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A chemically sulfated polysaccharide from *Grifola frondos* induces HepG2 cell apoptosis by notch1–NF-κB pathway

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ABSTRACT

Sulfated polysaccharides have been known to inhibit proliferation in tumor cells. However, the molecular mechanisms involved in sulfated polysaccharides-induced apoptosis are still uncharacterized. In this study, the effect of a chemically sulfated polysaccharide obtained from *Grifola frondosa* (S-GFB) on HepG2 cell proliferation and apoptosis-related mechanism were investigated. It was found that S-GFB inhibited proliferation of HepG2 cells in a dose-dependent manner with IC_{50} at 48 h of 61 μg ml^{-1}. The results of scanning electron micrographs indicated that S-GFB induced typical apoptotic morphological feature in HepG2 cells. Flow cytometric analysis demonstrated that S-GFB caused apoptosis of HepG2 cells through cells arrested at S phase. Western-blotting results showed that S-GFB inhibited notch1 expression, IκB-α degradation and NF-κB/p65 translocation from cytoplasm into nucleus. Simultaneously, the apoptotic mechanism of HepG2 cells induced by S-GFB was associated with down regulation of FLIP, and activation of caspase-3 and caspase-8. Taken together, these findings suggest that the S-GFB induces apoptosis through a notch1/NF-κB/p65-mediated caspase pathway.

1. Introduction

Grifola frondosa, which belongs to the polyporaceae family, is also called the king of mushrooms and the hen of the woods. Fruit bodies and liquid-cultured mycelium from this mushroom have been reported to contain many biologically active compounds, and it has become attractive as functional foods and a source of physiologically beneficial medicine (Bae, Kim, Lee, & Lee, 2011; Cui et al., 2007; Gu, Li, & Chao, 2006).

Various active substances isolated from *G. frondosa* have been recorded in the literature, and polysaccharides were one of the important substances (Hsieh, Liu, Tseng, Lo, & Yang, 2006; Xu, Liu, Shen, Fei, & Chen, 2010). However the polysaccharides extracted with alkali from *G. frondosa* are mainly insoluble in water and less suitable for pharmaceutical study, and little information was available regarding the water-insoluble polysaccharides from *G. frondosa*. Sulfated polysaccharides have a broad range of important bioactivities involving anticoagulant, antioxidant and antithrombotic activities (Huang et al., 2008; Yang, Ya, & Jeong, 2007). The sulfation of polysaccharides does not merely improve the water solubility, but also can change the chain conformation, leading to the alteration of their biological activities (Ma, Guo, Wang, Hu, & Shen, 2010).

In our previous study, we had produced a sulfated derivate of water-insoluble polysaccharide from *G. frondosa* mycelia by chemical modification (S-GFB), and investigated its anticoagulation activity (Cao et al., 2010). So far, few researchers only proved antitumor activity of sulfated polysaccharide obtained from *G. frondosa* (Shi, Nie, Chen, Liu, & Tao, 2007), the molecular mechanisms involved in tumor cells apoptosis induced by S-GFB were not understood. In this study, the effect of S-GFB on HepG2 cell proliferation and apoptosis-related mechanism were studied. We investigated the possible mechanisms of notch1, NF-κB, FLIP and caspase in S-GFB-induced HepG2 cells apoptosis.

2. Materials and methods

2.1. Materials

The strain of *G. frondosa* was obtained from Strain Preservation Center of Tianjin University of Science & Technology. Human liver cancer cells HepG2 and human normal liver cells L-02 were obtained from Tianjin Medical University (Tianjin, PR China). The preparation of sulfated polysaccharide was followed as our previous study described (Cao et al., 2010). HepG2 cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% heat inactivated (56 °C, 30 min) fetal bovine serum (FBS, GIBCO), 50 U ml^{-1} penicillin (GIBCO) and 50 U ml^{-1}

