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"Tissue" transglutaminase in animal development

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ABSTRACT The "tissue" transglutaminase is a multifunctional enzyme that in its cross-linking configuration catalyzes Ca^{2+}-dependent reactions resulting in post-translational modification of proteins by establishing ε(γ-glutamyl)lysine cross-links and/or covalent incorporation of biogenic amines (di- and poly-amines and histamine) into proteins. Several laboratories have shown that in Vertebrates, "tissue" transglutaminase (tTG) gene expression specifically characterizes cells undergoing apoptosis or programmed cell death (PCD). The Ca^{2+}-dependent activation of this enzyme leads to the formation of detergent-insoluble cross-linked protein polymers in cells undergoing PCD. This insoluble protein scaffold could stabilize the integrity of the dying cells before their clearance by phagocytosis, preventing the non-specific release of harmful intracellular components (e.g. lysosomal enzymes, nucleic acids, etc.) and consequently inflammatory responses and scar formation in bystander tissues. In this review we attempt to present an overview of the current knowledge on tTG expression and regulation in animal reproduction and development. The data available so far further strenghten the relationship existing between tTG expression and the induction of PCD.

KEY WORDS: *programmed cell death, apoptosis, embriogenesis.*

Programmed cell death and development

The generation of a multicellular organism from the zygote is achieved by the complex regulation of three major cellular events: mitosis, differentiation and death. Programmed cell death (PCD) leads to the controlled deletion of cells and is a key event in the regulation of cell number in tissue homeostasis (Hale *et al.*, 1996). PCD also plays an essential role in tissue re-modelling occurring during ontogenesis and metamorphosis (Beaulaton and Lockshin, 1982; Fesus *et al.* 1991; Milligan and Schwartz, 1997). In fact, the temporal and spatial regulation of PCD is essential to successfully achieve complete developmental program (Beaulaton and Lockshin, 1982).

In the course of development all tissue display PCD. This process confers to the organisms a profound plasticity, that allows them to address the wide array of developmental needs. In general, many more cells than needed are generated for any given function, and only those cells that undergo successful functional interaction receive positive stimuli and continue to develop the structure they belong, while the ones not properly interacting die (reviewed by Oppenheim, 1991). Furthermore, PCD is at the basis of the elimination of cells that have outlived their usefullness and is implicated in different processes such as the sculpturing of the body, for example in the digits formation in mammals, and the protection of organisms from cells that could be dangerous such as self-reactive T-cells (Beaulaton and Lockshin, 1982; Fesus *et al.*, 1991; Hale *et al.*, 1996; Milligan and Schwartz, 1997). From this picture is evident that the aim of programmed cell death is to eliminate those cells which are redundant and/or functionally inadequate without evokation of events such as inflammation or autoimmune response (Fesus *et al.*, 1991; Jacobson *et al.*, 1997). To undergo successful cell death three sequential processes are needed, a cell must: (i) receive a death signal; (ii) activate its endogenous death machinery and (iii) be cleared during or short after its death. These three processes are not unique and could be achieved in different ways and in response to different stimuli.

In vitro experiments have shown that, at least in some circumstances, the core machinery to execute the cell death program is already in place before the onset of the apoptotic signal and that the final events of apoptosis may be controlled at the post-translational level by activating the caspases (Nicholson, 1999). However, during ontogenesis PCD requires RNA and protein synthesis indicating that expression of specific genes is needed for death to

Abbreviations used in this paper: PCD, programmed cell death; tTG, "tissue" transglutaminase; PDI, protein disulphide isomerase; INZs, interdigital necrotic zones; AER, apical ectodermal ridge; RA, all *trans*-retinoic acid; FE, fertilization envelope; RAR, retinoic acid receptor.

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occur (Beaulaton and Lockshin, 1982; Fesus, 1999). In keeping with this assumption, essential elements of the apoptotic program such as Bax, CD95L and "tissue" transglutaminase (tTG; E.C. 2.3.2.13; also known as type II or C) are specifically induced before the onset of apoptosis (Fesus, 1999). Several laboratories have shown, both *in vivo* and *in vitro*, that there is a close relationship between the expression of tTG and the onset of programmed cell death (Piacentini, 1995; Amendola *et al.*, 1996; Oliverio *et al.*, 1997). The aim of this chapter is to review key aspects of the involvement of cell death in animal reproduction and ontogenesis, focusing on "tissue" Transglutaminase (tTG).

