Original Article

Baicalin induces apoptosis in hepatic cancer cells \textit{in vitro} and suppresses tumor growth \textit{in vivo}

Yang Yu\textsuperscript{1}, Mingyan Pei\textsuperscript{3}, Ling Li\textsuperscript{2}

\textsuperscript{1}Department of Traditional Chinese Medicine, The First Affiliated Hospital of The General Hospital of The People’s Liberation Army of China, Beijing 100048, China; \textsuperscript{2}Department of Pharmacy, Tongren Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200336, China; \textsuperscript{3}Department of Pharmacy, Heilongjiang Academy of Chinese Medicine Sciences, Harbin 150036, Heilongjiang, China

Received December 19, 2014; Accepted February 25, 2015; Epub June 15, 2015; Published June 30, 2015

Abstract: Baicalin is a flavonoid glycoside extracted from a kind of traditional Chinese drug, \textit{Scutellaria baicalensis}, and possesses multiple pharmacological activities. The present study was designed to investigate the effects and mechanisms of baicalin against hepatic cancer cell growth and survival. We found that baicalin inhibited the viability and proliferation of two widely used hepatic cancer cell lines, Hep G2 and SMMC-7721 cells, in a dose-dependent manner. Cell cycle analysis revealed an increase in the S-phase cell population following 48 h exposure to baicalin. The expression levels of Cyclin A, CDK2, and Cyclin D1 were downregulated by baicalin treatment. Moreover, baicalin induced apoptotic cell death in Hep G2 and SMMC-7721 cells, which was accompanied by upregulation of Bax, downregulation of Bcl-2, and cleavages of Caspase-9, Caspase-3, and PARP. Furthermore, baicalin significantly inhibited the growth of xenografts in nude mice. In conclusion, we demonstrated for the first time that baicalin inhibited hepatic cancer cell growth and survival both \textit{in vitro} and \textit{in vivo}, suggesting that baicalin may be a potential phytochemical flavonoid for hepatic cancer therapy.

Keywords: Hepatic cancer, baicalin, apoptosis

Introduction

Primary hepatic cancer or liver cancer is the sixth most common cancer globally and the second leading cause of cancer-related death [1]. Chronic inflammation and cirrhosis as a result of viral hepatitis B and C, fatty liver and alcohol abuse are usually considered the culprits of hepatic cancer which usually has a very poor prognosis. Clinically only a very limited number of patients are eligible for potentially curative treatment options such as surgical resection followed by orthotopic liver transplantation. To date, chemotherapy remains one of the major non-surgical therapeutic approaches for patients with advanced hepatic cancer [2]. However, due to drug resistance, systemic chemotherapy produces a disappointing low response rate, ranging between 10%-15% [3]. Moreover, many currently used anti-cancer agents have potent cytotoxic effects in normal cells [4]. Therefore, there is an urgent need to develop effective chemotherapeutic approaches for hepatic cancer patients.

Natural products have received recent interest in discovery of novel anti-cancer therapeutic agents as they have relatively few side effects and have long been used as alternative remedies for a variety of diseases including cancer [5]. Therefore, identifying naturally occurring agents is a promising approach for anticancer treatment. Baicalin (5,6-dihydroxy-7-O-glucuronide flavone) is a predominant flavonoid isolated from the roots of \textit{Scutellaria baicalensis} Georgi (Huang Qin), and its chemical structure has been clarified [6]. It has been reported that this compound exhibits various pharmacological activities, including anti-oxidation, anti-tumor, anti-inflammation and anti-proliferation [7-10]. Baicalin has displayed beneficial effects on several diseases models, such as hypoxia/reoxygenation caused cardiomyocytes injury [11], hepatic cytotoxicity [12], iron-overloaded mouse [13], rheumatoid arthritis [14] and so on. Moreover, baicalin shows certain therapeutic effects on hepatic fibrosis by immunoregulating the imbalance between profibrotic and antifibrotic cytokines [15].
Baicalein (5, 6, 7-trihydroxyflavone), a flavonoid also presented in the roots of *Scutellaria baikalensis*, was recently found to inhibit growth and invasion of hepatoma cells [16]. It has also been shown that approximately 90% of baicalein administered is metabolized to baicalin [17], and hence, it is necessary to investigate the role of baicalin in any particular medical condition. In the present study, we evaluated the effect of baicalin on hepatic cancer growth and survival in vitro and in vivo, and investigated the underlying molecular mechanisms.

