Effect of Shuang Huang Lian (双黄連) on hepatitis B virus surface antigen secretion

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Abstract

Shuang Huang Lian (双黄連, SHL) is one of the Chinese traditional medicines composed of the extracts of Lonicerae Flos, Scutellariae Radix, and Forsythiae Fructus and has been used for the treatment of various chronic and acute diseases including viral hepatitis. It also has shown efficacy in infections caused by antibiotic resistant bacteria. SHL and the extracts of each constituent plant medicine were tested for suppression of hepatitis B virus surface antigen (HBsAg) secretion in an experimental model for chronic HBV infection using the PLC/PRF/5 cell line. As a result, SHL as well as the extracts of Lonicerae Flos and Scutellariae Radix suppressed the secretion of HBsAg without cytotoxicity.

Key words Shuang Huang Lian, Chinese traditional medicines, chronic hepatitis B, HBV, HBsAg, Scutellariae Radix, Lonicerae Flos.

Abbreviations ELISA, enzyme linked immunosorbent assay ; HBV, hepatitis B virus ; HBsAg, hepatitis B virus surface antigen ; RPHA, reverse passive hemagglutination test.

Introduction

The medicines developed from the wisdom of ancient people have been recognized nowadays as being the alternative therapy for diseases resistant to the modern chemotherapy. The Chinese traditional medicine is one of the classic medical systems practiced for thousands of years using natural resources as the curative agents. Among several Chinese traditional medicines Shuang Huang Lian (双黄連, SHL) has been adopted as a medicine for the treatment of various acute and chronic diseases as well as infections caused by antibiotic-resistant microrganisms. It is indicated for remission of fever, detoxication and enhancement of immunological reactions. This medicine is composed of the extracts of the flowers of *Lonicera japonica* THUNB. (Lonicerae Flos), the roots of *Scutellaria baicalensis* GEORGI (Scutellariae Radix), and the fruits of *Forsythia suspensa* VAHL (Forsythiae Fructus). The intravenous infusion of SHL has shown to be safe and effective for the treatment of acute bronchiolitis [1] and is also used for the treatment of chronic hepatitis in China. Taking into account that hepatitis B virus (HBV) infection has been a serious problem in public health since it may give complications such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma through persistent infection, SHL was submitted to a test to confirm its therapeutic basis on HBV infection. The experiment was conducted in a system of a PLC/PRF/5[2] cell line that secretes HBsAg, one of the HBV infection markers that appear in blood of infected patients, and the effectiveness of the medicine was evaluated by the suppression of HBsAg secretion in the cell culture.

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Materials and Methods

Cells : The human hepatocellular carcinoma cell line, PLC/PRF/5, was grown and maintained in Dulbecco's modified Eagle medium supplemented with heat inactivated 10 % and 2 % fetal bovine serum, respectively. The cultures were incubated at 37°C in 5 % CO_2 atmosphere. The cells contain several integrated copies of the HBV genome and secrete a small amount of HBsAg, apparently in the form of 22 nm particles.[3,4]

Chemicals and reagents : Glycyrrhizin was kindly supplied by Minophagen Pharmaceutical Co., (Osaka, Japan). Dulbecco's modified Eagle medium was purchased from Nissui Co. (Tokyo, Japan), and the reversed passive hemagglutination (RPHA) test kit from Kokusai Shiyaku Co. (Kobe, Japan). Chlorogenic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and all other reagents used were of analytical grade.

Solutions of SHL : The intravenous solution of SHL was a product of the pharmaceutical factory of the First Hospital of Harbin Medical School (Harbin, China). Two preparations of SHL were tested : the intravenous solution (SHL-*s*), which was added into the culture medium without previous treatment, and a liophilized powder (SHL-*l*) that was dissolved in distilled water to a concentration of 10 mg/ml (stock solution) prior to add into the culture medium.

Constituent plant medicine extracts : The plant medicines Scutellariae Radix (the root of *Scutellaria baicalensis* GEORGI, Labiatae), Forsythiae Fructus (the fruit of *Forsythia suspensa* VAHL., Oleaceae) and Lonicerae Flos (the flower of *Lonicera japonica* THUNB., Caprifoliaceae) were purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan).

