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Therapeutic basis of glycyrrhizin on chronic hepatitis B

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Abstract

Glycyrrhizin, a major component of a herb (licorice), has been intravenously used for the treatment of chronic hepatitis B in Japan and improves liver function with occasional complete recovery from hepatitis. This substance modifies the intracellular transport and suppresses sialylation of hepatitis B virus (HBV) surface antigen (HBsAg) in vitro. This study was designed to clarify the pharmacological basis for its effectiveness. The structure–bioactivity relationship of glycyrrhizin, glycyrrhetic acid 3-*O*-monoglucuronide and glycyrrhetic acid was determined, and glycyrrhetic acid was found to be the most active of them. The amounts of three substances bound to the liver were evaluated in guinea pigs after intravenous administration of glycyrrhizin. Glycyrrhizin and glycyrrhetic acid 3-*O*-monoglucuronide were detected at concentrations of 31.8–1.3 μg/g of liver, but glycyrrhetic acid was not detected. When glycyrrhizin attained these concentrations in the cellular fraction of the PLC/PRF/5 cell culture, it suppressed the secretion of HBsAg as reported previously. These results indicated that glycyrrhizin administered intravenously might bind to hepatocytes at the concentration at which glycyrrhizin could modify the expression of HBV-related antigens on the hepatocytes and suppress sialylation of HBsAg.

Keywords: Glycyrrhizin; Chronic hepatitis B; Hepatitis B surface antigen

1. Introduction

Glycyrrhizin, a major component of the herb *Glycyrrhiza uralensis* (licorice), has been clinically used for the treatment of chronic hepatitis B in Japan. More than 100 million doses of glycyrrhizin injection have been annually administered intravenously to patients with chronic hepatitis and allergy. Studies on the therapeutic effects of glycyrrhizin report that it normalizes elevated serum transaminases levels (ALT and AST), and improves liver histology in patients with chronic hepatitis B, resulting in occasional complete recovery from chronic hepatitis B virus (HBV) infection (Fujisawa et al., 1973; Suzuki et al., 1983). Glycyrrhizin is a supplement of interferon for the treatment of patients with chronic hepatitis B and has also been used to treat non-responders to interferon therapy. There have been

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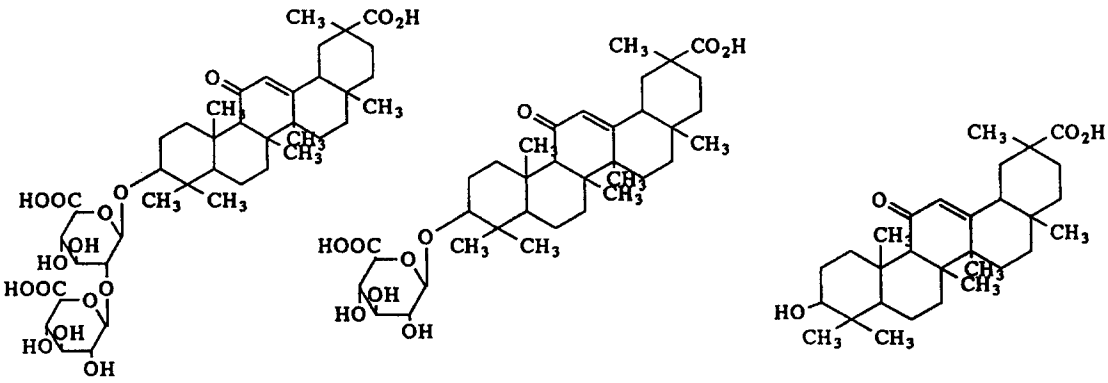

Fig. 1. Structure of glycyrrhizin, glycyrrhetic acid 3-*O*-monoglucuronide and glycyrrhetic acid.

several reports on the immunoregulatory activities of glycyrrhizin (Abe et al., 1982; Finney and Somers, 1958; Kimura et al., 1992; Okimatsu et al., 1982; Nose et al., 1994; Shinada et al., 1986; Zhang et al., 1990, 1992).

