Formononetin Induces Apoptosis of Human Osteosarcoma Cell Line U2OS by Regulating the Expression of Bcl-2, Bax and MiR-375 In Vitro and In Vivo

Wei Hu   ZengMing Xiao

Spine and Osteopathy Ward, The First Affiliated Hospital of Guangxi Medical University, Nanning, China

Key Words
Formononetin • Bax • Bcl-2 • U2OS • MiR-375

Abstract
Background/Aims: Phytoestrogens are known to prevent tumor progression by inhibiting proliferation and inducing apoptosis in cancer cells. Formononetin is one of the main components of red clover plants, and is considered as a typical phytoestrogen. This study investigates formononetin induction of apoptosis of human osteosarcoma cell line U2OS by regulating Bcl-2 and Bax expression in vitro and in vivo. Methods: U2OS cells were treated with different concentrations of formononetin and the proliferation of the cells was measured using an MTT assay. Cell apoptosis was examined by flow cytometry. The levels of miR-375, Bax and Bcl-2 protein expression in treated cells were determined by Western blot and RT-PCR. The antitumor activity of formononetin was also evaluated in vivo in nude mice bearing orthotopic tumor implants. Results: High concentrations of formononetin significantly suppress the proliferation of U2OS cells and induce cell apoptosis. Moreover, compared to control group the expression of Bcl-2 and miR-375 decreases with formononetin in the U2OS cells, while Bax increases. Conclusion: Formononetin has inhibitory effects on the proliferation of U2OS cells, both in vitro and in vivo. This antitumor effect is directly correlated with formononetin concentration.

Introduction

Osteosarcoma, the most common non-hematologic primary malignant neoplasm of the bone, is characterized by the development of bone or osteoid substance by the tumor cells [1]. The disease is mainly found in young patients between 10 and 25 years old, and it is one of the most frequent causes of cancer-related deaths in childhood [2]. The 5-year survival rate for patients with non-metastatic OS is 70% after surgery-based adjuvant chemotherapy [3].
Phytoestrogens, sometimes called dietary estrogens, are a diverse group of naturally occurring non-steroidal plant compounds that have been proposed as the natural alternatives to estrogen. They are divided into four main classes: isoflavones, stilbenes, lignans and coumestans. Both 5, 7-dihydroxy-4′-methoxyisoflavones and formononetin are isoflavones.

Plant-derived phytoestrogens display estrogentic properties due to the similar molecular structures between phytoestrogens and estrogens binding to the estrogen receptor (ER) [4]. There is also evidence to suggest that osteosarcoma is an ER-positive cancer and that phytoestrogens can mediate estrogen-like effects in human osteosarcoma cells [5].

We previously observed that low concentrations of phytoestrogens acted solely as estrogen agonists, while at higher concentrations they had multiple biological effects [6-8]. A recent study showed that apoptosis was induced in human osteosarcoma cells by 5, 7-dihydroxy-4′-methoxyisoflavones. The study demonstrated a dose-dependent inhibition of proliferation and induction of apoptosis of U2OS cells, as well as down regulation of Bcl-2, up regulation of Bax and decreased expression of miR-375.

Materials and Methods

Drugs and animals
Formononetin was dissolved in dimethyl sulfoxide to make a 100 mM stock solution and stored at 4°C for later use. Nude mice were housed in a specific-pathogen-free room with alternating 12 h periods of light and darkness, a constant temperature of 18 – 23°C and 55–65% humidity.

Cell culture
The human osteosarcoma cell line, U2OS, was obtained from the Shanghai Institute of Cell Biology. Cells were cultured in RPMI-1640 with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin and maintained at 37°C and 5% CO₂.

Cell survival assay
The effect of formononetin on the viability of U2OS cells was measured using an MTT assay. Cells were plated at a density of 4 × 10⁴ cells/well into 96-well plates. After overnight incubation, cells were treated with formononetin at different final concentrations (20, 40 and 80 μM), and the control group cells (0 μM) were treated with equal volumes of RPMI-1640 with 10% FBS. After cultivation for 0 h, 24 h, 48 h and 72 h, the cells were incubated in MTT (0.5 mg/mL final concentration) for 4 h, and added into 150 μL dimethyl sulfoxide for 15 min. Finally, the optical density (OD) was measured at 490 nm on a microplate reader (Bio-Rad, USA).

Flow cytometry
The effects of formononetin on cell apoptosis was evaluated using a flow cytometry assay. U2OS cells were plated in a 6-well plate (5×10⁴ cells/well) and incubated with 0, 20, 40 and 80 μM formononetin for 48 h. The cells were then collected, washed with ice-cold PBS and stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). The apoptotic ratio was determined by a FACS Aria flow cytometer. Early apoptotic cells were positive for Annexin V-FITC and negative for PI.

