

Changes in the PP1 mRNA Level and PP1 Activity of Ascites Hepatomas at Different Cell Growth Rates

Mostafa Saadat

Department of Biology, College of Sciences, Shiraz University, Shiraz 71454, Iran

ABSTRACT

It is now well established that the type Ia of serine/threonine protein phosphatases (PP1) may be implicated in malignant phenotype. Although the PP1 mRNA level increased in livers at preneoplastic stages of hepatocarcinogenesis and all of examined rat ascites hepatomas, unexpectedly, dramatically decreased in some of primary hepatomas. In order to elucidate the low level of PP1 mRNA in some of primary hepatomas and investigate any correlation between cell growth rates and change(s) of both PP1 mRNA and PP1 activity, the present experiments were done. The most important aspects of the present study are:" 1) The PP1 mRNA level may decreased in ascites hepatoma AH-109A cells harvested from a highly bloody ascites compared to ones harvested from a milky ascites. 2) The spontaneous PP1 activity in particulate fraction of AH-7974F showed a positive correlation with cell growth rate. 3) There was no correlation between proliferation rate of AH-7974F and the amount of PP1 catalytic subunit as well as potential PP1 activity in particulate fraction. Therefore, it is suggested that the PP1 mRNA level may decreased in the primary hepatomas with low proliferation rate which were under very bad nutritional conditions. *Iran. Biomed. J. 2: 33-37, 1998.*

Keywords: Protein Phosphatases, PP1, Enzyme Activity, Catalytic Subunit, Hepatoma.

INTRODUCTION

The reversible phosphorylation of serine, threonine, and tyrosine residues of proteins is recognized to be a major mechanism for the control of intracellular events in eukaryotic cells. The phosphorylation and dephosphorylation triggers conformational changes in regulatory proteins that alter their biological properties. The level of phosphoproteins is determined by the relative activities of protein kinases and the protein phosphatases [1, 2]. At present, serine/threonine protein phosphatases are classified into four groups, termed PP1, PP2A, PP2B, and PP2C [1]. The type 1 protein phosphatases (PP1) is inhibited by nanomolar concentration of two small heat- and acid-stable proteins, termed inhibitor-1 and inhibitor-2 and preferentially dephosphorylate the γ -subunit of phosphorylase kinase [1]. Four isoforms of the PP1 catalytic subunit have been identified in human (termed PP1 α , PP1 β , PP1 γ , and PP1 δ) and rat (termed PP1 α , PP1 β , PP1 γ , and PP1 δ) by analysis of cDNA clones [2, 3].

During several years ago, it is reported that mRNA level of PP1 was increased under various conditions such as during regeneration of rat liver from 6h after partial hepatectomy [4], in late of G1 phase of synchronized NIH3T3 fibroblasts [5], 3-6 weeks after administration of diethylnitrosamine (DEN) during chemical hepatocarcinogenesis according to the Solt-Farber model [6], all strains of examined rat ascites hepatoma cell lines [6-10] and in some of primary hepatomas [3, 6, 8], compared with their controls. Recently, we are reported that in the particulate and nuclear fractions of ascites hepatomas, potential PP1 activity (PP1 activity after treatment with Co^{2+} /trypsin) and the amount of PP1 catalytic subunit were remarkably increased compared with either regenerating or normal livers as controls [11]. Therefore, these alterations of PP1 in its mRNA level, protein content of its catalytic subunit and PP1 activity in subcellular fraction of hepatomas may be implicated in malignant phenotype. However, the decreases of the PP1 mRNA level in some primary hepatomas were unexpected [3, 6, 12]. In the present study, in order to elucidate this low level of PP1 mRNA in some

of primary hepatomas, it is tried to determined PP1 mRNA level, PP1 enzyme activity and protein content of catalytic subunit of PP1 a in poorly differentiated ascites hepatomas at different cellular growth rates.

MATERIALS AND METHODS

Hepatoma Cells. Male Donryu rats weighing 100-150 gr (5 weeks old) and ascites hepatoma cell lines AH-109A and AH-7974F [13] were used in the experiments. The ascites hepatomas were inoculated intraperitoneally into rats and all of ascites fluid containing hepatoma cells was harvested, and washed with physiological saline (3 times, 4°C) to remove erythrocytes.

