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Effect of an extract from *Phyllanthus amarus* on hepatitis B surface antigen gene expression in human hepatoma cells

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Summary

It has been suggested that *Phyllanthus amarus* may be helpful in the treatment of hepatitis B virus infection. We studied the effect of an aqueous extract of *P. amarus* on the cultured hepatoma cell line $HepA_2$. This cell line had been transfected with tandemly arranged HBV DNA and continued to synthesize and secrete both HBsAg and HBeAg. Extract of *P. amarus* reversibly inhibited cellular proliferation and suppressed HBsAg production but not HBeAg production in $HepA_2$ cells. We also found that *P. amarus* suppressed HBsAg gene expression at mRNA level in a time-dependent manner, and selectively abolished the HBsAg gene promoter driven CAT activity. Our results demonstrate that *P. amarus* contains some active components which can suppress the HBsAg gene expression in human hepatoma cells. Such suppression may contribute the antiviral activity of *P. amarus* in vivo.

Antiviral agent; Gene regulation; Promoter activity

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; β-gal, β-galactosidase; DMEM, Dulbecco's modified Eagle's medium; kb, kilobase; *P. amarus*, *Phyllanthus amarus*.

Introduction

Hepatitis B virus (HBV) is a causative agent for hepatitis. The viral genome can integrate into patients' liver cell chromosomes and a significant fraction of such patients later develop liver cirrhosis as well as hepatocellular carcinoma (Beasly et al., 1981; Marion et al., 1980; Chakraborty et al., 1980; Brechot et al., 1980; Edman et al., 1980; Sung et al., 1982; Shafritz et al., 1981; Szmuness et al., 1978). Development of a new therapeutic agent is, therefore, of great potential importance in the management of these patients. Recently, antihepatitis activity has been identified in the plant *Phyllanthus amarus* which was originally classified as *Phyllanthus niruri* and widely used in south India as a traditional medicine for jaundice (Thyagarajan et al., 1982). Venkateswaran et al. found that an aqueous extract of this plant not only inhibits endogenous DNA polymerase activity of HBV but also interferes with the binding of the HBV surface antigen (HBsAg) to the HBsAg antibody (anti-HBs) (Venkateswaran et al., 1987). In a clinical trial, Thyagarajan et al. further showed that an oral preparation of this plant significantly enhanced the seroconversion rate of HBsAg in chronic carriers of HBV (Blumberg et al., 1989; Thyagarajan et al., 1988).

In this paper, we report that *P. amarus* inhibits cell proliferation and selectively suppresses HBsAg gene expression in a cultured human hepatoma cell line HepA2 which is a stable HBV DNA transfected HepG2 cells.

Materials and Methods

Cell culture. HepA2 was a clonal derivative of human hepatoma cell line G2 (Aden et al., 1979) which was transfected with tandemly arranged HBV DNAs. The viral DNA had been integrated into a cellular chromosome and was stably maintained in the Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and antibiotics (100 units/ml of penicilline and streptomycin) at 37°C in 5% CO_2/95% air.

Preparation of the aqueous extract of P. amarus. Dried whole plant (40 g) collected from south India was pulverized in a Waring blender and mixed with 200 ml of distilled water. This plant was examined by Prof. G. Webster (Dept. of Botany, University of California at Davis, USA) and confirmed to be identical to the species used by Thyagarajan et al. for their clinical study. The mixture was shaken periodically at 75°C for 2 h and filtered through a nylon mesh. The filtrate was centrifuged at 8000 rpm for 1 h in a Beckman JR10 rotor at 20°C. The supernatant was lyophilized and resuspended into distilled water at the concentration of 50 mg/ml and then filtered through a 0.25 μm filter (Millipore) for cell culture assay.

Assay for HBsAg and HBeAg in culture medium. Culture fluids were collected

and the presence of HBsAg and HBeAg was measured using the enzyme immunoassay kit (Ever New, Taipei, Taiwan).

RNA isolation and Northern blot analysis. Total cellular RNA (20 μg), extracted by the guanidine isothiocyanate/cesium chloride method (Glisin et al., 1974), from control and *P. amarus* extract-treated HepA2 cells was subjected to electrophoresis on a 1.2% agarose/formaldehyde gel and transferred to nitrocellulose paper. After hybridization with the cloned HBsAg DNA as a probe (Lo et al., 1985), the blot was washed with a solution of 0.1 × SSC (1 × SSC, 0.15 M sodium chloride, 0.015 M sodium citrate (pH 7.0)), 0.1% SDS at 65°C for 1 h and autoradiographed at −70°C with intensifying screen (Quanta IV, Du Pont) for 12 h.