Pompei et al. (1979) reported that glycyrrhizic acid, a derivative of glycyrrhizin, inhibits the growth of several DNA and RNA viruses and inactivates herpes simplex virus particles. Glycyrrhizin inhibits varicella-zoster virus infection (Baba and Shigeta, 1987), human immunodeficiency virus (HIV) infection (Ito et al., 1987, 1988), and hepatitis A virus (HAV) infection (Crance et al., 1994). We have reported that glycyrrhizin modifies the intracellular transport and suppresses sialylation of HBsAg at the trans-Golgi area (Shiraki et al., 1991, 1992; Takahara et al., 1994). However, it is not clear whether glycyrrhizin acts directly against HBV and/or whether the glycyrrhizin functions in patients with chronic hepatitis B.

In this study we have examined the structure–bioactivity relationship of glycyrrhizin and its related compounds (Kanaoka et al., 1983; Kitagawa et al., 1993a,b) for its effect on the suppression of HBsAg secretion and also the correlation of the glycyrrhizin concentration between assays in vitro and in vivo. Although a human hepatocellular carcinoma cell line (PLC/PRF/5) (Alexander et al., 1982; Takahara et al., 1994) may not be the most suitable source of hepatocytes, it may offer the best means of focusing on the effect of glycyrrhizin-related compounds on the secretion of HBsAg in its culture. To elucidate the therapeutic basis of glycyrrhizin in patients with chronic hepatitis B, we conducted the present study using guinea pigs administered intravenously with glycyrrhizin. We confirmed that the concentration of glycyrrhizin was maintained so highly in the hepatocytes that it could suppress intracellular transport and secretion of HBsAg in the PLC/PRF/5 cell culture (Takahara et al., 1994).

2. Materials and methods

2.1. Cells and drugs

PLC/PRF/5 cells were grown and maintained in Dulbecco's minimum essential medium (D-MEM) supplemented with 10% and 3% fetal bovine serum (FBS), respectively. PLC/PRF/5 cells secrete HBsAg into the culture supernatant. Glycyrrhizin, glycyrrhetic acid 3-*O*-monoglucuronide and glycyrrhetic acid (Fig. 1) were kindly supplied by Minophagen Pharmaceutical Co.

2.2. Effect of glycyrrhizin on HBsAg secretion

PLC/PRF/5 cells were grown in 24-well plastic plates and the amount of secreted HBsAg was determined by the reverse passive hemagglutination (RPHA) test (Shiraki et al., 1991; Takahara et al., 1994). PLC/PRF/5 cells were plated at a concentration of 3×10^4 cells per well and the amount of HBsAg secreted in 24 h was determined by the mean titer of 6 wells assessed by the RPHA test. To compare the effect of three chemical compounds on HBsAg secretion, the cells were pretreated with various concentrations of each compound for 2 h. Then the cultures were further treated with the respective concentrations of each compound for 24 h for the next assay. The culture supernatants were harvested and centrifuged at 3000 rpm for 10 min. The amounts of HBsAg in the resulting supernatants were assessed by the RPHA test. The cytotoxicity was monitored by the trypan blue exclusion test.

2.3. Determination of the amount of glycyrrhizin in the PLC/PRF/5 cells

To determine the amount of glycyrrhizin bound to the PLC/PRF/5 cells, the cells were grown in Petri dishes (10 cm in diameter) and treated with various concentrations of glycyrrhizin. The cells were scraped by a rubber policeman and washed with phosphate-buffered saline (PBS) three times by centrifugation at 3000 rpm for 10 min. The cell pellet was used for the determination of the amount of glycyrrhizin as reported (Ishida et al., 1990). Briefly, the methanol-soluble fraction of the cells was analyzed by high pressure liquid column chromatography and the amount of glycyrrhizin was determined by comparison with a standard sample (Ishida et al., 1993).

2.4. Determination of the amount of glycyrrhizin in the liver of guinea pigs administered intravenously with glycyrrhizin

Glycyrrhizin was administered intravenously to Hartley guinea pigs to determine the amount of glycyrrhizin and its metabolites in serum, bile and liver by the excess and subclinical therapeutic doses. When the glycyrrhizin dose in humans is deduced from body surface and body weight, it corresponds to 4 and 1 mg per guinea pig, respectively. An excessive dose of 20 mg glycyrrhizin in 2 ml was injected intravenously in guinea pigs weighing 300 g, and serum and liver were taken 1 and 4 h later under ether anesthesia for the analysis of glycyrrhizin and its metabolites. A subclinical dose of 1 mg of glycyrrhizin in 0.1 ml was administered, and serum, bile and liver were used to identify the amount of glycyrrhizin. The obtained liver was rinsed in PBS thoroughly and wiped before extraction. The amount of glycyrrhizin and its related compounds was determined as reported (Ishida et al., 1990).

