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INVOLVEMENT OF CASPASE-3 IN THE CLEAVAGE OF TERMINAL TRANSFERASE

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To investigate the *in vivo* role of caspase-3 in Terminal Transferase metabolism DMSO-treated RPMI-8402, a human pre-T cell line was used. In DMSO treated samples ^{3}H-dGTP incorporation and TdT phosphorylation occurs after 4 hours of treatment. After 8 hours cells undergo TdT proteolysis in addition to its inactivation. The cleavage of TdT into 32- and 58-KDa proteolytic fragments occurred simultaneously with the activation of Caspase-3, but preceded changes associated with the apoptotic process described after 48 hours of treatment. The Caspase-3 peptide inhibitor V, used as a specific inhibitor, prevented TdT proteolysis prolonging its activity and rescued cells from apoptosis. Our experiments suggest that TdT is a nuclear substrate for Caspase-3, the main apoptotic effector protease in many cell types, and that the cleavage of TdT represents a primary step in a signal cascade leading to pre-T cell apoptosis.

Intrathymic T-cell development proceeds through a series of differentiating events before competent mature T cells emerge in the periphery (1). These events can be monitored by the expression of cell surface markers and the configuration of the T cell receptor (TCR) (2,3). TCR are generated in differentiating T-cells by a series of somatic site-specific DNA recombination reactions, collectively referred to as V(D)J recombination. The complexity of the TCR repertoire is dependent on N region diversity and the enzyme implicated in N-nucleotide addition is Terminal deoxynucleotidyl Transferase (TdT) (4,5). This enzyme catalyzes the polymerization of deoxyribonucleotides to the 3'-hydroxyl end of an oligonucleotide primer in the absence of a DNA template (6). TdT is normally detected in cortical thymocytes and in bone marrow cells for a very short period during lymphocyte development in adult higher vertebrates. TdT is also highly expressed in certain malignant leukaemia cells (7). Based on its *in vitro* function and tissue specificity, TdT has been thought to be essential for B- and T-cell development and differentiation. Recently, the function of TdT was clarified by the generation of TdT-deficient mice using gene targeting. Thymocytes lacking this enzyme are unable to form a complete and functional TCR (8,9). In fact, during a genetic rearrangement of immunoglobulin (Ig) and TCR genes, extranucleotides are inserted at V-J, V-D and D-J junctions, enhancing Ig and TCR diversity in N region. B- and T-cells derived from TdT deficient mice lacked the N region. These data confirm that TdT is a DNA polymerase that synthesizes the N region. During the early stages of T cell development, genes encoding for the TCR are assembled somatically via a series of ordered gene rearrangements triggered by the

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specialized stromal microenvironment present in the thymus. Thymocytes failing to rearrange their TCR genes, or expressing TCR α/ß combinations with inappropriate specificities, are generally arrested at discrete developmental control point and eventually eliminated by apoptosis (10,11).

Apoptosis or programmed cell death (PCD) represents a physiological mechanism for tissue remodelling and cell renewal, and it plays an important role in inflammatory and autoimmune events (12). Although the mechanism of cell death is still not completely known, progress has been made in identifying key elements in this process. In this respect, the cloning of *ced-3*, a gene encoding a protease with homology to the mammalian interleukin-1β-converting enzyme (ICE), provided the first indication that cysteine proteases are critical components of the cell death machinery (13-15). This observation led to the identification of a growing family of cysteine proteases with homology to CED-3, called now caspases. To date, more than 14 caspases have been cloned and partially characterized in mammals. The role of individual caspases in apoptosis remains to be clarified because multiple caspases are often co-expressed in the same tissue. Although the evidence available suggests that caspases play a major role in apoptosis, they are not the only effectors of cell death (16). Here we demonstrate the role of the caspase-dependent signal transduction pathway mediating pre-T thymocyte death. In fact, TdT is specifically cleaved by Caspase-3 into two major fragments of 32- and 58-KDa and this precedes biochemical and morphological changes occurring in the last phase of programmed cell death, such as DNA cleavage, chromatin condensation and apoptotic body formation.