RNA Extraction and Northern Blotting. Total RNA was extracted and purified from samples using a single step method of acid guanidium thiocyanate-phenol-chloroform extraction [14]. Total RNA (20 mg/lane) were electrophoresed on a 1.2% agarose gel containing 6% formaldehyde and transferred onto nitrocellulose membrane (Schleicher & Schulle, Dassel, Germany). After being baked at 80°C for 2h under vacuum, the filters were prehybridized in 5x SSPE (1x SSPE; 0.15M NaCl, 0.01M NaH₂PO₄, 1.0M EDTA), 5 x Denhardt's solution, 100 mg/ml denatured DNA, and 50% formamide at 42°C for 2h. For hybridization, the full length of rat PP1 cDNA [3, 7] probe was labeled with [α -³²P] dCTP using the random primer method, and incubated with membranes in the same solution at 42°C overnight. Following hybridization, the filters were washed as described in the figure legends. Washed and dried filters were exposed to XRP-5 X-ray film at -80°C in cassettes with intensifying screens [15].

Preparation of Subcellular Fractions. The subcellular fractionation was carried out at 0-4°C as described previously [10]. In brief, hepatoma cells (2×10^7) or rat liver (0.1 g) were homogenized with 5x2 strokes using a Potter homogenizer in 1 ml of homogenizer solution (150 mM Tris-HCl, pH 7.0, 2 mM EGTA, 4 mg/ml leupeptin, 0.1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride (= PMSF)) and then centrifuged at 800 \times g for 10 min to remove unbroken cells and nuclei. The resulting supernatant was further centrifuged at 100,000 \times g for 60 min to separate cytosol from particulate fraction. The resulting pellet was rinsed with

homogenizer solution, then resuspended with 1 ml of homogenizer solution containing 1% Nonidet P-40 and used as particulate fraction.

Assay of Type 1 Protein Phosphatase. Assay of PP1 was carried out by the method of Cohen et al. with a slight modification as described previously [16]. Briefly, ³²P-labeled rabbit skeletal muscle phosphorylase a (10^6 cpm/nmol) was used as substrate. After pre-incubation, reaction was carried out for 10 min and terminated by adding 20 mM selenic acid in 0.01 M H₂PO₄. PP1 is the activity that is sensitive to inhibitor-2. No PP2B, PP2A, or PP2C activity is detected under these conditions, as described previously [16]. Treatment with Co²⁺/trypsin was carried out as described elsewhere [16]. It is generally believed that conversion of the inactive/latent high-molecular-weight PP1 forms to the active M_r ~35,000 catalytic subunits can be achieved by the Co²⁺/trypsin treatment [12, 16].

All samples were diluted 50 times with reaction buffer containing 1 mg/ml bovine serum albumin and then assayed. The spontaneous and potential activities of PP1 represent ones before and after treatment with Co²⁺/trypsin, respectively. One unit of PP1 activity was defined as the amount of enzyme which catalyzes the release of 1 mmol of phosphate from ³²P-phosphorylase a per min at 30°C.

Western Blot Analysis. Samples were subjected to sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) on a 13% polyacrylamide gel according to the method of Laemmli [17]. The proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated with the anti-PP1 antisera and the immunoreactive bands were visualized using an ECL Western blotting detection kit as described previously [18].

Other Methods. Protein concentration was measured by the method of Bradford [19] with a Bio-Rad protein assay kit using bovine serum albumin as a standard. The immunoreactive bands were scanned with a Personal Densitometer to determine the relative amounts of PP1 catalytic subunit. The statistical significance of the differences between the mean of the samples was determined by Student's t-test [20]. A probability of P<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

It has been known that a tumor increases exponentially in size, then the growth become slow and the tumor eventually shifts into a quasi-stationary state [21-23]. It is showed that the mRNA levels of PP1 in poorly differentiated rat ascites hepatoma cell lines were affected by several factors, including harvest time, transplantation rate, percent of free cells, and sex. The PP1 mRNA level in the ascites hepatomas showed a negative correlation with harvest time [9]. Previously, AH-7974F harvested in exponential growth phase (day 7.0 after transplantation) and quasi-stationary phase (day 8.5 after transplantation) and it is found that the PP1 mRNA level in the quasi-stationary phase of AH7974F significantly decreased compared to that of in exponential growth phase [9].

