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원 저

Apoptotic Effects of *Sipimikwanjung-tang* of *Sasang* Constitutional Medicine in Human Hepatoblastoma Cells

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Objective : This study on *Sipimikwanjung-tang* was undertaken to evaluate its antioxidant capacities and antiperoxidation activities in rat liver tissues. *Sipimikwanjung-tang* which has been one of the prescriptions in *sasang* constitutional medicine is usually applied for the therapy of various liver diseases. It is elucidated that *Sipimikwanjung-tang* has antioxidants on liver tissue of rat and the cytotoxic effects on human hepatoblastoma Hep G2 cells.

Methods : *Sipimikwanjung-tang* extract in antioxidant effects of Hep G2 cells is evaluated by MTT assay, DAPI staining, DNA fragmentation assays and FACScan analysis.

Results : *Sipimikwanjung-tang* induced apoptosis in Hep G2 cells, and induced G1 and G2M arrest of the cell cycle as well as a significant increase in PARP and caspase-3 activity. It induced an increase in H_2O_2 generation and the subsequent NF-κB activation and also induced cell apoptosis through the caspase-3-dependent pathways in the low concentration of *Sipimikwanjung-tang* extracts. However, the high dose of Sipimikwanjung-tang extract in Hep G2 cells inhibited TGF-β1-induced apoptosis via increase in cellular H_2O_2 formation and NF-κB activation in human hepatoblastoma Hep G2 cells.

Conclusion : From this study, the possibility that Sipimikwanjung-tang extracts apply to antioxidant and apoptotic treatment of disease is revealed.

Key Words: *Sipimikwanjung-tang*, SKT, hepatoblastoma cells, Hep G2 cells, caspase-3, TGF-β1, NF-κB

Introduction

Sasang constitutional medicine has been used especially in Korea for the therapy of various diseases. *Sipimikwanjung-tang* which has been one of the prescriptions in Sasang constitutional medicine is usually applied for the therapy of liver diseases. Some results already showed that *Sipimikwanjung-tang*(SKT) extract might have a beneficial effect in patients with liver diseases and liver cancer[1-2]. However, to date, it has not been scientifically evaluated in human whether the prescription may constitute a plausible therapeutic for liver diseases including hepatoblastoma.

Sipimikwanjung-tang is made from 13 medicinal herbs, i.e. *Radix Cynanchi Wilfordii, Radix Polygoni Multiflori, Rhizoma Alpinae Officinarum, Rhizoma Zingiberis Siccatum, Pericarpium Citri Nobls, Pericarpium Citri Mobles Viride, Rhiaoma Cyperi,*

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Table 1. The Components of *Sipimikwanjung-tang*(十二味寬中湯,SKT)

Herbal Name	Scientific Name	Weight(g)
白何首烏	*Radix Cynanchi Wilfordii*	4
赤何首烏	*Radix Polygoni Multiflori*	4
良薑	*Rhizoma Alpinae Officinarum*	4
乾薑	*Rhizoma Zingiberis Siccatum*	4
陳皮	*Pericarpium Citri Nobls*	4
靑皮	*Pericarpium Citri Mobles Viride*	4
香附子	*Rhiaoma Cyperi*	4
益智仁	*Fructus Alpinae Oxyphyllae*	4
厚朴	*Cortex Magnoliae*	2
枳實	*Fructus Immatuurus Poncirl*	2
木香	*Radix Saussurea*	2
大腹皮	*Pericarpium Arecae*	2
大棗	*Fuctus Zizyphi Jujubae*	2
Total Amount		42

Fructus Alpinae Oxyphyllae, Cortex Magnoliae, Fructus Immatuurus Poncirl, Radix Saussurea, Pericarpium Arecae, Fructus Zizyphi Jujubae (Table. 1).

In this study we have examined the cytotoxic effects of the *Sipimikwanjung-tang*(SKT) extracts composed of the above herbs on human hepatoblastoma Hep G2 cells. It has been suggested that *Sipimikwanjung-tang*(SKT) extracts exhibited a modest hepatoprotective effect on the liver-damaged injury in rats[1-2]. The *Sipimikwanjung-tang*(SKT) extract shows antioxidant activities and inhibits lipid peroxidation. Furthermore, it also played a role in regulating NO synthesis and liver enzyme in rat liver tissue. In addition to these hepatoprotective effects, *Sipimikwanjung-tang*(SKT) extract also inhibited lipid peroxidation in rat liver microsomes[1-2]. Therefore, the prescription can be used in the treatment of liver disease. However, to date there has been little study to elucidate the anticancer effect in Hep G2 cells. So, in this paper, the anticancer effect of *Sipimikwanjung-tang*(SKT) extract has been studied for elucidating the mechanism of the effect in the level of cellular condition

So, it was examined whether the prescription has the anticancer effect on hepatoblastoma cells or not. The objectives of this study were: (1) to carry out a scientific assessment of the prescription on the effect for liver diseases, and especially to evaluate the therapeutic potential about liver cancer treatment, (2) to explore its mechanism of the action. And hopefully, based on this examination, the beneficial effects of the prescription, *Sipimikwanjung-tang*(SKT) extract, could be determined, and the dosage of this prescription could be revaluated for optimal therapy. It has been elucidated that the prescription has the cytotoxic effects on human hepatoblastoma Hep G2 cells. The method of MTT assay, DAPI staining, DNA fragmentation assays and FACScan analysis of propidium iodide-staining cells showed that *Sipimikwanjung-tang*(SKT) extract induced apoptosis in Hep G2 cells, and induced G1 and G2M arrest of the cell cycle as well as a significant increase in caspase-3 activity. And also, it was revealed that the high concentration of *Sipimikwanjung-tang*(SKT) extract induced an increase in H_2O_2 generation and the subsequent NF-κB activation.