MATERIALS AND METHODS

Cell Cultures

The human TdT positive pre-T (RPMI-8402) cell line (17) was maintained in continuous suspension culture in RPMI-1640 medium supplemented with 10% FBS, 4 mM L-Glutamine, 100 mM Na-Pyruvate, and 25 mM Hepes. Cells were grown at 2.5 x 10^5/ml, with more than 98% viability as determined by trypan blue dye exclusion test. During log phase growth, cells were treated with 1.5% (v/v) DMSO (gas chromatography grade) for up to 72 hrs. Inhibition of caspase-3 activity was obtained by bringing the medium to 40 μM caspase-3 inhibitor V [Z-DQMD-FMK; Z-Asp (Ome)-Gln-Met-Asp (Ome)-CH_2F], a potent cell permeable and specific irreversible inhibitor of caspase-3 (18). Inhibitor for Caspase 8, 6 and Granzyme B [Ac-IETD-CHO; Ac-Ile-Glu-Thr-Asp-H (aldehyde)] and inhibitor for Caspase 1 and 4 [Ac-YVAD-CMK; Ac-Tyr-Val-Ala-Asp-CMK] were used to evaluate the specificity of Caspase-3 effects [19]. Caspase inhibitors were supplemented with or without DMSO.

TdT Enzymatic Activity Determination

TdT enzymatic activity was determined as previously described (20,21). Briefly, cells ($2x10^7$), washed with RPMI 1640 medium, were lysed in 250 μl of 0.3 M KPi, pH 7.4, by freeze thawing or sonication. The lysate was cleared by centrifugation at 15,000 rpm for 30 min. at 4°C and aliquots assayed using 0.5 mM d (pA) 50, as initiator, 5 mM ^{3}H-dGTP (50-150 cpm/pmol) as substrate, 8.0 mM $MgCl_2$, 1.0 mM ß-mercaptoethanol, 0.1 mg/ml BSA, 0.2 M K-cacodylate buffer pH 7.5. Products of reaction were scored as acid-insoluble radioactivity on glass fibre filters.

SDS-PAGE and Immunoblot Analyses

Proteins from untreated and treated samples were subjected to a SDS-4% polyacrylamide stacking gel and SDS-10% polyacrylamide separation gel, and then electrophoretically transferred to a 0.2 μm nitrocellulose membrane. The transfer buffer contained 25 mM Tris-HCl, 192 mM glycine, 0.037% (w/v) SDS and 20% (v/v) methanol. The membranes were blocked with TBS (20 mM Tris-HCl, 150 mM NaCl, (pH 7.5) containing 5% (w/v) skim milk and 0.1% Tween 20 for 1 hour. The membrane was then incubated overnight at 4°C with the appropriate dilutions of rabbit polyclonal anti-TdT or anti-Caspase-3 and anti-human CD3 monoclonal antibody as primary antibodies. This was followed with horseradish peroxidase-conjugated anti-rabbit IgG as secondary antibody and then the membranes were developed using the ECL™ system. A prestained SDS-PAGE standard protein marker (Sigma) was used to calibrate molecular mass.

TdT "in vivo" phosphorylation

$3x10^7$ of untreated, DMSO and DMSO plus caspase-3 inhibitor-treated RPMI-8402 cells were incubated in 3 ml of DMEM phosphate free medium containing 10% FCS and 0.5 mCi/ml/$1x10^6$ of $^{32}PO_4$ for 8 hours at 37°C. Washed cells were homogenized in 1 ml of Pi 0.2 M (pH 7.4). Immunoprecipitation was performed by adding to 2 ml of sample, recovered as previously described (20), 10 µl of conjugated Protein-A sepharose gel monoclonal anti-human TdT, at a concentration of 2mg/ml, and then incubated overnight in the cold. The immunoprecipitated proteins where washed after centrifugation. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules. CA), then equals amounts of protein was electrophoresed by standard procedures and then autoradiographed on Kodak X-OMAT film.

