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Short communication

Inhibition of hepatitis B virus by D-fraction from *Grifola frondosa*: Synergistic effect of combination with interferon-α in HepG2 2.2.15

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Abstract

In this study, D-fraction extracted from *Grifola frondosa* (GF-D) and its combination with human interferon alpha-2b (IFN) were investigated for the inhibitory effect on hepatitis B virus (HBV) in HepG2 2.2.15 cells (2.2.15 cells). HBV DNA and viral antigens were analyzed by a quantitative real-time polymerase chain reaction and end-point titration in radioimmunoassays, respectively. The results showed that GF-D or IFN alone could inhibit HBV DNA in 2.2.15 cells with the 50% inhibitory concentration (IC_{50}) of 0.59 mg/ml and 1399 IU/ml, respectively. We further investigated the combination of GF-D and IFN for anti-HBV activity and found that they synergistically inhibited HBV replication in 2.2.15 cells. In combination with 0.45 mg/ml GF-D, the apparent IC_{50} value for IFN was 154 IU/ml. This 9-fold increase in antiviral activity of IFN suggested that GF-D could synergize with IFN. These results indicate that GF-D, in combination with IFN, might provide a potentially effective therapy against chronic HBV infections.

Keywords: Antiviral; *Grifola frondosa*; Interferon; Hepatitis B virus; Combination

Chronic hepatitis B virus (HBV) infection is endemic in Asia, and diseases caused by HBV are among the major public health problems in the world. It is estimated that nearly 300 million people worldwide are suffering from chronic HBV infection (Kao and Chen, 2002). Cytokines, such as interferon alpha-2b (IFN), can directly inactivate intracellular HBV and/or activate T cells to destroy the virus-infected hepatocytes to control HBV infection. Unfortunately, immunomodulatory therapy with IFN is confronted with many limitations on therapeutic efficiency (Manesis and Hadziyannis, 2001). Although the nucleoside analogs have been shown to be highly effective in the elimination of HBV DNA from serum, mutants may escape upon drug withdrawal (Liaw et al., 2000). Neither IFN nor lamivudine (3TC) can eradicate the hepatitis virus from the body. The unsatisfactory therapeutic results of IFN and 3TC enhance the need to search for new effective anti-HBV agents.

Grifola frondosa is a *Basidiomycete* fungus belonging to the family *Polyporaceae*. The polysaccharide from *Grifola frondosa* has shown particular promise as an immunomodulator by stimulating the immune efficiency of macrophages, dendritic cells (Hishida et al., 1988; Mayell, 2001), and as adjuncts in cancer and HIV infection treatment (Kodama et al., 2005). About 85% HIV-infected patients, treated with the fruit bodies of *Grifola frondosa*, were reported to show an increased sense of well-being with regard to various symptoms and secondary diseases caused by HIV. *Grifola frondosa* appeared to work at several levels, i.e. directly inhibiting HIV replication, or stimulating the body's defense system against HIV (Nanba et al., 2000). A protein isolated from *Grifola frondosa* possessed antiviral activity against the tobacco mosaic virus in plants (Chen et al., 2004).

In this study, the anti-HBV activity of D-fraction from *Grifola frondosa* (GF-D) and combined with IFN were investigated in HepG2 2.2.15 cells (2.2.15 cells).

D-Fraction was prepared, as reported previously (Kodama et al., 2005), from the powdered fruit bodies of *Grifola frondosa* (Huayi Biotech Co., Henan, China). Briefly, the hot-water soluble fraction from the powder of *Grifola frondosa* was concentrated, and alcohol was added at the final concentration of 80%, and then centrifuged. Acid-soluble matter was removed from the precipitates. After sodium hydroxide was added and centrifuged, alkaline-soluble matter was obtained and then dialyzed to remove low molecular weight substances. The concentrations of polysaccharide and protein were determined by the anthrone method and the Lowry-Folin method, respectively.