Transglutaminases: a complex family of multifunctional enzymes?

Transglutaminases (TGases) belong to a family of intra- and extra-cellular enzymes which were originally described to catalyze Ca^{2+}-dependent reactions, resulting in the post-translational modification of proteins by establishing ε(γ-glutamyl)lysine cross-linkings and/or covalent incorporation of di- and poly-amines into proteins (Folk and Finlayson, 1977; Folk, 1980; Greenberg *et al.*, 1992). However, it has been recently shown that in peculiar catalytic instances (absence of the acyl-acceptor ε-amino group of lysine or primary amino group of a polyamine) the acyl-enzyme intermediate may react with water resulting in the deamination of the γ-carboxamide group of the protein-bound glutamine (Schmidt *et al.*, 1998, 1999). In addition, a protein disulfide isomerase (PDI) activity for "tissue" transglutaminase has also been recently proposed (Chandrashekar *et al.*, 1998). TGase1 enzyme also forms ester bonds between specific glutaminyl residues of involucrin and an analog of omega-hydroxyceramides. This is an essential biochemical event in the formation of the thick (5 nm) lipid envelope on the surface of keratinocytes and consequently of the physiological skin barrier function (Nemes *et al.*, 1999).

Although the *in vivo* relevance of all these biological activities has yet to be defined, their unusual complexity makes this group of proteins a very interesting subject for future investigations. For all living organisms, the need for a TGase activity seems to be an essential feature that has been highly conserved during the course of evolution; in fact, proteins showing a TGase-like activity are present from simple unicelluar organisms to Vertebrates (Waffenschmidt *et al.*, 1999).

A number of different members of the TGase family have been identified in various organisms (Table I); among the six distinct genes coding for different forms of intracellular TGases described in Vertebrates the well characterized are:

1) The A catalytic sub-unit of the blood-clotting Factor XIII which have been shown to catalyze the intermolecular cross-linking of fibrin to other plasma proteins, important for wound healing (Hale *et al.*, 1996);
2) TGase1, a membrane associated enzyme responsible for the formation of the cornified cell envelope of the epidermis (Polakowska *et al.*, 1994);
3) TGase2 an ubiquitous soluble enzyme implicated in cell death and other cellular processes (Greenberg *et al.*, 1992; Gentile *et al.*, 1992).

Common to all these enzymes is the cross-linking activity which might determine the olygomerization of substrate protein(s) which acquire peculiar features of resistance to breakage and chemical attack (Folk and Finlayson, 1977; Folk, 1980). While a large number of TGase protein substrates have been identified, endoproteases capable of hydrolyzing these cross-links have yet to be identified in Vertebrates, thus suggesting a peculiar role for these irreversible post-translational modifications of proteins (Piacentini and Colizzi, 1999).

"Tissue" transglutaminase

The "tissue" transglutaminase (tTG; E.C. 2.3.2.13) or type-II transglutaminase (TGase2) gene is widely expressed in adult individuals and encodes a protein with a molecular weight of about 80 kDa (Folk and Finlayson, 1977; Folk, 1980). The very complex array of functions proposed in general for TGases become even more intrigated when tTG's activities are considered. This protein is a multifunctional enzyme with two characterized biological functions: cross-linking and G-protein (Nakaoka *et al.*, 1994;

TABLE I

BIOCHEMICAL PROPERTIES OF VARIOUS TGASES

Type of TGase	Source of isolation	Molecular weight (kDa)	Protease activation	Ca^{2+} dependence	Reference
Filarial parasite	*Brugia malayi*	22-25	No	Yes	Mehta *et al.* (1990), Singh and Mehta (1994)
	Dirofilaria immitis	57.1	No	Yes	Chandrashekar *et al.* (1998)
Microbial	*Streptoverticillum*	37.8	No	No	Kanaji *et al.* (1993), Duran *et al.* (1998
	Escherichia coli	110	No	?	Schmidt *et al.* (1998)
Epidermal	Human	77	Yes	Yes	Kim *et al.* (1993)
Karatinocyte	Human	90	No	Yes	Polakowska *et al.* (1991)
Plasma (Factor XIII)	Human	360	Yes	Yes	Ichinose and Davie (1988), Bottenus *et al.* (1990)
Prostate	Rat	65	No	Yes	Seitz *et al.* (1991)
	Human	77	No	Yes	Gentile *et al.* (1995), Dubbink *et al.* (1998)
Tissue	Guinea pig	76	No	Yes	Ikura *et al.* (1988)
	Horsoeshoe crab	87	No	Yes	Tokunaga *et al.* (1993)
	Human	85	No	Yes	Gentile *et al.* (1994)
	Red sea bream	78	No	Yes	Yasueda *et al.* (1995)
	Soybean leaves	80	No	No	Kang and Cho (1996)

Melino and Piacentini, 1998). Moreover, a PDI and a deaminating activity have recently been proposed for the same protein (Chandrashekar *et al.*, 1998).

