Icariin induces apoptosis in mouse MLTC-10 Leydig tumor cells through activation of the mitochondrial pathway and down-regulation of the expression of piwil4

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Abstract. The Leydig cell tumor, derived from interstitial cells, is a rare neoplasm. In most cases, Leydig cell tumors are benign, however, if the tumor is malignant, no effective treatments are currently available. In this study, we aimed to evaluate the effects of icariin on the growth of the mouse Leydig tumor cell line MLTC-1 and to examine its underlying mechanism. Icariin caused a dose-dependent decrease in the viability of MLTC-1 cells, which coincided with an increase in cell apoptosis through regulation of the expression of Bcl-2/Bax and cytochrome c; activation of caspase-9 and -3. Moreover, the pro-apoptotic effect of icariin on MLTC-1 cells is related to piwil4, since icariin induced a decrease in piwil4 protein expression and piwil4 silencing significantly enhanced the cytotoxic effects of icariin in MLTC-1 cells. These findings suggest a novel anticancer effect of icariin in Leydig cell tumor through activation of the mitochondrial pathway and down-regulation of the expression of piwil4.

Introduction

Testicular neoplasms include germ cell tumors and sex cord-stromal tumors. Germ cell tumors comprise 95% of all testicular neoplasms, while the remaining 5% of testicular tumors are sex cord-stromal tumors, which are derived from somatic cells, Sertoli and Leydig cells. The Leydig cell tumor is classified as an interstitial cell tumor, which are commonly benign; however, in adults, ~10% of cases are malignant (1-4). The etiology of Leydig cell tumor in humans remains unknown. Currently, in most standard therapy for benign Leydig cell tumors is considered to be orchiectomy. Recently, by retrospectively analyzing the long-term follow-up of a series of patients with Leydig cell tumors electively treated with testis sparing surgery, Glannarin et al (5) demonstrated that testis sparing surgery with frozen section examination has an excellent long-term oncological outcome. Therefore, testis-sparing surgery may be chosen for treating benign Leydig cell tumors to maintain fertility, especially in boys and young men (5-9). If the tumor is malignant, orchiectomy with retroperitoneal lymph node dissection is recommended, since chemotherapy and radiation therapy show limited efficacy in the treatment of malignant Leydig cell tumors. Survival time of patients with this malignancy range from 2 months to 17 years, and the mean survival time is 2 years (10,11). To improve the survival time of patients with malignant Leydig cell tumor, and to decrease the serious consequences, such as testicular dysfunction and infertility that is caused by the current treatment for benign Leydig cell tumors, alternative treatments need to be explored.

Icariin (C33H40O15; molecular weight, 676.67), a flavonoid isolated from Herba Epimedii, is considered to be the main active component responsible for the actions of Herba Epimedii. Many studies have shown that it has a wide range of pharmacological and biological activities on endocrine, cardiovascular, genital, bronchial, urinary and immune systems (12-15). In vitro studies have demonstrated that icariin possesses antitumor activity (16,17). Here we evaluated the effects of icariin on the growth of the mouse Leydig tumor cell line, MLTC-1 and examined its underlying mechanism.

Materials and methods

Materials. Icariin (purity >98%) was purchased from China's National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). RPMI-1640 and fetal bovine serum (FBS) were purchased from HyClone. Caspase-3, -8 and -9 detection kit, the Annexin V apoptosis detection kit, the protein extraction kit and the BeyoECL Plus Western blotting detection reagent were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The BLOCK-IT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP and Lipofectamine 2000 were purchased from Invitrogen (USA). Anti-piwil4 (sc-67593), caspase-9 and -3 inhibitor (DEVD-CHO and z-LEHD-fmk) were purchased from...
Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Bcl-2, anti-Bax, anti-cytochrome c and anti-β-actin antibodies were purchased from Bios (Beijing, China).

**Cell culture.** MLTC-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. All the cells were placed in an incubator, containing 95% air and 5% CO₂ at 37°C, and the media were replaced every 2 days.

**MTT assay.** The activity of icariin on cell proliferation was assessed by the MTT assay. In brief, MLTC-1 cells were plated in 96-multwell plates at a density of 5x10⁴ cells/well. After adherent, icariin at indicated doses was added to the wells and incubated for 24 or 48 h. At the indicated time after the treatment, the supernatant was removed and an MTT solution (5 mg/ml final concentration) was added. After 4 h incubation at 37°C, the MTT solution was removed and formazan was dissolved in DMSO. MTT reduction in living cells was quantified on a FACSVantage flow cytometer using the Annexin V Apoptosis Detection Kit. Briefly, MLTC-1 cells were treated with control medium or 50 µg/ml icariin for 24 or 48 h. PI vs. Annexin V-FITC gates of 100% surviving, and the number of living cells in the treated groups was expressed as a percentage of the control groups.

