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Tissue transglutaminase is a caspase substrate during apoptosis. Cleavage causes loss of transamidating function and is a biochemical marker of caspase 3 activation

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Abstract

Tissue transglutaminase (tTG) is a Ca^{2+}-dependent cross-linking enzyme that participates in the apoptotic machinery by irreversibly assembling a protein scaffold that prevents the leakage of intracellular components. In the present study a single-chain antibody fragment (scFv) detecting tTG is described. We demonstrate that TG/F8 scFv, selected from a phase display library of human V-gene segments by binding to guinea-pig liver tTG, can react with human tTG both in Western blot and in immunohistochemistry. The specific detection of tTG by TG/F8 in human thymocytes is verified by mass spectrometric analysis of the purified protein. Furthermore, we demonstrate that in lymphoid cells tTG is cleaved by caspase 3 during the late phase of apoptotic death, concomitant to DNA fragmentation, and that such cleavage causes loss of cross-linking function. We propose tTG cleavage as a valuable biochemical marker of caspase 3 activation during the late execution phase of apoptosis.

Keywords: apoptosis; tissue transglutaminase; single chain antibody fragment; caspase 3

Abbreviations: mAb, monoclonal antibody; PARP, poly(ADP-ribose) polymerase; scFv, single-chain antibody Fv fragment; tTG, tissue transglutaminase

Introduction

The process of apoptosis can be divided into three phases: initiation, execution and degradation.[1] Initiation involves a series of intracellular signaling molecules that are specific for a particular death stimulus. The signals emerging from the different initiation pathways converge on a death execution phase that seems common to all apoptotic systems and is evolutionary conserved.[2,3] The activation of a cascade of proteolytic enzymes and the subsequent selective degradation of a limited number of protein targets is characteristic of this stage. In particular, the caspase family of cysteinyl containing aspartate-specific proteases has been shown to play a pivotal role in the disintegration process that manifests the apoptotic phenotype.[2,4,5] So far 14 members of the caspase family of proteases have been described, including members predominantly involved in inflammation.[4–10] They are synthesized as inactive pro-enzymes and converted by an apoptotic signal into active enzymes whose most distinctive catalytic feature is an almost absolute requirement for aspartic acid in the substrate P_1 position.[11] The conversion of caspase proenzymes into the catalytically competent heterodimeric form is achieved by a cleavage at Asp-x bonds mediated by intermolecular autoproteolysis,[12–15] digestion by other caspases, or proteases with a similar specificity. Receptor-mediated recruitment and activation of 'regulatory' caspases, such as caspases-8 and -10, can indeed activate an 'effector' protease such as caspases-3, -6 and -7. Once activated, some family members may further cleave and activate the same caspases responsible for their activation, and thus setting up a protease amplification cycle (reviewed in[6]). Proteolysis is likely the key event in shutting down cell function by disabling a number of enzymes involved in signal transduction, in DNA repair, in cell cycle progression, and in the maintenance of the cytoskeleton network. These cleavage events lead to the disassembly of structural components of both nucleus and cytoskeleton, while promoting detachment of cell from the surrounding tissue (reviewed in[16]).

Conversely, besides proteolysis the dying cells can exhibit a polymerizing activity that crosslinks intracellular proteins and is linked to activation of tissue transglutaminase.[17–19] Tissue transglutaminase (tTG) is a Ca^{2+}-dependent enzyme that catalyses the formation of isodipeptide bonds between the ε-NH_2 side chain of a lysine residue and the γ-amide side chain of a glutamine residue (for reviews[20,21]). Its activation in cells undergoing apoptosis leads to the irreversible assembly of a cross-linked protein scaffold that prevents the leakage of intracellular components, thus reducing inflammation and autoimmunity.[19,22] Although tTG is present in the cytoplasm of viable cells, it may not be active as a crosslinking enzyme as a result of a tight post-synthetic regulation

operated by GTP,[23] Ca^{2+},[21,23] nitric oxide via S-nitrosylation,[24] and possibly by free putrescine and other polyamines.[25] In addition, tissue transglutaminase has been reported to function as a G-protein that participates in receptor signaling. In this context receptor-stimulated GTP binding might switch the function from transglutamination to receptor signaling.[26] The GTP-binding activity of tTG has been related to regulation of cell cycle progression[27] and cell viability.[28] On these grounds, it has been proposed that expression in viable cells of tTG in its G-protein configuration may have a role in prevention of cell death.[19] In the present study, a new monoclonal reagent detecting tTG is described. We demonstrate that a single-chain antibody fragment (scFv), selected from a phage display library of antibody fragments derived from human V-gene segments[29] by direct binding to immobilized guinea-pig liver tTG, can react with human tTG both in Western blot and in immunohistochemistry. The tTG identity of the protein species identified by the scFv in human thymocytes extracts is verified by mass spectrometric analysis. Moreover, evidence is provided that tTG is cleaved during apoptosis in a caspase 3-dependent mode, and that such cleavage occurs in the late phase of cell death, concomitant to DNA fragmentation, causing loss of cross-linking function.

Results

Selection of a human antibody fragment (TG/F8) specific for tTG from guinea-pig liver

Tissue transglutaminase is highly homologous across the various animal species. The human enzyme shows a 81% homology with the guinea-pig enzyme and a $>84\%$ homology with the mouse tTG.[30] A high degree of homology between molecules of different species is often the limiting factor in the successful generation of conventional monoclonal antibodies because of the mechanism of tolerance of self antigens. To overcome this problem, we used phage display technology, which makes it possible to by-pass *in vivo* immunization. A human scFv phage library[29] was subjected to four rounds of panning of the phage on guinea-pig liver tTG. The secreted scFv fragments from bacterial cultures were subsequently screened for binding to the same antigen by ELISA. One scFv yielding a good ELISA signal, clone TG/F8, was selected and its specificity and properties were studied. As shown in Figure 1, TG/F8 reacted with guinea-pig liver tTG both in native (Figure 1A) and unfolded (Figure 1B) form. A constant amount of TG/F8 scFv was challenged with varying amounts of tTG in soluble form; the immune complexes formed were resolved on a native-PAGE, where the protein complexes are preserved in the absence of SDS, and detected by

