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Apoptotic and proliferating hepatocytes differ in prothymosin α expression and cell localization

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Abstract

Prothymosin α is an acidic protein, reported to be involved in cell proliferation and apoptosis, although its precise function in both processes are still unknown. Due to the importance of these processes in the pathogenesis of hepatic diseases and the need to understand the molecular mechanisms underlying these diseases we aimed to investigate the behavior of this protein in liver growth and apoptosis, in two models of hepatocytes in culture. Prothymosin α expression varied throughout the hepatocyte cell cycle, according to its progression. Proliferating hepatocytes showed increased expression of the protein, while apoptotic ones showed decreased levels. The subcellular location of prothymosin α differed according to the different phases of the cell cycle. Thus, it appeared with a stippled and widely dispersed pattern throughout the nucleus in quiescent and proliferating hepatocytes, while it became cytoplasmic in mitotic and late apoptotic cells. These results are in agreement with the idea that high levels of prothymosin α need to be present in the nucleus for proliferation, and programmed cell death requires low levels of prothymosin α outside of the nucleus. The differences in prothymosin α expression and localization during hepatocyte proliferation and apoptosis suggest that this protein may have a pleiotropic function that depends not only on its availability but also on its various localizations in different subcellular compartments.

Key words: liver, hepatocytes, apoptosis, proliferation, prothymosin α

Introduction

Prothymosin α (PTA) is a highly acidic nuclear protein of aproximately 110 amino acids, which was first isolated from rat thymus [1]. This protein has been initially associated with regulation of immunity, but many evidences suggest that it participates in cell proliferation, although its role remains to be elucidated. The increase in PTA mRNA levels during cellular proliferation has been demonstrated in many cell types [2–7]. The role of PTA in cell proliferation has also been evidenced in myeloma cells that were not able to divide after depletion of PTA by treatment with antisense RNA or synthetic antisense DNA oligomers [8]. Over expression of PTA in NIH3T3 and HL-60 cells was shown to stimulate cell division due to the shortening of the G1 phase [9, 10].

Recently, caspase-3 mediated fragmentation of PTA was detected in apoptosis and the cleavage site was located within the nuclear localization signal of the protein [11]. This fragmentation correlates with the nuclear escape of the protein, detected in programmed cell death of HeLa cells [12].

The deregulation of hepatocyte growth and death generates liver diseases [13–17]. In consequence, it is important to understand the molecular bases of pathogenesis in the liver, and to have markers that would allow to characterize the

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sequential evolution of the aforementioned cell processes. PTA was detected to be over expressed in regenerating liver tissues and hepatocellular carcinoma [18–20]. In this paper, we investigate the fate and meaning of PTA during apoptosis and proliferation in cultures of hepatocytes. Here we show that in apoptotic hepatocytes PTA mRNA levels were decreased and the protein looses its nuclear compartmentalization to become cytoplasmic. On the other hand, during proliferation PTA expression was increased, and it is present in the nucleus, with the exception of mitotic cells that show cytoplasmic location of the protein. Our data suggest that PTA may have roles in hepatocyte proliferation and apoptosis, depending on its levels of expression and its subcellular localization. Accordingly, PTA may be involved in the pathogenesis of hepatic diseases caused by deregulation of these processes.

Materials and methods

Materials

TGF-β1, HGF and collagenase type IV were from Sigma. Fetal calf serum (FCS) was from Gibco. Cycloheximide (CHX) was from Calbiochem. α^{32}P-deoxy cytidine tri phosphate (α^{32}P-dCTP, 3000 Ci/mmol) and γ^{32}P-adenosine tri phosphate (γ^{32}P-ATP, 3000 Ci/mmol) were from NEN Perkin Elmer. The anti-PTA antibodies were obtained as previously described [3]. The Texas Red labelled anti-rabbit IgG antibodies were from Jackson Immuno Research.

Culture of clone-9 (C-9) cells, apoptosis induction

These cells (isolated from normal rat liver) were obtained from the European Collection of Cells Cultures (ECACC). They were cultured in Ham's F12-K medium, 10% FCS, penicilin (100 UI/mL) and streptomycin (100 μg/mL), at 37 °C and 5% CO_2. Apoptosis was induced with 2.5 ng/mL of TGF-β1 and 300 μM of CHX, incubated for 48 hr. We have previously determined the optimal conditions for apoptosis induction. Proliferating and quiescent cells were obtained from subconfluent monolayers or by contact inhibition and serum deprivation, respectively.

Hepatocytes isolation, culture, induction of apoptosis and proliferation

Hepatocytes were isolated from Sprague-Dawley male rats (150–200 g) by the two-step perfusion procedure, using 0.025% of collagenase (Type IV, Sigma), as previously described [21]. They were plated at a density of 10^5 cells per cm^2 in minimum essential and 199 mediums (4:1 vol/vol), supplemented with penicilin (100 UI/mL), streptomycin (100 μg/mL), insulin (5 μg/mL), bovine serum albumin (BSA, 1 mg/mL), dexamethasone (10^{-6} M), 10% FCS, and incubated for 4 hr at 37 °C and 5% CO_2 (attachment period). After this period the medium was replaced by the same fresh medium, but supplemented with hydrocortisone-21-hemisuccinate 0.6 mM. This post-attachment medium was replaced every 24 hr. Apoptosis was induced immediately after the attachment period by addition of 1 ng/mL TGF-β1 or CHX 300 μM, both incubated for 24 hr. Proliferation was induced by the addition of 2.5 ng/mL HGF after culturing the cells for 24 hr, and incubated for a period of 6 days. Quiescent hepatocytes were obtained from 24 hr cultures. We have previously determined the optimal conditions for apoptosis or proliferation induction.

