Original Article
Scutellarininduces human hepatocellular carcinoma cells apoptosis through increasing autophagy

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Abstract: Hepatocellular carcinoma (HCC) is one of the most lethal malignant tumors worldwide. It is urgent to develop novel effective agents in the field of liver cancer therapy. Although Scutellarin (Scu) has shown great potential as a new approach for cancer treatment, the inhibitory role of Scu inhepatocellular carcinoma cells and its underlying mechanisms are poorly understood. In this study, cell viability and growth was evaluated by CCK-8 assay. Apoptosis was examined by Hoechst 33258 staining, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and flow cytometry, respectively. Apoptosis-related markers and autophagy were determined by Western blot. Our results showed that Scu decreased the cell viability dose dependently and time dependently. Moreover, compared with control group, treatment with Scu induced HepG2 cells apoptosis, concomitantly with declined Bcl/Bax ration and caspase cascade activation. Immunofluorescence and Western blot analysis revealed a significant increase in autophagy, as evidenced by increased expression of autophagy marker LC3B-II and Beclin-1 and decreased expression of p62. Importantly, inhibition of autophagy by autophagy inhibitor markedly attenuated Scu-induced cell injury and apoptosis in HepG2 cells. Taken together, our results demonstrated that Scu induced autophagy-mediated cell death in HepG2 cells. These findings provide more convinced evidences for developing Scu as a novel therapeutic drug for treating liver cancer.

Keywords: Scutellarin, HepG2 cells, apoptosis, autophagy

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumor worldwide, and accounts for 695.900 death per year [1]. Of note, half of these deaths are occurred in China [2], where HCC ranks as the second highest cancer-related mortality rate [3, 4]. A relatively small number of patients with HCC are qualified for conventional therapies, including radiotherapy, chemotherapy, and surgical intervention [5]. However, most patients particularly with tumor thrombi in the major trunk of the portal vein are associated with low survival rate [6]. Despite advances in transcatheter arterial chemoembolization and regional hepatic arterial infusion chemotherapy within the last decades [7, 8], the prognosis of HCC patients remains extremely poor, because of high rate of the

occurrence of drug resistance. Thus it is crucial to develop novel therapeutic drugs for the treatment of HCC.

Autophagy is an intracellular degradation process for the degradation and recycling of organelles or protein to the lysosome [9]. Under normal conditions, constitutive autophagy help protect cells during starvation by regulating protein turnover or eliminating damaged organelles [10]. Once the cell damage is irreversible, autophagy is destructive intervening [11]. Deregulated autophagy contributes to the pathogenesis of various cancer diseases, including hepatocellular carcinoma [12, 13]. Notably, autophagy is suggested to be another programmed cell death different from classical apoptosis pathway [14]. However, the mechanisms whereby autophagy regulates hepatocellular carcinoma

cells survival and death are not well understood.

Recently, a variety of natural compounds derived from traditional Chinese herbs have been recognized as valuable resources for developing new agents for cancer treatment [15]. Scutellarin (Scu; molecular weight = 462.21), also known as scutellarin 7-0-β-D-glucuronide, is awell-known flavonoidisolated fromtraditional Chinese herb Erigeron breviscapus, and has been used in clinic in numerous Chinese medical formulas for cancer therapy [16, 17]. Previous studies demonstrated that Scu was able to induce cell death in colon cancer cells [18], human Burkitt lymphoma Namalwa cells [19], human breast cancer cell line MCF-7 [20] and human tongue squamous carcinoma cells [21]. In addition, Scu functions as a potential chemosensitization agent in 5-fluorouracil (5-FU)-induced colon cancer cell apoptosis [22]. However, the effect of Scu against hepatocellular carcinoma cells is not fully examined. Therefore, the aim of this study were to investigate whether Scu influence the survival of hepatocellular carcinoma cell line HepG2, and to understand the underlying mechanisms. We showed the pro-apoptotic effect of Scu on HepG2 cells through promoting autophagy.

Materials and methods

Materials and reagents

Scutellarin (Scu) was purchased from Yunnan Plant Pharmaceutical Factory (Purity>98.5%; Yunnan, China) and was dissolved in dissolved in dimethyl sufoxide (DMSO). The final concentration of DMSO in all experiments did not exceed 1%. Dulbecco modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and Hoechst 33342 were obtained from Invitrogen (CA, USA). All other reagents utilized were purchased from Sigma Chemical Co. (St.Louis, USA) unless otherwise specified.

