Icariin induces S-phase arrest and apoptosis in medulloblastoma cells

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Abstract: Medulloblastoma is the most common type of malignant brain tumor in children. Despite a relatively high long-term survival rate, complications still represent a great burden for the majority of patients receiving traditional therapy. Therefore, the development of new effective treatments and drugs is urgently needed. A cell counting kit-8 (CCK-8) and colony formation assay were used to evaluate medulloblastoma cell proliferation and colony formation, respectively. Cell cycles and apoptosis were assessed by flow cytometry. A western blot was performed to determine the levels of protein expression. Xenograft model of medulloblastoma was established to evaluate the in vivo anticancer effects of icariin. The CCK-8 assay showed that icariin decreased cell viability in a dose- and time-dependent manner. The colony formation assay indicated that icariin potently inhibited the colony formation ability of Daoy and D341 cells. Icariin-induced proliferation inhibition may be due to S-phase arrest in medulloblastoma cells. In addition, icariin induced apoptosis in a dose-dependent manner, as shown by the results of annexin V/propidium iodide (PI) double staining and Hoechst 33342 staining. Icariin progressively inhibited tumor growth and induced apoptosis in a mouse model. Moreover, cell cycle regulators Cyclin A, CDK2, and Cyclin B1, and apoptosis-related proteins caspase-3, caspase-9, poly (ADP-ribose) polymerase (PARP), and Bel-2 were modulated in response to treatment with icariin in vitro and in vivo. Our results suggest that icariin may exert anticancer effects. Thus, it is a promising drug for medulloblastoma treatment.

Key words: Icariin, cell cycle, apoptosis, medulloblastoma.

Introduction

Medulloblastoma is the most common type of malignant brain tumor in children. Although patients with this malignancy have a relatively high long-term survival rate, they are still plagued by complications, partially due to the intense therapies administered to ensure eradication of the tumor (1). Clinically, medulloblastoma is characterized by high invasiveness and metastasis through cerebrospinal fluid (CSF). Over the years, increasing incidence rates have been observed for medulloblastoma, despite advances in treatment (2). The high incidence rate has necessitated the development of new effective treatments and drugs.

Plant-derived natural products occupy a very important position in the field of cancer chemotherapy (3). Clinically, the newly developed natural products have greatly advanced chemotherapy. However, the quest for novel therapeutic compounds for cancer treatment is a never-ending venture. Icariin is a key active component of Epimedium species. It is most widely applied in traditional Chinese medicine, with its main application being in the kidneys (4). Icariin possesses antioxidative, neuroprotective, and immunomodulatory activity (5-7). In particular, it exhibits a variety of functions in tumorigenesis in colorectal cancer (8), hepatocellular carcinoma (3,9), gallbladder cancer (10), and chondrosarcoma (11). The mechanisms underlying icariin-mediated anticancer effects vary based on the type of cancer. However, icariin has been reported to be closely highlighted in the intervention of p38 mitogen-activated protein kinases(MAPK) and phosphatidylinositol 3-kinase/Akt(P13K/Akt) signal pathways, inhibition of phosphodiesterase 5, and regulation of nuclear receptors (12-14). For example, icariin can inhibit H2O2-activated p38 activity, thereby protecting neurons from H2O2-induced injury (15). Icariin exerts neuroprotective effects on corticosterone induced apoptosis in primary cultured rat hippocampal neurons via inhibition of p38 activity (12). It may also activate PI3K signaling to suppress glycogen synthesis kinase(GSK) expression, leading to dephosphorylation of amyloid beta-protein(Aβ)-induced tau protein and prevention of injury to neurons in Alzheimer’s disease (13). Interestingly, these studies indicate that icariin plays an extensive role in the central nervous system. This indication led us to examine whether icariin could maintain its key role in medulloblastoma.

Because of the pivotal role of icariin in human tumorigenesis and the central nervous system, we hypothesized that icariin could be a key mediator in medulloblastoma development. To test our hypothesis, we investigated the effects of icariin on medulloblastoma cell proliferation in vitro and on tumor growth in vivo. We also sought to identify the possible mechanisms that contributed to icariin-mediated medulloblastoma development.

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Materials and Methods

Drug and antibodies

Icariin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) to obtain a 100mM stock concentration. The stock solution of icariin was then diluted with the culture medium to a variety of concentrations according to the experimental design. An equal volume of DMSO (final concentration <0.1%) was added to the controls in all experiments. The primary antibodies used for western blotting were rabbit anti-Bax, anti-Bcl-2, anti-caspase-3, anti-caspase-9, anti-PARP, anti-cyclin A, anti-CDK2, cyclin B1, and mouse anti-β-actin. All primary and secondary antibodies were purchased from Santa Cruz Biotechnolohy (Santa Cruz, CA, USA).