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Recombinant human IFN alpha-2b (IFN) was obtained from Hualida Bioengineering Co. Lamivudine was provided by GlaxoSmithKline, Inc. Fetal bovine serum (FBS) was purchased from Hyclone laboratories. The neomycin analogue (G_{418}), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Co. Trypsin, penicillin, streptomycin, and Dulbecco's modification of Eagle's medium (DMEM) were purchased from Gibco Co. (Fig. 1).

The 2 2.2.15 cells, stably transfected with a head-to-tail dimer of HBV DNA, were kindly donated by Dr. Zhao (the NO.302 Hospital of PLA, Beijing, China), and used as the model system of HBV. The cells were plated on 24-well cell culture plates at a concentration of 2×10^5 cells/ml, and routinely cultured with DMEM supplemented with 10% FBS, 2 mmol/l glutamine, 100 units/ml penicillin G, 100 mg/l streptomycin, and 380 mg/l G_{418}.When cells reached confluence (day 4), FBS was reduced to 2%. Cells were incubated at 37 °C in an atmosphere of 5% CO_2. In these experiments the culture medium containing, either GF-D or IFN alone, or the mixture of the two compounds, were added to each well of the plate in triplicate on day 2 after the cells were plated. This step was repeated on day 5.The medium without antiviral compounds was also changed on day 5. 3TC was used as an active control compound. The resulting cell culture supernatants were harvested on day 9 and stored at −80 °C for subsequent experiments.

The cytotoxicity of each compound alone, or in combination, was evaluated using the MTT assay method, which had been previously used to measure the cytotoxicity of compounds.

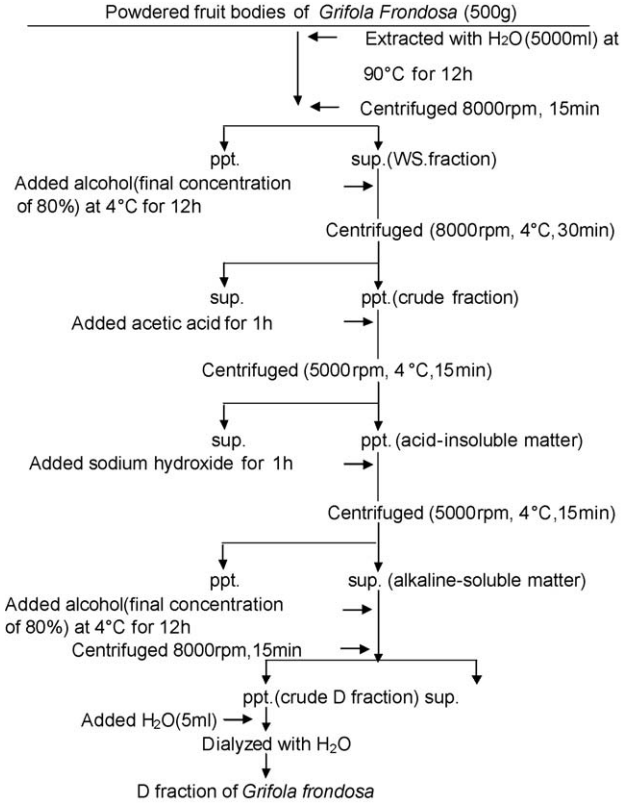

Fig. 1. Preparation of GF-D.

The cells were treated for MTT activity as described (Liu et al., 2003), and the monolayers were also observed microscopically for the estimation of CPE (i.e. rounding and other marked morphologic changes with respect to the control cells).

The quantity of HBV DNA in the suspension on day 9 was determined by real-time polymerase chain reaction (ABI PRISM 7000 Sequence Detector, PE Biosystems) based on the TaqMan technology (Chen et al., 2001). PCR was performed using HBV DNA real-time PCR Assay kit (Daan Gene Co., Ltd. of Sun Yat-sen University) according to the Manufacturer's instruction.

