Effects of *Scutellaria baicalensis* Georgi on Macrophage-Hepatocyte Interaction Through Cytokines Related to Growth Control of Murine Hepatocytes

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The aim of this study is to elucidate the effects of *Scutellaria baicalensis* Georgi (SbG) extract and its constituents on macrophage-hepatocyte interaction in primary cultures. By using trans-well primary Kupffer cell culture or conditioned medium (CM) from murine macrophage RAW264.7 cell line (RAW cells), effects of SbG on hepatocyte growth were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide and trypan blue exclusion assay. Cytokine production, antibody-neutralization studies, and molecular mechanisms of transforming growth factor (TGF)-β1 gene expression were elucidated on SbG-treated RAW264.7 cells. In addition, recombinant human TGF-β1 (r-human TGF-β1) was added to elucidate the mechanisms of SbG effects on cultured hepatocytes. Immunohistochemistry using anti-NF-κB antibody was used to determine the possible signal transduction pathways in primary hepatocyte culture. The results showed that SbG stimulated the proliferation of cultured hepatocytes, possibly through NF-κB, but not of Toll-like receptor 4 activation; whereas SbG-RAW-CM and SbG in trans-well significantly suppressed the proliferation of hepatocytes. Antibody-neutralization studies revealed that TGF-β1 was the main antimitotic cytokine in SbG-treated RAW cells CM. The growth stimulation effect of SbG on cultured hepatocytes was inhibited by exogenous administration of r-human TGF-β1. Furthermore, SbG induced NF-kB translocation into the nuclei of cultured cells. In the RAW264.7 line, SbG and baicalin stimulated TGF-β1 gene expression *via* NF-κB and protein kinase C activation. We conclude that SbG stimulates hepatocyte growth *via* activation of the NF-κB pathway and induces TGF-β1 gene expression through the Kupffer cell–hepatocyte interaction, which subsequently results in the inhibition of SbG-stimulated hepatocyte growth. Exp Biol Med 231:444–455, 2006

Key words: liver regeneration; *Scutellaria baicalensis* Georgi; interleukin-6 (IL-6); cDNA microarray; macrophage

Introduction

The liver is an organ containing many cell types that perform different biological functions. Some functions are mainly achieved by one cell type, while others are achieved with the aid of more than one cell type. For example, hepatocytes are known to execute the metabolic and detoxification processes, whereas activation of Kupffer cells and quiescent hepatic stellate cells (HSCs) to a myofibroblast phenotype are key events in liver inflammation and fibrosis (1–3). There is evidence that nerve growth factor expressed in hepatocytes during fibrotic liver injury may regulate activated HSCs through induction of apoptosis (4). Moreover, interaction between nonparenchymal cells and parenchymal cells has also been demonstrated in liver regeneration (5, 6). Nevertheless, information concerning

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how medicinal herbs affect such paracrine-regulated growth control of the hepatocytes remains lacking.

In the past decade, the prevalence and costs of alternative medicine have been increasing (7, 8). Sho-saiko-to (TJ-9, Japanese name), or Xiao-tsai-hu-tang (Chinese name), is a well-known herbal medicine commonly used for the treatment of human liver diseases in Asia. In animal studies, its therapeutic effects on hepatic fibrosis are postulated to operate through the inhibition of oxidative stress in hepatocytes and hepatic stellate cells (9, 10). Sho-saiko-to has been shown in a randomized controlled trial to have chemoprevention effects on carcinogenesis of human hepatocellular carcinoma, especially for those patients without hepatitis B virus infection (11). Previous investigations have shown that *Scutellaria baicalensis* Georgi (SbG) has antiproliferation activity in different kinds of cells (11–13). However, controversy remains as a result of the observation that active constituents of TJ-9 induced liver regeneration by increasing the production of hepatocyte growth factor and suppressing the production of transforming growth factor-β (TGF-β) (10). The exact mechanisms by which these compounds affect Kupffer cell–hepatocyte interaction are unclear.

It is well known that cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) secreted from nonparenchymal cells are major factors in priming quiescent hepatocytes to proliferative status during liver regeneration, whereas TGF-β plays an important role in terminating the regeneration process (5, 14, 15). TGF-β belongs to a superfamily of multifunctional cytokines that mediates differentiation, growth, and apoptosis in a variety of cell types. Besides, overexpression of TGF-β1 in a pathologic situation may produce large amounts of extracellular matrix and thereby cause hepatic fibrosis (1, 14). However, it remains unclear whether and how SbG modulates TGF-β–related growth control between parenchymal and nonparenchymal cells of the liver.

In our previous cDNA microarray analysis on mice regenerating liver, there was a good correlation between global gene expression profiles and liver responses to a SbG-containing remedy. Such correlation was verified by *in vivo* biological studies, and the results showed that the aforementioned remedy inhibited the hepatocytes growth through downregulation of the expression of immediate early gene encoding c-myc binding protein and cell cycle–related genes (16). Furthermore, the mechanisms of such growth inhibition are postulated to operate through the activation of macrophage-secreting cytokine pathway (17). Therefore, the aim of this study is to investigate the modulating effects of SbG and its constituents on interaction of different cell types in the liver, specifically on macrophage-hepatocyte interaction related to growth control of rodent cultured hepatocytes.

Materials and Methods

Cell Culture and Reagents. Primary mouse hepatocytes, primary mouse liver Kupffer cells and mouse macrophage cell line RAW264.7, denoted as RAW cells here (American Type Culture Collection TIB 71), were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) at 37°C, 5% CO_2, supplemented with 2 m*M* L-glutamine, 10% fetal calf serum (5% for RAW cells), nonessential amino acid (Gibco-BRL), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1.5 μg/ml fungizone (Gibco-BRL). NF-κB inhibitor (aspirin) and protein kinase C (PKC) inhibitor [1-(5-isoquinolinesulfonyl)-2-methylpiperazine] (H7) were purchased from Sigma Chemical Company (St. Louis, MO). MEK-1 inhibitor [2-(2′-amino-3′methoxyphenyl)-oxanaphthalen-4–1] (PD98059), and P38 kinase inhibitor [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole] (SB203580) were obtained from Calbiochem (La Jolla, CA).

Herb Preparation and Liquid Chromatography. SbG was purchased from a local wholesale distributor (Taipei, Taiwan, R.O.C.). The experimental herbal preparation was prepared per the following procedures. A 27-g SbG was extracted with 2700 ml distilled water and boiled at 100°C until the total volume was reduced to 750 ml. The extracts were filtered through layers of gauze, the residues discarded, and the filtrates were kept at –20°C, followed by lyophilization of the samples.

Ingredients in the herbal extract, such as baicalin (molecular weight, 446.37; 100% purity), baicalein (molecular weight, 270.20; 99.67% purity), wogonin (molecular weight, 284.27; 99.47% purity), and chrysin (molecular weight, 254.24; 96% purity), were purchased from Nacalai Tesque (Kyoto, Japan). Herbal extract dissolved in distilled water and its major constituents dissolved in methanol were separated using an Alltima reversed-phase C_{18} column (250 × 4.6 mm i.d.; particle size, 5 μm; Deerfield, IL) equipped with a chromatographic pump (PM-80; Bioanalytical Systems, West Lafayette, IN), an injector (Rheodyne 7125, Rohnert Park, CA), and an ultraviolet detector (Varian, Walnut Creek, CA). Samples were injected through the mobile phase comprising 10 m*M* monosodium phosphoric acid–acetonitrile (69:31, v/v, pH 3.0), with a flow rate of 1 ml/min and detected by an UV wavelength at 270 nm. Output data from the detector were integrated *via* an EZChrom chromatographic data system (Scientific Software, San Ramon, CA).