Cell lines and cell culture

Human medulloblastoma cell lines Daoy and D341 were purchased from the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (CAS, Shanghai, China). Cells were maintained in high-glucose Dulbecco’s modified eagle medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100μg/mL streptomycin, and 100μg/mL penicillin (HyClone, Logan, UT, USA). Cells were cultured at 37°C in a 5% CO₂, humidity controlled incubator. Culture medium was refreshed every two days.

Western blot analysis

Cells with different treatments were harvested after two-day culture and lysed in IP lysis buffer (Beyotime, Nantong, China), denatured for 5 min at 95°C, and resolved on the SDS/PAGE gels. Prior to loading, total protein concentration was determined using a BCA kit (Pierce, Rockford, IL, USA). Equal amounts of extract were loaded into each lane in a 12% SDS-PAGE and transferred to polyvinylidenedifluoride (PVDF) membranes (Bio-Rad, Hercules, CA). Thereafter, the membranes were blocked with TBS/0.1% Tween-20 (TBST) supplemented with 5% skim milk for 1h before proceeding to primary antibody incubations overnight. After incubation with primary antibodies, membranes were washed with TBST twice and subjected to secondary antibody incubation at 37°C for 1h. Membranes were then processed for chemiluminescent detection using an ECL detection kit (Pierce, Rockford, IL, USA). β-Actin was used as a loading control.

Cell counting kit-8(CCK-8) assay

The proliferation of Daoy and D341 cells was assessed using a CCK-8 assay (Beyotime, Nantong, China) according to the protocol provided by the manufacturer. In summary, the cells were cultured in a 96-well plate according to the protocol provided by the manufacturer. After determination, cell proliferation was terminated by the addition of 10μL of CCK-8 solution to each well, followed by incubation at 37°C for 2 h. Absorbance was measured by an ELISA reader at a wavelength of 450 nm.

Colony formation assay

In summary, cells treated with a corresponding concentration of icariin were seeded in a 6cm dish in triplicate (1000 cells per dish) and incubated at 37°C for two weeks until clones formed. Clones were then washed with PBS twice, fixed with 4% paraformaldehyde for 15min, and subjected to staining with crystal violet (0.5% crystal violet, 1% paraformaldehyde, and 20% methanol in PBS) for 30min. Clones with a cell number over 50 were included as a colony. The colonies on each plate were counted. Colony size was also measured in each dish.

Cell cycle analysis

Icariin-treated cells were harvested after two days of culture and gently resuspended into single cell suspensions in 2% FBS-containing PBS. The cells were then fixed with cold 70% ethanol overnight. After fixation, cells were washed with cold PBS twice, resuspended in RNase A solution, and incubated at 4°C for 30min. The suspension was added to 0.05 mg/mL propidium iodide (Beyotime) and incubated at 4°C for 30min. Cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA).

Cell apoptosis determination

Cell apoptosis was analyzed using the annexin V/PI apoptosis kit according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Daoy and D341 cells were seeded in a 6-well plate (1×10⁴ cells/well) and treated with different concentrations of icariin (according to the experimental design) for 48h. The cells were collected after seeding, washed with cold PBS, and resuspended in 100μL binding buffer at a concentration of 1×10⁴ cells/mL. Then, 5μL of annexin V-FITC and 5μL of PI working solution (100μg/mL) were added to the 100-μL aliquots of the cell suspension. After 15min of incubation in the dark at room temperature, 400μL of the binding buffer was added to the cell suspension. Samples were then analyzed by flow cytometry. Each sample was tested three times.

Hoechst 33342 staining was used for morphological examination of cells. After 24h of culture, cells treated with different concentrations of icariin were washed twice with PBS and fixed in 4% paraformaldehyde for 10min. Thereafter, cells were stained with Hoechst 33342 for 5min and subjected to observation under a fluorescence microscope. The apoptotic cells that exhibited compact, condensed, and fragmented nuclei were counted and quantified.

