Minireview

'Tissue' transglutaminase in cell death: a downstream or a multifunctional upstream effector?

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Abstract Apoptotic cells show morphological modifications which occur as the result of complex molecular mechanisms involving several proteins including 'tissue' transglutaminase (tTG). Although tTG was originally thought to be responsible for the protein crosslinks which prevent the leakage of intracellular components, thereby reducing inflammation and autoimmunity, recent evidence indicates that tTG is a multifunctional enzyme involved in the complex upstream regulation of the apoptotic machinery: (i) it functions as a GTP-binding protein to transduce signals; (ii) it binds/crosslinks only specific cytosolic and nuclear substrates, suggesting highly specific actions, e.g. on intermediate filaments and in cell cycle control; (iii) it is finely tuned by Ca^{2+}, GTP, *S*-nitrosylation, polyamines. In light of these recent discoveries, the role of tTG in the regulation of the crucial balance between survival and death is clearly complex.

Key words: Apoptosis; Cell death; Transglutaminase; Protein cross-link; Retinoblastoma protein; Caspase; *S*-Nitrosylation

1. Introduction

Cells undergoing apoptosis are characterised by typical ultrastructural modifications which involve complex molecular mechanisms regulated at both the transcriptional and the post-transcriptional levels [1,2]. While most proteins involved in the apoptotic pathway have been shown to follow common pathways, see for example Fig. 1, the role of 'tissue' transglutaminase (tTG or type 2 TGase; EC 2.3.2.13) has not been fully defined. In this review, we will discuss some recent developments on the role of tTG.

tTG belongs to the transglutaminase family which includes intracellular and extracellular enzymes catalysing Ca^{2+}-dependent reactions resulting in the formation of the ε(γ-glutamyl)lysine crosslinks and/or to the covalent incorporation of di- and polyamines and histamine [3]. tTG, like all TGases [4], is formed by an N-terminal domain, a catalytic domain and two β-barrels (Fig. 2). The catalytic pocket is identical to that of the cysteine proteases (Fig. 3), with the exception of the absence of H_2O. This water-depleted structure prevents the complete reaction from occurring and therefore the structure must revert to its original conformation using a different amino group (lysine or polyamines) with a long aliphatic chain. The product of the reaction is a pseudo-isodipeptide bond. Diamines and polyamines may also participate in crosslinking reactions through the formation of *N*,*N*-bis(γ-glutamyl)polyamine bonds [5]. The establishment of these covalent crosslinks leads to the post-translational modification and, in many instances, the oligomerisation of substrate proteins. The resulting protein polymers have peculiar features of resistance to breakage and chemical attack and can release polypeptides only through the proteolytic degradation of protein chains. In fact, endoproteases capable of hydrolysing the crosslinks formed by TGases have been described only in leech saliva [6].

Although the tTG gene is constitutively expressed in some cell types localised in select mammalian tissues, the onset of apoptosis in vivo is often characterised by the induction of the tTG gene. The presence and activity of the enzyme has been shown to increase in apoptotic cells in numerous in vivo and in vitro cell death models [7–9]. The activation of the tTG protein in the dying cells results in the assembly of a highly crosslinked intracellular protein net stabilised by both spermidine-derived and ε(γ-glutamyl)lysine crosslinks. Previous studies have suggested that the activation of tTG leads to the assembly of intracellular crosslinked protein polymers which irreversibly modifies cell organisation contributing to the wide ultrastructural changes occurring in cells undergoing apoptosis [7,8]. This extensive tTG-dependent protein polymerisation stabilises apoptotic cells before their clearance by phagocytosis, thus contributing to the prevention of inflammation in the surrounding tissues [10]. Thus, tTG can be said to play a role 'downstream' in the effector/degradation phases of apoptosis.