**Flow cytometry for cell apoptosis analysis.** MLTC-1 cells were treated with control medium or 50 µg/ml icariin for 24 or 48 h, then the cells were harvested for the Annexin V assay using the Annexin V Apoptosis Detection Kit. Briefly, treated cells were centrifuged for 10 min to remove the media. Then the cells were rinsed with 1X binding buffer supplied by the manufacturer. The rinsed cells were resuspended in 200 µl of binding buffer. Subsequently, 5 µl of Annexin V and 10 µl of PI were added and incubated at room temperature for 15 min. Flow cytometric analysis was carried out using a FACSVantage SE flow cytometer (Becton-Dickinson, USA).

**Statistical analysis.** Data are expressed as the mean ± SD. Statistical differences were evaluated by using ANOVA. The level for a statistically significant difference was set at P<0.05.

**Results**

**Effects of icariin on cell growth.** MLTC-1 cells were treated with 12.5, 25, 50 and 100 µg/ml of icariin for 24 or 48 h. The MTT assay results demonstrated that 12.5 µg/ml icariin did not significantly affect cell viability (P>0.05), while at concentrations above 12.5 µg/ml, the cell viability of MLTC-1 cells was inhibited in a time- and dose-dependent manner. As shown in Fig. 1, 24-h treatment with 25, 50 and 100 µg/ml icariin significantly reduced cell viability to 82.20±3.89% (P<0.05), 67.79±5.29% (P<0.01) and 55.93±6.72% (P<0.01), respectively. Treatment with 25, 50 and 100 µg/ml icariin after 48 h caused a reduction in cell viability to 65.93±5.72% (P<0.01), 48.90±5.30% (P<0.01) and 30.77±6.24% (P<0.01), respectively. The effective icariin concentration for 50% inhibition (EC50) of the MLTC-1 cell viability after 48 h was 50 µg/ml, which was thus the concentration chosen for the subsequent experiments.

**Icariin treatment (50 µg/ml) induces apoptosis in MLTC-1 cells.** To quantitatively assess the effects of 50 µg/ml icariin on MLTC-1 cells, MLTC-1 cells were treated with medium (control) or with 50 µg/ml icariin for 24 or 48 h. PI vs. Annexin V-FITC
fluorescence staining was then measured by flow cytometric analysis (Fig. 2). In the 24 h (Fig. 2A) and 48 h (Fig. 2C) vehicle-treated control group, 7.6±0.6% and 8.2±0.56% cells excluded PI and were positive for Annexin V-FITC binding, which represent the apoptotic cells. After exposure to 50 µg/ml icariin for 24 h (Fig. 2B) and 48 h (Fig. 2D), the percentage of apoptosis increased to 17.1±1.5% (P<0.01) and 24.7±1.6% (P<0.01), respectively.

Icariin affects the cell cycle progression of MLTC-1 cells. We determined the cell cycle phases in the cells with or without 50 µg/ml icariin treatment for 24 or 48 h by flow cytometric analysis (Fig. 3). The percentage of cells in the G1 and G2 phase after 24 or 48 h of icariin treatment was similar to that of the control groups (P>0.05). However, compared with
Figure 3. Effect of icariin on the cell cycle distribution of MLTC-1 cells. Representative flow cytometric analysis plot of the (A) 24-h control, (B) 50 µg/ml icariin for 24 h, (C) 48-h control and (D) 50 µg/ml icariin for 48 h treatment groups. Cells were fixed with 70% ethanol and stained with PI, followed by flow cytometric analysis. (E) Percentage of cells in the indicated phases of the cell cycle after 50 µg/ml icariin-treatment as determined by flow cytometry. The data are expressed as the means ± SD (n=3). *P<0.05, compared to the 24-h control group; **P<0.05, compared to the 48-h control group.

