells by activating common apoptotic pathways has raised the interesting possibility that single mutations that disable apoptosis might be the cause of multidrug resistance. Similarly, for tumor cells to progress and metastasize to distant sites, they must circumvent stressful conditions encountered in form of the hypoxia, nutrient factor deprivation, and altered cell adhesion. In addition, tumor cells must survive in a foreign environment and overcome and evade continual immune attack. Each hurdle imposes further selection pressure that enables certain tumor cells to survive by disabling apoptotic pathways, such that by the time a tumor metastasizes, it is highly resistant to pharmacologic and physiologic death-inducing signals. Thus, the ability of tumor cells to circumvent apoptosis allows them not only to grow and survive in foreign tissue sites (i.e., metastasize) but also to develop a drug-resistant phenotype (4). This may explain, in part, why metastatic tumors are highly resistant to chemotherapeutic drugs and conversely why cell lines selected *in vitro* for their resistance to chemotherapeutic drugs are more metastatic than the parental cell lines. Table 1 summarizes the effect of the altered expression/function of some key apoptosis-related proteins on the sensitivity of cancer cells to chemotherapeutic drugs.

In addition to gene silencing and mutations that lead to the inactivation of apoptotic pathways in cancer cells, the activation of prosurvival signal transduction pathways involving Ras, Akt/PTEN, or NF-κB can also contribute to intrinsic or inducible drug resistance (Table 1). These pathways are usually activated by specific external survival signals that activate their corresponding receptors. These survival signals include the growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). In the absence of survival signals, cells usually enter apoptosis by default. In many cancer cells, these prosurvival signal transduction pathways are constitutively activated as a result of aberrant expression, mutations, or interaction with certain regulatory proteins, which confers an apoptosis-resistant phenotype on the cells. For example, certain cell adhesion molecules such as integrins can strongly influence the ability of neoplastic cells to migrate, proliferate, and undergo apoptosis by mediating signal transduction and gene expression (5, 6).

Integrins, which are composed of α and β subunits, serve as heterodimeric transmembrane receptors for the extracellular matrix (ECM). They are expressed on most cell types and by sensing the immediate environments of cells, influence the decision whether cells live or die. In keeping with this, integrins play an important role in causing cellular resistance to apoptotic stimuli, especially to stimuli that activate the intrinsic pathway (also referred to as the stress pathway or the mitochondrial pathway). In particular, environmental insults, including chemotherapeutic drugs, ultraviolet radiations, and growth factor deprivation, can lead to activation of the intrinsic pathway, resulting in the activation of caspase 9 and consequently the cleavage of vital cellular proteins and cell death. Integrins can protect cell viability in response to stress signals at several levels (Figure 1). For example, ligation of integrin α5β1 or αvβ3 leads to the increased expression of the anti-apoptotic protein Bcl-2 and increased resistance to serum withdrawl (7, 8). Conversely, integrin-mediated cell survival can be effectively blocked by dominant-negative Shc, phosphoinositol (PI)-3 kinase, or protein kinase B/Akt constructs, suggesting that the PI-3 kinase/Akt pathways play a major role in these events. The activation of Akt can result in the transcriptional regulation of the Bcl-2 homolog Bcl_{xl}. This probably occurs via the activation of NF-κB because several prosurvival Bcl-2 proteins are regulated by this nuclear transcription factor. The translocation of NF-κB to the nucleus is driven by the ligation of integrins; however, the particular integrin heterodimer that results in the activation of NF-κB appears to be cell-type specific. Activated Akt can directly phosphorylate and inactivate human caspase 9. Activated Akt can also promote cell survival by phosphorylating nuclear transcription factors, such as FKHR and the apoptosis scaffold protein Apaf-1, resulting in the inhibition of pro-apoptotic factor expression and apoptosome formation. In addition to PI-3 kinase/Akt pathway activation, integrin-mediated Ras activation can lead to the activation of the Raf/MEK/ERK pathway (Figure 1). Raf-mediated phosphorylation of the pro-apoptotic factors Bad or Bax results in their binding to and the sequestration resultant of the members of the 14-3-3 protein family during an anti-apoptotic response. Similarly, the phosphorylation of Bcl-2 by mitogen-activated protei

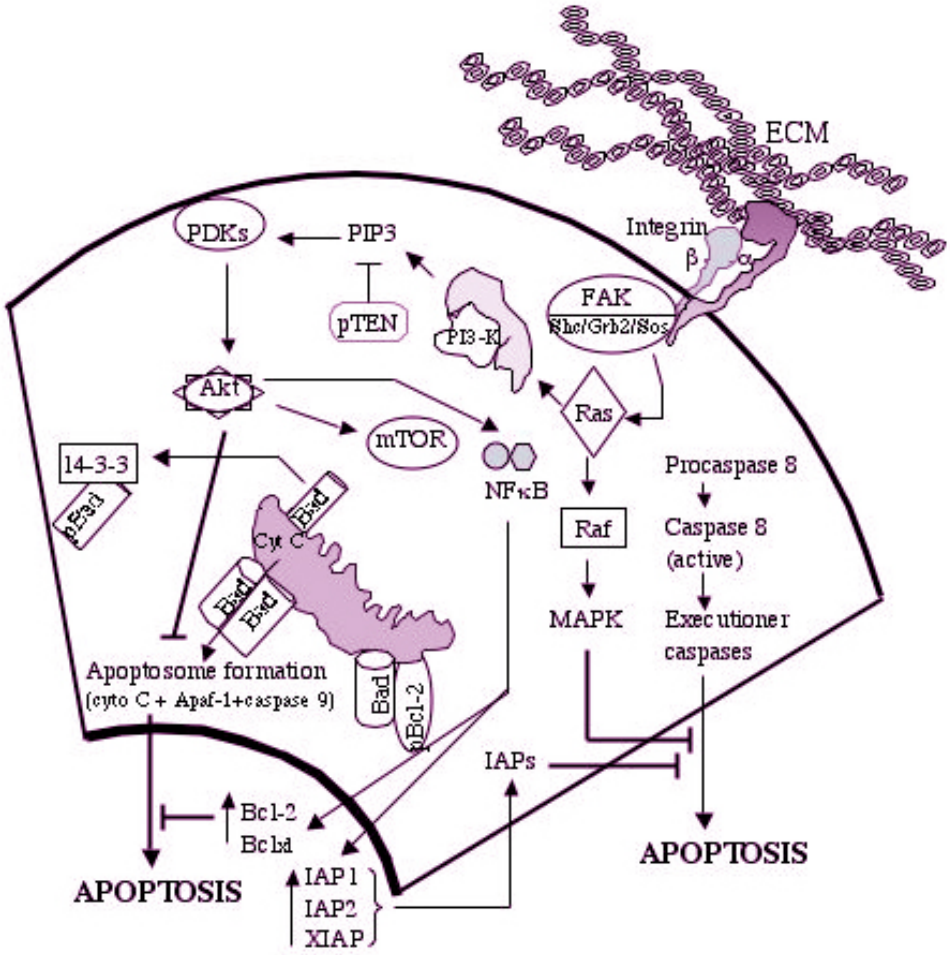

Figure 1. Cell-survival signaling pathways mediated by ECM-ligated integrins. Integrin signaling is dependent on the non-receptor tyrosine kinase activity of the FAK that initiates the down-stream signaling events leading to cell survival functions.