Materials and methods

1. Preparation of extracts

The air-dried plants of medicinal herbs were obtained from Medicinal Resources Research Center of Wonkwang University and Korea Plant Extract Bank and and also were purchased commercially. One hundred gram of each herb was extracted with 1,000ml distilled water for 2hr at 100℃, respectively, and then centrifuged at 3,000 rpm for 20min. The supernatant was filtered, dried using freeze dryer, stored at -70℃ until used.

2. MTT reduction assay for cell viability

Cell viability was measured with blue formazan that was metabolized from colorless 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

by mitochondrial dehydrogenases, which are active only in live cells. Hep G2 cells were preincubated in 24-well plates at a density of 1×10^6 cells per well for 24 hr, then washed with Phosphate-buffered saline(PBS). Cells with various concentrations of *Sipimikwanjung-tang*(SKT) extracts were treated with H_2O_2 or hypoxia for 1 or 2hr, and grown in 0.5 mg/ml MTT at 37℃. One hour later, 200μl of solubilization solution was added to each well and absorption values were read at 540nm on an automated SpectraMAX 250(Molecular Devices, USA) microtiter plate reader. Data were expressed as the mean percent of viable cells vs control.

3. Cell culture and growth factor

Hep G2 cells (American Type Culture Collection) were cultured in Dulbecco' s modified Eagle' s medium (DMEM) containing 10%(v/v) fetal bovine serum(FBS; Gibco BRL), 100U/ml penicillin G and 100g/ml streptomycin at 37℃ in a humidified atmosphere of air/CO_2(19:1). Human recombinant lyophilized transforming growth factor-1(TGFβ-1, 2mg)(Sigma) was dissolved in 1ml of 4mM HCl containing 0.1% bovine serum albumin, and divided into aliquots before storage at -70℃.

4. DNA fragmentation assay

Hep G2 cells were cultured in 100mm dishes in 10ml medium at plating densities of 1×10^6 cells/dish, and incubated at 3℃ in a humidified atmosphere of air/CO_2 (19:1) for 24 h prior to addition of experimental agents. Cells were then exposed to *Sipimikwanjung-tang*(SKT) extracts for another 24hr. After the treatment, cells were washed twice with PBS and harvested with incubation buffer (10mM EDTA, 50mM Tris-HCl, pH 8.0, 0.5% (v/v) sarkosyl and 1 g/ml proteinase K). The cells were incubated at 56℃ for 3hr, and then RNase (final concentration of 50g/ml) was added to the incubation buffer for a further 1h incubation. The DNA was extracted by phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and then precipitated with ethanol. The extracted DNA was separated and stained by electrophoresis in 2% agarose gel with ethidium bromide.

5. DAPI Staining

Hep G2 cells were seeded onto a chamber (Nunc, U.S.A.) precoated with 1% poly-D-lysine at a density of 1×10^5 cells/well and grown for 2hr. Cell cultures were exposed to *Sipimikwanjung-tang*(SKT) extracts for 24hr, then fixed with 4.0% paraformaldehyde in phosphate buffer for 10min. The cells were then stained with fluorescent 4,6-diamidino-2-phenylindole(DAPI) (1g/ml in methanol) for 15min. The number of nuclei in six fields per well was counted with a fluorescent microscope. The cells which showed the condensed nuclei were considered as cell in apoptotic status.

6. FACScan flow cytometric assay

The cells were seeded onto six-well plates, serum starved, and treated with or without the *Sipimikwanjung-tang*(SKT) extracts for 24hr. The cells were then washed twice with ice-cold PBS, and collected by centrifugation at 200g for 5min at 4℃. The cells were fixed in 70% (v/v) ethanol at 4℃ for 30 min. After fixation, the cells were treated with 0.2 ml DNA extraction buffer(0.2M Na_2HPO_4 and 0.1M citric acid buffer, pH 7.8) for 30min and then centrifuged and resuspended in 1 ml propidium iodide staining buffer(0.1% Triton X-100, 100g/ml RNase A, 80g/ml propidium iodide in PBS) at 37℃ for 30min. The cells were detected using a cytofluorometer, and analyzed by FACScan and CellQuest program(Becton Dickinson).

7. Preparation of cytosolic extracts and measurement of caspase-3 activity

Cells were seeded onto six-well plates for 24hr and

then treated with *Sipimikwanjung-tang*(SKT) extracts as a vehicle for 30 min. Then, vehicle or TGFβ-1 was added to the cells for another 16 hr. After the incubation period, cells were washed twice with ice-cold PBS, and then collected by centrifugation at 200×g for 5min at 4 ℃. The cell pellet was resuspended in lysis buffer (25 l/106 cells). After a 10-min incubation on ice, the cell homogenates were centrifuged at 10,000×g for 1 min and supernatants were removed for determination of caspase-3 activity. Proteolytic reactions were performed in a total volume of 100μl reaction buffer containing 50 μl of cytosolic extracts and 5μl DEVD-pNA, obtained from a commercial assay kit (Caspase-3 Colorimetric Assay Kit, R&D Systems). The reaction mixture was incubated at 37℃ for 1-2hr, and then the formation of p-nitroanilide was measured at 405 nm by an ELISA reader.

