Volume 284, number 1, 109–112 FEBS 09816 June 1991

ADONIS 0014579391005313

Degradation of cells dying by apoptosis leads to accumulation of ε(γ-glutamyl)lysine isodipeptide in culture fluid and blood

Laszlo Fesus[1], Edit Tarcsa[1], Noemi Kedei[1], Francesco Autuori[2] and Mauro Piacentini[2]

[1]*Department of Biochemistry, University School of Medicine, Debrecen H-4012, Hungary* and [2]*Department of Biology, 2nd University of Rome ('Tor Vergata'), 00173 Rome, Italy*

Received 1 March 1991; revised version received 16 April 1991

ε(γ-Glutamyl)lysine isodipeptide, the end-product of proteolytic digestion of proteins cross-linked by transglutaminase, was detected in culture fluid of neonatal rat hepatocytes and plasma of adult rats. The concentration of the isodipeptide was significantly increased in both when high rate of apoptosis with phagocytosis of dying hepatocytes was produced either by epidermal growth factor in the culture or by lead nitrate-induced hyperplasia with subsequent involution in rats. Specific induction of tissue transglutaminase and the consequent formation of highly cross-linked protein envelopes in apoptotic cells have been previously demonstrated by us in both systems.

Transglutaminase; Hepatocyte; Apoptosis; Phagocytosis; ε(γ-glutamyl)lysine; Isodipeptide

1. INTRODUCTION

Transglutaminase-catalyzed modification of proteins results in covalent cross-linkage by ε(γ-glutamyl)lysine or, under special circumstances, bis-γ-glutamylpolyamine bonds [1,2]. Stabilization of fibrin by blood coagulation factor XIII [3] and formation of cross-linked protein envelopes in cells by keratinocyte or tissue transglutaminase during terminal differentiation [4] or apoptosis [5], respectively, are examples of extensive formation of ε(γ-glutamyl)lysine bonds. The active cellular program of death by apoptosis is characterized by the requirement of protein synthesis, increased concentration of intracellular Ca^{2+}, activation of a Ca^{2+}- and Mg^{2+}-dependent endonuclease and changes of cell-surface carbohydrates which permit recognition, phagocytosis and then complete elimination of apoptotic cells [6–8]. We have previously shown that during involution of hyperplasia of rat liver and death of neonatal rat hepatocytes, tissue transglutaminase is induced, then highly cross-linked protein envelopes are formed in the apototic hepatocytes by the Ca^{2+}-activated enzyme [5,9,10]. The ε(γ-glutamyl)lysine bond is resistant to proteolytic degradation [11] which means that free ε(γ-glutamyl)lysine isodipeptide should appear whenever transglutaminase-cross-linked polymers are degraded. Since apoptotic cells are continuously produced, phagocytosed and rapidly digested within phagolysosomes under normal conditions, a constant isodipeptide concentration should be present both in cultures of cells with a basal apoptosis rate and in plasma under in vivo conditions. Testing this prediction we have found that it is indeed the case. Furthermore, when an additional wave of apoptosis and degradation of apoptotic cells was induced in cultures of neonatal rat liver cells or in rat liver, the extracellular level of isodipeptide concentration was temporarily increased.

Correspondence address: L. Fesus, Department of Biochemistry, University School of Medicine, Debrecen, H-4012 Hungary. Fax: (36) (52) 13847.

2. MATERIALS AND METHODS

Primary cultures of neonatal rat liver cells were prepared from newborn rats and grown on collagen-coated surface in RPMI medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (Sigma), 10 mM HEPES, 2 g/l bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin in a humidified atmosphere with 5% CO_2 at 37°C, as described [10,12]. Routinely, 120×10^3 cells/cm^2 were plated on a 25 cm^2 flask and the medium (5 ml) was replaced daily. In part of the experiments epidermal growth factor (EGF; Sigma) was added in a concentration of 10^{-9} M at the fourth day of the culture, 8 h following the last change to fresh medium. The culture fluids collected at 24-h intervals were concentrated 5 times by lyophilization. Immunohistochemical staining of neonatal hepatocytes was performed as described [10].