Caspase-3 activity

After apoptosis induction, caspase-3 activity was assessed with the EnzCheck caspase-3 Assay Kit 1 from Molecular Probes, following the manufacturer's instructions.

Analysis of DNA fragmentation

Approximately 1–2 $\times$ 10^6 cells were harvested, washed twice with PBS (phosphate buffer saline) and DNA was extracted with the Wizard Genomic DNA Purification Kit from Promega. DNA fragmentation analysis was done as previously described [22].

Analysis of DNA content by flow cytometry (hypoploid cells)

After apoptosis induction, the cells were harvested and washed twice with 0.1% BSA in PBS. They were fixed in 70% ethanol for 1 h at 4 °C, washed twice with 0.1% BSA in PBS and resuspended in the same buffer. After the addition of DNA extraction buffer (192 mM PO_4HNa_2; 4 mM citric acid; pH:7.8), incubation at room temperature (RT) for 5 min and centrifugation, the cells were stained with propidium iodide (50 μg/mL, Sigma) and treated with ribonuclease A (RNAse A 0.5 mg/mL, Sigma) for 30 min at RT. Cells were analyzed on a flow cytometer (Coulter-Epics XL) containing an argon laser (488 nm). The red fluorescence of propidium iodide, proportional to DNA content, was collected through a 620 $\pm$ 15 nm band pass filter.

Hepatocyte proliferation detection

Approximately 10^5 cells per well, cultured in 96 wells microplates, were induced to proliferate. After this period,

proliferation was detected with 5-Bromo-2′deoxy-uridine (BrdU) Labelling and Detection Kit from Boehringer, following the manufacturer's instructions.

PTA northern blot

After different treatments, cells were harvested in denaturalising solution for RNA extraction by the guanidinium isothyocianate-phenol method of Chomczynski [23]. After quantification at 260 nm, 25 μg of total RNA were fractionated by formaldehyde agarose gel electrophoresis. The ribosomal RNA 28S and 18S bands were visualized by ethidium bromide staining under UV light (VersaDoc^{TM} system and the Quantity One® program). RNA was transferred by capillary blotting to a charged nylon membrane (Immobilon ^{TM}-Ny^{+}, Millipore) and fixed under UV light. The filter was hybridised to a ^{32}P-labeled cDNA probe of human PTA. The correct hybridisation of this human PTA probe with PTA rat gene has been previously tested [5]. The cDNA subcloned in BamHI sites of a pGEM$_4$ vector was obtained after digestion with the restriction enzyme (Gibco) and purification with Sephaglass Band Prep Kit (Amersham Pharmacia Biotech), and labelled by random priming with the NEBlot® Kit (New England Biolabs). Hybridisation was performed at 42 °C overnight in pre-hybridisation buffer containing the labelled probe (1×10^6 cpm/mL), the membrane was washed 6 times and exposed to an X-ray film for different times. Densitometric analysis of the bands was performed to evaluate changes in mRNA levels. A rat 18S ribosomal RNA oligonucleotide probe 5′-ACGGTATCTGATCGTCTTCGAACC-3′ (Amersham Pharmacia Biotech) was used to asses the amount and integrity of the total RNA loaded on each gel. This oligonucleotide was end labelled by T4 polynucleotide kinase (Promega) and hybridised with filters at 50 °C for 24 hr. The membrane was washed and revealed as before.

Localization of PTA by confocal microscopy

Cells were grown in coverslips and treated as previously described for the obtaining of quiescent, proliferating and apoptotic hepatocytes. They were fixed with 4% paraformaldehyde for 1 h at 4 °C and permeabilized with 0.2% Triton-X100 during 5 min. The incubation with a 1:20 dilution of the anti-PTA antibody was at RT for 24 hr. After 3 washes with PBS, the cells were incubated with the labelled secondary antibody (anti-rabbit IgG-Texas Red) in a 1:100 dilution for 1 h at RT, and washed. The coverslips were mounted with 90% glycerol in PBS and evaluated under confocal microscopy (MRC-1024, BioRad).

Statistical analysis

The results of the experiments were expressed as mean ± standard error (SE). Means obtained in different experiments were compared by the ANOVA test. The differences between means were considered statistically significant when p resulted lower than 0.05.

Results

Culture of C-9 cells, apoptosis induction and detection

To obtain apoptotic C-9 cells, subconfluent monolayers were incubated for 48 hr with 300 μM CHX or 2.5 ng/mL TGF-β1. Apoptosis was determined by detection of caspase-3 activity and flow cytometry. Statistically significant caspase-3 activity was detected in cells incubated with CHX ($p < 0.001$) and TGF-β1 ($p < 0.001$) respectively, compared to the control quiescent cells. In addition, statistically significant increments in the percentage of hypoploid cells, determined by flow cytometry, were detected in apoptotic hepatocytes induced by CHX ($p < 0.001$) or TGF-β1 ($p < 0.001$), respective to the controls (Table 1).

