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Original Articles

Effect of *Samul-tang (Siwu-tang)* on Procollagen Synthesis in Cultured Murine Hepatic Non-parenchymal Cells

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Object : This study was done to evaluate the inhibitory effect of *Samul-tang (Siwu-tang)* on collagen production by cultured murine hepatic non-parenchymal cells.

Methods : Hepatic non-parenchymal cells were cultured from normal Sprague-Dawley rats and established in a primary cell culture on uncoated plastic culture plates.

The *Samul-tang (Siwu-tang)* was treated into the cell culture media for 72 hours and the cells were harvested for analysis. Analyses were done on cell proliferation, [3H]thymidine incorporation assay and procollagen type 1 C-peptide.

Results : The cultured cells resembled fibroblasts in shape and produced procollagen which is consistent to fibrogenesis in vivo. Proliferation of the non-parenchymal cells was inhibited slightly and the [3H]thymidine incorporation assay showed a dose-dependent decrease by *Samul-tang (Siwu-tang)* treatment. Production of procollagen type 1 C-peptide was decreased by low-concentration treatment of the *Samul-tang (Siwu-tang)*, but increased by high-concentration treatment.

Conclusion : It seemed that the cells were responding to the *Samul-tang (Siwu-tang)* in low-concentration, thus producing less collagen. However, when the drug was administered with high enough concentration to cause excessive stimulation of cells, it seemed that the activated cells might overly produce procollagen, the precursor of collagen, thus aggravating fibrosis of the liver. So, it is considered that the proper concentration of *Samul-tang (Siwu-tang)* is important when treating patients with liver cirrhosis based on the patients' status. *(Korean J of Oriental Med 2003;24(4):120-126)*

Key Words: *Samul-tang(Siwu-tang)*, liver cirrhosis, procollagen, non-parenchymal cell, fibrosis

Introduction

Liver cirrhosis is a common consequence of chronic liver diseases, including viral hepatitis, and results from the activation of non-parenchymal cells of the liver, especially the hepatic stellate cells (HSCs)[1]. The number of HBV carriers throughout the world is considered to be exceeding 200 million, the majority of them located in Southeast Asia and Africa[2]. Its prevalence is reported to be much higher among patients with certain kinds of diseases, such as Down's syndrome, lepromatous leprosy, leukemia, Hodgkin's disease, polyarteritis nodosa, etc[2]. According to a report published recently in Korea, HBsAg-positive patients accounted for 5.7% among all outpatients, and the prevalence was higher among the elderly[3]. Chronic hepatitis B is considered to be one of the major causes of liver cirrhosis and of hepatoma. Thus, liver cirrhosis is still considered to be a center of concern in the countries where HBV is prevailing.

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Various effects of herbs and herb prescriptions on chronic liver diseases have been researched recently through experimental and clinical studies[4-12], but studies on collagen synthesis, which is one of the major processes to develop liver cirrhosis, have not been well examined using herbs and herb formulae.

Samul-tang (Siwu-tang, SMT), a formula to improve blood formation and to enhance blood circulation in the body based on oriental medical concepts, has been frequently prescribed to patients. The liver is the viscera for blood storing and circulation. Thus *SMT* is considered to be beneficial for liver diseases.

We cultured hepatic non-parenchymal cells of rats through a primary cell culture technique and observed the inhibitory effect of *SMT* on procollagen synthesis.

Materials and Methods

1. Non-parenchymal cell isolation

4-wk-old Sprague-Dawley rats were used for the primary cell culture. The rats were treated according to the guidelines of the Kyunghee Medical Center. All procedures were performed with the rats under ether anesthesia. Livers were perfused with collagenase. The liver was then gently homogenized and incubated further in collagenase for 30 minutes at 37 ℃. The suspension was harvested and the remaining remnants underwent the same procedure once more. Then the gathered suspension containing various cells was seeded on uncoated plastic tissue culture dishes and serial culture was done in Dulbecco' s modified Eagle medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics in a 95% air, 5% CO_2 humidified atmosphere at 37 ℃. Non-proliferable cells like hepatocytes and blood cells were gradually eliminated through serial culture; we used 3rd passage hepatic non-parenchymal cells in this study(Fig. 1).