Liver hyperplasia and subsequent involution was initiated by the intravenous injection of lead nitrate (10 μmol/body weight) into male Wistar rats weighing 150–200 g as published [5,13]. Anticoagulated blood samples with 0.109 M trisodium citrate (ratio 9:1) were obtained from individual rats by opening the abdominal cavity under anesthesia and drawing blood from the vena cava inferior; plasma was prepared within 20 min by centrifugation. Body weight and liver weight were regularly recorded.

Concentrated culture fluid and blood samples were deproteinized by using Centrifree partition device (Amicon) with a molecular weight cut off of 5000. According to our published method for the quantitation of ε(γ-glutamyl)lysine [14] preliminary separation of amino acids and peptides by ion-exchange chromatography and on a silica column was followed by derivatization with phenylisothiocyanate (PITC) and separation of amino acid and peptide derivatives by HPLC on a μBundapack C18 column. ^{3}H-labelled ε(γ-glutamyl)lysine was used as

an interior standard throughout the procedure. Elution position of the isodipeptide was regularly established by standard $\epsilon(\gamma$-glutamyl)lysine (Serva). Calculation was based on peak areas as compared to ones obtained by various quantities of the standard isodipeptide and on the recovery determined from isotope dilution [14].

3. RESULTS AND DISCUSSION

We have reported earlier that $\epsilon(\gamma$-glutamyl)lysine isodipeptide in a concentration of up to 30–50 nmol/l could be detected in culture fluids of regularly growing Chinese hamster ovary (CHO) cells and that the extracellular accumulation of the isodipeptide was due to intracellular degradation of cross-linked proteins formed by tissue transglutaminase [15]. The significance of this finding in relation to the function of tissue transglutaminase was not clear, however. The latter has been recently coupled to the formation of a highly cross-linked protein scaffold in cells undergoing death by apoptosis [5,9,10]. The apoptotic cells are phagocytosed and degraded in adjacent cells [6–8]. In an effort to link the appearance of protein-free $\epsilon(\gamma$-glutamyl)lysine in biological fluids to apoptosis we first started to test a cell culture system in which, contrary to CHO cells, both apoptosis and the phagocytosis of apoptotic cells occur.

Primary cultures of neonatal rat liver cells consists of hepatocytes and fibroblasts [10,12]. At days 5–6 of the culture a spontaneous rate of apoptosis (0.2–0.4%, identified by morphology) develops. Addition of EGF to the cultured cells at day 4 induces a rapid and transient proliferation of hepatocytes followed by a wave of apoptosis (4–5% observed at time points between 36–60 h) which results in the dropping of cell numbers (Fig. 1, insert). Proliferation of hepatocytes is paralleled by a 10-fold increase in tissue transglutaminase mRNA followed by the appearance of transglutaminase protein in apoptotic cells (Fig. 2A) with an increase of enzyme activity and the formation of SDS-insoluble, cross-linked protein envelopes [10]; time points of peak values are shown in the insert of Fig. 1. The apoptotic cells are phagocytosed by the neighbouring cells where they are degraded (Fig. 2B and C). High concentration of isodipeptide, between 5.0–10.0 μmol/l, was observed in the culture fluid even without the EGF treatment (Fig. 1); in fact, this value is two orders of magnitude higher than the maximal ones seen in CHO cell cultures [15]. The isodipeptide level raised sharply reaching 25.0–30.0 μmol/l concentrations at 48 h after EGF treatment, that is when the highest rate of apoptotic body formation was seen in the culture (Fig. 2). Since the culture fluid was changed every 24 h the concentrations in all cases developed within the preceding 24 h. Several control experiments were carried out to investigate factors which could affect the results. The concentration of the isodipeptide in the complete medium was less than 100 nmol/l before adding it to the cells (less than 2% of the values observed after its interaction with cells); this comes from the fetal calf serum. Isodipeptide is not formed or degraded in the culture medium itself and it is not taken up by cells ([15] and data which are not shown).