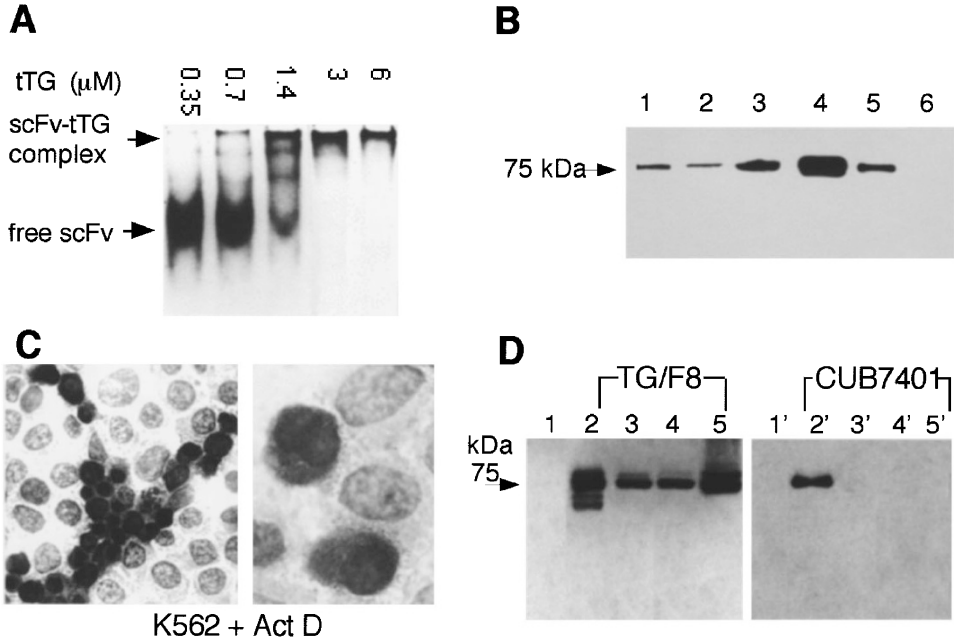

Figure 1 Immunoreactivity of scFv TG/F8. (**A**) ScFv TG/F8 reacts with soluble guinea-pig liver tTG. Fifty μg of scFv TG/F8 were incubated with the indicated concentrations of guinea-pig liver tTG and the immune complexes resolved on a native polyacrylamide gel and transferred on nitrocellulose filter. The presence of free scFv molecules and of scFv-tTG complexes were detected by immunoblot with anti *myc* mAb followed by peroxidase-labeled goat anti mouse IgG1 antibody. On these bases a binding affinity of 1.4 μM was calculated. (**B**) ScFv TG/F8 reacts with guinea-pig liver tTG and detects a 75 kDa protein in human and murine cells by immunoblot. Guinea-pig liver tTG (1 μg; lane 1) and 25 μg protein/lane of crude extracts from human lymphocytes (lane 2), murine thymocytes (lane 3), human thymocytes (lane 4) and murine splenocytes (lane 5) were probed with scFv TG/F8. In lane 6, human thymocyte extract was probed with a control scFv-containing bacterial supernatant as negative control. (**C**) ScFv TG/F8 reacts with human apoptotic cells in immunohistochemistry. K562 human leukemia cells were treated with 5 μg/ml Actinomycin D (Act D) for 24 h, applied to a glass slide by cytocentrifugation and stained with TG/F8 followed by APAAP. Apoptotic cells, identified by their condensed nuclei, show a strongly stained cytoplasm compared to cells with non apoptotic morphology. Original magnification $\times 400$ (left) and $\times 1000$ (right). (**D**) Comparison between TG/F8 scFv and CUB7401 mAb reactivity in Western blot. Guinea-pig liver tTG (1 μg; lanes 2 and 2'), tTG purified from human thymocytes (1 μg; lanes 3 and 3'), human thymocytes extract (25 μg; lanes 4 and 4'), and K562 cells extract (25 μg; lanes 5 and 5') were probed with scFv TG/F8 (lanes 2–5) and with mAb CUB7401 (lanes 2'–5'). As negative control, 1 μg guinea-pig liver tTG was probed with control scFv-containing bacterial supernatant (lane 1) and with control IgG1 mAb (lane 1'), respectively

immunoblot with an anti scFv-tag antibody. The result (Figure 1A) shows that TG/F8 binds soluble tTG with a calculated binding affinity of 1.4 μM. TG/F8 binding affinity to tTG in ELISA was also calculated and found to be higher than 1 nM (not shown), suggesting a better reactivity with the immobilized antigen. Additionally, TG/F8 showed a good reactivity with guinea-pig liver tTG in immunoblotting (Figure 1B, lane 1). Sequencing of the V-genes of TG/F8 identified human V_H segment DP2[31] and V_λ segment DPL-16,[32] with V_H-CDR3 sequence of HDKHNMLM, and V_λ-CDR sequence of NSRDSSGNH.

TG/F8 detects a transglutaminase-related molecule in human cells

As antibodies to highly conserved antigens are often reactive across the different animal species, the reactivity of TG/F8 was analysed on human and murine cell-extracts by immunoblot, and on human cell lines by immunohistochemistry. As shown in Figure 1B, a single band with the same electrophoretic mobility (75 kDa) as guinea-pig liver tTG (Figure 1B, lane 1) was detected by immunoblot both in human (Figure 1B, lanes 2, 4) and murine thymocytes and splenocytes (Figure 1B, lanes 3 and 5). TG/F8 was found reactive on human cells also by immunohistochemistry. It detected a cytoplasmic antigen in cytocentrifuge preparations of K562, HL60, and Jurkat leukemia cells (data not shown). Moreover, as shown in Figure 1C, K562 erythroleukemia cells undergoing apoptosis resulted more intensely stained. A stronger immunopositive staining by anti-tTG antibodies of apoptotic bodies as compared to viable cells was reported by others and correlated with an increased tTG expression during apoptotic cell death.[17,33]

The immunochemical data here reported are suggestive but not proof of human tTG recognition by TG/F8. To further demonstrate the specificity of TG/F8, the molecule recognized by TG/F8 in human thymocytes was purified and characterized. A three-steps purification protocol was developed (see Materials and Methods) in which the chromatographic fractions containing the TG/F8 antigen were identified by immunoblot. As shown in Figure 1D, the purified protein (lane 3) showed the same electrophoretic mobility as the guinea-pig liver tTG (Figure 1D, lane 2) and as the TG/F8 antigen in thymocytes (Figure 1D, lane 4) and K562 (Figure 1D, lane 5) crude extracts. This protein was tested for transglutaminase activity by the standard assay that quantifies the incorporation of [^{3}H]-putrescine into dimethylcasein.[34] A weak enzymatic activity was consistently measured, although it was always 50 fold less than the activity obtained from the same amount of guinea-pig liver tTG (data not shown).

