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REVIEW ARTICLE

Some lessons from the tissue transglutaminase knockout mouse

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Abstract Transglutaminase 2 (TG2) is an inducible transamidating acyltransferase that catalyzes Ca^{2+}-dependent protein modifications. It acts as a G protein in transmembrane signaling and as a cell surface adhesion mediator, this distinguishes it from other members of the transglutaminase family. The sequence motifs and domains revealed in the TG2 structure, can each be assigned distinct cellular functions, including the regulation of cytoskeleton, cell adhesion, and cell death. Though many biological functions of the enzyme have already been described or proposed previously, studies of TG2 null mice by our laboratory during the past years revealed several novel in vivo roles of the protein. In this review we will discuss these novel roles in their biological context.

Keywords Tissue transglutaminase · Knockout mice · Macrophage · Liver · Heart · Neutrophils

Tissue transglutaminase is a unique enzyme with various biological activities

Transglutaminases (TG2s) (Folk and Chung 1985) are a family of thiol- and Ca^{2+}-dependent acyl transferases that catalyze the formation of a covalent bond between the γ-carboxamide groups of peptide-bound glutamine residues and various primary amines, including the ε-amino group of lysine in certain proteins. The reaction results in post-translational modification of proteins by establishing ε-(γ-glutamyl)lysine cross-linkages and/or covalent incorporation of polyamines and histamine into proteins. Eight distinct enzymatically active transglutaminases have so far been described (Grenard et al. 2001). TG2 is very unique among them because besides being a transglutaminase it also possesses GTPase (Nakaoka et al. 1994), protein disulfide isomerase (Hasegawa et al. 2003), and protein kinase (Mishra et al. 2007) enzymatic activities. TG2 is localized predominantly in the cytoplasm; however, substantial amounts of the protein are also found in the nucleus, plasma membrane, and in the extracellular matrix (Fésüs and Piacentini 2002). Physiologically, the transamidation activity of TG2 is latent and is often manifested in various pathological states accompanied by rise in $[Ca^{2+}]$ (Lorand and Graham 2003). The binding of GTP or Ca^{2+} inhibits, respectively, the transamidation or GTPase function of TG2, which are mutually exclusive enzymatic activities in vivo (Monsonego et al. 1998).

Numerous recent observations point to a role of TG2 in cell–matrix interactions (Lorand and Graham 2003). Although TG2 has no leader sequence, hydrophobic domains, or posttranslational modifications for targeting to the endoplasmic reticulum or Golgi apparatus, it is present on the surface of various cell types and in the extracellular matrix (Thomázy and Fésüs 1989; Upchurch et al. 1991). Outside the cell TG2 interacts with the major extracellular protein fibronectin (Hang et al. 2005) and with integrins through a direct noncovalent interaction with the β1 and β3 integrin subunits and formation of stable ternary complexes with integrins and fibronectin (Akimov et al. 2000). This interaction induces integrin clustering regardless of integrin–ligand interaction and might modifiy integrin signaling (Janiak et al. 2006; Herman et al. 2006).

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To study the in vivo function of TG2, TG2 knockout mice have been developed in two laboratories (DeLaurenzi and Melino 2001; Nanda et al. 2001). These animals were found viable, to grow up to normal size and weight with no apparent abnormalities in organ functions including the extracellular matrix or the heart (where the need for its G protein function has been most expected). However, when our laboratory started to examine these mice more carefully, several abnormalities became visible. These abnormalities revealed some new biological functions related directly or indirectly to TG2.

Tissue transglutaminase enhances the in vivo apoptosis of both thymocytes and red blood cells using its transamidating activity

T cells differentiate in the thymus and due to the thymic selection processes 95% of the cells produced die without reaching the periphery. Previous studies in our laboratory have shown that TG2 is induced during the in vivo apoptosis program of the mouse thymus and appears in the dying thymocytes (Szondy et al. 1997; Szegezdi et al. 2000). When apoptotic bodies containing TG2-crosslinked proteins are taken up by phagocytic cells and digested, the $\varepsilon(\gamma$ -glutamyl)lysine crosslink formed by TG2 is not digested because it is resistant to proteolysis (Fesus and Tarcsa 1989). This dipeptide is released from phagocytes and appears in the circulation (Fesus et al. 1991). Since we detected elevated concentrations of the dipeptide during clearance of a high number of thymocytes (Szondy et al. 1997), we postulated that transamidation activity of TG2 may participate in mediating or facilitating of thymocyte apoptosis, and may contribute to the stabilization of dying cells (Piacentini et al. 1994).

