FEBS 29498 FEBS Letters 579 (2005) 3297–3302

Minireview

Transglutaminase 2 in the balance of cell death and survival

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Accepted 23 March 2005

Available online 7 April 2005

Edited by Péter Friedrich

Abstract Transglutaminase 2 (TG2), a multifunctional enzyme with Ca^{2+}-dependent protein crosslinking activity and GTP-dependent G protein functions, is often upregulated in cells undergoing apoptosis. In cultured cells TG2 may exert both pro- and anti-apoptotic effects depending upon the type of cell, the kind of death stimuli, the intracellular localization of the enzyme and the type of its activities switched on. The majority of data support the notion that transamidation by TG2 can both facilitate and inhibit apoptosis, while the GTP-bound form of the enzyme generally protects cells against death. In vivo studies confirm the Janus face of TG2 in the initiation of the apoptotic program. In addition, they reveal a further role: the prevention of inflammation, tissue injury and autoimmunity once the apoptosis has already been initiated. This function of TG2 is partially achieved by being expressed and activated also in macrophages digesting apoptotic cells and mediating a crosstalk between dying and phagocytic cells.

Keywords: Transglutaminase; Apoptosis; Signalling pathway; Phagocytosis; Autoimmunity

1. Introduction

During the last 15 years tremendous progress has been made in revealing the molecular mechanisms of apoptosis and other forms of natural cell death. It became evident that there are multiple pathways that mediate either death or survival of cells with the participation of pro- and anti-apoptotic protein families, such as caspase, Bcl-2 and death receptor proteins. However, members of either of these families may mediate opposite effects: e.g., some caspases are not only killer enzymes but may mediate cell proliferation as well [1], Bax, Bak and BH3-only proteins of the Bcl-2 survival protein family initiate apoptosis [2], while triggering the Fas death receptors can also promote proliferation [3].

Transglutaminase 2 (TG2) is a unique member of an enzyme family (EC 2.3.3.13) because in addition to its primary enzymatic activity of Ca^{2+}-dependent transamidation of polypeptide chains through their glutamine and lysine residues (or through polyamines), it also binds GTP (which blocks transamidation) and may act as a G protein. In addition, it also has a protein disulfide isomerase activity and may function even as a protein kinase [4–7]. Besides acting intracellularly, TG2 can also be secreted by unidentified mechanisms into the cellular environment, where it may participate in cell adhesion processes and stabilization of the extracellular matrix [8].

Some years ago we have described that TG2/tissue transglutaminase is induced and activated in cells undergoing apoptosis in the liver and thymus forming highly cross-linked protein polymers and proteinaceous shells which were resistant to detergents as well as chaotropic agents and could be isolated from tissues [9,10]. We postulated that transamidation activity of TG2 may be one of the mediators or facilitators of apoptosis, and may contribute to the stabilization of dying cells. [11,12]. While several additional data have provided further evidence for a pro-apoptotic activity of TG2, recent results have raised the possibility also for a survival role. Can these seemingly contradictory sets of data be reconciled?

2. Pro-apoptotic functions of TG2

Accumulation of TG2 in various cells undergoing apoptosis upon divergent stimuli has been demonstrated both in vivo and under cell culture conditions [13–15]. The induction of the enzyme may be mediated through various nuclear receptors and response elements including retinoids and tumor growth factor β (TGFβ) [16–18]. Since cell penetrating specific inhibitors of transglutaminases were not available, alternative approaches had to be used to clarify the role of TG2 in the apoptosis process. In U937 cells overexpression of the enzyme primed cells for suicide, while by inhibiting its expression using anti-sense transglutaminase constructs the rate of apoptosis could be significantly reduced [19]. Similarly, overexpression of TG2 in neuroblastoma cells resulted in a 4–5-fold more rapid death as compared to the wild-type cells [20]. This suggested a direct role of the enzyme in the death program. Using cell permeable synthetic substrates it was also demonstrated that in HL-60 and U937 cells the enzyme could transamidate the actin and retinoblastoma (Rb) proteins following the initiation of apoptosis [21,22]. However, in our yet unpublished experiments an active site mutant of TG2 could also promote cell death in these cells suggesting that

