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Immunohistochemical location of prothymosin alpha in regenerating human hepatocytes and hepatocellular carcinomas

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Abstract. In the present paper we analysed the presence of prothymosin alpha (ProT) in human liver. In normal liver, ProT immunostaining was found in the nuclei of bile duct cells, but not in the hepatocytes. In contrast an intense immunoreactivity was observed in regenerative hepatocytes of chronic hepatitis, cirrhosis and in hepatocellular carcinomas. In all cases the immunostaining was restricted to the nuclei, but the nucleoli were always negative. Similar results were obtained for proliferating cell nuclear antigen. These findings confirm that ProT is related to cell proliferation and provides a new immunohistochemical proliferation marker for routinely processed samples.

Key words: Prothymosin alpha – Cell proliferation – Chronic hepatitis – Cirrhosis – Hepatocarcinoma

Introduction

Prothymosin alpha (ProT) is an acidic polypeptide originally isolated from rat thymus (Haritos et al. 1984a). Thymosin alpha 1 (a sequence of ProT obtained by proteolitic modification during the process of biochemical extraction: Hannapel et al. 1982) has been associated with regulation of cellular immunity (Low et al. 1979; Zatz and Goldstein 1985). However, a growing body of evidence suggests that ProT plays an essential role in cell proliferation. ProT shows a wide distribution in many tissues (Haritos et al. 1984b; Economou et al. 1988) perhaps related to a widespread function and not restricted to the immune system; it is highly conserved (Makarova et al. 1989; Oates and Erdos 1989); its mRNA is found in proliferating cells but not in resting cells (Eschenfeldt and Berger 1986); and higher levels of ProT mRNA are found in the fetus and pups of rats than in adults (Dosil et al. 1990). Further, ProT antisense oligomers inhibit myeloma cell division (Sburlati et al. 1991) and ProT gene transcription is regulated by the proto-oncogene *myc* (Eilers et al. 1991). We have previously demonstrated ProT immunoreactivity in proliferating cells of lymphoid (Roson et al. 1990b) and non-lymphoid tissues (Roson et al. 1990a), and the aim of the current communication was to study the immunohistochemical expression of ProT in normal human liver and in the pathological liver processes that involve hepatocytic proliferation such as chronic hepatitis, cirrhosis and hepatocellular carcinomas. A comparative study between ProT and proliferating cell nuclear antigen (PCNA) was also made.

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Materials and methods

Samples of normal human liver, chronic hepatitis, cirrhosis and hepatocellular carcinomas ($n = 10$ each group) were obtained from biopsies or recent autopsies. The categories of the different entities selected for this study where those in which great cell proliferative activity was expected. Thus, only cases of chronic active hepatitis, cirrhosis (macronodular or mixed type) with moderate or severe activity, and moderately or poorly differentiated hepatocellular carcinomas were studied. All the hepatocarcinomas were associated with cirrhosis and no cases of the fibrolamellar variant were included. Specimens were immersion-fixed in 10% buffered formalin for 24 h, dehydrated and embedded in paraffin. The avidin-biotin-peroxidase complex (ABC) procedure was employed. After conventional blocking steps (0.3% hydrogen peroxide: Merck, Darmstadt, Germany, for 10 min, and goat or rabbit normal serum: Dakopatts, Glostrup, Denmark, diluted 1:10, for 30 min), the sections 4 μm thick were consecutively incubated in: a) 0.1% streptavidin (Sigma Chemical Co, St. Louis, Mo.) and 0.01% d-biotin (Sigma) for 30 min. each, to block endogenous biotin; b) 0.1 mg/ml IgG fractions purified from antiserum anti-thymosin alpha 1, obtained by F. Dominguez, or monoclonal antibody anti-proliferating cell nuclear antigen (PC10 Dakopatts) at a dilution 1:10, both for 1 h; c) biotinylated goat anti-rabbit immunoglobulins (Dakopatts) or biotinylated rabbit anti-mouse immunoglobulins (Dakopatts), both at a dilution of 1:400, for 30 min; d) avidin-biotin-peroxidase complex (Vectastain Elite Kit, Vector, Burlingame, Calif.) prepared