Source of materials

Human RPMI-8402 cell line, described by Huang et al. (17) is a thymic lymphoma showing immunological features of pre-T cells. The cells are TdT positive as previously analysed and can be induced to differentiate and to apoptotic death by DMSO (21,22). DMSO and all cell culture materials were from Sigma (St. Louis, MO. USA). Protein-A sepharose-conjugated monoclonal antibody against TdT protein and the rabbit polyclonal antibody against 32-kDa calf thymus TdT protein were from Supertechs (Rockville, MD. USA). Anti-Caspase-3 polyclonal antibody (sc-7148), reacting with the p11 and p20 subunits and the precursor of Caspase-3 and the anti-human CD3 monoclonal antibody (sc-1179) were from Santa Cruz Biotechnology (Santa Cruz, CA. USA). Horseradish peroxidase-conjugated secondary antibodies, nitrocellulose membrane, immunochemical and all reagent grade materials were from Sigma. γ-$^{32}PO_4$ orthophosphate in aqueous solution (HCl-free, 10 mCi/ml) was from Amersham Pharmacia Biotech (Milan. Italy). Caspase-3 inhibitor V and Caspase-1 Inhibitor II (Inhibitor for Caspase 1 and 4) were from Calbiochem (Inalco. Milan. Italy). Inhibitor for Caspase 8, 6 and Granzyme B was from Peptide Institute, INC. (Osaka, Japan). Products for electrophoresis were from Bio-Rad Laboratories (Munich, Germany).

RESULTS

T-cell receptors (TCRs) are generated by a stochastic gene rearrangement process during thymocyte ontogeny, producing thymocytes bearing useful, as well as unwanted specificities, which are subsequently eliminated via a thymocyte-specific apoptotic mechanism (2,3). The starting point of this study is that DMSO induces, in the first 48 hours of treatment of RPMI-8402 human pre-T cells changes of surface's immunophenotype from $CD10^+$, $CD2^+$, $CD7^+$, $CD3^-$, $CD4^-$, $CD8^-$ to $CD10^+$, $CD2^-$, $CD7^-$, $CD3^-$, $CD4^-$, $CD8^-$, associated with cell growth arrest. Moreover, DMSO induces, between the first 24 hours, an increase of TCR synthesis, followed by a down-regulation of TdT from more than 90% positive untreated cells down to less that 30% after 72 hours. All described events precede apoptotic cell death that is recognizable after 48 hours (21,22). CD3 antigens, even if not expressed on the cell surface, is detectable at cytoplasmic level without substantial changes of its amount due to DMSO treatment (23), and for this motif used as control of protein synthesis. Here we describe the role of caspase and caspase inhibition in TdT regulation and cell death progression. First of all our study proves that Caspase-3 is specifically involved in the control of TdT, in fact, using other inhibitors such as Caspase-1 Inhibitor II (Inhibitor for Caspase 1 and 4) and inhibitor for Caspase 8, 6 and Granzyme B the activity and the presence of TdT was not down regulated (data not shown). Evaluating the course of ^3H-dGTP incorporation (Figure 1), unstimulated cells seem unnoticeably influenced by the medium condition. DMSO alone induced evident changes of TdT activity. In fact, after 4 hours of DMSO treatment a peak of nucleotide incorporation was present, demonstrating higher TdT activity, which turn down, under to basal levels, after 12 hours. At 24 hours TdT appears mostly inactivate. These data are in accordance with previous results (22). DMSO/caspase-3 inhibitor-treated cells display an increase in TdT activation in the first 4 hours, as observed with DMSO-treated cells, but remain consistent at 12 hours and sustained up to 24 hours of treatment. Since TdT phosphorylation is an early step in the

