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Induction and activation of tissue transglutaminase during programmed cell death

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During the involution of lead nitrate-induced hyperplasia in rat liver a significant increase of transglutaminase activity, enzyme concentration, transglutaminase messenger RNA and protein-bound ε-(γ-glutamyl)lysine (product of transglutaminase action) coincided with programmed death (apoptosis) of hepatocytes. Immunohistochemical examination showed the appearance of transglutaminase in apoptotic hepatocytes. An increased transglutaminase level was also detected during glucocorticoid-induced apoptosis of rat thymocytes.

Transglutaminase; mRNA; ε-(γ-Glutamyl)lysine; Hepatocyte; Thymocyte; Apoptosis

1. INTRODUCTION

The biochemical mechanism of programmed cell death (apoptosis), a basic cellular phenomenon occurring during embryonic development, normal cell turnover, hormone-induced atrophy, involution of hyperplasia, elimination of preneoplastic cells, and regression of tumors [1–5], has not been fully clarified. Transglutaminases cross-link proteins through ε-(γ-glutamyl)lysine bonds [6]. Two of the transglutaminases have been shown to play essential roles in protecting biological integrity by formation of high molecular mass protein polymers: fibrin and α_2-plasmin inhibitor are cross-linked by blood coagulation factor XIII [7,8] as the final stage of clot formation. Cornified envelopes are formed by keratinocyte transglutaminase in the epidermis [9,10] during terminal differentiation of epidermal cells. No definite role has been assigned to a third, tissue type transglutaminase [6,11–13]. Here we report results which suggest that tissue transglutaminase-catalyzed protein cross-linking is part of the biochemical pathway of programmed cell death.

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2. MATERIALS AND METHODS

Lead nitrate was injected intravenously into male Wistar rats in a dose of 10 μmol/100 g of body weight. Livers were perfused in situ, removed, weighed, then homogenized [4,14] to determine protein [15] and DNA [16] concentrations as well as transglutaminase activity [17]. Concentration of transglutaminase protein was assayed by a sandwich ELISA system [14]. The apoptotic index was calculated by screening formalin-fixed sections stained with haematoxylin-eosin [3,4]. The concentration of protein-bound ε-(γ-glutamyl)lysine was determined following described principles [17,18]: extensive digestion of

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the protein fraction precipitated by trichloroacetic acid; separation of the dipeptide by ion-exchange chromatography, then on a silica column by HPLC; modification by phenylisothiocyanate followed by quantitation on HPLC using a C18 column and eluting with acetic acid/methanol; the presence of radioactive ϵ-(γ-glutamyl)lysine throughout the procedure to calculate recovery; applying γ-glutamylamine cyclotransferase, the specific enzyme for breaking ϵ-(γ-glutamyl)lysine [19], to identify the position of cross-linking during final analysis [20].

Total cellular RNA was isolated by the LiCl-urea method [21] from liver powdered under liquid N_2. RNA samples were denatured by 45% formamide, spotted on nylon membrane and baked at 82°C for 60 min under vacuum. A recombinant gt11 plasmid containing 1.6 kb cDNA for mouse tissue transglutaminase [22] was labeled by nick-translation using biotin (Biotin kit, Bethesda Res. Lab.). After 24 h of hybridization in the presence of 40% formamide, 4 × SSC (1 ×: 0.3 M NaCl, 0.03 M Na-citrate), 5 × Denhardt's solution, 5% dextran sulphate, 0.18% SDS, 5 mg/ml salmon sperm DNA blots were washed 3 times by 0.5 × SSC, 0.1% SDS at 56°C then developed using streptavidin and biotin labeled by alkaline phosphatase.

Immunohistochemistry was carried out by the indirect immunoperoxidase method. Endogenous peroxidase activity was blocked by 0.1 M periodic acid treatment followed by 0.02% $NaBH_4$ reduction. Primary antibody (affinity purified rabbit IgG raised against human tissue transglutaminase [14]) or preimmune serum was applied followed by horseradish peroxidase conjugated goat anti-rabbit IgG (Bio-Rad) developing the reaction with diaminobenzidine substrate.