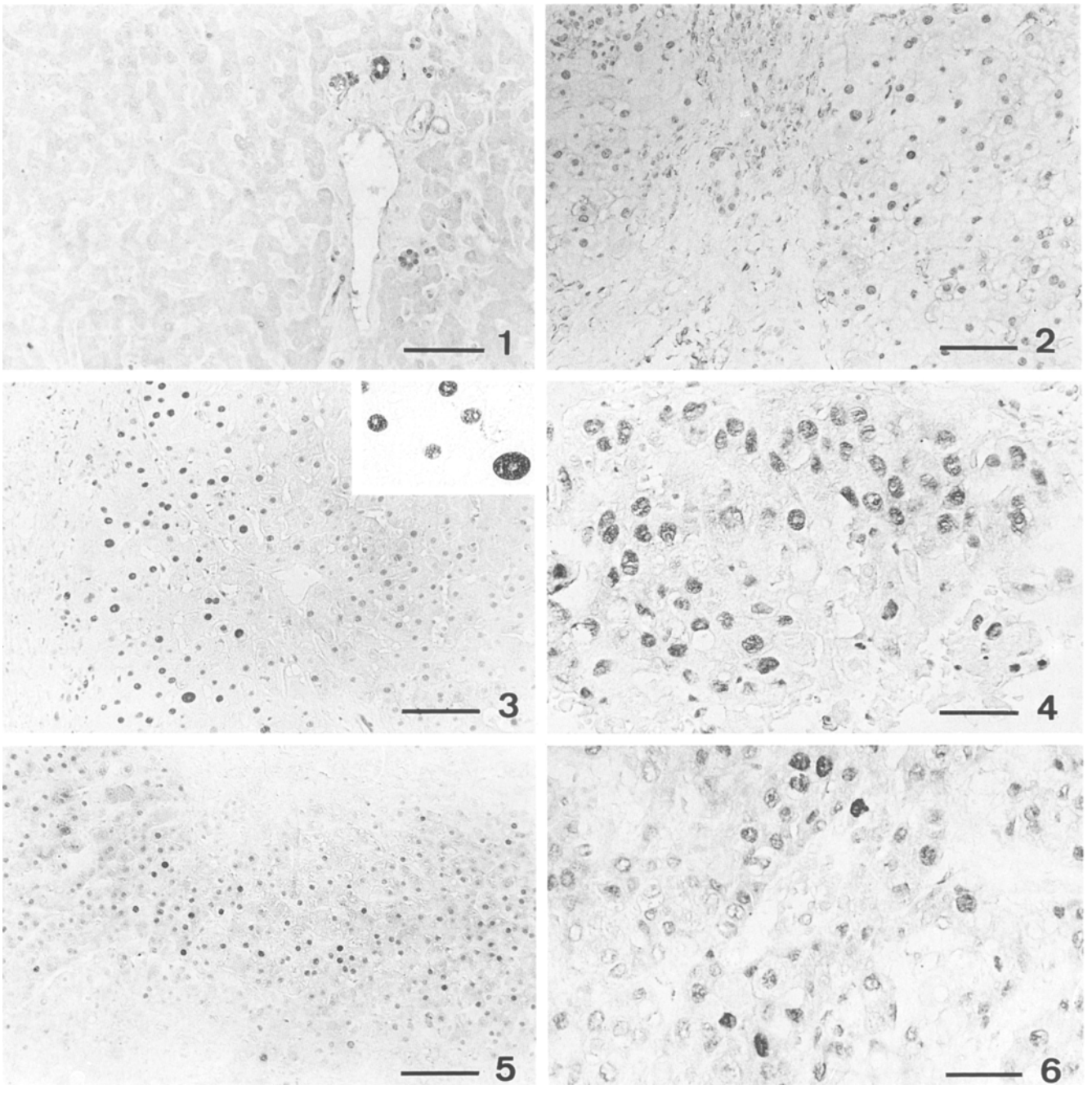

Fig. 1. Normal liver shows prothymosin alpha (ProT) immunoreactivity in the nuclei of bile duct epithelial cells. The hepatocytes are not immunostained. × 125, bar = 100 μm

Fig. 2. ProT immunoreactivity in chronic active hepatitis. Positivity is found in the nuclei of numerous regenerative hepatocytes. × 150, bar = 80 μm

Fig. 3. In cirrhosis ProT immunoreactivity is also seen in the nuclei of hepatocytes. The intensity of immunostaining decreases from the fibrotic septum *(left)* to the centre of the regenerative nodule *(right)*. *Inset:* Note that nucleoli are negative. × 125, bar = 100 μm; *inset* × 350

Fig. 4. ProT immunostaining in hepatocellular carcinoma. Positivity is diffusely distributed and confined to the nuclei of a great number of tumour cells. × 250, bar = 50 μm

Fig. 5. Proliferating cell nuclear antigen (PCNA) in cirrhosis also shows positivity in the nuclei of regenerative hepatocytes. Nucleoli are not spared. × 125, bar = 100 μm

Fig. 6. PCNA in the same hepatocarcinoma case of Fig. 4. A great number of nuclei are also positive, but the intensity of immunostaining is weaker compared to ProT. × 250, bar = 50 μm

according to the protocol provided by the manufacturer, for 30 min; e) 0.06% (w/v) solution of 3,3′ diaminobenzidine-tetrahydrochloride (Sigma) with 0.003% (v/v) hydrogen peroxide for 5 min. Between steps, the sections were washed with 0.01 M phosphate buffered saline (PBS) pH 7.4 and after step e), with distilled water. All dilutions were made in PBS. No counterstaining was done. Controls were performed by either omitting essential steps of the reaction or preadsorbing the non-commercial antibody anti-ProT with synthetic thymosin alpha 1 overnight at 4° C. In neither case was immunoreactivity seen.