This 'downstream role' concept originated from the peculiar form of cell death occurring in the skin where keratinocytes differentiate, before dying, to producing all ingredients necessary for tissue function such as TGases and their substrates. The dead keratinocytes play a crucial role in the structure of the organism, as they confer resistance and elasticity, and serve as a barrier against water. Morphological, biochemical and molecular studies [11] have shown that this process occurs in stages and is characterised by the sequential expression of several proteins. The keratins K5 and K14 are the main products of the proliferant basal cells and are assembled as 10-nm long intermediate filaments which extend from the desmosomes toward the nuclear lamina and, along with microtubules and the microfilaments, form the cytoskeleton of the epithelial cells. Normally, keratinocytes lose their mitotic activity when they migrate to the spinous layer where they begin

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Abbreviations: DAG, 1,2-diacylglycerol; PKC, protein kinase C; ICE, interleukin 1β converting enzyme; TGase, transglutaminase; tTG, 'tissue' TGase; CE, cornified envelope; pRB, retinoblastoma protein; SPR, small proline reach protein

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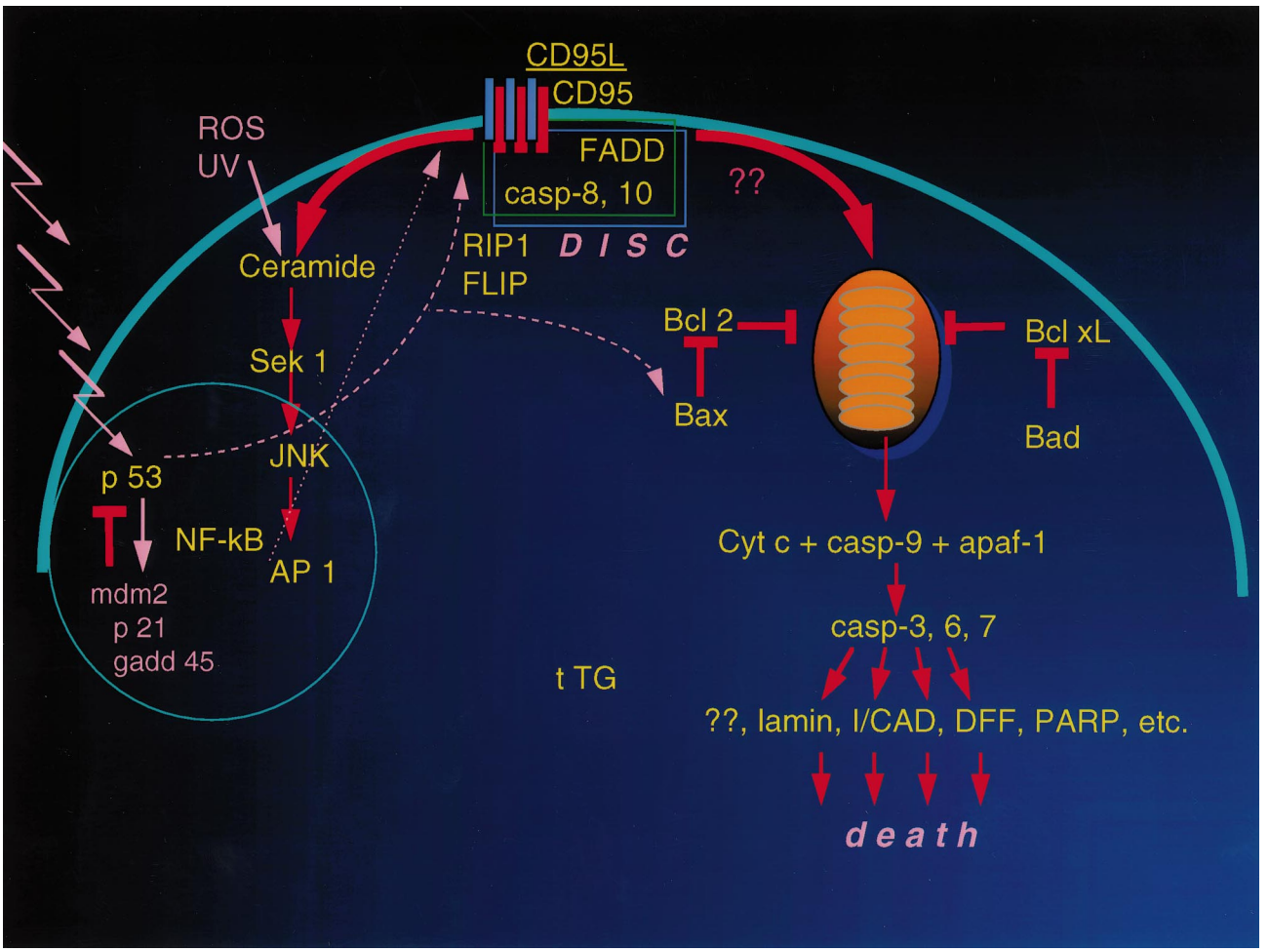