Culture of primary hepatocytes, induction and detection of apoptosis and proliferation

Apoptotic primary hepatocytes (PH) for different experiments, were obtained after induction with 300 μM CHX or 1.0 ng/mL TGF-β1, incubated for 24 hr. Apoptosis was detected by caspase-3 activity, flow cytometry and DNA laddering. Statistically significant caspase-3 activity was detected in hepatocytes induced with CHX ($p < 0.001$) or TGF-β1 ($p < 0.001$) respectively, compared to the control quiescent hepatocytes. Using flow cytometry, the percentage of hypoploid cells was observed to rise significantly, in cells treated with CHX ($p < 0.001$) or TGF-β1 ($p < 0.001$), respectively (Table 1). Figure 1 shows DNA ladderings of PH obtained by using both apoptotic agents. On the other hand, proliferating PH for different experiments were obtained by induction with 2.5 ng/mL HGF, incubated for 6 days. Proliferation was confirmed by significant detection of BrdU incorporation ($p < 0.001$) in treated hepatocytes, compared to the quiescent controls (Fig. 2).

PTA expression changes in apoptotic or proliferating hepatocytes

To determine variations in PTA expression during *in vitro* hepatocyte proliferation and apoptosis, in PH and C-9 cells,

Table 1. TGF-β1 and CHX induce apoptosis in hepatocytes

Cell type Cell state	Clone-9 Quiescent	Clone-9 Apop (TGF-β1 2.5 ng/mL 48 hs)	Clone-9 Apop (CHX 300 μM 48 hs)	Primary hepatocytes Quiescent	Primary hepatocytes Apop (TGF-β1 1.0 ng/mL 24 hs)	Primary hepatocytes Apop (CHX 300 μM 24 hs)
Caspase-3 (FlAU) mean $\pm$ SE (n)	186.5 $\pm$ 72.8 (3)	958.5 $\pm$ 137.9 *** (3)	1478.3 $\pm$ 157.1 *** (3)	374 $\pm$ 84 (3)	624 $\pm$ 90.5 ** (3)	5821 $\pm$ 557.2 *** (3)
% sub-G0 mean $\pm$ SE (n)	4.3 $\pm$ 0.4 (3)	13.4 $\pm$ 0.4 *** (3)	36.3 $\pm$ 0.8 *** (3)	17.5 $\pm$ 0.4 (3)	25.4 $\pm$ 1.4 *** (3)	29.6 $\pm$ 0.4 *** (3)

PH were treated with 1.0 ng/mL TGF-β1 or 300 μM CHX for 24 hr, and subconfluent monolayers of C-9 cells were treated with 2.5 ng/mL TGF-β1 or 300 μM CHX for 48 hr. After harvesting the cells, caspase-3 activity and the percentage of subdiploid population by flow cytometry were determined as described in *Materials and methods*. Results are expressed as the mean $\pm$ standard error obtained from three independent experiments. FlAU: fluorescence arbitary units; (***) $p < 0.001$; (**) $p < 0.01$; % sub-G0: percentage of subdiploid cells; n: is the number of replicates.

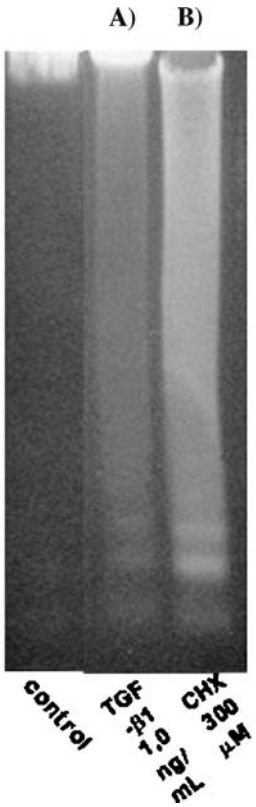

Fig. 1. TGF-β1 and CHX induce DNA laddering in apoptotic primary hepatocytes. Apoptotic primary hepatocytes induced by TGF-β1 (A) or CHX (B). PH were treated with 1.0 ng/mL TGF-β1 or 300 μM CHX for 24 hr. Cells were harvested, DNA was extracted (as described in *Materials and methods*) and electrophoresed on a 1% agarose gel containing ethidium bromide. The gel was visualised under UV light.

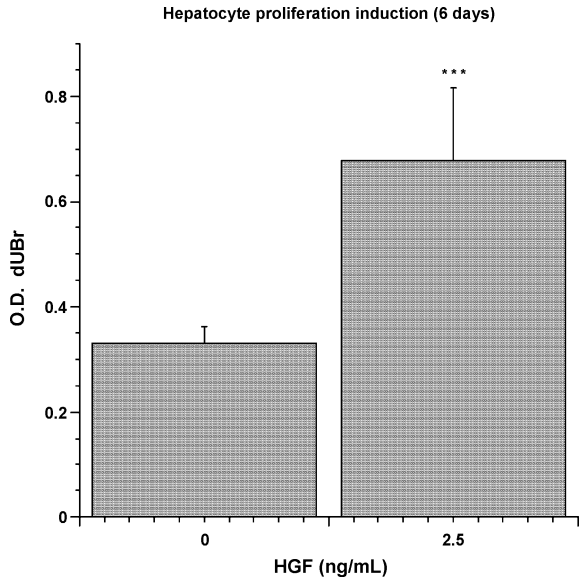

Fig. 2. HGF induces hepatocyte proliferation. Primary hepatocytes were stimulated with 2.5 ng/mL HGF 24 hr after plating and further incubated during 6 days, in the presence of the growth factor. BrdU incorporation was determined as described in *Materials and methods*. O. D. BrdU: optical density obtained from BrdU incorporation into the newly sinthesized DNA; (***) $p < 0.001$. Results are expressed as the mean $\pm$ standard error obtained from eight independent experiments.