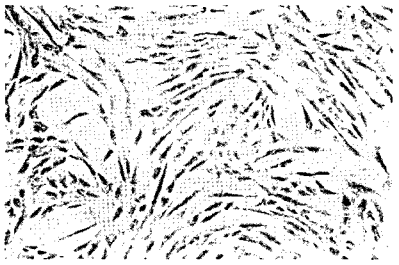

Fig. 1. 3rd passage hepatic non-parenchymal cells with fibroblast-like shape.

2. Herb formula preparation

Samul-tang (Siwu-tang, SMT) 100g[13], purchased at the Hospital of Oriental Medicine, Kyunghee Medical Center and consisting of 25 g of each Rehmanniae Radix, Angelicae gigantis Radix, Cnidii Rhizoma and Paeoniae Radix, was decocted twice with 1,000 ml of distilled water for 2 hours each time. Then it was filtered and followed by concentration with Rotary evaporator (Buchi, RE121, Switzerland). This concentrated material was dried with a freeze dryer (EYELA, Japan) and 37.23 g of dried powder was obtained. This powder was dissolved in cell culture medium with desired concentrations.

3. Cell count

Serial culture of primary cultured rat hepatic non-parenchymal cells was done and 3rd passage cells were seeded on culture dishes. The cells were cultured in media treated with different concentrations of *SMT*. Culture media was changed with fresh new one every 48 hours. The cells were cultured for 72 hours and then harvested for cell count. For cell counting, a hemocytometer was used and the cell proliferation was calculated.

4. [^{3}H]-thymidine incorporation assay

3rd passage hepatic non-parenchymal cells were

seeded on culture dishes and cultured for 24 hours. Then the cells were cultured for 72 hours until harvested in the media containing the *SMT* of different concentrations. 16 hours prior to cell harvest, 5 uCi of [^{3}H]thymidine (Amersham Pharmacia Biotec, UK) was added into the media containing the *SMT*. Cells were washed twice with PBS and then lysed by adding 0.5 N NaOH 0.5 ml/well for 30 minutes. This lysate was then put into a liquid scintillation vial and 5 ml of liquid scintillator solution (Aqualuma Plus, Lumac. LSC BV, Netherlands) was added to it. Then the radioactivity (count per minute: CPM) was counted after spending overnight at 4 ℃ using β-scintillation counter (LS9000, Beckman Instrument Inc., Fullerton, CA., USA).

5. Procollagen assay

Procollagen assay was conducted with Procollagen Type I C-Peptide EIA Kit (TaKaRa, Japan) according to the indications of the provider.

6. Statistical analysis.

One-way ANOVA was applied for the descriptive statistics and the Scheffe test was applied for the multiple comparison. Nonlinear regression was used to evaluate the optimum concentration of herb formulae for inhibiting collagen production. SPSS 10.0 version was used for the analysis.

Result

1. Cell Count

We used 3rd passage primary cultured hepatic non-parenchymal cells of rats in this study. We divided them into 6 different groups; the maximum concentration of herbal medicine in the media was 100 ug/ml. This herbal medicine was treated for 72 hours until harvest. The results showed a concentration-dependent decrease in the cell count, but was not statistically significant (Table 1).

2. [^{3}H]-Thymidine Incorporation Assay

Non-proliferable cells such as blood cells and hepatocytes were eliminated through serial culture. The 3rd passage hepatic non-parenchymal cells were cultured for 72 hours until harvested in media containing different concentrations of *SMT*. 16 hours prior to cell harvest, 5 uCi of [^{3}H]-Thymidine was added into the media. The cells were harvested and the radioactivity was measured by β-scintillation counter. The radioactivity of the hepatic non-parenchymal cells appeared to decrease in a concentration-dependent manner, but all the groups showed no statistically significant results except the *SMT* 100 ug/ml group (Table 2, $p<0.05$).