Figure 4. The expression of Bcl-2, Bax and cytochrome c (Cyt-C) in icariin-stimulated MLTC-1 cells. Cells were treated without or with 50 µg/ml icariin for 24 and 48 h, respectively. Bcl-2, Bax and cytochrome c-specific bands were detected by Western blotting. (A) Representative Western blotting of Bcl-2, Bax and cytochrome c. (B) Relative expression of Bcl-2, Bax and cytochrome c in different groups by normalizing to β-actin protein level. Protein levels of the control (24 h) group were set as 1. Values represent the means ± SD (n=3). *P<0.01, compared to the 24 h control group; **P<0.01, compared to the 48 h control group.
the control group, the percentage of cells in the S phase was slightly decreased by 50 µg/ml icariin after 24 (13.04±0.89% vs. 16.68±1.75%, P<0.05) or 48 h (11.57±0.81% vs. 17.16±3.26%, P<0.05) of treatment.

Icariin induces an alteration in Bcl-2, Bax and cytochrome c expression in MLTC-1 cells. Because of the essential role of the Bcl-2 and Bax proteins in the regulation of apoptosis, we examined the effects of icariin on the expression of these regulatory factors. As shown in Fig. 4, 50 µg/ml icariin treatment decreased the expression of the apoptosis inhibitory protein Bcl-2, while it increased the expression of the pro-apoptotic protein Bax. Also, 50 µg/ml icariin treatment, especially at 48 h, induced a significant increase in the release of the mitochondrial activator of apoptosis, cytochrome c.

Icariin induces differential caspases activation related to the apoptosis in MLTC-1 cells. The activity of caspases-3, -8 and -9 in the different groups was detected to investigate whether caspase proteins were activated by icariin in MLTC-1 cells. As shown in Fig. 5, 50 µg/ml icariin treatment activated the expression of both caspase-9 and caspase-3, while not of caspase-8. Pretreatment of MLTC-1 cells with the caspase-3 inhibitor DEVD-CHO (25 µmol/l) only inhibited icariin-induced activation of caspase-3, while, pretreatment of caspase-9 inhibitor z-LEHD-fmk (25 µmol/l) inhibited both caspase-3 and -9 activity. Values are means ± SD (n=3). *P<0.01, compared to 24 h control group; **P<0.01, compared to 48 h control group.

Piwil4 is involved in icariin-induced apoptosis in MLTC-1 cells. To further investigate whether piwil4 is related to icariin-induced apoptosis, the piwil4-pcDNA™ 6.2-GW/EmGFP miR (piwil4 miR RNAi) vector was constructed to down-regulate the expression of piwil4 in MLTC-1 cells. Compared to the negative control vector, piwil4 miR RNAi vector reduced piwil4 protein levels to 23±4.5% (Fig. 7), suggesting that piwil4 miR RNAi vector effectively inhibited piwil4 expression in MLTC-1 cells.

Subsequently, the effect of piwil4 silencing on the 50 µg/ml icariin-induced alterations of cell viability and cell apoptosis were assessed by MTT and flow cytometry analysis, respectively. Compared to parental MLTC-1 cells or MLTC-NC cells, piwil4 silencing exhibited no direct effect on cell viability (P>0.05). However, on the condition of icariin stimulation, a decrease in cell viability was observed (P<0.05). Similarly, piwil4 silencing showed no effect on cell apoptosis compared...
to parental MLTC-1 cells or MLTC-NC cells (data not shown), but enhanced the pro-apoptosis effect induced by icariin (Fig. 8B and C). These results indicate that piwil4 plays a role in the icariin-induced cytotoxicity in MLTC-1 cells.

Discussion

Several studies have been performed to explore new treatments for Leydig tumors, such as imatinib and cordycepin. Imatinib,
Cordycepin, a constituent from the mycelia of *Cordyceps sinensis* (CS), is considered an active component (CS), is considered an active component with antitumor effects in mouse melanoma and lung carcinoma cells and in human oral cancer cells (21,22). In addition, Jen et al have demonstrated that cordycepin exhibited an antitumor effect in Leydig cell tumors (23).

In this study, we showed that icariin could inhibit the growth of MLTC-1 Leydig tumor cells in vitro. A cell proliferation assay indicated that icariin in a concentration range from 25 to 100 μg/ml had time- and dose-dependent growth inhibitory effects on MLTC-1 cells. The decrease in cell proliferation may be due to an increase in cell apoptosis. In line with this expectation, compared to controls, the percentage of early- and late-apoptotic cells increased to 17.1±1.5% and 24.7±1.6% by exposure to 50 μg/ml icariin for 24 and 48 h, respectively.