Plasmid construction, transfections and enzyme assays. The plasmid pSpCAT contains the 410 base pair *Bgl*II–*Bgl*II fragment of HBV, which was inserted between *Bgl*II and *Hin*dIII site of pSVO[H]CAT[Bgl] (Chang et al., 1987). This sequence corresponds to map position 2828 to 25 of HBV (adw subtype, with the *Eco*RI site number 1). Rous sarcoma virus long terminal repeat driven β-galactosidase (pRSV-gal) was kindly given by Dr. Ming-der Tsai, Baylor College of Medicine, Houston, USA.

HepA2 cells were cotransfected with pSpCAT and pRSV-gal by the calcium phosphate precipitation method (Ausubel et al., 1987). CAT activity was assayed by the scintillation counting method (Neumann et al., 1987). Briefly, cell lysate was added to a scintillation vial, and mixed with 100 mM Tris-HCl (pH 7.8), 1 mM chloramphenicol and 0.2 μCi [^{3}H] acetyl CoA (6.4 Ci/mmol). The reaction mixture was gently overlaid with water immiscible scintillation fluid and counted at selected time intervals. β-Galactosidase activity was assayed according to Nielsen et al. (Mirldrn et al., 1983). Cell lysate was mixed with 0.8 mg/ml *o*-nitrophenol-β-galactose, 100 mM phosphate buffer (pH 7.4), 10 mM KCl, 1 mM $MgCl_2$ and 50 mM β-mercaptoethanol and then incubated at 30°C. The reaction was stopped by adding 0.4 vol. of 1 M Na_2 CO_3 and β-galactosidase activity was measured at 420 nm.

Results

Cell growth of HepA2 cells

HepA2 human hepatoma cells maintained a linear rate of growth after conversion from 10% fetal calf serum-supplemented DMEM to serum free medium. They exhibited approximately three cell doubling over a 4-day period and then reached stationary state when maintained in serum free medium alone. In marked contrast, HepA2 cell growth was dramatically suppressed following the addition of aqueous extract of *P. amarus* (1 mg/ml) (Fig. 1). The extract inhibited HepA2 cell growth almost completely compared with a control. This growth inhibitory action of *P. amarus* extract did not result from

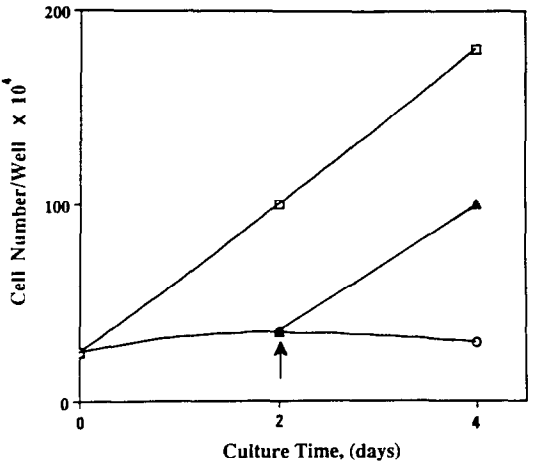

Fig. 1. Effect of *P. amarus* on cell growth of serum-starved human hepatoma cells HepA2. Cells were plated at a density of 1×10^5 cells/cm^3 in DMEM with 10% fetal calf serum and were allowed to attach overnight. Cells were then washed three times with phosphate buffered saline (pH 7.0). The medium was changed to DMEM without serum. After 2 days of serum starvation, the medium was changed to DMEM, either alone (□) or plus *P. amarus* (1 mg/ml) (○) and designated as day 0. The medium was changed every 2 days and the cell number in each well was determined by hemocytometer. In another set of cells, *P. amarus* was removed after 2 days treatment (as arrow indicates) and changed to the control medium. The cells were counted two days later (▲).

toxicity since treated cells still remained viable and resumed a normal growth after replating into a fresh medium.