mRNA levels were studied by Northern Blot. Figures 3A and 4A show typical auto radiographies of PTA and ribosomal RNA 18S hybridizations. In PH, PTA messenger is increased ($p < 0.001$) during proliferation, but decreased ($p < 0.001$) during apoptosis (Fig. 3B), compared to the untreated control cells. For C-9 cells, as for the case of PH, PTA expression increased ($p < 0.001$) in proliferation and decreased ($p < 0.001$, for TGF-β1 and $p < 0.01$, for CHX) in apoptosis (Fig. 4B). In summary, in both cell types studied, PTA expression increases during proliferation and decreases in apoptosis.

PTA shows different subcellular localization in apoptotic or proliferating hepatocytes

To determine if PTA localizes in different subcellular compartments during hepatocyte proliferation and apoptosis, we studied the protein localization by immunocytochemistry and confocal microscopy. Although nuclei were not stained to observe the typical DNA fragmentation of apoptosis, apoptotic cells were identified by their typical morphology. In the case of PH, quiescent cells (Fig. 5A) showed the presence of PTA in the nucleus, with a punctuate pattern and a more intense staining in the perinuclear zone. In proliferating preparations (Fig. 5B) the nucleus from certain cells, probably next to division, presented more intense fluorescence or increased expression of the protein. On the contrary, the mitotic cells found in these preparations showed cytoplasmic PTA. On the other hand, the protein showed cytoplasmic location in those cells with the typical morphologic characteristics of late apoptosis, and apoptotic bodies also showed intense staining for PTA. Typical early apoptotic (Fig. 5C) cells, showed perinuclear staining for PTA and very weak nuclear fluorescence, indicating a lower expression of the protein in the nucleus of these cells. In the case of C-9 cells, PTA localization showed a similar pattern to that obtained in PH. PTA was found nuclear in quiescent cells (Fig. 5D) with a dotted pattern and strong perinuclear fluorescence. On the other hand, in proliferating preparations (Fig. 5E) dividing C-9 cells evidenced cytoplasmic PTA, while cells which have already entered division, showed more intense staining in the nucleus. For C-9 cells with morphologic changes typical of apoptosis (Fig. 5F) PTA resulted strongly cytoplasmic and apoptotic bodies also showed intense red fluorescence for the protein. Some cells, probably in early apoptosis, presented weak nuclear staining. We also observed cells showing perinuclear PTA, but prac-

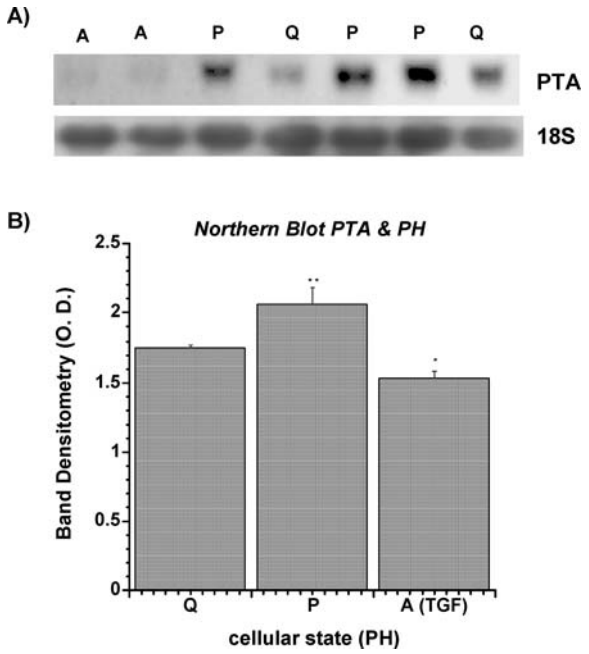

Fig. 3. PTA mRNA levels change in apoptotic and proliferating primary hepatocytes. PH were stimulated with 2.5 ng/mL HGF during 6 days (proliferating), or incubated in the presence of 1 ng/mL TGF-β1 for 24 hr (apoptotic). RNA was extracted, electrophoresed, transfered and hydridised with a PTA probe as described in *Materials and methods*. rRNA 18S was used to ensure equality of loading among samples. (A) Autoradiography of PTA and RNA 18S hybridisation; A: apoptotic cells by TGF-β1, P: proliferating by HGF, Q: quiescent controls. (B) Optical densities (OD PTA/18S) obtained from densitometries of the bands in autoradiographies. Results are expressed as the mean $\pm$ standard error obtained from four independent experiments.

tically no fluorescence in the nucleus, indicating that these cells might have entered apoptosis and, in agreement with the rest of the results, have low expression of the protein.