The tTG-related antigen detected in human thymocytes is tissue transglutaminase

The TG/F8 antigen was then analyzed in Western blot with CUB 7401, an anti guinea-pig liver tTG mAb that can detect the human enzyme.[35,36] Figure 1D shows that CUB 7401 mAb, although reactive with tTG from guinea-pig liver (Figure 1D, lane 2′) is unable to recognize the TG/F8 antigen both as purified protein (Figure 1D, lane 3′) and in crude extracts (Figure 1D, lanes 4′ and 5′). This observation together with the low enzymatic activity found in the purified TG/F8 antigen prompted the definitive characterization by mass spectrometric analysis of the TG/F8 reactive protein, purified to homogeneity. The final preparation contained less than 5% contaminating proteins, as assessed by SDS–PAGE comparing the coomassie blue staining pattern with the immunoblot staining pattern (not shown). The set of peptide masses obtained from mass spectrometric measurement of the protein tryptic products (Table 1) was searched against the SWISSPROT database taking advantage of the specificity of the proteolytic enzyme used for the hydrolysis and the taxonomic category of the sample. All searches were performed against a non-redundant sequence database. The above set of 13 masses was compared to the theoretically predicted peptide sets for each protein in the explored database. The number of measured masses that coincided within the given masses accuracy of 0.1% were recorded and the protein that received the highest number

Table 1 Mass spectrometric analysis. SWISSPROT-search of incognite protein. The first protein assigned is tissue transglutaminase

Protein mass range		50–100 kDa	
Cleavage agent		Trypsin	
Peptide mass accuracy		0.1%	
Peptide charge state		Protonated	
Number of peptides required for match		10	
Number of uncleavaged sites		2	
Number of peptides used in search		13	
Human protein-glutamine gamma glutamyltransferase (E.C.2.3.2.13)			
Human Tissue Transglutaminase			
Matched peptides listed accordingly to their molecular mass:			
1234.4	581–590	1635.8	465–478
1267.3	203–213	1788.4[a]	274–289
1313.5	264–273	1835.1[a]	223–240
1342.6	675–687	2014.8	272–289
1513.4	551–562	2501.3	297–317
1588.9	36–48	2700.3	274–296

12 specified mol weights matched. Not found 2255.9; [a]reduced form

of peptide matches (12) was the tissue transglutaminase, thus leading to the positive identification of the protein sample. The next human protein in the rank presented less than eight peptide matches and molecular weights not in agreement with that observed by SDS–PAGE analysis. We can therefore conclude that the TG/F8 antibody fragment detects tTG in human cells.

Tissue transglutaminase in human thymocytes undergoing apoptosis

Previous papers report an increased transglutaminase activity in glucocorticoid-treated rat thymocytes *in vitro*[17] and mouse thymocytes *in vivo*.[37] To investigate tTG in human thymocytes undergoing apoptosis, isolated cells from thymic tissue were incubated with dexamethasone for 6 and 17 h. Transglutaminase activity was then measured and the protein level evaluated by Western blot. Execution of the apoptotic signal was monitored by detection of PARP cleavage at the 6 h time point (not shown), and by analysis of DNA fragmentation at later time points (Figure 2B). Although the apoptotic mechanism was efficiently triggered, the basal level of transglutaminase activity did not change (data not shown). Moreover, as shown in Figure 2A no detectable increase of the tTG protein was observed. Instead, in addition to the expected 75 kDa tTG band, a new 48 kDa signal was detected in the 17 h sample (Figure 2A) when DNA fragmentation was evident (Figure 2B).

To better correlate the presence of the 48 kDa TG/F8 band with the presence of apoptotic bodies, control and dexamethasone-treated thymocytes were layered onto discontinuous Percoll gradients, which separate cells according to their density. All samples were then analyzed for tTG expression by immunoblot and for DNA fragmentation by agarose gel electrophoresis.

Three cell fractions, designated A, B, C from the lowest to the highest density, can be recovered following the protocol described in Tiso *et al*.[38] The high density one, fraction C, is enriched in late stage apoptotic cells and shows the largest presence of fragmented DNA. Figure 2C,D, demonstrate the correlation between presence of the 48 kDa TG/F8 positive fragment and apoptotic characteristics such as DNA fragmentation.

Tissue transglutaminase in human tumor cells undergoing apoptosis

The observation that immunostaining by TG/F8 was stronger in cytospin preparations of apoptotic leukemia cells even when cell death was induced by protein synthesis inhibitors (Figure 1C) suggested that modifications other than increased expression can influence tTG in apoptosis. To investigate the relationship between protein synthesis and appearance of the 48 kDa TG/F8 positive band, different leukemia cells were treated with a variety of apoptotic stimuli and tTG was analyzed by Western blot. As shown in Figure 3A for HL60 myelomonocytic leukemia cells, the 48 kDa TG/F8 band was