3. Procollagen Assay

Procollagen type I c-peptide was measured in the media of the hepatic non-parenchymal cells after treating the *SMT* with different concentrations. The production level of procollagen was the lowest in the *SMT* 6.25 ug/ml group, but the procollagen levels were increased consistent to the *SMT* concentration and that of the 100 ug/ml group was higher than that of the non-treated group, though not statistically significantly

Table 1. Number of Non-Parenchymal Cell after *Samul-tang (Siwu-tang, SMT)* Treatment

SMT(ug/ml)	0	6.25	12.5	25	50	100
Cell No.($\times 10^5$)	7.55±0.20	7.60±0.40	6.93±0.28	6.90±0.50	6.85±0.30	6.13±0.28

Values represent mean ± S.E
1×10^4 cells were seeded in 6 well plate and cultured for 72 hrs

Table 2. [^{3}H]-Thymidine Incorporation Assay of Non-Parenchymal Cell after *Samul-tang (Siwu-tang, SMT)* Treatment

SMT(ug/ml)	0	6.25	12.5	25	50	100
CPM	67818±2448	69853±1667	67438±828	65573±1173	61410±1430	57288±813*

CPM: count per minute
Isotope volume added: 5 uCi/well
1×10^4 cells were seeded and cultured for 72 hrs
Values represent mean ± S.E.
*: $p<0.05$

Table 3. Procollagen Assay of Non-Parenchymal Cell after *Samul-tang (Siwu-tang, SMT)* Treatment

SMT(ug/ml)	0	6.25	12.5	25	50	100
procollagen(ng/ml)	282.0±2.0	149.0±32.0	156.5±38.5	186.0±2.0	237.0±39.0	310.0±50.0

1×10^4 cells were seeded and cultured for 72 hrs.
Values represent mean ± S.E.

(Table 3).

Discussion

The number of HBV carriers throughout the world is considered to be exceeding 200 million, the majority of them located in Southeast Asia and Africa[2]. A recent report revealed that 5.7% of outpatients were HBV positive carriers in Korea[3]. The chance to develop hepatoma is about 100 times higher in patients with chronic hepatitis B than others, and more than half of the patients with liver cirrhosis are associated with HBV. Though the death rate by liver cirrhosis is decreasing along with economic development and medical progress, it is still very high[14]. This explains the importance of developing an effective therapeutic way for liver cirrhosis.

Symptoms associated with abdominal mass, jaundice, tympanism and flatulence in Oriental Medicine are similar to those of the liver cirrhosis according to recent concepts[15]. Thus, some herb formulae described in those chapters are assumed to have a therapeutic effect on liver cirrhosis. Many experimental and clinical studies of liver diseases in Oriental medicine have recently been performed mainly on the effect of the Artemisiae Capillaris Herba and the formulae containing the herb[4-6,9,10].

Animal models by physical and chemical method and in vitro models by hepatoma cell lines for liver cirrhosis are applied frequently in the study of herbal medicine[11,12]. Though the non-parenchymal cells are proven to be the major source of collagen production in the liver, thus have the property of developing liver cirrhosis, studies using that system have been rarely performed on Oriental medicine.

The liver viscera in Oriental medicine is regarded to have the property to deposit blood and to make the blood circulate well. The symptoms of blood deficiency in the liver viscera are similar to those of liver cirrhosis. Thus, the *SMT*, one of the main herb formulae to improve the blood deficiency state, is assumed to be helpful to treat chronic liver diseases. Based on this hypothesis, we performed this study to evaluate the inhibitory effect of *SMT* on collagen synthesis.

Liver fibrosis is characterized by excessive accumulation of extracellular matrix, especially the type 1 collagen in the liver, as a consequence of the breakdown of the balance between the fibrogenesis and fibrolysis. The HSC (hepatic stellate cell, lipocyte, fat storing or Ito cell) plays the key role in hepatic fibrosis[16]. Collagen is found mainly in the portal area of the liver, and infrequently in the space of Disse and central vein.

When liver cirrhosis is developed, collagen is found throughout the hepatic lobule and the deposited collagen disturbs the blood circulation and the material exchanges of the hepatocytes. External stimulation to the HSCs of the liver, such as endotoxins and cytokines, is thought to be the cause of overproduction of collagen, though the liver also has some ability to produce it[17]. In the process, the Kupffer cells are known to activate the HSCs to produce collagen by producing tumor necrosis factor alpha (TNF-α) and nitric oxide (NO)[18].