Fig. 1. Simplified scheme of the cascade of events in apoptosis.

to synthesise several proteins of intermediate filaments (desmoplachine I and II and desmoglein I) including specific keratins of the epidermal differentiation, K1 and K10 instead of K5 and K14, which are then assembled in bunches. In the more advanced stages of differentiation, the cells acquire keratohyalin granules, which are composed mainly of a filaggrin precursor. A series of proteins (loricrin, trichohyalin and SPRs) are simultaneously deposited and polymerised by the TGases from underneath the plasma membrane in order to build a collective structure called the cornified envelope (CE) [11]. From a biochemical point of view, the CE is the most insoluble component of the cornified epithelium and is a complex structure in terms of protein and lipid composition. Many proteins are CE precursors and function as transglutaminase (TGase 1 and TGase 3) substrates. Although the insolubility of the CE is accompanied by crosslinks ε(γ-glutamyl)-lysine, catalysed by Ca^{2+}-dependent TGases [11] and by disulfur bridges, the sequence of events that leads to the formation of the CE is not clear. Nonetheless, the action of TGases is highly specific [12,13]. Due to the presence of crosslinks, they are resistant to proteolysis. Mutations in TGase 1 result in the loss of activity or the loss of proteolytic activation, and thus cause the skin disorder lamellar ichthyosis, which is characterised by polygonal scales and follicular keratosis [14].

Although studies clearly indicate that TGases play a 'downstream' role in the process of skin keratinisation, the role and position of tTG in the apoptotic pathway is not so evident. Can these observations made in a skin model be applied to a different type of TGase in a more broad biochemical process? Several recent findings seem to suggest a more complex organisation of events.

2. 'Tissue' transglutaminase and its substrates in apoptosis

During apoptosis, the tTG gene is transcribed, the enzyme is synthesised and prompted to crosslink. The de novo transcription of the tTG gene is induced by several factors (retinoic acid, prostaglandin E2, interleukin 6, tumour growth factor β) which also modulate apoptosis. This regulation by multiple factors might be typical of the effector elements of the cell death program. In fact, the sequence analysis of fragments of the cloned genomic DNA has revealed the presence of several binding sites for transcriptional factors known to modulate apoptosis, thus indicating that the transcription of a putative apoptotic 'effector' gene can be controlled by a multifunctional promoter [15–17]. The retinoid receptors involved in the regulation of tTG and apoptosis have been recently identified. The induction of apoptosis in both human neuroblastoma cells and in lymphocytes is under the specific control of RARα and RARγ [18,19].