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streptomycin (GIBCO). L-02 cells were cultured in RPMI-1640 medium (GIBCO) containing 10% FBS, 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin. All cells were cultivated at 37 °C with 5% CO_2. The NF-κB, FLIP, Bcl-2, caspase-3, caspase-8, β-actin anti-mouse IgG antibodies, notch1 and the notch1 inhibitor (DAPT) were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell viability assay

Viability of cells was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) assay. HepG2 and L-02 cells (5×10^4 ml^{-1}/well) were seeded into each well of a 96-well flat-bottomed culture plate in their exponential growth phase. After incubation for 24 h, various concentrations of S-GFB were added into each well and each concentration was repeated in five wells. After another 48 h incubation, the medium was aspirated and 0.5 μg ml^{-1} MTT was added to cells at a concentration. Cells were incubated at 37 °C for another 4 h and the formazan product was solubilized with dimethylsulfoxide (DMSO). The absorbance was read at 570 nm on an enzyme linked immunosorbent assay (ELISA) microplate reader (Multiskan EX, Labsystems, Helsinki, Finland). Each test was performed in triplicate experiments.

2.3. Scanning electron microscopy analysis

Briefly, the cell suspension (1×10^5 ml^{-1}) was inoculated on cover slips which were partitioned previously into the wells of 6-well plates. After 24 h, HepG2 cells were treated with 61 μg ml^{-1} S-GFB for 0 h, 24 h and 48 h, and then, pre-fixed with 2.5% glutaraldehyde at 4 °C for 1 h. The cells were then rinsed thoroughly in PBS and post-fixed in 1% O_sO_4 at 4 °C for 1 h, and scanning electron microscopy was applied to investigate the effect of the pro-damage on S-GFB-treated cell surface morphology.

2.4. Flow cytometry analysis

HepG2 cells were plated in a 6-well plate and treated with S-GFB (61 μg ml^{-1}) for 0, 12, 24 and 48 h, and cells were collected and washed with cold PBS and fixed by ethanol (70%, v/v). Then, cells were dissolved in 1 ml PBS (containing RNase, PI, Triton X-100 and EDTA, pH 7.4), and incubated at 37 °C for 30 min, followed by incubation in the dark, 4 °C for 1 h. The samples were performed with a flow cytometry (Becton, Dickinson, USA).

2.5. Immunofluorescence imaging of NF-κB p65

Briefly, the cell suspension (1×10^5 ml^{-1}) was inoculated on cover slips which were partitioned previously into the wells of a 6-well plate. After 24 h, HepG2 cells were treated with 61 μg ml^{-1} S-GFB for 0 and 48 h. Cells were fixed with 3% formaldehyde in phosphate buffered saline (PBS, 8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 0.24 g l^{-1} NaH_2PO_4, 1.44 g l^{-1} Na_2HPO_4, pH 7.4) for 20 min, and washed thrice with PBS. Washed cells were permeabilized using 0.2% Triton X-100 and blocked in 2% BSA (B.M.) in PBS. Then cells were washed thrice with PBS, and incubated with the antibody NF-κB p65 (dilution 1:200) with 2% BSA in PBS at 37 °C for 1 h. The resulting cells were washed thrice with PBS and incubated with fluorescein FITC-labeled polyclonal goat anti-mouse IgG antibody (dilution 1:200) at 37 °C for 1 h. Cells were stained with propidium iodide (PI) (Sigma) and scanned by LSCM after washed with PBS. All images were acquired using the same intensity and photodetector gain.

2.6. Western blotting analysis

In order to investigate the potential apoptosis induction mechanisms, the expression of characteristic apoptosis protein was detected by Western blot. Protein extracts of HepG2 cells treated with or without S-GFB were prepared by lysing cells in the buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris–Cl, 1 mM phenylmethylsulfonyl Xuoride) with occasional vortexing. In brief, cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The nitrocellulose membranes were then blocked in a blocking buffer (Tris = 20 mM, pH 7.6, NaCl = 150 mM and Tween 20 = 0.1%) containing 5% non-fat dry milk powder and incubated with the indicated antibodies overnight at room temperature. The membranes were subsequently washed and incubated for 1 h at room temperature with the appropriate secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). Immunoreactive bands were detected by DAB kit (Zhongshan Goldenbridge, Beijing, China). Western blot analysis was carried out by the method as described previously (Cao et al., 2009).

