

Differential expression of tissue transglutaminase during in vivo apoptosis of thymocytes induced via distinct signalling pathways

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Abstract A significant increase in the expression and activity of tissue transglutaminase (tTG), one of the effector elements of apoptosis, was observed during involution of thymus elicited by treatment with either anti-CD3 antibody or dexamethasone or by irradiation. The blood plasma concentration of $\epsilon(\gamma\text{-glutamyl})\text{-lysine}$ isodipeptide, the end-product of the digestion of transglutaminase cross-linked proteins, was also elevated in each of these cases. tTG was localized in cells of the cortical layer of the thymus and immunofluorescence double staining revealed that the enzyme appeared in the apoptotic cells. None of these observations could be made when apoptosis was induced by *fas*-receptor stimulation. The lack of tTG activity in *fas*-stimulated cells was accompanied with a less organized apoptotic morphology. Our data suggest that distinct signalling pathways, which induce apoptosis within the same cell type, can differentially regulate the expression of tTG, and this enzyme may be involved in structural stabilization of the apoptotic cells.

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Key words: T lymphocyte; Apoptosis; Signal transduction; Tissue transglutaminase

1. Introduction

The cell-autonomous process of apoptosis was originally defined by morphological criteria (cellular shrinkage, membrane blebbing, chromatin condensation and fragmentation) established in living tissues [1]. The intense genetic, biochemical and cellular studies of recent years have revealed that distinct molecular pathways of apoptosis may exist beyond this characteristic morphology in various cells and even in one cell type [2,3]. One of the critical issues in the biochemical characterization of apoptosis is to dissect these pathways clarifying which of the effector elements (e.g. endonucleases, proteases, transglutaminases) are activated and how they are regulated by various death signals [3]. Maturing thymocytes are very suitable for such studies because their death program

can be initiated via several distinct molecular pathways [2]: stimulation of TCR and CD3 induces changes in second-messenger systems [4], glucocorticoids bind to steroid receptors [5], while inhibition of topoisomerase II or irradiation causes direct DNA damage [6,7]. Each of these pathways appears to induce distinct sets of genes. The transcripts RP-2 and RP-8 are expressed in thymocytes following treatment with glucocorticoids [8]. DNA damage leads to *p53* induction [9,10]. The immediate-early gene, *nur 77* is induced in response to TCR signals but not by glucocorticoids or ionizing radiation [11]. *Fas* stimulation, on the other hand, induces apoptosis without the involvement of new macromolecular synthesis [12].

Characteristic biochemical features of apoptosis have been observed in all the four forms of thymocyte death mentioned above [5–7,12–14], suggesting that common effector elements of apoptosis are activated by distinct molecular signaling pathways. The induction and activation of the Ca^{2+} -dependent tTG has also been implicated in the molecular events of apoptosis [for references see [15]]. Transglutaminases catalyze a protein cross-linking reaction in which gamma-carboxamide groups of peptide bound glutamine residues serve as acyl donors and primary amino groups of peptide-bound lysine function as acceptor substrates [16]; it has been suggested that enzymatic cross-linking of proteins by tTG leads to the assembly of a stable protein scaffold in the dying cells [15]. However, it is not known which and how apoptotic pathways switch on the catalytic action of transglutaminase. Data presented in our paper suggest that induction and activation of tTG is part of the biochemical pathway of thymocyte apoptosis induced by dexamethasone (DXM), TCR activation and DNA damage but not of that initiated by *fas*-receptor stimulation, and the lack of tTG activity is accompanied with a less organized apoptotic morphology.

2. Materials and methods

2.1. Materials

Anti-CD3 and anti-*fas* monoclonal antibody were purchased from Pharmingen (San Diego, CA), anti-tTG antibody was a gift of Dr. Paul Birckbichler (Oklahoma City, OK). Horseradish peroxidase-labelled goat anti-mouse and anti-rabbit IgG, dexamethasone-21-acetate, *N,N'*-dimethyl-casein were from Sigma Chemical Co. (St. Louis, MO). Luminol and bovine serum albumin were from Reanal (Budapest, Hungary), PVDF membrane was from Millipore Corp. (Bedford, MA) while [^3H]putrescine (26 Ci/mmol) was purchased from Amersham. All other reagents were of analytical grade and obtained from commercial sources.