In correlation with the above suggestions closer examination of the in vivo apoptosis program of the thymus has revealed that the thymus disappears slower in the $TG2^{-/-}$ animals than in their wild-type counterparts following injection of various apoptotic stimuli (Szondy et al. 2003). Though this was partially the result of an impaired phagocytosis of apoptotic cells, determination of the percentage of accumulated dead and viable cells suggested that the in vivo rate of apoptosis was also delayed. Since isolated thymocytes do not upregulate TG2 in vitro, though they die when are exposed to various apoptotic stimuli (Szegezdi et al. 2000), we could not study in vitro the role of TG2 in the enhancement of apoptosis program of thymocytes. Some of our in vivo and in vitro results, however, suggest that lack of latent TGFβ activation by macrophages in the absence of TG2 cross-linking activity (Kojima et al. 1993), which TGFβ would be needed to accelerate thymocyte apoptosis, might be partially responsible for the phenomenon (Szondy et al. 2003).

During their daily life, erythrocytes are exposed to a variety of stress situations. On average they pass once a minute through the lung, where they are exposed to oxidative stress. More than once an hour, they travel through kidney medulla, where they face osmotic shock. Red blood cells (RBCs) are deformed to squeeze through small capillaries. The loss of erythrocyte cell integrity is pathological; rupture releases hemoglobin to extracellular fluid which may be filtered at the glomerula of the kidney, precipitate in the acid lumen of the tubules, obliterate the tubules and thus lead to renal failure. To avoid these complications, in aged red blood cells an apoptosis like, caspase-independent death program is initiated (Bratosin et al. 2001). Though erythrocytes constantly express TG2, in old RBCs both protein levels and the in vitro activity of TG2 are increased (DeLaurenzi and Melino 2001). In addition, transglutaminase-catalyzed polymers were isolated from patients with Köln disease or sickle cell anemia—diseases in which the lifespan of RBCs is known to be greatly reduced (Lorand and Graham 2003), suggesting a role of TG2 also in the erythrocyte cell death program. Indeed, TG2 null red blood cells died slower than their wild-type counterparts, when they were exposed to apoptotic stimuli (Sarang et al. 2007). The apoptosis enhancing effect of TG2 was related to its transamidating activity. TG2 affected both the speed of phosphatidylserine exposure which is needed for the recognition of dying cells (Fadok et al. 1992), and the activity of calpain, which plays a key role in the activation of the proteolysis during the caspase-independent death program of red blood cells (Bratosin et al. 2001).

These data demonstrated that TG2, as we originally proposed, might indeed facilitate the death program of certain cell types using its transamidating activity, though certainly other mechanisms, through which TG2 accelerates death, might also exist (Rodolfo et al. 2004). Since, however, thymocytes died in the absence of TG2 and the loss of TG2 did not affect the longevity of erythrocytes, our data implied that TG2 does not play an initiator role in the apoptosis program in these cell types.

Tissue transglutaminase is a cell surface protein participating in the "third synapse" created between apoptotic cells and macrophages