Western blot
U2OS cells were treated with 0, 20, 40, and 80 μM formononetin for 48 h. Protein was extracted and subjected to Western blot assay for quantitative analysis of protein expression. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The membranes were blocked in TBST (Trisbuffered solution, pH 7.6, 0.05 % Tween 20) containing 5 % non-fat dried milk at room temperature overnight. Membranes were probed with primary antibodies: anti-B-cell CLL/lymphoma 2 (Bcl-2) (1:1000) and anti-Actin (1:5000). After being washed five times, the blots were subsequently incubated with appropriate secondary antibodies coupled to horseradish peroxidase at room temperature for 1.5 h and then developed in electrochemiluminescence Western blot detection reagents (ZSGB-BIO, China).
Reverse transcription-polymerase chain reaction (RT-PCR)

U2OS cells were incubated with various concentrations of formononetin for 48 h. Cells were then transferred into Trizol (Gibco-BRL, Grand Island, NY, USA) in a tube. The cells were then lysed in TRIzol reagent (Gibco-BRL) to extract total RNA.

RNA was reverse transcribed to single-strand cDNA with the RevertAid First Strand cRNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD, USA), according to the manufacturer's instructions. Bax and miR-375 levels were determined using a quantitative reverse transcription-polymerase chain reaction with specific primers for BCL2-associated X protein (Bax), miR-375, glyceraldehyde-3-phosphate dehydrogenase and miRNA U6 using SYBR Green qPCR Master Mix (Fermentas Life Sciences, Hanover, MD).

Statistics

Data are expressed as mean ± standard deviation. The Statistical Package for Social Sciences (SPSS) 13.0 software (SPSS, Chicago, IL, USA) was used for statistical analyses including one-way ANOVA and Student’s $t$-test. A probability (p)-value < 0.05 was considered statistically significant.

Results

Formononetin inhibits proliferation in human osteosarcoma U2OS cells

To demonstrate the inhibitory effect of formononetin on the proliferation of human osteosarcoma cells, U2SO cells were treated with different concentrations of formononetin (0, 20, 40 and 80 μM) for 0 h, 24 h, 48 h and 72 h, and then evaluated by MTT assay. At the same culture time, as the concentrations of formononetin gradually increased compared with the control group, the U2SO cells were significantly inhibited. For the same concentration of formononetin, at longer time periods, the formononetin also significantly inhibited the growth of U2SO cells compared with the control group (Fig. 1).

Formononetin induces apoptosis in human osteosarcoma U2OS cells

Flow cytometry was used to demonstrate that formononetin can induce apoptosis in U2OS cell lines. After formononetin treatment of U2SO cells, we found an apparent increase in number of apoptotic cells with 80 μM formononetin (p < 0.05) (Fig. 2). The results showed that formononetin-induced cell apoptosis increases with increased formononetin dose.

Formononetin downregulates Bcl-2 expression in human osteosarcoma U2OS cells

The level of Bcl-2 protein with different concentrations of formononetin (0, 20, 40 and 80 μM) was determined in U2SO cells using a Western blot assay. By comparing the integrated optical density level of the protein bands, we found that high concentrations of formononetin significantly lowered Bcl-2 expression compared with the control group (Fig. 3).

Fig. 1. Effects of formononetin on cell proliferation of U2OS cells. U2OS cells were treated with formononetin for 0, 24, 48 and 72 h. Cell viability was determined by MTT assay. Data are shown as mean ± SD compared with the control group ** p < 0.05.
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Fig. 2. Effects of formononetin on early apoptosis of U2SO Cells. Early apoptosis rate of U2SO stained with Annexin V-FITC and PI was tested by flow cytometry. Data are shown as mean ± SD compared with the control group ** p < 0.05.

Fig. 3. Effect of formononetin on the expression of Bcl-2 proteins in U2SO cells. (a) U2SO cells were treated with 0, 20, 40, and 80 μM formononetin for 48 h. The activation of Bcl-2 was determined. (b) Dose-dependent effect of formononetin on Bcl-2 with respect to formononetin concentrations in (a) above. Means at 40 and 80 μM are different from the control; ** p<0.05. These are representative data from 3 independent experiments.

Table 1. Expression of Bax and miR-375 after treatment with formononetin determined by real-time PCR (mean ± SD). Data were obtained from three independent experiments performed in triplicate. Compared with control vehicle (0 μM) ** p<0.05

<table>
<thead>
<tr>
<th>Group</th>
<th>Bax / GAPDH</th>
<th>miR-375 / U6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 μM)</td>
<td>0.721±0.041</td>
<td>0.923±0.022</td>
</tr>
<tr>
<td>20 μM</td>
<td>0.732±0.043</td>
<td>0.913±0.016</td>
</tr>
<tr>
<td>40 μM</td>
<td>0.993±0.034**</td>
<td>0.696±0.022**</td>
</tr>
<tr>
<td>80 μM</td>
<td>1.423±0.057**</td>
<td>0.412±0.018**</td>
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Formononetin upregulates Bax expression and decreases miR-375 expression levels in human osteosarcoma U2SO cells