In order to know more precisely the correlation between hepatoma cell growth rate and PP1 enzyme activity, the spontaneous and potential activity of PP1 in cytosolic and particulate fractions of the hepatomas was measured and compared with those of rat liver (Fig. 1). Both spontaneous and potential PP1 activities in cytosolic fractions of AH-7974F (harvested 7.0 and 8.5 days after inoculation), remain constant at those of normal liver. On the other hand the spontaneous PP1 activity in particulate fraction was significantly increased ($t_{df,6}+2.98$; $P<0.05$) in AH-7974F which harvested day 7.0 compared to normal liver but markedly decreased and reached to its normal level at slower proliferating rates of the AH-7974F which harvested day 8.5 after inoculation ($t_{df,6} = -0.81$; $P>0.05$). Interestingly, AH-7974F cells, irrespective of their harvest time, showed high level of potential PP1 activity in particulate fractions compared to normal liver, that confirm our previous findings [10, 11].

To get more insight into the alteration of PP1 activity in hepatoma, Western blot analysis was performed using specific antisera against the carboxyl-terminal peptide of PP1. It should point out that the intensities of immunoreactive PP1 bands were proportional to the serial dilutions of rat liver extracts (Fig. 2). As show in figure 3, Western blot analysis demonstrated that the amounts of particulate PP1 catalytic subunit in AH-7974F were kept at high levels, which confirming our previous data [11]. However,

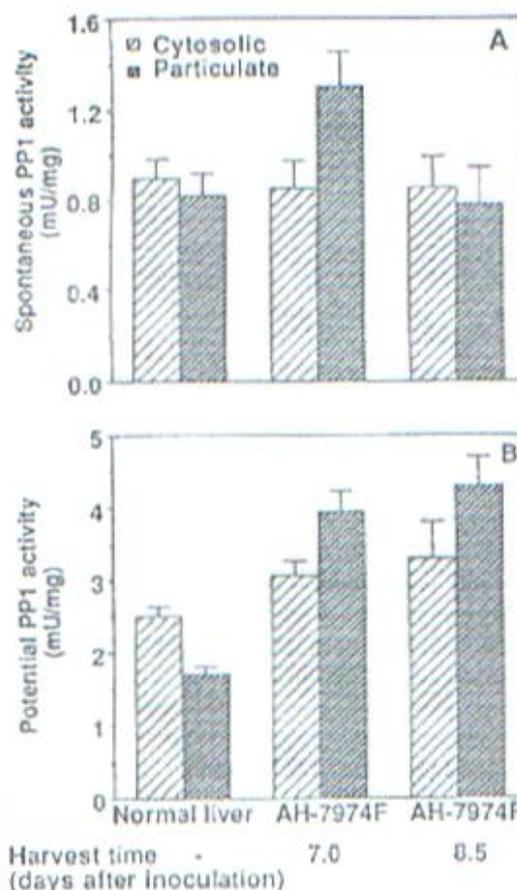


Fig. 1. PP1 activities at different growth stages of neoplastic cells. Spontaneous (A) and potential (B) PP1 activities in cytosolic and particulate fractions of AH7974F at exponentially and quasi-stationary stages, which were harvested 7 and 8.5 days after inoculation, respectively and those of normal liver are shown. Values of normal rat liver and hepatomas represent averages of 6 and 4 assays respectively.

there is no correlation between the hepatoma proliferation rate and the amounts of PP1 in either particulate or cytosolic fractions (Fig. 3).

Figure 4 represents the PP1 mRNA level in normal liver, AH-109A harvested from highly bloody ascites 12 days after transplantation and AH-109A harvested at the same time from a milky ascites. It is clear that PP1 mRNA was increased in AH-109A harvested from milky ascites compared to the bloody one and normal liver. Therefore, low level of PP1 mRNA in AH-109A harvested from bloody ascites may be elucidated by the effect of bad nutritional conditions.