Discussion

Although PTA has been involved in proliferation and apoptosis of many cell types, the precise role of the protein in these processes remains to be elucidated. To our knowledge the basic behaviour of PTA in hepatocytes undergoing proliferation or cell death has not been studied. In this work, we studied the expression levels of the protein and its subcellular localization in hepatocytes to obtain preliminary data that could contribute to elucidate the biologic activity of PTA in hepatocyte growth and programmed death.

Clone-9 cell line was selected as a model of hepatocytes in culture from normal rat tissue to compare their results with the obtained in primary rat normal hepatocytes. Our results suggest that it is a good model analogous to primary hepatocytes for different experiments.

To determine changes in PTA expression in proliferating and apoptotic hepatocytes, mRNA levels of the protein were studied by Northern Blot. Primary proliferating hepatocytes showed a significant increase in this protein expression, in agreement with the results of other authors [24–27]. The overexpression of PTA in proliferating PH in vitro is reported for the first time in this work. Other studies in liver [19, 20] had demonstrated that PTA is increased in hepatic regeneration and hepatocellular carcinoma. Accordingly, our results suggest that PTA increments are likely related to normal or pathologic cellular proliferation. On the contrary, apoptotic PH showed decreased levels of PTA mRNA. Our results are coincident with studies on other cell types, reporting that in apoptotic cells there is a depletion of PTA due to the proteolytic cleavage of the protein [12]. In cells undergoing apoptotic death, the decrease of mRNA levels, which reflects a reduced synthesis of PTA, is further augmented by its degradation. These results suggest that the low levels of PTA that may exist in the cells to undergo apoptosis might be generated by the cleavage of the preexisting protein and the reduced synthesis of new molecules of PTA. To check if the results we obtained in PH were reproducible in other cells we used clone-9 cells derived from normal Sprague Dawley rat liver cells. In clone-9 cells, as for primary hepatocytes, mRNA levels of PTA were elevated in proliferating cells, and decreased in apoptotic ones, although magnitudes of changes for the cell line were much more pronounced than those detected on primary cells.

Physiological PTA depletion, produced by caspases cleavage or decreased synthesis of the protein, or experimentally induced by treatment with antisense oligonucleotides, promotes the change from proliferation to apoptosis. The high levels of PTA produced in cancerous cells probably contribute not only to maintain its high proliferative state, but also may protect them from entering the apoptotic pathway, preventing tumoral cell death. Further research would be required in order to explore the possibility of controlling tumoral cell growth through the manipulation of PTA levels.

We have also investigated the subcellular localization of PTA by immunocytochemistry and confocal microscopy. PTA was detected in nucleus of primary hepatocytes under quiescence or proliferation, but the intensity of staining was much more strong in growing cells, indicating an increased expression of the protein, in agreement with our results obtained by Northern Blot. Other authors had also reported the increased expression of PTA in proliferating cells [24, 28] and although its precise function is still unknown, it has been mainly related to the proliferative process [25, 26]. PTA nuclear location has been widely described and although it is detected under some circumstances in the cytoplasm where the protein is synthesized, it is considered as a nuclear protein [29, 30]. It has also been described a nuclear localization signal in PTA molecule, that mediates the active nuclear import and the accumulation of the protein in the nucleus [31]. The nuclear

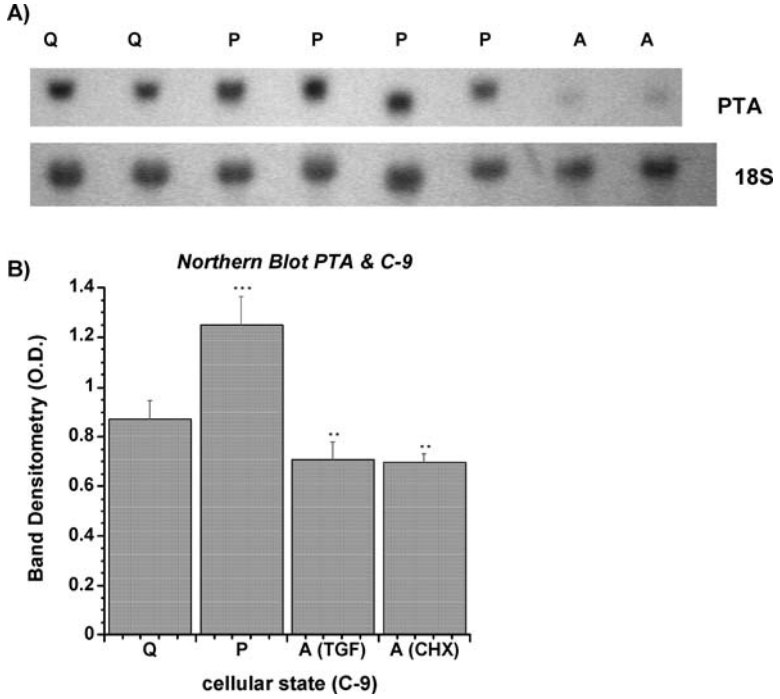

Fig. 4. PTA mRNA levels change in apoptotic and proliferating clone-9 cells. Apoptotic C-9 were obtained incubating cultured cells with 2.5 ng/mL TGF-β1 or 300 μM CHX for 48 hr. Proliferating or quiescent cells were obtained from subconfluent monolayers or by contact inhibition and serodeprivation, respectively. RNA was extracted, electrophoresed, transfered and hydridised with a PTA probe as described in *Materials and methods*. rRNA 18S was used to ensure equality of loading among samples. (A) Autoradiography of PTA and RNA 18S hybridization A(TGF): apoptotic cells by TGF-β1, A(CHX): apoptotic cells by CHX, P: proliferating by HGF, Q: quiescent controls. (B) Optical densities (OD PTA/18S) obtained of densitometries of the bands in autoradiographies. Results are expressed as the mean $\pm$ standard error obtained from five independent experiments.