(MAP) kinases, such as Erk-1/2 and c-Jun N terminal kinase (JNK), protects it from ubiquitin-targeted degradation, thus causing Bcl-2 to accumulate` and promoting cell survival (9).

Similarly, the ligation of integrins can induce the phosphorylation of focal adhesion kinase (FAK), which allows FAK to recruit Src homology 2(SH2) containing proteins, such as Src, Fyn, p85 subunit of PI-3 Kinase, and phospholipase C (Figure 1). This recruitment of Src provides more SH2 docking sites by phosphorylating additional tyrosine residues on the FAK molecule and initiates signaling pathways, such as Ras/Erk, PI-3 Kinase/Akt, and Crk/Dock180/Rac, which contribute to conferring an apoptosis resistant phenotype on cells (10, 11)**.** Moreover, Src kinase activation is also known to play a role in the metastatic spread of carcinoma cells (12). Similarly, integrin-mediated signaling via FAK can result in increased expression of the inhibitors-of-apoptosis proteins (IAPs), specifically cIAP1, cIAP2, and XIAP, therby protecting cells from apoptosis (13). In addition to binding to and inactivating executioner caspases, IAPs can regulate cell division and signaling via the transforming growth factor-beta (TGF-β) receptor to SMAD, NF-κB, and JNK pathways (14).

From this discussion, it is therefore clear that the interaction of integrins with their ligands in the ECM can trigger multiple pathways that together increase cell survival by protecting the cells from stress-induced apoptosis, including chemotherapy-induced apoptosis (Figure 1). However, the integrin-ligand interactions that trigger these pathways are strongly influenced by certain factors. In general, integrins bind to their ligands in the ECM with a low affinity (10^6-10^9 liters/mole). Particularly, when diffusely distributed over the cell surface integrins fail to promote stable cell adhesion. In response to certain stimuli, integrins cluster in form of focal contacts and promote stable adhesion of cells to the ECM. Alternatively, certain proteins can bind directly to cytoplasmic or extracellular domains of the integrins and enhance their affinity for the ECM, thereby promoting cell signaling (15,16). Several of these proteins can modulate integrin affinity or interaction with the ECM (17). Recently, a similar association between tissue

transglutaminase (TG2) and the β1 and β3 subfamilies of integrins has been reported (18, 19). It is estimated that 40% of □1 integrins can form complexes with TG2 in a 1:1 ratio (20). Although, TG2 is one of the most extensively studied members of the transglutaminases family, its precise physiologic function remains largely a matter of speculation. In this review, we will discuss the current knowledge on emerging role of TG2 in protecting cells from apoptosis and its implications in conferring drug-resistant and metastatic phenotypes on cancer cells.

3. TISSUE TRANSGLUTAMINASE AS THE ENZYME WITH A SPLIT PERSONALITY

TG2, also referred to as the cytosolic, type II, or liver transglutaminase, is a unique member of the transglutaminase enzyme family because in addition to catalyzing the calcium-dependent posttranslation modification of proteins, it can bind to and hydrolyze guanosine triphosphate (GTP) with an affinity similar to those of the α subunits of large heterodimeric G proteins and small Ras-type G proteins (21, 22, 23, 24). TG2/G_h is involved in coupling the α_{1b}- and α_{1d}-adrenoreceptors, thromboxane, and oxytocin receptors to phospholipase C, thereby mediating inositol phosphate production in response to the agonistic activation. In its GTP/GDP-bound form, TG2 cannot catalyze protein cross-linking reactions, but calcium can reverse this inhibition and regulate the transamidation functions of the TG2 protein. Such calcium-dependent activation of TG2 has been implicated in diverse biologic functions, such as differentiation, receptor-mediated endocytosis, cell adhesion, and induction of apoptosis.

Although, it is predominantly a cytosolic protein, TG2 can also be secreted outside the cell (by an unknown mechanism), where it can stabilize the ECM by making the matrix resistant to mechanical and proteolytic degradation (25). Several ECM proteins, such as fibronectin, vitronectin, collagen, osteonectin, and osteopontin, can serve as substrates for TG2-catalyzed cross-linking reactions. Such TG2-catalyzed cross-linking of ECM proteins plays an important role in the deposition and stabilization of the ECM, which in turn helps promote the attachment and spreading of several cell types (26). Another important property of TG2 is that it binds to fibronectin with high affinity (27). This property of TG2 may be of particular significance in cancer biology because the membrane-associated TG2 can easily interact with fibronectin and integrins, which play crucial roles in the cell signaling that promotes cell survival, cell growth, and metastatic functions. Similarly, under certain conditions, TG2 can translocate to the nucleus presumable with the help of the importin α-3 protein, where it functions as a G protein or as a transamidase activated by nuclear calcium to cross-link histones, retinoblastoma protein (pRb), and SP1 transcription factor (21, 28). Taken together, these properties of TG2 clearly suggest that it is a multifunctional protein that, in addition to catalyzing protein cross-linking reactions, can affect cell-cell and cell-matrix interactions and thus participate in cell signaling mechanisms.

3.1. TG2 as a pro-and anti-apoptotic protein

An initial link between TG2 and apoptosis was suspected by Laszlo Fesus and his coworkers (29) based on their observation that lead-induced hypertrophy of the liver in rats was associated with an increased expression of TG2. Since then, many reports have supported the finding that TG2 is involved in apoptosis (30 and the references therein). In particular, cells undergoing apoptosis show an induced expression of TG2, which primes the cells to undergo apoptosis. Its inhibition (by means of an antisense approach) results in a decreased propensity for cell death.

