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Suppressive effects of destruxin B on hepatitis B virus surface antigen gene expression in human hepatoma cells

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Abstract

Destruxin B, a cyclodepsipeptide was originally identified as a plant pathogen from the fungus, *Alternaria brassicae*. We examined the antiviral activity of destruxin B and found that it suppresses the expression of the hepatitis B viral surface antigen (HBsAg) gene in human hepatoma Hep3B cells which carry an integrated viral gene in its chromosome. In contrast, destruxin B shows no cytotoxic effect on the viability of the cells. Furthermore, it can be shown that destruxin B can reversibly suppress HBsAg production by Hep3B cells in a concentration-dependent manner with EC_{50} of 0.5 μM. Northern blot analysis indicates that the suppression of HBsAg gene expression by destruxin B is mainly at the mRNA level. Destruxin B not only suppresses the endogenously expressed HBsAg in the Hep3B cells but also suppresses the HBsAg produced either from the stable transfected HBV DNA in another human hepatoma HuH-7 cell line which carry no endogenous HBV genome. These results suggest that destruxin B may have future potential for development as a specific anti-HBV drug. © 1997 Elsevier Science B.V.

Keywords: Fungus metabolite; Cyclodepsipeptide; Antiviral agent; Gene regulation

1. Introduction

Hepatitis B virus (HBV) is a small DNA virus. The HBV genome is a circular and partially double stranded DNA consisting of 3.2 kb (Robinson et al., 1974; Robinson, 1977). Infection by HBV commonly results in acute and chronic hepatitis and is also associated with high risks of developing liver cirrhosis and hepatocellular carcinoma (Tong et al., 1971; Szmuness, 1978). Immunization against HBsAg has been shown to be an effective way to prevent both vertical and hori-

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zontal viral infection (Sung et al., 1982; Lo et al., 1988), but has no effect on the existing 200 million chronic carriers around the world. Therefore, development of new antiviral drugs to eradicate HBV in chronic carriers is still urgently needed.

Cultured human hepatoma Hep3B cells contain one or two copies of integrated HBV DNA and continually secrete HBsAg into the culture medium (Brechot et al., 1980). We have previously shown that the HBsAg gene expression in Hep3B cells can be regulated by various agents, including retinoic acid, insulin and glucocorticoids (Hsu et al., 1993; Chou et al., 1989; Kaspa et al., 1986). This cell line, therefore, provides a convenient assay system to screen biologically active components from natural resources which may exhibit anti-HBV activity.

Destruxin B (Fig. 1), was originally identified as a plant pathogen from the fungus *Metarhizium anisopliae* (Kodaira, 1961). More than 20 structurally related destruxins have been isolated which present a variety of biological activities (Suzuki et al., 1970; Pais et al., 1981; Suzuki and Tamura, 1971; Gupta et al., 1989). For example, the destruxin E was shown to disturb macromolecular syntheses and to have antiviral activity on leukemic cells (Odier et al., 1984; Quiot et al., 1985; Odier et al., 1992). Destruxin B has also been reported as a specific inhibitor of vacuolar-type H^+ translocating ATPase (Muroi et al., 1994). Recently we have demonstrated that desmethyldestruxin B2 from *Metarhizium anisopliae* strongly suppresses the secretion of HBsAg in human hepatoma Hep3B cells (Chen et al., 1995).

Since the fungus *Alternaria brassicaea* is another good source of cyclohexadepsipeptide destruxins (Ayer and Pena-Rodriguez, 1987), we have identified homodestruxin B and destruxin B from *A. brassicaea* , which can also suppress HBsAg production by the Hep3B cells (Sun et al., 1994). In the present study, we discuss destruxin B which can directly suppress gene expression of HBsAg in Hep3B cells.

2. Materials and methods

2.1. Materials

HBsAg enzyme immunoassay (EIA) kits were purchased from Ever New (Taipei, Taiwan). α-Fetoprotein enzyme immunoassay (EIA) kits were purchased from GENEMED Biotechnology (Los Angeles, CA). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM) balanced salt solutions were obtained from Gibco/BRL (Gaithersburg, MD). [(α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). Sea Kem LE-agarose was purchased from FMC Bioproducts (Rockland, MA). Other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

Stock cultures of human hepatoma cells, Hep3B (Brechot et al., 1980), Hep3B/C16 (Hsu et al., 1993), and HuH-7 (which have no HBV genomes integrated in its chromosome) were maintained in DMEM medium supplemented with 10% fetal calf serum and antibiotics (100 units/ml each of penicillin and streptomycin) in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. The hepatoma cell line Hep3B/C16 is a clonal derivative of the human hepatoma cell line Hep3B which was stablely transfected with a cloned 2.4 kb DNA fragment corresponding to the intact transcriptional unit of HBsAg