Animals and Partial Hepatectomy. Male BALB/c mice, 6–8 weeks old, weighing 20–25 g, were purchased from the animal center of the National Science Council in Taiwan, R.O.C. They were fed a standard laboratory diet, provided water *ad libitum*, and treated under the regulations set forth in the *Guide for the Care and Use of Laboratory Animals*. The studies were approved by a committee for

experimental animals organized at National Yang-Ming University.

Under ketamine (50 mg/kg, ip) anesthesia, a midline incision was made and a two-thirds partial hepatectomy (PHx) was performed, as described by Higgins and Anderson (18). Resected livers were frozen in liquid nitrogen before storage at −70°C. Mice were allowed to recover with free access to food and water until they were sacrificed, at intervals of 15 mins, 4, 24, 36, 48, 72, 96, 120, 168, and 240 hrs after PHx ($n \geq 6$ in each group at different time points.

Bromodeoxyuridine (BrdU) Incorporation and DNA Flow Cytometric Analysis. For *in vivo* BrdU incorporation studies, BrdU pulse-labeling and anti-BrdU immunofluorescence were used to detect DNA replication foci in regenerating livers. In brief, 200 mg/ml BrdU (no. B-5002; Sigma Chemical) was injected ip 2 hrs before surgical removal of liver remnants at different intervals after PHx. After mice were sacrificed, liver remnants were removed and immediately frozen in liquid nitrogen, followed by homogenizing the liver tissues for flow cytometric analysis. Cell cycle analysis using double stain, namely, fluorescein isothiocyanate (FITC) for BrdU and propidium iodide (PI) for DNA content, was performed as described previously (19). In brief, 0.5 ml (v/v) 4 m*M* citrate buffer (pH 7.2) was added to the prepared samples, and this was followed by incubation with 0.1 ml mouse anti-BrdU monoclonal antibody (no. M-0744; DAKO, Copenhagen, Denmark) at 4°C for 30 mins. The samples were then incubated with 0.1 ml rabbit anti-mouse immunoglobulins-FITC (no. F-0313; DAKO) at 4°C for another 30 mins. After the samples were treated with solution B (v/v) (50 μg/ml PI, 3% polyethyleneglycol 6000, 0.1% Triton X-100, 0.4 *M* NaCl, pH 7.2) and incubated at 4°C for 30 mins, the cell supernatants were detected using a flow cytometer (Epics Profile; Coulter Electronics, Hialeah, FL) supplemented with an Omnichrom 500 series (150 mW; Ominicon, NY) argon ion laser (25 mW at 488 nm) and a 610-nm red fluorescence filter (3802055; Coulter Electronics). All data were analyzed with an offline software (Cytology; Coulter Electronics) and presented as the percentage of BrdU(+) in S-phase cells. The results were double-checked by immunostaining on tissue sections.

Isolation of Mice Hepatocyte and Kupffer Cells for Primary Culture. Isolation of hepatocytes from male BALB/c mice was prepared, with some modification, as described previously (20). After ligating the central vein, the anesthetized and heparinized mouse was cannulated with a catheter through the portal vein. The liver was perfused with calcium-free Hanks' balanced salt solution (HBSS; Gibco-BRL) supplemented with 0.5 ml 0.5 *M* ethylene glycol-bis (β-aminoethylether)N,N,N′,N′-tetraacetic acid (EGTA) (Sigma Chemical) 10 ml 0.5M HEPES (11344-033, Gibco-BRL) and 0.5 ml 50 mg/ml gentamycin sulfate (Sigma Chemical) at 37°C at a rate of 5 ml/min, followed by perfusion with collagenase-containing perfusion solution, which contained DMEM (Gibco-BRL) supplemented with 10 ml 0.5 *M* HEPES, 0.5 ml 50 mg/ml gentamycin sulfate, and Type IV collagenase (20 mg/50 ml; Sigma Chemical) followed by centrifugation at 50 *g* (3 min for 2 cycles). After isolation, hepatocytes were cultured with DMEM with 25 ml fetal bovine serum (Hyclone), 5 ml penicillin streptomycin (Gibco-BRL), and 0.3 ml fungizone (Gibco-BRL). The culture plates should be coated with 2 mg/ml collagen Type I (Sigma Chemical) in 12.5 ml 0.013 *M* HCl. Isolation Kupffer cells from BALB/c mice were obtained as described previously (21). The perfusion process to obtain Kupffer cells was the same performed for isolation of primary hepatocytes, as mentioned above, with centrifugation at 300 *g* (10 min for 2 cycles). After perfusion, the liver cell suspension was centrifuged with gradient medium (30% histodenz; Sigma Chemical, in HBSS) followed by centrifugation at 800 *g* (30 min) to obtain Kupffer cells and hepatocytes.

To elucidate the role of Toll-like receptor 4 (Tlr4) in SbG-modulated hepatocyte growth, a defective lipopolysaccharide signaling mice (C3H/HeJ) was used (22). The Tlr4 sequence from C3H/HeJ mice has been confirmed in our laboratory.

Collection of Conditioned Medium (CM) from Mouse RAW Cells. To elucidate the effect of SbG on RAW cells to produce growth-related cytokines, 1×10^3 cells were incubated separately in DMEM containing 10% bovine calf serum (BCS) (JRH Bioscience, Lenexa, KS) with or without SbG (0.1–10 μg/ml) at 37°C for 48 hrs. The CM, denoted as SbG-RAW-CM, was collected, filtered, and stored at −70°C until use.

Assessment of Cell Number for CM-Treated and Trans-Well Cultured Hepatocytes. After a total of 1×10^4 hepatocytes/well was seeded onto a 96-well plate or trans-well system overnight, cells were incubated with SbG extracts or their constituents on trans-well cultures or with SbG-RAW-CM at 37°C for another 48 hrs, respectively. In SbG-RAW-CM–treated experiments, cell number was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)–based colorimetry, in which the absorbance was set at the wavelength of 570 nm (EL311SX reader; Bio-Tek Instruments, Inc., Winooski, VT). The optic density was normalized into cell numbers using linear regression curves obtained from different cell numbers seeded 1 day before each experiment. Because it was difficult to count cell number by MTT assay in a trans-well system, cell number in such a system was evaluated by counting the trypan blue–stained, trypsin-digested hepatocytes with a hemocytometer. To prevent contamination of lipopolysaccharide (LPS) on drug preparation, cells in each experiment were prepared in the presence of 10 μg/ml of polymyxin B (Sigma Chemical).