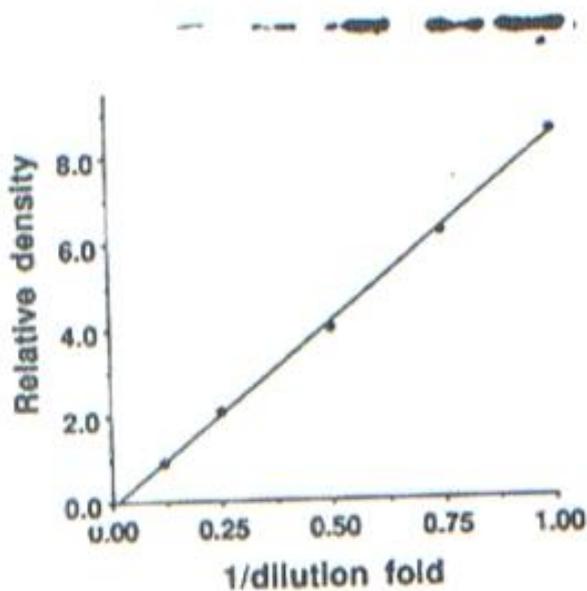


Fig. 2. Correlation of intensities of immunoreactive PP1 bands and amounts of PP1 catalytic subunit. Serially diluted extracts from normal rat liver were subjected to Western blot analysis using antisera against carboxyl-terminal peptide of PP1 catalytic subunit. The immunoreactive bands were scanned with a densitometer, values were normalized to that of the extract diluted 8-fold, and the calculated relative densities were plotted against 1/[dilution fold].



Fig. 3. Western blot analysis of PP1 at different growth rates of hepatoma cells. Samples of 20 mg protein from normal rat liver (lanes 1 and 2), AH-7974F at exponentially (lanes 3 and 4) and quasi-stationary stages (lanes 5 and 6) that harvested 7.0 and 8.5 days after inoculation, respectively, were used for immunoblot analysis. The immunoreactive PP1 bands in cytosolic (A) and particulate (B) fractions of AH-7974F at different growth rates were compared with each other as well as with those of the normal rat liver. In order to compare amounts of PP1 between hepatomas harvested at different growth rates and normal liver, the bands were scanned and values were normalized against those of normal liver. The relative amounts of PP1 in cytosolic fractions of AH7974F harvested at exponentially and quasi-stationary phases are 1.0 and 1.0, respectively; and corresponding values for particulate fractions are 2.9 and 2.9, respectively.

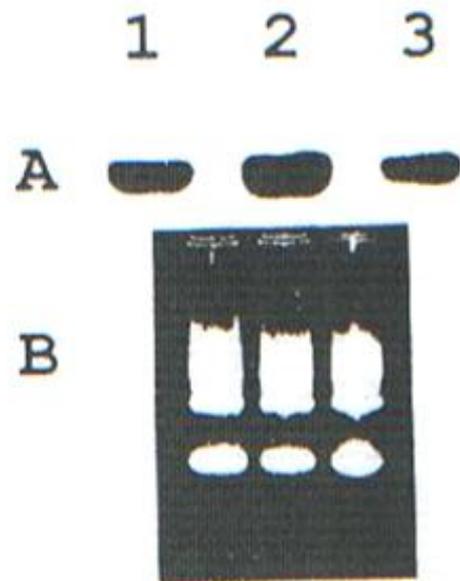


Fig. 4. Effect of blood in ascites on PP1 mRNA level. (A) AH-109A harvested from highly bloody (lane 1) and milky (lane 2) ascites 12 days after inoculation; lane 3 showed normal rat liver. Specific activity of the probe was approximately 5×10^8 cpm/mg DNA. (B) The ethidium bromide-stained gel showed equal amounts of total RNA loaded to gel and RNA not degraded.

Taken together, the present data showed that PP1 mRNA level and the spontaneous PP1 activity in particulate fraction may decrease in poorly differentiated ascites hepatoma cells under critical nutritional conditions, while the amount of PP1 catalytic subunit and potential PP1 activity remained constant at high level compared to the control livers. It should be noted that the primary hepatomas like other solid tumors require plenty of blood supply, but actually the rate of neovascularization often fails to keep pace with tumor growth. Therefore, it is common for subpopulations of cells within solid tumors to experience nutritional poverty. It is suggested that the PP1 mRNA level may decrease in the primary hepatomas which were under very bad nutritional conditions. Further experiments may elucidate the control mechanism(s) of PP1 gene expression in both hepatoma and hepatocyte systems.