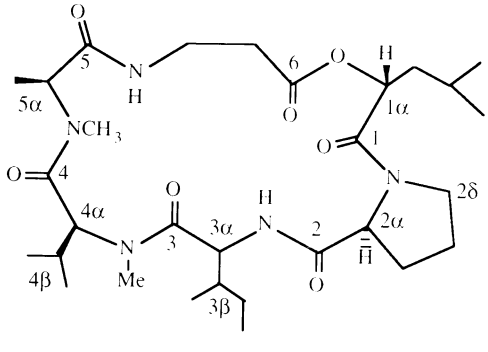

Fig. 1. Chemical structure of destruxin B.

with map position 2839–1896 of HBV (ayw subtype, with the EcoRI site number 0). The cultures were passaged by trypsinization every four days. Cells were plated either in 24-well plates at a density of 1.0×10^5 cells/well or in 100 mm culture dishes at a density of 1.5×10^6 cells/dish in DMEM medium containing 10% fetal calf serum.

2.3. Preparation of destruxin B

The fungus, *A. brassicae*, was grown on a medium containing 10% V-8 juice, 1% glucose, 0.3% $CaCO_3$. The culture flasks were allowed to stand at 26°C for 28 days. The culture broth was extracted with EtOAc and the pure destruxin B was purified by repeated silica gel column chromatography and reversed phase HPLC (Gupta et al., 1989). The purity of destruxin B was judged by mass spectrometry and ^{1}H-NMR spectroscopy. For bioassay, destruxin B was first dissolved in ethanol, filtered through a 0.25 μm fluoropore filter (Millipore), and added to cell cultures.

2.4. Determination of HBsAg and α-fetoprotein production

Human hepatoma cells cultured in DMEM with 10% FCS for 24 h were changed to serum-free DMEM medium with or without various concentrations of destruxin B and incubated for 48 h. The HBsAg or α-fetoprotein in culture media was measured by enzyme immunoassay (EIA) kits. The viability of cells was determined using a hemocytometer and trypan blue dye exclusion.

2.5. RNA isolation and northern blotting analysis

Total cellular RNA was isolated by centrifugation through cesium chloride (Glisin et al., 1974). The RNA (20 μg) was denatured in 6.5% formaldehyde and fractionated by electrophoresis in a 1% agarose gel. The RNA was transferred to a nitrocellulose filter by capillary blotting and immobilized by heating at 80°C for 2 h (Thomas, 1980). The membrane filter was prehybridized for 6 h at 42°C in a solution containing 35% formamide, 5x Denhardt's reagent (1x = 0.02% Ficoll, 0.2% BSA, 0.02% polyvinylpyrrolidone), 5x SSPE (1x = 0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA, 1% SDS, and 500 μg/ml denatured salmon sperm DNA, pH 7.4). A full-length 2.1 kb HBsAg cDNA was labeled with [α-^{32}P]dCTP using a random primers kit. Denatured ^{32}P-labeled probes (10^6 cpm/μg) were added directly to the prehybridization buffer, and hybridization was carried out at 42°C for 36 h. The membrane filter was washed twice in 0.2x SSC (1x = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.2% SDS at 42°C for 15 min, and once in 0.1x SSC, 0.1% SDS at 65°C for 15 min (Thomas, 1980). Autoradiography was performed at −70°C with an intensifying screen (Quanta IV, Du Pont) for 12 h on Kodak SAFET X-ray film.

2.6. Plasmid construction and transfection

The plasmid pC16 consists of a 2329 bp *Bgl II* (2839)-*Bgl II* (1986) DNA fragment of HBV genome. This DNA fragment contains the entire HBsAg coding sequence plus a 300 bp upstream promoter region of HBsAg gene (see Fig. 5 (A)). The *Bgl II*-*Bgl II* fragment of HBV viral DNA was inserted into the *Bam* HI site between *Kpn* I and *Sac* I of pSV2 neo (Chang et al., 1987). HuH-7 cells were transfected with pC16 DNA, using the calcium phosphate precipitation method (Graham and Van der Eb, 1973). Cells were transfected in DMEM medium supplemented with 10% FCS for 16 h. After DMSO shock, cells were changed to serum-free DMEM medium with destruxin B for two days. The amount of HBsAg in the media was determined by enzyme immunoassay (EIA) kits.