Antibody Neutralization in CM from Mouse RAW Cells. To elucidate the effect of SbG on RAW cells to produce growth-related cytokines and to determine how these cytokines affect the growth of cultured hepatocytes,

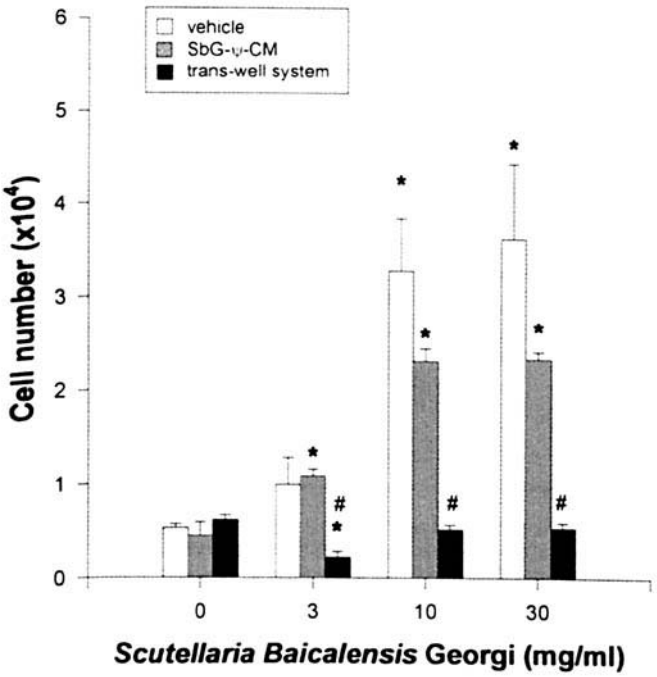

Figure 1. Effects of *Scutellaria baicalensis* Georgi (SbG) on macrophage-hepatocyte interaction. The isolated hepatocytes were cultured overnight, followed by treatment of vehicle (□), SbG-RAW-CM (■), and treatment in a trans-well system (■) for another 48 hrs. The cell number of hepatocytes in primary culture was evaluated by MTT or trypan blue–exclusion assay (trans-well system), as described in Materials and Methods. *, $P < 0.05$ vs. SbG (0 mg/ml); #, $P < 0.05$ versus SbG alone-treated group (one-way ANOVA followed by Dunnet's *t* test).

different neutralizing antibodies (R&D System, Minneapolis, MN) against TNF-α, IL-6, and TGF-β were used.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR). For transcription analysis, total RNA was isolated from 1×10^6 RAW cells and reversed-transcribed into cDNA, and then PCR amplification of the cDNA was performed, as previously described (23). Three micrograms of cellular RNA was used as a template along with RT-generated cDNA. The sequences of PCR primers are as follows: mouse TGF-β1 (439 base pairs [bp]) (sense, 5′-CTGTCCAAACTAAGGCTC GC-3′; antisense, 5′-CGTCAAAAGACAGCCACTCA-3′) and mouse β2-microglobulin (β2U, internal control) (222 bp), (sense, 5′-TGACCGGCTTGTATGCTATC-3′; antisense, 5′-CAGTGTGAGCCAGGATATAG-3′). Thermal cycle conditions were 94°C for 30 secs, denaturing; 54°C for 30 secs, annealing; and 72°C for 60 secs, extension; and this cycle was repeated 25 times. The PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. For semiquantitative analysis, more than three independent samples were used, and each experiment was performed to obtain a TGF-β1 to β2-microglobulin ratio. The data in each experiment are expressed as mean ± SEM.

Real-Time RT-PCR. Quantitative real-time PCR was performed using a LightCycler machine (Roche, Mannheim, Germany) with SYBR Green dye, and the threshold cycle numbers were calculated using LightCycler software (version 3.5; Roche). The TGF-β1 forward primer sequence was 5′-ACTATTGCTTCAGCTCC-3′, and its reverse primer sequence was 5′-GTGTTGGTTGTAGAGGG-3′. The conditions for amplification were as follows: 1 cycle at 95°C for 6 mins, followed by 70 cycles at 95°C for 5 secs and 59°C for 5 secs and 72°C for 8 secs. Reactions were performed in triplicate, and threshold cycle numbers were averaged.

Table 1. Effects of Cytokine-Neutralizing Antibodies on the Growth Inhibition of Primarily Cultured Hepatocytes Treated by Extract of *Scutellaria baicalensis* Georgi[a]

Treatment	SbG (μg/ml)	Percentage of inhibition (%)
Condition medium alone	0.0	22 ± 6.5
	0.1	37 ± 9.7
	1.0	43 ± 4.0
	10	46 ± 6.4*
+ anti–IL-6	0.0	28 ± 18.4
	0.1	26 ± 5.6
	1.0	37 ± 14.6
	10	39 ± 19.1
+ anti–TNF-α**	0.0	51 ± 14.2
	0.1	44 ± 4.0
	1.0	19 ± 5.9
	10	28 ± 3.9
+ anti–TGF-β1**	0.0	46 ± 3.3
	0.1	29 ± 5.3
	1.0	22 ± 8.1
	10	17 ± 7.8*
+ anti–IL-6 + anti–TNF-α	0.0	65 ± 14.2
	0.1	64 ± 8.9
	1.0	47 ± 3.3
	10	23 ± 4.0*
+ anti–IL-6 + anti–TGF-β1	0.0	25 ± 6.8
	0.1	40 ± 20.7
	1.0	23 ± 17.4
	10	24 ± 9.0
+ anti–TNF-α + anti–TGF-β1	0.0	37 ± 14.7
	0.1	37 ± 10.1
	1.0	23 ± 4.5
	10	19 ± 8.2
+ anti–IL-6 + anti–TNF-α + anti–TGF-β1	0.0	35 ± 8.4
	0.1	24 ± 3.4
	1.0	32 ± 13.4
	10	32 ± 10.0

[a] Macrophage cell line RAW264.7 with different doses of *Scutellaria baicalensis* Georgi (SbG) were treated with or without various cytokine-neutralizing antibodies (anti–IL-6, anti–TNF-α, anti–TGF-β1) at 37°C for 24 hrs. The conditioned media were added to cultured hepatocytes for another 48 hrs and MTT assay was performed. Data are expressed as mean ± SEM of four separate experiments.
* $P < 0.05$ versus no SbG treatment (one-way ANOVA); ** $P < 0.005$ versus conditioned medium alone (two-way ANOVA).

Cytokine Production from RAW Cells. Cytokines such as TNF-α, IL-6, and TGF-β1 from RAW cells were measured using a commercially available enzyme-linked immunosorbent assay kit (BioSource, Inc., Camarillo, CA).

Table 2. Cytokine (pg/ml) Secretion from RAW264.7 Induced by Major Constituents and Crude Extracts of *Scutellaria baicalensis* Georgi[a]

		IL-6	TNF-α	TGF-β1
SbG	0.0 μg/ml	6.6 ± 1.08	12 ± 2.1	37 ± 7
	0.1 μg/ml	6.4 ± 1.12	45 ± 2.3*	59 ± 9
	1.0 μg/ml	11.4 ± 2.35*	62 ± 6.0*	69 ± 8*
	10 μg/ml	16.1 ± 2.42*	86 ± 7.4*	85 ± 5*
DMSO		7.5 ± 1.25	24 ± 4.6	25 ± 2
Baicalin	10^{-6} *M*	8.8 ± 2.34	21 ± 3.8	32 ± 8
	10^{-5} *M*	7.0 ± 1.27	44 ± 11.8*	40 ± 10*
Baicalein	10^{-6} *M*	9.4 ± 2.48	28 ± 3.9	46 ± 10*
	10^{-5} *M*	18.4 ± 3.81*	19 ± 1.8	86 ± 10*
Chrysin	10^{-6} *M*	4.8 ± 0.58	29 ± 8.0	66 ± 5*
	10^{-5} *M*	10.0 ± 2.77	17 ± 1.1	118 ± 10*
Wogonin	10^{-6} *M*	14.0 ± 3.32*	27 ± 9.1	60 ± 3*
	10^{-5} *M*	5.3 ± 0.69	26 ± 4.9	99 ± 9*

[a] SbG, *Scutellaria baicalensis* Georgi. Macrophage cell line RAW264.7 (1×10^3) was incubated at 37°C for 2 days with or without treatment. Conditioned media were then collected for evaluating various cytokines. In pure compound experiments, DMSO was used for vehicle (solvent) control. Data were presented as mean ± SEM and were analyzed by one-way ANOVA followed by Dunnet's *t* test.
* $P < 0.05$ compared with no treatment in SbG group or DMSO in pure compound groups.