**Methods**

**Drugs and antibodies**

Baicalin was obtained from the National Institute for Control of Pharmaceuticals and Biological Products (Beijing, China), and the purity was at least 95% as confirmed by HPLC. Baicalin was dissolved in dimethyl sulfoxide (DMSO) in a 100 μmol/L stock solution and stored at -20°C, and diluted to appropriate concentrations with culture medium immediately before experimental use. An equal volume of DMSO with final concentration of 0.08% was added to the controls. Antibodies to the following proteins were used for western blot analysis: Cyclin A (Epitomics, Burlingame, CA, USA); CDK2, Cyclin D1, Bax, Bcl-2, cleaved Caspase-3 (Asp175), cleaved Caspase-9 (Asp330), cleaved PARP (Asp214), and β-actin (Cell Signaling, Danvers, MA, USA).

**Cell culture**

Human hepatic cancer cell lines Hep G2 and SMMC-7721 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Hep G2 cells were maintained in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), SMMC-7721 cells were maintained in RPMI-1640 (Gibco) supplemented with 10% FBS. All cell lines were cultured at 37°C in a humidified incubator containing 5% CO₂.

**Cell viability assay**

The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay was used to measure drug sensitivity. Briefly, Hep G2 and SMMC-7721 cells were seeded into 96-well plates (Corning, USA) at a density of 5 × 10³ cells per well, incubated overnight, and then treated with various concentrations (0, 10, 20, 40, and 80 μmol/L) of baicalin for 24, 48, and 72 h. Thereafter, 20 μL of MTT solution (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and the plates were incubated at 37°C for 4 h. The formed formazan crystals were dissolved in 100 μL of DMSO after removal of the supernatant. The optical density (OD) was recorded at 490 nm using a microplate reader (Bio-Tek, Winooski, VT, USA). The percentage of cell viability was calculated as: (OD of baicalin-treated group/OD of control group) × 100%.

**Colony formation assay**

Hep G2 and SMMC-7721 cells were seeded onto 6-well plates (Corning, USA) at a density of 500 cells per well. After adherence, cells were treated with various concentrations (0, 10, 20, and 40 μmol/L) of baicalin for 48 h, and then cultured for 14 days. Thereafter, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich) for 20 min. The stained colonies were captured and counted under a microscope (Leica, Wetzlar, Germany).

**Flow cytometry analysis of cell cycle**

Hep G2 and SMMC-7721 cells were treated with various concentrations (0, 10, 20, and 40 μmol/L) of baicalin for 48 h. Cells were then harvested, fixed in 70% ethanol, and stored overnight at 4°C. Thereafter, cells were resuspended in staining buffer of 1 mg/mL PI and 10 mg/mL RNase A. Following incubation for 30 min in the dark at room temperature, cells were analyzed by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA, USA). The fractions of the cells in G0/G1, S and G2/M phases were analyzed with dedicated software (Becton Dickinson).

**Flow cytometry analysis of apoptosis**

Apoptosis was analyzed using an Annexin V/PI apoptosis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, Hep G2 and SMMC-7721 cells were treated with various concentrations (0, 10, 20, and 40 μmol/L) of baicalin for 48 h. Cells were then harvested, washed twice with cold PBS, and resuspended in 100 μL of binding buffer containing 5 μL of Annexin V-FITC and 1 μL of PI solution (100 μg/mL). Following incubation for
Baicalin on hepatic cancer cell growth and survival

Figure 1. Baicalin inhibits the viability and proliferation of hepatic cancer cells. (A) Hep G2 and SMMC-7721 cells were treated with varying concentrations (0, 10, 20, 40, and 80 μmol/L) of baicalin for 48 h, and cell viability was determined by MTT assay. (B) Hep G2 and SMMC-7721 cells were treated with 20 μmol/L of baicalin for 24 h, 48 h, and 72 h, and cell viability was determined by MTT assay. (C) Representative photographs of colony formation are shown. Baicalin caused a concentration-dependent decrease in both colony numbers (D) and colony size (E). Values represent the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
Baicalin on hepatic cancer cell growth and survival

30 min in the dark at room temperature, cells were analyzed by flow cytometry.

