0014-4754/96/110942-08$1.50 + 0.20

Transglutaminase induction by various cell death and apoptosis pathways

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Abstract. Clarification of the molecular details of forms of natural cell death, including apoptosis, has become one of the most challenging issues of contemporary biomedical sciences. One of the effector elements of various cell death pathways is the covalent cross-linking of cellular proteins by transglutaminases. This review will discuss the accumulating data related to the induction and regulation of these enzymes, particularly of tissue type transglutaminase, in the molecular program of cell death. A wide range of signalling pathways can lead to the parallel induction of apoptosis and transglutaminase, providing a handle for better understanding the exact molecular interactions responsible for the mechanism of regulated cell death.
Key words. Apoptosis; transglutaminase; signalling; gene expression; promoter elements; retinoic acids.

Introduction

Biomedical studies of recent years have revealed the existence of a basic, evolutionarily conserved mechanism of regulated cell death which usually occurs under physiological conditions and is often programmed [1–5]. A cascade of specific genes must be expressed in order for the cells to die naturally [1, 6, 7]. Among the effector elements Ca^{2+}-dependent transglutaminases have often been observed to be induced during the molecular process of natural cell deaths [2]. Transglutaminases catalyse Ca^{2+}-dependent cross-linking reactions of proteins in which γ-carboxamide groups of glutamine residues serve as acyl donors and primary amino groups of protein-bound lysines, or small molecular weight amines function as acceptor substrates [8, 9]. The result of the reaction is usually the formation of $\epsilon(\gamma$-glutamyl)lysine linkage(s) between polypeptide chains. When diamines or polyamines participate in such cross-linking reactions, the formation of *N,N*-bis(γ-glutamyl)polyamine cross-bridges occurs [10]. Transglutaminase-dependent cross-linking of proteins leads to protein polymerization that confers stability as well as resistance to mechanical disruption and chemical attack [11, 12]. In mammals, five transglutaminase genes have been identified so far, encoding biochemically and immunologically distinct transglutaminases (for citations, see Piacentini et al.) [13]: blood coagulation Factor XIIIa, epidermal, keratinocyte, prostate and tissue type transglutaminases. The latter is the most ancient form (found even in simple metazoans and nematodes) from which the others supposedly emerged during evolution [12, 14]. The various transglutaminases have been implicated in a wide range of biological phenomena occurring in both extracellular and intracellular compartments and including blood coagulation, wound healing, terminal differentiation and cell death. Although these functions appear divergent, they all converge toward protection of cell and tissue integrity [11, 12, 15]. The first reports of the participation of a transglutaminase in the apoptosis process were published almost 10 years ago [16, 17]. However, the involvement of transglutaminase-catalysed cross-linking of proteins in terminal differentiation and ageing had been suggested even earlier [18–20].

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Transglutaminases in natural forms of cell death

Naturally occurring forms of cell death can be divided into at least three major groups (fig. 1). Although the three forms differ somewhat in morphology as well as in some characteristic molecular features, they share common basic elements [1, 2, 21, 22]. These include elimination of the nuclei by internucleosomal cleavage of DNA, efficient clearance of the dying cells without leakage of intracellular macromolecules and inflammation, involvement of various forms of intracellular proteases and control of death by negative regulatory elements such as bcl-2 and related proteins. Not surprisingly, the major biochemical effector elements of cell death are enzymes catalysing irreversible changes in DNA (endonucleases/DNAses) and proteins (proteases) [23–25]. The covalent cross-linking of cellular proteins by transglutaminases is also one of those biochemical reactions which cannot be reversed in living systems [11]. Products of the transglutaminase genes, most particularly the ubiquitous tissue transglutaminase, have been implicated in various forms of natural death; these include not only apoptosis elicited through distinct molecular pathways (these will be discussed in a later section) but both specific and programmed forms of natural death (fig. 1b).