We used different concentrations of formononetin to treat the U2SO cells, and determined the Bax and miR-375 expression levels by RT-PCR. The results showed that high concentrations of formononetin gradually upregulated Bax and decreased miR-375 expression levels in a dose-dependent manner (Table 1).
Effect of formononetin on tumor growth in vivo

We tested the inhibitory effect of formononetin in tumor-bearing nude mice. We found that tumor weight gradually decreased with increasing concentrations of formononetin. At the end of the treatment, the tumor weight of nude mice in the 80 mg/kg formononetin group was remarkably reduced compared with the control group. The 80 mg/kg formononetin treatment inhibitory rate was 39.53% compared with the control group (Table 2).

Discussion

The estrogen receptor is usually expressed in mammary glands, uterus, ovaries, and in areas such as the brain in osteoblasts is also expressed in addition, the abnormal expression is associated with the low age, high incidence of tumor [9]. Estrogen is effective through combined with estrogen receptor of the target cells. Osteosarcoma is a kind of hyperactivity, abnormal proliferation of osteoblast disease, occurs in 10–20 of teenagers, which is the most active [10]. The occurrence and development of osteosarcoma is caused by cumulative multi-stage, multiple factors. A large number of clinical and experimental evidence suggests that the estrogen in hormone sensitive organs plays an important role in osteosarcoma development [11]. Dohi et al. observed the expression of the ER in 23 out of 28 osteosarcoma cases, providing evidence that osteosarcoma may be a hormone-dependent cancer [12]. Kallio et al. found that osteoblast apoptosis was induced by E2 through ER-mediated through osteosarcoma U2OS cell line experiments in vitro experiments [13]. Estrogen has been shown to promote proliferation of human osteosarcoma cells, and thus anti-estrogen therapy may be an effective tool in treating osteosarcomas [14-16].

Phytoestrogens have a similar molecular structure to estrogens, thus phytoestrogens can produce either estrogenic or anti-estrogenic effects. It is generally believed that phytoestrogens combine with the estrogen receptor of osteosarcoma cells, affecting a variety of signal transduction pathways and cell metabolism, resulting in altered cell proliferation, differentiation, apoptosis, invasion and migration ability [17-20]. Formononetin is another important phytoestrogen found in red clover. Our MTT assays showed that formononetin markedly inhibited the growth of U2OS cells at concentrations higher than 20 μM, suggesting a potential anti-proliferative effect on the human osteosarcoma cells. We also used flow cytometry to verify that U2OS cells treated with various formononetin concentrations for 48 h gave a dose-dependent increase in apoptosis with formononetin treatment. These results demonstrated that formononetin induced the apoptosis of U2OS cells, consistent with the MTT data. In vivo, formononetin also prevented tumor growth of U2OS cells in nude mouse xenografts.

MiR-375 is located in human chromosome 2 q35 district on cryba2 and Ccdc108 genetic regions and was the one of earliest discovered members of the miRNA family. Some research shows that abnormal expression of miR-375 is associated with the development of a variety of tumors, such as melanoma, pancreatic cancer, gastric cancer, prostate cancer and breast cancer [21-25]. In recent years, some studies have identified miR-375 as the first miRNA
with the capacity to enhance ER signaling in cells. Our previous studies also suggested that different concentrations of phytoestrogens had different effects on the proliferation and apoptosis of ER cells \textit{in vitro} and \textit{in vivo}, suggesting that these effects might be related to the level of miR-375. Our research showed that, as formononetin concentration gradually increases, the expression of miR-375 gradually decreased, possibly due to miR-375 and ER-positive U2OS cells forming pathways that were inhibited by formononetin.

The expression of Bcl-2 and Bax were shown to have changed significantly through western blot and PCR experiments. The mitochondrial-mediated apoptotic pathway was considered as a key center of apoptosis, and is exclusively regulated by the Bcl-2 family of proteins [26]. Bcl-2 and Bax both belong to Bcl-2 family proteins. Bcl-2 is one of the most important repressors for apoptosis, while Bax can promote apoptosis, which are critical factors in determining whether a cell will live or die [27]. We found that Bax protein expression dose-dependently increased, whereas Bcl-2 protein expression decreased. Thus, we could conclude that after treatment with formononetin, the intrinsic apoptotic pathway was activated by the altered expression of Bax and Bcl-2 proteins, and this could contribute to the commitment of U2SO cells to apoptosis.

In summary, our results suggest that formononetin has inhibitory effects on the proliferation of U2SO cells, both \textit{in vitro} and \textit{in vivo}. This antitumor effect is directly correlated with formononetin concentration. Considering that red clover plants are widely used clinically, health professionals should take special caution with regard to the variation in anti-estrogenic effects.

**Disclosure Statement**

The authors declare no conflict of interest.

**References**