3. RESULTS AND DISCUSSION

Liver hyperplasia was induced in rats by a single intravenous injection of the liver mitogen lead nitrate [4]. 3–4 days following treatment a marked liver enlargement with a maximum of almost twice the original size can be observed due mostly to the increase in the number of hepatocytes [4]; the change is reflected in values of liver DNA content (fig.1a). From day 5 onward, when the amount of lead in the liver is returning to normal values, a process of functional de-adaptation of the liver takes place, that is the cell number (liver DNA) returns to values observed before treatment (fig.1a). The mechanism of the involution process is the initiation of controlled death of almost every second hepatocyte [4]. The appearance of apoptotic bodies (figs 1b, 3c) reaching peak values simultaneously with decrease of liver DNA indicates the process. Enzyme activities in serum that increase during liver cell cytolysis remained unaltered [3,4] at any time of apoptosis (not shown).

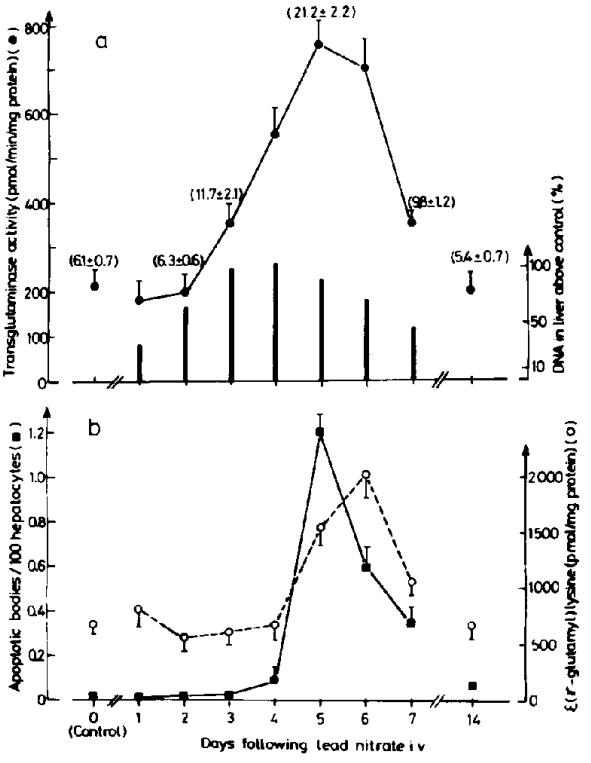

Fig.1. DNA content, transglutaminase activity and quantity (a), apoptotic index and protein-bound ϵ-(γ-glutamyl)lysine concentration (b) in rat liver following intravenous injection of lead nitrate. Means ± SE are shown.

Measuring transglutaminase activity in liver homogenates at various time intervals following lead nitrate administration an increase was observed starting on the third day, leveling off with a peak on the 5th–6th day when it was 3–4-times higher than that in control livers (fig.1a). The ac-

tivity dropped on day 7, then returned to normal values on day 14. The maximum activity and maximum incidence of apoptotic bodies (fig.1) coincided with involution. Using a sandwich ELISA system developed for the quantitation of tissue transglutaminase [14], it could be established that a higher level of transglutaminase protein appears simultaneously with increased enzyme activity (fig.1a; numbers above closed circles). On Western blots of liver samples two transglutaminase bands could be visualized with small molecular mass difference representing isoforms of tissue transglutaminase in rodents [23]. The intensity of both bands was increased when the liver samples undergoing apoptosis were analysed (fig.2b). The level of transglutaminase messenger RNA was also found to be higher in involuting liver as compared to controls or those with hyperplasia (fig.2a). This indicates that the induction of transglutaminase gene expression occurs in liver parallel to deletion of hepatocytes and the regulation is pretranslational. It has been shown that apoptosis depends upon synthesis of new RNA and protein species [5].

By immunohistochemical techniques tissue transglutaminase can be detected in endothelial cells of normal rat liver: endothelial lining of sinusoids gives a positive reaction (fig.3a) which is not observed using nonimmune serum (fig.3b). The presence of transglutaminase protein could not be demonstrated in hepatocytes of either normal or proliferating liver. During the involution of lead nitrate-induced hyperplasia intracellular apoptotic bodies (fig.3c, arrowhead) and apoptotic hepatocytes (fig.3c, arrow) can be observed. An intense positive reaction of apoptotic bodies with anti-transglutaminase antibody was observed (fig.3d), i.e. the excess amount of transglutaminase detected biochemically during involution seems to be present in the apoptotic cells.