8. Preparation of Cell Extracts and Western Blotting

Test medium was removed from culture dishes and cells were washed twice with ice-cold phosphate-buffered saline(PBS), scraped off with a rubber policeman, and centrifuged for 10 min at 4℃. The cell pellets were resuspended in an appropriate volume(4×10^7 cells/ml) of lysis buffer containing 20mM Tris-HCl, pH7.5, 137mM NaCl, 1mM phenylmethylsulfonylfluoride(PMSF), 10g/ml aprotinin, 10 g/ml leupeptin, and 5g/ml pepstain A. The suspension was then sonicated. Protein concentration of samples was determined by Bradford assay (Bio-Rad, Hemel, Hempstead, UK) and samples equilibrated to 2 mg/ml with lysis buffer.

Protein samples containing 50g of protein were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride(PVDF) membranes(Millipore, Bedford, MA). Membranes were incubated for 1 hr with 5% dry skim milk in TBST buffer(0.1 M Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween-20) to block nonspecific binding, and then incubated with rabbit anti-caspase-3(1:2;000; Calbiochem, San Diego, CA). Subsequently, membranes were incubated with secondary antibody streptavidin-horseradish peroxidase conjugated affinity goat anti-rabbit IgG (Zymed, USA). Caspase-3 and PARP proteins were detected by chemiluminescence detection system according to the manufacturer' s instruction(ECL; Amersham, Berkshire, UK). The band intensity was quantified with a densitometric scanner(PDI, Huntington Station, NY).

9. Measurement of H_2O_2 generation

Intracellular H_2O_2 levels were measured by the fluorescent probe DCFH-DA. This cell-permeable dye, once inside the cells, is cleaved by endogenous esterase into DCFH. The intracellular non-fluorescent form of DCFH is oxidized, commonly by H_2O_2, into the fluorescent form, DCF. Hep G2 cells were seeded onto six-well plates for 24 hr and then incubated in the absence or presence of TGFβ-1 or *Sipimikwanjung-tang*(SKT) extracts for 4hr. Then 30min before termination of the incubation, DCFH-DA(10M) was added to the cells, and incubated for the last 30 min at 37℃. Cells were then harvested for the detection of H_2O_2 accumulation using FACScan flow cytometric analysis.

10. Assay for nuclear translocation of NF-κB

DNA binding activities of NF-κB were determined using electrophoretic mobility shift assay(EMSA). Hep G2 cells were incubated in the absence or presence of *Sipimikwanjung-tang*(SKT) extract, *Sipimikwanjung-tang*(SKT) extract plus catalase or NF-κB competitor for 1 hr, and then cells were washed twice with ice-cold PBS and collected in buffer A, containing 10mM HEPES(pH7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM dithiothreitol and 0.2mM phenylmethane sulphonyl

fluoride for 10 min on ice and centrifuged at 2000 rpm for 3min. The pellet was re-suspended in buffer C containing 20mM HEPES(pH7.9), 25% glycerol, 420 mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM dithiothreitol and 0.2mM phenylmethane sulphonyl fluoride, vortexed vigorously, and allowed to stand on ice for 20 min. The supernatants with soluble nuclear proteins were collected by centrifugation at 14,000 rpm for 5 min, and stored at −70℃. For NF-κB−DNA binding assay, each nuclear extract(2㎍) was incubated with a 35-base pair double-stranded ^{32}P-labeled probe encoding the κB consensus sequence(5'-AGT TGA GGG GAT CCC CCC AGG C-3') in binding buffer containing 10mM Tris-HCl, 40mM NaCl, 10% glycerol, 1mM EDTA, 1mM dithiothreitol, 1% Nonidet P-40, 1% deoxycholate, 3g/ml polydeoxyinosinic-deoxycytidylic acid at room temperature for 3 min. Then samples were applied to native 5% polyacrylamide gels and analyzed on autoradiography. For competition assay, 20-fold molar excess unlabeled consensus oligonucleotide was added at 30min prior to the addition of the labeled probe. Components of NF-κB proteins were identified by supershift assay using antibodies against p50 or p65 antibodies.

11. Data Analysis

All data were expressed as the mean ± SEM. For single variable comparisons, Student's t test was used. For multiple variable comparisons, data were analyzed by one-way analysis of variance(ANOVA) followed by Scheffe's test. *p* values less than 0.05 were considered significant.

Results

In this study, we have examined the effects of *Sipimikwanjung-tang*(SKT) extract in human hepatoblastoma Hep G2 cells, by means of MTT assay and cell morphology identification and in situ morphological assay of apoptosis using DAPI staining. The effect of *Sipimikwanjung-tang*(SKT) extract on the cytotoxicity of cells was evaluated using the MTT assay. Shown as Fig. 1, 24hr exposure to *Sipimikwanjung-tang*(SKT) extract showed a little decrease on the viability of Hep G2 cells below the concentration of 100 ㎕/㎖, but the viability of cells decreased dramatically over the concentration of 150㎕/㎖. The concentration required to inhibit growth of Hep G2 cells by 50% (IC_{50}) was approximately 150㎕/㎖(Fig. 1).

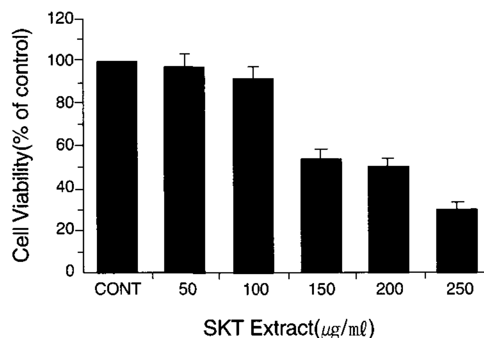

Fig. 1. Effect of the *Sipimikwanjung-tang*(SKT) extract on the cell viability of Hep G2 cells. The cells(1×10^6cells/㎖) were treated with various concentrations(50-250㎍/㎖) of the *Sipimikwanjung-tang*(SKT) extract and the cells were tested for viability by MTT assay for 24hr incubation after the treatment of the *Sipimikwanjung-tang*(SKT) extract.