Hepatitis B e antigen (HBeAg) and Hepatitis B surface antigen (HBsAg) in clarified tissue culture medium were analyzed on day 9. HBsAg and HBeAg in the supernatant were quantified by the Solid Phase Radioimmunoassay Kit for quantification of HBsAg and HBeAg (China Institute of Atomic Energy Physics, Beijing) according to the Manufacturer's instruction.

The combination assay was performed essentially according to the published method, but with some modification (Briolant et al., 2004). The cell monolayer was added with DMEM containing an appropriate concentration of the combination of IFN (100–1400 IU/ml) and GF-D (0.1–0.6 mg/ml). Three wells were used for each concentration of IFN in a constant ratio with that of GF-D. The cell culture supernatants were harvested on day 9 for subsequent experiments.

To determine the synergistic or antagonistic interactions of the compounds, we used the isobologram method (Briolant et al., 2004). The fractional inhibitory concentration (FIC) for each combination of IFN and GF-D was calculated as follows: $FIC_{GF\text{-}D}$ = (concentration of GF-D in the combination at the end point)/(concentration of GF-D alone required to achieve the end point) and FIC_{IFN} = (concentration of IFN in the combination at the end point)/(concentration of IFN alone required to achieve the end point). The combinations resulting in an additive antiviral effect ($FIC_{GF\text{-}D} + FIC_{IFN} = 1$) are represented by straight line (unity line) on the isobologram. When the combination results in synergy, i.e. stronger antiviral effects than the sum of the individual effects ($FIC_{GF\text{-}D} + FIC_{IFN} < 1$), the FIC value falls below the unity line. When the combination results in an antagonistic antiviral effect ($FIC_{GF\text{-}D} + FIC_{IFN} > 1$), the FIC value rises above the unity line.

HBV DNA polymerases were isolated from the HBV virus and the HBV polymerase assay was performed as previously described (Davis et al., 1996; Gaillard et al., 2002). HBV polymerase activity was monitored by measurement of the incorporation of α-^{32}P-labeled deoxynucleoside triphosphate (dNTP) into acid-precipitable products. Assays were performed in 40 μl of a solution containing 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 0.2 mg of activated calf thymus DNA/ml, 100 mM unlabeled dNTPs (e.g., dATP, dGTP, and dTTP), various concentrations of α-^{32}P-labeled dNTP (500 Ci/mmol) and various concentrations of GF-D. HBV polymerase (5 μl, 0.1 μg) was added to start the reaction. Assays were routinely performed at 30 °C for 30 min. Then, 35 μl of the reaction mixture was transferred onto 3 MM paper (Whatman Inc.), dried briefly and washed three times in 5% trichloroacetic acid plus 1% sodium pyrophosphate. The incorporated radioactivity was measured in a Beckman scintillation counter.

Table 1
Cytotoxicity and antiviral activity of substances in 2.2.15 cell

Compounds	CC_{50}	IC_{50}			TI_{50}		
		HBsAg	HBeAg	DNA	HBsAg	HBeAg	DNA
GF-D (mg/ml)	5.37 ± 0.82	>2[a]	0.77 ± 0.21	0.59 ± 0.33	<2.69	7.0	9.1
IFN (IU/ml)	83753 ± 9254	>12800[b]	>12800[c]	1399 ± 739	<6.54	<6.54	59.9
3TC (μM)	>30 ± 4.6	–	–	0.31 ± 0.13	–	–	>96.8

All data represent mean ± S.D. of three separate experiments. CC_{50}, 50% cytotoxic concentration, is defined as a drug concentration causing 50% inhibition of 2.2.15 cells vs. the untreated culture; IC_{50}, 50% inhibition concentration, is defined as a drug concentration resulting in 50% inhibition of HBV replication vs. the untreated culture; TI_{50}, therapeutic index, is calculated as the ratio of CC_{50} to IC_{50}.

[a] At 2.0 mg/ml, 27.4% inhibition.

[b] At 12800 IU/ml, 19.2% inhibition.