Results

In normal human liver ProT immunoreactivity was confined to the nuclei of bile ductular epithelial cells and occasional sinusoidal cells. Virtually no hepatocytic immunostaining was noted (Fig. 1). In contrast in chronic active hepatitis a great number of hepatocytes showed nuclear immunostaining for ProT (Fig. 2). Similar results were obtained in cirrhosis where the positivity was more intense in the periphery of the regenerative nodules (near the fibrotic septa) than in the central areas (Fig. 3). Hepatocellular carcinomas also showed many cells immunostained for ProT randomly distributed throughout the tumour mass (Fig. 4). In all cases of chronic active hepatitis, cirrhosis and hepatocellular carcinomas positivity for ProT was found in the nuclei, and the intensity of immunostaining was variable from one cell to another. Nucleoli were always spared (Fig. 3, inset).

PCNA immunoreactivity was also restricted to proliferating liver cells (Figs. 5, 6). The immunostaining pattern was similar to that observed for ProT. However, both in cirrhosis and hepatocarcinomas, some differences were found. Thus, with PCNA the nucleoli were in general immunoreactive, the intensity of immunostaining for PCNA was frequently weaker than that for ProT (demonstrated in consecutive sections from the same tissue), and aberrant cytoplasmic PCNA positivities were occasionally observed (not shown).

Discussion

As has been previously reported, a relationship between ProT and cell proliferation is undoubted (Eschenfeldt and Berger 1986; Sburlati et al. 1991). The present immunohistochemical study confirms that ProT is expressed by proliferating cells of human liver (demonstrated in chronic hepatitis, cirrhosis and hepatocarcinomas) but not by resting human hepatocytes. The resting concentration of ProT in the liver of different animal species is very low (Haritos et al. 1984b; Tsitsiloni et al. 1989) and it was found that ProT concentration rises after partial hepatectomy in rats (Bustelo et al. 1991). However, at our knowledge, there are no previous reports concerning immunohistochemical demonstration of ProT in human liver. ProT concentration in several tissues is roughly reciprocal in its relationship to parathymosin, which reaches its highest concentration in liver (Haritos et al. 1984b; Clinton et al. 1989; Brand and Heinickel 1991). We have recently showed that parathymosin is expressed by virtually all the nuclei of normal rat and human hepatocytes (Garcia-Caballero et al. 1993), and our results here are not surprising. The ProT positivity found in the nuclei of bile duct epithelial cells of normal liver is also unsurprising because of the well-known capacity for cell renewall in this epithelium.

Different immunohistochemical markers have been employed to study liver proliferation in various pathological conditions (Seki et al. 1990; Kawakita et al. 1992a, b; Kayano et al. 1992; Terada and Nakanuma 1992). The most widely used is PCNA described by Miyachi et al. (1978). PCNA is an auxiliary protein of DNA polymerase-delta (Bravo et al. 1987; Prelich et al. 1987) which plays a critical role in cell proliferation (Jackulski et al. 1988). It is known that both ProT and PCNA are found in cells within the cell cycle but not in the G_0 state. PCNA has the advantage over other procedures for estimating hepatocyte proliferating rates (such as incorporation of tritiated thymidine or 5-bromo-2′-deoxyuridine, or immunostaining with monoclonal antibody Ki 67) of being a simple technique that can be used on routine histopathological material, thus enabling retrospective studies to be made. The antibody to ProT also works well in routine material. Moreover, it has an important advantage over PCNA–PC10; it works well even after prolonged fixation in formalin, whereas as far as 85% of immunoreactivity with PCNA-PC10 is lost after 48 h fixation (Takahashi et al. 1992). Fixation problems could also explain the weak immunostaining frequently observed with PCNA-PC10, as well as the cytoplasmic positivity found in some cases (Benjamin and Gown 1991; Takahashi et al. 1992).

Our findings in chronic active hepatitis are similar to those reported by Kawakita et al. (1992a). However, the distribution of positivity for ProT in cirrhosis (primarily near fibrotic septa) and hepatocarcinomas (diffusely throughout the neoplasm) is in good agreement with the results obtained by Kawakita et al. (1992b) using antibodies to PCNA/cyclin and DNA polymerase-alpha. These authors also showed that no hepatocytic nuclei were positive for PCNA/cyclin or DNA polymerase alpha in the controls (Seki et al. 1990; Kawakita et al. 1992b). Our results with ProT in normal liver are in good agreement with these findings.

Summing up, we have demonstrated that ProT is expressed by liver proliferating cells, providing us of a new tool for evaluating cell proliferation is samples routinely processed. Further cuantitative studies are under way in greater series to validate the usefulness of ProT as a prognostic factor.

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