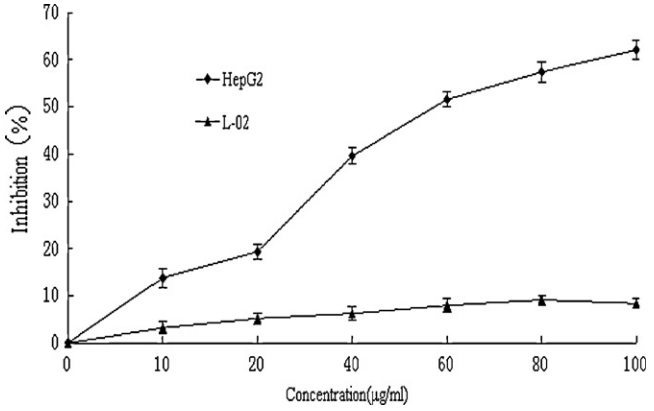

Fig. 1. Dose-dependent effect of S-GFB on human liver cancer cells HepG2 and human normal liver cells L-02. L-02 cells were cultured in RPMI 1640 medium and HepG2 cells were cultured in DMEM medium with S-GFB at the concentration of 0, 10, 20, 40, 60, 80, 100 μg ml^{-1} for 48 h, respectively. The viability of cells was determined by MTT assay. Data represent means ± SD of three independent experiments.

2.7. Statistical analysis

The experiments were repeated three times and the mean values were analyzed by a two-tailed unpaired *t*-test. The level of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of S-GFB on HepG2 cells inhibition

To identify the effect of S-GFB on cell viability, HepG2 cells and L-02 cells were cultured with the indicated concentrations of S-GFB for 48 h, and then cell viability was determined by MTT assay. The results are shown in Fig. 1, S-GFB inhibited the growth of HepG2 cells in a dose-dependent manner, with 50% inhibition (IC50) at 48 h of 61 μg ml^{-1}. In contrast, S-GFB had no obvious effect on the normal human liver L-02 cells. These results suggested that S-GFB had remarkably lower toxicity to the non-tumor cell lines and had high selectivity to tumor cells in vitro.

3.2. Effect of S-GFB on the morphology of HepG2 cells

To investigate the type of cells apoptosis induced by S-GFB, the cells were observed by scanning electron microscopy which allowed the identification of viable and apoptotic cells based appearance. The results showed that numerous microvilli on surface of the control group cells were kept intact, and the cells of S-GFB treated groups provided the morphological changes of

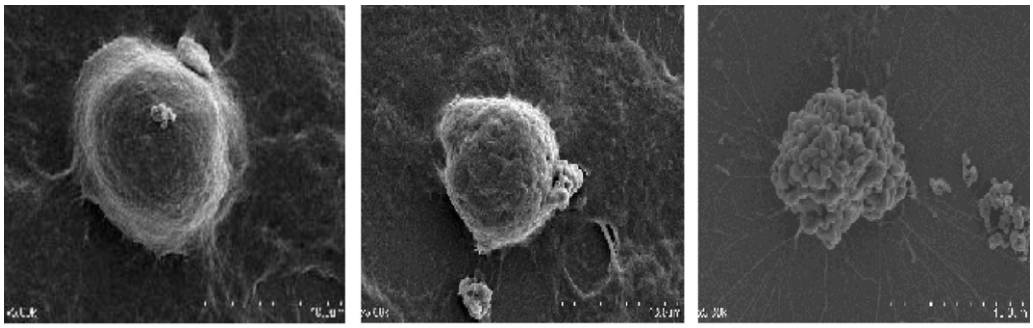

Fig. 2. Scanning electron micrographs of HepG2 cells after treatment with S-GFB for different times. (A) Untreated control cells; (B) cells treated with S-GFB for 24 h; (C) cells treated with S-GFB for 48 h. Results presented are representative of three independent experiments.

pre-apoptotic characteristics, such as loss of microvilli, blebbing formation and appearance of apoptotic bodies (Fig. 2).