We must assume that tTG is not active as a crosslinking

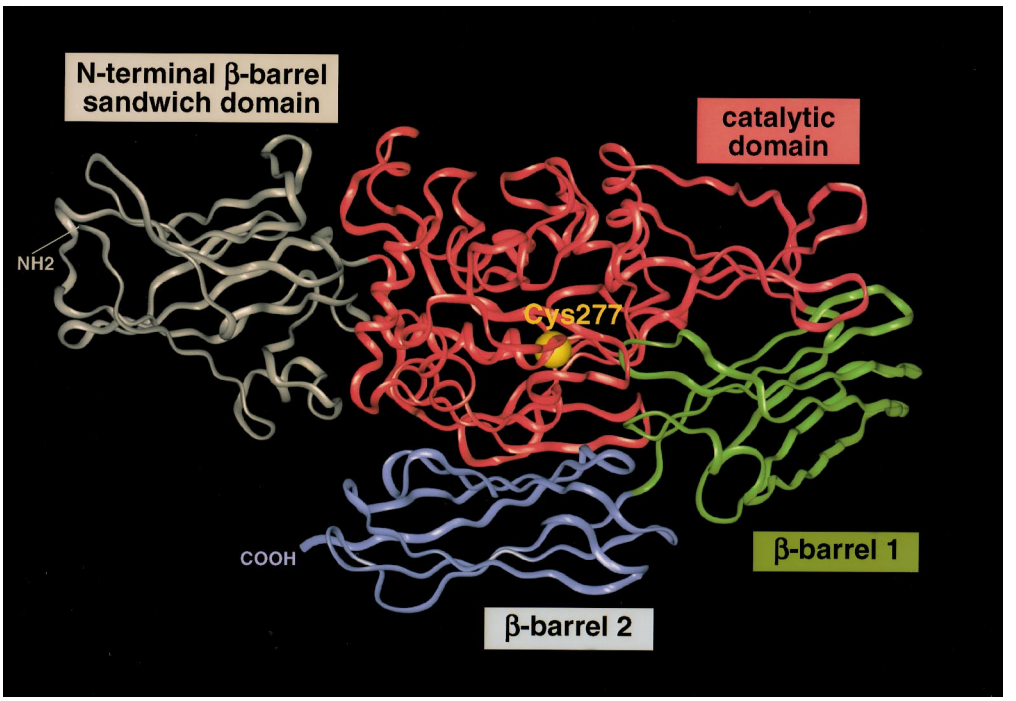

Fig. 2. Computerised homology model of 'tissue' transglutaminase based on the sequence identity with clotting factor XIII that has been resolved in its structure [4]. The model shows four domains depicted with different colours: N-terminal β-barrel sandwich domain (white), catalytic domain (red), β-barrel 1 (green), β-barrel 2 (purple). The N-terminal activation peptide of fXIII is absent in tTG. In TGase 1, mutations found in lamellar ichthyosis show the importance of the interface between the N-terminal domain and the central catalytic domain [14].

enzyme in viable cells, and we assume that the fine modulation of the tTG protein by GTP, Ca^{2+} [5] and nitric oxide by *S*-nitrosylation [20], and possibly other molecules, such as free putrescine and other polyamines [21], may explain how cells are able to survive in the presence of high tTG protein levels in their cytoplasm. Consistent with this hypothesis it is evident that tTG acts as the Gαh subunit, associated with the 50-kDa β subunit (Gβh), of the GTP-binding protein (Gh) in a ternary complex associated with the rat liver α1-adrenergic receptor [22]. Thus, the tTG/Gαh (this abbreviation indicates the double function of the protein) is a multifunctional protein, which by binding GTP in a GαhGTP complex, can