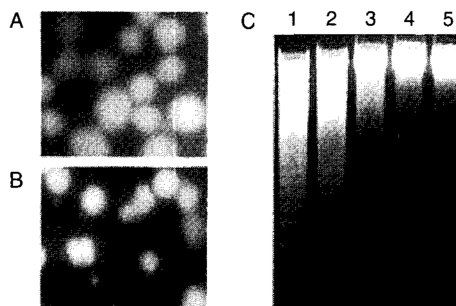

Fig. 2. Effect of *Sipimikwanjung-tang*(SKT) extrac on the morphology of Hep G2 cells. Hep G2 cells(1 $\times 10^6$cells/*ml*) were treated with various concentrations of *Sipimikwanjung-tang*(SKT) extract(40-250*μg/ml*) for 24hr. After the cells were stained with DAPI, and then subjected to cytospin, the morphologic changes were observed under a fluorescence microscope. (A):control, (B): 100*μg/ml* of *Sipimikwanjung-tang* extract; DNA fragmentation was done in Hep G2 cells after the treatment of *Sipimikwanjung-tang*(SKT) extract(50-250*μg/ml*). Hep G2 cells were exposed to the indicated concentration of *Sipimikwanjung-tang*(SKT) extract(50-250*μg/ml*) for 24hr. DNA was extracted, then separated by 1.2% agarose gel (contained ethidium bromide) electrophoresis, and visualized under UV light. C; lane 1, 50*μg/ml* of *Sipimikwanjung-tang*(SKT) extract; lane 2, 100*μg/ml*; lane 3, 150*μg/ml*; lane 4, 200*μg/ml*; lane 5, 250*μg/ml*.

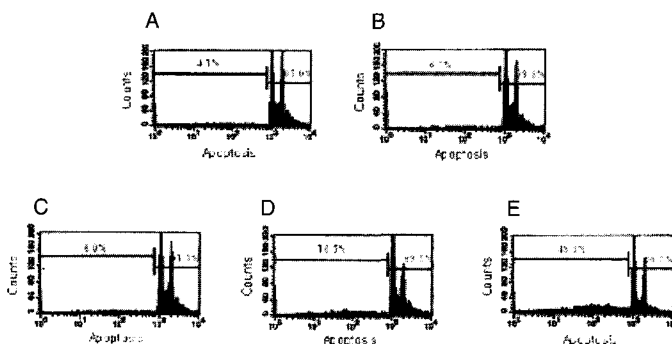

Fig. 3. Effect of *Sipimikwanjung-tang*(SKT) extract on apoptosis of Hep G2 cells by flow cytometric analysis. Flow cytometric analysis of DNA fragmentation following *Sipimikwanjung-tang*(SKT) extract treatment. Hep G2 cells were treated with various concentrations(40-200*μg/ml*) of *Sipimikwanjung-tang*(SKT) extract for 24hr. The harvested cells were fixed in fixed 100% ethanol and stained with propidium iodide, followed by flow cytometric analysis. The percentages of cells with hypodiploid DNA content represent fractions undergoing apoptotic DNA degradation. A; 40*μg/ml* *Sipimikwanjung-tang*(SKT) extract, B; 80*μg/ml*, C; 120*μg/ml*, D; 160*μg/ml*, E; 200*μg/ml*.

The results showed an apoptotic change after the treatment of *Sipimikwanjung-tang*(SKT) extract in a concentration of 50-250*μg/ml* on hepatoblastoma Hep G2 cells. In those concentration, the cells exposed to *Sipimikwanjung-tang*(SKT) extract showed a cytotoxic morphology and a apoptotic change. The cytotoxic effect, induced by *Sipimikwanjung-tang*(SKT) extract, was further examined using DNA ladder detection assays. The data showed that the extract profoundly induced DNA fragmentation in Hep G2 cells in concentration of 50-150*μg/ml*, indicating the occurrence of an apoptotic reaction(Fig. 2).

The effects of the extract on cytotoxicity were also examined using FACScan flow cytometric analysis.

The data of Fig. 3 showed that *Sipimikwanjung-tang*(SKT) extract had some apoptotic effect on Hep G2 cells, however the extract had no effect on the apoptosis on Hep G2 cells in the concentration of *Sipimikwanjung-tang*(SKT) extract over 120㎍/㎖(Fig. 3).

As demonstrated in Fig. 4, *Sipimikwanjung-tang*(SKT) extract had some influence on the phase distribution of the cell cycle progression. *Sipimikwanjung-tang*(SKT) extract significantly induced an increase in sub-G1 phase, indicating the induction of apoptosis in Hep G2 cells. The concentration-dependent response of *Sipimikwanjung-tang*(SKT) extract were analysed(Fig. 4).

The data showed that the extracts induced cell apoptosis in a concentration-dependent manner with 40-80㎍/㎖, respectively(Fig. 3). And the extracts showed a significant but modest increase in sub-G1 phase and G2/M phase arrest of the cell cycle(Fig. 4, 5).

