Anti-Proliferative Efficacy of Icariin on HepG2 Hepatoma and Its Possible Mechanism of Action

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Abstract: The aim of the present work was to explore the anti-hepatoma effects of icariin both in vitro and in vivo and to elucidate its potential mechanism of action. The MTT assay was applied to test the anti-proliferative effects of icariin in vitro. HepG2 bearing NMRI nu/nu mice were used to test the anticancer effects of icariin in vivo. Immunohistochemical assay and flow cytometry assay (FACS) were applied to detect the possible mechanisms of action of icariin. MTT assay illustrated that icariin inhibited the proliferation of HepG2 cells in a concentration dependent manner; meanwhile, icariin inhibited the tumor growth in HepG2 bearing NMRI nu/nu mice. The tumor weight was inhibited by 55.6% and tumor volume was inhibited by 47.2%. Icariin did not influence the spleen and body weights or blood parameters. Immunohistochemical analysis indicated that the expressions of both CD31 and Ki67 in the icariin treated group were significantly lower than those in the control group (p < 0.01). FACS assay showed that icariin dramatically decreased the percentage of CD4+ and CD8+ cells in bone marrow and CD19+ cells in blood on day 8. On day 17, the percentage of CD8+ cells in blood was lower than those in the control group. CD4/CD8 ratio in icariin group was significantly elevated in bone marrow on day 17. Icariin showed anticancer efficacy both in vitro and in vivo. The possible mechanism of action could be related to its anti-angiogenesis and anti-proliferative effects in tumors.

Keywords: Icariin; HepG2; CD31; Ki67; Flow Cytometry; MTT Assay; Immunohistochemical Assay.
Introduction

Hepatocellular carcinoma (HCC) is a primary cancer of the liver that is predominant in developing countries, with nearly 600,000 deaths each year worldwide (El-Serag et al., 2007). HCC shows great geographical variation, with a very high incidence in Asia and sub-Saharan Africa. Although the incidence is far lower in the US and Europe, the rates have been increasing in recent years (Lau and Lai, 2008). It is the fifth most common cancer in the world, and is the third highest cause of cancer-related mortality (Masuzaki and Omata, 2008). For years, partial hepatectomy and liver transplantation have been considered as the main curative treatments. Currently, these therapy methods compete with local ablative therapy as the first-line treatment option for small HCCs in patients with well-preserved liver function. Various locoregional therapies are used for patients who are not candidates for surgical cure because of severity of liver disease or advanced stage of the HCC. These modalities, which include local ablative therapy [radiofrequency ablation (RFA), microwave coagulation therapy (MCT) or percutaneous ethanol injection (PEI)], transarterial techniques [transarterial embolization (TAE), transarterial chemotherapy (TAC), transarterial chemoembolization (TACE), transarterial radioembolization (TARE)], and some forms of extracorporeal energy therapy (Lau and Lai, 2008). The goal of management is “cancer control” — a reduction in its incidence and mortality as well as an improvement in the life quality of patients with HCC and their families (Lau and Lai, 2008).

Icariin, 5-hydroxy 4′-methoxy 8-isopentenyl 3-O-α-rhamnosyl 7-O-β-glucosyl flavone (Fig. 1), is the major component in Herba epimedii, which is widely used in Chinese traditional medicine. Icariin possesses many biological effects. It has cardiovascular effects (Xu and Huang, 2007) and can protect hypoxia injured vascular endothelial cells (Ji et al., 2005). Icariin has also been reported to be protective on cerebral ischemia/reperfusion injured neurons (Li et al., 2005). What’s more, it also has testosterone mimetic properties and therapeutic potential (Zhang and Yang, 2006) and has a therapeutic effect on erectile dysfunction (Liu et al., 2005). Besides, icariin possesses potent antidepressant-like activities which are at least in part mediated by improving the abnormalities in the hypothalamic-pituitary-adrenal axis functions (Pan et al., 2007). Additionally, the bone-strengthening effects of icariin have
been documented in several studies (Chen et al., 2007; Zhang et al., 2008). Furthermore, icariin is also a concentration-dependent chemopreventor in protecting DNA against radical-induced oxidative damage (Zhao et al., 2007). As for its anticancer effects, studies in human hepatoma HepG2 (Wang et al., 2007) and human prostate carcinoma PC-3 cell line in vitro (Huang, et al., 2007), icariin has proved to inhibit cancer growth. Thus, in this study, the effect of icariin on HepG2 cells was studied both in vitro and in vivo to confirm its anticancer function.