There is a substantial amount of ϵ-(γ-glutamyl)lysine in proteins of normal liver (fig.1b). An increase of the cross-link concentration, up to 4-fold, was measured during involution of the enlarged liver. A high concentration of cross-link and high incidence of transglutaminase containing apoptotic cells coincided in time, i.e. simultaneously whith increased synthesis of transglutaminase, a higher level of enzyme product was measured.

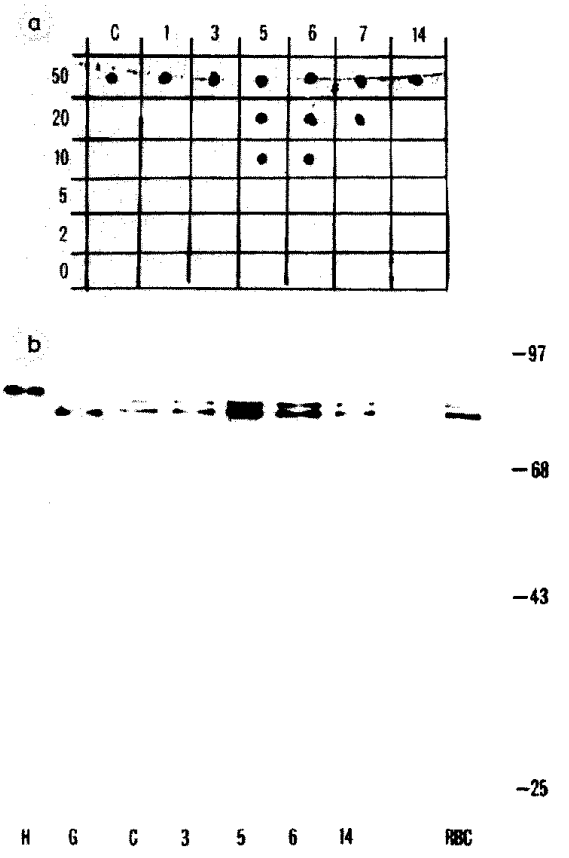

Fig.2. Transglutaminase messenger RNA (a) and enzyme protein (b) in liver samples following lead nitrate injection. (a) Numbers in the vertical row show the amount of RNA (ng) spotted. Numbers listed horizontally represent days following lead nitrate injection; C, control. (b) Western blot analysis was carried out as described [17]. Samples: H, purified human red blood cell transglutaminase (2 ng); G, purified guinea pig liver transglutaminase (3 ng); RBC, rat red blood cell extract following removal of hemoglobin (100 μg protein); C, control; 3, 5, 6, 14, liver samples (450 μg protein of each) obtained from animals 3, 5, 6, 14 days following treatment. Numbers to the right indicate positions of molecular mass reference (kDa) proteins.

Transglutaminases are Ca^{2+}-dependent enzymes [6,24]. It has been concluded that an increased Ca^{2+} concentration in apoptotic cells is necessary for completion of the death program [25]; the activation of an endogenous endonuclease by Ca^{2+} is part of the process [2]. Apoptosis manifests itself by the aggregation of organelles, digestion of the