Furthermore, in this study, several pharmacological

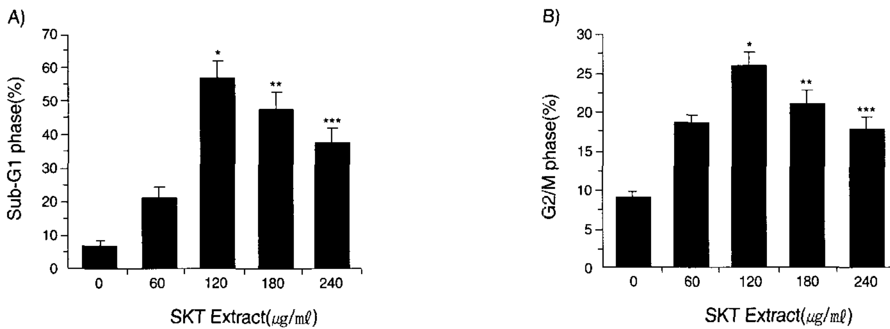

Fig. 4. Effect of *Sipimikwanjung-tang*(SKT) extract on cell cycle progression in Hep G2 cells. Cells were exposed to vehicle, *Sipimikwanjung-tang*(SKT) extract of the indicated concentration for 24hr. Then, the cells were harvested for the detection of cell cycle progression using FACScan flow cytometric analysis as described in the Materials and methods section. Data are expressed as the mean and SEM of four determinations. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with control.

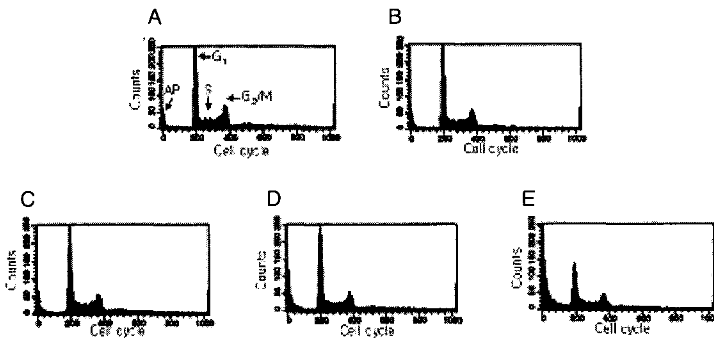

Fig. 5. Cell cycle analysis of Hep G2 cells treated with *Sipimikwanjung-tang*(SKT) extract. Hep G2 cells(1×106cells/㎖) were treated with various concentrations (40-200㎍/㎖) of *Sipimikwanjung-tang*(SKT) extract for 24hr. The cells were harvested, and DNA content was analyzed by FACS after propidium iodide staining of RNase-digested, fixed cells. A; 40㎍/㎖ *Sipimikwanjung-tang*(SKT) extract, B; 80㎍/㎖, C; 120㎍/㎖, D; 160㎍/㎖, E; 200㎍/㎖.

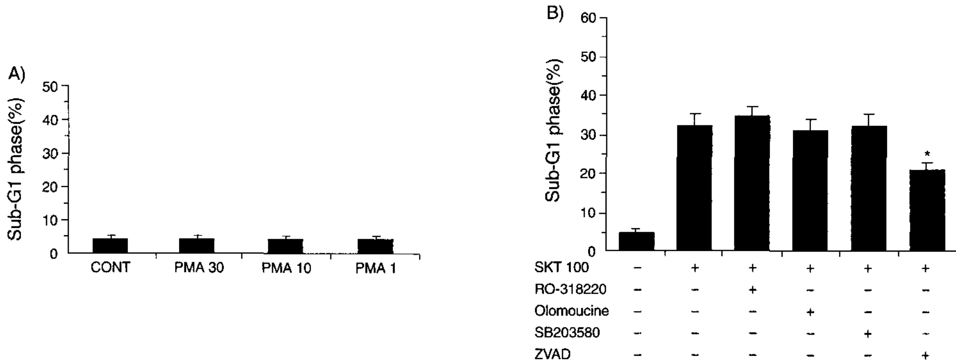

Fig. 6. Effect of several pharmacological agents and *Sipimikwanjung-tang*(SKT) extract on the regulation of apoptosis in Hep G2 cells. Cells were incubated without or with PMA for 24hr (A), or cells were preincubated with the indicated inhibitor for 30 min and then *Sipimikwanjung-tang*(SKT) extract was added to induce cell apoptosis using FACScan flow cytometric analysis as described in the Materials and methods section. Data are expressed as the mean and SEM of three determinations. *p<0.05 compared with control.

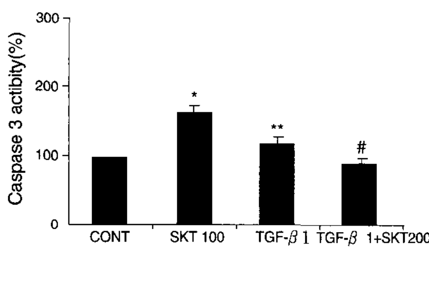

Fig. 7. Effect of *Sipimikwanjung-tang*(SKT) extract on caspase-3 activity. Cells were treated without (control) or with *Sipimikwanjung-tang*(SKT) extract, TGF-β1(10㎍/㎖) for 16 hr, and then cells were lysed for the detection of caspase-3 activity as described in the Materials and methods section. Data are expressed as mean±SEM of four determinations. *p<0.05 and **p<0.01 compared with the control, and #p<0.01 compared with TGF-β1 alone.