Xenograft model of Medulloblastoma

Eighteen 6-week-old athymic Balb/C nude mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Mice were randomly divided into three groups: control, treatment with 20mg/kg icariin, and treatment with 40mg/kg icariin (n=6 for each group). D341 cells (1×10⁶) were subcutaneously injected into the right flank of nude mice. Tumor diameters were measured twice a week and tumor volumes were calculated as described (16). Mice from each group were injected with corresponding concentrations of icariin daily. On day 27, all mice were sacrificed, and all tumors were dissected and
Icariin inhibited colony formation in both Daoy and D341 cells. A colony formation assay was performed to examine the anti-proliferative effects of icariin (0, 5, 10, and 20μM) on Daoy (A) and D341(B) cells. The mean colony sizes were smaller in Daoy (C) and D341 (E) cells. Total number of colonies was also observed to be less in a dose-dependent manner in both Daoy (D) and D341 cells (F). *p<0.05. **, p<0.01.

**Figure 2.** Icariin inhibited colony formation in both Daoy and D341 cells. A colony formation assay was performed to examine the anti-proliferative effects of icariin (0, 5, 10, and 20μM) on Daoy (A) and D341(B) cells. The mean colony sizes were smaller in Daoy (C) and D341 (E) cells. Total number of colonies was also observed to be less in a dose-dependent manner in both Daoy (D) and D341 cells (F). *p<0.05. **, p<0.01.

**Immunofluorescence assay**

Tissue from the tumor model was plated on coverslips, fixed with 4% paraformaldehyde(Sigma-Aldrich) for 10min, and permeabilized with 0.1% Triton X-100/PBS. Then, the slides were blocked with 1% BSA for 1 h, and incubated overnight at 4°C with the primary antibodies against PCNA and cleaved-caspase-3. Then, slides were incubated with FITC-conjugated secondary antibody (Beyotime) for 1h. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The fluorescence imaging was visualized using confocal laser scanning microscopy (Carl Zeiss, Oberkochen, Germany).

**Statistical analysis**

Data were obtained from at least three independent replicate experiments and expressed as mean± standard deviation(SD). The Student’s t-test was used to analyze the difference between two groups. P-values below 0.05 were considered statistically significant.

**Results**

**Icariin decreases viability of medulloblastoma cells in a dose- and time-dependent manner**

To investigate the effects of icariin on medulloblastoma cell proliferation and survival, we initially treated two medulloblastoma cell lines, Daoy and D341, with increasing concentrations of icariin (0 to 40μM/l) for 48h. We found that the icariin medium inhibited proliferation of both cell lines. Viability of both Daoy and D341 cells was suppressed in a dose-dependent manner (Figure 1A). We treated both Daoy and D341 cells with icariin (10μM/l, 10μM for short) for three consecutive days. Cell viability decreased overtime, and was inhibited by approximately 50% on day 3 for both cell lines (Figure 1B). These data suggest that icariin can inhibit medulloblastoma cell proliferation in a dose- and time-dependent manner.

**Icariin inhibited colony formation in both Daoy and D341 cells**

We further investigated the anti-proliferative effects of icariin on medulloblastoma cells Daoy (Figure 2A) and D341 (Figure 2B) by using a plate-well colony formation assay. Based on our observations in Figure 1, we treated both cell lines with 0, 5, 10, and 20μMicariin. In Daoy cells, the mean size of colonies formed by icariin-treated cells decreased in a dose-dependent manner (Figure 2C). Moreover, the mean number of colonies decreased (Figure 2D). We observed similar results for D341 cells (Figure 2E and 2F). These results supported the observations in Figure 1 and suggested that icariin can exert anti-proliferative effects in medulloblastoma cells.

**Icariin induces S-phase arrest and regulates cell cycle regulators in medulloblastoma cells**

In view of the icariin-mediated cell proliferation inhibition, cell cycle distribution was assessed using flow cytometry. Cell cycle progression was significantly inhibited in Daoy cells; cells prominently accumulated in S-phase and a decreased cell proportion in G1 phase was observed. Moreover, cells increasingly accumulated in S-phase with increasing concentration of icariin (Figure 3A). The cell cycle was also arrested in S-phase for D341 cells, with a prominent S-phase cell accumulation and a decreased proportion of G1-phase cells. The proportion of S-phase cells accordingly increased at increasing concentration of icariin (Figure 3B). Collectively, it can be concluded that cell cycle progression was inhibited by treatment with icariin in Daoy and D341 cells. The S-phase arrest was dose-dependent.