process of lymphoid differentiation (6,20) and there is considerable evidence for interaction between protein phosphorylation and proteolysis in death regulation (24-27) the study of *in vivo* TdT phosphorylation and proteolysis was carried out. Treated and untreated samples were labelled *in vivo* with $^{32}PO_4$. After 8 hours of treatment phosphorylated TdT was recovered from the lysate by immunoprecipitation, using a specific protein-A conjugated monoclonal antibody. SDS-polyacrylamide gel electrophoresis followed by autoradiography (Figure 2) revealed, in DMSO treated cells (lane B) the presence of two strongly phosphorylated bands corresponding to 32-kDa and 58-kDa TdT peptides. These results are consistent with data on the protein structure of TdT [28]. DMSO-treated samples (lane C), cultured in the presence of 40 μM caspase-3 inhibitor V, display the presence of one single band corresponding to intact phosphorylated 58-kDa TdT peptide. The untreated sample (lane A) did not show a sizeable level of phosphorylated peptides or cleaved forms of TdT. Figure 3 reports the western blot analysis of TdT during DMSO treatment with or without Caspase-3 inhibitor. Bands corresponding to 0-72 hours of treatment without inhibitor, show a decrease of the intact 58-kDa TdT form and the corresponding increase of the 32-kDa cleaved molecule detectable from 8 hours and more evident at 24-72 hours of DMSO treatment. In DMSO/caspase inhibitor-treated cells (Figure 3, bands 12, 24 and 48) and in untreated cells (Figure 3, band 0) it is possible to note the presence of the 58-kDa intact form of TdT, suggesting that the presence of the Caspase-inhibitor prevents the cleavage of TdT. In order to confirm this result we analysed by western blotting the Caspase-3 protein values during DMSO treatment. Figure 4 reports the immunoblotting of samples recovered at early times of treatment, showing the progressive increase of caspase that reaches a maximum between 4-8 hours. These data are confirmed in detail by another experiment analysing the complete range of treatment periods. In fact, densitometric analysis of the immunoblots of the proteins recovered from 0 to 72 hours of treatment (Figure 5) shows the time-dependent increase of caspase-3, reaching the highest level

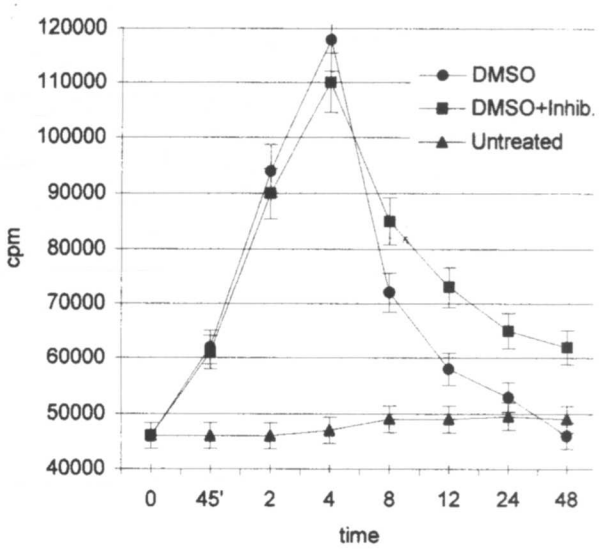


Fig. 1. *Kinetic analysis of ^{3}H-dGTP incorporation. DMSO induces an increase of TdT activity that reaches the greatest level at 4 hours of treatment. At 8 hours and then at 12-48 hours of DMSO treatment a great decrease of TdT activity can be recognized. Caspase-3 inhibitor V, supplemented as reported in materials and methods, affects TdT activity that appears more prolonged than DMSO-treated cells. In fact, at 12 hours of treatment is still detectable a great TdT-activity. Untreated cells did not show endogenous activation that remains almost at the same basal levels. The results reported represent the mean of four different experiments. The cpm indicates the mean of triplicate wells for each experiment (± 5%).*