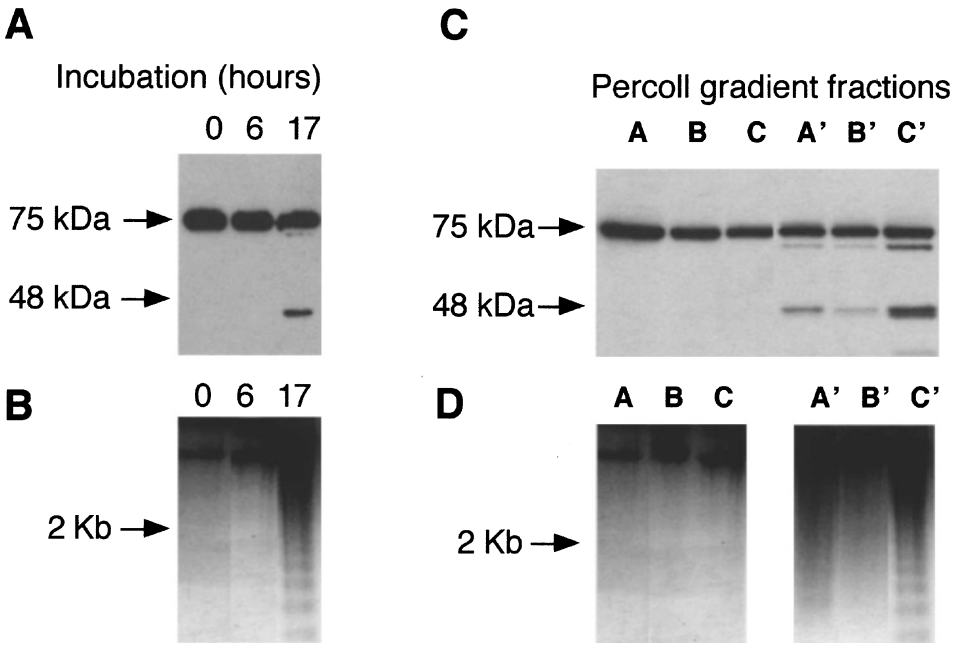

Figure 2 Tissue transglutaminase in human thymocytes undergoing apoptosis. A 48 kDa TG/F8-reactive protein species is detected concomitant with DNA fragmentation. (**A**) Western blot analysis of human thymocytes cultured with 10 μM dexamethasone for the indicated times. Twenty-five μg crude extract/lane were probed with TG/F8 scFv. (**B**) Evaluation of DNA fragmentation in human thymocytes cultured with 10 μM dexamethasone for the indicated times. The DNA is resolved on a 2% agarose gel. (**C**) Western blot analysis of control (A–C) and dexamethasone-treated (A'–C') human thymocytes following separation onto discontinuous Percoll gradients. Twenty-five μg crude extract/lane were proved with TG/F8 scFv. The three cell fractions recovered are designated A, B and C from the lowest to the highest density. Fraction C is enriched in apoptotic bodies. (**D**) Evaluation of DNA fragmentation in the same samples as in **C**

detectable when apoptosis was induced by protein synthesis inhibitors after 8 and 20 h of treatment with actinomycin D or cycloheximide, respectively. This result suggests that the 48 kDa TG/F8 band is the product of a post-synthetic modification (possibly a cleavage). In addition, the appearance of the 48 kDa band was found to be general in apoptosis as it could be detected in other leukemia lines (not shown) and with apoptotic stimuli as different as taxoids, which target microtubules, etoposide, that targets DNA (not shown), and CD95/Fas ligation (Figure 3A). Moreover, the correlation between appearance of the 48 kDa TG/F8 band and progression of DNA fragmentation, quantified by TUNEL assay, showed that the two phenomena follow the same kinetics (Figure 3B), indicating that tTG processing can be used as marker of late stage of apoptosis.

Tissue transglutaminase is cleaved in a caspase 3-dependent mode during apoptosis

The activation of the caspase family of proteases with the subsequent selective proteolysis of a limited number of protein targets is characteristic of apoptosis. We therefore investigated whether the observed processing of tTG was dependent on caspase activity. The availability of cell permeable specific peptide inhibitors allows the modulation of caspase activity in the living cell. The acidic tetrapeptide aldehyde Ac-DEVD-CHO is a potent inhibitor of caspase 3-like activity[39] whereas the peptide Ac-YVAD-CHO is a potent inhibitor of caspase 1-like activity.[40] We focused on caspase 3 because in the hierarchy of caspases it is the prototype of effector caspases, and investigated caspase 1 as a control, as it is involved in inflammation more than in apoptosis.[5,6] Isolated human thymocytes were incubated with dexamethasone in the presence of varying amounts of Ac-DEVD-CHO or Ac-YVAD-CHO. As shown in Figure 4A, the former abrogated the appearance of the 48 kDa TG/F8 band whereas the latter was minimally effective. These data indicate that an efficient caspase 3-like proteolytic activity is responsible for the processing of tTG.

Tissue transglutaminase is cleaved by caspase 3 *in vitro*

As reported for PARP and other caspase substrates,[39,41] the processing of tTG in apoptotic cells was abolished by nanomolar concentrations of Ac-DEVD-CHO and micromolar amounts of Ac-YVAD-CHO (Figure 4A), confirming the involvement of caspase 3 activity. To investigate whether

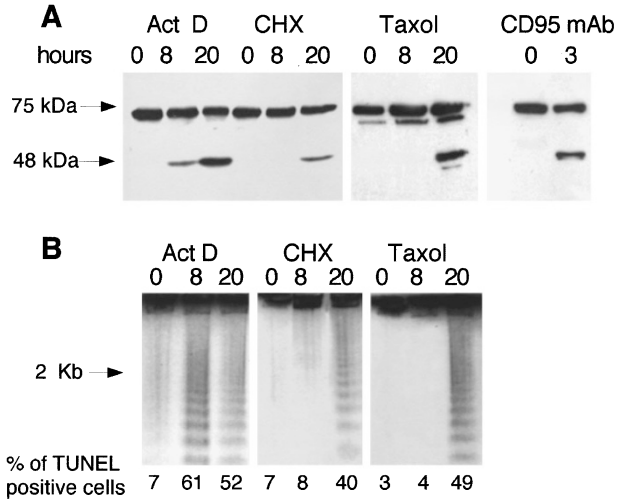

Figure 3 Tissue transglutaminase is processed in human cell lines during apoptosis induced by different stimuli. A 48 kDa TG/F8-reactive protein species is detected concomitant with DNA fragmentation with every stimulus used, including protein synthesis inhibitors. (**A**) Western blot analysis of human HL60 myelomonocytic cells cultured for the indicated times with Actinomycin D (Act D; 5 μg/ml), Cycloheximide (CHX; 50 μg/ml) and Taxol (1 μM). Jurkat T cell leukemia was instead incubated with CD95/Fas mAb (0.5 μg/ml) for 3 h. Twenty-five μg crude extract/lane were probed with TG/F8 scFv. (**B**) Evaluation of DNA fragmentation in HL60 cells cultured for the indicated times with Actinomycin D, Cycloheximide and Taxol. The DNA is resolved on a 2% agarose gel. Numbers indicate the percent of cells with fragmented DNA, as measured by flow cytometry directly after the TUNEL reaction