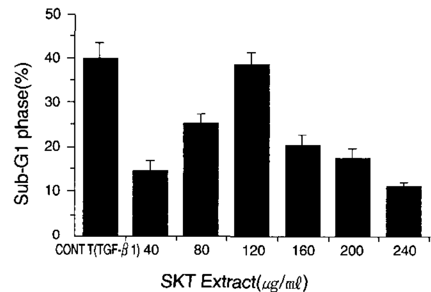

Fig. 8. Effect of *Sipimikwanjung-tang*(SKT) extract on TGF-β 1-induced apoptosis in Hep G2 cells. Cells were preincubated without (basal and control) or with *Sipimikwanjung-tang*(SKT) extract for 30min, and then vehicle (basal) or 10㎍/㎖ TGF-β1 was added to induce cell apoptosis using FACScan flow cytometric analysis as described in the Materials and methods section. Data are expressed as the mean and SEM of 4 determinations. *p<0.01 and **p<0.001 compared with TGF-β1 control.

agents were used to investigate the apoptotic mechanism. As demonstrated in Fig. 6, both the activation and down-regulation of protein kinase C by long-term exposure of low (30nM) and high concentration (1M) of phorbol 12-myristate 13-acetate(PMA) did not induce any apoptosis, revealing that the regulation of protein kinase C activity was not involved in the extracts(Fig. 6A).

Additionally, RO-318220 (3M, a protein kinase C inhibitor), olomoucine (60M) and SB203580 (10M, a p38 mitogen-activated protein kinase (MAPK, inhibitor) did not modify, while N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (zVAD-fmk;

50M) significantly inhibited the *Sipimikwanjung-tang* extract-induced apoptosis, suggesting the involvement of caspase activation in this reaction(Fig. 6B).

The caspase activation plays a pivotal role in the execution of apoptosis. Recent studies have demonstrated that caspase-3 is the major caspase activated in response to distinct apoptotic stimuli[3]. Furthermore, there are several lines of evidence suggesting that the activation of caspase-3 is involved in anti-tumor agent-induced apoptosis in Hep G2 cells[4].

We determined caspase-3 activity in this study, and the data showed that the *Sipimikwanjung-tang*(SKT) extractinduced a significant increase on the caspase-3 activity. This suggests that caspase-3 activity plays a role in *Sipimikwanjung-tang*(SKT) extract induced mechanism other than the other-mediated mechanism(Fig. 7).

The data showed that *Sipimikwanjung-tang*(SKT) extract did not induce any apoptosis in Hep G2 cells over the concentration of *Sipimikwanjung-tang*(SKT) extract 120㎍/㎖. Interestingly enough, the high dose of *Sipimikwanjung-tang*(SKT) extract over the concentration of the extract 120㎍/㎖ inhibited TGF-β1-induced apoptosis in a concentration-dependent manner(Fig. 8). The data demonstrated that TGF-β1 induced a significant increase in caspase-3 activity(Fig. 7).; however, it increased H_2O_2 generation in Hep G2 cells. The high dose of *Sipimikwanjung-tang*(SKT) extract completely abolished the caspase-3 activation induced by TGF-β1(Fig. 7). It is worth noting that the high dose of *Sipimikwanjung-tang*(SKT) extractinduced the increase in H_2O_2 generation in a concentration-dependent manner(Fig. 9).

It has been suggested that the activation of NF-κB contributes to antiapoptosis in a variety of cells. In addition, there are several lines of evidence suggesting that H_2O_2 is capable of inducing the activation of NF-κB in numerous types of cells[5]. In this study, using electrophoretic mobility shift assay to detect DNA binding activities of NF-κB, we found that the high dose of *Sipimikwanjung-tang*(SKT) extract significantly induced NF-κB activation. Furthermore, the high dose

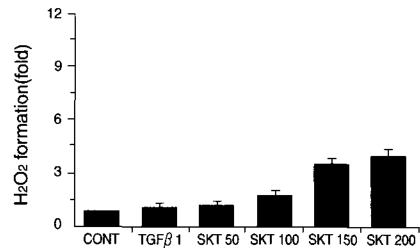

Fig. 9. Effect of TGF-β1 and on cytosolic H_2O_2 formation. Cells were incubated in the absence (control) or presence of TGF-β1 or *Sipimikwanjung-tang*(SKT) extractfor 4hr, and then H_2O_2 formation was detected using DCFH-DA fluorescent probe as described in the Materials and methods section. Data are expressed as the mean and SEM of four determinations. *p<0.01 and **p<0.001 compared with control.

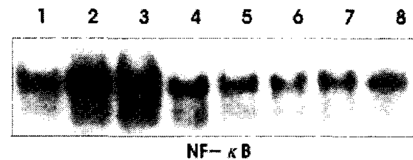

Fig. 10. Effect of *Sipimikwanjung-tang*(SKT) extract on NF-κB nuclear translocation in Hep G2 cells. Cells were incubated in the absence or presence of the indicated agents for 1hr, and then cells were prepared and nuclei were extracted for the detection of NF-κB nuclear translocation by EMSA technique as described in the Materials and methods section. Lane 1, control; lane 2, 10㎍/㎖ TGF-β1; lane 3, 200㎍/㎖ *Sipimikwanjung-tang*(SKT) extract;lane 4, 200㎍/㎖ *Sipimikwanjung-tang*(SKT) extract plus TGF-β1; lane 5, 4000U/ml catalase; lane 6, catalase plus 200㎍/㎖ *Sipimikwanjung-tang*(SKT) extract; lane 7, catalase plus 200㎍/㎖ *Sipimikwanjung-tang*(SKT) extract and TGF-β1; lane 8, NF-κB competitor.

of *Sipimikwanjung-tang* extract-induced effect was markedly inhibited by catalase, a H_2O_2 degrading enzyme, suggesting the involvement of a H_2O_2-dependent pathway(Fig. 10).