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Abbreviations: EGF, epidermal growth factor; HPR, *N*-(hydroxyphenyl)retinamide; Rb, retinoblastoma protein; TG, transglutaminase; TGFβ, tumor growth factor β

0014-5793/$30.00
doi:10.1016/j.febslet.2005.03.063

the transamidation function does not have an exclusive role in the apoptosis induction. One possible mechanism is the induction of death through the recently described BH3 domain of TG2 [23]. Cell permeable peptides mimicking this domain (but not its mutants forms) could induce conformational change and translocation of Bax to the mitochondria, release of cytochrome *c* and death of neuroblastoma cells. In addition, Bax acted as a substrate of TG2 at the mitochondrial level. Based on these results Piacentini and his co-workers have proposed an interaction between TG2 and Bax through their BH3 domains. A TG2-dependent polymerization of Bax may occur when the level of Ca^{2+} is increased at the mitochondrial level during the course of apoptosis. The covalent polymerization resulted may then stabilize the pore-forming and cytochrome *c*-releasing conformation of Bax. Clearly, this puts TG2 onto the stage of upstream regulatory players of the mitochondrial apoptosis pathway. It remains to be seen, however, how general this phenomenon is in terms of cell types and apoptotic signaling pathways.

In the late phase of apoptosis dropping of the normally high intracellular GTP concentration (which blocks transamidating activity of TG2) and the overall elevation of Ca^{2+} levels result in extensive protein cross-linking and formation of detergent insoluble protein scaffolds in cells containing high levels of TG2 [10]. Similar observations were made when TG2 containing cells were exposed to stimuli leading to loss of Ca^{2+} homeostasis and consequent necrosis [24]. TG2-dependent crosslinking stabilizes the dying cells before their clearance, inhibits leakage of intracellular components and may prevent scarring and inflammation [11,25].

3. TG2 may protect against apoptosis

Adhesion-dependent survival signaling of cells is mainly mediated by the adhesive glycoprotein fibronectin binding to cell-surface matrix receptors (primarily the $\alpha_5\beta_1$ integrins) through their Arg-Gly-Asp (RGD) sites. Synthetic RGD peptides inhibit this binding leading to detachment-induced apoptosis called anoikis. Griffin and his co-workers [26] have described a novel RGD-independent cell adhesion mechanism of osteoblasts and fibroblasts that rescues cells from anoikis induced by blocking the RGD-dependent survival signaling. This newly proposed pathway is mediated by externalized TG2 bound to fibronectin and cell surface heparan sulfate chains, is integrin-independent, requires the function of protein kinase Cα and leads to activation of Rho and the focal adhesion kinase. Cell adhesion to TG2- fibronectin does not require the transamidating activity of the enzyme, though at the high calcium concentration of the extracellular space TG2 is very likely in the active conformation [27].

According to Aeschlimann et al. [28] TG2 can also promote cell adhesion by regulating and being regulated by phospholipase C through binding to it with the non-transamidating GTP form of the enzyme [29]. This pathway also involves protein kinase Cα, Rho and focal adhesion kinase, but the mechanism is independent of the externalization and binding of TG2 to fibronectin. Therefore, it is still an open question how TG2 is involved in the extracellular matrix-dependent cells survival mechanism.

Cerione and his co-workers [30] have recently shown that in some cell lines treated with retinoic acid the retinoic acid-induced TG2 can protect also against apoptosis induced by the synthetic retinoid *N*-(hydroxyphenyl)retinamide (HPR). Phosphoinositide 3-kinase activity was required for both the retinoic acid-stimulated expression and GTP-binding activity of TG2 [31], while its induction was antagonized by the Ras-ERK pathway [32]. GTPase activity of TG2 was found sufficient to protect cells from HPR-induced death suggesting that the survival signaling does not require transamidating activity of TG2 [30]. On the other hand, in their next set of experiments it was shown that TG2 protects Rb from caspase-induced degradation in a transamidation dependent manner [33], and suggested that transamidation of Rb by TG2 is necessary for its ability to inhibit apoptosis. This apparent controversy may arise from the use of monodansylcadaverine which is a competitive but non-specific inhibitor of transglutaminases and is known to accumulate in membrane structures. Therefore, without direct demonstration of in situ changes in protein transamidation the use of monodansylcadaverine by itself is not sufficient to prove that the observed effect is linked to transamidation function of TG2.