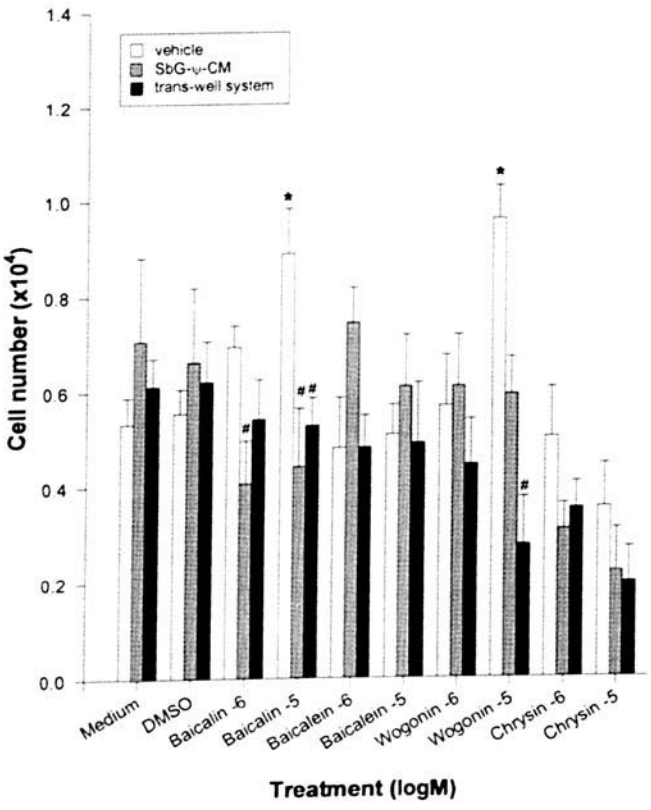

Figure 2. Effects of flavonoids in *Scutellaria baicalensis* Georgi (SbG) on macrophage-hepatocyte interaction. The isolated hepatocytes were cultured overnight, followed by treatment of vehicle (□), SbG-RAW-CM (■) for another 48 hrs. The cell number of hepatocytes in primary culture was evaluated by MTT or trypan blue–exclusion (trans-well system, ■) assay, as described in Materials and Methods. The flavonoids include baicalin, baicalein, wogonin, and chrysin. *, $P < 0.05$ vs. DMSO group; #, $P < 0.05$ versus hepatocyte-alone group (one-way ANOVA followed by Dunnet's *t* test).

Proliferation of Hepatocytes in Primary Culture. The growth of primarily cultured hepatocytes was assayed by BrdU pulse-labeling and anti-BrdU immunofluorescence flow cytometry, as mentioned above. In brief, isolated primary hepatocytes (5×10^5) were cultured overnight, treated with SbG (0, 10, or 30 mg/ml),

Table 3. Effects of Aspirin, SB203580, PD98059, and H7 on Baicalin-Treated RAW264.7 Cells[a]

Treatments (*M*)	TGF-β/β_2–microglobulin (Ratio of control)
Vehicle	1 ± 0.7*
Baicalin 10^{-5}	1 ± 0.7*
Baicalin 10^{-5} + inhibitors	
Aspirin	
10^{-6}	25 ± 2.7
10^{-5}	15 ± 1.7*
10^{-4}	5.7 ± 1.5*
SB203580	
10^{-6}	27 ± 3.9
10^{-5}	20 ± 2.6
10^{-4}	21 ± 3.2
PD98059	
10^{-6}	26 ± 4.9
10^{-5}	25 ± 6.0
10^{-4}	21 ± 2.8
H7	
10^{-6}	27 ± 5.1
10^{-5}	9.1 ± 2.3*
10^{-4}	11 ± 2.1*

[a] RAW264.7 cells (1×10^6 cells/well) were coincubated with baicalin (10^{-5} *M*) and either inhibitors (10^{-6}–10^{-4} *M*) including NF-κB inhibitors, aspirin; p38 MAPK inhibitors, SB203580; ERK/MAPKK inhibitors, PD98059; or PKC inhibitors, H7, for 6 hrs, and the cellular RNA was extracted for RT-PCR analysis as described in Materials and Methods. The data were presented as mean ± SEM and were analyzed by one-way ANOVA followed by Dunnet's *t* test.
* $P < 0.05$ compared with baicalin-treated group, and $n = 3$.

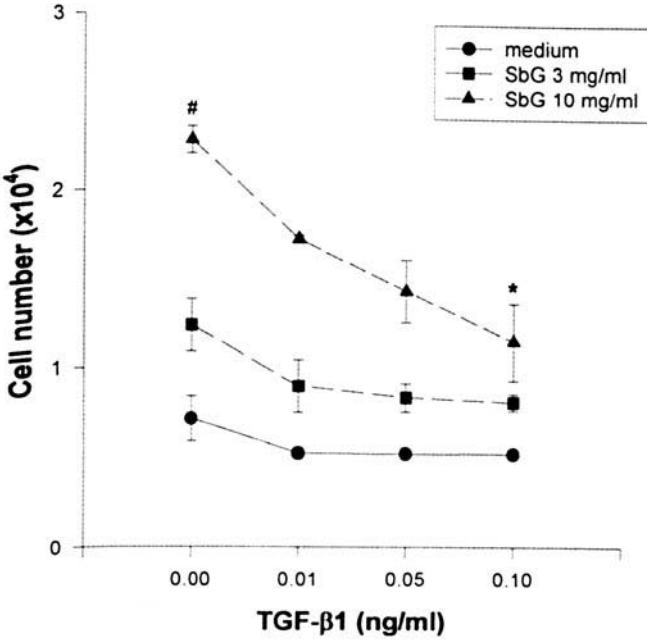

Figure 3. Effect of r-human TGF-1 on suppression of hepatocytes growth induced by extract of *Scutellaria baicalensis* Georgi (SbG). The isolated hepatocytes were cultured overnight, followed by treatment of SbG (0 mg/ml, ●; 3 mg/ml, ■; 10 mg/ml, ▲), and exogenous r-human TGF-β1 (0, 0.01, 0.05, and 0.1 ng/ml) was administered simultaneously. The cell number of hepatocytes in primary culture was evaluated by MTT, as described in Materials and Methods. *, $P < 0.05$ versus TGF-β1 (0 ng/ml) group; #, $P < 0.05$ versus SbG (0 mg/ml) group (one-way ANOVA followed by Dunnet's *t* test).