3. Results

3.1. Suppression of HBsAg production in Hep3B cells

Hep3B cells were plated into 24-well plates and allowed to attach overnight. The media was subsequently changed to serum free DMEM and various concentrations of destruxin B were added. After 48 h of incubation, HBsAg or α-fetoprotein

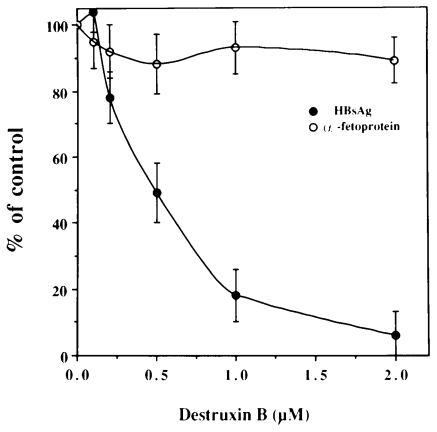

Fig. 2. Effect of destruxin B on the production of HBsAg and (α-fetoprotein in human hepatoma Hep3B cells. Hep3B cells were cultured and treated with various concentrations of destruxin B in serum free DMEM medium for another 48 h as described in Section 2. The amount of HBsAg and α-fetoprotein in culture medium was then determined by enzyme immunoassay. Control cells produced 15 ng of HBsAg/10^6 cells/48 h. Data are expressed as mean ± S.D. ($n = 3$).

in the media were determined. The results showed that destruxin B specifically suppressed HBsAg production with IC_{50} of about 0.5 μM (Fig. 2). In contrast to the suppression of HBsAg, the production of α-fetoprotein remained unaffected by destruxin B up to 2.0 μM. The suppressive effect of destruxin B on HBsAg production was reversible, since the treated cells resumed a normal HBsAg production after the drug was removed (Fig. 3).

3.2. Transcript of HBsAg in destruxin B treated Hep3B cells

To examine the change of HBsAg mRNA during the destruxin B treatment, the steady state level of HBsAg mRNA in the Hep3B cells was assayed by Northern blot hybridization using a cloned HBsAg cDNA as the probe. One major mRNA species, 4.0 kb in length which included the entire HBsAg gene with host flanking sequences, was detected in the Hep3B cells. The dramatic decrease of HBsAg mRNA in the Hep3B cells during destruxin B treatment suggested that the suppression of HBsAg gene expression in Hep3B cells was mainly at the mRNA level (Fig. 4). The suppression of HBsAg mRNA by destruxin B did not require de novo protein synthesis, since the protein synthesis inhibitor, cycloheximide, had no effect on the destruxin B induced suppression of HBsAg mRNA in the Hep3B cells (Fig. 4).

3.3. Suppression of transiently transfected HBsAg DNA in other human hepatoma cells

Since Hep3B cells carry an endogenous viral gene in its chromosome, suppression of HBsAg gene expression in Hep3B cells by destruxin B raised an interesting question about whether destruxin B could directly regulate viral gene expression or may act through host franking sequence of viral DNA integration site in the cells. In order to address this question, we transfected the plasmid pC16 that contains 300 bp promoter region of HBsAg with an intact HBsAg coding sequence into human hepatoma HuH-7 cells which carry no

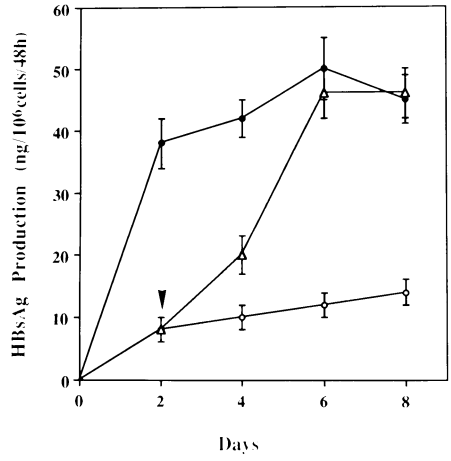

Fig. 3. Reversibility of HBsAg production by Hep3B cells after destruxin B treatment. Hep3B cells were treated without (closed circle) or with 1.5 μM of destruxin B (open circle) as described in Fig. 2 and designated as day 0. The medium with drug was changed every two days and the amount of HBsAg production in culture medium was determined by enzyme immunoassay. In another set of cells, destruxin B was removed after 2 days treatment (as arrow indicates) and changed to the fresh medium without drug, and the medium was changed every two days. The amount of HBsAg in culture medium was determined every two days later (open triangle).