In its protein cross-linking configuration tTG catalyzes a Ca^{2+} and thiol-dependent acyl-transfer reaction among polypeptide chains (Folk, 1980). tTG has been shown to be induced and activated in apoptotic cells in both physiological and experimental models (Fesus *et al.*, 1989, 1991; Piacentini, 1995; Ledda-Columbano *et al.*, 1991; Piacentini *et al.*, 1991; Oliverio *et al.*, 1997). In apoptosis the enzyme may have more than one function within the cascade of events that lead to the establishment of the apoptotic phenotype: i) an early regulatory function which, through the polymerization of substrate protein involved in the regulation of apoptosis induction, such as pRb, may influence the decision within the cell to undergo PCD or to survive (Oliverio *et al.*, 1997); ii) a late effect leading to the stabilization of dying cells through the formation of intracellular cross-linked protein polymers (Fesus *et al.*, 1989, 1991; Piacentini *et al.*, 1991). The cross-linking activity of tTG need to be activated by high Ca^{2+} levels paralleled by a decrease of the intracellular GTP levels. In fact, binding of GTP shifts the enzyme to a membrane bound rather than a cytosolic form, revealing its G-protein function as the $G_{(\alpha h)}$ subunit of the α1-adrenergic receptor. As a G-protein, tTG is involved in the transmembrane transmission of the α1-adrenergic and tromboxane receptors to their effector enzyme phospholipase C-delta1 (PLC-δ1). Coupling of tTG/$G_{(\alpha h)}$ to PLC activates the hydrolysis of membrane-bound inositol phospholipids leading to generation of the second messangers 1,4,5-triphosphate (IP3) and diacylglycerol and subsequent intracellular Ca^{2+} mobilization and protein kinase C activation. The GTPase activity of the enzyme seems to be involved in the deactivation of the signalling pathway, converting the enzyme from a GTP-bound to a GDP-bound state. In HeLa cells, the shift from tTG-GTP inactive form to tTG-GDP active form could be induced by treatment with retinoic acid, leading to a complex which shows high affinity for eIF-5A, one of the factors involved in mRNA export from the nucleous (Singh and Cerione, 1996).

tTg in invertebrate development

During the larval development in Invertebrates, such as nematodes, a wide rearrangement of the cellular structures takes place. tTG activity seems to be involved both in the formation of nematode embryos and in cell death waves involving the disappearence of specific larval structures.

Brugia malayi

Filarial nematodes are responsible for chronic infections worldwide and are a major cause of morbidity in endemic areas. Infection in humans is initiated by transmission of third-stage larvae (L3) by a blood suck arthropod vector and the larvae develop into adults over several months. After fertilization, females produce microfilariae that migrate into the blood stream or skin. A 25 kDa transglutaminase, reacting with monoclonal antibody against tTG, has been purified and characterized from adults worms (Metha *et al.*, 1990, 1992, 1996). The enzyme is present in female worms, but not in male, and *in utero* developing embryos contained very high amounts of tTG, especially during early stages of development. Treatment of females with tTG inhibitors cause impairment of embryos development and prevent their release; this seems to be due to the lack of incorporation of host protein into developing microfilariae. In fact, exudates of the peritoneal cavity of jirds, the site where adults worms reside and produce microfilariae, contain several proteins that are taken up by adult female and incorporated into the developing microfilariae (Metha *et al.*, 1990, 1992, 1996).