The novel finding of the studies on TG2 null mice was that while TG2 was clearly not required for the initiation of the apoptotic program, it was required for the proper phagocytosis of apoptotic cells. Though TG2 can promote phagocytosis from the side of apoptotic cells by facilitating

the phosphatidylserine exposure (Sarang et al. 2007), or by cross-linking the S19 ribonuclear protein that acts as chemotactic factor for macrophages (Nishiura et al. 1998), the main defect was found in macrophages. This was partially related to a defect in TGFβ activation (Szondy et al. 2003), as TGFβ is released by macrophages digesting apoptotic cells (Fadok et al. 1998) and promotes phagocytosis of apoptotic cells (Rose et al. 1995). Recently, however, we found that TG2 is also needed on the macrophage cell surface to promote the concentration of integrin $\beta 3$ receptors in the phagocytic cup (unpublished data). $\alpha_v\beta_3$ and β_5 receptors play a key role in the initiation of the uptake of apoptotic cells (Akakura et al. 2004), and in the absence of this concentration the integrin β_3 fails to properly activate the Rac signaling pathway and the actin cytoskeletal rearrangement.

TG2 participates in the crosstalk between dying and phagocytic cells to ensure tissue integrity

From the in vivo results obtained in our laboratory from the TG2 null mice, we would propose that the main role of TG2 in vivo in the context of the apopto-phagocytosis program is to ensure that once apoptosis has been initiated, it is finished without causing inflammation and apparent tissue injury. There are many ways through which TG2 can achieve this goal.

In many cell types TG2 enhances the rate of apoptosis either directly by acting within the apoptotic cells (Rodolfo et al. 2004; Sarang et al. 2007) or indirectly by acting in macrophages, where it activates TGFβ released by macrophages ingesting apoptotic cells (Kojima et al. 1993). TGF-β then enhances apoptosis in TGFβ-sensitive cells such as thymocytes (Szondy et al. 2003). In addition, it participates in the in vivo induction of TG2 in both macrophages and apoptotic cells forming an autoregulatory loop in the regulation of TG2 expression (Szondy et al. 2003). TG2-dependent cross-linking of proteins and formation of protective protenaceous shells will prevent the leakage of harmful cell content from the apoptotic cells (Piredda et al. 1997), while TG2 in macrophages will promote the speed of phagocytosis (Szondy et al. 2003). In apoptotic cells TG2 also promotes the formation of chemoattractants (Nishiura et al. 1998) and the exposure of phosphatidylserine (Sarang et al. 2007) that facilitate migration of macrophages to the apoptosis site and recognition of apoptotic cells, respectively. In addition, TGFβ (unpublished data, TG2) is also required for the proper downregulation of the proinflammatory cytokine formation by macrophages (Fadok et al. 1998; Falasca et al. 2005), which otherwise would induce an inflammatory response. These mechanisms ensure that all the unwanted cells are killed and removed fast without leading to necrosis and inflammation. If, however, necrosis still occurs TG2 promotes both tissue stability and repair (Nardacci et al. 2003).

In $TG2^{-/-}$ animals all these anti-inflammatory actions are compromised resulting in the appearance of inflammatory cells at the apoptosis sites in short term and leading on long term to autoimmunity (Szondy et al. 2003).

TG2 is required for proper differentiation and bacterial killing of neutrophils

Phagocytes are myeloid-derived leukocytes that are specialized in exiting the blood vessels, migrating through the connective tissues, and finding large, particular targets such as bacteria and fungi to ingest. Myeloid cells committed to mature toward neutrophils spend about 14 days in the bone marrow, where they mature completely to a terminally differentiated state. During this time period, the cells undergo remarkable morphologic and functional changes. A larger portion of the chromatin condenses and associates with the nuclear envelope to form filament-like structures. Meanwhile, the cytosol is filled up with proteins involved in nonoxidative (e.g., lactoferrin and gelatinase in specific granules) as well as oxidative (e.g., the NADPH oxidase system) killing and in destruction of microbes (Lopez-Boado et al. 2004; Ellison and Giehl 1991; Hampton et al. 1998). Neutrophils express TG2 (Kim and Rikihisa 2002).