Although evidence supporting the involvement of TG2 in apoptosis is convincing, TG2's exact physiologic significance remains elusive. Nevertheless, we do know that the involvement of TG2 in apoptosis is mediated by its cross-linking configuration, which requires millimolar concentrations of calcium. In the presence of high calcium concentrations, TG2 cross-links cellular proteins and induces their polymerization and the formation of detergent-insoluble structures. The protein scaffolds thus formed stabilize the structure of the dying cell before its clearance by phagocytosis, thereby preventing the release of intracellular components and a subsequent inflammatory response. It is likely that TG2 remains inactive under normal conditions; however, stressful conditions such as hypoxia, growth factor deprivation, and treatment with chemotherapeutic drugs perturb calcium homeostasis inside the cell and transform latent TG2 into its active cross-linking configuration, resulting in the extensive cross-linking of intracellular proteins. Interestingly, on the basis of studies performed in $TG2^{-/-}$ mice, it was recently suggested that TG2 is required for the phagocytosis of apoptotic bodies (31). This function of TG2 probably depends on its ability to activate TGF-β, a cytokine that plays an essential role in phagocytosis.

Interestingly, the expression of TG2 and apoptosis do not completely overlap, as directly evidenced by $TG2^{-/-}$ mice that do not display any phenotype that could be ascribed to perturbed apoptosis (32, 33). However, the possibility that some other isoform compensates for the loss of TG2 in these mice cannot be ruled out. Moreover, several rapidly dividing cancer cells that do not undergo apoptosis have been shown to express high levels of TG2 (34, 35, 36). More recent studies have provided direct evidence that the increased expression of TG2 can prolong cell survival by preventing apoptosis (37, 38, 39). As the causes of these discrepancies are revealed, it is becoming apparent that the activation of the pro-apoptotic or anti-apoptotic functions of TG2 depends upon its location within the cell (40). The available data suggest that a cytosolic fraction of TG2 is involved in inducing alterations that lead to apoptosis; data also suggest that while in the nucleus, TG2 can interact with and result in the transamidation of pRb which protects cells from apoptotic insults (39, 40). Similarly, in cell membranes, TG2 can associate with integrins and provide a binding site for fibronectin (18) by simultaneously binding to β integrins and fibronectin through a gelatin-binding domain (42-kDa fragment, module $I_6II_{1,2},I_{7-9}$) (18, 19, 20).

3.2. TG2: drug resistance, and metastasis

Depending on the cell type, 5-40% of β1 integrins on the cell surface can exist as a complex with TG2 in a 1:1 ratio (18, 19). This interaction of TG2 with integrins occurs primarily at the extracellular domains of integrin β subunits. It does not require cross-linking activity (41) and facilitates the adhesion, spreading, and motility of cells (19, 41, 42, 43). In view of these observations and the fact that the integrin-mediated signaling pathways play an important role in cell growth, cell survival, and metastasis, it is tempting to speculate that TG2 expression in cancer cells promotes signaling pathways that could affect not only the adhesive, migratory, and invasive functions of tumor cells but also their growth and survival.

Several years ago, we observed that macrophages collected from inflammatory sites or activated *in vitro* accumulate large amounts of TG2 (44, 45, 46). An important property of activated macrophages is that they readily migrate to inflammatory sites and kill infectious agents; this involves the production and release of a large number of soluble mediators, which have a potent cytotoxic effect on infectious targets (47). Nevertheless, the survival of activated macrophages that produce these cytotoxic mediators is not appreciably compromised. We now believe that the induction of TG2 in activated macrophages might play a role in macrophage migration to inflammatory sites and protecting macrophages against the cytotoxic mediators that they produce in response to inflammatory signals. Indeed, two recent studies have documented the direct involvement of TG2 in promoting the migration of normal cells. The first study suggested that the inhibition of TG2 expression could effectively block the transmigration of T lymphocytes across endothelial cells treated with interferon-γ and tumor necrosis factor-α (48). The second study showed that TGF-β-induced increase in cell surface expression of TG2 was responsible for augmenting the attachment and migration of retinal pigment epithelial cells on fibronectin-coated matrices (49). Moreover, Hox A7-mediated down-regulation of TG2 was recently shown to inhibit the interaction and migration of differentiated HL-60 cells on fibronectin-coated surfaces (43). More importantly, in an attempt to identify metastasis-associated proteins by proteomic analysis, Jiang et al. (50) identified TG2 as one of 11 proteins selectively amplified in metastatic human lung carcinoma.

We have previously observed that irrespective of their source or type, cancer cells resistant to chemotherapeutic drugs exhibit high levels of TG2 expression when compared with their parental cell line (51, 52, 53, 54). TG2 expression in such drug-resistant cancer cells is up-regulated at all three levels: mRNA, protein, and enzyme activity. A similar increase in TG2 expression was observed by Han and Park (55) in PC-14 lung cancer cells after their resistance to doxorubicin (i.e., PC-14/ADR cells). More importantly, these investigators observed that the down-regulation of TG2 by stable transfection with TG2-specific antisense or ribozyme rendered the drug-resistant PC-14/ADR cells sensitive to doxorubicin and other chemotherapeutic drugs, suggesting that TG2 plays a role in the acquisition of drug resistance. Similarly, EGF was recently shown to promote the expression of TG2 in breast cancer cell lines, an event that was associated with the protection of cells from doxorubicin-induced apoptosis (56).

Our studies on the regulation of TG2 functions in drug-resistant MCF-7 breast cancer cells suggested that TG2 is inert or latent in these cells because of deficient or defective intracellular calcium pools (39). Nevertheless, the activation of TG2 by pharmacologic agents, such as A23187 that increase intracellular calcium levels have induced massive apoptosis in MCF-7/DOX cells, despite their high resistance to chemotherapeutic drugs (40). These findings suggest that a cell's development of resistance, at least against multidrug resistance-related drugs, is associated with an increased expression of TG2 and that TG2 is present in an inactive form in these cells and could serve as a target of therapies that cause drug-resistant cancer cells to undergo apoptosis.