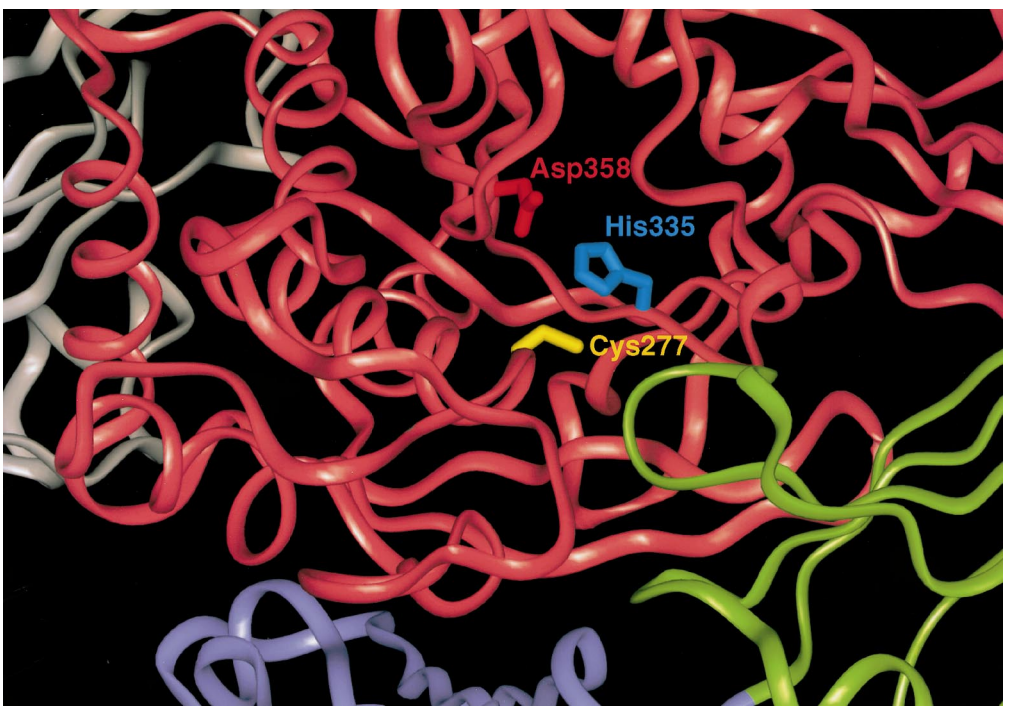

Fig. 3. Structural model of the active site of tTG in the catalytic domain shown above. The catalytic triade (Cys-277, His-335, Asp-358) is identical to other cysteine proteases, indicating a similarity in the mechanism of action. The colours of the four domains are as described above.

modulate receptor-stimulated phospholipase-C activation. The tTG/Gαh represents a novel class of GTP-binding proteins that participate in the receptor-mediated signalling pathway. The GTP binding activity of tTG/Gαh actively prevents the activation of the crosslinking activity of tTG. In fact, the Ca^{2+}-dependent crosslinking activity of tTG is finely regulated by GTP binding levels which in turn regulate secondary messengers, such as the production of inositol-1,4,5-triphosphate and *sn*-1,2-diacylglycerol (DAG) from phosphatidylinositol-4,5-biphosphate [22].

In light of these findings, we can hypothesise that tTG expression in its G-protein configuration in viable cells is related to the prevention of cell death. In fact, the DAG-mediated activation of protein kinase C, is one of the apoptosis-inhibiting pathways [23]. Nevertheless, cells constitutively expressing tTG are localised in tissue areas exposed to environmental and functional stress; and in order to avoid harmful consequences, they might keep the apoptotic machinery in place, ready to act whenever their integrity is affected. It has been shown that some of the biochemical features of apoptosis can be reproduced in anucleated cells, not requiring protein synthesis. Thus, we may speculate that in the course of evolution, the tTG gene acquired a cell-type-specific regulation, which allows for the accumulation of the enzyme under a crosslinking inactive configuration in cells particularly exposed to environmental stress. These findings raise the important question of the regulation of the effector genes during the priming/execution phase of apoptosis.

tTG has, therefore, at least two different functions that lead to opposite effects: life and death. The concept of multifunctional proteins controlling apoptosis vs. survival is neither unique nor novel, and highlights the complexity of these regulations. Indeed, proteins of the Ced-9/Bcl-2 family show opposite effects, and in some cases the same gene generates proteins with different effects by alternative mRNA splicing or post-translational modifications, e.g. Ced-9 or Bcl-X_L and Bcl-X_S [24,25]. Caspases, too, include proteins with or without death domains, able to balance each other, e.g. caspase 8 (FLICE) [26] and FLIP [27]. The case of cytochrome *c* is unique: the same protein is involved in mitochondrial electron transport (necessary for survival) or in the activation of caspase 9 (in conjunction with apaf 1 and ATP) [28], depending on the intracellular localisation (Fig. 1).