We used NB4 cells (Lanotte et al. 1991) to study the role of TG2 in the differentiation and function of neutrophils. TG2 expression was induced concomitant with initiation of neutrophil granulocyte differentiation, and part of the enzyme appeared in the nucleus (Balajthy et al. 2006). In line with these observations the transglutaminase-catalyzed cross-link content of both the cytosolic and the nuclear protein fractions increased. Inhibition of cross-linking activity of TG2 by monodansylcadaverine in the differentiating cells led to diminished nitroblue tetrazolium (NBT) positivity (one of the most evident indicators of neutrophil maturation beyond morphologic features), production of less superoxide anion, and decreased expression of GP91PHOX, the membrane-associated subunit of NADPH oxidase. These observations were confirmed by testing neutrophils isolated from $TG2^{-/-}$ mice, which also showed diminished NBT reduction capacity, reduced superoxide anion formation, and down-regulation of the gp91phox subunit of NADPH oxidase, compared with wild-type cells. In addition, $TG2^{-/-}$ mice exhibited increased neutrophil phagocytic activity toward bacteria and yeast, but had attenuated neutrophil chemotaxis and impaired neutrophil extravasation with higher neutrophil counts in their circulation during yeast extract-induced peritonitis (Balajthy

et al. 2006). Neutrophil extravasation, subsequent phagocytosis, and superoxide anion generation play a crucial role in host defense. Failure in elimination of exogenous or translocated endogenous bacteria due to impairment of these processes would contribute to persistent bacterial infection. On the other hand, failure of these processes during pathological inflammatory upregulation occurring in septic shock protects $TG2^{-/-}$ animals from the lethal consequences (Falasca et al. 2008). The results clearly suggest that TG2 may modulate the expression of genes related to neutrophil functions and is involved in several intracellular and extracellular functions of extravasating neutrophil, though the exact mechanism of these actions is still not clarified.

TG2 participates in the hepatic tissue regeneration program acting as a G protein in the alpha$_{1b}$-adrenergic receptor signaling pathway

Under physiological conditions, as few as one out of 2000–3000 hepatocytes divide to maintain the physiological liver mass. Liver damage or loss of liver mass can however extensively stimulate the regenerative capacity until the tissue mass has been restored. In a young adult mouse, approximately 95% of hepatocytes replicate during the first 3 days after partial hepatectomy and up to 75% of surgically removed liver mass can be regenerated within 1 week (Michalopoulos and DeFrances 1997). Accelerated parenchymal regeneration after necrogenic (CCl_4 administration) or surgical loss of liver tissue principally originates from the extensive proliferation of mature parenchymal liver cells (Fausto et al. 2006).

A wide variety of genes are differentially expressed during the first few hours after partial hepatectomy (the "priming phase"); many of these genes are involved in a cytokine network involving interleukin-6 and tumor necrosis factor (TNF)α receptors (Iwai et al. 2001; Akerman et al. 1992). Cytokines are important for regeneration, because hepatocytes in the normal liver exhibit only a minimal response to potent in vitro mitogens, such as transforming growth factor alpha, epidermal growth factor, and hepatocyte growth factor (HGF). However, growth factor infusion into animals preceded by a single TNF injection induces replication in up to 40% of hepatocytes in the normal liver (Webber et al. 1998).

Release of cytokines is followed by the release of growth factors such as Fas ligand, HGF, and norepinephrine within 2 h following liver injury (Michalopoulus and DeFrances, 1997; Desbarats and Newell 2000). HGF transmits its signals via tyrosine kinase receptors (Stuart et al. 2000), while norepinephrine acts on the alpha$_{1b}$-adrenergic receptors, which couple signals to the phospholipase C-δ1 (Feng et al. 1996) and to hepatocyte proliferation (Wu et al. 2000) via TG2 acting as G protein (Nakaoka et al. 1994). Though under physiological conditions engagement of Fas receptors triggers a Caspase 8 and Bid-dependent apoptosis in hepatocytes (Yin et al. 1999), following liver injury Fas ligand induces hepatocyte proliferation, and this effect is related to a simultaneous inhibition of the Fas-mediated cell death pathway (Desbarats and Newell 2000). Both HGF (Kosai et al. 1998) and norepinephrine (acting on beta$_2$-adrenergic receptors) (André et al. 1999) were reported to play a role in blocking Fas-mediated death pathway in hepatocytes. $TG2^{-/-}$ mice revealed that norepinephrine acting on alpha$_{1b}$-adrenergic receptors also blocks the Fas-mediated pathway in hepatocytes (Sarang et al. 2005). While, however, for inhibiting the Fas-mediated death pathway beta$_2$-adrenergic receptors seem to elevate the levels of $FLIP_L$, a protein which interferes with the autoproteolytical maturation of Fas-FADD-bound Caspase-8 (FLICE) proximal in the Fas apoptotic pathway, alpha$_{1b}$-adrenergic receptors enhance Bcl-x_L expression, which interferes with the action of Bid (Sarang et al. 2005). Thus, TG2 might be involved in the liver regeneration program acting as a G protein in the alpha$_{1b}$-adrenergic signaling pathway (1) to induce cell proliferation (Wu et al. 2000), (2) to promote HGF production (Broten et al. 1999), and (3) to inhibit Fas receptor-mediated cell death following liver injury. In line with these observations an increase in the liver TG2 expression was observed following CCl_4 exposure, and $TG2^{-/-}$ mice were more sensitive to CCl_4-induced hepatic injury (Nardacci et al. 2003).