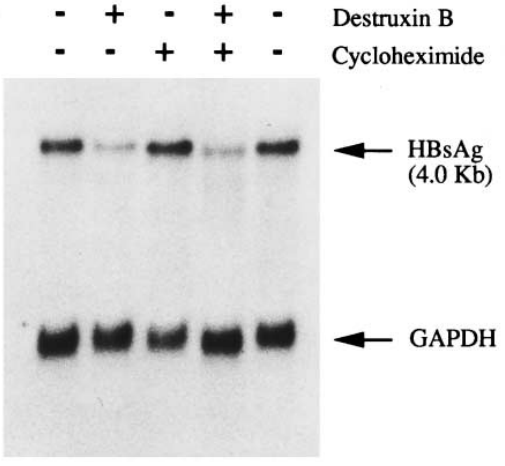

Fig. 4. Effect of destruxin B on the steady state mRNA level of HBsAg in Hep3B cells. Hep3B cells were seeded on 100 mm culture dish at a density 1.5×10^6 cells/dish and treated with 1.0 μM of destruxin B or cycloheximide (1.0 μM) in serum free DMEM medium for 24 h. Total RNA was extracted and analyzed by Northern hybridization with HBsAg DNA as described in Section 2. The constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control.

endogenous HBV DNA. As shown in Fig. 5, destruxin B significantly suppressed HBsAg production by HBsAg DNA transiently transfected HuH-7 cells. Furthermore, the suppressive effect of destruxin B on HBsAg gene expression was also observed in Hep3B/C16 cells (Fig. 6). These result suggest that destruxin B may directly suppress HBsAg gene expression in human hepatoma cells.

4. Discussion

Destruxins, a family of cyclodepsipeptides, were originally isolated as insecticides from fungus *Metarhizium anisopliae* (Suzuki et al., 1970). To date, up to 20 different members have been identified in this family. Many of these naturally occurring cyclic peptides exhibit a variety of biological activities including antibacterial and antitumor activity (Quiot et al., 1985; Muroi et al., 1994). In this study, we have shown the strong suppressive effect of destruxin B on HBsAg gene expression in human hepatoma cells.

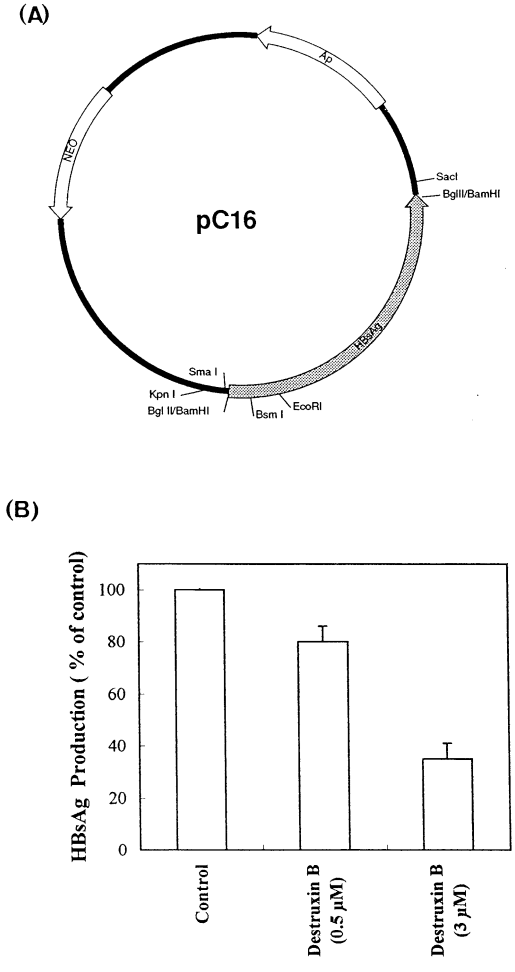

Fig. 5. Effect of destruxin B on the promoter activity of HBsAg gene in HuH-7 cells. (A) Schematic diagram of the plasmid pC16. The plasmid pC16 contains a 2329 bp *Bgl* II (2839)-*Bgl* II (1986) fragment of HBV DNA cloned into the *Kpn* I and *Sac* I sites of pSV2 neo. The HBV DNA fragment contains the HBsAg coding sequence (S) and the upstream promoter region (SP II). (B) HuH-7 were transfected with plasmid pC16 DNA, using the calcium phosphate precipitation method. Cells were transfected in DMEM medium supplemented with 10% FCS for 16 h. After DMSO shock, cells were changed to serum-free DMEM medium with destruxin B for 48 h. The amount of HBsAg in culture medium was determined by enzyme immunoassay. Control cells produced 15 ng of HBsAg/10^6 cells/48 h. Data are expressed as mean $\pm$ S.D. ($n = 3$).