3. Results

3.1. Effect of structure–bioactivity relationship of glycyrrhizin and its related compounds on HBsAg secretion

The amount of secreted HBsAg was stable after reaching confluence of the PLC/PRF/5 cell culture (data not shown) and therefore the 4th-day culture was used for the assays. Fig. 2 shows the role of the structure–bioactivity relationship of glycyrrhizin, glycyrrhetic acid 3-*O*-monoglucuronide and glycyrrhetic acid in suppressing HBsAg secretion into the culture medium. Although three compounds suppressed the secretion of HBsAg, glycyrrhetic acid showed the most potent activity among them. No significant cytotoxicity was observed with any compound at any concentrations examined (data not shown).

3.2. Amounts of glycyrrhizin and its related compounds in liver administered with glycyrrhizin

Excess (20 mg per guinea pig) dose of glycyrrhizin was administered intravenously into guinea pigs weighing 300 g and the amounts of three compounds were determined, as shown in Table 1. Glycyrrhizin was mainly detected but glycyrrhetic acid was not detected in the liver of guinea pigs administered with an excessive dose of glycyrrhizin. The concentration of glycyrrhizin

was substantially higher in serum than in the liver. This suggested that some of the glycyrrhizin was metabolized to glycyrrhetic acid 3-*O*-monoglucuronide in the liver but not to glycyrrhetic acid.

As shown in Table 2, glycyrrhizin was detected at a concentration of 4.2 and 1.3 μg/g of liver at 1 and 4 h, respectively, after administration of 1 mg glycyrrhizin per guinea pig as a subclinical dose. Serum and bile concentrations of glycyrrhizin were also determined at the same time, as shown in Table 2. Serum glycyrrhizin concentration decreased but, by contrast, bile glycyrrhizin concentration increased with time. Both these concentrations were much higher than that in the liver.

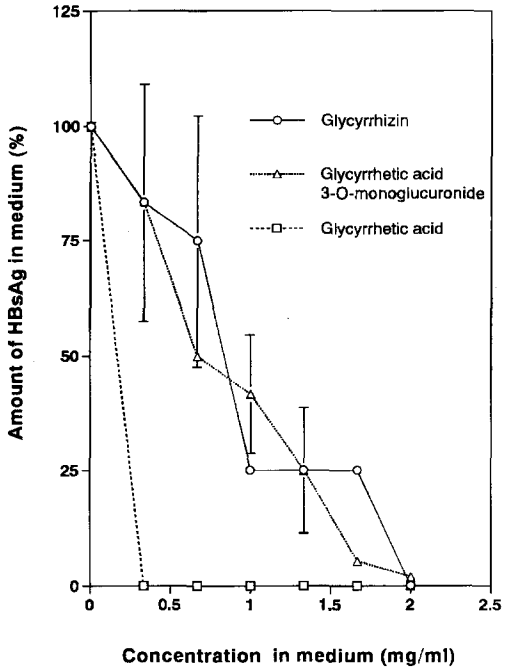

Fig. 2. Structure–bioactivity relationship of glycyrrhizin, glycyrrhetic acid 3-*O*-monoglucuronide and glycyrrhetic acid for the secretion of HBsAg in the PLC/PRF/5 cell culture. The amounts of secreted HBsAg were assessed by the RPHA test after 2-fold serial dilution and expressed as the mean percentage ± S.D. ($N = 6$) of those in untreated cells. Circles, triangles and squares represent the amount of HBsAg in the culture supernatant treated with glycyrrhizin, glycyrrhetic acid 3-*O*-monoglucuronide, and glycyrrhetic acid, respectively.