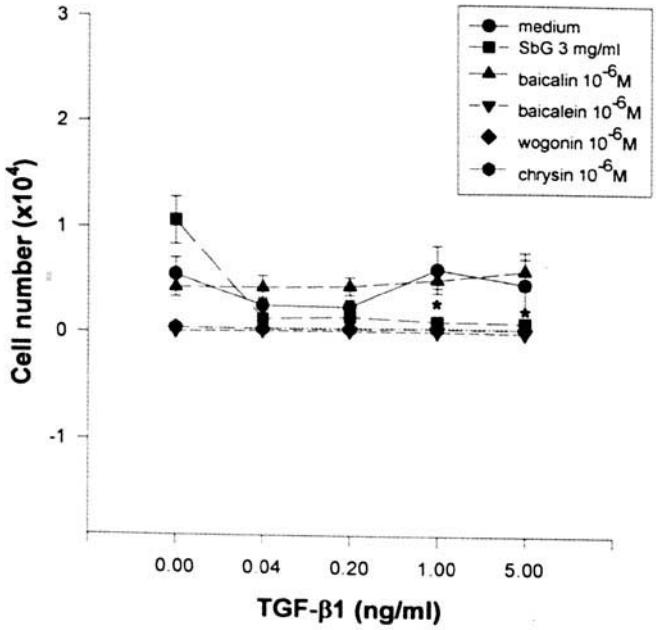

Figure 4. Effect of r-human TGF-β1 on suppression of hepatocyte growth induced by flavonoids extracted from *Scutellaria baicalensis* Georgi (SbG). The isolated hepatocytes were cultured overnight, followed by treatment of vehicle (●), SbG (3 mg/ml, ■) of flavonoids (10^{-6} *M*, ▲ ▼ ♦ ●), and exogenous r-human TGF-β1 (0, 0.04, 0.20, 1.00, and 5.00 ng/ml) was administrated simultaneously. The cell number of hepatocytes in primary culture was evaluated by MTT, as described in Materials and Methods. The flavonoids include baicalin, baicalein, wogonin, and chrysin. *, $P < 0.05$ versus TGF-β1 (0 ng/ml) group (one-way ANOVA followed by Dunnet's *t* test).

incorporated with BrdU for another 24 hrs; then cells were trypsinized, washed with phosphate-buffered saline, and, finally, the cell suspensions were ready for immunofluorescence flow cytometric analysis. The data were also double-checked by immunohistochemistry.

Signal Transduction Related to SbG-Modulated Cell Growth on Cultured Hepatocytes. To elucidate the possible signal transduction pathway involved in SbG-stimulated hepatocyte growth, some signal transduction factors, such as PKC and serine-threonine protein kinase–encoded AKT, were investigated with chelerythrin (24) and wortmannin (25), respectively. After blocker treatment for 1 hr, SbG (0, 3, 10, and 30 mg/ml) were added for another 48 hrs, followed by MTT assay on cultured hepatocytes.

Double-Stained Immunohistochemistry for NF-κB Translocation. To provide direct evidence of NF-κB activation, double-immunostaining with FITC for NF-κB (p65) and PI for nuclei staining were performed on 4 hr–cultured hepatocytes. In brief, following collagen Type IV mounting on the slides, primary hepatocytes were seeded onto the slides for 4 hrs, followed by overnight cultures with fresh complete medium. After incubation with medium alone, SbG (10 mg/ml), or baicalin (5×10^{-6} *M*) for 2 or 4 hrs, cells were fixed with 2.5% paraformaldehyde for 15 mins in 4°C. After blocking with 5% bovine serum albumin for 1 hr, cells were incubated with anti–NF-κB primary antibody (anti-P65; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr, followed by incubation with secondary antibody (donkey anti-rabbit immunoglobulin G (H+L)-FITC; Jackson ImmunoResearch, West Grove, PA) for 1 hr with PI (1μ*M*) for 10 mins. Then the cells were mounted with 50% glycerol and observed under a fluorescence microscope (Zeiss, Hamburg, Germany).

Statistical Methods. Results were expressed as the mean ± SEM. Differences between groups on each time point were identified by one-way analysis of variance (ANOVA) followed by Dunnet's *t* test. The interaction between concentration–response curve and different treatments was analyzed by two-way ANOVA or general linear models procedure followed by post-hoc Dunnet's test (SPSS 12.0.1 for Windows, Release 12.0.1; SPSS Inc., Chicago, IL). *P* values of ≤0.05 were considered statistically significant.

Results

SbG-Containing Remedy Suppressed the Proliferative Capacity in Regenerating Mouse Liver. Our previous cDNA microarray data showed that an herbal remedy containing SbG downregulated the expression of immediate early gene encoding c-myc binding protein and cell cycle–related genes. To correlate the information obtained from cDNA microarray and the liver responses

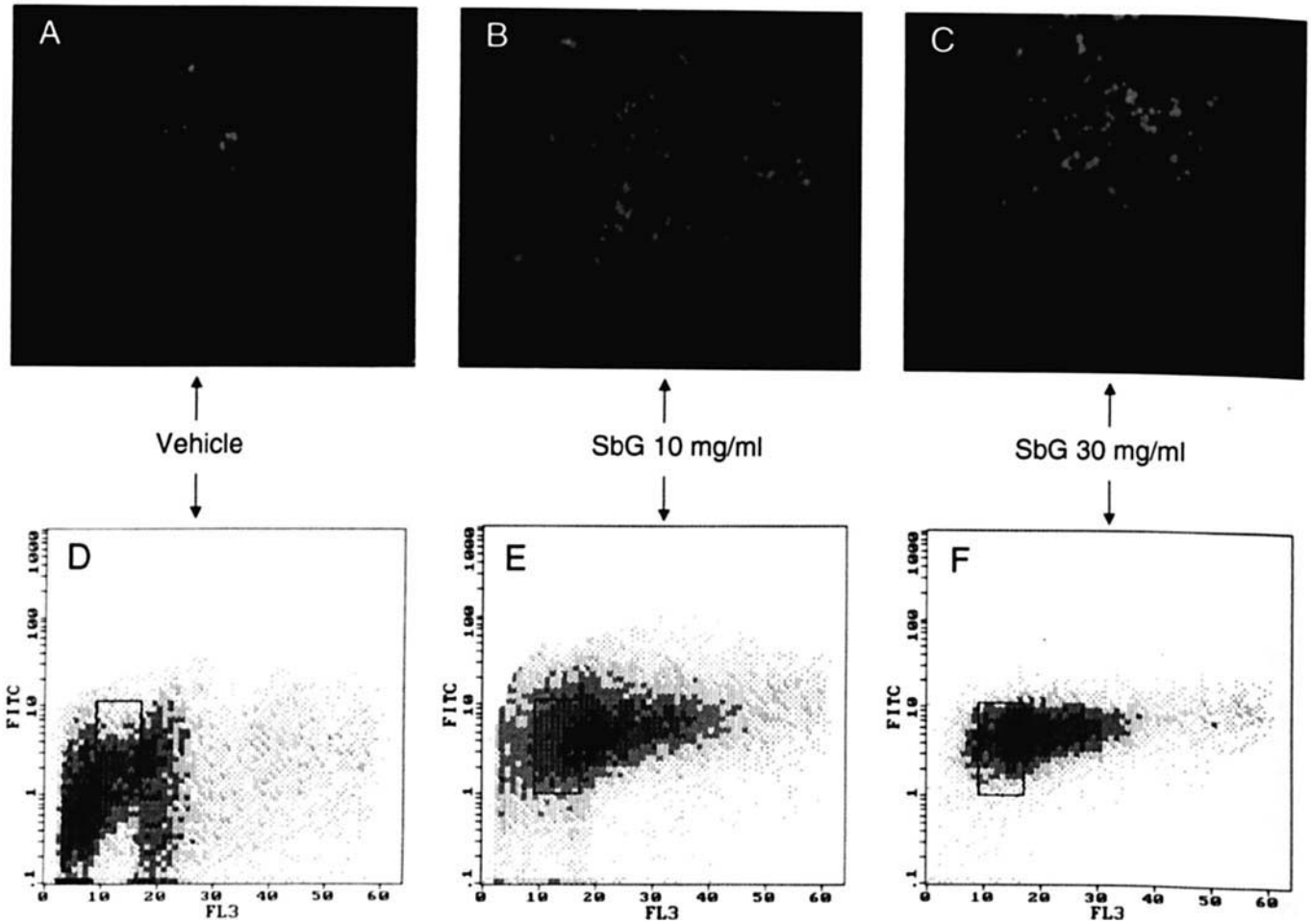

Figure 5. Effects of *Scutellaria baicalensis* Georgi (SbG)–stimulated cell growth in cultured hepatocytes. The isolated hepatocytes were cultured overnight, followed by treatment of SbG (0, 10, and 30 mg/ml) and incorporated with bromodeoxyuridine for 24 hrs for immunohistochemistry assay (vehicle, A; SbG 10 mg/ml, B; SbG 30 mg/ml, C) and double-checked by flow cytometry (vehicle, D; SbG 10 mg/ml, E; SbG 30 mg/ml, F).