In breast cancer cell lines epidermal growth factor (EGF) could inhibit doxorubicin-induced apoptosis while upregulated TG2 [34]. Expression of exogenous TG2 could mimic the survival advantage of EGF. In addition, the observation that transfection of cells with transamidation-defective TG2 before EGF treament could block the death-preventing effect of EGF argued for the role of transaminidation in the protection against death.

4. What determines the differential effect of TG2 on cell fate?

The above results clearly suggest that TG2 has the capability both to facilitate and to prevent apoptosis. These two opposing activities may be separated in the sense that each occurs distinctly depending on the specific biochemical pathways of apoptosis in different cell types, the kind of stimuli, the intracellular localization of the enzyme and the type of activity of TG2 switched on. It has been proposed that the observed upregulation of TG2 in some cells dying by apoptosis upon distinct stimuli is a cellular regulatory mechanism to block or delay the onset of death rather than reflecting a direct participation of the enzyme in apoptosis [33]. On the other hand, the BH3 domain of TG2 can clearly mediate cell death at the mitochondrial level in other types of cells. TG2–Bax interaction might be anti-apoptotic at first by blocking Bax action on the mitochondria and preventing cytochrome *c* release [23]. After apoptosis induction, however, the calcium-activated TG2 polymerizes Bax to a pore-forming complex. Alternatively, it cannot be excluded that the TG2–Bax complex is already proapoptotic similarly to other BH3-only proteins without transamidating activity, and transamidation results only in further stabilization of the proapoptotic conformation.

It has also been shown that TG2 modulates apoptosis in a stimulus-dependent manner [35]. It potentiates apoptosis in response to osmotic stress with increased in situ transamidating activity, and protects cells against heat shock-induced apoptosis without increased transamidation, very likely acting as a G protein.

Johnson and her co-workers [36] have designed experiments to see how the intracellular localization and transamidating activity of TG2 modulates its effect on thapsigargin-induced

apoptosis. These studies utilized externally added TG2 variants targeting different cellular compartments and it was found that cytosolic TG2 was pro-apoptotic by virtue of its transamidating activity. On the other hand, its nuclear localization attenuated apoptosis in human embryonic kidney cells without a requirement for transamidation but being dependent on a non-covalent interaction between TG2 and Rb as opposed to the need of transamidation of RB to protect human promyelocytic leukemia cells [33].

Secreted TG2 can protect cells from anoikis in an RGD independent manner without a requirement for the G protein or the transamidating activity [26]. Regarding transamidation and G protein function the so far published data show that the G form of the enzyme has not been found proapoptotic in any system while its transamidating activity was more often required for facilitating cell death than for survival. One cannot exclude the possibility that the newly discovered biochemical properties of TG2, namely its protein disulfide isomerase and protein kinase activity [6,7], are also important in determining how TG2 influences cell death.

5. Messages from the living tissues

All the results discussed in the above sections have been obtained from cell culture experiments, and it is still a question, how relevant these data are to the in vivo settings. TG2 knock out mice have been developed in two laboratories [37,38], and 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). Cells taken from these animals did not show any defect in apoptosis in either way, that is they were not less or not more resistant to death stimuli than their normal counterpart. Certainly, these observations may question both the pro-apoptotic and pro-survival function of TG2. One explanation may be the possibility that induction of other transglutaminases my compensate for the loss of TG2 in these mice, as we also found induction of TG1, 3, 5 and 7, in addition to TG2, in the thymus following injection of various apoptotic stimuli (our yet unpublished observation). Alternatively, for the in vivo induction of TG2 in apoptotic cells the tissue environment is also required, as we did not find induction of TG2 in apoptotic thymocytes under in vitro conditions, while it was highly elevated in vivo [39,18]. The lack of TG2 induction in vitro might explain while TG2+/+ and TG2−/− died with similar kinetics in vitro. In line with this possibility we found that TG2+/+ red blood cells that express TG2 constitutively [40] expose phosphatidylserine, an early sign of apoptosis [41], faster than TG2−/− red blood cells if exposed to an apoptotic stimulus implying that at least in these cells the presence of TG2 facilitates apoptosis-related events.