3.3. Effect of S-GFB on cell cycle distribution and apoptosis ratio of HepG2 cells

Cell-cycle phase distribution and apoptosis rates were analyzed by flow cytometry with PI staining. The percentage of cells in G_1, S and G_2/M phase, respectively, was calculated using Multi-cycle software. The results presented in Table 1 show a significant accumulation of cells in the S phase (from 17.18% to 60.85%), and that the number of apoptotic cells increased (from 0.13% to 43.53%) in a time-dependent manner at a concentration (61 $\mu g\,ml^{-1}$) of S-GFB.

3.4. Effect of S-GFB on NF-κB/p65 and IκB-α

Immunofluorescence was carried out to determine whether the entering nucleus of NF-κB took an effect on HepG2 cells apoptosis induced by S-GFB. Under the LSCM, the intensity of NF-kB/p65 fluorescent signal in nuclei treated by S-GFB was obviously weaker than that of the control group, the NF-κB/p65 protein expression was reduced in the nucleus while increased in cytoplasm (Fig. 3A). Simultaneously, the result of Western blot showed that the expression of NF-κB/p65 in nucleus was decreased in HepG2 cells treated with S-GFB, and the decrease was in a time-dependent manner, but the total was almost invariable (Fig. 3B).

3.5. Effect of S-GFB on notch1

In order to investigate whether notch1 is involved in S-GFB-induced apoptosis, we firstly analyzed the effect of S-GFB on notch1 in HepG2 cells by Western blot analysis. As shown in Fig. 4A, notch1 decreased in a time-dependent manner.

To explore the functional involvement of notch1 down regulation in S-GFB-induced apoptosis, notch1 inhibitor (DAPT) was used for treating HepG2 cells with S-GFB, then cell apoptosis was determined by flow cytometry (Fig. 4B). The result showed that apoptosis induced by S-GFB was further increased by DAPT. Overall, the decrease of notch1 was associated with S-GFB-induced apoptosis in HepG2 cells.

Table 1
Effect of S-GFB on cell cycle distribution and apoptosis rate of HepG2 cells ($n = 3$). HepG2 cells were treated with S-GFB (61 $\mu g\,ml^{-1}$) for 12, 24 and 48 h.

Time (h)	Apoptosis (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
Control	0.13	70.37	17.18	12.45
12	5.07	60.80	27.56	11.82
24	8.60	60.61	30.72	8.48
48	43.53	39.15	60.85	0.00

3.6. Roles of notch1 on entering nucleus of NF-κB/p65 in S-GFB-induced apoptosis

To decide whether S-GFB hinder NF-κB/p65 from entering the nucleus through notch1 pathway, HepG2 cells were treated with S-GFB for 48 h in the presence or absence of notch1 inhibitor (DAPT). Then the expression of NF-κB/p65 in nucleus was evaluated by Western blot analysis. The expression of NF-κB/p65 in the nucleus was further reduced in HepG2 cells treated with S-GFB and DAPT compared with treatment with S-GFB alone (Fig. 5). In short, the results indicated that the reduction of notch1 hindered NF-κB p65 from entering the nucleus in S-GFB-induced apoptosis of HepG2 cells.

3.7. Effect of S-GFB on FLIP and caspase activities

Biochemical events such as the reduction of FLIP gene (a target gene of NF-κB) expression occur during NF-κB-mediated cell apoptosis. This study therefore investigated whether S-GFB-induced cell apoptosis was also followed by the biochemical events. FLIP expression was detected after a different time exposure of S-GFB. As shown in Fig. 6A, the expression of FLIP was reduced significantly in a time-dependent manner.