2.2. Experimental animals

Male NMRI, 4-week-old mice with a body weight of 27.1 ± 1.5 g purchased from LATI (Gödöllő, Hungary) were used. For the induction of thymic involution and apoptosis mice were treated i.p. with 0.5

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Abbreviations: ABC-AP, avidin-biotin-alkaline phosphatase complex; DXM, dexamethasone; FITC, fluorescein isothiocyanate; GR, glucocorticoid receptor; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; PMSF, phenylmethyl sulphonyl fluoride; TCR, T-cell receptor; TdT, terminal-deoxynucleotidyl-transferase; TEM, transmission electron microscopy; TRITC, tetramethyl-rhodamine isothiocyanate; tTG, tissue transglutaminase; TTBS, Tris-buffered saline with 0.005% Tween

mg dexamethasone-21-acetate or 12.5 μ g anti-fas or 80 μ g anti-CD3 monoclonal antibody dissolved in 0.2 ml of physiological saline, or were exposed to 5 Gy irradiation.

2.3. Determination of transglutaminase activity

Thymus was collected from control or treated animals at various time points after treatment, washed extensively with PBS and homogenized in 0.1 M Tris-HCl, pH 7.5, containing 0.25 M sucrose, 0.5 mM EDTA, and 1 mM PMSF. tTG activity in homogenates was measured by detecting the incorporation of [3 H]putrescine into *N,N'*-dimethyl-casein as it was described previously [17]. Enzyme activity was calculated as nmoles of [3 H]putrescine incorporated into casein in 1 h by 1 mg cellular protein.

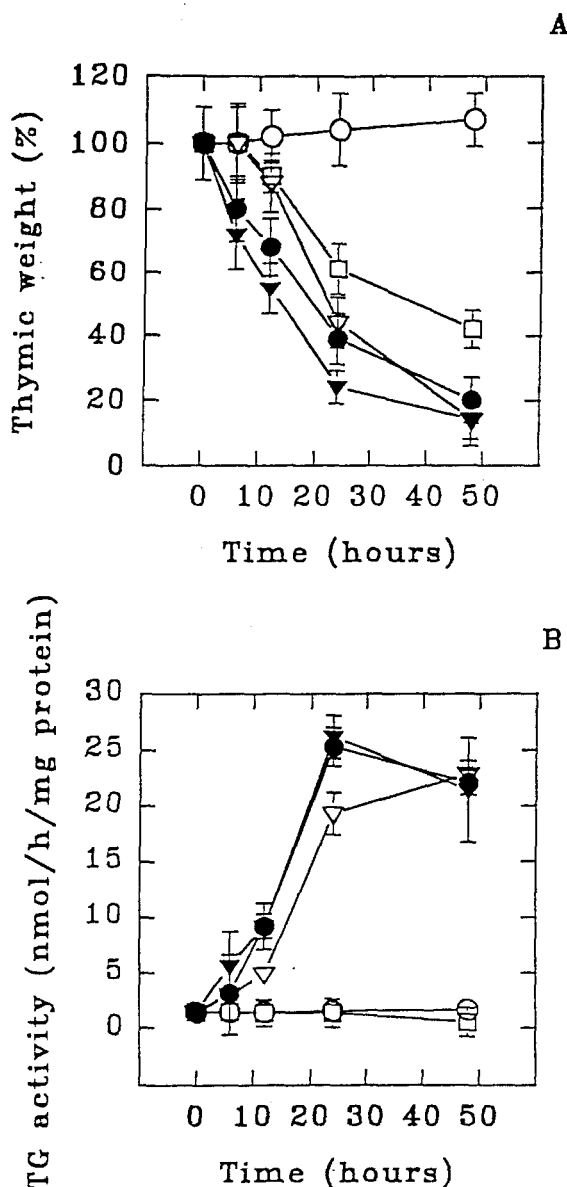


Fig. 1. Changes in the activity of tTG during in vivo apoptosis of thymocytes induced via distinct signalling pathways. Changes in thymic weight (A) and tTG activity (B) at various time points after in vivo induction of apoptosis by anti-fas antibody (□) dexamethasone-21-acetate (▼), irradiation (▼) or anti-CD3 antibody (●) as compared to treatment with physiological saline alone (○). Data represent mean \pm SD of three determinations.

2.4. Western blot analysis

Thymus was collected from control or treated animals at various time points after treatment, washed extensively with PBS and homogenized in 0.1 M Tris-HCl, pH 7.5, containing 0.25 M sucrose, 0.5 mM EDTA, and 1 mM PMSF. Homogenates containing 1 mg/ml protein were denatured in Laemmli buffer [18]. The proteins were then separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore). Membrane was blocked and probed with a monoclonal antibody against tTG, diluted 1:100. tTG was detected by using a horseradish peroxidase-labelled second antibody and the Amersham ECL system.