**Hoechst 33342 staining**

Apoptotic cells were identified on the basis of morphological changes in their nuclear assembly by observing chromatin condensation and fragment staining with Hoechst 33342, as previously described [18]. After treatment with various concentrations of baicalin for 48 h, Hep G2 and SMMC-7721 cells were fixed with methanol: acetic acid (3:1), washed with PBS and then stained with 5 μg/mL of Hoechst 33342 stain for 10 min. Nuclear DNA staining was observed using a fluorescence microscope. In each group, five microscopic fields were randomly selected and counted.

**Western blot analysis**

After treatment with various concentrations of baicalin for 48 h, Hep G2 and SMMC-7721 cells were suspended in a lysis buffer (2% mercaptoethanol, 20% glycerol, and 4% SDS, in 100 mM Tris-HCl buffer, pH 6.8). Equal amounts of lysate (50 μg) in each lane, as determined by the bicinchoninic acid (BCA) assay, were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature, and incubated with TBST containing primary antibody overnight at 4°C, followed by incubation with secondary antibody for 1 h at room temperature. The blots were detected using enhanced chemiluminescence (ECL) kit (Amersham) and visualized by exposure to X-ray film. β-actin was used as a control to verify equal protein loading.

**In vivo efficacy of baicalin**

Six-week old athymic nude mice (with an initial body weight of ~20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed under pathogen-free conditions with controlled temperature (22°C), humidity, and a 12 h light/dark cycle. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Tongren Hospital, Shanghai Jiaotong University School of Medicine. Each nude mouse was subcutaneously injected of 3 × 10⁶ of Hep G2 cells in a volume of 100 μL in the right flank to initiate tumor growth. After inoculation for one week, these mice were randomly assigned to three groups (7 mice/group). One group was treated with vehicle (10% DMSO and 90% propylene glycol) intraperitoneally (i.p.), and the other two groups were administered an i.p. injection of baicalin at 50 mg/kg and 100 mg/kg daily. After treatment for three weeks, all nude mice were sacrificed, and the tumors were removed and weighed. Immunohistochemical staining was performed on paraffin-embedded tumor sections using the avidin-biotin complex protocol. Briefly, cleaved Caspase-3 (Asp175), or cleaved Caspase-9 (Asp330) was applied to the sections. The slides were stained with 3, 3’-diaminobenzidine (DAB, Sigma-Aldrich), and then dehydrated, cleared, and mounted. Immunostaining was captured under a microscope.

**Statistical analysis**

Statistical analysis was performed using SPSS 16.0 software package (SPSS Inc, Chicago, IL, USA). All data were expressed as mean ± standard deviation (SD) from three independent experiments. Differences between two groups were analyzed by Student’s t-test and a p value of less than 0.05 was considered statistically significant.

**Results and discussion**

**Baicalin reduces viability and proliferation of hepatic cancer cells in a dose-dependent manner**

It has been reported that baicalin was more cytotoxic than baicalein in Hep G2 hepatic cancer cells [19]. Thus, the toxic effect of baicalin on hepatic cancer was initially examined in vitro using two kinds of hepatic cancer cell lines Hep G2 and SMMC-7721. MTT assays showed that baicalin exerted a concentration-dependent and time-dependent killing of both cell lines (Figure 1A, 1B). The IC50 values of baicalin for both cell lines were not more than 40 μM, thus cells were treated with baicalin in the range of the drug concentrations (0-40 μM) in vitro. As shown in Figure 1C, the colony forming ability of Hep G2 and SMMC-7721 cells in the presence of baicalin was detected with the plate colony formation assay. The colony count indicated that baicalin had induced a concentration-dependent decrease in colony formation (Figure
also, the size of single colony in baicalin-treated cells was much smaller than in control cells (Figure 1E). These findings support the fact that baicalin exerts a significant influence on hepatic cancer cell proliferation as described previously [20].
Baicalin on hepatic cancer cell growth and survival