to such remedy after partial hepatectomy (16), BrdU incorporation and cell cycle analysis by flow cytometry in tissue homogenates of remnant livers were conducted. The BrdU(+) levels in synthetic phase fraction in 24-, 36-, and 48-hr herb-treated regenerating livers were 6.3% ± 0.7%, 9.2% ± 0.8%, and 7.8% ± 0.1%, and in vehicle-treated livers, these levels were 12.0% ± 0.5%, 18.4% ± 2.5%, and 4.6% ± 1.0%, respectively ($P \leq 0.05$ by one-way ANOVA followed by Dunnet's t test).

Antiproliferative Effect of SbG-Treated RAW Cells-CM (SbG-RAW-CM) on Cultured Hepatocytes. To elucidate the effects of SbG on macrophage-hepatocyte interaction, SbG-RAW-CM and primary Kupffer cells–hepatocytes trans-well cultures were used. In primary hepatocyte culture, SbG stimulated cell proliferation in a concentration-dependent manner, and such effect was suppressed by pretreatment of SbG-RAW-CM or in trans-well culture (Fig. 1) The results indicated that there existed some soluble factors mediating antimitogenic effects on SbG-stimulated proliferating hepatocytes.

Cytokines Secreted from RAW Cells. To elucidate the roles of cytokines affecting hepatocyte growth, antibody-neutralization studies were performed. The results showed that after SbG-RAW-CM was blocked with anti–TGF-β1 antibody, the percentage of growth inhibition of SbG-RAW-CM–treated hepatocytes was significantly decreased from 44% ± 4.0% to 17% ± 7.8%. Interestingly, after SbG-RAW-CM was blocked with anti–IL-6 and anti–TNF-α antibodies, the percentage of growth inhibition of cultured hepatocytes was also significantly decreased, from 65% ± 14.2% to 23% ± 4.0% (Table 1). Although there were increased levels of cytokines such as TNF-α, IL-6, and TGF-β1 detected in SbG-RAW-CM, the expression level of TGF-β1 was much higher than that of the other two cytokines (Table 2). These results indicated that the presence of TGF-β1 in SbG-RAW-CM markedly suppressed the growth of hepatocytes, while SbG-RAW-CM without the presence of TNF-α and IL-6 remained mitogenic activity on cultured hepatocytes.

Effects of Pure Compounds in SbG on Cell Growth of Primary Hepatocyte Culture. To further identify which compounds in SbG played the major role in

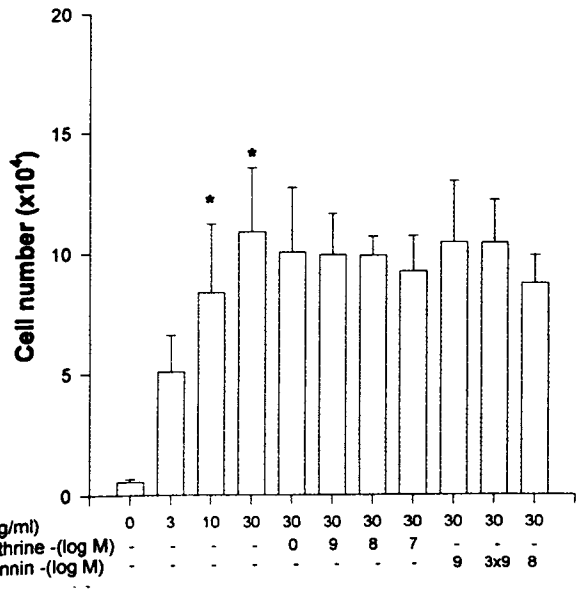

Figure 6. Effects of *Scutellaria baicalensis* Georgi (SbG) on signal transduction pathways in primary hepatocyte culture. Isolated hepatocytes were cultured overnight, followed by the treatment of SbG (0, 3, 10, and 30 mg/ml). For blocking protein kinase C and AKT pathway, chelery (10^{-9} to 10^{-7} *M*) and wortmannin (10^{-9}, 3×10^{-9}, and 10^{-8} *M*) were pretreated 1 hr before SbG (30 mg/ml) administration, respectively. The cell number of hepatocytes in primary culture was evaluated by MTT, as described in Materials and Methods. *, $P < 0.05$ versus SbG (0 mg/ml) group. #, $P < 0.05$ versus DMSO group (one-way ANOVA followed by Dunnet's *t* test).

the above-mentioned effects, four major SbG-derived pure compounds were used. The results demonstrated that baicalin and wogonin were the ones involved in such macrophage-related hepatocyte growth inhibition (Fig. 2).

Baicalin-Induced TGF-β1 Gene Expression on RAW Cells. To investigate the molecular mechanisms concerning baicalin-induced TGF-β1 gene expression, the RAW264.7 cell line was used. Results from real-time RT-PCR showed that baicalin at 10^{-9}, 10^{-7}, and 10^{-5} *M* induced TGF-β1 mRNA expression up to 183% ± 26%, 422% ± 36%, and 669% ± 62%, respectively, compared with housekeeping gene expression (data not shown). Receptor antagonization studies indicated that the signal transduction pathway involved in such TGF-β1 upregulation was related to the activation of NF-κB and PKC pathway (Table 3).

Effect of Exogenous r-Human TGF-β1 on SbG-Stimulated Hepatocyte Proliferation. To test the hypothesis that SbG stimulated the proliferation of cultured hepatocytes and that SbG-induced macrophage-secreted TGF-β would inhibit such phenomenon, only exogenous r-human TGF-β1 was administered in cultured hepatocytes to avoid interference of many factors, such as TNF-α and IL-6 detectable in SbG-RAW-CM (Table 2). The results showed that quiescent hepatocytes (SbG 0 ng/ml) were more resistant to exogenous administration of r-human TGF-β1 (0–0.1 ng/ml). On the contrary, the growth of SbG (10 mg/ml)-treated hepatocytes was significantly inhibited by the treatment of r-human TGF-β1 in a concentration-dependent fashion (Fig. 3). In Figure 4, we demonstrate a significant growth reduction in higher concentrations of TGF-β (1 and 5 ng/ml) treatment only in the SbG (3 mg/ml) group, compared with the vehicle group, while there was no significant growth inhibition by r-human TGF-β1 in the pure compound–treated groups.

SbG Stimulated the Proliferation of Cultured Hepatocytes. To study the effect of SbG on hepatocyte growth, cell proliferation assay was investigated by flow cytometry and double-checked by BrdU immunostaining. The results showed that SbG stimulated cell proliferation in a concentration-dependent manner. By BrdU immunostaining, there was an increase of BrdU(+) cells in SbG 10 mg/ml and 30 mg/ml groups, compared with the vehicle groups (Fig. 5A–C). By flow cytometry, the fraction of BrdU(+)–S-phase was 15.5% in vehicle (Fig. 5D), 22.0% in SbG (10 mg/ml; Fig. 5E), and 38.0% in SbG (30 mg/ml) treatment groups (Fig. 5F).