[c] At 12800 IU/ml, 26.3% inhibition.

Statistical analysis of the data was carried out with one-way analysis of variance (ANOVA) using SPSS10.0 software. All data are presented as mean ± S.D. Mean, S.D., CC_{50} and IC_{50} values were calculated using the computer program Pharm PCS.

GF-D showed potent antiviral activity against HBV. The IC_{50} of GF-D on HBV DNA and HBeAg were 0.59 mg/ml and 0.77 mg/ml in 2.2.15 cell, respectively (Table 1). However, GF-D could only cause a 20.4% inhibition of HBsAg at a dose of 2.0 mg/ml. The IC_{50} and TI_{50} of IFN for HBV DNA were 1399 IU/ml and 59.9, respectively. The IC_{50} of 3TC for HBV DNA was 0.31 μM.

When 2.2.15 cells were treated with both IFN and GF-D, a synergistic effect was observed. The different dose combinations of IFN with GF-D used in this study showed no significant cytotoxic effect ($P > 0.05$) in the 9-day cell exposure experiments.

As shown in Fig. 2, 0.15 mg/ml GF-D (1/4 IC_{50}) in combination with 670 IU/ml IFN (1/2 IC_{50}), or 0.24 mg/ml GF-D (1/2.5 IC_{50}) with 420 IU/ml IFN (1/3 IC_{50}), or 0.45 mg/ml GF-D (1/1.3 IC_{50}) with 154 IU/ml IFN (1/9 IC_{50}) all inhibited HBV DNA replication by 50%.

To determine the mechanism of action, the inhibitory effect of GF-D on HBV DNA polymerase was assayed in vitro. GF-D exhibited the inhibitory activity on HBV polymerase (Fig. 3). GF-D significantly inhibited HBV polymerase activity in dose-dependent manner (0.125 mg/ml: 11.5%; 0.25 mg/ml: 19.6%; 0.5 mg/ml: 34.6%; 1.0 mg/ml: 48.7%; 2 mg/ml: 52.1%). The calculated IC_{50} of GFPS for HBV polymerase was 1.38 mg/ml.

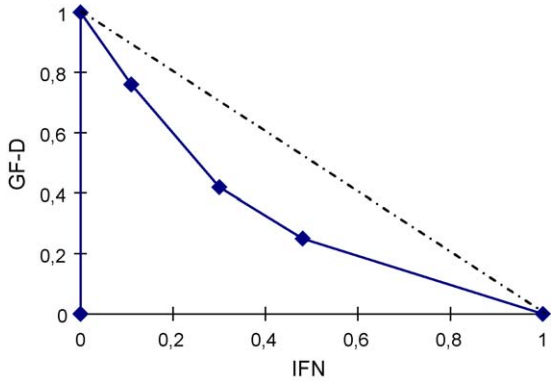

Fig. 2. Isobologram: synergy of the combinations of IFN and GF-D in antiviral activity against HBV DNA in 2.2.15 cells. $FIC_{GF\text{-}D} + FIC_{IFN} = 0.73$, 0.72, 0.87 and <1. FIC: fractional inhibitory concentration.

In this study, we have demonstrated that GF-D has antiviral activity against HBV, and the combination of GF-D and IFN showed synergistic effect in 2.2.15 cells. The IC_{50} value of 3TC on HBV DNA was 0.31 μM.GF-D consisted of polysaccharide (71.4%) and protein (21.6%), which were determined by the anthrone method and the Lowry-Folin method.

Higher fungi have been tested for antibacterial activity, but only recently has it been realized that some mushrooms also possess antiviral properties. For example, *Fomes fomentarius* has been shown to have an antiviral effect on the tobacco mosaic virus (Aoki et al., 1993) and an extract of *Lentinus edodes* has been found to inhibit the replication of the herpes simplex virus (HSV), the poliovirus, the measles virus, the mumps virus (Sorimachi et al., 1990; Sarkar et al., 1993), and the human immunodeficiency virus (Tochikura et al., 1988).