HBsAg production

Cells were plated into 24-well plate and allowed to attach overnight. The medium was changed to DMEM without serum and plus various concentrations of *P. amarus* extract for 48 h. When the HBsAg and HBeAg in the culture medium was examined, marked suppression of HBsAg was observed. In contrast, the HBeAg, another HBV marker produced by HepA2 cells was not significantly affected (Fig. 2). The *P. amarus* extract itself did not interfere with the enzyme immunoassay of HBsAg determination.

A negligible amount of HBsAg remained in the cell lysate and were detected after drug treatment indicating that the *P. amarus* extract indeed suppressed the HBsAg production but did not block the secretion process of HBsAg in HepA2 cells.

Transcript of HBsAg

To examine the change of HBsAg mRNA during the *P. amarus* treatment, the steady state level of HBsAg mRNA was assayed by Northern blot hybridization using a cloned HBsAg sequence as the probe. One major mRNA band, 2.1 kb in length, could be detected. The dramatic decrease of HBsAg mRNA in HepA2 cells during the *P. amarus* treatment suggested that *P.*

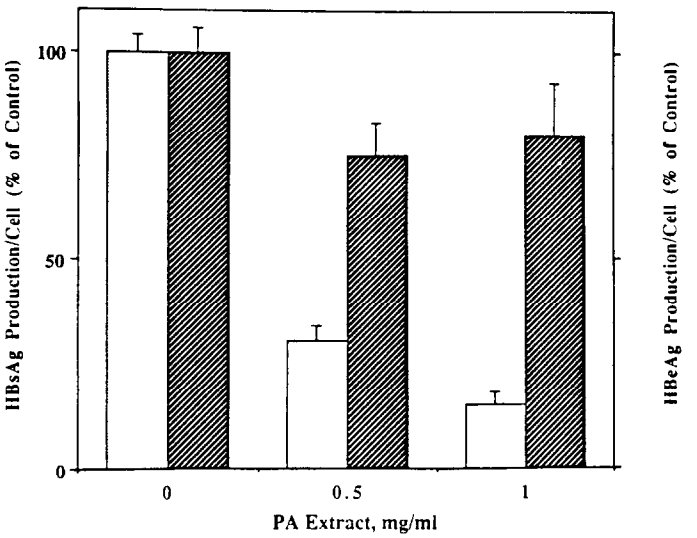

Fig. 2. Effect of *P. amarus* on HBsAg and HBeAg production of HepA2 cells. Cells were plated and cultured in the absence of serum and treated with *P. amarus* for 48 h as described in the legend of Fig. 1. The cell number of each well was determined by hemocytometer and amounts of HBsAg and HBeAg in the culture medium was determined by enzyme immunoassay. Control cells produced 15 ng of HBsAg/10^6 cells/48 h and O.D. 1.2 of HBeAg/10^6 cells/48 h. Data expressed as the mean $\pm$ S.D. ($n = 3$).

amarus suppression of HBsAg gene expression in HepA2 cells was mainly on the mRNA level. The 2.1 kb transcript of HBsAg in HepA2 cells significantly decreased after 12 h of *P. amarus* treatment (Fig. 3).

Promoter activity of HBsAg gene in human hepatoma cells

To test the effect of *P. amarus* extract on HBsAg gene promoter activity, the

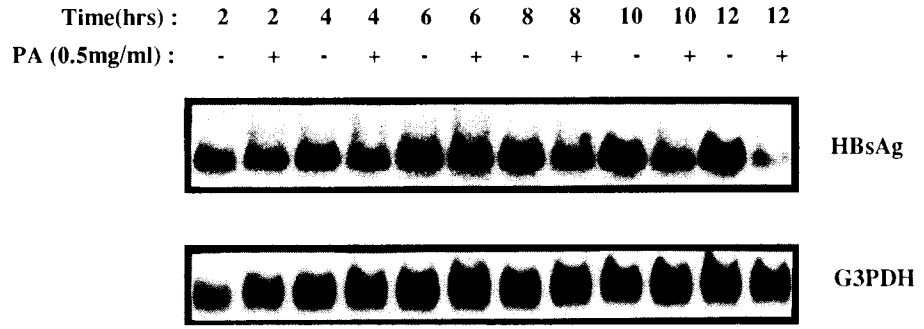

Fig. 3. Time-course of *P. amarus* effect on mRNA level of HBsAg in HepA2 cells. HepA2 cells were serum starved for 2 days. RNA from control (−) and *P. amarus* (0.5 mg/ml) treated (+) cells were extracted at 2, 4, 6, 8, 10 and 12 h after drug treatments and analyzed by Northern hybridization with HBsAg DNA and glycerol-3-phosphate dehydrogenase (G3PDH) cDNA as described in Materials and Methods. The experiment presented here was performed three times with similar results.