TG2 affects mitochondrial ATP synthesis in the heart possibly acting as protein disulfide isomerase

Since alpha$_{1b}$-adrenergic receptors are also expressed by cardiac cells (Ramarao et al. 1992), and Fas signaling was shown to participate in heart failure (Yamaguchi et al. 1999; Jeremias et al. 2000), we decided to investigate whether alpha$_{1b}$-adrenergic receptors might play a similar role in protecting against Fas-mediated death in cardiac cells during ischemia/reperfusion injury as they do in hepatocytes (Szondy et al. 2006). While, however, loss of alpha$_{1b}$-adrenergic receptors in knockout animals did not affect the infarct size, the incidence of ventricular fibrillations or the postischemic cardiac functional parameters, $TG2^{-/-}$ hearts were more sensitive to ischemia/reperfusion injury suggesting an adrenergic receptor-independent protective role of TG2. Interestingly, already the preischemic values in heart rate, coronary flow, aortic flow, and aortic pressure of the $TG2^{-/-}$ control group were significantly reduced when compared to those of the wild-type values. Considering that high-energy phosphate content was

reported to affect the cardiac functional parameters before and after ischemia/reperfusion (Fenchel et al. 1988), and it also mirrors the degree of cellular damage, we tested the high-energy phosphate content of the wild-type versus $TG2^{-/-}$ hearts before and after ischemia/reperfusion. We found a significant reduction in the high-energy phosphate content present in the $TG2^{-/-}$ mice already before ischemia, which was further reduced following ischemia/reperfusion. Prompted by these and previous results, which showed that TG2 co-localizes with mitochondria and its over-expression leads to mitochondrial hyperpolarization (Piacentini et al. 2002), together with Piacentini's group we measured the membrane polarization ($\Delta\psi$) on mitochondria isolated from hearts of control and $TG2^{-/-}$ mice and found a defect in the function of the respiratory chain complex V. In line with these observations a defect in maintaining ATP levels was also detected in the skeletal muscle following prolonged exercise.

$TG2^{-/-}$ mice show a phenotype resembling that of the maturity onset diabetes of young (MODY) (Bernassola et al. 2002). Defects in glucose-induced insulin release are thought to be linked to defective respiratory chain activation and loss of ATP production (Maechler and Wollheim 2001) implying a broader involvement of TG2 than cardiac or skeletal muscle in the mitochondrial function. Though the precise role of TG2 in maintaining the mitochondrial respiratory chain function is not clear yet, the protein disulfide isomerase activity of it, which contributes to the formation of disulfide bridges in proteins of mitochondrial respiratory complexes, might be involved (Mastroberardino et al. 2006).

Concluding remarks

TG2 is emerging as a well-characterized, multifunctional molecular player in various cellular processes, ranging from intracellular signaling to apoptosis and pathological conditions such as autoimmune and Huntington diseases. We are convinced that further studies of TG2 null mice will still reveal further novel biological functions of this enigmatic protein.

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