Cell growth primarily depends on cell cycle progression in mitosis and is regulated by cell cycle regulators, including cyclins and cyclin-dependent kinases (CDKs) (17,18). According to our observations above, we assessed whether key cell cycle regulators were altered in response to icariin treatments. As shown in Figure 3C, levels of cell cycle regulators cyclin B1, cyclin A, and CDK2 gradually decreased with increasing concentrations of icariin in both Daoy and D341 cells. These results were consistent with the observations obtained from flow cytometry. In our study, the cell cycle was significantly arrested in S-phase, indicating the dysregulation of G1/S transition. Dysregulation of G1/S transition may be attributed to the alterations of cyclin A and CDK2 expression, which is closely related to G1/S transition and S-phase (19). Concomitantly, the proportion of cells in G2-phase decreased, leading to alteration of cyclin B1, which is closely related with this phase (19). Overall, our results indicated that the cell cycle...
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arrest mirrored the cell growth inhibition.

Icariin induces cell apoptosis in Daoy and D341 cells

To further examine whether icariin induces apoptosis in Daoy and D341 cells, both cells were exposed to different concentrations of icariin (0, 5, 10, and 20μM) for 48h. Icariin treatment caused the proportion of cells in G1-phase to decrease, while increasing the proportion of cells in S-phase in both Daoy cells (A) and D341 cells (B). The S-phase arrest was even prominent with increasing icariin concentration. Further assessment of cell cycle regulators revealed that G1/S transition-related cyclin A and CDK2 and G2/M phase-related cyclin B1 were all altered in response to icariin treatment in both cell lines (C).

Cells in the Q3 quadrant (FITC−/PI−) were alive, whereas those in the Q4 (FITC+/PI−) and Q2 (FITC+/PI+) quadrants were early and late apoptotic cells, respectively (20). Our results showed that cell survival rate gradually decreased with increasing doses of icariin. Early apoptotic and late apoptotic cells increased in an icariin dose-dependent manner in Daoy cells (Figure 5B). Likewise, survival decreased with increased early and late cell apoptosis in D341 cells (Figure 5C). We then assessed the apoptosis-related proteins in both cell lines. It was shown that Bax, cleaved-caspase-3, cleaved-caspase-9, and cleaved-PARP were progressively upregulated with increasing doses of icariin. In contrast Bcl-2 was down regulated in both cell lines (Figure 5D). Bax, cleaved-caspase-3, cleaved-caspase-9, and cleaved-PARP are widely reported to be associated with pro-apoptotic activities, whereas Bcl-2 is considered the main anti-apoptotic factor (20,21). These results confirmed that icariin significantly induced apoptosis in Daoy and D341 cells.

Icariin inhibited tumor growth in a xenograft model of medulloblastoma

To investigate whether icariin could exhibit anticancer effects in vivo, we initially established a xenograft model of medulloblastoma with D341 cells. Mice from each group were periodically administered either of the two treatments (20mg/kg or 40mg/kg) or no treatment. Tumor size was measured twice a week. On day 27, mice were sacrificed and tumor weight was determined. It was shown that mice treated with 20mg/kg icariin exhibited overall smaller tumor sizes than control mice. Tumor sizes were even smaller in mice periodically treated with 40mg/kg icariin (Figure 6A). Mean tumor weight in the 20mg/kg group was only 55% of that in the control group. In addition, the mean tumor weight was further decreased, with a value of approximately 22% of that of the control mice (Figure 6B).
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Volume was also calculated from the periodic measurement of tumor dimensions. As shown in Figure 6C, tumor volume showed group-based differences on day 13. Tumors rapidly grew in the control group. However, the growth rate decreased in mice treated with 20mg/kg icariin, and even more so in mice treated with 40mg/kg icariin (Figure 6C). These results strongly suggest that treatment with icariin can suppress tumor growth in vivo.

Furthermore, we performed immunofluorescent analysis of proliferating cell nuclear antigen (PCNA) and cleaved-caspase-3 in the dissected tumor tissue. PCNA is a direct indicator of cell proliferation (22), whereas cleaved-caspase-3 indicates cell apoptosis (23). As shown in Figure 7A, PCNA was significantly suppressed in 20mg/kg- and 40mg/kg-treated tumor tissue. On the contrary, cleaved-caspase-3 increased in tumor tissue treated with icariin. Furthermore, we analyzed the main apoptosis-related proteins in these tumor tissues using western blotting. Consistent with the results in Figure 5C, our results showed that the anti-apoptotic factor Bcl-2 decreased in icariin-treated tumor tissue, whereas pro-apoptotic factors Bax, cleaved-caspase-3, cleaved-caspase-9, and cleaved-PARP increased in response to various concentrations of icariin (Figure 7B). These observations confirmed the anti-proliferative and pro-apoptotic effects of icariin in vivo.