The classical example of the functional involvement of a transglutaminase in a specific form of natural death is

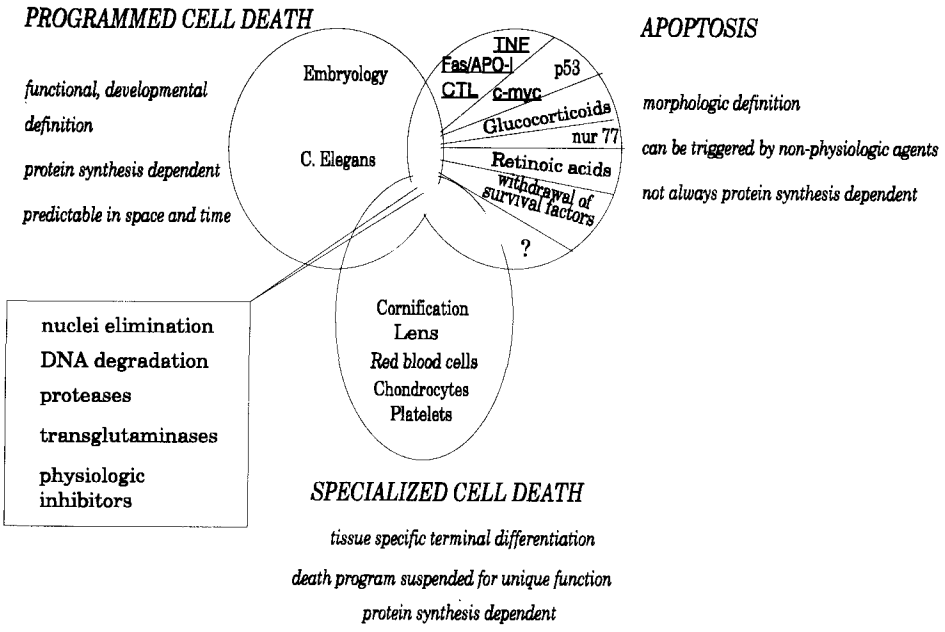

Figure 1. Schematic representation of various forms and some common features of natural cell death forms. Underlined apoptosis pathways have not been found to lead to the induction of transglutaminase.

the epidermis where, in the stratum spinosum and granulosum, terminally differentiating keratinocytes express both the membrane-bound (type 1) and a cytosolic (type 3) form of the enzyme as well as specific substrate proteins such as involucrin, loricrin and cornifin [18, 26–28]. Parallel to the disintegration of the nucleus, the rise in the level of intracellular Ca^{2+} activates these enzymes, which in turn create highly cross-linked cornified envelopes, mechanically and chemically stable sheaths at the outside, protective layer of the skin. Interestingly, apoptotic cells in the basal layer of skin that occasionally forms [e.g. following ultra violet (UV) radiation] express tissue transglutaminase (type 2) upon the initiation of the death process [26, 29]. One of the proteins incorporated into the cornified envelopes of keratinocytes by type 1 transglutaminase is involucrin which is also used by type 2 transglutaminase and in apoptotic forms of cell death [30, 31].

The process of terminal differentiation of lens epithelial cells shows classical features of physiologic cell death, including transglutaminase-catalysed cross-linking of proteins [19, 32]. Increased rate of apoptosis with elevated transglutaminase activity may be one of the crucial elements in cataract formation [19, 33]. Terminally differentiated red blood cells and chondrocytes are loaded with tissue-type transglutaminase induced at specific times in the differentiation program [19, 34]. Platelets originating from megakaryocytes by fragmentation (which is quite similar to the fragmentation of the cytosol during the early phase of apoptosis) contain high levels of the cellular form of FXIII, the plasma form of transglutaminase. The latter enzyme is also present and activated during the apoptotic process induced by tumour necrosis factor in U937 promonocytic leukemia cells (Nemes et al., unpublished observation).

In embryogenesis, cells at the interdigital webs of the limb bud, which die at specific times in development, express high levels of tissue transglutaminase [35, 36]; both death and expression of transglutaminase are augmented by retinoic acid [37]. A covalent dimer of interleukin-2, produced in vitro by the action of a nerve-derived transglutaminase, has been shown to induce apoptosis via the activation of the p53 protein [38, 39]; oligodendrocytes are established growth inhibitors of neuronal cells in higher vertebrates, and in their presence neuronal cells do not grow. According to an intriguing finding cellular transglutaminase may also be responsible for the proper processing and production of the activated form of transforming growth factor-β1 [40], which is capable of inducing apoptosis in a variety of cell types [41–45].