Figure 3. Baicalin induces apoptosis in hepatic cancer cells. A. Apoptotic morphological changes, including abnormal nuclear morphology, cell number reduction and apoptotic body formation, induced by baicalin (10, 20 and 40 μmol/L) treatment for 48 h, were observed by Hoechst 33342 staining in Hep G2 and SMMC-7721 cells. B. Statistical analysis of apoptotic cells in Hep G2 and SMMC-7721 cells. C. Hep G2 and SMMC-7721 cells were analyzed by flow cytometry with Annexin V-FITC/PI staining after baicalin treatment. Annexin V vs PI plots from the gated cells showed the populations corresponding to viable (Annexin V-/PI-), and necrotic (Annexin V-/PI+), early (Annexin V+/PI+), and late (Annexin V+/PI+) apoptotic cells. D. Statistical analysis of different cell population in Hep G2 and SMMC-7721 cells. E. Western blot analysis of Bax, Bcl-2, Caspase-3, Caspase-9, and PARP protein levels in Hep G2 and SMMC-7721 cells after baicalin treatment. Values represent the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01.

Baicalin induces cell cycle arrest at S phase in Hep G2 and SMMC-7721 hepatic cancer cells

To assess whether baicalin affects cell cycle progression, flow cytometric analysis was carried out. As shown in Figure 2A, 2B, an increasing number of cells was arrested in the S phase as the increasing concentration of baicalin in both Hep G2 and SMMC-7721 cell lines. Concomitantly, the percentages of cells in G1
Baicalin on hepatic cancer cell growth and survival

Figure 4. Baicalin suppresses tumor growth in xenografted nude mice by causing apoptosis. A. Tumor dimensions were periodically measured using calipers. B. Tumors were excised from the animals and weighed. C. Photographs of 7 tumors from each group are shown. D. Photographs of Caspase-3 and Caspase-9 immunostaining are shown. *P < 0.05, **P < 0.01.

Phase and G2/M phase were decreased. These results suggest that baicalin-inhibited proliferation of hepatic cancer cells partly as a result of an accumulation of cells in the S phase of the cell cycle. The S phase is associated with DNA synthesis and the mitotic preparation period, which plays a crucial role in cell cycle progression. The complex formation of Cyclins with
Baicalin on hepatic cancer cell growth and survival

CDKs results in an active agent that phosphorylates substrates involved in cell cycle progression [21]. The mitosis-promoting factor, which comprises a complex of Cyclin A and CDK2, is thought to be the key controller of the progression from S to mitosis [22]. Western blot analysis showed that Cyclin A and CDK2 were dose-dependently decreased by baicalin treatment. In addition, Cyclin D1, a key regulator in G1 phase progression [23], was also downregulated in both Hep G2 and SMMC-7721 cell lines. Thus, it is tempting to speculate that the reduction of Cyclin A, CDK2, and Cyclin D1 levels is responsible for the perturbation of the S/G2 phase transition with a decrease of cells in G1 phase and an increase of cells in S phase.