2.5. Immunohistochemistry and co-localization of tTG and apoptotic nuclei in thymus sections

For immunohistochemical staining and immunofluorescence thymus was fixed with freshly prepared formaldehyde and embedded in paraffin. Sections (5 μ m thick) were cut and picked up on slides then dewaxed with xylene, hydrated and finally digested with 10 μ g/ml proteinase K for 30 min at room temperature to unmask antigenic structures. Affinity purified polyclonal rabbit antibody raised against human tTG [19] was used to detect enzyme protein. Biotinylated donkey anti-rabbit link antibody (Amersham, Buckinghamshire, UK) and ABC-AP (Dakopatts, Glostrup, Denmark) was applied to develop the colour reaction. Fragmented DNA was labelled with digoxigenin-11-dUTP using calf-thymus TdT [20] and later detected by TRITC-conjugated sheep-anti-digoxigenin f(ab) fragments (all from Boehringer Mannheim, Mannheim, Germany). For immunofluorescent staining a FITC-conjugated swine anti-rabbit antibody (Dakopatts) was applied. Controls were performed by using normal rabbit serum instead of primary rabbit antibodies and omission of TdT, respectively. Photographs were taken by a Leitz Orthoplan microscope equipped with Ploemopak epi-illuminator and using dual excitation filter.

2.6. Transmission electron microscopy

Samples (1 mm³) of the thymus (control or treated with various apoptotic stimuli) were collected and routinely processed for transmission electron microscopy (TEM). Random serial photographs (1500–60 000 \times) were taken without prior knowledge of the actual treatment which the animals had prior dissection.

2.7. Preparation of plasma samples and determination of the concentration of ϵ (γ -glutamyl)lysine isodi-peptide

Citrated platelet poor plasma was produced from whole blood anti-coagulated with 0.109 M trisodium citrate (ratio 9:1) by centrifugation at 2000 \times g for 10 min. All samples were stored at -20°C and thawed at $+37^{\circ}\text{C}$ immediately prior to analysis. Pooled plasma were obtained from 3 mice for each time point. To determine the concentration of ϵ (γ -glutamyl)lysine isodi-peptide several purification and analytical steps were carried out as described [21] except that instead of proteolytic digests, protein-free plasma sample prepared by centrifugation at $+4^{\circ}\text{C}$ in a Centrifree partition device (Amicon, cut-off 5000) was used as a starting material. The reproducibility of the method is reflected in a coefficient of variation of less than 10%.

3. Results

3.1. Transglutaminase is induced in thymus after induction of apoptosis in vivo via the TCR, GR and P53 signal transduction pathways but not via fas-receptor stimulation

Mice were injected with DXM, anti-fas or anti-CD3 antibody or exposed to 5 Gy irradiation to induce apoptosis in the thymus. Each treatment resulted in a very similar time course of thymic involution (Fig. 1A): irradiation induced the highest rate of cell death followed by that elicited by anti-fas, anti-CD3 and DXM treatments. With anti-fas and anti-CD3 treatment or following irradiation a significant decrease in thymic weight was detected already at 6 h. At 48 h following each treatment 80% of the thymus disappeared and no further decrease was found in the thymic weight at later time points (data not shown). As shown in Fig. 1B each treat-