Caenorhabditis elegans

In the nematode *Caenorhabditis elegans* a protein of about 61 kDa possessing a Ca^{2+} dependent tTG activity has been characterized (Madi *et al.*, 1998). Immunohistochemistry has revealed the expression of the enzyme in the intestinal cells of the adult wild type animals. tTG activity has been detected as higher in adult animals but it was also high in the L1 animals, when compared to other larval stages. This activity could be associated to the cell death occuring at this stage. A lower tTG activity, compared with wild type animals, has been measured in *ced-3*, *ced-4* and *ced-9* mutants, in which the mutations cause the survival of all cells that normally die. Analysis of *ced-2* and *ced-5* phagocytosis mutants, in which the mutations allow cell death but not engulfment, showed a lower enzyme's acivity. It is interesting to note that in *ced-3* mutants lower enzyme's activity was not parallelled by a low level of $\varepsilon(\gamma$-glutamyl)lisine cross-link and moreover in *ced-5* and *ced-5/ced-7* double mutants, in which the cells are programmed to die but not engulfed and persists as dead corpse, the levels were found to be high and several immunopositive corps are detectable in the head of the adults (Fig. 1), where normally most neuronal cells die. The data indicate that expression of tTG in *C. elegans* might be related to the nematode's developing death program and could be positioned dowstream to *ced-3* and *ced-4* gene's products and upstream to the action of those involved in engulfment of dead cells.

tTg in vertebrate development

Vertebrate reproduction, from gametogenesis to embryo implantation and sculpture, is widely dependent on PCD. "Tissue" transglutaminase has been shown to be expressed in many tissues at different stages of development (Table II). In the next sections we have attempted to review the current knowledge on the localization and regulation of the enzyme.

TABLE II

"TISSUE" TRANSGLUTAMINASE EXPRESSION IN DEVELOPING MAMMALIAN ORGANS

Organ	Cell type
Skin	Peridermal Basal Hair Follicle Endothelial
Neural Tube	Neurons
Nothocord	Mesodermal
Limb	Mesenchymal Epithelial (AER) Dying chondrocytes
Lung	Epithelial Extracellular matrix
Blood	Red cells

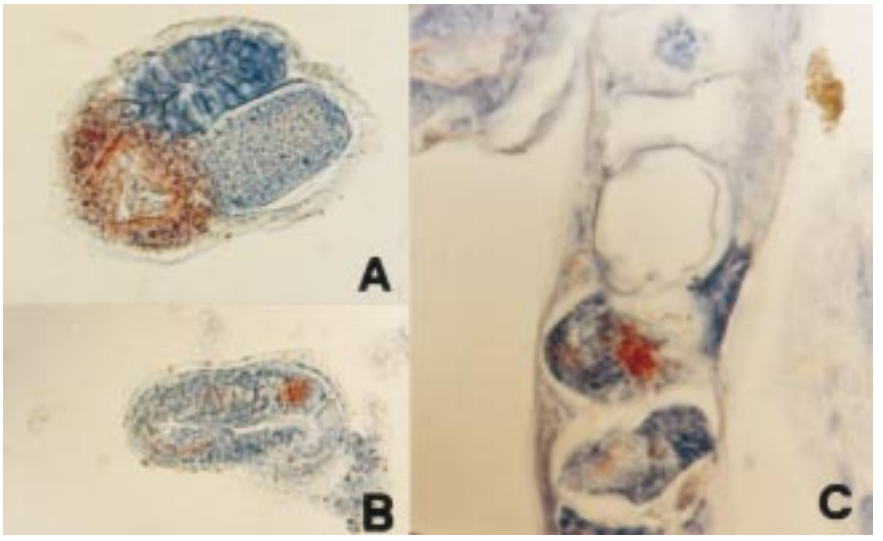

Fig. 1. Immunohistochemical localization of transglutaminase in *C. elegans*. (A) *Cross section of a wild type animal showing an immunopositive intestinal cell.* **(B-C)** *Embryos of the ced-5/ced-7 double mutant showing transglutaminase-positive corpses, corresponding to cells that died but are not engulfed.*

Vitellogenesis

A typical example of the role played by PCD is the involution occurring post-vitellogenesis in female frog liver. The massive wave of apoptosis that takes place in this organ is strictly regulated by sex hormones such as testosterone, estradiol and progesterone. Frogs are seasonal breeders, ovulation and egg deposition occurring in early spring. In autumn, gonads are engaged in a recovery phase and gamete production is slowly resumed (Rastogi *et al.*, 1983; Lofts, 1984); in the ovary, about one thousand small follicles are rescued and brought to vitellogenesis. Vitellogenine accumulates in the oocytes which became fully mature in the next winter (Whittier and Crews, 1987). Ovarian recovery depends greatly on liver contribution since this organ, under the influence of ovarian steroids, secretes vitellogenins and possibly other substances which are delivered through the bloodstream to the ovary (Gobetti *et al.*, 1985).