*Baicalin enhances apoptosis in Hep G2 and SMMC-7721 hepatic cancer cells*

Previous studies have revealed that baicalin inhibits the growth of cancer cells through the induction of apoptosis [24-27]. Therefore, we further examined the effect of baicalin on apoptosis of hepatic cancer cells. As shown in Figure 3A, morphological changes in the apoptotic cells were revealed by the Hoechst 33342 staining. The nuclei were stained weak homogeneous blue in the untreated Hep G2 and SMMC-7721 cells, whereas in baicalin-treated cells, bright chromatin condensation and nuclear fragmentation were observed indicating that apoptotic cells was increased by baicalin treatment (Figure 3B). To further confirm these results, we evaluated the effect of baicalin on cell apoptosis by flow cytometry using Annexin V-FITC and propidium iodide (PI) staining (Figure 3C). Both the early and late stages of apoptosis were obvious increased in both Hep G2 and SMMC-7721 cell lines after baicalin treatment compared with untreated cells (Figure 3D). These results suggest that baicalin-inhibited proliferation of hepatic cancer cells partly as a result of an induction of apoptosis. Furthermore, we identified several apoptosis-related factors mainly involved in baicalin-induced apoptotic process by western blot analysis. As shown in Figure 3E, the expression of the pro-apoptotic protein Bax was upregulated by baicalin treatment in both Hep G2 and SMMC-7721 cell lines, whereas the expression of Bcl-2, which belongs to the Bcl-2 family of anti-apoptotic proteins, was concomitantly downregulated. Moreover, the protein levels of cleaved-Caspase-3, cleaved-Caspase-9 and cleaved-PARP1 were upregulated by baicalin treatment. Apoptosis is a type of programmed cell death that is caspase-dependent [28]. Activated Caspase-9 cleaves and activates effector caspases such as Caspase-3, that then cleave a variety of cellular proteins and cause cell death [29]. Poly-ADP-ribose polymerase (PARP), a member of the PARP enzyme family, is an abundant DNA-binding enzyme that detects and signals DNA strand breaks [30]. The presence of cleaved-PARP1 is one of the most used diagnostic tools for the detection of apoptosis in many cell types [31]. Additionally, mitochondrial membrane permeability during apoptosis is regulated directly by the Bcl-2 family of proteins, thereby regulating release of apoptogenic factors from the intermembrane space into the cytoplasm [32-34]. Taken together, it could be concluded that the mechanism of baicalin promoting hepatic cancer cell apoptosis may be via the activation of the mitochondrial-mediated intrinsic caspase pathway.

*Baicalin suppresses tumor growth in xenografted nude mice*

To further confirm the effect of baicalin in vivo, we measured the tumor growth in xenograft tumor models in which Hep G2 cells were injected intraperitoneally (i.p.) into nude mice. When transplant tumors reached a mean group size of approximately 100 mm³, mice were treated with various dose of baicalin (i.p., 50, and 100 mg/kg) daily for three weeks. Compared with the control group, baicalin showed a significant inhibitory effect on tumor size (Figure 4A). After treatment for three weeks, all nude mice were sacrificed, and the tumors were removed and weighed. The average tumor weight was significantly less in baicalin-treated mice than in control mice (Figure 4B, 4C), suggesting that baicalin could suppress the growth of xenografts in nude mice. To determine whether the reduced tumor growth rate following baicalin treatment could be explained by the induction of apoptosis, we detected the expression of Caspase-3 and Caspase-9 in tumor sections by immunohistochemical staining. As shown in Figure 4D, the expression levels of cleaved-Caspase-3 and cleaved-Caspase-9 were obviously stronger in baicalin-treated mice than in control mice, as measured by positive immunostaining. These results confirm that baicalin could inhibit tumor growth by causing apoptotic cell death. To our knowledge,
this is the first study to evaluate the potential role of baicalin in xenograft animal model in vivo. A significant reduction of tumor mass was observed after a 3-week treatment. In addition, no significant difference emerged in the average body weight of baicalin-treated mice, compared to that of the control mice (data not shown), indicating that mice receiving baicalin treatment do not show obvious toxicities. Collectively, baicalin has good potential as an antitumor drug for future treatment of hepatic cancer.

Conclusions

From the data, we found that the natural flavonoid baicalin conspicuously inhibited cell growth by inducing apoptosis in human Hep G2 and SMMC-7721 hepatic cancer cells. The in vivo effect of baicalin on hepatic cancer tumors strongly supported baicalin as a potential new drug for the treatment of hepatic cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Ling Li, Department of Pharmacy, Tongren Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200050, China. Tel: +86-021-62524259; Fax: +86-021-6252-259; E-mail: liling_l1@163.com

References

Baicalin on hepatic cancer cell growth and survival