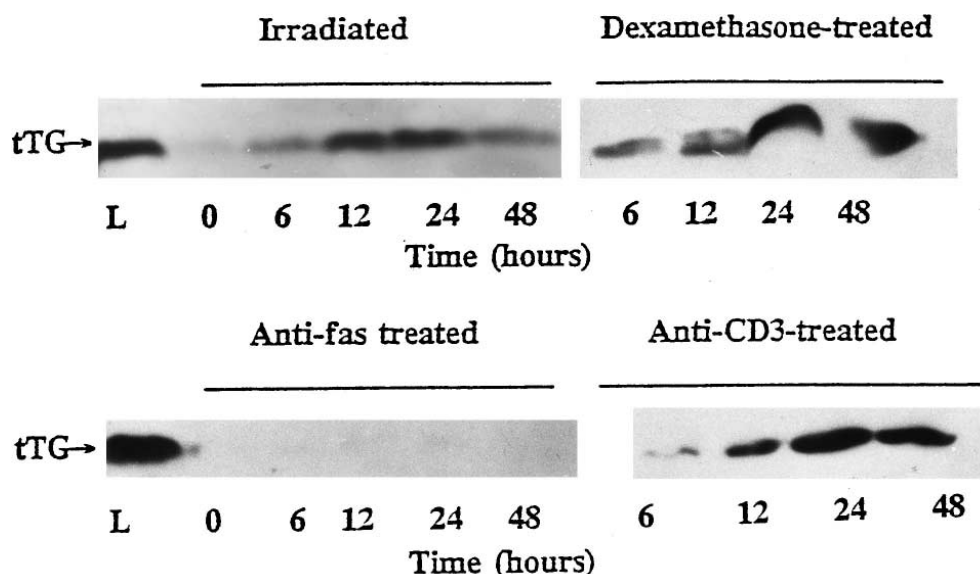


Fig. 2. Changes in transglutaminase expression studied with Western blot analysis during in vivo apoptosis of thymocytes induced via distinct signalling pathways. L: tissue homogenate from mouse liver where tTG is constitutively expressed. tTG indicates the position of the electrophoretic migration of mouse tTG (77 kDa).

ment, with the exception of anti-*fas*, resulted in a significant increase in tTG activity from an initial 1.45 ± 1.2 with a peak activity of 25.3 ± 2.1 , 19.3 ± 1.8 and 26.2 ± 3.1 nmol/h/mg protein following anti-CD3, DXM treatment and irradiation, respectively. The first significant increase in activity ($P < 0.05$ by Student's *t* test) was observed after 6 h and reached the peak value at 24 h in each case.

Fig. 2 shows changes in the expression of tTG determined by Western blot analysis after induction of apoptosis with irradiation, anti-CD3 or DXM. As time progressed more and more tTG was expressed, reaching maximum amounts at 24 h. There was no change in the expression of tTG if apoptosis was induced via *fas*-receptor stimulation.

3.2. tTG is expressed primarily in the apoptotic cells of the cortical layer of the thymus

Thymus sections at various time points after each treatment were stained for tTG protein. The presence of tTG in thymus of control animals was limited to the endothelial cells lining the vessels (section not shown) where tTG is constitutively expressed [22]. As the apoptotic process induced by DXM was accelerated and the involution of the thymus progressed, more and more cells became tTG positive. All these tTG-positive cells were localized within the cortical layer of the thymus (Fig. 3). The cortical layer of the thymus is known to contain the immature $CD4^+CD8^+$, $bcl-2^-$ thymocytes which are sensitive to apoptotic stimuli [23]. Similar pictures were found with anti-CD3 treatment or after irradiation, while in thymus treated with anti-*fas* antibody tTG expression was not observed (these sections are not shown).

To prove that cells expressing tTG were indeed those which entered the apoptotic program, thymic cells were immunostained for both tTG and apoptotic nuclei using the TdT reaction for the latter. As shown in Fig. 4A most of the cells were negative for the presence of both tTG and apoptotic nuclei in thymus of control animals, though a very few positive cells were seen representing those undergoing spontaneous apoptosis. At 12 h after DXM treatment (Fig. 4B) the