In post-vitellogenesis involuting livers, specific induction of the tTG gene is observed in parenchymal cells undergoing PCD, coupled with specific protein accumulation in hepatocytes showing the morphological features of apoptosis. The hormone-dependent increase of both PCD and tTG is reproduced in ovariectomized frogs. In fact, treatment of castrated animals with testosterone, estradiol and progesterone inhibits the induction of both tTG and PCD, thus indicating that *in vivo* a drop in the circulating sex hormone is the signal favouring the involution phase of the maternal frog liver after mating. It is interesting to note that tTG cleavage products are detected during the late stages of the liver involution suggesting a proteolitic processing of the enzyme protein (Assisi *et al.*, 1999). In keeping with this first evidence of a tTG processing during PCD *in vivo* a recent report confirmed that tTG can act as a caspase substrate in the course of apoptosis (Fabbi *et al.*, 1999).

Fertilization

The fertilization envelope (FE) is a complex macromolecular aggregate assembled by the addition of cortical granule contents to the vitelline layer. Two cortical granule enzymes, peroxidase and protease, in addition to a transglutaminase have been proposed to be required for the correct assembly of the FE which displays a trilaminar structure where a dense layer is sandwiched between two coating layers of paracrystalline material (Mozingo and Chandler, 1991). The addition, during *Sea urchin* fertilization, of an active-site transglutaminase inhibitor (1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride or L-682777) affects the appearance of the FE and its subsequent development in a concentration-dependent manner (Cariello *et al.*, 1994). In addition to a wrinkled appearance of the FE, after treatment with the TGases inhibitor, numerous denuded cells and empty FEs are also observed, thus indicating that the inhibition of TGase(s) renders the FE fragile. The *Sea urchin* zygote cleavage (the 2-cell stage) is also affected by treating fertilized egg with this transglutaminase inhibitor (Cariello *et al.*, 1994). In view of the abnormalities with regard to FE wrinkling and deranged cell division obtained with other transglutaminase inhibitors, it can be hypothesized that transglutaminases may be involved at various stages in fertilization and early cleavage.

A TGase responsible for crosslinking of egg envelope (chorion) proteins and probably participating in the post-fertilization chorion hardening has been purified from unfertilized egg chorions of *Oncorhynchus mykiss* (Ha and Iuchi, 1997). The purified enzyme was a monomeric protein having the molecular mass of 76 kDa. Although the amino acid composition of the purified TGase does not correspond with those of the previously characterized TGases of fishes, such as chum salmon and Red sea bream (Yasueda *et al.*, 1995; Sano *et al.*, 1996), the biochemical properties of the chorion TGase were consistent with the suggested function on polymerization of chorion proteins, resulting in chorion hardening.

Implantation

One of the most striking examples of massive and rapid tissue disruption occurring under physiological conditions takes place during the interstitial implantation of some mammalian embryos, the formation of hemo-chorial placenta and post-partum involution of uterus. The large remodelling occurring in maternal tissues during embryo implantation and development involves sequential depletion of decidual cells as well as giant cells in late pregnancy and after delivery of the epithelial cells from uterine mucosa (Welsh and Enders, 1985, 1991a,b; Parr *et al.*, 1987). The formation of the hemo-chorial placenta in rats takes place in specialized regions of the endometrium, defined as implantation chambers. During the first days after implant (5-10 days), the embryo is located in the antimesometrial part of the implantation chamber and does not reach the mesometrial part of the chamber until day 10. In order to get access to the maternal blood supply, the conceptus induces the disruption of several maternal tissues and starts to invade the extracellullar matrix beneath the uterine epithelium (Welsh and Enders, 1985, 1991a,b). Death of the decidual cells characterizes both the early and late events of pregnancy. It starts when the trophoblast has established the definitive connection with the maternal blood supply and the conceptus starts to occupy the implantation chamber. Throughout pregnancy the deletion of the