Previous studies have shown an association between cell progression and cancer, and targeting the cell cycle has become an appreciated strategy for tumor treatment (24,25). The cell cycle profile was analyzed in MLTC-1 cells with icariin treatment. The percentage of cells in the S phase was slightly decreased with 50 μg/ml icariin stimulation for 24 or 48 h, while alterations of the G0/G1 and G2/M cell populations showed no significant differences between the icariin-treated and the control groups. These results demonstrate that icariin causes an alteration in cell cycle progression of MLTC-1 cells. Collectively considering the results from cell apoptosis and cell cycle assays, it was speculated that the inhibition of tumor cell proliferation by icariin was mainly due to an increase in cell apoptosis.

The proteins of the Bcl-2 family, including Bcl-2 and Bax, are key regulators of apoptosis and may act as balancing factors for the cell apoptotic program (26). Bcl-2 related proteins are primarily localized in the outer mitochondrial nuclear membranes and the endoplasmic reticulum (27). Bcl-2 may function as a protective factor against apoptosis triggered by cytochrome c release from the mitochondria. Bax, a pro-apoptotic protein, is located in the cytosol or loosely attached to membranes. In response to apoptotic stimulus, Bax translocates form the cytosol to the mitochondria, resulting in cytochrome c release and caspases activation (28,29). The Bcl-2 and Bax proteins are sensitive to apoptotic agents, and the ratio of Bcl-2 and Bax is considered to be a good predictive marker for cell will survival or death (30). Here, results of Western blotting indicated that icariin lowered the Bcl-2/Bax protein expression ratio, implying that the antitumor effects of icariin are elicited through regulation of the expression of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax. In addition, accompanying the Bax expression increase, cytochrome c was elevated by icariin stimulation, which further confirms the role of Bcl-2/Bax expression in the icariin-induced MLTC-1 cell apoptosis.

Caspases, the important members of the apoptotic machinery, are a large family of proteases, including the upstream initiators (caspase-2, -8, -9, -10, -12) and the downstream effectors (caspase-3, -6, -7) (28). Among these caspases, caspase-8 is activated in death receptor-mediated apoptosis, while caspase-9, as the upstream caspase in the cytochrome c pathway for apoptosis, is triggered by the release of cytochrome c from mitochondria (31).

In order to examine the important roles of caspase-8 and -9 in apoptosis as described above, we next evaluated whether caspase-8, -9 as well as the downstream effector, caspase-3 are involved in the apoptotic effects of icariin. It is evident that icariin treatment resulted in activation of caspase-9 and -3, other than caspase-8. To further confirm the change of caspase-9 and -3 in the icariin-treated MLTC-1 cells, the caspase-9 or -3 inhibitor was added to the media. The results demonstrate that the caspase-3 inhibitor only inhibited icariin-induced activation of caspase-3, while the caspase-9 inhibitor inhibited both caspase-3 and -9 activity, which indicated that the effector caspase-3 is triggered by the upstream molecule, caspase-9. These results suggest that icariin can induce apoptosis by activating caspase-9, and the downstream effector, caspase-3.

Piwi4 belongs to the Ago/Piwi family, which is composed of two conserved domains, PAZ and PIWI. The Ago/PIWI proteins have been shown to interact with the ribonuclease (RNase) III enzyme Dicer that catalyzes the maturation of miRNA and siRNA. Within the family, four members of the PIWI-like family, PIWIL1, PIWIL2, PIWIL3 and PIWIL4, have been identified in humans in previous reports, while in the mouse, there are only three Piwi subfamily genes, piwi1, piwi2 and piwi4 (32-35). In regards to piwi4 and its tissue distribution, research has mainly focused on its role in spermatogenesis (36-38).

We find that piwi4 is expressed in MLTC-1 cells, and interestingly, MLTC-1 cell apoptosis induced by icariin is related to piwi4. Western blotting results illustrate that the relative expression of piwi4 protein was decreased by treatment with 50 μg/ml icariin in a time-dependent manner. To examine whether piwi4 is involved in icariin-induced apoptosis, we used miRNAi vector to down-regulate the expression of piwi4 in MLTC-1 cells. MTT and flow cytometry results demonstrate that piwi4 silencing enlarged the effect of icariin on cell viability and cell apoptosis, which indicated that piwi4 may be an anti-apoptotic factor, while icariin could exert its antitumor activity by down-regulating piwi4 expression.

In summary, we have shown that icariin has pro-apoptotic activity in MLTC-1 cells by regulating the expression of Bcl-2/Bax, the release of cytochrome c and the activity of caspase-9 and -3. Additionally, piwi4, an important regulator of spermatogenesis, is involved in the icariin-induced cell apoptosis. In reference to its antitumor activity, icariin may be a therapeutic candidate on testicular Leydig cell tumors.

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References