Is transglutaminase involved in the death pathway of cells in the nematode *Caenorhabditis elegans*?

C. elegans, the well-known model organism of developmental genetics, has provided some fundamental principles for cell death studies. During the ontogenesis of hermaphrodite nematode 1090 somatic cells are generated, of which 131 cells undergo programmed death at predictable sites and time points. Cell death (*ced*) mutants have been successfully used to define the molecular pathway of programmed cell death; several key

points of this pathway have been conserved during evolution. Loss of function mutations of *ced-3* (the gene of a cysteine proteinase which is homologous to a group of apoptosis- related enzymes) and *ced-4* gene, as well as the gain of function mutation of the negative regulator *ced-9* gene (a functional homologue of the mammalian cell death suppressor gene *bcl-2*), cause all cells that normally die to survive [1, 46]. Furthermore, several *ced* genes are required for the engulfment of dead cells. We reasoned that if transglutaminase-mediated cross-linking of proteins is an essential and general feature of natural forms of cell death, it must be part of the death process described in *C. elegans*. It could be demonstrated that there is a transglutaminase protein in *C. elegans*; the enzyme is constitutively expressed in some cells of the adult worm and induced in those programmed to die (Madi et al., unpublished observation). Studying the *ced* genes so far recognized, none of them was found to be a transglutaminase. However, several of the *ced* mutant organisms show distinct and characteristic changes in the activity of the enzyme – including significant elevation in the level of protein-bound $\epsilon(\gamma$-glutamyl)lysine cross-links in mutants where the cells die but are not eliminated because of the loss of genes involved in phagocytosis of the cell corpses – providing indirect evidence that it is part of the basic cell death program.

Biochemical pathways of apoptosis leading to the induction of tissue transglutaminase in mammalian cells

At least four separate signalling pathways – activation of T cell receptor (TCR) by antigen or anti-CD3 antibody, steroid receptor stimulation, DNA damage and *fas* receptor stimulation – can lead to morphologically indistinguishable forms of apoptosis in mouse thymocytes. The apoptotic program induced in each of these cases works via different signal transduction pathways [47]. Stimulation of TCR induces changes in second messenger systems [48], glucocorticoids bind to steroid receptors, while inhibition of topoisomerase II or irradiation causes direct DNA damage. Each of these pathways appears to induce distinct sets of genes. The transcripts RP-2 and RP-8 are expressed in thymocyes following treatment with glucocorticoids [49]. DNA damage leads to p53 induction; thymocytes lacking p53 are resistant to the lethal effects of ionizing radiation or etoposide, but not to other treatments [50, 51]. The immediate-early gene *nur 77*, on the other hand, is induced in response to TCR signals but not by glucocorticoids or ionizing radiation [51]. *Bcl-2*, the physiologic inhibitor of cell death, has only minimal effects on TCR-dependent deletion of autoreactive T cells [52]. Antisense inhibition of *nur 77* expression prevents apoptosis in TCR-stimulated cells but not if death was induced by other stimuli [53]. CD4 + CD8 + thymocytes also express fas/apo-1 receptor, and following the administration of an anti-*fas* antibody they enter the apoptotic program [36, 54]; the fas/apo-1 apoptosis pathway is not regulated by *bcl-2* either [52].