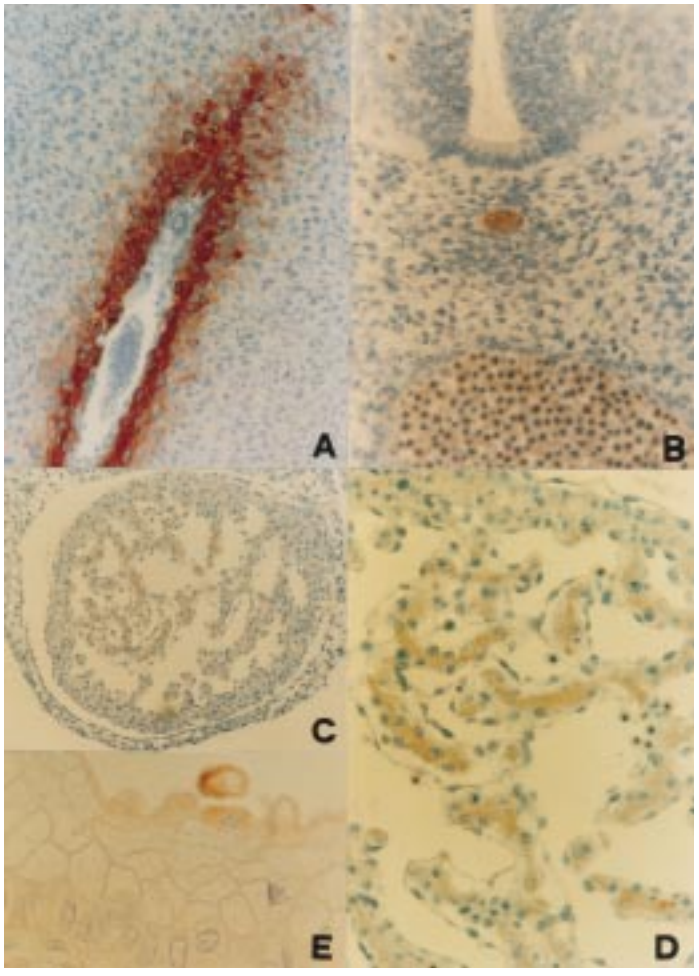

Fig. 2. "Tissue" transglutaminase expression in embryonal tissues. *Immunohistochemical localization of "tissue" transglutaminase in rat embryos (A-D) and human skin (E).* **(A)** *Cross section of the implantation chamber at the 10th day of pregnancy showing intense tTG staining of decidual cells lining the embryo.* **(B)** *Cross section of a 12 d.p.c. embryo showing tTG expression in the notochord and in nucleated red blood cells in the aorta.* **(C-D)** *Low and high magnification of 14 d.p.c. fetal lung showing tTG localization in the extracellular matrix.* **(E)** *Human trunk skin at 16 weeks estimated gestational age. tTG positivity is localized in the cytoplasm and in the plasma membrane of periderm cells.*

decidual cells is always localized in the stromal district lining the embryo (Welsh and Enders, 1985, 1991a,b; Parr *et al.*, 1987). We have shown that the maternal tissues lining the rat embryo are strongly positive for tTG (Fig. 2 A). The gene is expressed in the decidual cells adjacent to the implantation chamber showing the morphological features of apoptosis. tTG protein localization follows a gradient which diplays its maximum in those cells localized in strict contact with embryonic tissues (Fig. 2 A). The morphological analysis of the decidual tTG positive cells confirmed the apoptotic nature of the event. The remodelling of the maternal stroma is an essential requisite for survival and development of the mammalian embryo; however, although many potential functions have been proposed, the role of the decidual tissue is still unclear. The features of this phenomenon suggest that apoptosis in cells nearby the conceptus might be induced by the fetus itself during the implant, releasing active apoptotic factor/s. Noteworthy, are several factors such as TGFβ, TNFα, prostaglandin, which have been shown to induce tTG and apoptosis in other tissues (Buttyan *et al.*, 1989; Fesus *et al.*, 1991; Rotello *et al.*, 1991; Mastino *et al.*, 1992; Oberhammer *et al.*, 1992;) and are actively released in the interstitial space between mother and fetus (Khan *et al.*, 1991; Manova *et al.*, 1992; Tabizadeh, 1992).

Organogenesis

In the developing embryo of Vertebrates programmed cell death is precisely controlled and occurs in many tissues during organogenesis including: neural tube, heart, palate, duodenal mucosa, kidney and limb buds. In limb development a syncronous wave of cell death is observed and occurs predominantly in the interdigital web and in the apical ectodermal ridge (AER). Characterization of the genes involved in the regulation of these PCD events and evidence for an involvement of tTG, in various model systems, were presented during the past few years.