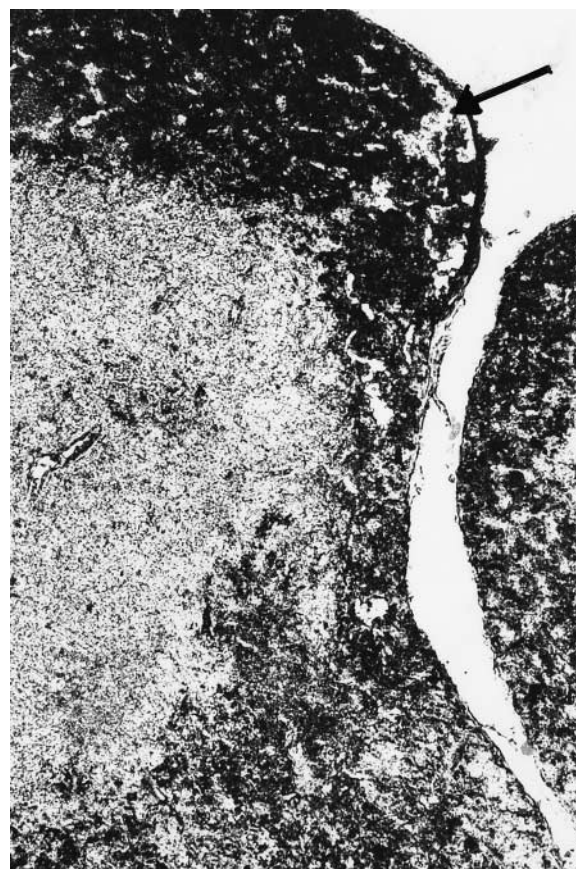
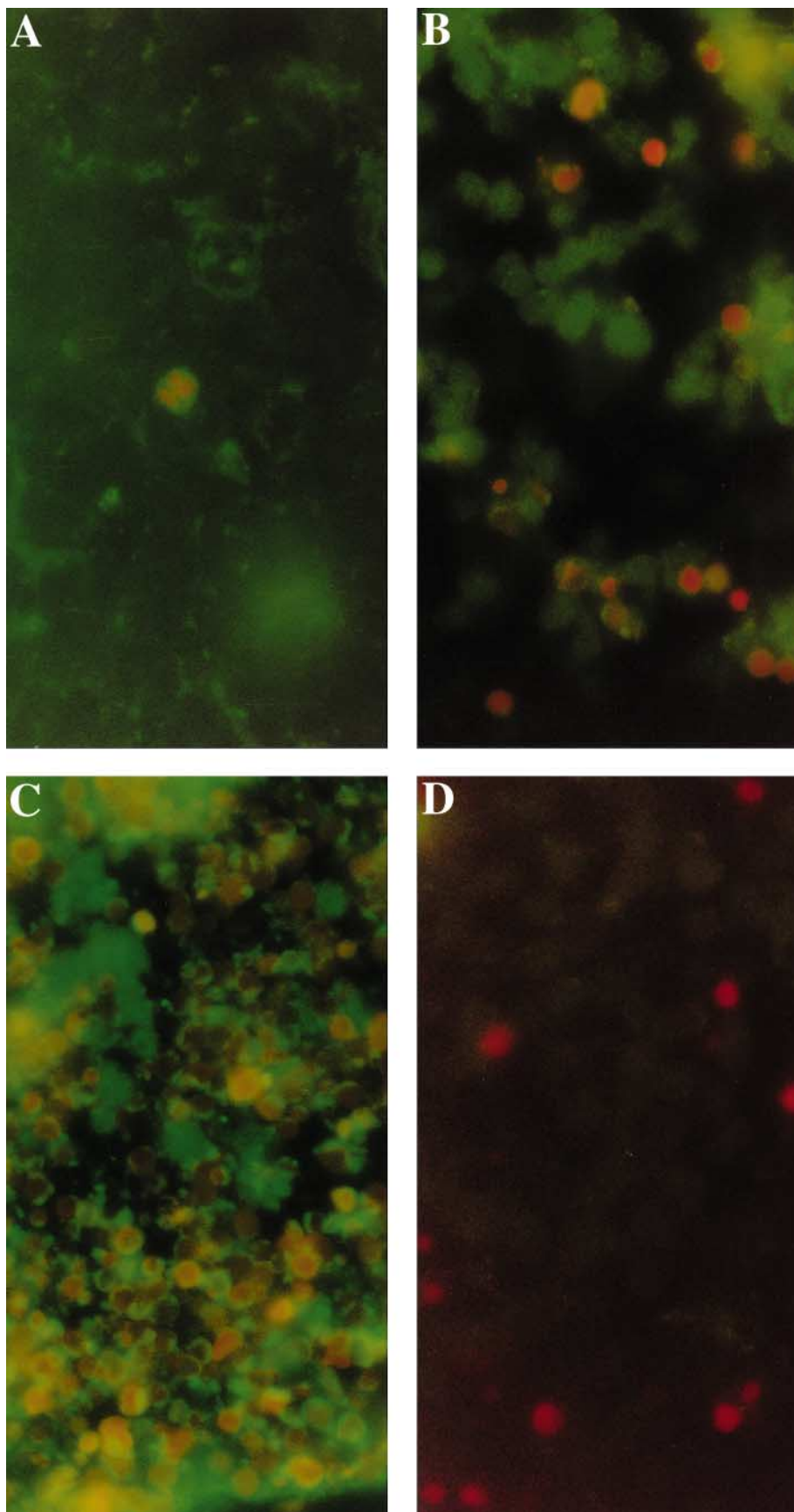


Fig. 3. Immunolocalization of tTG in mouse thymus at 24 h after the induction of apoptosis by DXM. Thymus sample was collected from a DXM-treated animal at 24 h after treatment, and immunostained for tTG as it was described in Section 2. Arrow points out to the cortical layer which became strongly tTG positive after apoptosis induction. This layer contains the apoptosis-sensitive $CD4^+CD8^+$ thymocytes (23). (Magnification $\times 160$).