Discussion

Medulloblastoma is the most common type of malignant brain tumor in children. It frequently causes cancer-related death in children (24). Despite recent advancements in understanding the molecular mechanisms underlying the prevalence of medulloblastoma and the availability of combined treatment with surgery, chemotherapy, and radiation, the 5-year survival rate for medulloblastoma patients is only 70-80%. Unfortunately, the survival rate of young children and infants with medulloblastomas is even lower (25). In addition, patients receiving the current treatments will experience inevitable side effects and lifelong consequences. Thus, it is necessary to find novel therapies that not only improve the cure rate, but also lack harmful side effects. Previous studies have widely reported that icariin, a natural product from Epimedium species, has anti-depressive, neuroprotective, and immunomodulatory activities (5-7), particularly in human tumorigenesis (3,8-11). The present study is the first to report that icariin can inhibit tumor growth in medulloblastoma, both in vitro and in vivo. Icariin inhibited cell proliferation in medulloblastoma by inducing S-phase arrest and eventually causing cell apoptosis.

Previously, icariin was shown to induce apoptosis in human hepatoma SMMC-7721 cells in a dose- and

![Figure 5. Icariin decreases cell survival and induces early/late apoptosis. (A) Annexin/PI staining was performed to assess living, early, and late apoptotic cells. Cell survival decreased, whereas early and late apoptosis increased in both Daoy cells (B) and D341 cells (C) with treatment with varying concentrations of icariin. (D) Apoptosis-related proteins were evaluated by western blotting. The expression of pro-apoptotic proteins Bax, cleaved-caspase-3, cleaved-caspase-9, and cleaved-PARP increased, whereas the expression level of anti-apoptotic protein Bcl-2 decreased with the dose of icariin. *p<0.05. **, p<0.01.](image)

![Figure 6. Icariin inhibits tumor growth in a xenograft model of medulloblastoma. The mice were then intraperitoneally administered 0.2 mL of vehicle (PBS) or icariin (20 and 40 mg/kg) every day for up to 4 weeks. (A) Compared to the control group, tumor sizes were visually smaller in the mice treated with 20mg/kg icariin, and even more so in mice treated with 40mg/kg icariin (Figure 6C). These results strongly suggest that treatment with icariin can suppress tumor growth in vivo.](image)

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*Figure 6.* Icariin inhibits tumor growth in a xenograft model of medulloblastoma. The mice were then intraperitoneally administered 0.2 mL of vehicle (PBS) or icariin (20 and 40 mg/kg) every day for up to 4 weeks. (A) Compared to the control group, tumor sizes were visually smaller in the mice treated with 20mg/kg icariin, and even more so in mice treated with 40mg/kg icariin. (B) Measurement of the mean tumor weight also showed that tumor weight was significantly decreased in mice treated with 20mg/kg icariin, and even more so in mice treated with 40mg/kg icariin. (C) Tumor volume showed group-based differences. Tumors grew at a high rate in the control group. However, tumor growth was slowed down in mice treated with 20mg/kg icariin, and even more so in mice treated with 40mg/kg icariin. *p<0.05. **, p<0.01.
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The present study, we found that the anticancer effect of icariin in human medulloblastoma was associated with apoptosis-related proteins and cell cycle regulators. Treatment of cells with icariin dose-dependently decreased the expression of cyclin A and CDK2, key regulators of G1/S transition and the S-phase (19) (Figure 3). The proportion of cells in G2-phase also decreased; this was further evident by the decreased cyclin B1 expression, which positively regulates G2-phase progression (19). Cell cycle arrest leads to eventual cell apoptosis (Figure 4). The expression of pro-apoptotic factors Bax, cleaved-caspase-3, cleaved-caspase-9, and cleaved-PARP was increased, whereas anti-apoptotic factor Bcl-2 expression decreased in response to increasing concentrations of icariin (Figure 5D and Figure 7B). Overall, we can conclude that the anticancer effects of icariin may be accomplished by modulating cell cycle progression and cell apoptosis-related proteins. Icariin exerts neuroprotective effects on corticosterone induced apoptosis in primary cultured rat hippocampal neurons (12). However, our results indicated that icariin induces apoptosis in human medulloblastoma cells. We speculate that icariin may have differential roles in neurons. Icariin may exert anti-apoptotic effects on normal neurons while exerting an opposite function on rapidly growing neurons.

In summary, we identified that icariin effectively induces apoptosis in medulloblastoma cells, likely by modulating the cell cycle and apoptotic pathways. All of the data above suggest that icariin is a promising drug for treating human medulloblastoma. However, further studies are required to understand the detailed mechanisms underlying the apoptosis and cell cycle arrest induced by icariin in medulloblastoma cells.

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