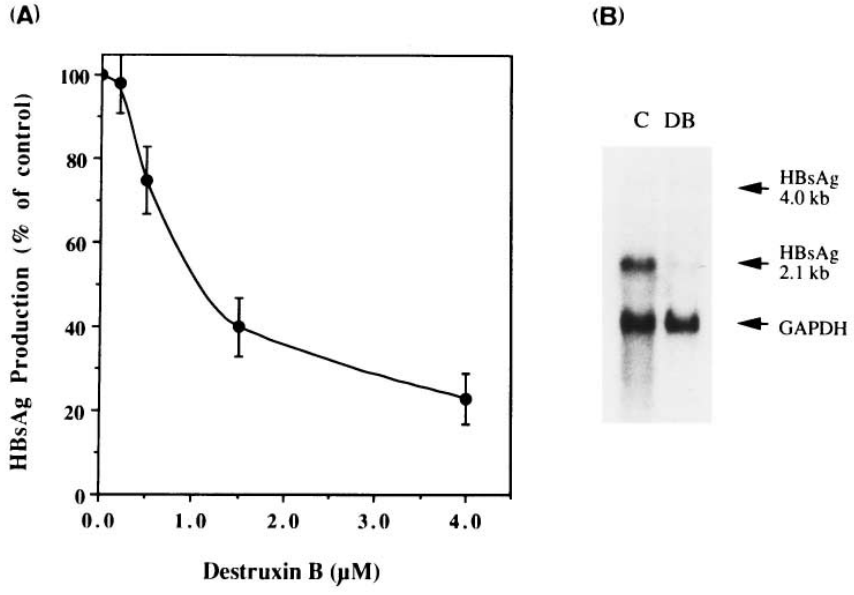

Fig. 6. Effect of destruxin B on the expression of HBsAg in Hep3B/C16 cells. (A) Hep3B/C16 cells were plated in DMEM with 10% fetal calf serum and allowed to attach overnight. The cells were then washed and treated with various concentrations of destruxin B in serum free DMEM medium for another 48 h. The amount of HBsAg in culture medium was determined by enzyme immunoassay. Control cells produced 15 ng of HBsAg/10^6 cells/48 h. Data are expressed as mean $\pm$ S.D. ($n = 3$). (B) Hep3B/C16 cells were treated with (DB) or without (C) destruxin B (4 μM) for 24 h. Total RNA was extracted and anaiyzed by Northern hybridization with HBsAg DNA as described in Section 2. The constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control.

The suppressive activity of destruxin B on HBsAg production in human hepatoma Hep3B cells is highly specific and is not due to the general cytotoxic effect of the drug. Several lines of evidence support this conclusion. First, prolonged treatment (> 6 days) of destruxin B, up to 2.0 μM, does not change the proliferation rate of the cells (Sun et al., 1994). Second, destruxin B suppresses the production of HBsAg in a concentration-dependent manner but has no effect on the production of another human hepatoma marker protein, α-fetoprotein, in the Hep3B cells. Third, after removal of the drug, the cells completely recover their capability to produce HBsAg.

The suppression of HBsAg by destruxin B observed in this study is not due to the interference by destruxin B on the binding of HBsAg to its specific antibody as suggested by (Venkateswaran et al., 1987) to elucidate the antiviral mechanism of *P. niruri*. Destruxin B does not require de novo protein synthesis to exert its suppressive activity either. Destruxin B not only suppresses the production of HBsAg from endogenously integrated HBV genome in the Hep3B cells, but also suppresses the production of HBsAg from transiently transfected HBV DNA in a HBV negative human hepatoma HuH-7 cells. Our observations strongly suggest that destruxin B may directly suppress HBsAg gene expression in human liver cells.

The mechanism of how destruxin B suppresses HBsAg gene expression in the Hep3B cells is not yet clear. Destruxin B may directly travel into the cell and alter the transcriptional machinery of HBsAg gene as glucocorticoid does (Kaspa et al., 1986). Alternatively, destruxin may target the cell membrane, delivering an inhibitory signal to the nucleus to suppress HBsAg gene expression as insulin does (Ting et al., 1989; Chou et al., 1989). The synthetically lineal destruxin B analog completely loses its antiviral activity suggesting that the cyclic peptide backbone is essential for this biological activity (data not shown). The cyclic

backbone may stabilize an unique structure of destruxin B for its specific interaction with cellular macromolecule. Identification of the cellular target of destruxin B is currently under investigation.

Our study has established a rapid and effective biological assay system for screening antiviral components from natural resources. Elucidating the mechanism of anti-HBsAg activity of those natural products will greatly enhance our understanding on the regulation of viral gene expression in the cell and provide a new avenue to developing novel antiviral agents in the future.

Acknowledgements

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