Limb

Annulin is a protein expressed in epithelial annuli in developing limbs of the grasshopper embryo (Singer *et al.*, 1992). Annuli comprise narrow circumferential bands of epithelial cells at the boundaries of limb segments and the expression of annulin precedes the first morphological signs of segmentation. Bands arise in a stereotyped order and, within a band, expression occurs in an ordered circumferential progression. Annulin has a molecular weight of about 97 kDa and appears to be intracellular and peripherally associated with the inner leaflet of the cell membrane. The nucleotide and deduced amino acid sequences indicate that the annulin protein appears to be a transglutaminase (Singer *et al.*, 1992). Its expression pattern within the limb and the embryo is associated with areas undergoing morphogenetic rearrangements, movements, or rapid cell division. It may stabilize cells under mechanical stress or participate in these morphogenetic activities.

To study the tTG expression during mouse embryo development Nagy and collaborators (Nagy *et al.*, 1998) generated transgenic mice carrying the β-galactosidase gene under the control of the mouse tTG promoter region (3.8 kb of the 5' flanking DNA). The results indicate that in E13.5 embryos β-gal activity was localized in auricolar and peri-orbital tissue, in the snout and particularly in the developing limbs. Detailed comparative analysis, between β-galactosidase expression and tTG immunoreactivity in the limb structure of E13.5 embryos, showed a colocalization of the transgene expression with endogenous tTG in the developing digits and in the area corresponding to the interdigital web.

Retinoic acid (RA) plays a major role in the formation of the interdigital necrotic zones (INZs). In fact, mice lacking the retinoic acid receptor (RAR) gamma gene and one or both alleles of the RAR beta gene (i.e., RAR beta+/-/RAR gamma-/- and RAR beta-/-/RAR gamma-/- mutants) display the interdigital web (soft tissue syndactyly), caused by the persistence of the interdigital mesenchyme (Ghyselinck *et al.*, 1997). The INZs mutants show a marked decrease in the number of apoptotic cells accompanied by an increase of cell proliferation. Moreover, the expression of a number of genes (BMP-2, BMP-4, Bcl2, Bax, and p53) known to be involved in the establishment of the INZs, the patterning of the autopod and/or the initiation of apoptosis was unaffected. In contrast, the INZs mutant displayed a specific down-regulation of tTG promoter

activity upon the removal of one or both alleles of the RAR beta gene from the RAR gamma null genetic background. These findings, coupled with the presence of several RA response elements in the tTG's promoter region, suggest that RA might increase the amount of cell death in the INZs through a direct modulation of tTG expression.

Mesoderm-derived cell lineages

Studies carried out in chiken and mouse showed that tTG is expressed in several mesoderm-derived cell lineages. In chiken embryos of 7.5 days expression of the tTG gene has also been investigated by means of both *in situ* hybridization and immunoistochemical analysis (Thomazy and Davies, 1999). Marked expression of tTG was found in cells undergoing programmed cell death both in the hind and fore limb. Moreover, expression of the tTG gene has also been observed in cartilagenous core of the hind limb metatarsals and phalanges and mRNA is present in the hypertrofic chondrocytes of the prospective primary ossification center, whereas the less mature proliferating condrocytes of the metaphyseal region did not express the gene. The epiphyseal regions of developing bones exhibited the presence of tTG's mRNA and protein. Protein accumulation is detectable both in cytoplasm and extracellular matrix, and seems to be due to an active secretion from viable cells preeceding the destruction of hyperthrophic chondrocytes. The presence of tTG in epiphysis and (pre)articular mesenchym indicates that transglutaminase might contribute to ossification and joint formation.

Expression of tTG mRNA has been also detected in developing skeletal muscle at a stage in which myoblasts are arranged into bundles but not yet fused to form myotubes. This data correlates well with observations on *in vitro* cultured chick embryonic myoblasts (Kang *et al.*, 1995). In fact, during myogenesis tTG expression was modified, showing that the enzyme level decreases at the initiation of fusion and then increases gradually. Furthermore, the distribution of tTG was also drammatically changed from a diffuse pattern to a definitively striated sarcomeric banding pattern, and was found to be associated to myosin in sarcomere (Kang *et al.*, 1995). tTG expression was detected in the precartilagineous blastema and in hypertrophyc chondrocytes and in the periarticular mesenchyme, but not in the proliferating chondrocytes. We have observed (unpublished observations) marked tTG expression in the notochord of developing mouse embryo (Fig. 2 B) that might be related to the apoptotic wave leading to the disapperance of this structure during the normal development of the individual. It is interesting to note that an intense staining is also observed in nucleated red blood cells in the aorta (Fig. 2 B).