Preparation of the extracts : The plant medicines were extracted following the general procedure of preparation of SHL. Scutellariae Radix (375 g) was extracted with hot water (1 L each time) three times (2 h for the first and 1 h for the following extractions). The extract fluids were combined, filtered and concentrated under reduced pressure to a volume of 1 L. The concentrated extract was acidified to pH 1-2 with 2 N HCl and allowed 1 h at 80°C and 12 h at room temperature. The acidic extract was filtered, and the residue was suspended in 6-8-fold distilled water and adjusted to pH 7 with 40 % (v/v) NaOH aqueous solution. Equal volume of ethanol was added with constant stirring and the solution was filtered. The filtrate fluid was acidified to pH 2 with 2 N HCl, kept 30 min at 60°C and 12 h at room temperature. It was filtered and the residue was washed with ethanol until pH 4, dried and used as Scutellariae Radix extract (final yield, 35.6 g). Forsythiae Fructus (750 g) and Lonicerae Flos (375 g) were extracted twice (1 h each extraction) with hot water (total volume of 5 L) after soaking in water for 30 min. The extracts were combined and concentrated to half volume at 80°C to the density 1.2-1.25. It was cooled to 45°C and 75 % (v/v) ethanol was added with stirring. It was kept 12 h at room temperature, filtered and the filtrate was concentrated to evaporate the ethanol, then lyophilized, and used as the extract of Forsythiae Fructus (final yield, 102.2 g) and Lonicerae Flos (final yield, 43.5 g). All extracts were dissolved in distilled water to a concentration of 10 mg/ml prior to use in the experiment.

Assay for HBsAg : The cells were seeded into the 24-well tissue culture plate at a density of 10^4 cells/well grown to confluence. The culture medium of the confluent cells was pretreated for 2 h and treated for 24 h with medium containing SHL or the plant medicine extracts. The concentrations of SHL-*l* and plant medicine extracts in the culture medium were 0.5, 1.0 and 2.0 mg/ml and those of SHL-*s* were 2.5, 5.0 and 10 % (v/v). Then the medium was harvested, centrifuged (10,000 rpm for 5 min) and the amount of HBsAg released into the medium was determined by the reversed passive hemagglutination test (RPHA). The cytotoxity was assessed by the trypan blue exclusion test. Each extract was tested in six replicate wells. Controls for a normal secretion of HBsAg were run without any test sample and controls for the suppression of HBsAg secretion contained glycyrrhizin (0.5, 1.0 and 2.0 mg/ml).[5]

Results and Discussion

The suppressive effects of SHL on the HBsAg secretion were observed in cultured PLC/PRF/5 cells.

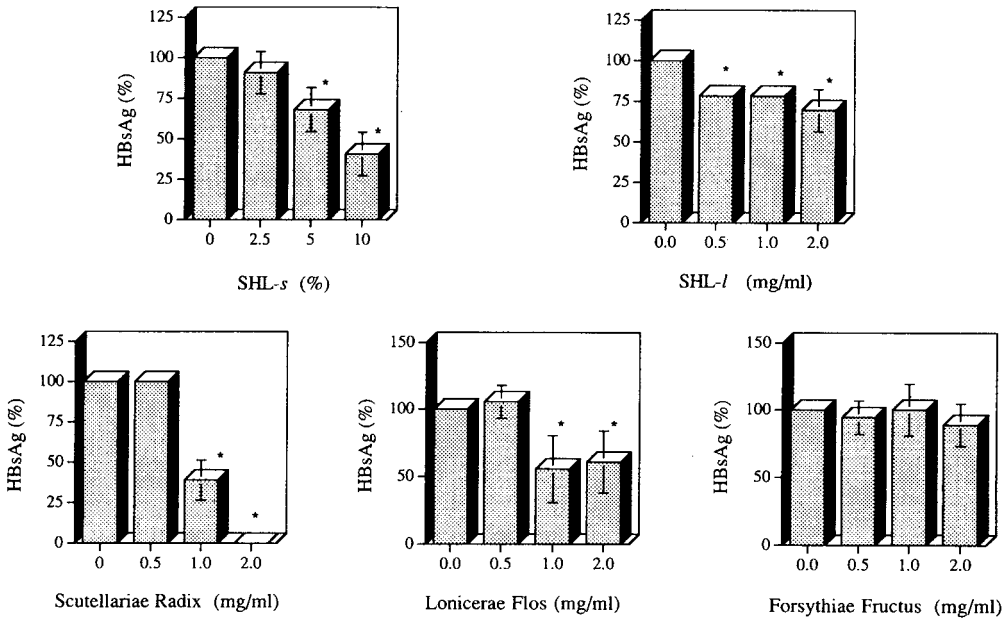

Fig. 1 Effects of SHL and the extracts of its constituent plant medicines on the secretion of HBsAg in cultured PLC/PRF/5 cells. *Significantly different from the untreated cell culture, $p<0.05$, data analyzed by Dunnett's method.