Caspases-3, apoptosis executioner, is responsible for degradation of cellular proteins, and caspase-3 is known to be activated by caspase-8 and/or caspase-9. To determine whether a caspase-8-dependent pathway is operating in the present system, caspase-3 and caspase-8 were analyzed. As shown in Fig. 6B, treatment of HepG2 cells for 48 h with S-GFB resulted in an obvious increase in caspase-8 and caspase-3. Interestingly, we observed that the expressions of caspase-8 and caspase-3 were increased in a time-dependent manner, the apoptosis of HepG2 cells was induced ultimately by the increase of caspase-3. The result indicates that activation of caspase-8 and caspase-3 is involved in S-GFB induced apoptosis.

4. Discussion

In recent years, studies of the biological activities of polysaccharides have been of particular interest. However, little information of anti-tumor activities was available regarding the water-insoluble polysaccharides from *G. frondosa*, only Shi proved antitumor activity of sulfated polysaccharide obtained from *G. frondosa* (Shi et al., 2007). Although sulfated polysaccharides have been tested for potential anti-tumor effect, the mechanism is not clear. The aim of this study was mainly to determine antitumor activity of S-GFB, which was derived from water-insoluble polysaccharide of *G. frondosa* mycelia. The present study focused upon S-GFB-induced

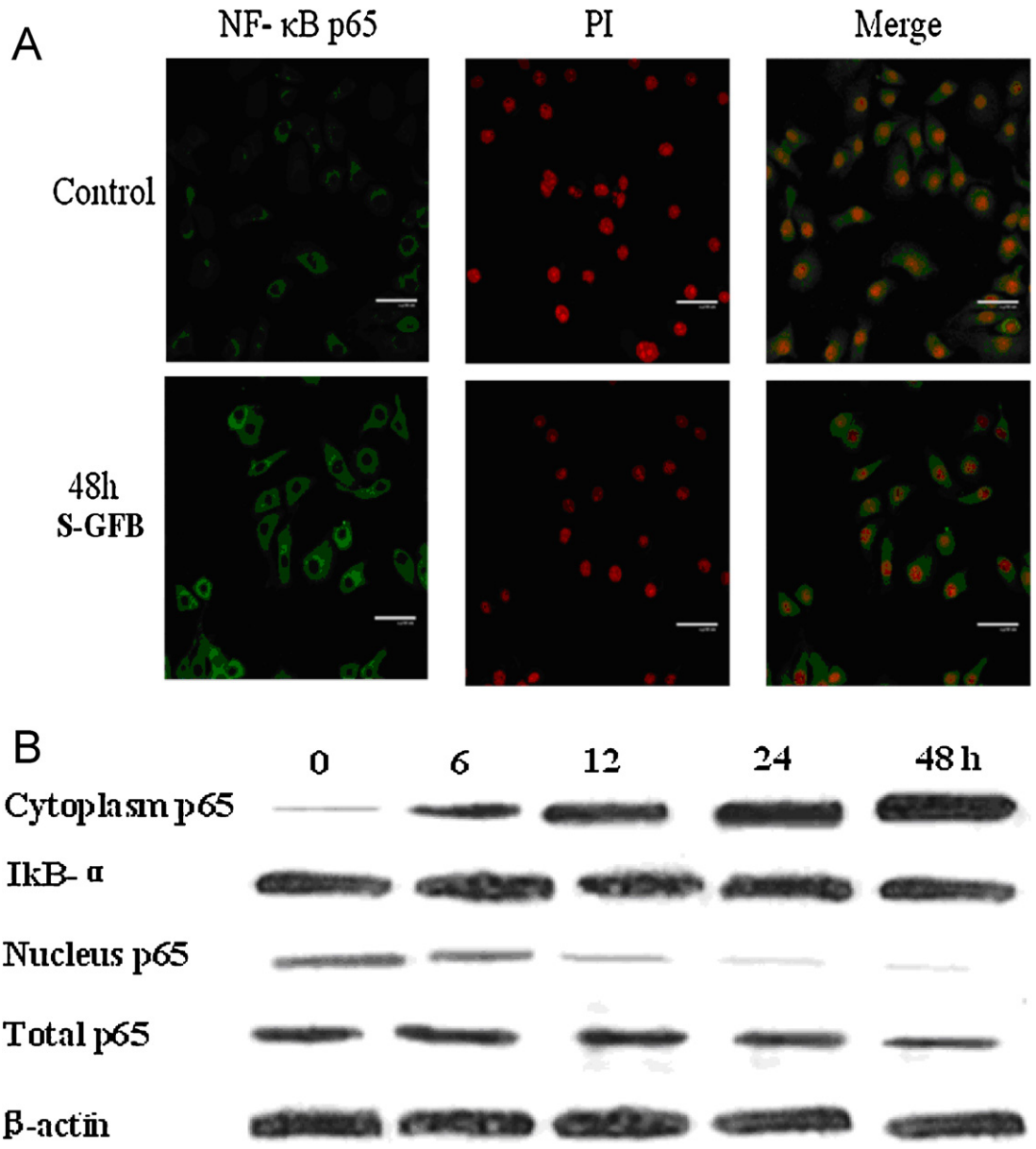

Fig. 3. The role of NF-κB/p65 in S-GFB-induced HepG2 cells apoptosis. (A) Immunofluorescence staining demonstrating the effect of S-GFB on subcellular localization of NF-κB p65 in HepG2 cells. (B) Western blot analysis of the protein level of NF-κB p65 (cytoplasm, nucleus and total) and IκB-αrespectively. β-Actin was used as an equal loading control. The data shown are representative of three independent experiments.

apoptosis of HepG2 cells, and probed the underlying intracellular signaling pathways.