Signal Transduction Involved in SbG-Stimulated Hepatocyte Growth. Figure 6 showed that pretreatment of PKC- and AKT-inhibitors did not significantly inhibit SbG-enhanced hepatocyte growth. To provide direct evidence of NF-κB activation, double-stained immunohistochemistry, namely, FITC for NF-κB (p65) and PI for nuclei staining, was performed on 4 hr–cultured hepatocytes. Figure 7 shows that SbG (10 mg/ml, Fig. 7C) induced NF-kB translocation into the nuclei of cultured cells, while there were no such translocation in the vehicle group (Fig. 7A and B) or the baicalin (5×10^{-6} *M*; Fig. 7D) group. One of the upstream factors of NF-κB activation, Tlr4, was supposed to be involved in SbG effects on hepatocyte cultures. However, by using LPS-responsive cells isolated from Tlr4-mutant (C3H/HeJ) and wild-type (C3H/HeN) mice, there was no significant difference in SbG-stimulated hepatocyte growth between these two lines (Fig. 8). This indicated that SbG-stimulated hepatocyte growth was not necessarily mediated *via* Tlr4.

Discussion

There is consensus that cell-cell interaction between macrophage (Kupffer cells) and hepatic parenchymal cells plays an important role in regulation of growth control of hepatocytes. There are three major points that should be addressed in this study, namely, (1) to our knowledge, this is the first study to investigate the modulatory effects of SbG, a major component of TJ-9, on macrophage-hepatocyte interaction; (2) this work includes biological studies on crude extracts and their major constituents; and (3) this study incorporates *in vivo* genomics bioinformation and *in vitro* experimental verification. All of these are novel findings linked to clinical observations. Since co-culture of macrophages and hepatocytes resulted in liver cell cytotoxicity, we used RAW cells-CM and Kupffer cells trans-well system to study macrophage-hepatocyte interaction. It has been accepted that studies using CM and a

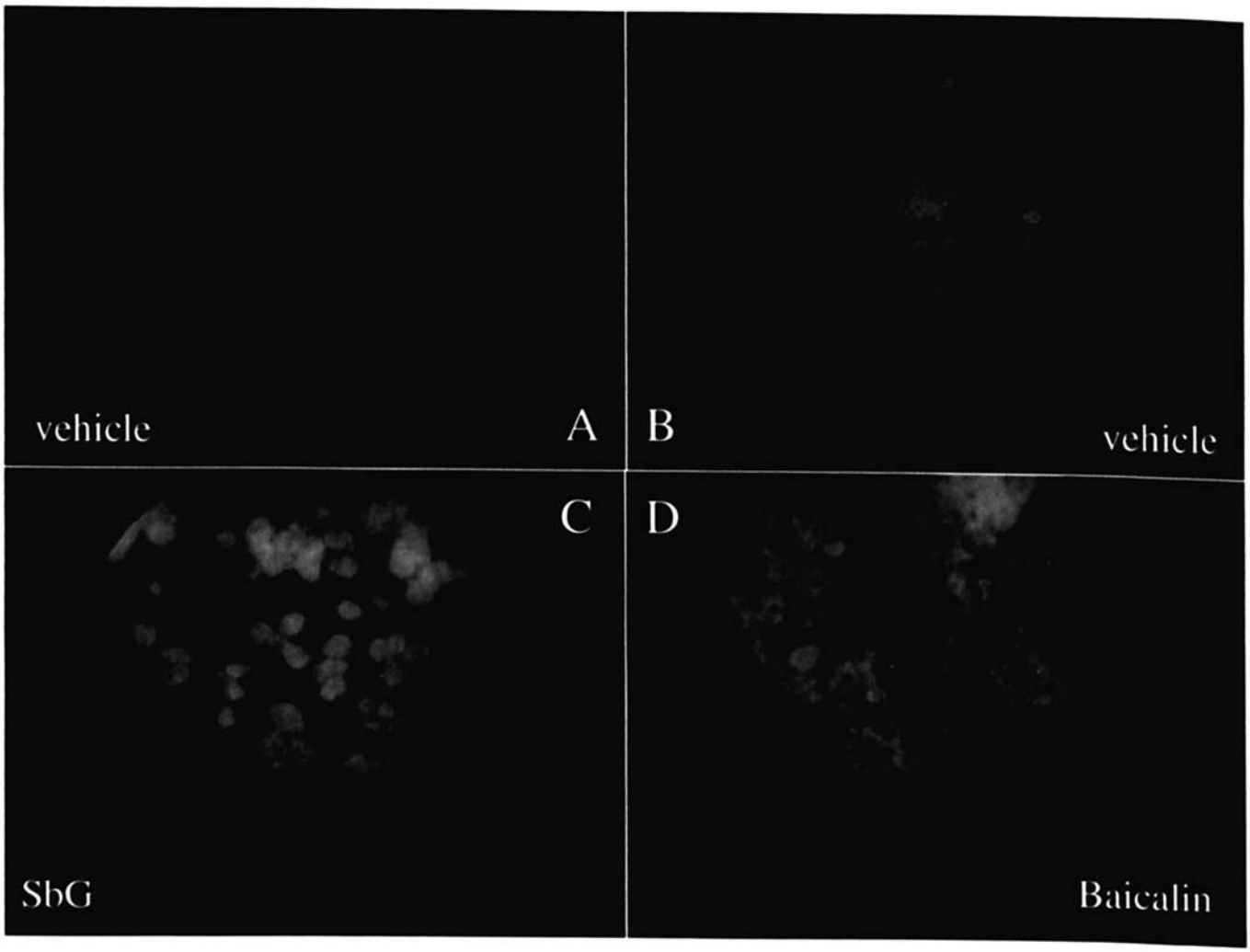

Figure 7. Effects of *Scutellaria baicalensis* Georgi (SbG) and baicalin on NF-κB activation. Primarily cultured hepatocytes were seeded onto collagen-mounted slides and treated by medium alone (A, B), SbG 10 mg/ml (C), or baicalin 5×10^{-6} *M* (D) for 4 hrs followed by double-stained immunohistochemistry, namely, FITC for NF-κB (p65) and PI for nuclei staining (B), as described in Materials and Methods.

trans-well system are feasible for studying the cell-cell interaction (26–28) In this study, Kupffer cell responses to SbG were double-checked and molecular mechanisms were further elucidated in mouse macrophage cell line RAW264.7 for TGF-β gene expression.

Previous investigations have shown that many flavonoids stimulate RAW cells, as well as Kupffer cells in the liver, to secrete many cytokines (29, 30). Our results demonstrated that SbG induced many cytokines, such as TNF-α, IL-6, and TGF-β1, on RAW cells. It is interesting to note that the amount of TGF-β1 secretion is much higher than that of either TNF-α or IL-6 (Table 2). The former is an inhibitory regulator of hepatocyte proliferation, while the latter are positive regulators. Antibody neutralization studies indicate that the presence of TGF-β1 in SbG-RAW-CM markedly suppresses hepatocyte growth, while SbG-RAW-CM without the presence of TNF- and IL-6 remains mitogenic activity, even in the presence of TGF-β1. This indicates that SbG alone, without the presence of TNF-α and IL-6, would stimulate proliferation in cultured hepatocytes (discussed below). It is also of note that there was 22% inhibition of cell growth in cultured hepatocytes treated with vehicle (SbG 0 μg/ml)-RAW-CM, indicating that there were some factors secreted from RAW cells present. The exact explanation for this condition requires further investigation.