The HBsAg titer was 1 : 64 in the control culture for a normal secretion of this antigen. The results are expressed as the percentage of the control culture without the test sample. Fg. 1 shows the amounts of HBsAg (%) in the culture supernatant as determined by RPHA. The amounts of HBsAg in the presence of SHL-*s* were 90.9, 68.2 and 40.9 % at concentrations 2.5, 5.0 and 10 % (v/v) respectively. In the presence of SHL-*l* the amount of HBsAg was 78.3 % at 0.5 and 1.0 mg/ml and 69.6 % at 2.0 mg/ml. The presence of Scutellariae Radix extract completely suppressed the secretion of HBsAg at 2.0 mg / ml and reduced to 38.9 % at 1.0 mg/ml. No suppression was observed at 0.5 mg/ml. Similarly, but with less potency, Lonicerae Flos extract reduced the amount of HBsAg to 55.6 % at 1.0 mg/ml and 61.1 % at 2.0 mg/ml with no effect at 0.5 mg/ml. The suppressive effect was not observed in any of the concentration of the extract of Forsythiae Fructus. One of the compounds present in SHL, chlorogenic acid was also tested in the same experimental system. This compound showed a potent suppressive effect of 96.7 % at a concentration of 0.5 mg/ml. In a control assay for suppression of HBsAg secretion, glycyrrizin showed suppression of 33 %, 87 % and 100 % at concentrations 0.5, 1.0 and 2.0 mg/ml, respectively.

Actually interferon represents a promising agent to treat chronic hepatitis B[6,7] and several experimental studies have been done with a wide variety of antiviral drugs.[8-11] Glycyrrhizin is a constituent of *Glycyrrhiza* sp., a Chinese traditional medicine, which has shown to be effective in chronic hepatitis B resistant to interferon treatment [5] and the mechanism of this action could be at least in part, the improvement of immunological status and inhibition of the glycosylation of HBsAg in the cell.[12]

The clinical use of SHL in China has shown the efficacy of this medicine in patients with respiratory diseases, acute diseases of internal organs, and infectious diseases with appreciable efficacy in those resistant to antibiotic therapy. It has anti-microbial activity of broad spectrum, effective against parainfluenza virus,[13] influenza virus (A3) and respiratory syncytial virus.[14] Various pharmacological activities such as increase in heart beating rate and respiratory function have been reported in animal experiments.[15] On the other hand, the plant medicines Lonicerae Flos, Scutellariae Radix, and Forsythiae Fructus that com-

pose this formula are known to have antimicrobial, antidotal and febrifuge properties. Also, Scutellariae Radix is one of the herbal medicines of choice for jaundice.[16] The intravenous administration of SHL has also shown efficacy in the treatment of chronic hepatitis in China, although a randomized clinical trial has been performed only in patients with acute bronchiolitis so far.[1]

In the present experiment both SHL-*s* and SHL-*l* were confirmed to suppress the secretion of HBsAg without cytotoxicity and this effect seems to be due to the plant medicine constituents, Scutellariae Radix and Lonicerae Flos. The extract of Scutellariae Radix was the one that showed the most potent suppression of HBsAg secretion in cultured cells compared to the other two extracts. This plant medicine is known to have antibacterial, antiinflammatory, and antithermic actions which are related to its flavonoid constituents such as baicalin, baicalein and wogonin. Lonicerae Flos that bears antibacterial properties also has flavonoids such as luteolin and lonicerin as chemical constituents. Compounds such as chlorogenic acid, baicalin and phillyrin are present in SHL injection.[17] Among them, chlorogenic acid, a compound common to many plant species, completely suppressed the HBsAg secretion in this experimental system. The results show in part the therapeutic basis of SHL for the treatment of HBV infection. Other effective substances may be present, moreover, the interaction among drugs is an important factor for the effectiveness of a Chinese pharmaceutical formulation composed of two or more drugs. Kong *et al.*[1] reported that the action of SHL on the treatment of acute bronchiolitis was not due to bacterial inhibition. Antiinflammatory, immune enhancing, and other unknown actions of SHL are presumed to be involved. The systemic effects such as regulation of body fluids and stimulation of the immune system would be the additional mechanisms of actions of SHL.

和文抄録

"双黄連"は金銀花，黄芩および連翹の3種類の生薬から抽出された複方剤である。中国では臨床にも応用されており，呼吸系疾患，内科急性疾患，外科感染性疾患，耳鼻科と歯科感染性疾患など，広範囲に渡り効果がみられ，抗生物質に耐性をもつ菌株に対しても良好な作用を有するとされる。本実験では慢性B型肝炎の実験モデルとされるPLC/PRF/5細胞を用いて双黄連およびその構成生薬の熱水抽出エキスによるB型肝炎ウイルス表面抗原（HBsAg）の分泌における作用を調べた。その結果，双黄連ならびに金銀花および黄芩のエキスにHBsAg分泌抑制作用が見出された。なお，これらのエキスには細胞毒性は認められなかった。

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