The spread of cancer to distant sites (metastasis) is a multistep process and is the most common cause of cancer-related deaths. Key steps in the metastatic process are the detachment of malignant cells from the primary tumor, the invasion of malignat cells through the tissue into the blood vessel lumen, and the proliferation of malignant cells in distant tissue sites. The invasion of ECM is crucial for a number of physiologic and pathologic processes, including metastasis, arthritis, embryo implantation, wound healing, and early development. Invasion itself is a multistep process that involves the attachment of cells to the ECM, the degradation of the structural components, and migration of cells through the ECM. Although most metastatic cells die during migration or upon reaching the new microenvironments (distant tissues), some cells become dormant or growth-arrested and survive in these stressful microenvironments for extended periods. They remain protected from death and, in fact, survive multiple rounds of chemotherapy administered for the very purpose of eradicating them. Some of these cells begin to proliferate years later and form secondary and tertiary metastatic tumors that become hard to treat and result in the death of the patient.

Although there is a consensus that drug resistance and metastasis represent different phenotypes, there are several reasons to believe that they share some common pathways. For example, during advanced stages, cancer cells accumulate a large number of genetic alterations that can render them more resistant to apoptosis (3). This property endows tumor cells with not only an increased ability to grow and survive in foreign tissue environments (i.e., metastasize) but also an increased likelihood of having a drug-resistant phenotype. Moreover, cancer cell lines selected *in vitro* for resistance to drugs are more metastatic *in vivo*, whereas cancer cells isolated from metastatic sites, in general, exhibit a higher resistance to chemotherapeutic drugs. In view of these observations and the results reported by Aoudjit and Vuori (57) showing that the culture of the highly metastatic breast cancer cell line MDA-MB-231 on fibronectin-coated surfaces rendered the cells resistant to chemotherapy-induced apoptosis, we reasoned

that metastatic cancer cells may have a high basal expression of TG2. Because, TG2 has a high binding affinity for fibronectin (29) and associates with β integrin (11 12), its presence on the cell surface can not only promote the binding and motility of cancer cells on fibronectin-coated surfaces but can also render them resistant to chemotherapy-induced apoptosis by up-regulating cell survival and anti-apoptotic signaling pathways.

We recently reported that the metastatic breast cancer cell line MDA-MB-231expresses high basal levels of TG2. Importantly, two single-cell clones (MDA231/cl.9 and MDA231/cl.16) derived from this cell line by the limiting dilution method showed a 10- to 15-fold difference in TG2 levels. TG2-deficient MDA231/cl.9 cells exhibited a higher sensitivity to doxorubicin and were less invasive than the TG2-positive MDA231/cl.16 cells (35). More importantly, several sub lines derived from an immortal but normal breast epithelial cell line, MCF10A, representing various stages in the progression of breast cancer from benign hyperplasia to atypical hyperplasia to carcinoma *in situ* and fully malignant invasive tumors that can metastasize, showed increased TG2 expression as they advanced from a noninvasive to an invasive phenotype (35). Moreover, lymph node metastases obtained from patients with breast cancer showed significantly higher levels of TG2 expression compared with the primary tumors of the same patients (35). These results suggest that the development of drug-resistant and metastatic phenotypes in breast cancer cells is associated with an increased expression of TG2 and that TG2 may play a role in conferring these phenotypes.

3.3. TG2 and integrin connection

It is likely that the increased expression of TG2 in drug-resistant and metastatic tumor cells is involved in protecting cells from apoptosis by promoting interactions between integrins and fibronectin thereby activating cell-survival signaling pathways. In tumor cells, several factors that promote the interaction between cell-surface integrins and their ECM ligands, including fibronectin, have been shown to affect signaling pathways that can influence not only the cell adhesive, migratory, and invasive functions but also cell survival and proliferation (58, 59, 60, 61). For example, the culture of α5β1 integrin expressing cells on fibronectin was associated with an increased expression of the anti-apoptotic protein Bcl-2 and protection from stress-induced apoptosis (8). Similarly, the activation of Shc in response to α5β1-fibronectin interaction modulated the adhesion and motility of MCF-7 breast cancer cells (58) and induced activation of the Akt pathway that, in turn, led to the increased resistance of cells to apoptosis (59). More importantly, the culture of TG2-positive breast cancer cells on fibronectin-coated surfaces led to the activation of FAK, an event that allowed FAK to recruit SH2-containing proteins such as Src, Fyn, p85 subunit of PI3K, and phospholipase C. The recruitment of Src provides more SH2 docking sites by phosphorylating additional tyrosine residues on the FAK molecule, which initiates signaling pathways such as Ras/Erk, PI3K/Akt, and Crk/Dock180/Rac that render the cells resistant to apoptosis (10, 11). Indeed, the activation of Src kinase is known to play an important role in the metastatic spread of carcinoma cells (12). In this context, it is interesting to note that TG2-transfected fibroblasts showed a strong activation of FAK in response to their culture on fibronectin or its gelatin-binding fragment (18). We have observed a similar activation of FAK in TG2-expressing drug-resistant MCF-7 cells following their culture on fibronectin-coated surfaces. No such activation of FAK was evident in TG2-negative drug-sensitive MCF-7 cells when cultured on either fibronectin- or bovine serum albumin (BSA)-coated surfaces or in drug-resistant MCF-7 cells cultured under identical conditions but on BSA-coated surfaces (J. Herman and K. Mehta, unpublished). More importantly, the incubation of TG2-positive cells on fibronectin-coated surfaces was able to rescue them from serum-deprived apoptosis. From these observations, it is apparent that the presence of TG2 can effectively promote integrin-mediated cell survival signaling pathways. Whether the association of β integrins with TG2 on the surface of cancer cells promotes their interaction with the ECM directly or as a result of its high binding affinity for fibronectin remains to be determined.

As pointed out earlier, some proteins can directly bind to integrin cytoplasmic or extra cellular domains and enhance their affinity for the ECM ligands, which promotes cell signaling (15, 16). For example, expression of cytohesin-1 that binds to the β2 integrin can enhance the binding of αLβ2 to its ligand (ICAM-1) (62). Similarly, by interacting with β1 integrin, a heavy chain component of 4F2 antigen (CD98hc) promotes integrin-dependent cell signaling, cell migration, and protection from apoptosis (63). It is likely that the interaction of TG2 with -β1, -β3, and/or -β5 integrins promotes cell-survival signaling by enhancing the direct binding of integrins with their ECM ligands. Alternatively, because of its high affinity for fibronectin, integrin-associated cell surface TG2 can bind and promote integrin-mediated signaling by serving as a bridge between integrin and the ECM. The recent identification of the fibronectin-binding sequence (amino acids 81-140) within the first β-sandwich domain of the TG2 protein (64) should help clarify whether TG2 promotes the direct or indirect interaction of integrin and fibronectin.