localization of the protein has been related with transcription-related processes [27] and was observed in the mitotic spindle during mitosis [32]. Here we localized PTA in the cytoplasm of primary hepatic cells in division, with high intensity of fluorescence, which indicates a high cytoplasmic expression of the protein. These results are coincident with Enkemann *et al.* [12], reporting that PTA was detected in the cytoplasm of other types of dividing cells. This cytoplasmic location of PTA is transient, because it is actively transported to the nucleus after the nuclear membrane is established. Apoptotic primary hepatocytes presented cytoplasmic PTA and the apoptotic bodies also showed intense staining for the protein. This cytoplasmic localization can be explained by the cleavage by caspases in the nuclear localization signal, which prevents PTA to reach the nuclear compartment [11, 33]. Some cells in the apoptotic preparations, probably in early apoptosis, showed very weak staining for the protein with more intense perinuclear fluorescence. These results, that indicate a lower expression of PTA in the nucleus of these cells, are in agreement with the mRNA levels detected in this work. This stage would be previous to the rupture of the nuclear membrane and the cleavage of PTA by caspases, because once these events start, the protein would begin to accumulate in cytoplasms.

For clone-9 cells, PTA was localized as in PH cells. Proliferating and quiescent cells presented nuclear PTA, with more intense staining for the growing cells owed to the increased expression of the protein. Mitotic cells showed cytoplasmic PTA with high fluorescence intensity or high PTA expression. Cells in apoptosis presented cytoplasmic PTA because of the protein exit to the cytoplasm or the impossibility to enter the nucleus. Early apoptotic cells showed weak staining in the nucleus and some perinuclear staining, evidencing low expression of the protein and yet no cytoplasmic accumulation.

Our results obtained in hepatocytes demonstrate the nuclear location of PTA and the increased expression during proliferation, providing with more evidences for the potential participation of PTA in hepatocyte proliferation, functioning in the nucleus. The facts that PTA exits cytoplasm, cannot enter the nucleus or is cleaved by caspases during apoptosis, suggest that this protein must not be present in the nucleus, probably carrying out its function, for hepatocyte apoptosis to proceed. Probably the permanence of PTA in the nucleus would exert an antiapoptotic effect. Other evidences suggest an antiapoptotic role for PTA. It was found to negatively regulate caspase-9 activation by inhibiting the formation of the apoptosome. It may act as an oncogene by