Table 1
Content of glycyrrhizin in the livers of guinea pigs administered with an excessive dose of glycyrrhizin (20 mg per guinea pig)

	1 h later	4 h later
Liver (μg/g of liver)		
Glycyrrhizin	31.8 ± 7.6	22.5 ± 4.3
Glycyrrhetic acid 3-*O*-monoglucuronide	1.68 ± 0.74	2.83 ± 0.92
Glycyrrhetic acid	Not detected	Not detected
Serum (μg/ml)		
Glycyrrhizin	153.3 ± 33.4	64.8 ± 28.7
Glycyrrhetic acid 3-*O*-monoglucuronide	Not detected	Not detected
Glycyrrhetic acid	Not detected	Not detected
Bile (μg/ml)		
Glycyrrhizin	248.8	336.3
Glycyrrhetic acid 3-*O*-monoglucuronide	Not detected	13.0
Glycyrrhetic acid	Not detected	Not detected

The amounts of the three compounds in the liver, serum and bile were determined after intravenous administration of glycyrrhizin at a dose of 100 mg/kg of body weight. The values represent the mean ± S.D. for four guinea pigs. The content of the three compounds was determined in the bile of a guinea pig.

Together with these results, glycyrrhizin and glycyrrhetic acid 3-*O*-monoglucuronide were detected at a concentration of 31.8–1.3 μg/g of liver, but glycyrrhetic acid was not detected at 1 and 4 h after intravenous administration of glycyrrhizin. Glycyrrhizin in serum was quickly secreted into the bile through hepatocytes, and some were metabolized to glycyrrhetic acid 3-*O*-monoglucuronide in the hepatocytes and secreted into the bile.

Table 2
The content of glycyrrhizin in the liver, serum and bile after administration of a subclinical dose of glycyrrhizin (1 mg/guinea pig)

	1 h later	4 h later
Liver (μg/g of liver)	4.157 ± 0.77	1.34 ± 0.45
Serum (μg/ml)	38.2 ± 10.7	9.4 ± 1.8
Bile (μg/ml)	117.8 ± 18.5	34.7 ± 5.8

The content of glycyrrhizin in the liver, serum and bile was determined after intravenous administration of glycyrrhizin at a dose of 2 mg/kg of body weight. The values represent the mean ± S.D. for four guinea pigs.

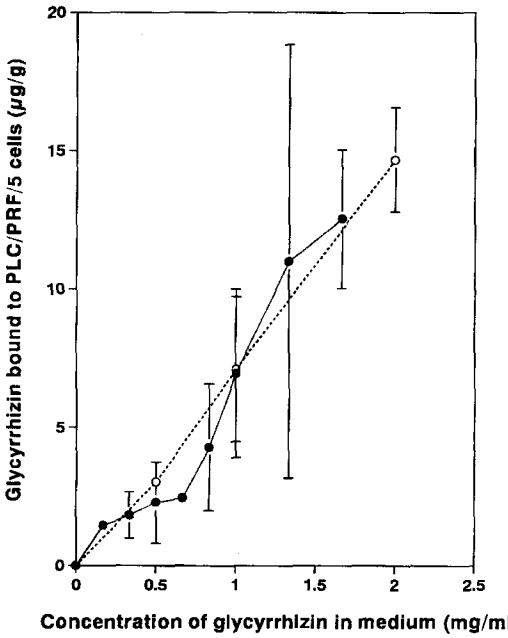

Fig. 3. Relationship between glycyrrhizin concentrations of the medium and the amount of glycyrrhizin bound to the PLC/PRF/5 cells in two independent experiments. Glycyrrhizin concentration (mg per PLC/PRF/5 cells (g)) in the cellular fraction was determined as described in the text and expressed as the mean concentration ± S.D. for three cultures.

3.3. Amount of glycyrrhizin bound to PLC/PRF/5 cells

Fig. 3 shows the relationship between glycyrrhizin concentrations of the medium and the amounts of glycyrrhizin bound to the PLC/PRF/5 cells. The amount of glycyrrhizin bound to the PLC/PRF/5 cells increased dose-dependently with the glycyrrhizin concentration of the medium. The effective concentration of glycyrrhizin for 50% reduction in HBsAg secretion (EC_{50}) in the medium was 0.71 ± 0.07 mg/ml in three independent experiments and it corresponded to 2.4 and 3.9 μg/g of PLC/PRF/5 cells in two experiments. The glycyrrhizin concentration at which HBsAg secretion was suppressed in the PLC/PRF/5 cell culture was lower than that in liver after intravenous administration. This indicated that glycyrrhizin would inhibit the secretion of HBsAg in the liver of patients with chronic hepatitis B at the concentration detected in this experimental system.