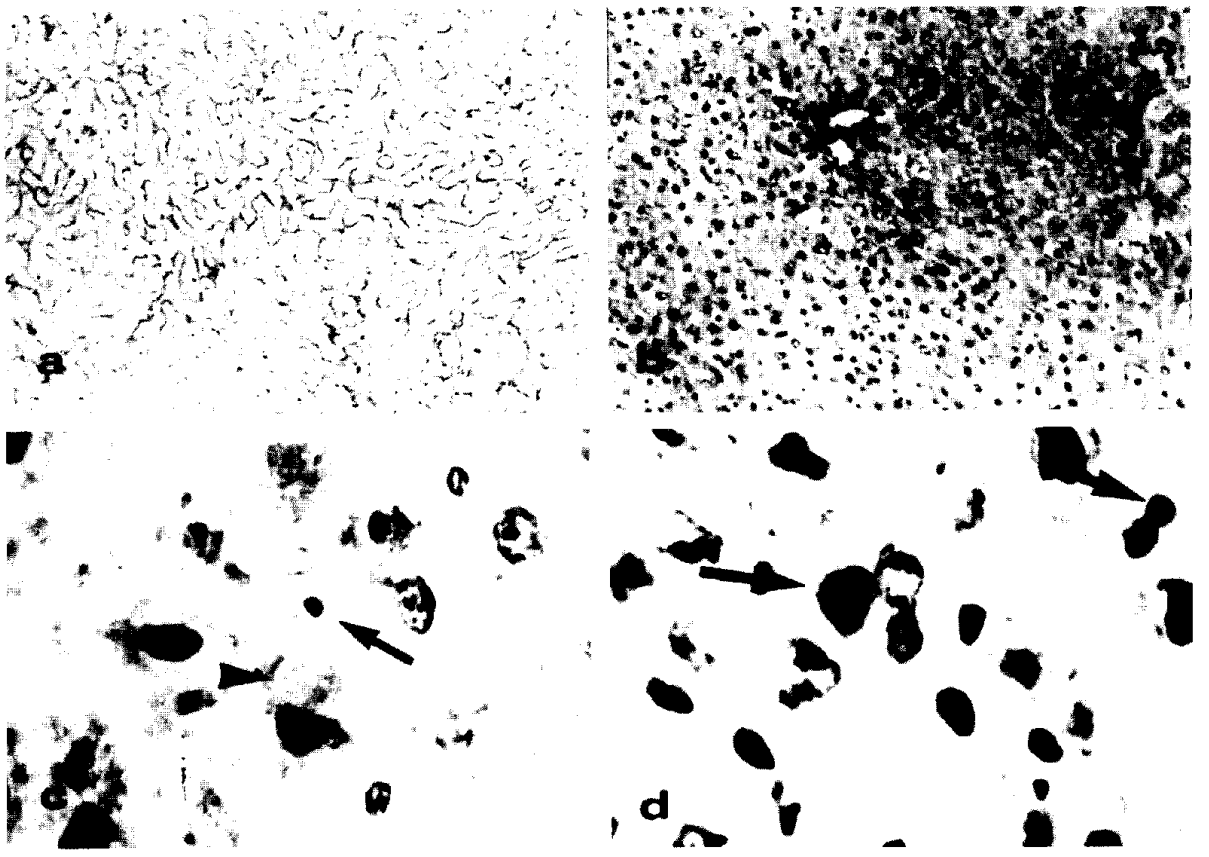

Fig.3. Immunohistochemical analysis of rat liver sections 4 days following lead nitrate injection. 6 μm cryostat sections (a,b,d) fixed in absolute acetone ($-20°C$, 10 min) and 5 μm paraffin sections fixed with 4% formaldehyde (c). Except for c, slides are counterstained with Mayer's heamalum; c is a heamalum-eosin stained section.

nuclei, shrinkage and fragmentation [1]. The probable consequence of transglutaminase activation is an extensive cross-linking of cytoplasmic and membrane proteins providing a way to maintain cellular integrity during the formation of the apoptotic bodies. Consequently, outpouring of potentially harmful intracellular molecules and abrogation of tissue continuity are prevented until phagocytosis of apoptotic bodies takes place. In terminally differentiating epidermal cells induction and activation of keratinocyte transglutaminase and its activation by raised intracellular Ca^{2+} concentrations are key elements in the formation of the cross-linked cornified envelope [9,10].

There was no change of transglutaminase level (table 1) during liver cell necrosis initiated with galactosamine [26]. Studying glucocorticoid-induced apoptosis of rat thymocytes transglutaminase level increased substantially in glucocorticoid-treated cells as compared to untreated ones (table 1).

It is concluded that the function of the ubiquitous tissue transglutaminase is related to its induction and activation as part of the biochemical program for deletion of cells through the process of apoptosis. The enzyme may be used as a marker to detect apoptotic cells among normal ones and it may serve as a tool to learn how the program of this basic cellular phenomenon is initiated under a variety of conditions including embryonic development, hormone-induced atrophy, normal cell turnover, involution of hyperplasia, elimination of preneoplastic cells and regression of tumor [1,3,5,27].

Table 1

Amount and activity of transglutaminase in necrotic liver and cultured thymocytes of rats

Experiment	Time following treatment (h)	Activity (pmol/min per mg)	Enzyme protein (ng/mg protein)
Liver			
Control	2	256.6	5.2
	12	242.2	6.1
Galactosamine	2	224.7	6.0
treatment	6	259.5	5.4
	12	230.4	6.0
Thymocyte			
Control	2	40.1	1.4
	12	52.7	1.7
Glucocorticoid	2	58.1	1.3
treatment	4	310.9	5.6
	12	390.6	8.1

Experimental conditions were as described by others [2,26]. Transglutaminase activity and concentration were assayed as described elsewhere [14,17]. Mean of at least 4 results is shown (variation coefficients were within 10%)

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