Moreover, it is well-known that the inflammatory cytokine TGF-β secreted by stroma cells surrounding the tumor, can contribute to the processes of tumorigenesis and metastasis (65, 66). In this regard, it is worth noting that TGF-β can regulate the expression of TG2 activity in several cell types (67). Conversely, TG2 is known to regulate the activation of TGF-β by cross-linking the latent TGF-β1-binding protein to the ECM (68). Therefore, it is reasonable to believe that TG2-mediated activation of TGF-β1, which in turn can induce the expression of TG2, may play a role in conferring metastatic potential on cancer cells. Oxidative stress can also induce the expression of TG2 (69). Thus, the oxidative stress induced in response to chemotherapeutic drugs or a tumor's intrinsic oxidative stress may help promote chemoresistance in tumor cells

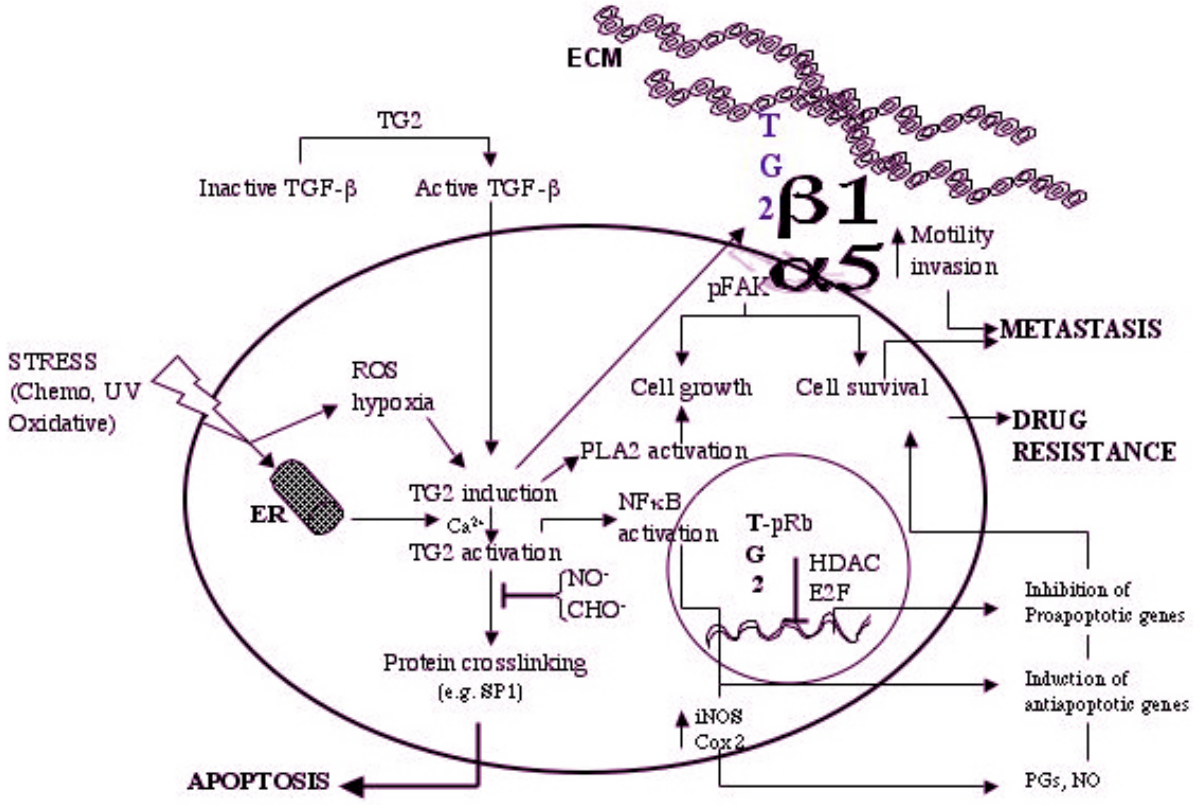

Figure 2. TG2-mediated pro-apoptotic and anti-apoptotic functions. Stressful conditions (e.g., ultraviolet radiations, chemotherapeutic agents) can generate reactive oxygen species (ROS) that result in the induction of TG2. Extreme stressful conditions can further trigger Ca^{2+} release from the endoplasmic reticulum (ER), leading to the activation of TG2 and massive cross-linking of intracellular proteins. Extensive cross-linking of proteins, in turn, initiates the apoptotic process. In the nucleus, TG2 can interact with pRb and protect cells from apoptosis. TG2, by inducing the polymerization of the alpha-inhibitory subunit of NF-kappaB, can activate this transcription factor, leading to the transcriptional regulation of several key genes involved in protecting cells from apoptosis. Similarly, TG2 can translocate to cell membranes in association with beta-integrins. In this form, TG2 can serve as a co-receptor for integrins and promote their binding to fibronectin. TG2-dependent interaction between integrins and fibronectin can lead to the activation of cell-survival and antiapoptotic signaling pathways. In the extracellular environments, TG2 can transform latent TGF-beta into its active form, which in turn can up regulate the expression of TG2. Also, some free radicals (e.g., CHO^- and NO^-) can bind and damage the cysteine residue in the active site, leading to the inactivation of TG2 cross-linking functions.

because of the tumor's ability to up-regulate TG2 expression. In a recent report, Antonyak *et al.* (56) further observed that the ligation of EGF receptor induces TG2 expression in several breast cancer cell lines. Interestingly, whereas the EGF-induced TG2 expression could protect cells from doxorubicin-induced cytotoxic effects, the exposure of cells to a TG2 inhibitor or the expression of a dominantly negative form of TG2 inhibited the EGF-mediated protection from doxorubicin-induced apoptosis (56). The mechanism by which EGF-induced TG2 contributes to the development of chemoresistance is not well understood. TG2 can also induce the activation of nuclear transcription factor NF-κB via a novel mechanism that involves the cross-linking of the inhibitory factor Iκ-Bα (70). The cross-linked Ik-Bα fails to bind to NF-κB, leading to its translocation to the nucleus where it induces the transcriptional regulation of various anti-apoptotic and cell-survival genes. Indeed, in aggressive tumors (chemo resistant and metastatic), constitutive activation of NF-κB has been well documented by various investigators (71, 72 and the references therein]. On the basis of these observations, we postulate that TG2 confers drug resistance and metastatic phenotypes by constitutively activating anti-apoptotic and cell-survival signaling pathways. A schematic representation of TG2's involvement in cell-survival and cell death signaling pathways is depicted in Figure 2.