4. Discussion

Glycyrrhizin has been used to treat patients with chronic hepatitis B, but the mechanism for the improvement of liver function remains to be determined. We have shown the glycyrrhizin action in vitro on HBsAg (Takahara et al., 1994). In this study, we have clarified the effect of the structure–bioactivity relationship of glycyrrhizin and its related compounds on the inhibition of HBsAg secretion, and the possible pharmacokinetic effect of glycyrrhizin on the liver. Glycyrrhizin resulted in the accumulation of HBsAg in the trans-Golgi area and a suppression of the secretion of HBsAg in vitro. Furthermore, glycyrrhizin may modify the interaction between the cell surface and HBV (Takahara et al., 1994). Similarly, glycyrrhizin inhibits adsorption and penetration of HAV to the PLC/PRF/5 cells (Crance et al., 1994). Thus, glycyrrhizin treatment of hepatocytes modifies the intracellular transport and the surface nature of the hepatocytes.

Of the three compounds examined (Fig. 2), glycyrrhetic acid inhibited the secretion of HBsAg most strongly. However glycyrrhetic acid was not detected in the liver fraction even after administration of an excessive dose of glycyrrhizin (Table 1). From the structure–bioactivity relationship and the guinea pig study, glycyrrhizin was the main compound in the liver fraction after intravenous administration (Table 1) and it is suggested that glycyrrhizin would play the most important role in the treatment of patients with chronic hepatitis B.

The concentration of glycyrrhizin may range from 1.3–4.2 to 22.5–31.8 μg/g of liver after administration of a clinical dose. These values are consistent with those observed in rat liver after intravenous administration (Ishida et al., 1990). Glycyrrhizin was captured by the liver and secreted directly into bile, after it had been partially converted to glycyrrhetic acid 3-*O*-monoglucuronide. Human liver homogenates can convert glycyrrhizin to glycyrrhetic acid 3-*O*-monoglu-

curonide but not further on to glycyrrhetic acid (Akao et al., 1991). Thus the results are consistent with the converting enzyme activity observed in the human and rat liver (Akao et al., 1990, 1991). These concentrations of glycyrrhizin in the liver corresponded to those at which the secretion of HBsAg in the PLC/PRF/5 cell culture was suppressed, as can be deduced from Figs. 2 and 3. This indicated that glycyrrhizin could inhibit the secretion of HBsAg and exhibit its action in the hepatocytes chronically infected with hepatitis B virus, as observed in the previous study (Takahara et al., 1994).

It is not excluded that glycyrrhizin is converted to unknown derivatives by the liver cells. However, most of glycyrrhizin is excreted or concentrated from plasma into the bile without modification. We assume that glycyrrhizin may be actively transported from plasma via the liver cells into the bile.

Although glycyrrhizin has been widely used for the clinical treatment of chronic hepatitis B in Japan, the precise mechanism of its effect on HBV has not been elucidated. Glycyrrhizin treatment improves the liver function without rebound of the transaminase level after cessation of interferon treatment. If glycyrrhizin directly enhances the immune system (Abe et al., 1982; Finney and Somers, 1958; Kimura et al., 1992; Okimatsu et al., 1982; Nose et al., 1994; Shinada et al., 1986; Zhang et al., 1990, 1992) and thereby improves liver function, liver damage should be exaggerated by immune lymphocytes activated by interferon as well as glycyrrhizin. Immune activation by glycyrrhizin may not directly prevent liver damage. If, on the other hand, glycyrrhizin modifies the intracellular transport and the surface structure of the hepatocytes (Crance et al., 1994; Takahara et al., 1994), the interaction between hepatocytes and lymphocytes may be altered in such a way that there is no rebound of transaminases after interferon treatment. Although we have come to the conclusion that glycyrrhizin may act directly on hepatocytes in addition to its immune activation, the precise mechanism by which glycyrrhizin improves liver function needs to be further assessed.

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