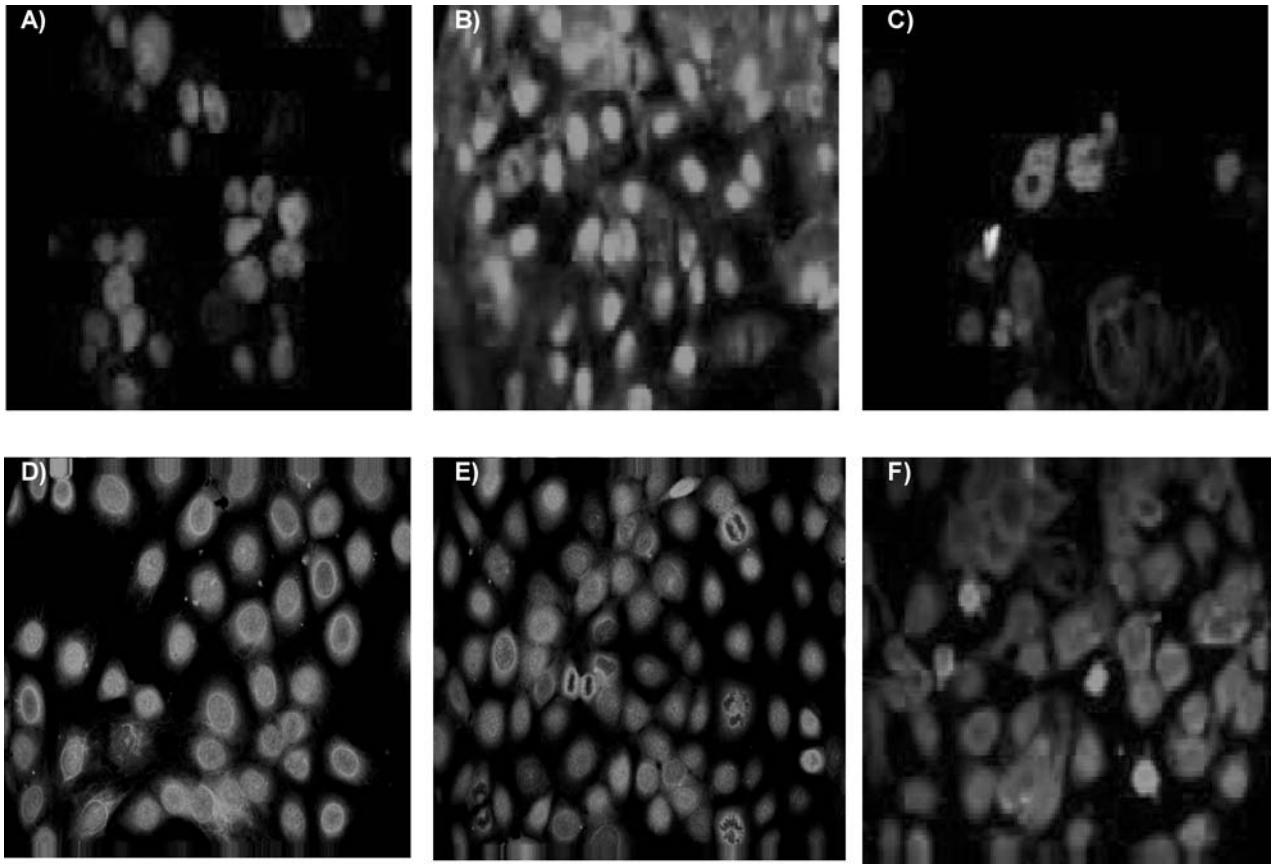

Fig. 5. PTA changes its localization in quiescent, proliferating and apoptotic hepatic cells. Cells grown in coverslips were treated for induction of apoptosis or proliferation as previously described, fixed with paraformaldehyde and permeabilized with Triton-X100. PTA was detected with a rabbit anti-PTA antibody and an anti-rabbit-Texas Red secondary antibody. The preparations were evaluated by confocal microscopy. PTA immunostaining of quiescent (A), proliferating (B) and apoptotic (C) primary hepatocytes and quiescent (D), proliferating (E) and apoptotic (F) clone-9 cells.

virtue of inhibiting caspase activation via the mitochondrial pathway [34].

The increases in PTA mRNA levels detected in proliferating hepatocytes are in agreement with the intensities of fluorescence detected by confocal microscopy in cells entering the cell cycle or in mitotic cells. Although the immunocytochemistry allowed us to correlate the levels of mRNA with the expression of the protein, to assure that the incremented mRNA levels are translated to protein it would be necessary to determine the levels of PTA by Western Blot. For apoptotic hepatocytes, the decreased levels of mRNA detected by Northern Blot, which imply a decreased synthesis of the protein, were also reflected by immunochemistry with a weak nuclear staining for PTA. The intense staining for PTA detected in apoptotic bodies would come from the already synthesized protein, probably cleaved by caspases that concentrates in these bodies.

In conclusion, the differences in PTA expression and localization during hepatocyte proliferation and apoptosis suggest that PTA may have a pleiotropic function that depends not only on its availability but also on its various localizations in different cell compartments. Our data suggest that PTA could be used not only as a marker of cell proliferation but also as a marker for hepatocyte apoptosis and that it may be involved in the pathogenesis of liver diseases by deregulating any of these processes.

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References

1. Haritos AA, Goodall GJ, Horecker BL: Prothymosin alpha: isolation and properties of the major immunoreactive form of thymosin alpha 1 in rat thymus. Proc Natl Acad Sci 81: 1008–1011, 1984