The biochemical characterisation of the crosslinked protein polymers formed by tTG in dying cells has revealed the presence of several intracellular proteins (actin, annexin, vinculin, fibronectin and other unknown proteins) [29,30]. Recently, the retinoblastoma protein (pRB) and troponin have also been shown to undergo tTG-dependent polymerisation in apoptosis [31,32]. In addition, tTG binds β-tubulin, glutathione transferase P1-1 and histone H2B (Piredda, Melino and Piacentini, unpublished results). Most of these proteins are cleaved by caspases and calpains during the cell death execution phase, thus indicating that tTG and thiol proteases share several target proteins. The presence of a cysteine active site is essential for the catalytic activity of tTG (Fig. 3), calpains and caspases [33,34]. This finding supports the biochemical similarities existing between tTG and papain, a thiol protease from which tTG is likely derived [35]. Considering that papain, like caspases, cleaves proteins at the Asp residues, these observations suggest a papain-like ancestral thiol protease as the original cell 'executioner'. In the course of evolution, this gene might have evolved into the different classes of executioners (caspases, calpains and tTG) able to mediate the highly regulated form(s) of death present in multicellular organisms.

3. Is tTG an essential effector element of apoptosis?

A definitive role for tTG in apoptosis has not yet been firmly established, although tTG expression does not seem to be a late epiphenomenon. A crucial answer to this will come from the experiment with the knock-out mouse which is being carried out in our laboratory using constructs disrupting exon 4 or exon 5, forming the active site of tTG. It will soon be clear whether tTG is essential in the central pathway of apoptosis, and thus in embryonic development, or whether it modulates apoptosis in an ancillary mode in a tissue- and inducer-specific fashion. Interesting clues supporting an important role for tTG in apoptosis derive from transfection studies carried out in various mammalian cells. Human neuroblastoma SK-N-BE(2) [36], BALB-C 3T3 [37] and L929 fibroblasts [10] transfected with a full length tTG cDNA show a large reduction in their proliferative capacity paralleled by an increase in both spontaneous and induced apoptosis. Transfection of neuroblastoma cells with a segment of the human tTG complementary DNA in antisense orientation results in a pronounced decrease of both spontaneous as well as induced apoptosis [36]. Consistent with our data, it has recently been reported that apoptosis induced by expression of polyglutamine stretches in COS-7 cells, mimicking the expansion of CAG (glutamine) repeats, which is at the basis of the pathogenesis of various neurodegenerative diseases, is suppressed by tTG inhibitors [38]. These findings indicate an important biochemical event for the induction of the structural changes featured in cells dying by apoptosis in the tTG-catalysed irreversible crosslinking of intracellular protein. As discussed in the previous paragraph, recent evidence indicates that in the early stages of the death pathway, a regulated tTG-mediated post-translational modification of specific protein substrates might play a role in the commitment to apoptosis. We have recently shown that the polymerisation of pRB, which precedes apoptosis in U937 cells, is mediated by its post-translational modification catalysed by tTG [31], thus suggesting that the activation of this crosslinking enzyme might determine an irreversible commitment to death. While pRB plays a key role in cell cycle control, the absence of functional pRB may result in apoptosis rather than in uncontrolled cell proliferation. Overexpression of functional pRB may induce apoptosis or rescue cells from death, depending on the system; homozygous pRB-null mice die during gestation showing massive induction of apoptosis during liver erythropoiesis and neuronal development [39]. The functional implications of the tTG-dependent polymerisation of pRB occurring during the early phases of apoptosis are not clear. In keeping with the possibility that pRB polymerisation might lead to its functional inactivation, we showed that the tTG-dependent polymerisation of pRB is paralleled by the rapid disappearance of E2F-1, which occurs when the transcription factor is not protected by pRB binding from entering the Ub/proteasome pathway [31].

In keeping with its close relation to cell death, tTG has already been shown to be involved in several pathologies: from AIDS, to autoimmunity, to tumours, to neurodegenerative diseases [10,35,38,40,41].

In conclusion, the idea that cell death by apoptosis was a genetically regulated event has been confirmed by the identification of several genes participating in the process. However, despite the exponential increase in the number of studies on gene-dependent cell death, a single 'killer' gene has yet to be identified in mammalian cells. A number of distinct enzymes (i.e. caspase, calpains and tTG) might work together to achieve the fast and immunologically silent removal of apoptotic bodies. Under controlled physiological conditions, it is very likely that different effector elements have complementary integrated functions in different cell compartments.

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