number of tTG-positive cells in the cortical layer was significantly increased. However, the majority of tTG-positive cells

were negative for DNA fragmentation at this time point; in a typical experiment the ratio of TdT^-tTG^+ , TdT^+tTG^+ and

Fig. 4. Co-localization of tTG and apoptotic nuclei in thymic sections. Thymus was collected at 0 (A), 6 (B) and 24 h (C) after treatment with DXM, or 6 h following the injection of anti-fas antibody (D). Thymus sections were stained for both tTG (green) and apoptotic nuclei (red) as was described in Section 2. Note that the appearance of tTG expression is prior to that of the apoptotic nuclei (B), tTG is co-localized with the apoptotic nuclei (appears as yellow) of DXM-treated thymocytes (C) and lack of tTG expression in the anti-fas-treated thymus though the apoptotic nuclei are present in comparable number to that of the DXM-treated one at the same time point (D). (Magnification $\times 800$).

TdT⁺tTG⁻ cells were 10:4:0.5, respectively. At 24 h (Fig. 4C) the number of tTG-positive cells were further increased and tTG positivity was found in apoptotic thymocytes containing fragmented DNA; the ratio of TdT⁻tTG⁺, TdT⁺tTG⁺ and TdT⁺tTG⁻ cells were 6:5:0.5, respectively. Similar pictures were found if the animals were irradiated or treated with anti-CD3 antibody (sections not shown). These observations suggest that tTG is induced in the pre-apoptotic thymocytes and the induction of the expression of the enzyme is prior to endonuclease activation.

As shown on Fig. 4D the number of apoptotic nuclei is also increased in the thymus of animals treated with anti-fas antibody. However, there was no change in the pattern or intensity of the expression of tTG providing further evidence that

the enzyme is not induced during apoptosis initiated through fas stimulation.

3.3. Elevated levels of plasma $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isodipeptide indicate activation of tTG in the dying cells of involuting thymus

Covalent $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ crosslinks are formed between polypeptide chains of proteins when tTG is active within cells. Since proteases do not cleave the $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ crosslink formed by tTG [16] the amount of isodipeptide released into blood circulation following phagocytosis and degradation of apoptotic bodies may reflect the rate of apoptosis in tissues including lymph nodes and T cells [24,25]. As the involution of thymus was initiated the concentration of the isodipeptide