2. Roson E, Garcia-Caballero T, Heimer EP, Felix AM, Dominguez F: Cellular distribution of prothymosin a and parathymosin in rat thymus and spleen. J Histochem Cytochem 38: 1889–1894, 1990
3. Roson E, Gallego R, Garcia-Caballero T, Heimer EP, Felix AM, Dominguez F: Prothymosin alpha expression is associated to cell division in rat testis. Histochemistry 94: 597–599, 1990
4. Gallego R, Roson E, Garcia-Caballero T, Fraga M, Forteza J, Dominguez F, Beiras A: Prothymosin alpha expression in lymph nodes and tonsils. An optical and ultrastructural study. Acta Anat 143: 219–222, 1992
5. Zalvide J, Cancio E, Alvarez CV, Regueiro BJ, Dominguez F: Prothymosin alpha mRNA levels are invariant throughout the cell cycle. J Biol Chem 267: 8692–8695, 1992
6. Alvarez CV, Zalvide JB, Cancio E, Regueiro BJ, Vega FV, Dominguez F: Regulation of prothymosin alpha mRNA levels in rat pituitary tumor cells. Neuroendocrinology 57: 1048–1056, 1993
7. Alvarez CV, Zalvide JB, Cancio E, Dieguez C, Regueiro BJ, Vega FV, Dominguez F: Prothymosin alpha mRNA is expressed in competent and proliferating rat thyroid cells (FRTL-5) but is not sufficient to elicit cell progression throughout the cell cycle. J Mol Endocrinol 11: 249–256, 1993
8. Sburlati AR, Manrow RE, Berger SL: Prothymosin alpha antisense oligomers inhibit myeloma cell division. Proc Natl Acad Sci 88: 253–257, 1991
9. Wu CL, Shiau AL, Lin CS: Prothymosin alpha promotes cell proliferation in NIH3T3 cells. Life Sci 61: 2091–2101, 1997
10. Rodriguez P, Vinuela JE, Alvarez-Fernandez L, Buceta M, Vidal A, Dominguez F, Gomez-Marquez J: Overexpression of prothymosin alpha accelerates proliferation and retards differentiation in HL-60 cells. Biochem J 331: 753–761, 1998
11. Evstafieva AG, Belov GA, Kalkum M, Chichkova NV, Bogdanov AA, Agol VI, Vartapetian AB: Prothymosin alpha fragmentation in apoptosis. FEBS Lett 467: 150–154, 2000
12. Enkemann SA, Wang RH, Trumbore MW, Berger SL: Functional discontinuities in prothymosin alpha caused by caspase cleavage in apoptotic cells. J Cell Physiol 182: 256–268, 2000
13. Kitamura T, Watanabe S, Sato NJ: Gastroenterol Hepatol Supl 1: S96–99, 1998
14. Benedetti A, Marcucci L: The significance of apoptosis in the liver. Liver 19: 453–463, 1999
15. Kanzler S, Galle P: Apoptosis in the liver. Semin Cancer Biol 10: 173–184, 2000
16. Rust C, Gores G: Apoptosis in liver disease. Am J Med 108: 567–574, 2000
17. Schuchmann M, Galle P: Apoptosis in liver disease. European J Gastroenterol Hepatol 13: 785–790, 2001
18. Fraga M, Garcia-Caballero T, Dominguez F, Perez-Becerra E, Beiras A, Forteza J: Immunohistochemical location of prothymosin alpha in regenerating human hepatocytes and hepatocellular carcinoma. Virchows Archiv A Pathol Anat Histopathol 423: 449–452, 1993
19. Wu CG, Boers W, Reitsma PR, van Deventer SJ, Chamuleau RA: Overexpression of prothymosin alpha, concomitant with c-myc, during rat hepatic carcinogenesis. Biochem Biophys Res Commun 32: 817–821, 1997
20. Wu CG, Habib NA, Mitry RR, Reitsma PH, van Deventer SJ, Chamuleau RA: Overexpression of hepatic prothymosin alpha, a novel marker for human hepatocellular carcinoma. Br J Cancer 76: 1199–1204, 1997
21. Pazo JA, Rodriguez ME, Vega F, De la Cruz L, Guibert E, Mediavilla MG, Furno G, Rodriguez JV: Primary Culture of Rat Hepatocytes After Cold Storage in the University of Wisconsin Solution: A Tool to Study the Effects of Hypothermic Preservation. Cell Preservation Technology 1: 189–199, 2002
22. Carneiro C, Alvarez CV, Zalvide J, Vidal A, Dominguez F: TGF-β1 actions on FRTL-5 cells provide a model for the physiological regulation of thyroid growth. Oncogene 16: 1455–1465, 1998
23. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159, 1987
24. Smith MR, al-Katib A, Mohammad R, Silverman A, Szabo P, Khilnani S, Kohler W, Nath R, Mutchnick MG: Prothymosin alpha gene expression correlates with proliferation, not differentiation, of HL-60 cells. Blood 82: 1127–1132, 1993
25. Castro JM, Barcia MG: Localization of prothymosin alpha in the nucleus. Biochem Biophys Res Commun 224: 140–146, 1996
26. Vareli K, Tsolas O, Frangou-Lazaridis M: Regulation of prothymosin alpha during the cell cycle. Eur J Biochem 238: 799–806, 1996
27. Vareli K, Frangou-Lazaridis M, van der Kraan I, Tsolas O, van Driel R: Nuclear distribution of prothymosin α and parathymosin: Evidence that prothymosin is associated with RNA synthesis processing and parathymosin with DNA replication. Exp Cell Res 257: 152–161, 2000
28. Conteas CN, Mutchnick MG, Palmer KC, Weller FE, Luk GD, Naylor PH, Erdos MR, Goldstein AL, Panneerselvam C, Horecker BL: Cellular levels of thymosin immunoreactive peptides are linked to proliferative events: evidence for a nuclear site of action. Proc Natl Acad Sci 87: 3269–3273, 1990
29. Manrow RE, Sburlati AR, Hanover JA, Berger SL: Nuclear targeting of prothymosin alpha. J Biol Chem 266: 3916–3924, 1991
30. Clinton M, Graeve L, El-Dorry H, Rodriguez-Boulan E, Horecker BL: Evidence for nuclear targeting of prothymosin and parathymosin synthesized in situ. Proc Natl Acad Sci 88: 6608–6612, 1991
31. Gomez-Marquez J, Segade F: Prothymosin alpha is a nuclear protein. FEBS Lett 226: 217–219, 1988
32. Vareli K, Frangou-Lazaridis M: Prothymosin α is localized in mitotic spindle during mitosis. Biol Cell 96: 421–428, 2004
33. Evstafieva AG, Belov GA, Rubtsov YP, Kalkum M, Joshep B, Chichkova NV, Sukhacheva EA, Bogdanov AA, Petterson RF, Agol VI, Vartapetian AB: Apoptosis-related fragmentation, translocation, and properties of human prothymosin alpha. Experim Cell Res 284: 211–223, 2003
34. Jiang X, Kim HE, Shu H, Zhao Y, Zhang H, Kofron J, Donnelly J, Burns D, Ng SC, Rosenberg S, Wang X: Distinctive roles of PHAP proteins and prothymosin-alpha in a death regulatory pathway. Science 29: 223–226, 2003