Apoptosis is a process of cell suicide, characterized by specific morphological changes such as condensation of chromatin, loss of microvilli, blebbing formation, and appearance of apoptotic bodies. The results of this study revealed that S-GFB-induced apoptosis in HepG2 cells, and had high selectivity to tumor cells in vitro.

Notch signaling pathway is an evolutionarily highly conserved mechanism for cell–cell communication, which is important for many types of cell fate determination in a wide range of organisms (Artavanis, Rand, & Lake, 1999). The notch1 signaling pathway has been shown to regulate the differentiation and growth of carcinoid tumor cells. However, the molecules mechanism that notch1 mediate signaling, as well as their potential roles in regulating the growth of carcinoid tumors, have not been characterized. In the present study, we demonstrated that S-GFB suppressed notch1 signaling in HepG2 cells by its interaction with ICN1 and thus recruitment to the RBP-J recognition motif on downstream gene promoters of notch signaling (Beverly, Felsher, & Capobianco, 2005; Jundt, PrÊbsting, Anagnostopoulos, & Mathas, 2004).

Transcription factor Rel/nuclear factor-kappaB (NF-κB) proteins are a family of transcription factors and are of central importance in inflammation and immunity (Tak & Firestein, 2001), NF-κB transcription factors also regulate the expression of hundreds of genes that are involved in regulating cell growth, differentiation, development, and apoptosis (Barkett & Gilmore, 1999; Karin & You, 2000). The NF-κB plays a key role in regulating of gene transcription and is involved in the governance of cellular proliferation and apoptosis (Ghosh, May, & Kopp, 1998; Kodama, Asakawa, Inui, Masuda, & Nanba, 2005). In most cell types, NF-κB is retained in an inactive form in the cytoplasm by combining with IκBα which is its inhibitory protein. IκBα may be induced phosphorylation and degradation by the trimeric IκB kinase (IKK). The degradation of IκBα can free NF-κB to enter cell nucleus and activate transcription of target genes. We therefore hypothesized that IκBα/β may be the essential downstream factor in notch1-mediated growth