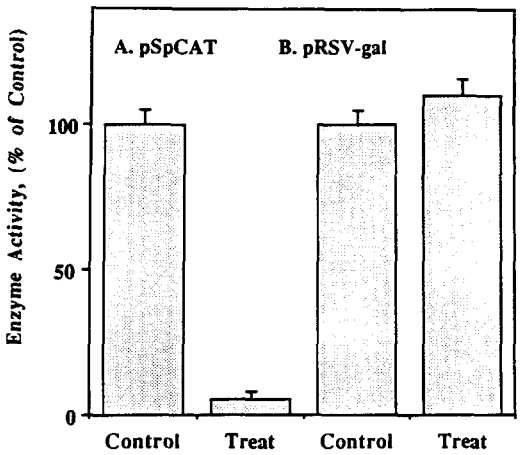

Fig. 4. Effect of *P. amarus* on the promoter activity of HBsAg gene and Rous Sarcoma Virus long terminal repeat. Cells were cotransfected with pSpCAT and RSV-gal as described in Materials and Methods. Cell lysates were prepared by repeatedly freezing and thawing. The CAT and β-gal activity in the cell lysate were assayed as described in Materials and Methods. Data expressed as the mean ± S.D. ($n=3$).

transient CAT expression system was employed in which the promoter of major S antigen in the native HBV organization was used to drive the expression of CAT gene. The result showed a high CAT activity in the control HepA2 cells after transfection with pSpCAT. However, *P. amarus* treatment could almost completely block major S antigen promoter driven CAT expression. In contrast, *P. amarus* extract had no effect to the Rous sarcoma virus long terminal repeat driven β-galactosidase expression in HepA2 cells (Fig. 4).

Discussion

In this study, we have demonstrated that an aqueous extract of *P. amarus* could reversibly inhibit cell growth and suppress the gene expression of HBsAg in human hepatoma HepA2 cells.

Venkateswaran et al. have previously shown that an extract of *P. amarus* inhibited the endogenous DNA polymerase and the binding of HBsAg to its specific antibody in vitro (Venkateswaran et al., 1987). Similar observations were made on the replication of woodchuck HV in vivo (Venkateswaran et al., 1987). However, when duck hepatitis B virus infection was examined, Niu et al. were unable to reproduce these effects (Niu et al., 1990). Nevertheless, like the situation in woodchucks, the *P. amarus*-treated ducks showed a reduction in the levels of hepatitis B surface antigenaemia. In the clinical trial of Thyagarajan et al., the oral preparation of *P. amarus* was also shown to increase the clearance rate of the HBsAg carrier state in human subjects (Blumberg et al., 1989; Thyagarajan et al., 1988). These results are consistent

with the hypothesis that *P. amarus* specifically suppresses the gene expression of HBsAg in human liver cell as we have demonstrated here.

The active principle(s) and the mechanism of *P. amarus* actions toward HBsAg gene expression are still unknown. It has been argued that surface antigen binding or chelating properties of *P. amarus* would be an important mechanism in the clearance of the HBsAg in serum. However, using cultured human hepatoma cells as a model system, we have clearly shown here that *P. amarus* can specifically suppress gene expression of HBsAg.

Three lines of evidence support such a suggestion. First, *P. amarus* treatment only suppresses the HBsAg but not another hepatitis B viral maker, HBeAg, in media. Such suppression was not due to the interference by *P. amarus* of the binding of HBsAg to its specific antibody as we tested in our ELISA assay system. Second, suppression of HBsAg production by *P. amarus* in HepA2 cells can be reflected at the mRNA level in a time-dependent manner. In contrast, *P. amarus* has no effect on the transcript of a housekeeping gene glycerol-3-phosphate dehydrogenase. Third, *P. amarus* completely abolished the major surface antigen promoter driven CAT activity, but did not alter the Rous sarcoma virus long terminal repeat driven β-galactosidase activity.

Our results establish a novel biological assay for screening antiviral activity from natural resources in the future. To identify active component in *P. amarus* and to elucidate the mechanism of its antiviral activity are currently under our investigation.

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