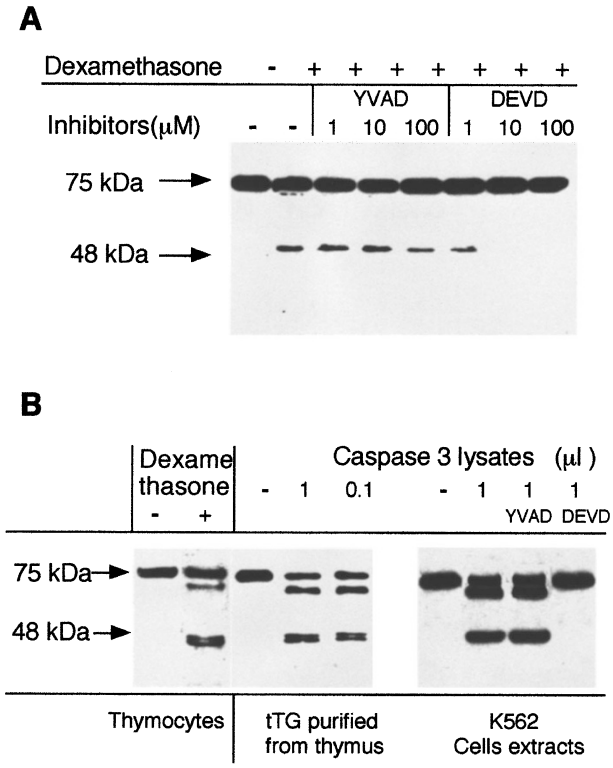

Figure 4 Tissue transglutaminase is a caspase substrate. (**A**) tTG is processed in a caspase 3-dependent mode during apoptosis. Western blot analysis of human thymocytes cultured with 10 μM dexamethasone for 17 h in the presence of the indicated concentrations of the synthetic tetrapeptide inhibitors of caspase 1 (Ac-YVAD-CHO) and of caspase 3 (Ac-DEVD-CHO). Twenty-five μg crude extract/lane were probed with TG/F8 scFv. The appearance of the 48 kDa TG/F8-reactive band is dependent on an efficient caspase 3-like proteolytic activity. (**B**) tTG is cleaved by recombinant caspase 3 in a cell free system, yielding the 48 kDa TG/F8-reactive protein species. Control (−) and caspase 3-containing bacterial lysates were added to tTG purified from thymocytes and to K562 cell extracts for a 1 h digestion. YVAD and DEVD indicate the presence of the corresponding caspase inhibitor during digestion. Samples were then analyzed by Western blot with TG/F8 scFv. For comparison, the tTG processing pattern generated in thymocytes during dexamethasone-induced apoptosis is shown in the left section of panel

tTG was a direct substrate of caspase 3 or a substrate of different proteolytic activities triggered by caspase 3, purified human thymocyte tTG was incubated *in vitro* with control or caspase 3-expressing bacterial lysates. Purified tTG was cleaved by recombinant caspase 3 (Figure 4B), and *in vitro* cleavage fragments were identical to those detected in intact apoptotic cells following treatment with dexamethasone. Addition of caspase 3 to non apoptotic K562 cell extracts also generated the same pattern of cleavage. Addition of the specific tetrapeptide inhibitor Ac-DEVD-CHO prevented tTG cleavage *in vitro* as well.

Occasionally, both tTG and its cleavage product appeared as a doublet. These forms were most likely produced by oxidative degradation during sample preparation, since they increased with repeated freezing and handling of the samples, specially of the purified enzymes, as described for purified erythrocyte tTG.[42]

These results demonstrate that tTG is cleaved during apoptosis in human lymphoid cells and that tTG cleavage can be a valuable marker of caspase 3 activation during the execution phase of apoptosis.

Cleavage of tissue transglutaminase causes loss of cross-linking activity

To assess whether the caspase 3-dependent cleavage of tTG caused loss of enzymatic function, postnuclear extracts from intact K562 cells and guinea-pig liver tTG were both challenged *in vitro* with control and recombinant caspase 3-containing bacterial lysates, and subsequently evaluated for the presence of protein cross-linking activity. Addition of caspase 3 lowered the activity of tTG in K562 extracts from 0.08 to 0.045 u/μg, and the activity of guinea-pig liver tTG from 1.5 to 1.0 u/μg (mean values from three experiments; S.E. never exceeding 10%). Similarly, when K562 cells undergoing apoptosis were examined, extracts from cells in the late stage of taxol-induced apoptosis showed a reduced tTG activity (0.04 u/μg) compared to untreated samples (0.08 u/μg) and to treated samples harvested when DNA fragmentation was still undetectable (0.085 u/μg). In addition, the presence in culture of Ac-DEVD-CHO peptide partly counteracted the decrease of tTG activity during late apoptosis. Taken together these results indicate that tTG cleavage by caspase 3 causes loss of transamidating function.

Consistent with these data is the observation that the 48 kDa TG/F8 fragment, purified from dexamethasone-treated thymocytes with the same protocol used for the whole enzyme (see Materials and Methods), shows no enzymatic activity (data not shown).