A significant increase in the expression and activity of tissue transglutaminase was observed during involution of thymus elicited by treatment with either anti-CD3 antibody or dexamethasone or by irradiation (Szondy et al., unpublished observation). The blood plasma concentration of $\epsilon(\gamma$-glutamyl)lysine isodipeptide, the end-product of the digestion of transglutaminase cross-linked proteins of apoptotic bodies [55], was also elevated in each of these cases, showing that the induced enzyme was activated. Tissue transglutaminase was localized in cells of the cortical layer of the thymus, and immunofluorescence double labelling revealed that the enzyme appeared in the apoptotic cells. None of these observations could be made when apoptosis was induced by fas/apo-1 receptor stimulation in vivo and when the four stimuli were applied in cell culture conditions. These data show that distinct signalling pathways, inducing apoptosis within the same cell type, differentially regulate the expression of tissue transglutaminase; the enzyme is not induced through the fas/apo-1 apoptosis pathway. Furthermore, the in vivo tissue environment is essential for the induction of tissue transglutaminase, at least during the apoptosis of thymocytes.

Direct evidence for the induction of tissue transglutaminase in the apoptosis process was originally provided by studying the involution of induced hyperplasia of the liver following the withdrawal of mitogens. It was shown that high levels of the enzyme selectively appeared in the dying hepatocytes [16] where it was activated to form highly cross-linked protein polymers extractable from hepatocyte preparations by treatment with detergents and chaotropic agents [56]. The number of transglutaminase cross-linked protein envelopes isolated from either resting or involuting liver correlated well with the number of apoptotic cells observed by conventional histology. It is not known exactly which (either negative or positive) factors regulate active cell death in the liver; several studies suggest that transforming growth factor β (TGF-β) and related peptides are involved in the initiation of in situ apoptosis of hepatocytes [41, 42]. TGF-β has been shown to be a strong inducer of the tissue transglutaminase gene in several types of cells including hepatocytes [43, 44], and parallel induction of the enzyme and apoptosis has been observed [45]. Furthermore, TGF-β is a crucial regulator of the interaction of cells with their extracellular matrix and is capable of signalling to the individual cell whether it fits into the tissue architecture or should die [57, 58].

In many cases normal cell deaths occur because cells fail to obtain the extracellular signals they need to suppress

the death program [7]. A large number of apoptosis model systems have been developed on the basis of initiation of death by removal of survival factors for cells in culture or living tissues. Transglutaminase induction has not been widely studied in such systems. The notable exceptions are hormone-dependent tissues such as the prostate and mammary gland. Androgen ablation causes cell loss by apoptosis in the rat ventral prostate, and in the dying, androgen-dependent prostatic glandular epithelial cells (but not in the surviving basal epithelial or stromal cells), tissue-type transglutaminase is induced [59, 60]. Similarly, the induction and activation of this enzyme occur in the dying cells of the mammary secretory epithelium following forced weaning, as we have demonstrated by RNA in situ hybridization, immunohistochemical and immunoblotting methods, and cross-linking measurements [61]. It has been proposed that in both the prostate and lactating mammary gland specific hormone-dependent morphogens, synthesized in the stroma, maintain the basal signal transduction pathways to ensure the repression of the apoptosis genes, including transglutaminase [62]. Hormone-deprivation may alter the synthesis of these critical morphogens and lead to apoptosis of epithelial cells by disrupting the signalling pathways, changing the phosphorylation status of BZip and/or CREB proteins (which are suspected to be responsible for the repression of the apoptosis genes), and thereby resulting in the induction of death genes [62]. On the other hand, it has also been suggested that a cell cycle G1-like state is associated with programmed cell death of mammary epithelial cells involving the activity of AP-1, cFos and JunD and that apoptosis occurs without S phase induction [63].

In some forms of apoptosis – e.g. those induced by TNF, fas/apo-1, cytotoxic T cells or cytotoxic chemotherapeutic agents [36] – transglutaminase induction is not typically observed, and protein cross-linking does not occur unless one form of the enzyme is constitutively expressed (authors' unpubl. data). For example, initiation of apoptosis in Chinese hamster ovary cells by switching on the expression of a transfected *c-myc* gene [64] does not lead to the induction of a transglutaminase; however, the activation of the constitutively expressed tissue transglutaminase does occur, as judged by the sharp increase in the cellular content of cross-linked proteins during the death process (Balajthy and Fesus, unpublished data).