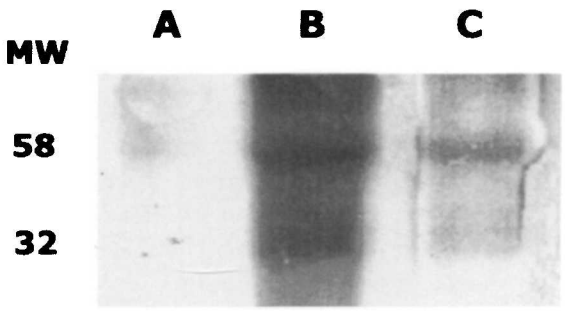


Fig. 2. *In vivo phosphorylation assay. TdT was recovered by immunoprecipitation after 8 hours of DMSO treatment. Treated sample (Lane B) shows two over-phosphorylated bands corresponding to 58- and 32-kDa TdT peptides that are unnoticeable in untreated cells (lane A). In the presence of caspase-3 inhibitor, treated cells (lane C) mostly display the intact phosphorylated 58-kDa form of TdT.*

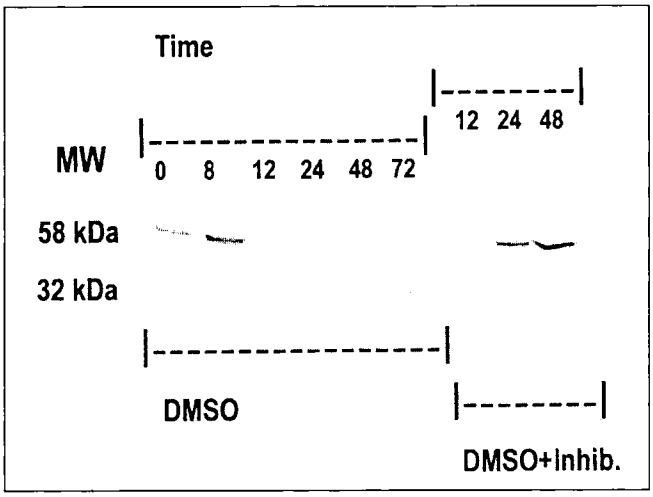
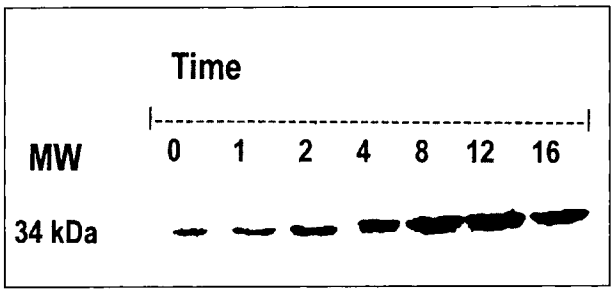


Fig. 3. *Immunoblot analysis showing the different amount of the 58- and 32-kDa TdT peptides in DMSO, DMSO/caspase inhibitor and in untreated cells. In DMSO-treated cells it is possible to note the increase of the low molecular form of TdT starting after 8 hours. DMSO-cells, cultured in the presence of Caspase-3 inhibitor V, show the exclusive presence of the intact form of TdT, demonstrating that the proteolysis of TdT is due to caspase activity. Results reported correspond to one of three experiments that generated the same results. MW; Molecular weight.*

Fig. 4. *Caspase-3 expression measured at the first part of the treatment by Western blot analysis. DMSO treatment induces an immediate increase of Caspase-3 level up to a maximum at 8 hours of treatment. Data represent one out of four independent experiments.*