Recent evidence indicates that many factors (TRAIL, Fas ligand, etc.) are involved in nonparenchymal cell-mediated cytotoxicity of liver parenchymal cells. For example, Ochi *et al.* (31) have demonstrated that TRAIL-expressing NK cells play a critical role in self-hepatocyte killing. Accordingly, we used exogenous r-human TGF-β1 administration in primary hepatocyte culture to elucidate the role of TGF-β on SbG-stimulated hepatocyte growth, so that the above-mentioned confounding factors could be eliminated. There is evidence that conversion of the latent form of TGF-β1 to its active form is a critical step regulating many biological functions, including suppression of hepatocyte proliferation (32). Gressner *et al.* (33) have reported that the TGF-β1 concentration (<0.25 ng/ml) in the CM of myofibroblasts is effective in induction of apoptosis of rat

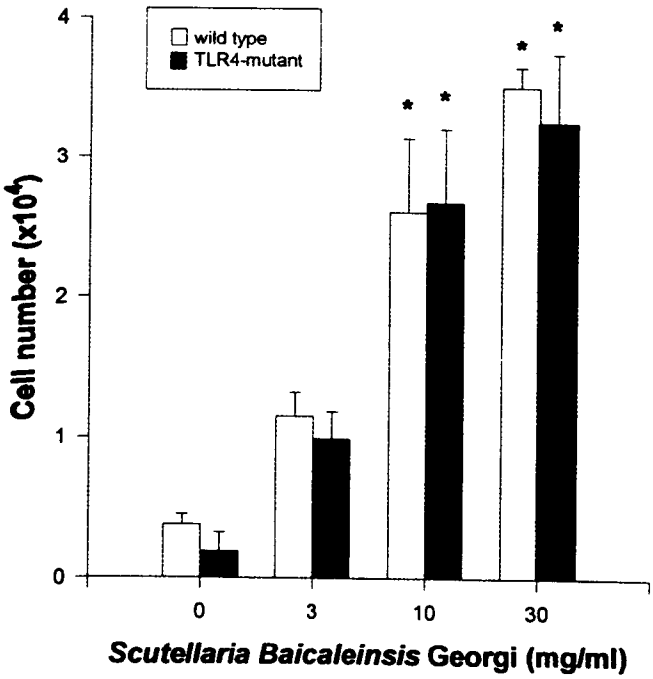

Figure 8. The role of Toll-like receptor 4 (Tlr4) on *Scutellaria baicalensis* Georgi (SbG)–modulated hepatocyte growth. The hepatocyte of Tlr4 wild-type (C3H/HeN, □) and mutant (C3H/HeJ, ■) mice were isolated and cultured overnight, followed by the treatment of SbG (0, 3, 10, and 30 mg/ml). The cell number of hepatocytes in primary culture was evaluated by MTT assay, as described in Materials and Methods. *, $P < 0.05$ vs. SbG (0 mg/ml) group (one-way ANOVA followed by Dunnet's *t* test).

liver parenchymal cells, which supports our observations in this study. It is interesting to find that exogenous administration of r-human TGF- β1 with mouse cross-activity results in no suppression of SbG(−)–hepatocyte proliferation (Fig. 3). This indicates that primary cultured mouse hepatocytes are TGF-β1 resistant. There are two possibilities for this indication, namely, (1) the TGF-β1 used is not an active form; and (2) quiescent hepatocytes are more resistant to TGF-β1. The fact that the activity of commercially available r-human TGF-β1 is measured by its ability to inhibit the IL-4–dependent proliferation of mouse HT-2 cells make the former possibility less likely (34). Furthermore, it has been reported (35) that primary cultured rodent hepatocytes without insulin, epidermal growth factor treatments remained in the G0 phase, which made the second possibility more reasonable.

Hepatocytes normally remain in proliferative quiescence (35). Regeneration requires priming of hepatocytes to achieve competence for proliferation. Priming requires the cytokines TNF-α and IL-6 in addition to other agents that prevent cytotoxicity. Our results demonstrated that SbG stimulated the proliferation of cultured hepatocytes in a concentration-dependent manner, indicating that the process of G0–G1 transition had occurred. Accordingly, the involvement of priming factor such as TNF-α and IL-6 has been speculated. The fact that SbG-RAW-CM without the presence of TNF-α and IL-6 remained mitogenic activity on cultured hepatocytes (Table 1) indicates that there are factors other than TNF-α and IL-6 working in our system. Furthermore, it is interesting to note that SbG, but not pure compounds (baicalin, baicalein, wogonin, and chrysin), make cultured hepatocytes respond to exogenous r-human TGF-β1 administration. Three possibilities have been proposed. First, the activity of flavonoids in stimulating hepatocyte growth is not as prominent as that of SbG extract. Second, there are some active factors other than the above-mentioned flavonoids playing the role of cultured hepatocytes. Third, synergic effects of these flavonoids are not observed in a model treated with a single compound. The discrepancy between biological responses to crude extracts and pure compounds is commonly observed in many herbal remedies with regard to clinical aspect, for which more evidence is needed to prove or disprove such discrepancies. Previous reports showing that active TGF-β1 impaired liver regeneration in mice by LPS (*in vivo* model) and induced liver parenchymal cell apoptosis by hepatic myofibroblast (conditioned medium) do not conflict with our findings, since in both models, the hepatocytes are also primed (6, 33).

Previous investigations have shown that SbG has a broad spectrum of biological activities, including metabolic and anticancer activity (36). Recently, baicalin and baicalein were reported to exhibit growth inhibitory activity toward the human hepatoma cell lines and pancreatic cancer cell lines (37). Our data demonstrated that SbG stimulated the proliferation of cultured hepatocytes, which were inhibited by addition of Kupffer cells-CM or by co-culturation with Kupffer cells in a trans-well system. This might explain the activities of SbG in *in vivo* and clinical observations. There are several signal transduction pathways involved in TGF-β1 gene expression, such as NF-κB, ERK/MAPKK, P38 MAPK, PKC, and so on (38–40). Our results showed that SbG and baicalin stimulated TGF-β1 synthesis in a dose-dependent manner through NF-κB pathways in the RAW264.7 cell line. Interestingly, it is noteworthy that NF-κB activation is involved in SbG-stimulated hepatocyte growth but is not mediated *via* Tlr4, which has been generally postulated to regulate signaling in liver regeneration (5).

There are many pure compounds extracted from SbG, and each of them has different effects on hepatocyte growth. For example, baicalin induces hepatocyte growth after partial hepatectomy through enhancing of the expression levels of HGF and suppression of TGF-β level, thus increasing the cell numbers in the synthetic phase (10). On the other hand, wogonin inhibits the expression of IL-6, by which the hepatocytes are triggered to proliferate. Both baicalein and wogonin inhibit the growth of hepatic stellate cells (40). Although the hepatocyte responses to different compounds are different and sometimes are contradictory to those responses to other compounds, our data indicating baicalin as a TGF-β inducer might explain the clinical observation that acute hepatitis, fibrosis, and interstitial

pneumonia have been sporadically reported in patients treated with TJ-9 (41).

Since studies on cell-cell interaction are so complicated, using the genomics approach is reasonable to elucidate the overall gene expression profiles for cell-cell, drug-drug, and drug-cell interactions; studies using this approach are underway. In summary, SbG and some of its constituents have proliferative effects on cultured hepatocytes and, simultaneously, have antiproliferation effects on hepatocytes *via* TGF-β1 secretion from Kupffer cells. Thus, the overall effects of SbG depend on the balance between the opposite effects resulting from different cell types. The knowledge of modulatory effects of medicinal herbs on macrophage-hepatocyte interaction might bring into perspective the new strategy on clinical application of complementary and alternative medicine.

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