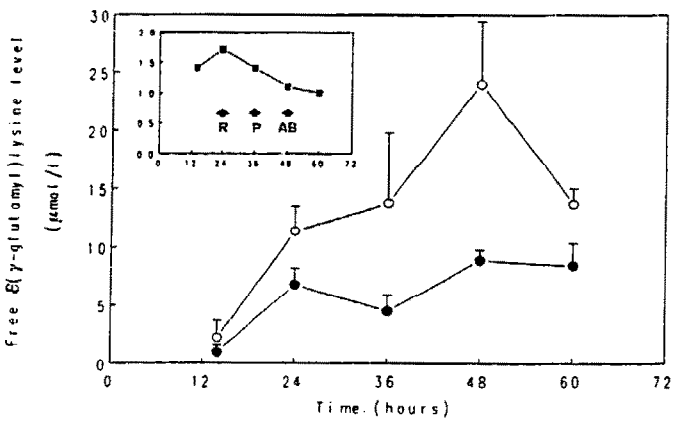

Fig. 1. Concentration of $\epsilon(\gamma$-glutamyl)lysine isodipeptide in the medium of primary cultures of neonatal rat liver cells treated (○) or not treated (●) with EGF. Typical changes of cell number (ordinate, $\times 10^6$) in the culture at various time points (hours at the abscissa) following EGF treatment (at 4th day of the culture corresponding to '0' time point on the figure) are shown in the insert in parallel with the previously reported [10] time course of the developing peak values of tissue transglutaminase mRNA (R), enzyme protein (P) and number of cross-linked apoptotic bodies (AB). Data are means ± SEM of determinations in at least 4 separate cultures.

Free $\epsilon(\gamma$-glutamyl)lysine could also be detected in plasma of untreated adult rats in a concentration range of 1.8–3.2 μmol/l (Fig. 3). The interpretation of this value is not easy since little is known about the in vivo clearance mechanism of the isodipeptide. A preliminary report, based on studies with intravenously injected radioactive isodipeptide, has suggested that it may take place in kidney tubules [16]. We have recently found that in human plasma a small portion (about 1% under normal conditions) of the amount of blood isodipeptide may come from plasmin degradation of cross-linked fibrin [17]. Most of it may be derived from degradation of apoptotic cells as a result of the steady-state cell turnover in tissues. If this assumption is correct increasing rate of apoptosis in some cell compartments should elevate the level of the isodipeptide in the blood.

A large portion of hepatocytes die by apoptosis during the involution of liver which follows hyperplasia induced by the liver mitogen lead nitrate [9,13]. According to our published results [5,9] the involution is accompanied by induction of tissue transglutaminase mRNA and enzyme protein, the enzyme is detected in apoptotic hepatocytes and its activation leads to the formation of highly cross-linked protein envelopes; changes of liver weight and time points of peak values are indicated on Fig. 3. During the involution phase apoptotic cells within phagolysosomes can be frequently seen (Fig. 2D and E). Parallel, significantly increased