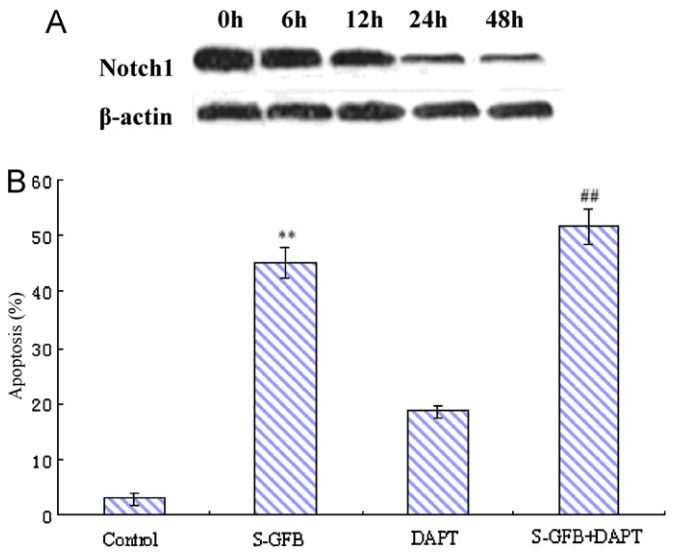

Fig. 4. Effect of S-GFB on notch1 in HepG2 cells apoptosis. (A) Effect of S-GFB on notch1 expression in HepG2 cells. Cells were treated with 61 μg ml^{-1} S-GFB for the indicated time periods and Western blot analysis of the protein level of notch1 expression. β-Actin was used as an equal loading control. (B) Effect of DAPT (notch1 inhibitor) on S-GFB-induced HepG2 cells apoptosis. (The cells were treated by S-GFB for 48 h.) The percentage of apoptotic cells was determined by flow cytometry as described in materials and methods. Data represent means ± SD of three independent experiments. $^{**}p < 0.01$ compared with control; $^{\#\#}p < 0.01$ compared with S-GFB alone.

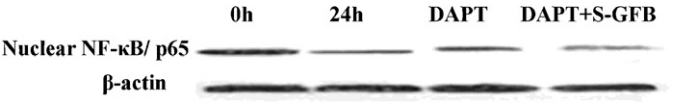

Fig. 5. Roles of notch1 on entering nucleus of NF-κB/p65 in S-GFB-induced apoptosis. Cells were treated with 61 μg ml^{-1} S-GFB for 48 h in the presence or absence of DAPT (notch1 inhibitor) NF-κB/p65 in nucleus and total NF-κB/p65 in HepG2 cells were evaluated by Western blot analysis. β-Actin was used as an equal loading control.

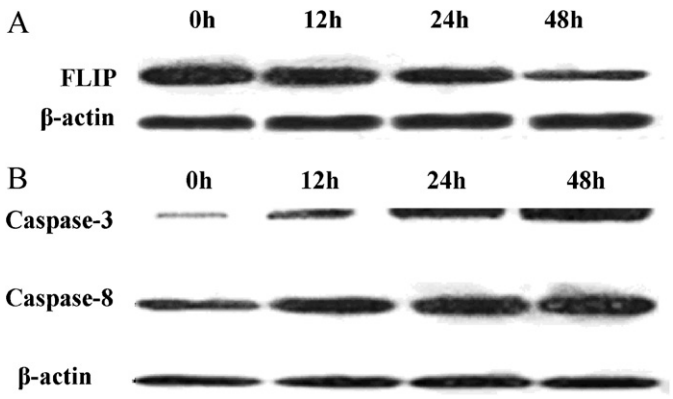

Fig. 6. Effect of S-GFB(61 μg ml^{-1}) on FLIP, caspases-3 and caspase-8 in HepG2 cells. Cells were treated with 61 μg ml^{-1} S-GFB for the indicated time periods, activation of FLIP, caspases-3 and caspase-8 was evaluated by Western blot analysis. β-Actin was used as an equal loading control. (A) Effect of S-GFB on FLIP in Hep2 cells; (B) effect of S-GFB on caspases-3 and caspase-8 in Hep2 cells.

regulation of carcinoid tumors (Pahl, 1999). A constitutive activation of the notch pathway through IKK has been implicated in this activation of NF-κB in HeG2 cells. In our present study, upon investigating the effect of notch1 inhibitor (DAPT) on nucleus NF-κB/p65 expression induced by S-GFB, it turned out that DAPT decreased the expression of nucleus NF-κB, indicating that impaired notch1 activation hindered its downstream target NF-κB/p65 from entering the nucleus in S-GFB-induced apoptosis of HepG2 cells. This study demonstrated that S-GFB prevented the entering nucleus of NF-κB p65 through notch1.