Discussion

In Sasang costitutional medicine, *Sipimikwanjung-tang*(SKT) extract has been one of the herbal prescription administered to Soeumin with general weakness, both limb weakness, urinary incontinence, impotence, edema, depression, indigestion, vomiting, diarrhea, ascites, chronic liver diseases, cancer as well as hepatoma[6-10]. The principles underlying *Sipimikwanjung-tang*(SKT) extractas well as many other traditional prescriptions were established on the basis of clinical experience and Sasang constitution. To date, the effect of many prescriptions as well as the harmful effects in most of these prescription drugs have not been identified. In this study, we have elucidated the pharmacological effects of *Sipimikwanjung-tang*(SKT) extract, especially as regards the role in the apoptotic reaction in hepatoblastoma Hep G2 cells, to evaluate their potential as a clinical medication.

At first, positive DAPI staining, DNA ladder assay were examined to determine apoptosis. In addition, the cells become rounder and apoptotic bodies are identified. According to these criteria, the present study revealed apoptosis in Hep G2 cells in response to the *Sipimikwanjung-tang*(SKT) extract. Apoptosis was also detected and quantified using FACScan flow cytometric analysis. The data were consistent with those of morphologic assay. Furthermore, it also revealed that *Sipimikwanjung-tang*(SKT) extract in a low dose was the most potent ingredient to cause apoptosis in Hep G2 cells, and high dose of *Sipimikwanjung-tang*(SKT) extractshowed the antiapoptotic effect. The high dose of *Sipimikwanjung-tang*(SKT) extract might have the necrotic effect on Hep G2 cells.

Two fundamental forms of cell death, that is to say, apoptosis and necrosis occurred after the apoptotic stimuli were also observed in a variety of cell types. Filipovic and colleagues[11] have suggested that oxidant-induced apoptosis activates poly(ADP-ribose) polymerase(PARP), and that the subsequent ATP and NAD depletions contribute to necrotic cell death in renal epithelial cells. Li and colleagues[12] observed that β-lapachone induced cell death in a spectrum of human carcinoma cells; it induced apoptosis in human ovary, colon, and lung cancer cells, and necrotic cell death in four human breast cancer cell lines. Therefore, it has been suggested that the necrosis might be secondary to the apoptosis in response to several apoptotic stimuli, or that these two different types of cell death share the same signaling events in their mechanisms of action.

In this study, it was evident by FACScan analysis that *Sipimikwanjung-tang*(SKT) extract showed a significant increase in apoptotic change and that the prescription induced a significant increase in Sub G1 phase and a modest increase in G2/M arrest(Fig. 4B), which is characterized by abnormal metaphase morphology, of the cell cycle. It has been suggested that disturbance in cdc2 kinase activity and tubulin assembly/disassembly contributes to the increase in G2/M arrest in numerous types of cells. It has been demonstrated that taxol stabilizes microtubules and induces G2/M arrest as well as cell apoptosis coincident with cdc2 activation[13]. These data have shown that the preponderance of apoptotic nuclei was most likely blocked at the G_1 phase. Apoptosis and proliferation are linked by cell cycle regulators, and apoptotic stimuli affect both cell proliferation and death. Cell cycle components such as p53, pRb and E2F, have been shown to participate in both cell cycle progression and apoptosis. p53, pRb, and E2F have also been acted as G_1 regulators. p53 and E2F are involved in the elimination of abnormal cells through apoptosis. In

constrast pRb induces G1 arrest and suppresses apoptosis. We examined mechnism for cell cycle arrest by *Sipimikwanjung-tang*(SKT) extract through futher studies of common components of apoptotic and cell cycle machinery. Furthermore, TGFβ-1 induced an increase in the cell population in the G2/M phase and a transient increase in cdc2 expression at an early phase of apoptosis in hepatoma FaO cells. Treatment with olomoucine blocked TGFβ-1-induced apoptosis in these cells[14].

Several molecular signals have been suggested to be involved in the regulation of apoptosis in various types of cells. Protein kinase C, a family of serine/threonine protein kinases, is involved in signal transduction pathways that regulate cell proliferation, apoptosis, differentiation and numerous cellular responses[15]. Its central role in these processes, such as tumor initiation, progression, invasion, angiogenesis and response to antitumor agents, makes it a potential therapeutic target in cancer treatment. We treated Hep G2 cells with long-term exposure to low or high concentration of PMA to activate or inactivate protein kinase C; however, cells demonstrated a nearly unaltered cell cycle distribution. Furthermore, RO-318220, a non-selective protein kinase C inhibitor, was employed to examine the functional involvement of this enzyme. However, cells pre-treated with or without RO-318220 did not behave distinctly differently in their viability against *Sipimikwanjung-tang*(SKT) extract treatment, suggesting that protein kinase C was not responsible for Hep G2 cell death incurred by *Sipimikwanjung-tang*(SKT) extract. p38 MAPK, another serine/threonine protein kinase, belongs to the MAPK superfamily and appears to play a crucial role in cell apoptosis, cytokine production and transcriptional regulation in response to diverse extracellular stimuli, including irradiation, heat shock, cytokines and chemotherapeutic agents[16].

In this work, SB203580, a selective inhibitor of p38 MAPK, did not influence the *Sipimikwanjung-tang* extract-induced effect. In a parallel experiment using Western blot detection, *Sipimikwanjung-tang*(SKT) extract showed little effect on the basal level of phosphorylated p38 MAPK expression(data not shown). These data indicate that *Sipimikwanjung-tang* extract-induced apoptosis was not accomplished through the activation of p38 MAPK.