Cell culture

Human hepatocellular carcinoma cell line HepG2 and nonmalignant hepatocytes LO2 obtained from American Type Culture Collection (ATCC, MD, USA), were routinely cultured in DMEM supplemented with 10% FBS and 100

U/mL penicillin and 0.1 U/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell viability assay

The cellular viabilitywas examined by Cell counting kit-8 (CCK-8, Dojindo Laboratories, Japan) according to the manufacturer's instructions. Briefly, HepG2 or LO2 cells were seeded in 96-well plates (2×10^3 cells/well) and rendered quiescent by replacing the medium with 0.2% FBS/DMEM for 24 h. Then, the cells were treated with different concentrations of Scu or were continuously incubated for different times according to the experimental design. The medium were replaced by the fresh medium containing 10 µl of CCK8, followed by incubation at 37°C for 1 h. The absorbance value was read at 450-540 nm using a SPECTRA MAX190 spectrophotometry (Sunnyvale, CA, USA).

Hoechst 33258 dye staining

Hoechst 33258 dye staining was used to observe apoptotic morphology of HepG2 cells. After treatment, the cells were carefully washed with phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde for 30 min, and then stained with 20 µg/ml of Hoechst 33258 for 10 min at room temperature. Morphological changes wereobserved by a laser-scanning confocal microscopy (LSM 710, Carl Zeiss, München, Germany).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

After appropriate treatment, the cells were fixed with 4% paraformaldehyde for 30 minat room temperature and then permeabilized using 0.1% Triton X-100 for 5 min. After washed with PBS, HepG2 cells were incubated with TdT-UDP nick end labeling (TUNEL) reaction mixture (DeadEnd fluorometrictunel system, Promega, WI, USA) for 1 h at 37°C. Cells were then viewed using HepG2 cells apoptosis was detected using the FITC-Annexin V and PropidiumIodide (PI) Double StainingKit (BD Biosciences, CA, USA) according to the manufacturer's protocol. The cells were washed, trypsinized and centrifuged. After washed with PBS, cells were incubated with Annexin V-FITC and PIat room temperature in dark for 15 min. The apoptotic cells were counted by flow cytometry analyses, on an
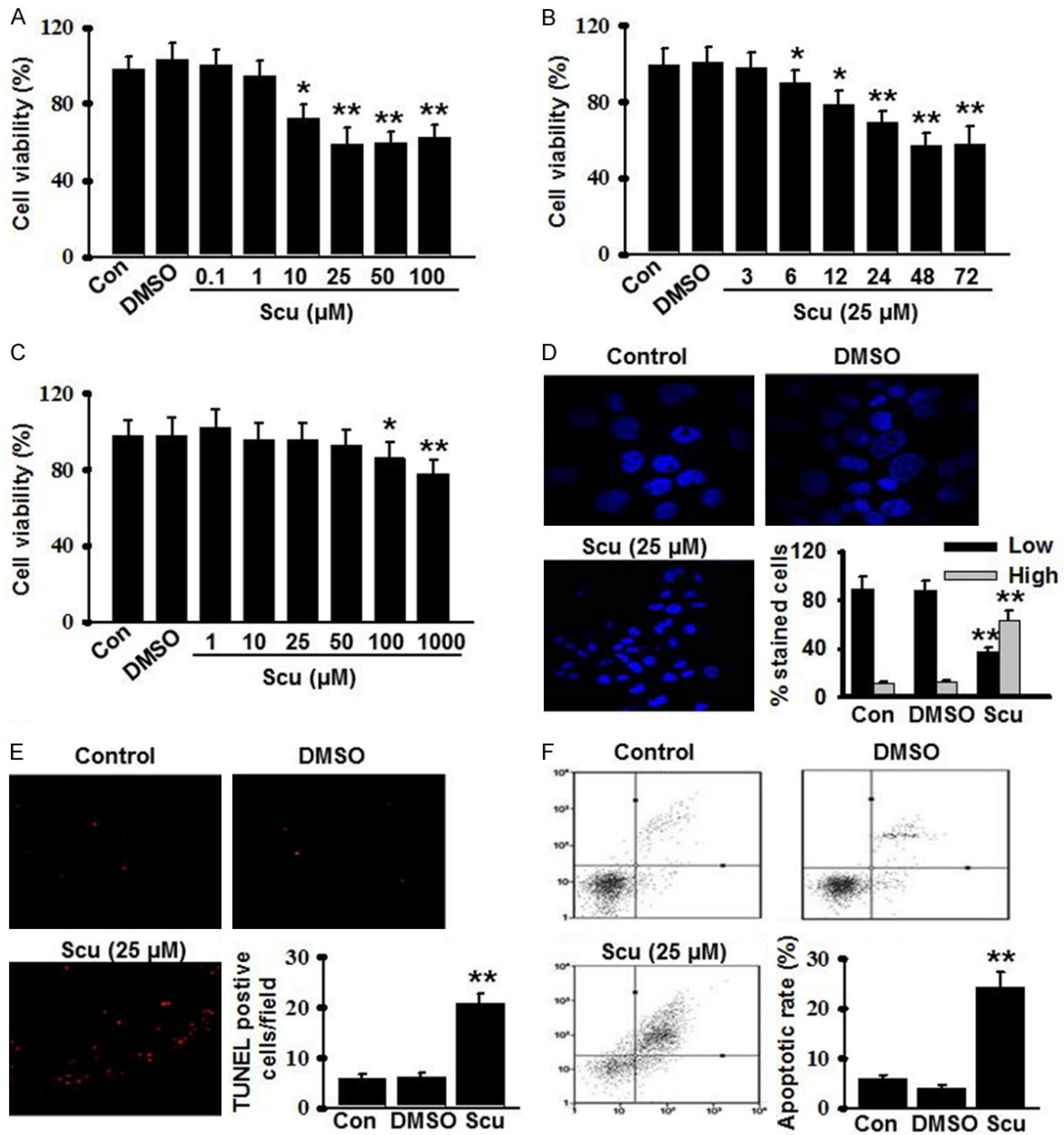
Figure 1. Scu induced cell injury and apoptosis in HepG2 cells. (A) HepG2 cells were seeded in 96-well plates and cultured in different concentrations of Scutellatrin (Scu) (0.1, 1, 10, 25, 50, 100 μM) for 48 h. Then the cell viability was determined by CCK-8 assay. (B) The cells were incubated with 25 μM Scu for different times (3, 6, 12, 24, 48, 72 h) and cell viability was examined by CCK-8 assay. (C) LO2 cells were treated with different concentrations of Scu as indicated for 48 h, and then cell viability was determined. (D-F) HepG2 cells were treated with or without Scu (25 μM) for 48 h, the apoptosis incidence was detected by Hoechst 33258 staining (D), TUNEL assay (E) and flow cytometry (F), respectively. All data are presented as mean ± SEM. *P<0.05, **P<0.01 vs. control, n=6.