Materials and Methods

Materials

Icariin was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products. DMEM medium and fetal calf serum were obtained from Gibco (USA). MTT and DMSO were purchased from Sigma (USA). Rat anti-mouse CD31 monoclonal antibody was purchased from BD Pharmingen (USA), goat anti-rat HRP-conjugated antibody from Southern Biotech (USA). Ki67 immunohistochemistry kit was obtained from Dako (DK). FITC rat anti-mouse CD4, PE rat anti-mouse CD8a and APC rat anti-mouse CD19 antibodies were all purchased from BD Pharmingen (USA).

Animals

BDF1 mice (Charles River, Sulzfeld, Germany) and NMRI:nu/nu mice (in-house breeding of EPO-GmbH, Germany) received a standard pellet diet (Sniff, Soest, Germany) and water ad libitum. They were maintained under standard environmental conditions (20–25°C, 12 hours of light/dark cycle). All animal experiments were performed according to the German animal protection law with approval by the local responsible authorities.

MTT Assay

HepG2 cells were passaged regularly. 2.78 × 10⁴ tumor cells/ml were incubated with DMEM medium containing 10% of fetal calf serum. The cells were seeded in 96-well plates, 5,000 cells in each well. The plates were incubated with humidified atmosphere, 37°C 50 ml/L CO₂ for 24 hours. Icariin was first dissolved in DMSO (final concentration below 1%), PBS was used to adjust the icariin concentrations. Afterwards, the corresponding icariin solutions were added to the wells and the final concentrations were 0.1, 1, 10, 100 and 1000 µg/ml. The plates were kept in the incubator for 72 hours. After incubation, 25 µl of 5 mg/ml MTT solution (5 mg/ml, freshly made) was added to each well. After 4 hours incubation, the plates were centrifuged to discard the supernatant, 200 µl of DMSO was added to dissolve the sediments. The plates were read with the Sunrise ELISA reader (version 4.51), at 570 nm wave length. The experiment was repeated three times. All IC50 values were graphically determined.
Anti-Cancer Experiment

Seventeen male NMRI nu/nu mice were injected with $10^7$ of HepG2 cells using matrigel subcutaneously, and then randomly divided into two groups, 6 mice in the control group, 11 mice in the icariin group. The treatment started on the following day. The mice in the control group were given PBS intragastrically (i.g.), while the mice in the icariin group were given 80 mg/kg of icariin solubilised in PBS with 10% tween 80. Blood parameters were tested on the day 7 and day 35. The tumor volume was measured twice per week. All the treatment lasted for 35 days. Tumor growth inhibition is expressed as percentage of treated/control values. The mice were sacrificed on day 35. Tumors were stripped, weighed, and then snap frozen for further study. Spleens were also stripped and weighed in the same way as the tumors.

Immunohistochemical Analysis of CD31

Tumors were cut into 5 µm slices and kept in a $-20^\circ$C freezer for use. Sections were dried for 10 min, and fixed with 4% paraformaldehyde for 15 min, then washed with PBS for 10 min. Peroxidase (Dako) was used for blocking 5 min at room temperature (RT), and 20% goat serum for 30 min at RT in a humid chamber (HC). The primary rat-anti-mouse CD31 monoclonal antibody (1:100 in antibody dilution media) was added for 2 hours at RT in HC. After washing, secondary goat-anti-rat HRP-conjugated antibody (1:100 in PBS Southern Biotech) was added, 30 min at RT in HC, followed by DAB-substrate (Dako) 1 drop reagent in 2 ml buffer for 3 min. The slides were rinsed with water for 5 min, and counterstained with hematoxylin. From each slide, 6 pictures were taken ($100\times$ magnification). The Axiovision software was applied to mark the positively stained parts to evaluate the total area.

Immunohistochemical Analysis of Ki67

The determination of Ki67 positive cells was performed according to the protocol of the Ki67 immunohistochemistry kit. From each slide, 10 pictures were taken ($400\times$ magnification). The Axiovision software was applied to randomly mark three areas in a picture and to count Ki67 positive and negative cells. The average rate of Ki67 positive cells in one tumor sample was calculated.