The promoter region of transglutaminase genes

The above set of examples clearly shows that the induction of a transglutaminase, particularly that of the tissue-type enzyme, is part of many of the molecular pathways of natural cell death; the induction can be elicited either by agents acting on nuclear receptors or through cell surface receptors and signalling pathways which activate nuclear transcriptional factors. Therefore, the final details of the death-related induction mechanism of transglutaminases lie in molecular interactions at the promoter sites of the transglutaminase genes. Analysis of these interactions has just been started. There is no report yet of studies of the promoters of either the plasma or the epidermal transglutaminase gene. The characterization of the 5′ region of the keratinocyte transglutaminase gene has already been started; the sequence predicts the participation of several well-known response elements in its regulation [65]. A relatively detailed study suggests that signalling systems including protein kinase C, retinoic acid/retinoid-X receptors, and Jun/Fos regulate the transcription of the type 1 transglutaminase gene.

Analysis of the sequence of the tissue-type transglutaminase promoter [66, 67] also shows the existence of several potential response elements, as one may expect seeing the many data related to multiple and differential regulation of the enzyme in living tissues and the death process. One such element probably responds to IL-6, which stimulates tissue transglutaminase expression [68]; IL-6 triggers apoptosis in some hemopoietic cell lines [69]. Although the above detailed signalling pathways, which induce both apoptosis and the expression of tissue transglutaminase, suggest that there must be response elements for a number of molecules (including p53, glucocorticoids, *nur 77*, TGF-β and others), these have not been found or characterized in detail as yet. One notable exception is the identification and characterization of a versatile tripartite retinoid response element which is located 1.7 kb upstream of the transcription start site in the mouse tissue transglutaminase gene promoter and which is induced optimally by retinoid receptor panagonists [70]. It is very likely that this response element plays a crucial role in the regulation of the enzyme in natural forms of cell death elicited by retinoic acids.

Induction of apoptosis and tissue transglutaminase by retinoic acids

All-*trans* and 9-*cis* RA are vitamin A derivatives present in living mammalian tissues. Both are physiological ligands for the retinoic acid receptors (RARs, RXRs) which belong to the steroid/thyroid/retinoid nuclear receptor family [71]. These receptors are ligand-dependent transcription factors which bind to specific hormone response elements (RARE, RXRE) on DNA and transactivate specific target genes [72]. All-*trans* RA and 9-*cis* RA are equipotent in activating RAR, while all-*trans* RA activation of RXR is 50-fold weaker than that of 9-*cis* retinoic acid [73]. Retinoic acid receptors can function in the form of either RAR/RXR heterodimers or of RXR/RXR homodimers in the presence of

retinoic acids [74]. RXR, however, can also form heterodimers with various members of the steroid/thyroid/retinoid receptor family. These complex interactions and the existence of multiple retinoic acid nuclear receptors (RARα, β and γ) as well as retinoid X receptors (RXRα, β and γ), differentially expressed in various tissues and cell types, explain the pleiotropic effects of retinoids in practically all cells [75]. Various sets of recent data clearly show that apoptosis and tissue transglutaminase are co-regulated in different settings of both cell death and retinoic acid receptor complexes.

Retinoids appear to be generalized regulators of tissue (type 2) transglutaminase expression. Rats rendered vitamin A-deficient have a marked depression, which can be rapidly reversed by administration of all-*trans* retinoic acid, in the level of tissue transglutaminase mRNA and activity in many tissues [76]. These effects could be reproduced in vitro [35]. The induction of type 2 transglutaminase often occurs in parallel with retinoid-induced cell death. One example is the interdigital web in developing limbs in mammals and some birds, where the expression of the enzyme was found to be focal and restricted to apoptotic cells [35, 36]; these regions of the limb bud are associated with selective expression of RARβ [77]. In tracheobronchial epithelial cells it was found that concomitant induction of both apoptosis and tissue transglutaminase by retinoids is mediated through RARα [78]. Using receptor-selective synthetic retinoids and HL-60 cell sublines with different retinoid responsiveness, it has been shown that ligand activation of RARs is sufficient to induce differentiation and global suppression of *bcl-2* expression, whereas ligand activation of retinoid X receptors (very likely in the structure of RAR-RXR heterodimers) is necessary and sufficient for the induction of both apoptosis and transglutaminase expression [79, 80].