3.4. TG2 as a host's response to tumor

Results of a recent study we conducted suggest that TG2 expression in the stroma surrounding the tumor is strongly associated with a node-negative status in patients with breast cancer. A similar increase in TG2 expression in the stroma was observed by Haroon *et al.* in subcutaneously implanted rat mammary tumors (73). Treatment of these tumors with enzymatically active TG2

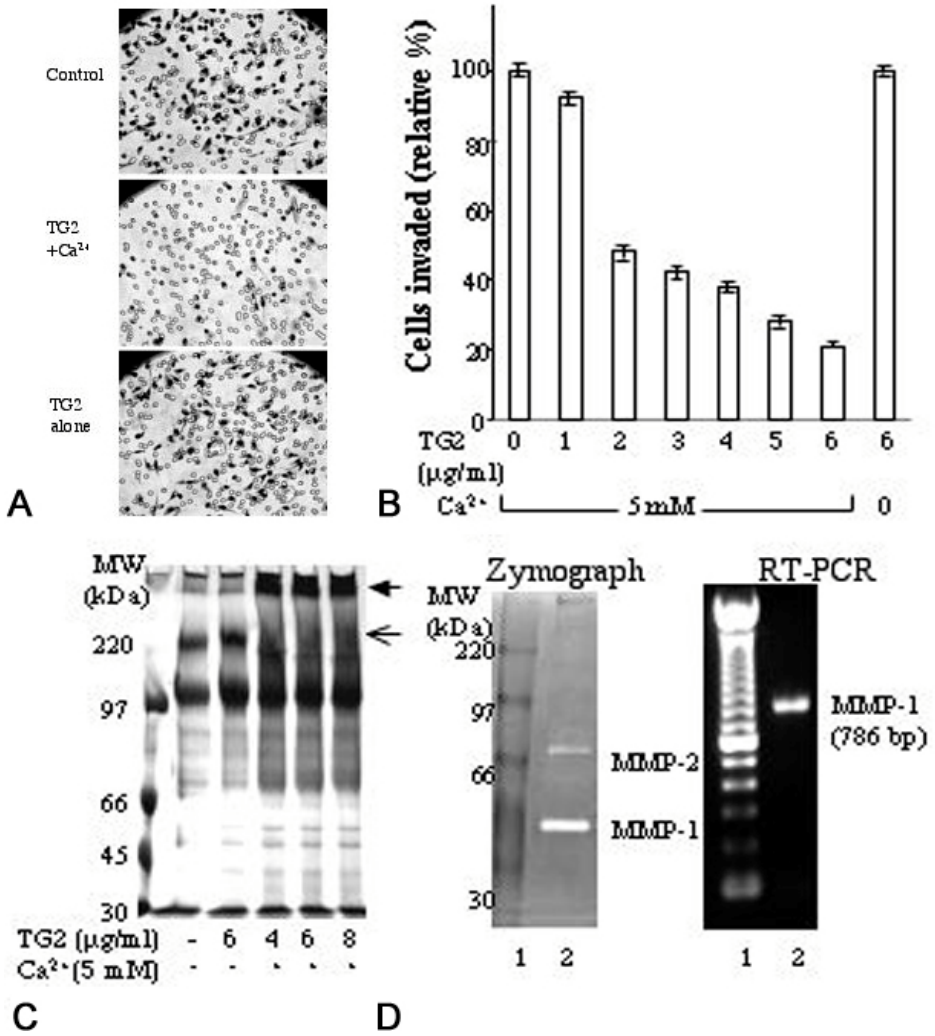

Figure 3. TG2 inhibits the invasion of MDA-MB-231 cells through a Matrigel-coated Transwell membrane. A) Matrigel contents were incubated with buffer alone (control) or buffer containing purified guinea pig liver TG2 in the presence or absence of Ca^{2+} before being coated onto the Transwell membranes. The MDA-MB-231 cells were compared for their ability to invade through the TG2-pretreated or untreated Matrigel-Transwell membranes. Representative fields with cells that migrated under the membrane were photographed. B) Matrigels containing increasing amounts of the purified TG2 were preincubated with Ca^{2+}. Another tube containing Matrigel plus TG2 without Ca^{2+} served as the control. Matrigel contents pretreated with various amounts of TG2 were layered onto the Transwell membranes and compared for their ability to support the invasion of MDA-MB-231 cells. The number of cells that migrated through the Matrigel were plotted per field. C) Equal volumes of Matrigel were incubated with buffer alone or buffer containing varying concentrations of purified TG2 in the presence (+) or absence (-) of Ca^{2+}. Forty microliter reactants, each were fractionated on 6% SDS-PAGE. The gel was stained with Coomassie brilliant blue and viewed for TG2-induced changes in protein bands. D) Basal levels of MMP-1 and MMP-2 in MDA-MB-231 cells were determined by zymogram performed on MDA-MB-231 cell supernatant or by RT-PCR.

significantly delayed tumor growth when compared with the tumors treated with catalytically inactive TG2 mutant. On the basis of these observations, Haroon et al. concluded that TG2 might constitute a distinct part of the host response to growing tumors. Additionally, by cross-linking the component proteins, TG2 may stabilize the ECM and affect tumor growth (73).

It is likely that local injury caused by the growing tumor elicits the host's response and induces cytokine production, which promotes wound healing and restricts the invasion of cells by producing new or stable ECM (74). Indeed, TG2 is capable of cross-linking several constituent proteins in the ECM, which can render the ECM more resistant to proteases and mechanical disruptions (75). TG2 can also enhance the stability of and strengthen the ECM by facilitating the activation of TGF-β. Our recent *in vitro* data shown in Figure 3 clearly support this and suggest that TG2-mediated cross-linking of ECM proteins renders the ECM resistant to the invasion of MDA-MB-231 cells, regardless of the fact that these cells actively produce matrix metalloproteinases (MMP), such as MMP-1 and MMP-2 (Figure 3D).