Discussion

The transamidating activity of tTG has been described as playing a role at a downstream stage in the apoptotic pathway, where it is responsible for the extensive protein polymerization that stabilizes the apoptotic body. Tissue transglutaminase in its G-protein configuration has been hypothesized to play a role also upstream in the apoptotic signal transduction pathway (reviewed in[19]). In the present study, with the help of the new monoclonal antibody fragment TG/F8, we provide evidence that tTG is cleaved during apoptosis in a caspase 3-dependent mode, and that such cleavage occurs in the late phase of cell death, concomitant to DNA fragmentation, causing loss of cross-linking function.

The anti-tTG TG/F8 scFv has been generated using phage display technology, which enabled us to overcome immune tolerance to highly conserved intracellular proteins by-passing *in vivo* immunization. Moreover, phage display libraries potentially lead to a different spectrum of epitopes from natural immune systems, generating also reagents against the non immunodominant regions of a given antigen.[29] When this is the case, such reagents may become invaluable tools for mapping the functions of proteins. In Western blot analysis of human and murine tissues, scFv TG/F8, selected for direct binding to immobilized guinea-pig liver tTG, detects a protein species electrophoretically indistinguishable from the guinea-pig's (Figure 1B). In immunohistochemistry, it detects a cytoplasmic protein that appears to increase during apoptosis in human cell lines (Figure 1C), as reported for tTG in human and animal cells.[17,33,43] In addition, the purified thymocyte TG/F8 antigen is unequivocally identified as tTG by mass spectrometric analysis (Table 1). We can therefore conclude that TG/F8 detects tTG in human cells.

The observation that the TG/F8 antigen purified from thymocytes shows a very low cross-linking activity is no contradiction to its identification with tTG, as it could be the result of a regulatory element active in thymocytes. The majority of the tTG *in vivo* is indeed predicted to remain latent[21,44] and modulation of its activity by nucleotides was reported.[45] Several *in vitro* studies describe in detail the inhibitory effect of GTP binding on tTG activity[23,46] and its interplay with other regulatory elements.[47] In addition, GTP binding causes conformational changes in tTG that affect both enzymatic activity[23,48,49] and interactions with other proteins including proteolytic enzymes.[23,28,50] Conformational changes able to modulate tTG activity are also described following binding of sphingosylphosphocholine.[47] In any situation involving conformational changes, cryptic epitopes may be exposed which make tTG a better target for select monoclonal antibodies while other epitopes may be hidden. This seems the likely explanation of the lack of reactivity with the purified thymic TG/F8-tTG by the CUB 7401 anti tTG mAb, as well as of other observations made possible by the TG/F8 scFv.

The observation that immunostaining by TG/F8 was stronger in cytocentrifuge preparations of apoptotic leukemia cells even when cell death was induced by protein synthesis inhibitors (Figure 1C) suggested that other modifications than increased expression could involve tTG in apoptosis. Western blot analysis of human thymocytes and leukemia cell lines undergoing apoptosis triggered by several stimuli acting through different mechanisms showed that tTG is cleaved in a caspase-dependent mode during apoptotic cell death. A new 48 kDa band was detected in apoptotic samples whenever DNA fragmentation became evident (Figures 2 and 3), and the presence of the acidic tetrapeptide aldehyde Ac-DEVD-CHO could efficiently prevent the phenomenon (Figure 4).

The reversible caspase inhibitor Ac-DEVD-CHO is a potent inhibitor of caspase 3 ($K_i < 1$ nM) whereas Ac-YVAD-CHO, which is caspase 1-selective, is a poor inhibitor of caspase 3 ($K_i < 12$ μM).[39] The observation that Ac-DEVD-CHO prevented cleavage of tTG at concentrations 100 times lower than Ac-YVAD-CHO (Figure 4A) indicates that a caspase 3-like activity is involved in processing tTG during apoptosis. Moreover, the finding that tTG cleavage is a common feature of cell death induced by stimuli as diverse as taxol, protein synthesis inhibitors, and CD95/Fas ligation is consistent with tTG being substrate of caspase 3. Indeed, in a possible model of hierarchy of caspases, caspase 3 together with other short prodomain-containing caspases is identified as a 'machinery' (downstream) protease, common to different death pathways.[6,51,52] Timing of tTG cleavage relative to PARP cleavage, used as marker of caspase 3 activation *in vivo*, showed that tTG cleavage occurs beyond the 5-h exposure to apoptotic drugs required to detect PARP cleavage (not shown). In particular, tTG cleavage was detectable simultaneously with DNA fragmentation in every apoptotic stimulus tested (Figures 2 and 3). These data demonstrate that tTG processing coincides with the presence of caspase 3 in its active form and is a late event in the execution phase of apoptosis.

tTG was tested as a substrate of recombinant caspase 3 *in vitro* because DEVD-based compounds cannot be assumed to inhibit only caspase 3, particularly in intact cells where intracellular inhibitor concentrations are not measured and where prolonged incubation times are required.[53] Purified human thymic tTG was cleaved by recombinant caspase 3 (Figure 4), and *in vitro* cleavage fragments were identical to those detected in intact apoptotic cells. The addition of caspase 3 to non apoptotic cell extracts also generated the same pattern of cleavage (Figure 4B). Sequence analysis of human tTG shows that there are two DxxD consensus sites for caspase 3; cleavage at one of these sites (aa 400–403) would be expected to product a protein fragment of 45 kDa. Although it cannot be excluded that *in vivo* tTG may also be a substrate of other closely related proteases activated by caspase 3. Taken together these results support a specific role for caspase 3 in tTG processing during apoptosis and propose tTG cleavage as a biochemical marker of caspase activation.