EPICS XL-MCL platform, using a XL System II (Beckman Coulter, CA, USA). The apoptosis percentagewas expressed as Annexin V/PI ratio.

Western blot analysis

HepG2 cell extracts were subjected to western blot analysis as previously described [23]. In

brief, the cells were washed with cold PBS three times, and lysed in RIPA lysis buffer (Beyotime, Jiangsu, China) containing protease and phosphatase inhibitor cocktail (Merck, Darmstadt, Germany). After centrifugation, the cell lysate were harvested and the protein content was quantified by Bicinchoninicacid (BCA) Kit (Beyotime). Equal amounts of protein were sep-

arated by 8%-12% SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). Membranes were blocked in 5% non-fat dry milk diluted with Tris Buffered Saline Tween-20 (TBST) (Tris-HCl 20 mM, NaCl 150 mM, pH 7.5, 0.1% Tween 20) at room temperature for 1 h and then probed overnight at 4°C with the following primary antibodies: Bcl-2, Bax, cleaved caspase-9, cleaved capase-3, cleaved PARP, LC3B-I/II and Beclin-1 (1:1000 dilution, Cell Signaling Technology, MA, USA), GAPDH and p62 rabbit (1:1000 dilution, Santa Cruz Biotechnology Inc., CA, USA). After washed with TBST, membranes were incubated with appropriate antibodies including HRP-conjugated anti-rabbit or anti-mouse or anti-goat (1:1000 dilution, Cell Signaling Technology). The proteins were visualizedby a chemiluminescence kit (Thermo Fisher Scientific Inc., IL, USA). The densitometry of the bands was quantified using the ImageJ software (NIH, Maryland, USA).