FACS Assay of CD4, CD8 and CD19 in Blood, Spleen and Bone Marrow

Thirty BDF1 mice were randomly divided into the control group and the icariin group with 15 mice each. Mice in the control group were injected i.g with 0.2 ml of saline. Mice in the icariin group were treated once daily with icariin, 80 mg/kg. The treatment lasted for 7 days. On the 8th, 13th and 17th day, blood was taken from the retro orbital plexus of mice to mini-collectors containing heparin, 5 mice per group. The mice were then sacrificed; spleens and thigh-bones were stripped out. Spleens were immediately smashed with cold PBS gently and pressed through a cell sieve (100 µm pore size). Thigh-bones were flushed
with 1 ml of cold PBS to take the bone marrow out. The blood, spleen and bone marrow cells were counted by Beckman Coulter, and adjusted to the concentration of $5 \times 10^6$/ml. Three color immunofluorescence staining protocol was applied in BD FACSCalibur flow cytometer. Sixty to 120 $\mu$l of blood, spleen cells solution and bone marrow solution were taken out separately in the FACS-round-bottom-tubes. The three corresponding antibodies, CD4 $1–2 \mu$l, CD8a $2 \mu$l, CD19 $2 \mu$l were added. Blood tubes were kept at room temperature in the dark for 15 min. Spleen and bone marrow tubes were kept in the refrigerator ($4^\circ$C) for 25 min. Two ml of diluted FACS lysis solution was added to blood tubes to destroy erythrocytes and the tubes were incubated for 10 min at room temperature, then centrifuged at 1000 r/min 5 min at $4^\circ$C, after that, washed with 3 ml of FACS buffer (PBS $+2\%$ FBS) and centrifuged at the same condition. For spleen and bone marrow tubes, after incubation with antibodies, 3 ml FACS buffer (PBS $+2\%$ FBS) was immediately added. The supernatant was removed and the tubes were analyzed. Cell quest software was applied to analyze the data. Histo-plot statistical assay was used for calculation.

Statistical Analysis

SPSS software, version 11.5 was applied to analyze the data. The data were expressed in the form of mean $\pm$ SD or percentage, the comparison between groups using t-tests or analysis of variance (ANOVA) or Wilcoxon tests where appropriate. $p \leq 0.05$ was considered statistically significant. All the data were analyzed by the software SPSS, version 11.5.

Results

MTT Assay

Figure 2 shows that icariin inhibited the proliferation of HepG2 cells in vitro in a dose dependent manner. According to the graph, the IC50 concentration was around 100 $\mu$g/ml.

Anti-Tumor Effect in Vivo

Icariin inhibited the tumor growth in HepG2 bearing NMRI nu/nu mice. The tumor weight inhibition rate was 55.6% (Table 1) and the tumor volume inhibition was 47.2%, and there is no significant difference in the volume of tumors between the two groups (Fig. 3). Icariin did not significantly influence the body weight and weight of spleens, as shown in Table 1.

Icariin did not significantly influence blood parameters, such as leucocytes, erythrocytes and thrombocytes in nude mice even after 7 or 35 days of the treatment (Table 2).

Immunohistochemical Assay of CD31 and Ki67

Immunohistochemical study indicated that the CD31 protein positive area percentage, and the Ki67 positive cells percentage in icariin treated group were both significantly ($p < 0.01$) lower than in those in the control group (Table 1, Figs. 4 and 5).
Figure 2. The results of MTT assay. Icariin inhibited the proliferation of HepG2 cells in a dose dependent manner. The IC50 concentration was around 100 µg/ml according to the graph.

Figure 3. Tumor volume changes in the control and the icariin groups. There is no significant difference in the volume of tumors between two groups. There is a significant difference in the tendency of the two curves, $F = 7.83$, $p = 0.0059$.