There are indirect data which suggest that transglutaminase-mediated processes are indeed involved in the in vivo apoptosis program. When the crosslinked apoptotic bodies are taken up by professional or non-professional phagocytic cells and digested in the lysosomes, the ε(γ-glutamyl)lysine crosslink formed by transglutaminase is not digested because it is resistant to proteolysis [42]. This dipeptide is released from phagocytes and appears in the circulation [43]. In line with the idea of the involvement of TG2 in the in vivo apoptosis program, we detected elevated concentrations of the dipeptide in the blood during clearance of a high number of apoptotic cells in the thymus or liver [39,43].

In correlation with these observations closer examination of the in vivo apoptosis program of the thymus has revealed that that thymus disappears slower in the TG2−/− animals than in their wild-type counterparts following injection of various apoptotic stimuli [14]. 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. The pro-death activity of TG2 was detected in neuronal cells as well, where ablation of TG2 reduced autophagy type of death in a model of Huntington's disease [44].

The novel finding of the studies on TG2−/− 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 could promote phagocytosis from the side of apoptotic cells by facilitating the phosphatidylserine exposure that is required for the recognition of apoptotic cells [45], or by crosslinking the S19 ribonuclear protein that acts as chemotactic factor for macrophages [46], the main defect was found in macrophages. This was partially related to a defect in TGFβ activation [47], as TGFβ was shown to promote phagocytosis of apoptotic cells [14,48]. In addition, these macrophages show detectable changes also in their cytoskeletal structure suggesting that signaling pathways that regulate cytoskeletal rearrangement during phagocytosis might also be affected by the absence of TG2. This possibility is under current investigation in our laboratory.

Interestingly, we found that in certain cells and conditions TG2 can protect cells against death in vivo. Injecting anti-Fas antibodies to study thymic apoptosis, we found that TG2−/− mice are more susceptible to Fas-induced death than their wild-type counterparts. This was found to be related to an impaired α1b-adrenergic signaling in the liver, in which TG2 participates as a G protein. In hepatocytes Fas engagement induces a Bid and mitochondria-dependent apoptosis [49], and we found that the impaired α1b-adrenergic signaling results in a decreased bcl-x_L expression in these cells. TG2−/− cardiac cells were also found to be more susceptible to ischemia reperfusion-induced injury than their wild-type counterparts; however, this is not related to the G protein activity of TG2 (our yet unpublished observation). In addition, TG2−/− hepatocytes were more sensitive also to carbon tetrachloride-induced liver injury as a result of a decreased tissue stability and repair in the absence of TG2; these observations were confirmed by human studies as well [50].