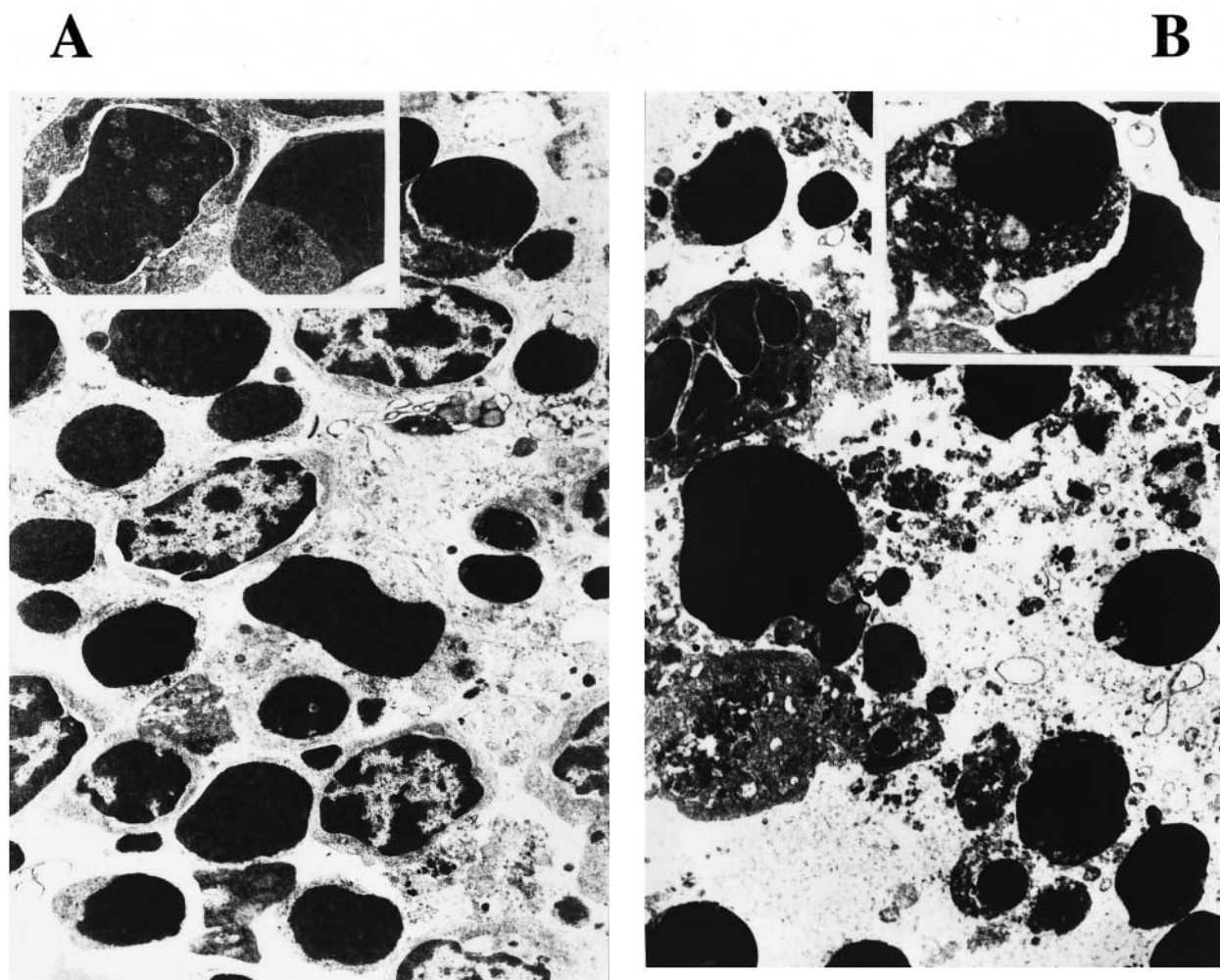


Fig. 5. Apoptotic morphology of thymocytes after DXM or fas treatment. (A) DXM-treated animal with lymphocytes undergoing the classic ('orderly') apoptotic changes. (Magnification $\times 5200$). Inset: two lymphocytes one with perinuclear space dilatation, the other with typical nuclear condensation (magnification $\times 13000$). (B) Apoptosis induced by fas-stimulation is characterized by a more irregular fragmentation of cells and sieve-like cytoplasmic changes. (Magnification $\times 5200$). Inset: two lymphocytes with various degrees of nuclear condensation but marked enlargement of the endoplasmic reticulum and/or lysosomes. (Magnification $\times 13000$).

Table 1
Changes in the plasma $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isodipeptide level at various time points after the induction of apoptosis by various treatments

Treatment	Isodipeptide level (nmol/ml)				
	Time after treatment (h)				
	0	6	12	24	48
Control	1.65	1.32	1.71	1.75	1.68
Dexamethasone		1.34	2.86	4.33	3.81
Anti-CD3 antibody		1.92	2.42	4.61	4.42
Irradiation		3.05	3.21	5.73	6.54
Anti- <i>fas</i> antibody		1.69	2.01	1.87	1.92

Data are values of one representative experiment and are the result of determination from pooled plasmas of three mice for each time point after various treatments.

in plasma was increased in case of each treatment with the exception of anti-*fas* antibody, where only a slight decrease was found at 12 h (Table 1). These findings suggest that tTG is not only induced but activated during apoptosis initiated by DXM, anti-CD3 treatment or irradiation. Though the time course of the decrease in thymic weight was very similar after these three treatments, the increase in the isodipeptide level was much more pronounced and earlier in irradiated animals than in the others. This observation may be explained by the selective action of DXM and anti-CD3 on lymphoid cells, while irradiation can induce apoptosis in other cell types as well (e.g. intestinal epithelial cells, bone marrow cells and others [26]). The slight but reproducible increase in isopeptide level found in anti-*fas*-treated animals, on the other hand, may reflect the apoptosis of other *fas* bearing cells which constitutively express tTG [27]. In these cells tTG can be activated by increases in intracellular Ca^{2+} triggered by *fas* engagement [28].

3.4. *Fas*-induced cell death is characterized with a less organized morphology

Thymus samples after various treatments were studied with TEM. A consistent feature at all magnifications used became obvious: animals treated with DXM (Fig. 5A), anti-CD3 antibody or irradiation (sections are not shown) displayed pronounced apoptotic changes of the lymphocytes, usually displaying the classical ultrastructural changes with relatively frequently started by the dilatation of the perinuclear space. Apoptosis that was induced by *fas*-receptor stimulation appeared more dramatic with conspicuous fragmentation of the cells resulting in a much less orderly, sometimes even chaotic morphology. The cytoplasm of the lymphocytes in this series often became sieve-like due to marked dilatation of lysosomes and RER (Fig. 5B).