Identification of autophagy marker

GFP-LC3 plasmid was obtained from Cyagen Biosciences Inc. (CA, USA). The plasmid was transfected transiently with Lipofectamine2000 reagent in serum free-Opti-MEMI medium according to the manufacturer's instructions. After being transfected overnight, transfection efficiency was detected by a fluorescence microscope. Subsequently, HepG2 cells were treated with or without Scu (25 µM) for 48 h and then observed under alaser-scanning confocal microscope (LSM 710, Carl Zeiss). Average punctuatedotsofeach cell were counted to quantify autophagy activities as previously described [24]. The percentage of GFP-LC3 positive cells with GFP-LC3 puncta was assessed by counting a least 100 cells, and the number of GPF-LC3 puncta per GFP-LC3 positive cell was assessed by counting a least 50 cells.

Statistical analysis

All data were presented as mean ± SEM. The significance of the observed differences of multiple groups was determined by one-way analysis of variance (ANOVA) or an unpaired Student's t test using SPSS 17.0 statistical software (SPSS Inc., IL, USA). *P* value less than 0.05 was considered to be statistically significant.

Results

Scu induced cytotoxicity and apoptosis in HepG2 cells

First, we investigated the effects of Scu on the growth of human hepatocellular carcinoma cell line HepG2. We examined the cell viability of HepG2 cells as assessed by the CCK-8 assay. As shown in **Figure 1A**, compared with the control group, Scu remarkably inhibited the growth of HepG2 cells dose-dependently, and the inhibitory effect of Scu reached to a peak value at 25 µM. Moreover, we treated HepG2 cells with Scu (25 µM) for different times. The growth of HepG2 cells was significantly depressed by Scu in a time-dependent manner. The maximal inhibition was observed at 48 h (**Figure 1B**). Therefore, concentration (25 µM) and the time (48 h) of Scu exposure were selected in the following experiments. LO2 cells are considered nonmalignant hepatocytes. In this study, to examine the safety of clinical use of Scu, we also treated LO2 cells with different concentrations of Scu. CCK-8 assay showed that Scu produced less much cytotoxicity on LO2 cells at concentration less than 100 µM (**Figure 1C**), while only exerted slight cytotoxicity on cell growth at concentration more than 100 µM. These results suggest that Scu may specifically inhibit the growth of hepatocellular carcinoma cells, but limited adverse effect on normal cells.

To validate that Scu exposure induced HepG2 cells apoptosis, we observed Hoechst 33258 staining that we named low and high. Low appears as Hoechst 33258 blue-pale, while high shows nuclear fragmentation. Compared with control group, the accumulation of nuclear fragmentation HepG2 cells was much more in Scu group, evidenced by intense staining (**Figure 1D**). In agreement with the Hoechst 33258 staining, the TUNEL assay revealed that Scu dramatically increased apoptotic cell death from 6.3±0.5% to 21.3±1.8% (**Figure 1E**). To further confirm the effect of Scu on HepG2 cells apoptosis, quantitative analysis of apoptosis by flow cytometry was performed. Incubation with 25 µM Scu for 48 h induced an apoptotic rate of 25.4±2.7% (**Figure 1F**), which suggest the increased apoptosis may contribute to the inhibition of cell viability in HepG2 cells.
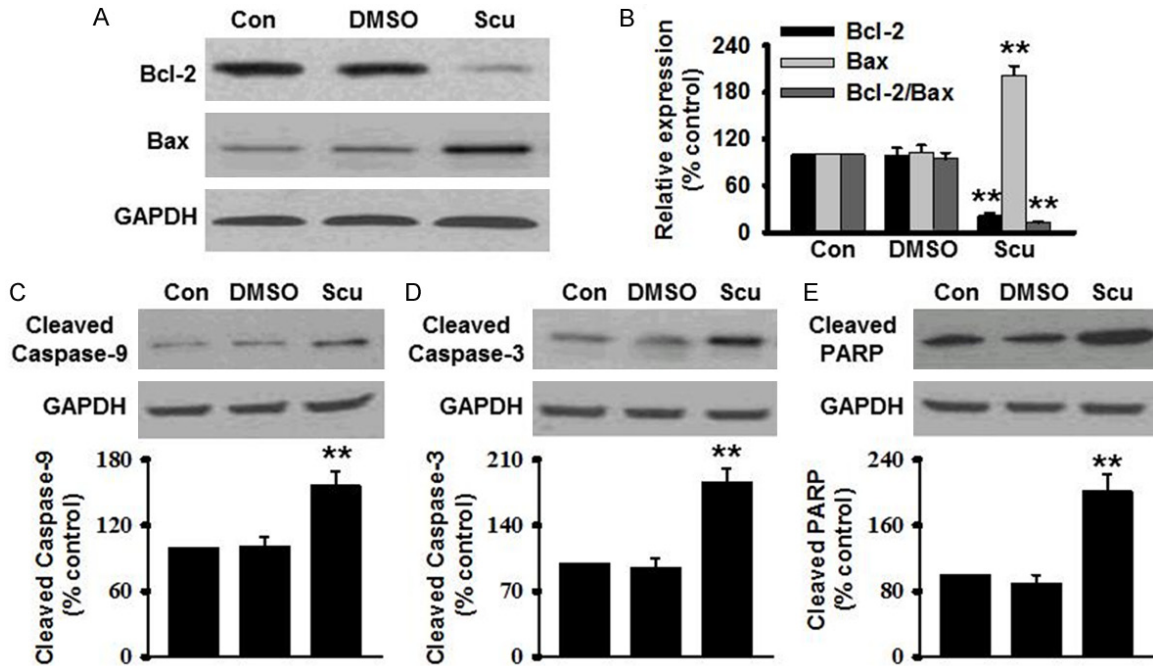
Figure 2. Effects of Scu on Bcl-2, Bax and caspase activation in HepG2 cells treated with Scu. (A-E) HepG2 cells were treated with 25 µM Scu for 48 h. Western blot analysis for Bcl-2, Bax (A and B), cleaved caspase-9 (C), cleaved caspase-3 (D) and cleaved PARP (E). The graphs represent the average of 6 independent western blot experiments for Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3 and cleaved PARP. Data are presented as mean ± SEM. **$P < 0.01$ vs. control, n=6.