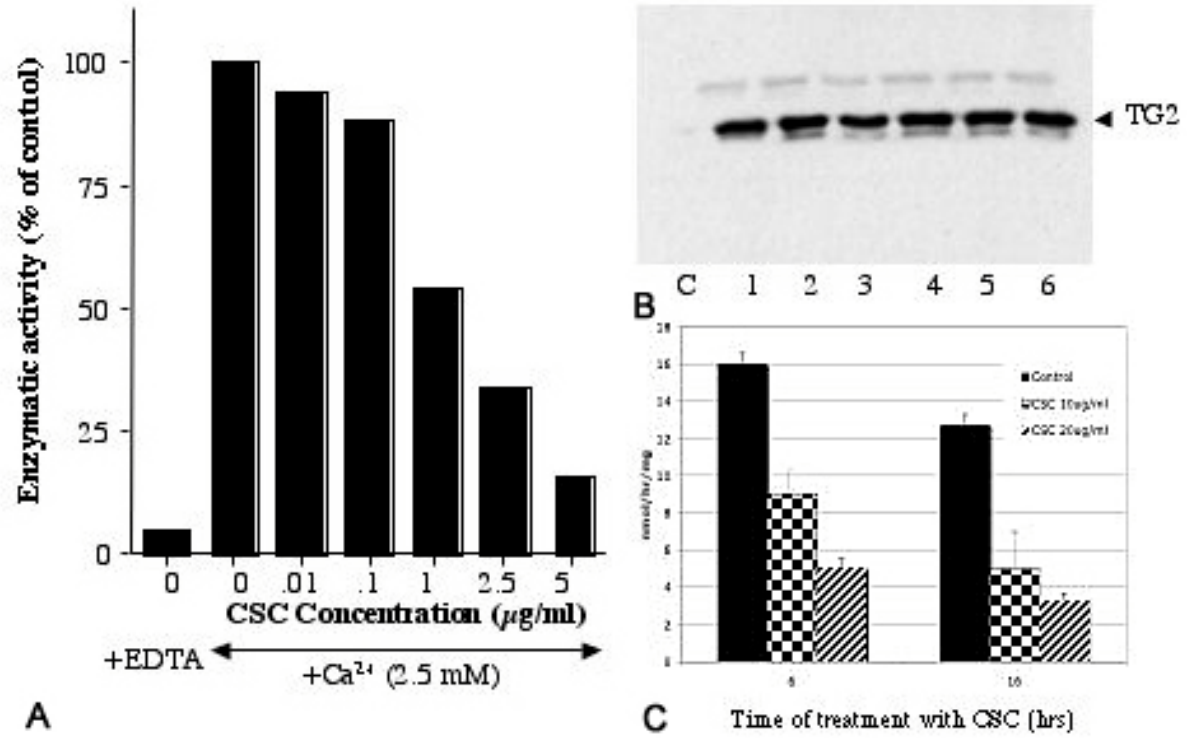

Figure 4. Effect of cigarette smoke condensates (CMC) on TG2 activity and levels A). Aliquots (0.5 μg each) of purified guinea pig liver TG2 were incubated with increasing amounts of CSC during a 15 min assay. The amount of radioactivity (CPMs due to ^{3}H- putrescine) incorporated into dimethylcasein in the absence of CSC, reflects fully active (100%) TG2 under the conditions employed and was used for calculating the percentage of inhibition induced by CSC at indicated concentrations. The enzyme was completely inactive in the absence of calcium (+EDTA). B) At the end of incubation, samples (50 μl each = 0.25 μg TG2 protein) were solubilized in 3X sample buffer and subjected to Western blotting using TG2-specific moAb. C) TG2-transfected HEK cells were cultured in presence or absence of CSC (10 or 20 μg/ml). After 6 and 16 hrs cell lysates were assayed for TG2 activity.

In conclusion, TG2 in the stroma surrounding the tumor may represent a part of the host response that prevents tumor cells from spreading to distant sites. However, the ability of tumor cells to produce proteases and other factors that could render TG2 inactive may overwhelm the ability of TG2 to prevent metastasis. Indeed, TG2 has been shown to be a good substrate for MMP-2-like proteases that are abundantly produced by metastatic tumors (76, 77). The selective induction of active TG2 in the stroma surrounding the tumor may thus offer a promising approach to limiting tumor growth and metastasis.

3.5. TG2 and carcinogenesis

An emerging paradigm in the field of carcinogenesis suggests that the interaction of integrins with their surrounding stroma plays an important role in tumorigenesis. We believe that the induction of TG2 in response to various stresses, such as oxidative stress, ultraviolet radiations, carcinogens, cigarette smoke, or viral infections can alter the interaction of epithelial cells with neighboring stroma. In one scenario, the induction of TG2 accompanied by elevated intracellular calcium levels (e.g., as a result of H_2O_2 generation) can lead to the apoptosis of the target cell. However, in some instances the catalytic functions of TG2 may be blocked, e.g., as a result of S-nitrosylation of the active-site cysteine residue (C277). Indeed, nanomolar concentrations of NO^{-} could effectively inhibit TG2 activity and anti-FAS antibody-induced apoptosis in Jurkat's cells (78). Similarly, tobacco smoke components inactivate TG2, representing an important biochemical alteration induced by smoking. Besides lung cancer, smoking is linked with the development of cancers of the larynx, oral cavity, pharynx, esophagus, pancreas, kidney, and bladder. Tobacco smoke contains more than 4000 individual compounds of which several are known carcinogens, mutagens, and tumor promoters (79). Moreover, water-soluble extracts of gas-phase cigarette smoke have been shown to contain powerful oxidizing elements, which can induce TG2 expression and mediate oxidative damage to proteins *in vitro* over several days or weeks. Each puff of smoke contains over 10 trillion free radicals, which may induce oxidative damage and thus initiate carcinogenesis. Several thiol-containing enzymes have been shown to be susceptible to inactivation by smoke components (80, 81). In this context, it is tempting to speculate that oxidative damage of the thiol group in the active-site cysteine residue (Cys277) of TG2 can render TG2 catalytically inactive. Indeed, using purified TG2 protein from guinea-pig liver, our preliminary results, revealed that cigarette smoke condensate (CSC) could effectively inhibit the enzymatic activity of TG2 in a dose-dependent manner (Figure 4). Importantly, when TG2-