**FACS Assay**

On the 8th day, the CD4 and CD8 positive cells in the bone marrow of the icariin group were significantly lower than in the control group ($p < 0.05$). The CD19+ cells on the 8th day in blood were also conspicuously lower than those in the control group ($p < 0.05$). On the 17th day, the CD8+ cells in blood were much lower than those in the control group ($p < 0.05$, Table 3).
Table 1. The Effects of Icariin on the Anti-HepG2

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Organ Weight</th>
<th>Tumor Weight</th>
<th>Spleen (g)</th>
<th>Tumor (g)</th>
<th>Body Weight (g)</th>
<th>Tumor Inhibition (%)</th>
<th>CD31 Positive Area (%)</th>
<th>Ki67 Positive Cells Ration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0.103 ± 0.011</td>
<td>0.287 ± 0.208</td>
<td>30.3 ± 3.7</td>
<td>4.75 ± 1.51</td>
<td>93.4 ± 1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Icariin</td>
<td>11</td>
<td>0.111 ± 0.015</td>
<td>0.128 ± 0.128</td>
<td>30.1 ± 2.6</td>
<td>1.35 ± 0.61*</td>
<td>63.8 ± 14.3*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For the histochemistry assay of CD31 and Ki67, 5 tumor samples in control group and 9 tumor samples in icariin group were analyzed. Compared to the control group, *p < 0.01.

Table 2. Blood Parameters of the Mice in Two Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Dose (mg/kg/d)</th>
<th>WBC day 7th (10^6/ml)</th>
<th>WBC day 35th (10^6/ml)</th>
<th>RBC day 7th (10^9/ml)</th>
<th>RBC day 35th (10^9/ml)</th>
<th>PLT day 7th (10^6/ml)</th>
<th>PLT day 35th (10^6/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>9.8 ± 1.7</td>
<td>6.8 ± 0.9</td>
<td>9.4 ± 0.8</td>
<td>9.2 ± 0.6</td>
<td>1238.4 ± 89.5</td>
<td>1162.0 ± 429.3</td>
<td></td>
</tr>
<tr>
<td>Icariin</td>
<td>10</td>
<td>8.7 ± 3.3</td>
<td>6.4 ± 2.2</td>
<td>9.3 ± 0.4</td>
<td>9.5 ± 0.4</td>
<td>1428.9 ± 155.4*</td>
<td>1303.8 ± 138.2</td>
<td></td>
</tr>
</tbody>
</table>

In blood parameter tests, 5 mice in group A were tested, 10 mice in group B were tested. Compared to the control group, *p < 0.05.
Figure 4. Immunohistochemical analyses of CD31. The brown stain in the pictures indicated the CD31 positive section. (A) and (B) are pictures of the control group and (C) and (D) are pictures of the icariin group. The expression of CD31 protein in icariin group was significantly lower than that in the control group, \( p < 0.01 \).

Compared to the control group, CD4/CD8 ratio in the icariin group was significantly elevated in bone marrow on the 17th day \( (p < 0.05, \text{Table 4}) \).

Discussion

Icarin is the major component in Herba epimedii, and possesses many bioactivities. Our studies showed that it can inhibit the growth of HepG2 cells \textit{in vitro}, which was in coincidence with the results of our previous study (Wang \textit{et al.}, 2007).

\textit{In vivo}, icariin displayed tumor inhibition efficacy both on tumor weight and tumor volume in our study. No obvious side effects of icariin were registered; the body weight, spleen weight and blood parameters were all shown to be normal.

Angiogenesis is crucial for the growth of solid tumor (Ziche and Gullino, 1982; Folkman, 1990). Microvascular density in primary tumors is closely associated with metastasis and prognosis in several tumors and is most predictive in tumors that induce significant angiogenesis (Nico \textit{et al.}, 2008). CD31, also known as PECAM-1 or endocam, has long been considered as a marker of blood vessels (Albelda \textit{et al.}, 1991). CD31 is a pan-endothelial marker, present in blood vessels and lymphatics, albeit at a lower level of expression on
Figure 5. Immunohistochemical analyses of Ki67. Ki67 positive cells are stained with dark brown color in nucleolus. (A) and (B) are representative pictures of the control group and (C) and (D) of the icariin group. The Ki67 positive cell percentage in icariin treated group was significantly lower than that in control group, $p < 0.01$.

lymphatic endothelial cells (Baluk et al., 2007). CD31 is specifically expressed on the surface of endothelial cells and is weakly expressed on lymphoid cells and platelets (Attoub et al., 2008). We have analyzed the effect of icariin on tumor angiogenesis in vivo by assessing the levels of CD31 expression through immunohistochemical assay. There was an obvious decrease in the mean positive area of CD31 in the icariin treated group in comparison with the control group. Thus, the inhibition of HepG2 tumor growth by icariin appears to be related to the anti-angiogenesis effect of tumors.