Scu induced cell apoptosis through activation of caspase pathway

It has been documented that cellular apoptosis was characterized by several apoptotic marker, including the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax [25]. As seen in **Figure 2A**, treatment of Scu remarkably decreased Bcl-2 expression, while increased Bax expression. Notably, these alterations resulted in an obvious decline in Bcl-2/Bax ratio (**Figure 2B**), which is a critical indicator for the survival or death fate of cell. Activation of caspase cascades is a hallmark of cell apoptosis. Western blot results showed that Scu activated caspase pathway, as evidenced by an increase in cleaved caspase-9 and caspase-3 (**Figure 2C** and **2D**). Expectedly, PARP cleavage in HepG2 treated with Scu was significantly increased (**Figure 2E**).

Scutreatment increased autophagy in HepG cells

Autophagy plays an important role in cell growth, survival, differentiation and homeostasis [26, 27]. To investigate whether Scu influence autophagy in HepG2 cells, we first examine the protein expression of LC3B-II, acrucial marker for autophagy, in HepG2 cells treated with or without Scu (25 µM) for 48 h. Scu treatment showed an increase in LC3B-II (**Figure 3A**). To corroborate the results in the western blot studies, we observed GFP-LC3 puncta in HepG2 following Scu treatment. As shown in **Figure 3B**, Scu exhibited a significant accumulation of GFP-LC3 puncta when compared with control untreated cells. However, the expression of p62, which is associated with autophagosomes and efficiently degraded in lysosomes, was instead decreased (**Figure 3C**). Beclin-1 plays an important role in the initiation of autophagosome formation and coupling of autophagy to apoptosis [23, 28]. To further determine the underlying molecular signaling pathway that regulates autophagy in Scu-treated HepG2 cells, we detected the protein expression of Beclin-1. Western blot showed that Beclin-1 expression was increased after Scu treatment (**Figure 3D**), indicating that Scu-induced autophagy may be dependent of Beclin-1.