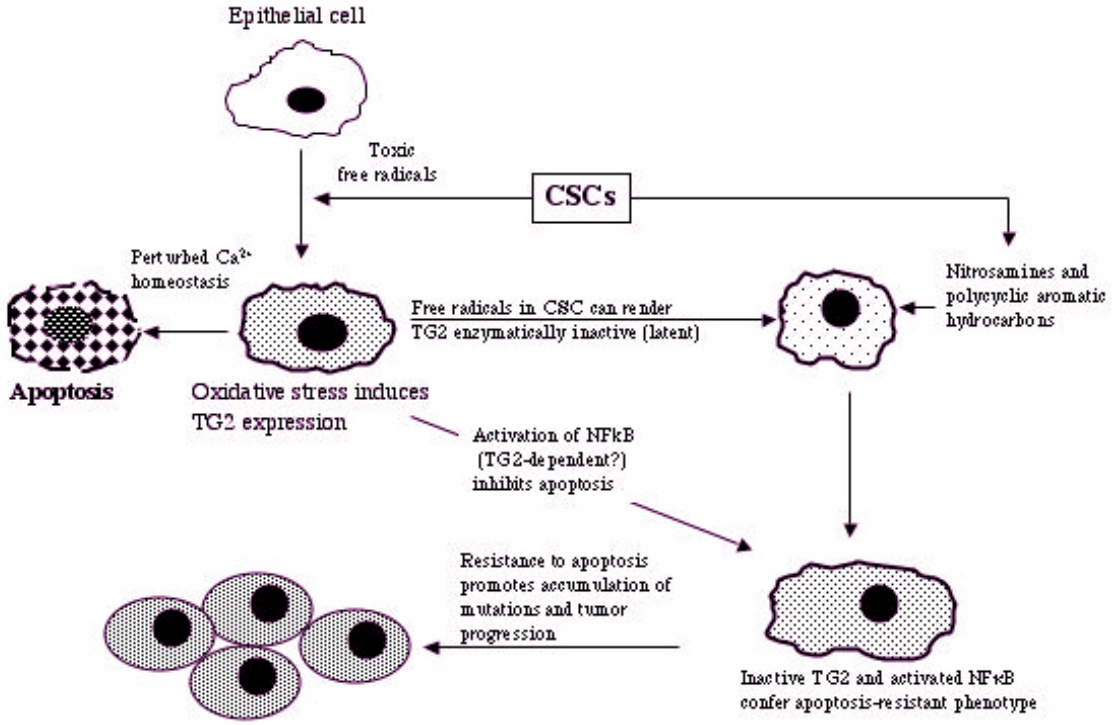

Figure 5. Hypothetical model for CSC-induced inactivation of TG2 and its role in carcinogenesis. CSCs have been shown to contain powerful oxidizing potential, which can lead to free radical production and TG2 induction in bronchial epithelial cells. The intracellular antioxidant defenses, such as glutathione and catalase, can mitigate further oxidative damage to the cells. TG2 expressing cells undergo apoptosis via Ca^{2+}-dependent activation of the enzyme. However, if intracellular oxidant load is capable of overcoming the intracellular antioxidant defenses, it can lead to oxidative damage and inactivation of thiol- containing enzymes, including TG2. The inactivation of TG2 can prevent the cells from undergoing apoptosis and can induce cell-survival and cell-growth signaling pathways by promoting the interaction of cell-surface integrins with ECM or other pathways as shown in Figure 2. The increased threshold of cells to undergo apoptosis can permit the accumulation of additional mutations in response to oxidative damage or other smoke-related carcinogens, thus increasing the tumorigenic potential of cells.

ransfected HEK-293 cells were cultured in the presence of CSC, they showed a significant decline in enzyme activity in a time and dose-dependent manner without appreciable changes in protein levels. A similar inhibition in factor XIIIa (plasma TG) catalytic activity by cigarette smoke components had been reported previously (82, 83). Similarly, the exposure of HEK-293 cells to CSC is associated with strong activation of NF-κB (84). Whether CSC-induced activation of NF-κB is TG2-dependent remains to be determined. On the basis of these observations, we believe that the inactivation of TG2's cross-linking functions can result in the failure of cells to undergo apoptosis in response to CSC or carcinogen-induced injury. The failure of cells to undergo apoptosis permits the accumulation of mutations and activates TG2-dependent cell-growth and cell-survival signaling pathways (as discussed in previous sections and Figure 2), leading to increased tumorigenic potential. A schematic representation of TG2's involvement in cigarette smoke-induced carcinogenesis is depicted in Figure 5.

4. SUMMARY AND PROSPECTIVE

TG2 is the most ubiquitous and multifunctional member of the transglutaminase family of enzymes which in addition to catalyzing Ca^{2+}-dependent post-translational modification of proteins, can also bind and hydorlyze GTP and serve as G protein in certain agonist-induced signaling pathways. TG2 can exert both pro- and anti-apoptotic effects depending on its localization within the cell. For example, in the membrane TG2 can serve as a survival factor by promoting interaction between cell surface integrin and the ECM. Similarly, in the nucleus TG2 by interacting with pRb can attenuate apoptosis. In the cytosol compartment, the stressful conditions which cause sudden perturbations in intracellular Ca^{2+} homeostasis, can convert TG2 into its cross-linking configuration leading to a massive cross-linking of cellular proteins and the onset of apoptosis. In the stroma, TG2 expression is upregualted probably as a part of host's response to the growing tumor in an attempt to restrict tumor growth and prevent it from spreading to distant sites. TG2-induced alterations in the ECM can attenuate the process of metastasis Therefore, induction of enzymatically active TG2 in the stroma surrounding the tumor, represents an interesting possibility for preventing progression of cancer to metastatic disease. Moreover, on the basis of recent observations that drug-resistant and metastatic breast cancer cells express high levels of TG2, we propose that TG2 expression may be involved in promoting the interaction of cancer cells with the ECM, leading to the activation of cell survival signaling pathways and thus conferring apoptosis-resistant

phenotype. Indeed, resistance to apoptosis is an important feature of aggressive cancers, enabling the cancer cells to withstand not only stressful environments in foreign tissue (metastasis) but also chemotherapy-induced cytotoxic effects. Recently it has been proposed that many anticancer drugs can kill tumor cells by activating common apoptotic pathways and mutations or changes that disable apoptosis could produce multidrug-resistance. Alternatively, TG2 can render cancer cells resistant to apoptosis by activating NF-κB like transcription factors that is known to regulate transcriptional activation of several metastasis and anti-apoptosis related genes. TG2 can also affect cell survival and cell growth functions by interacting with key regulatory proteins, such as phospholipase-δ_1, osteonectin, RhoA, multilineage kinases, and pRb. Therefore, elucidation of TG2-mediated signaling pathways that contribute in the development of drug-resistance and metastatic phenotypes may aid in the identification of promising molecular targets for effective treatment of aggressive form of tumors.

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