We have recently found that both all-*trans* and 9-*cis* retinoic acid induce apoptosis of mouse thymocytes in ex vivo cultures, 9-*cis* retinoic acid being 50 times more effective. The induction of apoptosis by retinoic acids is mediated by RARγ because the phenomenon can be reproduced only by RARγ-selective retinoic acid analogues and the cell death induced by either retinoic acids or RARγ analogues can be inhibited by RARγ-specific antagonists. In vivo administration of an RARγ analogue resulted in thymus involution with the concomitant activation of the apoptosis-related endonuclease and the induction of tissue transglutaminase. The RARγ pathway of apoptosis is RNA and protein synthesis-dependent, affects the CD4 + CD8 + double positive thymocytes and can be inhibited by addition of either Ca^{2+}-chelators or protease inhibitors. Using various RAR and RXR-specific analogues and antagonists, it was demonstrated that stimulation of RARα inhibits the RARγ-specific death pathway (which explains the lack of apoptosis stimulatory effects of all-*trans* retinoic acid at physiological concentrations), and co-stimulation of the RXR receptors can neutralize this inhibitory effect (which explains the apoptosis-inducing effect of 9-*cis* retinoic acid).

What is the role of transglutaminase-mediated cross-linking of proteins in dying cells?

Our efforts (which have included searching for substrate proteins, using antisense oligonucleotides, enzyme inhibitors and transfection approaches) to clarify the role of transglutaminases in natural forms of cell death have led to the conclusion that while in some cell types the activation of the enzyme is lethal, its role is not directly related to the act of killing in others [2, 13]. Ca^{2+}-ionophore treatment of cells expressing transglutaminase constitutively (endothelial cells, keratinocytes, glioma cells) leads to the formation of cross-linked protein envelopes and subsequent death. Overexpression of tissue transglutaminase either by treatment with retinoic acid or by stably transfecting various cell types (fibroblasts, L929 cells, neuroblastoma cells) with a constitutive expression plasmid, results in a significantly increased rate of cell death [3, 81]; (Fesus et al., unpublished observation). On the other hand, there are cell types in which the inhibition of the enzyme by specific inhibitors or the prevention of its expression by antisense nucleotides does not influence the development of death (e.g. apoptosis of neutrophils; Fesus and Susan, unpublished); however, blocking the action of the enzyme shifts cells toward the necrotic death pathway, including the release of intracellular macromolecules into the environment. Conversely, biochemical features of necrotic forms of cell death could be shifted toward the apoptotic type by stably transfecting transglutaminase complementary DNA (cDNA) into cells showing a necrotic response to tumour necrosis factor (Fesus et al., unpublished). These results suggest that there are conditions in which the induction of a transglutaminase is an important and beneficial part of the death process and should be facilitated to avoid leakage of macromolecules and inflammation even in those cases where the enzyme is originally not part of the killing pathway.

Conclusions

It is often claimed that most mammalian cells constitutively express all the proteins required to undergo programmed cell death or apoptosis [82]. Nevertheless, there are apoptosis genes induced in cells as the result of death stimuli; apoptotic regulation of fas ligand [46, 83] or bax protein [51, 84] are excellent examples. Similarly, transglutaminses are induced in most forms of natural cell death through various signalling pathways utilizing transcriptional factors either directly (p53, receptors of

glucocorticoids and retinoic acids) or indirectly (*nur 77*, TGF-β). The most detailed studies have been done with the tissue type transglutaminase, including the first analysis of potential promoter elements responsible for its expression in dying cells. Future results will tell how much of the transglutaminase-specific observations so far revealed can be generalized and used for understanding the molecular regulation of the apoptosis machinery.

Acknowledgements. The author's investigations have been supported by the Hungarian OTKA Fund T 012923, the Hungarian Ministry of Welfare T-01 408/1993 and the EU Biomed-1 Concerted Action ERBCI PDCT940217.

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