An ever-growing number of proteins have been found to undergo proteolysis at caspase consensus sites during apoptosis. However, it is unlikely that cleavage of any single protein is a necessary requirement for apoptosis, as demonstrated by the cleavage of PARP. In fact, the physiological significance of this event is still under investigation, since PARP-null mice develop normally.[54] It is more likely that the collective effect of cleavage of a limited number of key proteins is necessary to assure the ordered process of apoptosis while many cleavage events could be purely coincidental and have no active role in the process of cell death. The biological significance of tTG cleavage by caspases is not self-explanatory because a definitive role for tTG in apoptosis has not yet been firmly established. If a role as an upstream effector in prevention of cell death has to be credited to tTG in its G-protein configuration,[19] then its cleavage during apoptosis could play an active role in the process of cell death as an amplifier of execution signals. If a downstream role as stabilizer of the apoptotic body by protein polymerization is the main function of tTG, then its cleavage with subsequent loss of function in the late phases of apoptosis may have no role in the mechanism of cell death but function instead in preventing the generation of potentially harmful products. Spilling of active enzyme in the surrounding tissue could even favor the generation of immunogenic peptides in analogy to what described in celiac disease. In this case, the extracellularly released tTG is reported to interact with dietary gliadin, creating antigenic neoepitopes able to initiate an immune response in genetically susceptible individuals.[55–57] These reports describe the enzymatic deamidation of a foreign antigen, but it is tempting to speculate that self proteins could also be substrates for similar types of modifications, leading to pathological immune activation. The biological significance of tTG cleavage once the apoptotic body is stabilized could therefore be in preventing the harmful consequences of excess enzymatic activity in the surrounding tissue.

Materials and Methods

Antibodies

Antibody to scFv tag peptide was anti-myc, murine IgG1 clone 9E10, obtained from ATCC (Rockville, MD, USA) and used at the final concentration of 5 μg/ml. Anti-tTG mAb CUB 7401 (murine IgG1) was kindly provided as culture supernatant by Dr. PJ Birckbichler (Oklahoma Medical Research Foundation, Oklahoma City, USA). Peroxidase-labeled goat anti-mouse IgG1 and anti-mouse IgG (H+L) were obtained from Southern Biotechnology (Birmingham, AL, USA). Rabbit anti-mouse Ig and APAAP Mouse Monoclonal were purchased from Dako (Glostrup, Denmark).

Selection of scFv TG/F8

A human scFv phage library, kindly provided by Dr G Winter (MRC Centre for Protein Engineering, Cambridge, UK) was used to select recombinant antibodies by direct binding to antigen (e.g. guinea-pig liver tTG; Sigma, St. Louis, MO, USA) as described in Nissim *et al*.[29] For large scale scFv production, a single TF/F8 bacterial colony was grown at 37°C in 2 × TY medium (Difco Laboratories, Detroit, MI, USA) containing 100 μg/l ampicillin (Merck) and 0.1% glucose until the cell suspension reached OD_{600}=0.8. IPTG (Calbiochem, La Jolla, CA, USA) was then added to a final concentration of 1 mM and growth continued for 16–24 h at 30°C, shaking. After centrifugation (4000 *g*, 30 min), the supernatant was supplemented with protease inhibitors PMSF (1 mM; Sigma) and Leupeptin (10 μM; Calbiochem), filtered, and stored frozen in aliquots.

Sequencing of the TG/F8 V-genes was performed on a 377 ABI PRISM™ automated DNA sequencer (Applied Biosystem, Perkin Elmer Co., Foster City, CA, USA) using primers LMB3 and pHENseq,[58] and the ABI PRISM™ Big-Dyes (Applied Biosystem) terminator cycle-sequencing kit.

Electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis of proteins under native or unfolded configuration were performed according to Ornstein[59] and to Laemmli,[60] respectively. Native-PAGE was used to analyze TG/F8 scFv binding affinity to soluble guinea-pig liver tTG. Briefly, 2.5, 5, 10, 20 and 40 μg of guinea-pig liver tTG (respectively 0.35, 0.7, 1.4, 3 and 6 μM) were incubated with 50 μg of TG/F8 in 100 μl of 20 mM HEPES pH 7.0, 100 mM dithiothreitol, 0.02% Tween-20 for 30 min at room temperature. Samples were first loaded on a native polyacrylamide gel for the electrophoretic run and then transferred on a nitrocellulose filter for Western blot analysis.

Cell extracts were prepared by lysing with 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% v/v Triton-x-100, 150 mM NaCl, 1 mM PMSF and 10 μM leupeptin. After removal of nuclei by centrifugation at 400 $\times$ *g*, protein concentration was estimated by BCA Protein Assay Reagent (Pierce, Rockford, IL, USA), and 25 μg were loaded per lane. Proteins were fractionated by SDS-electrophoresis on 10% polyacrylamide gels under non reducing conditions and then transferred electrophoretically to nitrocellulose (Hybond-C Extra, Amersham, UK), unless coomassie brilliant blue- or silver nitrate-staining was required. The transferred antigens were detected by incubating the membrane, previously blocked overnight at 4°C with 4% milk in PBS, with TF/F8- or control scFv-containing bacterial supernatant supplemented with 5 μg/ml anti-myc mAb, for 1 h at room temperature. CUB 7401 mAb was used instead as culture supernatant. TBST buffer (10 mM Tris HCL, pH 8.0, 150 mM NaCl, 0.005% Tween-20) was used for washes and as a diluent of the peroxidase-labeled secondary antibody. Peroxidase was revealed by enhanced chemiluminescence (ECL, Amersham) detected by autoradiography (Hyperfilm, Amersham).

Analysis of DNA fragmentation

DNA fragmentation was visualized as described in Gangemi *et al*.[61] Briefly, pelleted cells were lysed in 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% (w/v) SDS, and 0.5 mg/ml Proteinase K (Boehringer, Mannheim, Germany) for 1 h at 50°C. RNase A (Sigma) was then added to a concentration of 0.25 mg/ml for 1 h at 50°C. Samples were then loaded onto a 2% agarose gel in TBE buffer and DNA visualized after soaking the gel in 1 μg/ml ethidium bromide. The amount of cells with fragmented DNA was measured by flow cytometry directly after the TUNEL reaction performed with the APO-DIRECT kit (Phoenix Flow Systems, Inc., San Diego, CA, USA).