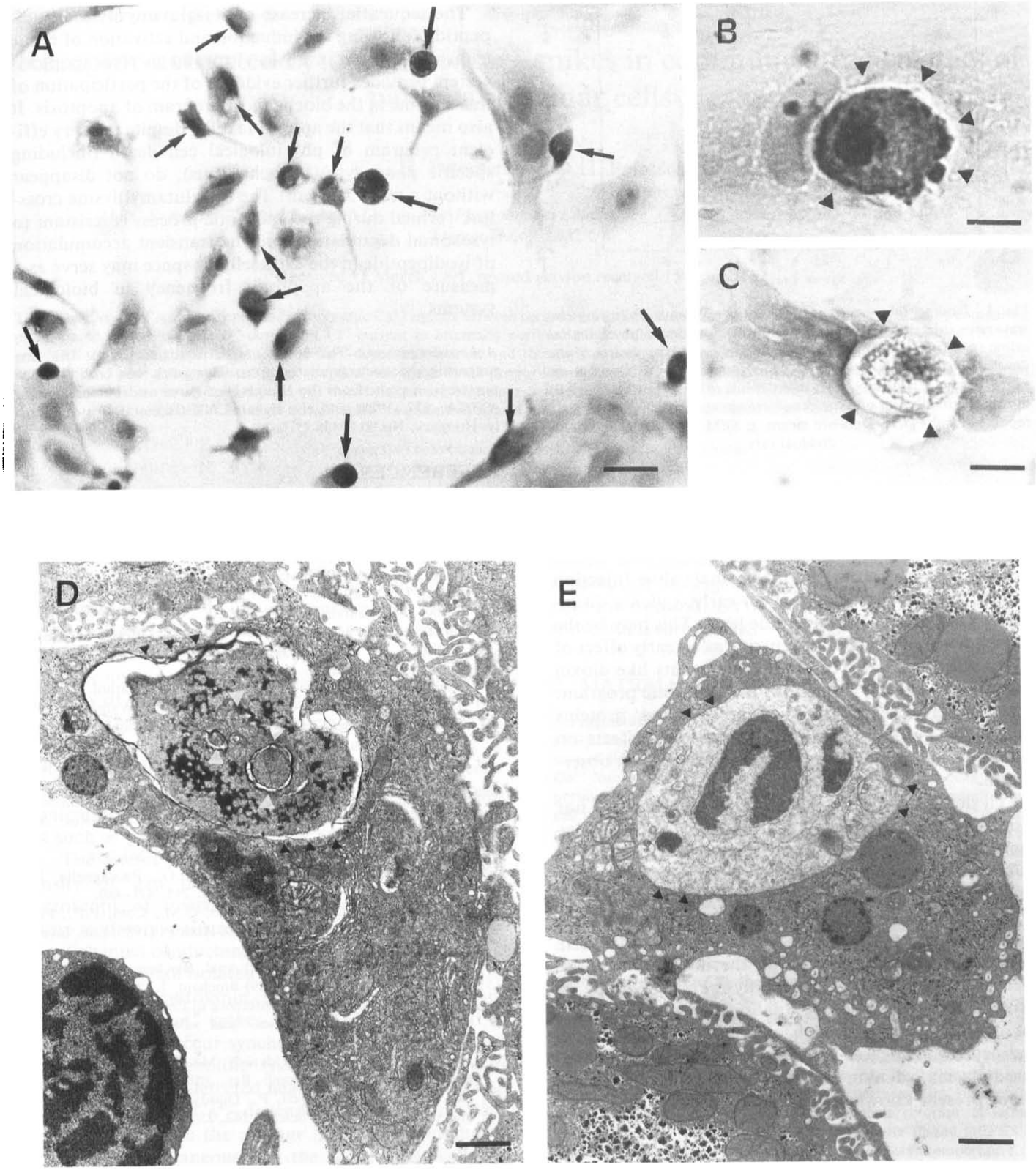

Fig. 2. Morphologic observation of in vitro and in vivo degradation of apoptotic bodies. Tissue transglutaminase immunopositive apoptotic bodies showing highly condensed chromatin (A, arrows, and B) and different stages of degradation of apoptotic bodies within phagolysosomes (B and C, arrowheads) 36 h upon EGF treatment. Kupffer cells engulfing apoptotic bodies with typical dilated endoplasmic reticulum (D, arrowheads) and condensed chromatin (E) 5 days upon injection of lead nitrate. Bars represent 20 μm (A), 5 μm (B and C), 2 μm (D and E).

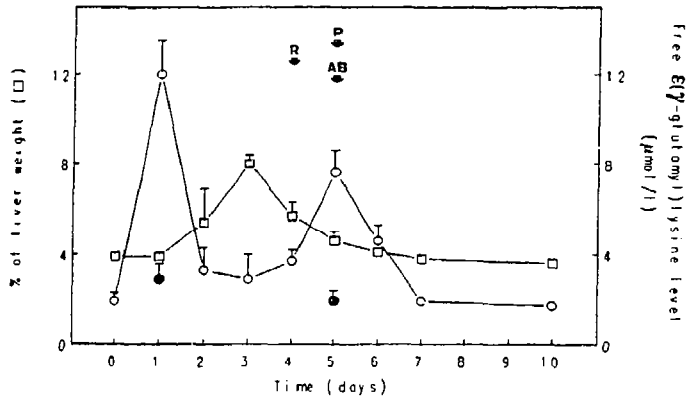

Fig. 3. Isodipeptide concentration in rat plasma following intravenous injection of lead nitrate (○) or solution of physiological saline (●). Typical changes of % liver weight and time course of the developing peak values of tissue transglutaminase mRNA (R), enzyme protein (P) and a number of cross-linked apoptotic bodies (AB) during the involution of hyperplasia are shown according to already reported results [5,9]. Data are means ± SEM of values found in individual rats.

isodipeptide levels with peak values about 5 times as much as in salt-injected controls occurred at the 5th day. At the time of normalized liver weight (day 10) the isodipeptide level returns to the normal value. Injection of lead nitrate also results in an early, quickly disappearing elevation of isodipeptide level. This may be the consequence of apoptosis occurring as an early effect of lead nitrate, similarly to other toxic agents like dioxin [18], in cells already primed for the apoptotic program. Alternatively, degradation of cross-linked proteins before the mitotic response or some toxic effects on kidney tubular cells may explain this surprising observation.