Inhibition of autophagy attenuated Scu-induced apoptosis

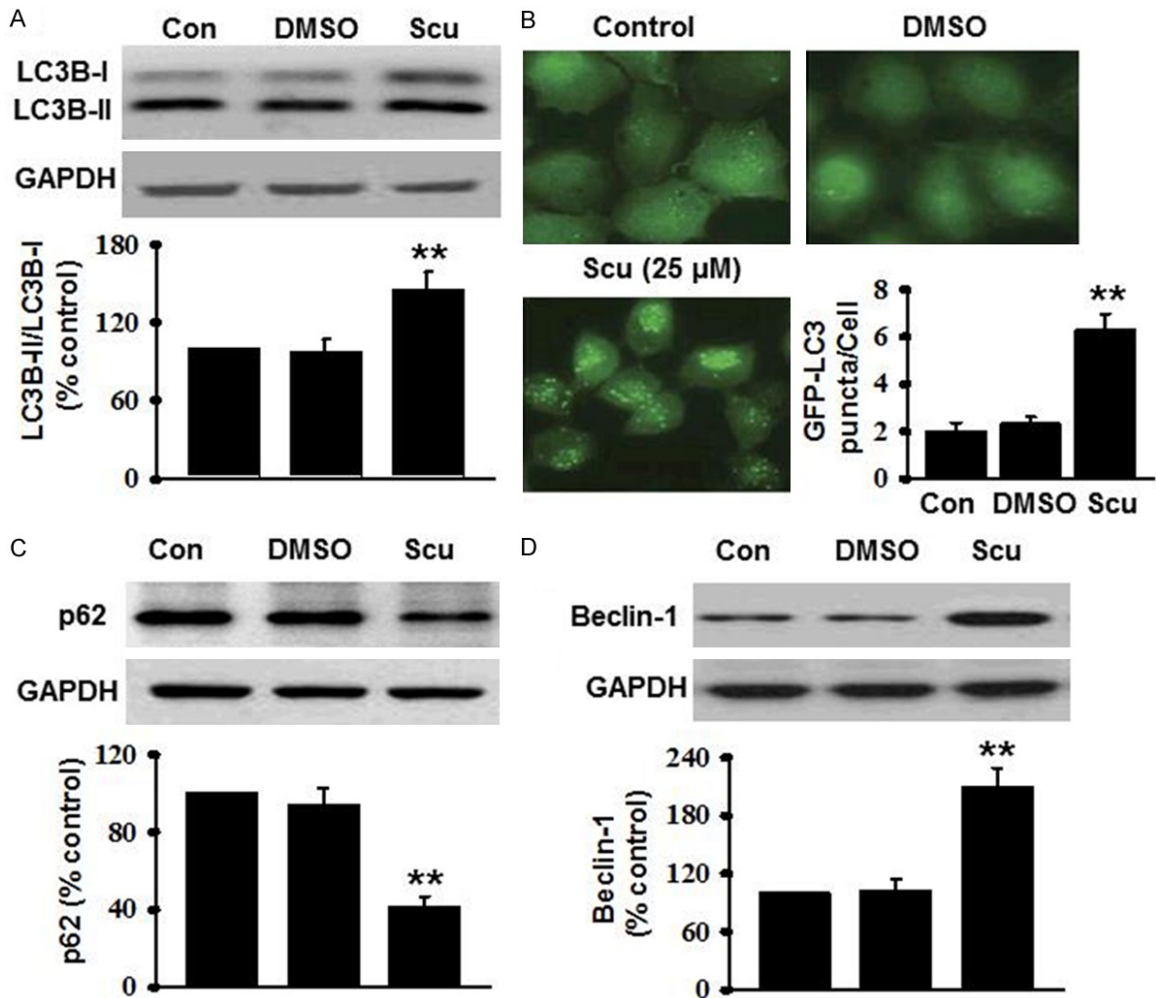To explore the possibility whether autophagy is involved in Scu-induced apoptosis in HepG2,

Figure 3. Scu exposure increased the expression of markers for autophagy in HepG2. (A) HepG2 cells were treated with 25 μM Scu for 48 h. The protein expression of LC3B-II was detected by western blot. (B) GFP-LC3 plasmid was transfected into HepG2 cells as described in method sections. Quantitation of GFP-positive punctawas shown on the lower right from three independent experiments. (C and D) Western blot images showing the expression of p62 (C) and Beclin-1 (D) in control, DMSO and Scu-treated cells. Histogram represents the relative intensity of p62 and Beclin-1 normalized to GAPDH. Values represent mean ± SEM from four to six independent experiments. **$P<0.01$ vs. control group.

we used an autophagy inhibitor, 3-MA, to inhibit autophagy and then examined cell viability and apoptosis incidence. As expected, pretreatment of 3-MA (5 mM) remarkably inhibited Scu-induced the increase in LC3B-II expression (**Figure 4A**). Strikingly, the inhibition of cell viability in HepG2 cells induced by Scu was almost abolished by 3-MA (**Figure 3B**). Similarly, 3-MA blunted the increase of apoptotic rate in HepG2 cells after being exposed to 25 μM Scu for 48 h (**Figure 4C** and **4D**). However, 3-MA produced no significant effect on cell viability and apoptosis in HepG2 cells. These results suggest that autophagy underlies, at least in part, the pro-apoptotic effect of Scuin HepG2 cells.