Immunohistochemistry

Fifty thousand cells per sample were seeded onto poly-L-lysine-coated glass slides (Sigma) by cytocentrifugation at 200 $\times$ *g*. Slides were then air dried and stored at -20°C. Before immunostaining, the samples were dipped for 10 min in cold acetone and thoroughly washed with washing buffer (TBS buffer: 10 mM Tris HCl pH 8.0, 150 mM NaCl). TG/F8- and control scFv-containing bacterial supernatant with the addition of 10 μg/ml anti-*myc* tag antibody were then applied for 1 h at room temperature, followed by rabbit anti-mouse antibody (Dako). Samples were then processed for APAAP reaction as described in Favre *et al*.[62]

Thymocyte tissue transglutaminase purification and functional analysis

Tissue transglutaminase was purified from human thymocytes with a three step protocol consisting of gel filtration, ion exchange chromatography, and again gel filtration, as follows. 10^{10} thymocytes were thoroughly washed in cold PBS and resuspended in 10 ml of cold buffer A (hypotonic phosphate buffer 0.025 M pH 6.5, 1 mM EDTA, and 5 mM mercapto-ethanol). Cells were then lysed by freezing and thawing and homogenized with a tissue grinder. All the following steps were performed at 4°C in a cold room. The homogenate was centrifuged at 17.000 r.p.m. for 30 min (BioFuge 17RS Haereus Instruments, Hanau, Germany) and the supernatant loaded on a Sephacryl S200 column (Pharmacia) measuring 1 m length and 1.5 cm diameter, previously equilibrated with buffer A. Proteins were eluted with 200 ml of the same buffer and each fraction was tested for its reactivity with TG/F8 scFv by dot blot. TG/F8-positive fractions were pooled and loaded on a DEAE Sepharose column (Pharmacia) (2 $\times$ 25 cm) previously equilibrated with buffer A. Elution buffer was buffer A with NaCl 0.4 M. All the fractions were tested for reactivity with TG/F8 scFv by dot blot and the positive ones were pooled and concentrated by an Amicon System (Amicon Co., Danvers, MA, USA). The concentrated fractions were then applied to a Superdex 200 column (Pharmacia) previously equilibrated in PBS and again the TG/F8 positive fractions were identified by immunoreactivity, pooled and stored at -20°C.

Tissue transglutaminase enzymatic activity was assayed by measuring incorporation of [1.4(n)-^{3}H]-putrescine dihydrocloride (14.4 Ci/mM; Amersham) into N,N′-dimethylcasein (Sigma) as described by Curtis and Lorand.[34]

Mass spectrometry

Trypsin, dithiothreitol, iodoacetamide, glycerol and α-cyano-4-hydroxycinnamic acid were purchased from Sigma. All other reagents and solvents were of the highest purity available from Carlo Erba (Milan, Italy). Mass spectrometric analysis was performed on the Coomassie blue-stained protein excised from a preparative SDS electrophoresis on a 10% polyacrylamide gel as previously described.[63] Enzymatic digestion was carried out with trypsin (15 mg/ml) in 50 mM ammonium bicarbonate pH 8.5 for 4 h at 4°C, followed by additional 18 h at 37°C with a new aliquot of the enzyme/buffer solution. Peptides were then extracted with 20 mM ammonium bicarbonate and 0.1% trifluoroacetic acid in 50% acetonitrile and lyophilized. MALDI mass spectra were recorded using a PerSeptive Biosystem Voyager DE Instrument. A mixture of analyte solution and α-cyano-hydroxycinnamic acid (10 mg/ml in acetonitrile/ethyl alcohol/0.1% trifluoroacetic acid (1 : 1 : 1, v/v/v) was applied to the metallic sample plate and dried under vacuum. Mass calibration was performed with insulin at 5734.5 Da and a matrix peak at 379.3 Da as internal standards. Raw data were analyzed by using computer software provided by the manufacturers and reported as average masses.

Cells and cell treatments

Human tumor cell lines HL60, K562, Jurkat were obtained from the ATCC and cultured at 5×10^5/ml in RPMI 1640 with 10% heat-inactivated FCS and antibiotics. Thymocytes, isolated by teasing thymic tissue obtained from children undergoing corrective cardiac surgery, were purified by density centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Apoptotic stimuli were 50 mg/ml Cycloheximide (Sigma), 5 μg/ml Actinomycin D (Sigma), 1 μM Taxol (Paclitaxel; Calbiochem), 10 μM Dexamethasone (Sigma), and 0.5 μg/ml CD95 mAb (clone CH-11; Immunotech, Marseille, France) applied for the indicated times. When required, varying amounts (from 100 to 1 μM) of the caspase inhibitors synthetic tetrapeptides Ac-DEVD-CHO and Ac-YVAD-CHO (Bachem AG, Bubendorf, Switzerland) were added to the cultures 30 min before addition of the apoptotic stimulus.

To isolate apoptotic cells, 10^8 thymocytes treated overnight with dexamethasone were layered onto discontinuous Percoll gradients, corresponding to densities of 1.094, 1.077, and 1063 g/cm^3, as described in Tiso *et al*.[38]

In vitro cleavage of purified human tTG

Purified tTG from human thymocytes was dialysed against 50 mM ammonium bicarbonate pH 8.0 and lyophilized. Protein was then solubilized in 10 mM HEPES-KOH pH 7.0, 5 mM $MgCl_2$, 5 mM EGTA, 10% v/v glycerol, and 1 mM dithiothreitol (caspase-buffer) at approximately 200 μg/ml. To test caspase 3 activity on human tTG, 400 ng purified tTG per sample were challenged with a 1 : 100 final dilution of control- and caspase 3-containing bacterial lysates, prepared as described in Brancolini *et al*.[64] When required, tetrapeptides Ac-DEVD-CHO and Ac-YVAD-CHO were added at varying concentrations (from 100 to 1 μM final). Reaction volume was 10 μl in caspase-buffer. Digestion was carried out for 1 h at 37°C and blocked by addition of 2 × SDS – PAGE sample buffer. Cleavage of tTG was assessed by immunoblot.

To assay for the cleavage of endogenous tTG in non apoptotic K562 cell lysate, cells were lysed with 10 mM HEPES-KOH pH 7.4, 2 mM EDTA, 5 mM dithiothreitol, 1% NP-40, 1 mM PMSF and 100 μg aliquots of cell lysate were challenged with control or caspase 3-containing bacterial lysate as described for the purified material.

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