Collagens (types I, II, III, IV and V) are synthesized as precursor molecules called procollagens. These contain additional peptide sequences, usually called "propeptides", at both the amino-terminal and the carboxy-terminal ends. The function of these propeptides is to facilitate the winding of procollagen molecules into a triple-helical conformation within the endoplasmic reticulum. The propeptides are cleaved off from the collagen triple helix molecule during its secretion, after which the triple helix collagens polymerize into extracellular collagen fibrils. Thus, the amount of the free propeptides reflects stoichiometrically the amount of collagen molecules synthesized.

Quantitative detection of collagen synthesis was first reported by Taubman et al.[19], who performed competitive radioimmunoassays for procollagen type I carboxy-terminal peptide (PIP) using polyclonal antibodies. Similar methods using PIP as a reference have been used in studying the correlation of collagen levels with certain health disorders, e.g. bone diseases[20], alcoholic liver diseases[21], and adenocarcinoma of the stomach[22].

To determine the proper concentration of herb extracts which shows effects without having direct toxicity to cells is very important when treating the extracts into the cell culture media. From the review of former studies, we selected the maximum dose as 100 ug/ml and divided experimental groups into 6.

When the herb extracts were added into the media for 72 hours, the cell counts decreased in a dose-dependent manner, but the values were not significant statistically(Table 1). The proliferation of the hepatic non-parenchymal cells cultured for 72 hours in a herb extract-treated media was measured by β-scintillation counter after treating 5 uCi/well of [^{3}H]thymidine into the culture media 16 hours prior to cell harvest. The radioactivity of the *SMT*-treated cells decreased dose-dependently, but a statistically significant result was not shown except in the *SMT* 100 ug/ml group (Table 2). Dose-dependent decreases in the cell count assay and in the [^{3}H]thymidine show that the *SMT* is effective in inhibiting both the proliferation and the activity of the collagen producing hepatic non-parenchymal cells. While the absolute volume of collagen produced in the low concentration groups ($\leq$50 ug/ml) was less than that of the normal group, the collagen volume in the high concentration group (100 ug/ml) was higher than that of the normal group(Table 3).

We found in procollagen assay that the production level of procollagen was the lowest in the *SMT* 6.25 ug/ml group, though not significant statistically. This non-significant result is thought to come from the wide internal variance in the *SMT* 6.25 ug/ml group. Considering that fact, we can assume that the result might be meaningful with repeated experiment. The procollagen levels in *SMT*-treated groups increased consistent to the concentration and that of the 100 ug/ml group was higher that the non-treated group(Table 3), though the cell counts and activity both decreased (Table 1 and 2). From this result we can assume that the number of non-parenchymal cells and their activity are not associated directly with the collagen-producing property. This means that though the cell count is decreased in some situations, like excessive stimulation of the cells, the activity of the cells to produce collagen might be increased. Here we can assume that the

procollagen-mRNA expression may be increased by stimulation exceeding a certain extent of concentration, even in the case of non-parenchymal cell decrease.

We needed an explanation for that phenomenon. Thus the function 'θ1exp(θ2 × concentration) +θ3' was selected to estimate the collagen synthesis depending on the *SMT* concentration and we obtained the function '111.91exp (0.000067 × concentration) - 111.47' through nonlinear regression. Based on this function, we assume that further experiment is needed, treating 6.25 ug/ml or less concentration of the *SMT*, to find the optimum level to inhibit the production of collagen.

The 95% confidence interval (CI) of min was 0.39-0.50 pg/cell(Fig. 2).

In conclusion, it is possible that the volume of herbal medicine should be reduced based on the stage of the patients' condition and on the severity of liver cirrhosis. According to that concept, it is necessary to adjust the volume of herbal medicine according to the patient' s condition, and further studies to elucidate the proper dosage to treat liver cirrhosis are needed.

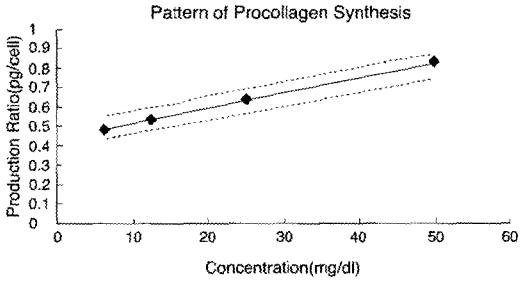

Fig. 2. Estimated pattern of Procollagen Synthesis according to the concentration of the *Samul-tang (Siwu-tang, SMT)*

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