In this study, GF-D exhibited an inhibitory effect on HBV polymerase. This result suggested that the mechanism of antiviral action of GF-D appeared to be a direct interference in the HBV replication at the level of DNA polymerase. This occurred at an early stage. It is possible that GF-D was a competitive inhibitor of endogenous HBV polymerase in a cell-free assay. It is necessary to prove the mechanism of action of GF-D in further experiments.

Polysaccharides possess a wide range of therapeutically important biological properties, such as inhibition of DNA polymerase, induction of interferon production, and virus-inhibitory activity (Cassady and Whitley, 1997). GF-D consisted mainly of the acid-insoluble polysaccharide. The antiviral activities of

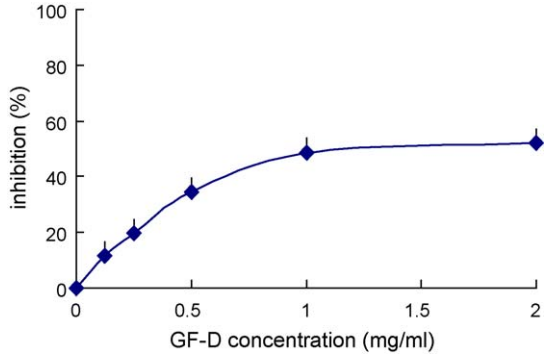

Fig. 3. Inhibitory effect of GF-D on HBV polymerase. GF-D inhibited HBV polymerase dose-dependently. Data are expressed as mean S.D. ($n = 3$).

GF-D may be linked to the biological properties of polysaccharide. Also the antiviral activity of GF-D may be related to the protein component of GF-D. Further isolation and extraction of the anti-HBV component from GF-D is needed in future experiments.

IFN was selective and effective in its inhibition of HBV DNA with an IC_{50} and TI_{50} of 1399 IU/ml and 59.9, respectively. These results are consistent with prior studies, which have demonstrated antiviral activity of IFN against HBV. In 2.2.15 cells, IFN was able to inhibit the production of HBV DNA, but the HBsAg was not significantly reduced (Hayashi and Koike, 1989). The IC_{90} and TI_{90} of IFN on HBV DNA were 989 IU/ml and 60, respectively (Korba, 1996). However, in HuH_7 cells, IFN treatment reduced the levels of HBsAg and HbeAg (Rang et al., 1999). Treatment of various stable HBV-expressing cell lines with IFN led to the inhibition of synthesis of one or several HBV products, depending on the type of cell and system used.

IFN could cause a marked reduction in the level of HBV DNA in the cytoplasm, through inhibiting HBV replication by blocking some steps in the RNA-primed assembly of core particles (Rang et al., 1999). The modes of anti-HBV action of GF-D and IFN are different. The combination studies showed that the combination of GF-D and IFN, had a synergistic action against HBV. In combination with 0.45 mg/ml GF-D, the apparent IC_{50} for IFN was 154 IU/ml. This 9-fold increase in IFN antiviral activity suggested that GF-D could synergize with IFN and increase the efficacy of IFN on HBV infections. The various reasons for the combination chemotherapy for HBV infections, include synergy of antiviral effects, antagonism of toxicities, and prevention of occurrence of resistant variants (Lewin et al., 2002). The combination of 3TC with IFN significantly enhances the antiviral effect of each compound on HBV replication in 2.2.15 cells. Low levels of 3TC could substantially increase the efficacy of IFN (Korba, 1996).

A combination of IFN and GF-D could also be used for the treatment for chronic hepatitis B. However, it is difficult to extrapolate the studies in 2.2.15 cells to clinical outcomes in humans. The data suggest that combination of IFN and GF-D may increase antiviral efficacy in a synergistic manner at low doses. However, the combination of IFN and GF-D should be further tested for the treatment of HBV infections in experimental animal models.

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