FADD-like interleukin-1b-converting enzyme inhibitory protein (FLIP) is known to specifically inhibit caspase-8 in the receptor-mediated apoptotic pathways, over-expression of FLIP protein has been documented in cancers (Griffith, Chin, Jackson, Lynch, & Kubin, 1998; Kreuz, Siegmund, Scheurich, & Wajant, 2001). FLIP is a target gene of NF-κB, inhibits apoptosis through interfering

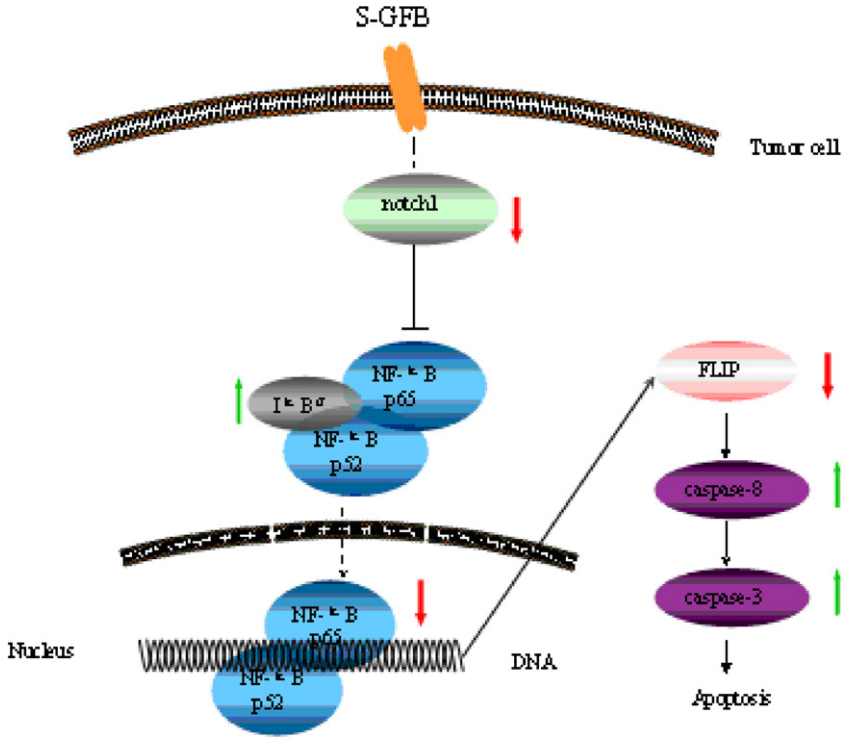

Fig. 7. A tentative scheme indicating the pathways involved in apoptotic effect of S-GFB in HepG2 cells.

with caspase-8 activation. In the study, the FLIP was decreased in HepG2 cells, and the decrease of FLIP gene expressions in HepG2 cells may be controlled by the NF-κB/p65, FLIP downregulation activates caspase-8 and the downstream effector caspase-3, leading to apoptosis (Kim, Maeng, Lee, & Hong, 2011; Sharifi, Eslami, Larijani, & Davoodi, 2009; Yang, Yaguchi, Yamamoto, & Nishizaki, 2007).

In summary, our results suggest that S-GFB induces apoptosis of HepG2 cells via notch1 signal, possibly involving inhibiting NF-κB p65 from entering the nucleus by downregulation notch1, activation of initiator caspase-8 by neutralizing caspase-8 inhibition due to c-FLIP as a result of decreased expression of c-FLIP, followed by activation of the downstream effector caspase-3. A speculated schematic diagram of S-GFB-induced cell apoptosis is depicted in Fig. 7.

Over the years, cancer therapy had witnessed many exciting developments, but cure of cancer has still remained as complex as the disease itself, since the mechanisms of tumor killing are still not fully realized. These results provide important new insights into the possible molecular mechanisms of S-GFB, in addition to its potential value as a novel candidate as an anti-tumor agent.

Acknowledgments

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