Cell death by apoptosis, contrary to necrosis, is not associated with autolysis and leakage of intracellular components with subsequent inflammatory reactions [6,7]. This is mainly due to the fast and specific phagocytosis of apoptotic cells followed by their proteolytic degradation in lysosomes [8]. We have not observed signs of autolysis or necrosis in the studied cell culture model system or during the involution of rat liver [5,9,13]. The ε(γ-glutamyl)lysine isodipeptide is resistant to proteolytic degradation and lysosomes do not contain enzymes which can split it [11,15]. The isodipeptide is released into the extracellular space and blood plasma reaching the kidney where it is (according to our present knowledge) catabolized [16].

The sequential increase of ε(γ-glutamyl)lysine isodipeptide following the induction and activation of tissue transglutaminase in an in vitro and in vivo apoptosis system provides further evidence of the participation of this enzyme in the biochemical program of apoptosis. It also means that the apoptotic cells, despite the very efficient program of physiological cell death (including specific phagocytosis mechanisms), do not disappear without a trace after all. The ε(γ-glutamyl)lysine cross-link formed during the apoptotic process is resistant to lysosomal degradation and the transient accumulation of isodipeptide in the extracellular space may serve as a measure of the apoptotic frequency in biological systems.

Acknowledgements: The authors wish to thank Dr. L. Dini for preparing the electron micrographs. This work was partially supported by a grant from the Hungarian Science and Research Fund (OTKA; 537; 1988) and the Italian CNR (Bilateral Projects, Italy-Hungary, No 90.02458.CT04).

REFERENCES

[1] Folk, J.E. (1980) Annu. Rev. Biochem. 49, 517–531.
[2] Piacentini, M., Martinet, N., Beninati, S. and Folk, J.E. (1988) J. Biol. Chem. 263, 3790–3794.
[3] Lorand, L., Losowsky, M.S. and Miloszewski, K.J.M. (1980) Progr. Haemost. Thrombos. 5, 245–290.
[4] Green, H. (1980) Harvey Lect. 74, 101–139.
[5] Fesus, L., Thomazy, V., Autuori, F., Ceru, M.P., Tarcsa, E. and Piacentini, M. (1989) FEBS Lett. 245, 150–154.
[6] Wyllie, A.H., Kerr, J.F.R. and Currie, A.R. (1980) Int. Rev. Cytol. 68, 251–306.
[7] Arends, M.J. and Wyllie, A.H. (1991) J. Exp. Pathol., in press.
[8] Savill, J.S., Wyllie, A.H., Henson, J.E., Walport, M.J., Henson, P.M. and Haslett, C. (1989) J. Clin. Invest. 83, 865–874.
[9] Fesus, L., Thomazy, V. and Falus, A. (1987) FEBS Lett. 224, 104–108.
[10] Piacentini, M., Autuori, F., Dini, L., Farrace, M.G., Ghibelli, L., Piredda, L. and Fesus, L. (1991) Cell Tissue Res. 263, 227–235.
[11] Loewy, A.G. (1984) Methods Enzymol. 107, 241–257.
[12] Armato, U., Romano, F., Andreis, P.G., Paccagnella, L., Marchesini, C. (1986) Cell Tissue Res. 245, 471–480.
[13] Columbano, A., Ledda-Columbano, G.M., Coni, P.P., Faa, G., Liquori, C., Santa Cruz, G. and Pani, P. (1985) Lab. Invest. 52, 670–679.
[14] Tarcsa, E. and Fesus, L. (1990) Anal. Biochem. 186, 135–140.
[15] Fesus, L. and Tarcsa, E. (1989) Biochem. J. 263, 843–848.
[16] Fink, M.L. (1987) Nobel Conference in Cellular and Molecular Biology; 'Transglutaminases and Protein Cross-linking Reactions', Miami.
[17] Harsfalvi, J., Tarcsa, E., Udvardy, M., Zajka, G., Szarvas, T. and Fesus, L. (1991) Thromb. Res., submitted.
[18] McConkey, D.J., Hartzell, P., Duddy, S.K., Hakansson, H. and Orenius, S. (1988) Science 242, 256–259.