4. Discussion

According to our current understanding of the molecular mechanism of apoptosis a variety of signaling processes, initiated by either the appearance of apoptosis factors or the disappearance of survival factors, induce and/or activate a set of apoptotic effector elements. The latter are usually enzymes which catalyze irreversible biochemical reactions such as DNA degradation, proteolysis and cross-linking of proteins [29]. Little is known, however, about the transmittance of the death signals to these effector elements. One of the reasons for the lack of detailed information is the complexity of most of the effector enzyme systems. It is still not clarified which of the candidate endonucleases [30–32] degrade DNA. Several

sets of proteases [2,3,5], including a family of interleukin-1 β -converting enzymes [13], have been linked to the death process. Additionally, these endonucleases and proteases are expressed constitutively and are only activated via apoptosis signalling pathways.

tTG may serve as a better candidate for studies designed to understand the link between the apoptosis signalling pathways and the apoptosis effector elements, since it is the only member of the transglutaminase enzyme family which has been implicated to participate in the apoptotic process [15] and it is one of the very few genes which have been found to be induced during apoptosis resulting in a significant accumulation of the enzyme protein in pre-apoptotic and apoptotic cells [15]. An additional advantage is the possibility of determining, with the help of measuring $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isodipeptide concentration in blood plasma, whether the enzyme was active or not in the dying cells. The data presented here are the first to show that within the same cell type various signals leading to cell death regulate differentially the expression and activity of tTG. While stimulation of both a cell surface (TCR), a nuclear receptor (steroid receptor) or a nuclear transcription factor (*p53*) induced the expression and activation of tTG in thymocytes, stimulation of the *fas* receptor did not.

The *fas*-mediated cell death pathway has been reported to have many unusual characteristics. Unlike others, *fas*-induced cell death does not necessarily require macromolecular synthesis, is not blocked by *bcl-2* protein alone [33,34] (though the lack of *bcl-2* inhibition is dependent on cell type [35,36]) and typical cytoplasmic changes of apoptosis can occur in the absence of the nucleus [37]. The *fas* signalling pathway involves cysteine proteinase(s). Deletion of the gene or inhibition of the interleukin-1 β -converting enzyme in mice renders thymocytes relatively resistant to *fas*-induced death [14,38] but not to other forms of apoptosis. The lack of the induction of tTG can be added now to the list of differences found between *fas*-dependent and other forms of apoptosis.

The mechanism of the regulation of tTG expression during steroid receptor-, TCR- and *p53*-dependent apoptosis is not yet clear. It seems to occur at transcriptional level, since transgenic mice carrying the bacterial β -galactosidase enzyme under the expressional control of tTG promoter express an increased level of the bacterial enzyme in the apoptotic thymus (our yet unpublished observation). Additionally, in all cases studied so far the mechanism of tTG induction in apoptotic cells has turned out to be transcriptional [15,39,40]. The regulation may involve the synthesis and/or activation of a so far unidentified transcriptional factor which results in a higher level of tTG mRNA and protein upon induction of apoptosis.

Alternatively, the regulatory regions of the *tTG* gene may contain a relatively large number of response elements (steroid, retinoid, *p53* and others) which can be utilized by the apoptotic stimuli. Further studies in our laboratory are in progress to differentiate between these alternatives.

The time course of the appearance of tTG after apoptosis induction in thymic sections, as compared to that of the apoptotic nuclei, shows that the induction of the enzyme is prior to the activation of the endonuclease. This may suggest that induction of the tTG may play role in the initiation of the apoptotic program induced by signals depending on new mRNA synthesis. Indeed, it was shown that transfection of tTG into balb-c 3T3 fibroblasts induces a high rate of apoptosis [41], and in some forms of apoptosis blocking the induction and of tTG has lead to the inhibition of the apoptotic program [42]. Generally, transglutaminase-dependent formation of stable cross-links leads to protein polymerization and confers resistance to breakage and chemical attack [16]. In several apoptosis models the inhibition of the enzyme resulted in an increased leakage of intracellular macromolecules [15], suggesting a potential role of the enzyme in stabilizing cellular structures until phagocytosis of the apoptotic bodies takes place. Further studies will reveal how the presented morphologic differences between the *fas*-mediated and tTG expressing forms of apoptosis are related to the protein crosslinking function of the enzyme.

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