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle [G(1), S, G(2), and mitosis], but is absent from resting cells [G(0)], makes it an excellent marker for determining the so-called growth fraction of a given cell population (Scholzen and Gerdes, 2000). We have analyzed the effect of icariin on tumor-cell proliferation in vivo by assessing the levels of the nuclear antigen Ki-67. There was a significant decrease in the mean number of Ki-67-positive cells in the icariin treated group in comparison with the control group. Thus, the
Table 3. Percentages of CD4, CD8, CD19 Positive Cells in Blood, Spleen and Bone Marrow of BDF1 Mice

<table>
<thead>
<tr>
<th>Sample</th>
<th>Group</th>
<th>CD4+ (%)</th>
<th>CD8+ (%)</th>
<th>CD19+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 d</td>
<td>13 d</td>
<td>17 d</td>
<td>8 d</td>
</tr>
<tr>
<td></td>
<td>Icarin</td>
<td>11.53 ± 2.11</td>
<td>13.66 ± 3.28</td>
<td>10.89 ± 2.81</td>
</tr>
<tr>
<td>Spleen</td>
<td>Control</td>
<td>19.63 ± 1.81</td>
<td>16.80 ± 1.91</td>
<td>13.05 ± 2.78</td>
</tr>
<tr>
<td></td>
<td>Icarin</td>
<td>18.28 ± 1.73</td>
<td>16.18 ± 2.02</td>
<td>12.57 ± 0.91</td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>Control</td>
<td>5.78 ± 1.47</td>
<td>7.43 ± 1.28</td>
<td>3.77 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>Icarin</td>
<td>4.00 ± 0.14*</td>
<td>8.97 ± 2.81</td>
<td>5.06 ± 0.78</td>
</tr>
</tbody>
</table>

n = 5, compared to the control group, *p < 0.05.

Table 4. CD4/CD8 Ratio in Blood, Spleen and Bone Marrow

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood CD4/CD8</th>
<th>Spleen CD4/CD8</th>
<th>Bone marrow CD4/CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 d</td>
<td>13 d</td>
<td>17 d</td>
</tr>
<tr>
<td>Control</td>
<td>1.42 ± 0.17</td>
<td>1.45 ± 0.14</td>
<td>1.30 ± 0.37</td>
</tr>
<tr>
<td>Icarin</td>
<td>1.30 ± 0.12</td>
<td>1.23 ± 0.13</td>
<td>1.36 ± 0.17</td>
</tr>
</tbody>
</table>

n = 5, compared to the control group, *p < 0.01.
inhibition of tumor growth by icariin appears to be due to, at least in part, a direct effect on the proliferation of tumor cells.

Tumor-antigen-specific CD4+ T cells have the ability to effectively eradicate established tumors. CD8+ T cells also play a central role in the host response to cancers (Appay et al., 2008). The role of CD8+ cells, in tumor clearance is well established and considered to be largely dependent on the production of IFN-γ and perforin-mediated or Fas-mediated cytotoxic activity (Dobrzanski et al., 1999). CD19 serves as a major positive response regulator in B cells (Wicks et al., 2007), and CD19 expression is restricted to the B cell lineage and follicular dendritic cells (Fujimoto and Sato, 2007). The main function of B lineage cells is to secrete antibodies in the blood and other body fluids making them inhospitable to foreign invaders. B cells also play two additional roles in the immune system. First, they can act as antigen-presenting cells to T lymphocytes and secondly, activated B cells can secrete certain lymphokines and other factors that influence the growth and activities of other immunologically important cells (Adnan et al., 2008). Our FACS analyses showed that icariin significantly decreased the CD4+ and CD8+ cells in bone marrow and CD19+ cells in blood on day 8. On day 17, the CD8+ cells in blood of the icariin treated group were lower than those in the control group. CD4/CD8 ratio thus in the icariin group was significantly elevated in bone marrow on day 17. The results suggested that icariin had no constant and remarkable influence on the CD4, CD8 and CD19 cells, in other words, its immune promoting effect should be further evaluated.

In summary, icariin showed anti-hepatoma efficacy both in vitro and in vivo, and no obvious side effects were detected. The possible anticancer mechanism can be related to its anti-angiogenesis effects of tumors and anti-proliferating effects on the tumor cells.

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References


Anti-hepatoma effect of icariin