6. Participation of TG2 in the crosstalk between dying and phagocytic cells to ensure tissue integrity

The above in vivo studies confirmed the Janus face activity of TG2 in the initiation of apoptosis. In certain cells, like thymocytes, neurons or red blood cells, TG2 facilitates apoptosis. In hepatocytes and cardiac cells it has a protective role against induction of massive cell death. Generally, however, from the in vivo results obtained in our laboratory we would propose that the main role of TG2 in vivo 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 (Fig. 1). It promotes apoptosis by either direct mechanism in certain apoptotic cells [19,20,23], or indirectly by promoting activation of TGFβ released by macrophages that can promote the death of various cells [14,51]. This ensures that all the unwanted cells are killed and fast without leading to necrosis. In apoptotic cells TG2 also promotes the formation of chemoattractants [46] and the exposure of phosphatidylserine that facilitate migration of macrophages to the apoptosis site and recognition of apoptotic cells, respectively. TG2 also participates in the activation of TGFβ which is required for the in vivo induction of TG2 in both macrophages and apoptotic cells [14,52]. TG2-dependent crosslinking of proteins and formation of protective protenaceous shells will prevent the leakage of harmful cell content from the apoptotic cells [11], while TG2 in macrophages will promote the speed of phagocytosis [14] and result in further formation of TGFβ (Fig. 2). In addition to promoing the rate of apoptosis, induction of TG2 and the efficiency of phagocytosis, TGFβ was found to be essential for the proper downregulation of proinflammatory cytokine production in macrophages as well [53]. If, however, necrosis still occurs TG2 promotes both tissue stability and repair [50]. In TG2−/− animals all these anti-inflammatory actions are compromised resulting in the appearance of inflammatory cells at the apoptosic sites in short term and leading on long term to autoimmunity [14].

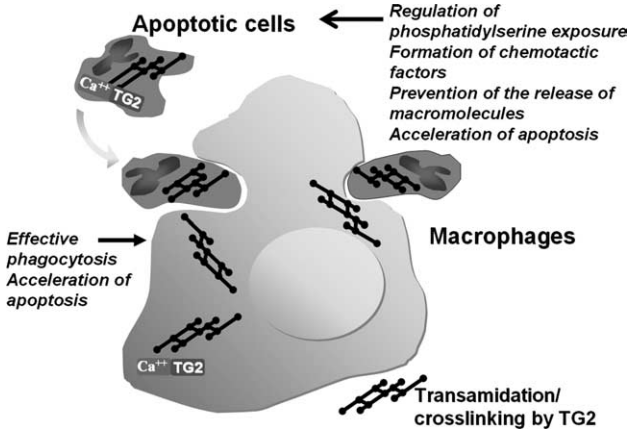

Fig. 1. Transglutaminase 2 expressed both in apoptotic cells and macrophages participates in various processes that ensure the fast recognition and removal of apoptotic cells, and prevention of the release of harmful cellular content from the dying cells.

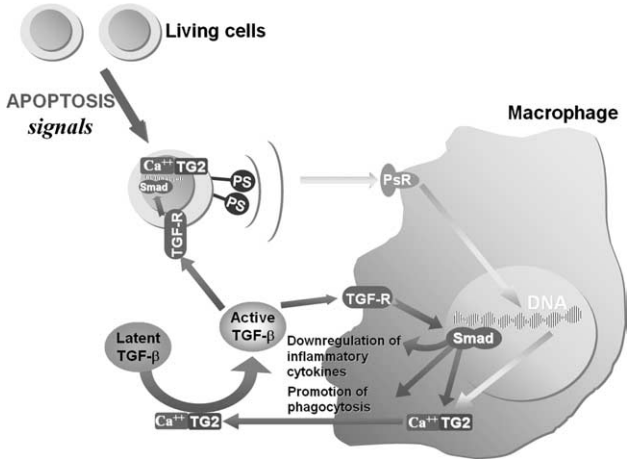

Fig. 2. Connection between tissue transglutaminase and TGF-β in the regulation of apoptosis and removal of apoptotic cells in the thymus. Recognition of apoptotic cells via phosphatidylserine receptors (PsR) triggers latent TGF-β release and activation by the simultaneously released TG2. Both macrophages and apoptotic cells possess TGF-β receptors (TGF-R). In apoptotic thymocytes TGF-β promotes apoptosis induced by specific signals and induces TG2, while in macrophages TGF-β promotes phagocytosis and downregulation of the formation of proinflammatory cytokines. Induction of TG2 by TGF-β in macrophages results in an autoregulatory loop leading to further TGF-β formation and release.

Acknowledgments: Works in the authors' laboratories have been supported by the Hungarian National Research Fund OTKA TS 044798, T043083, T 034, T04944, by EC QLK3-CT-2002-02017 and from grants of the Hungarian Ministry of Health.

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