Discussion

Previous studies showed that Scu exerted obvious anti-cancer effect in vivo and vitro [17, 20, 21, 29]. Interestingly, several studies also have evidenced that Scu protects against liver injury induced by diosbulbin B [16], brain ischemia/reperfusion [30] or lipopolysaccharide [31]. In particular, Xu et al. reported Scutellarin induced apoptosis in HepG2 hepatocellular carcinoma cells via attenuation STAT3 pathway [32]. Apart from this literature, however, there is no more information related to Scu in HepG2 cells apoptosis. Thus, whether Scu affects the survival and death of hepatocellular carcinoma cells
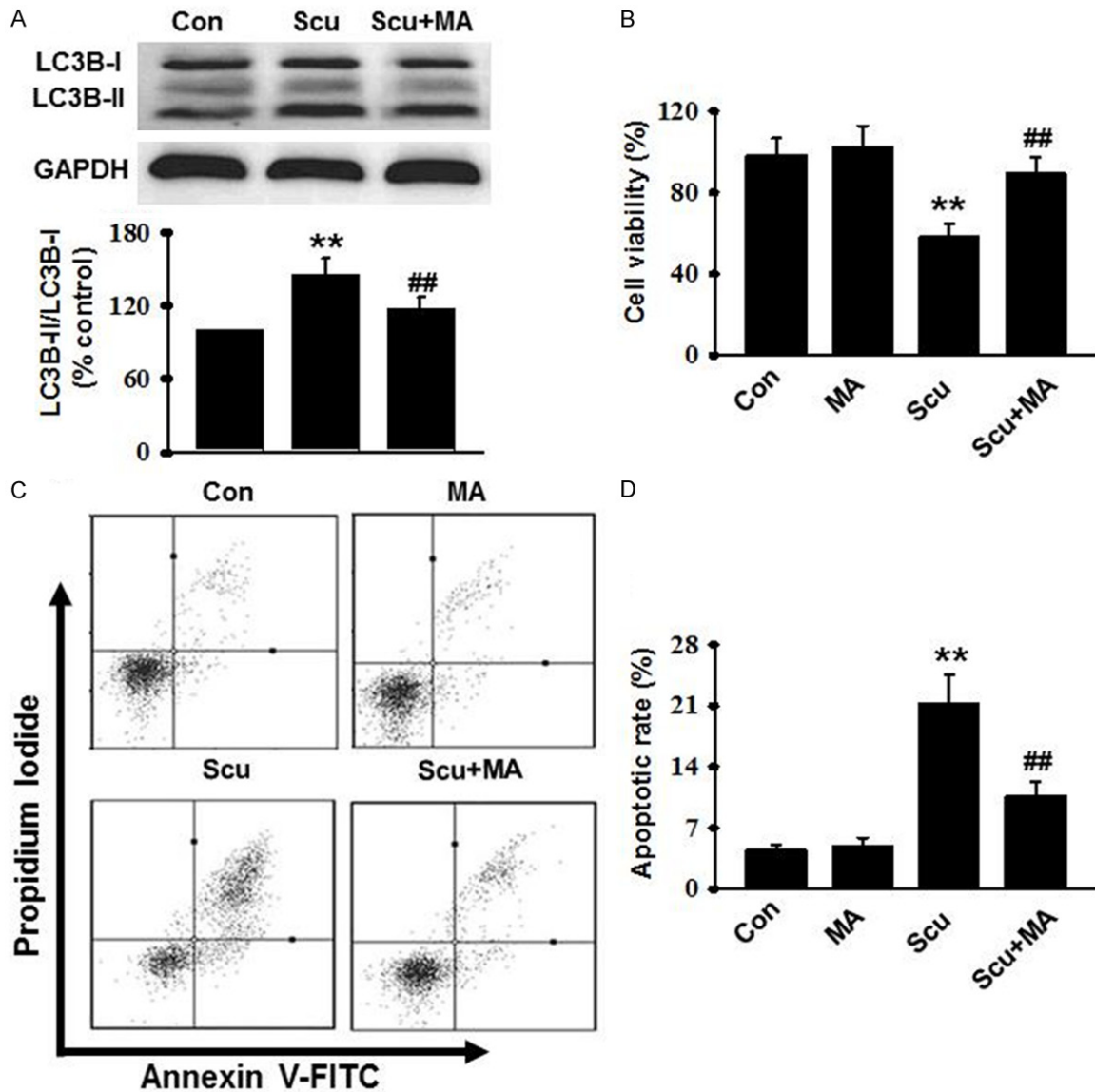
Figure 4. Inhibition of autophagy inhibited Scu-induced cell injury and apoptosis in HepG2. A. HepG2 cells were pretreated with 3-MA (5 mM) for 2 h, and then were cultured in the presence of Scu (25 μM) for 48 h. Western analysis of LC3B-II protein expression was performed. B. Cells were cultured in the absence or presence of Scu for 48 h with or without 3-MA. Cell viability was determined by CCK-8 assay. C. Apoptosis in HepG2 was determined by Annexin V/PI staining followed by flow cytometry. D. Quantitative analysis of the percentage of apoptotic cells. The data were expressed as mean ± SEM. **P<0.01 vs. control, ##P<0.01 vs. Scualone, n=6.