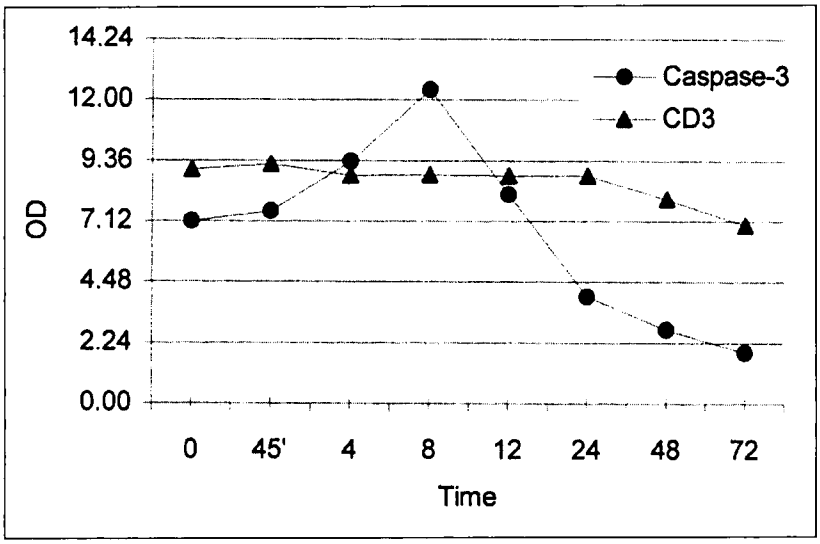


Fig. 5. *Densitometric analysis of Caspase-3 and CD3 quantity analysed by Western blot during 72 hours of DMSO treatment. As reported in figure 4 the up-regulation of Caspase-3 occurs at early time of treatment after which a decrease of the protein is seem. CD3 levels appear not directly influenced by treatment. In fact, it is possible to observe a light decrease starting from 48 hours associated with cell death progression. These experiments were performed at last three times and representative results are shown.*

at 8 hours and returning below the initial level after 24 hours. The amount of CD3 protein, analysed at the same treatment times, does not show noticeable differences in the amounts at early times of treatment. The decrease after 48 hours is due to the apoptotic process. The reported data indicate that the Caspase-3 increase is DMSO-dependent.

DISCUSSION

Many years have passed since Bollum described the biochemical properties of TdT (29), meanwhile, many Authors have tried to establish the precise role of this DNA polymerase. Numerous studies have indicated that TdT expression occurs during early T cell development and its main function consists in the diversification of the immune repertoire (1-5,30,31). However, the precise temporal relationship between TdT gene expression and T cell receptor gene rearrangement during *in vivo* mature T cell development in the thymus is still unknown. Although TdT shows *in vitro* DNA polymerization activity as a 58 kDa monomer, it has been speculated that the N region is synthesized by the orchestration of many proteins, including TdT (32). In particular, the biochemical mechanisms able to activate and inactivate the function of TdT *in vivo* are not yet fully elucidated. Here we demonstrate that Caspase-3 may be critically involved in the control of TdT activity, generating inactive 32-kDa peptides by proteolysis. The complete amino acid sequence of human TdT has been deduced from the cloned cDNA sequence (28). The active whole protein contains 508 amino acids, having a molecular weight of 58-kDa while the 32-kDa protein originates from carboxy-terminal residues 159-508. Even if previous results show the presence of several PKC-dependent phosphorylation sites along the whole peptite (21,28), the biologically active phosporylation sites and nuclear localization sites are present in the first 17 amino-terminal residues. TdT phosphorylation at these sites, modulating its function and/or localization, might be an early step in the overall process of lymphoid differentiation (4, 6, 9, 21, 28). Several authors (13, 32-34) have shown that caspases do not mediate the indiscriminate destruction of proteins during apoptosis, but a select set of proteins are targeted and cleaved at specific sequences. This cleavage usually inhibits the function of target proteins. Moreover, other reports indicate the involvement of caspases in the control of thymic selection (35-37). In particular, Caspase-3 is the effector caspase that is largely responsible for the morphological and biochemical cell changes that are the hallmark of apoptosis. Moreover the activation of Caspase-3 is indispensable for nuclear modification. The data reported in this paper prove that the TdT is a biological nuclear substrate for Caspase-3, the main apoptotic effector protease in many cell types, and that cleavage of TdT is an important step in thymocytes selection. In fact, the exclusive intranuclear localization of Terminal Transferase (38-41) and its cleavage during the apoptotic process (42-47) seems to indicate that activated Caspase-3 exerts a specific controlling role on this particular and specific *creative* DNA polymerase.

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