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REFERENCES

1. Cohen, P. (1989) The structure and regulation of protein phosphatases. *Ann. Rev. Biochem.* 58: 453-508.
2. Bollen, M., and Stalmans, W. (1992) The structure, roles, and regulation of type 1 protein phosphatases. *Crit. Rev. Biochem. Mol. Biol.* 27: 227-281.
3. Sasaki, K., Shima, H., Kitagawa, Y., Irino, S., Sugimura, T., and Nagao, M. (1990) Identification of members of the protein phosphatase 1 gene family in the rat and enhanced expression of protein phosphatase 1 a gene in rat hepatocellular carcinomas. *Jpn. J. Cancer Res.* 81: 1272-1280.
4. Kakinoki, Y., Kitamura, K., Matsuzawa, S., Mizuno, Y., Miyazaki, T., and Kikuchi, K. (1992) Gene expression and activities of protein phosphatases PP1 and PP2A in rat liver regeneration after partial hepatectomy. *Biochem Biophys. Res. Common.* 185: 291-297.
5. Nakamura, K., Koda, T., Kakinuma, M., Matsuzawa, S., Kitamura, K., Mizuno, Y., and Kikuchi, K. (1992) Cell cycle dependent gene expressions and activities of protein phosphatases PP1 and PP2A in mouse NIH3T3 fibroblasts. *Biochem. Biophys. Res. Common.* 187: 507-514.
6. Kitamura K., Mizuno Y., Hatayama, I., Sato, K., Tamura, S., Nagao, M., Tsuiki S., and Kikuchi K. (1992) mRNA levels of catalytic subunits of protein phosphatases 1, 2A, and 2C in hepatocarcinogenesis. *Jpn. J. Cancer Res.* 83: 66-70.
7. Kitamura, K., Mizuno, Y., Sasaki, A., Tsuiki, S. and Kikuchi, K. (1991) Molecular sequence analysis of cDNA for the catalytic subunit 1 a of rat kidney type 1 protein phosphatase and detection of the gene expression at high levels in hepatoma cells and regenerating livers as compared to rat liver. *J. Biochem. (Tokyo)* 109: 307-310.
8. Kitamura, K., Mizuno, Y., Hatayama, I., Sato, K. and Kikuchi, K. (1993) Selective high expression of protein phosphatase PP1 mRNA in poorly differentiated ascites hepatomas. *Int. J. Oncol.* 2: 237240.
9. Saadat, M., Kitamura, K., Mizuno, Y., Saeki, H., Kudo, T., and Kikuchi, K. (1994) Gene expression of protein phosphatases in rat ascites hepatoma cell lines. *Cancer Detec. Preve.* 18: 115-122.
10. Saadat, M., Saeki, H., Kudo, T., Mizuno, Y. and Kikuchi, K. (1995). mRNA levels and enzyme activities of protein phosphatases in drug-resistant rat ascites hepatomas. *Oncol. Res.* 7: 505-510.
11. Saadat, M., Mizuno, Y., Takizawa, N., Kakinoki, Y. and Kikuchi, K.: (1995) Neoplastic alterations in subcellular distribution of type 1a protein phosphatase in rat ascites hepatoma cells. *Cancer Lett.* 4: 165-170.
12. Kikuchi, K., Mizuno, Y., Kitamura, K., Kakinoki, Y., Nakamura, K., Matsuzawa, S. and Saadat, M. (1993) Expression and activities of protein phosphatases during carcinogenesis, regeneration, and cell cycle. *Adv. Prot. Phosphatases* 7: 221-236.
13. Yoshida, T. (1971) Comparative studies of ascites hepatomas. *Methods. Cancer Res.* 6: 97-157.
14. Chmczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate phenol chloroform extraction. *Anal. Biochem.* 162: 156-159.
15. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual. Second edition. New York. Cold Spring Harbor Laboratory.
16. Matsuzawa, S., Tamura, T., Mizuno, Y., Kobayashi, S., Okuyama, H., Tsukitani, Y., Uemura D. and Kikuchi, K. (1992) Increase in potential activities of protein phosphatases PP1 and PP2A in lymphoid tissues of autoimmune MRL/Mpj 1pr/1pr mice. *J. Biochem (Tokyo).* 111: 472-477.
17. Laemmli, U. K. (1970) Cleavage of structural proteins during the assemble of the head of bacteriophage T4. *Nature* 227: 680-685.
18. Takizawa, N., Mizuno, Y., Saadat, M. and Kikuchi, K. (1994) Selective increases in isoform PP1 of type-1 protein phosphatase in ascites hepatoma cells. *Jpn. J. Cancer Res.* 85: 274-278.
19. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
20. Parker, R. E. (1982) Introductory statistics for biology. Second edition. Edward Arnold Publishers LTD.
21. Patt, H. M. and Straube, R. L. (1956) Measurement and nature of ascites tumor growth. *Ann. NY. Acad. Sci.* 63: 728-737.
22. Watanabe, I. and Okada, S. (1967) Stationary phase of cultured mammalian cells (L5178Y). *J. Cell. Biol.* 35: 285-294.
23. Hatayama, I. and Sato, K. (1980) Changes in the glycogen phosphorylase isozyme pattern of AH-130 during cell growth. *Gann* 71: 875-880.