attracts our attention. In the present study, we found Scu decreased cell viability dose-dependently and time-dependently, while produced less much cytotoxicity on LO2 cells. Moreover, apoptosis incidence in HepG2 was enhanced after Scu exposure, evidenced by Hoechst 33258 staining, TUNEL assay and flow cytometry, all of which were in line with a previous study [32]. Additionally, the por-apoptotic effect of Scu was further confirmed by the apoptosis markers, including Bcl-2, Bax and caspasecascade activation.

Although many clinical and experimental studies have shown that hepatic apoptosis is regulated by autophagic activity, the mechanisms mediating their interaction remain to be determined [33]. Autophagy exerts dual effect in hepatocellular carcinoma cells, which likely could be a pro-survival or pro-apoptotic mechanism. For instance, autophagy-mediated cell apoptosis is the major mechanism in response to certain anti-cancer drugs, such as 5-FU and cisplatin [34-36]. On the contrary, autophagy promotes cell growth that attenuates the che-

mosensitivity to anti-cancer drugs [36]. In this case, the high autophagy activity instead enhances tumor adaptation to stress [9]. Presumably, the discrepancy is probably relatedto the complicated interplay between hepatic autophagy and apoptosis, which finally determines the degree of hepatic apoptosis and the progression of liver disease [33]. Recently, several Chinese medicinal herbs have been shown to induce autophagy-mediated apoptosis in hepatocellular carcinoma cells. For example, Baicalein, isolated from the Chinese medicinal herb Scu tellariae radix, has been found to inhibit the growth of HepG2 cells via induction of autophagy [37]. In addition, another traditional Chinese medicine, Sann-Joong-Kuey-Jian-Tang was reported to induce apoptosis and autophagy in HepG2 through activation of PI3K/Akt/mTOR and MAPK pathways [38]. These findings indicate that natural compounds that induce autophagy may be novel approach to treat with hepatocellular carcinoma. Based on the effects of Scu on HepG2 cells apoptosis, we opine that Scu mediates autophagy and subsequently promotes cell apoptosis in HepG2. To the best of our knowledge, our findings are the first to demonstrate that Scu triggers autophagy in HepG2 cells. During autophagy, the LC3B-I should be converted to the autophagosome-associated form LC3B-II, which then binds to adaptor protein p62 (also known as Sequestosome, SQSTM1) to facilitate autophagic degradation of ubiquitinated protein aggregates in the lysosomes [39]. In the present study, although we cannot differentiate between LC3B-I and LC3B-II by immunofluorescence staining, it is conceivable that this is due to the increased LC3B-II expression based on the western blot results. Similarly, the level of p62 was markedly increased after Scu treatment.

While autophagy has been found to be regulated by more than 30 autophagy specific genes (Atg), Beclin-1 plays an important role in the autophagy formation and coupling of autophagy to apoptosis [28, 40]. Genetic modification of Beclin-1 can cause dynamic changes of autophagy [41]. Previous study showed that overexpression of Beclin-1 could stimulate autophagy and inhibit tumor development [42]. Moreover, Furuya et al. reported that overexpression of Beclin-1 in gastric cancer cells enhanced caspase activity, leading to augmentation of apoptosis [43]. This was true in our

study, where, treatment of Scu induced apoptotic cell death in HepG2 plausibly by increasing autophagy and caspase activity. Importantly, we found that apoptosis was drastically inhibited after inhibitionof autophagy by 3-MA. These results further confirm the critical role of autophagy in HepG2 cells apoptosis after Scu exposure.

In summary, our results showed that Scu treatment induced cell injury and apoptosis in HepG2 cells. Additionally, the present study demonstrated for the first time that Scu could induce autophagy in HepG2 cells and inhibition of autophagy attenuated the pro-apoptotic effect of Scu. Our findings reveal a novel molecular mechanism whereby Scu induces HepG2 cells apoptosis, and provide a potential therapeutic agentfor treating hepatocellular carcinoma.

Disclosure of conflict of interest

None.

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