Recent studies have suggested that apoptosis requires the participation of endogenous cellular enzymes. Central to the apoptotic program is a family of cysteine proteases, termed caspases[17-18]. It appears that apoptotic processes promoted by a variety of stimuli converge on the activation of a member of the caspase family. In living cells caspases are present as inactive zymogens and become activated following the apoptotic stimuli. To date, more than ten distinct human caspase genes have been identified[19]. Among these caspases, the activation of caspase-3 is the crucial event in a variety of cells leading to the execution of apoptosis[17-18]. In the present study, it was found that caspase-3 participates in the apoptosis induced by *Sipimikwanjung-tang*(SKT) extract, suggesting a central role of caspase-3.

It has been recognized that TGF-β1 induces an apoptotic reaction when added to cultures of hepatoma cell lines, or primary cultures[20]. Therefore, TGF-β1 is thought to be a very important factor in growth regulation in hepatocytes and hepatoma cells. In this study, we also examined the effect of *Sipimikwanjung-tang*(SKT) extracton TGF-β1-induced apoptosis in Hep G2 cells. We found that high dose of *Sipimikwanjung-tang*(SKT) extract could efficiently prevent the TGF-β1-induced apoptosis. In the previous study, it has been suggested that *Sipimikwanjung-tang*(SKT) extract exhibits antioxidant activity in both in vitro and in vivo studies[1-2]. One possible route of high dose of *Sipimikwanjung-tang* extract-mediated antiapoptosis is via its antioxidant activity. However, in this study TGF-β1 did not markedly generate reactive oxygen species(ROS), demonstrating

that the antioxidant effect could not reasonably explain high dose of *Sipimikwanjung-tang* extract-mediated mechanisms. As a point of interest though, high dose of *Sipimikwanjung-tang*(SKT) extract could profoundly induce the formation of H_2O_2. There is accumulating evidence suggesting that H_2O_2 appears to serve as a messenger mediating the activation of NF-κB, which plays a crucial role in the apoptotic, antiapoptotic, immune and inflammatory responses in a many cells[21]. Furthermore, it has been suggested that diverse agents, such as calcium ionophore, tumor necrosis factor, interleukin-1 and lipopolysaccharide, thought to activate NF-κB by distinct intracellular pathways, might all act through a common mechanism involving the synthesis of ROS[22]. In the present study, high dose of *Sipimikwanjung-tang*(SKT) extract markedly induced the activation of NF-κB. This high dose of *Sipimikwanjung-tang* extract-induced effect on the NF-κB level was significantly attenuated by catalase, a H_2O_2 degrading enzyme, indicating that H_2O_2 serves as the upstream activator of NF-κB activation of baicalein action. Furthermore, we also tried to identify the effect of high dose of *Sipimikwanjung-tang*(SKT) extract on NF-κB nuclear binding activity in Hep G2 cells. We found that the dose of *Sipimikwanjung-tang*(SKT) extract had some relation with the effect on this transcription factor. It is worth noting that high dose of *Sipimikwanjung-tang*(SKT) extract(150$\mu g/ml$) significantly inhibited the low dose of *Sipimikwanjung-tang* extract-induced apoptosis in Hep G2 cells. This has considerable implications for the efficacy of *Sipimikwanjung-tang*(SKT) extract. That is the point what is optimal dose of *Sipimikwanjung-tang*(SKT) extract when that is administered to patients to need the anti-cancer action. In order to increase the anticancer action against Hep G2 cells, it seems that the optimal dose of *Sipimikwanjung-tang*(SKT) extract will be furthermore studied.

In summary, after general examination of anticancer effect of *Sipimikwanjung-tang*(SKT) extract in the present study, the low dose of *Sipimikwanjung-tang*(SKT) extract cause some cytotoxic effect on human hepatoma Hep G2 cells, however the high dose of *Sipimikwanjung-tang*(SKT) extract showed a little antiapoptotic effect against TGF-β1-induced apoptosis. The optimal dose of *Sipimikwanjung-tang*(SKT) extract for anticancer effect and its mechanism should be furthermore studied.

Conclusion

We studied the effects of *Sipimikwanjung-tang*(SKT) extract on apoptosis in Hep G2 cells. The results are as follows :

1. *Sipimikwanjung-tang*(SKT) extract showed a decrease on the viability of Hep G2 cells, and showed a cytotoxic morphology and a apoptotic change.
2. *Sipimikwanjung-tang*(SKT) extract showed a significant but modest increase in sub-G1 phase and G2/M phase arrest of the cell cycle.
3. *Sipimikwanjung-tang*(SKT) extract induced a significant increase on the caspase-3 activity.
4. The high dose of *Sipimikwanjung-tang*(SKT) extract inhibited TGF-β1-induced apoptosis.
5. TGF-β1 had little effect on H_2O_2 generation in Hep G2 cells
6. The high dose of *Sipimikwanjung-tang*(SKT) extract significantly induced NF-κB activation, suggesting the involvement of a H_2O_2-dependent pathway.

In conclusion, our study provides evidences that *Sipimikwanjung-tang*(SKT) extractinduces cell apoptosis through the caspase-3-dependent pathways in the low concentration of *Sipimikwanjung-tang*(SKT) extract, but, the high dose of *Sipimikwanjung-tang*(SKT) extract in Hep G2 cells inhibits TGF-β1-induced apoptosis via

increase in cellular H_2O_2 formation and NF-κB activation in human hepatoblastoma Hep G2 cells. And so, the optimal dose of *Sipimikwanjung-tang*(SKT) extract for anticancer effect and its mechanism should be furthermore studied.

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