Lung

At birth, the mammalian lung is not mature. The alveoli are not completely formed and during the first weeks of postnatal life, the alveolar septa undergo a marked reorganization, the amount of connective tissue decreases and the mature lung parenchyma is formed. It has been shown that, while enzyme activity and intracellular tTG are already present before term, the enzyme products and extracellular tTG appear between the second and third week of postnatal life in the rat lung parenchyma (Schittny *et al.*, 1997). In contrast, we show here that extracellular tTG is already detectable at the end of the fetal mouse development (Fig. 2 C, D). However, despite the difference in timing, it is interesting to note that tTG is expressed and externalized into the extracellular matrix of lung before the functional maturation of the organ. Release of intracellular tTG has been shown to occur under several pathological instances, particularly under those circumstances in which a fibrotic events is associated to the pathology (Piacentini *et al.*, 1999). Although is well known that tTG covalently and irreversibly crosslinks extracellular matrix proteins, it is difficult to hypothesize what role the enzyme might play on the organization of extracellular components, such as collagen, during development and whether its function is limited to its crosslinking activity. There is good rationale for hypothesizing a role for vitamin A-derivatives in lung development and tTG induction. In fact, vitamin A stores are high in fetal lung and decrease toward term; the activity, levels and expression of the cytosolic and nuclear receptor proteins for vitamin A undergo changes before and after birth in rat lung which parallel tTG expression (Schittny *et al.*, 1997).

Skin and its appendages

During human skin development, embryonic- and fetal-specific periderm cells and incompletely keratinized cells are replaced by keratinocytes that differentiate while stratifying to form the fully functional epidermis. The periderm is a transient epithelial layer destined to be sloughed off into the amniotic fluid and replaced by cornified epidermis (Polakowska *et al.*, 1994). Proliferating basal cells of fetal skin also develop into epidermal appendages such as hair follicles and glands. PCD has an important function in establishing the final architecture of the human epidermis and its appendages. It is interesting to note that tTG localization in fetal periderm, intermediate epidermal cells, and within appendages coincides with DNA fragmentation indicating that PCD is involved in deletion of these stage-specific cells and remodeling of appendages (Fig. 2 E). These data as well as the expression of other apoptosis-related gene suggest that terminal differentiation of epidermal cells might be a specialized form of apoptosis. It is of interest that the periderm blebbing process, occurring after the first trimester in human development, closely resembles the formation of apoptotic bodies and is coincident with the first detectable co-expression of both type 1 TG and tTG (Polakowska *et al.*, 1994). The coexistence of both TGases may indicate overlapping apoptotic and cell-specific effects of these enzymes.

Conclusions

From the data described above it is clear that the expression of a TGase, particularly the tTG, characterizes most, if not all, the stages of vertebrate development. Experiment carried out using pan-inhibitor (dansylcadaverine) of TGases have outlined the importance of the crosslinking activity already during the early embryonic cleavages (2-cell stage). A confirmation of the importance of type 1 TGase, the membrane-bound isozyme of the TGase family (Table I), was recently obtained by generating knock out mice for this gene. These mice show abnormal skin keratinization and its barrier function was markedly impaired (Matsuki *et al.*,1998). In fact, the stratum corneum was incomplete and cell envelope assembly was defective and mice died within 4-5 h after birth. These results clearly demonstrate that the TGase1 gene is essential to the development and maturation of the stratum corneum and to adaptation to the environment after birth. However, in light of the recent findings showing the ubiquitous expression of TGase1 in other tissues (Hiiragi *et al.*, 1999; Kim *et al.*, 1999), a

more detailed analysis of the internal organs could also reveal additional anormalities that may help to explain the severe phenotype observed in these mice. Considering the recent demonstration of the co-espression of tTG with other TGases in adult tissues (Kim *et al.*, 1999), one may wonder whether this redundancy is also present during embryonal development. So far, very few studies have tried to specifically address the role of the different TGases in vertebrate development and the only data available have been previously discussed on the co-expression of the type 1 and 2 enzyme in the developing skin. By using single and double knockout tecniques in parallel with functional studies on the protein substrates it should be possible to understand the specific role of the various TGases in vertebrate development. These studies are essential to verify the relative contribution of the various functions proposed for these enzymes, particularly for tTG, *in vivo*.

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