Embelin induces apoptosis through down-regulation of XIAP in human leukemia cells

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Abstract The present study was undertaken to determine the molecular mechanisms by which embelin induces apoptosis in human leukemia cells. Embelin resulted in loss of cell viability and inhibition of proliferation in a dose- and time-dependent manner, which was largely attributed to apoptosis. Embelin caused depolarization of mitochondrial membrane potential. Western blot analysis showed that the expression of anti-apoptotic proteins X-linked inhibitor of apoptosis (XIAP) was down-regulated by embelin. Embelin induced activation of caspase-9 and embelin-induced apoptosis was prevented by caspase inhibitors. Taken together, these findings suggest that embelin results in human leukemia cells apoptosis through caspase-dependent mechanisms involving down-regulation of XIAP.

Keywords Embelin · Apoptosis · X-linked inhibitor of apoptosis · Leukemia

Introduction

Acute leukemia represents the most aggressive hematologic malignancies and is prone to acquire resistance which results in relapse and death. Embelin is a novel cell permeable inhibitor of XIAP which was discovered by screening a library of natural products derived from traditional Chinese medicine [1]. They have multiple biological, pharmacological and medicinal properties including anti-inflammatory, antibacterial, antioxidant and antitumor [2–5]. There has been considerable interest in the antitumor activities of embelin, and previous studies have shown that embelin induces growth inhibition and cell apoptosis in human solid tumors [6–8]. However, the effect of embelin on human leukemia cells was seldom studied, and the precise mechanisms are not elucidated. The present study was therefore undertaken to determine the effect of embelin on human leukemia cells and its molecular mechanisms. Our data demonstrated that embelin induces apoptosis of human leukemia cells through caspase-dependent mechanisms involving down-regulation of XIAP.

Materials and methods

Main reagents

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Annexin -FITC-propidium iodide and JC-1 (CUSABIO, China); z-VAD-FMK, DEVD-CHO, embelin and antibodies (Sigma–Aldrich, USA).

Cell culture

HL60 cells were incubated in RPMI 1640 media, 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in humidified environment of 5% CO₂ and subcultured every 3–4 days to maintain exponential growth.

Cells were seeded in 96-well plates at a density of 1 × 10⁵ viable cells/well, and the cells were exposed to embelin at concentrations ranging from 3 to 300 ug/ml for 12, 24 and 48 h.
Measurement of cell viability, proliferation and cell apoptosis

Cell viability was evaluated using a MTT assay [9]. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. The cells were incubated for 4 h at 37°C, and the supernatant was removed, and the formed formazan crystals in viable cells were solubilized with 0.15 ml of dimethyl sulfoxide. A 0.1-ml aliquot of each sample was then translated to 96-well plates, and the absorbance of each well was measured at 570 nm with ELISA Reader. Data were expressed as a percentage of control measured in the absence of embelin.

Western blot analysis

Cells were harvested for various times after embelin treatment. Cell debris was removed by centrifugation at 1,200 rpm for 1 h at 4°C. The resulting supernatants were

Fig. 1 Effect of embelin on cell viability. Cells were exposed to 3–300 ug/ml embelin for various times (a) and for 24 h (b). Cell viability was estimated by MTT assay. Data are mean ± SEM of eight independent experiments performed in duplicate. * P < 0.05 compared with control without embelin

Fig. 2 Effects of embelin on cell apoptosis. Cells were exposed to 30 ug/ml for 24 h (a) or various times (b) and fluorescence intensity was measured by a flow cytometer. Data in b are mean ± SEM of three independent experiments performed in duplicate. * P < 0.05 compared with control without embelin

Fig. 3 a HL60 cells under 30 ug/ml embelin for 12 h, JC-1 dye under microscope (single filter) ×400. b HL60 cells under 30 ug/ml embelin for 24 h, JC-1 dye under microscope (single filter) ×400
resolved on a 12% SDS–PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 30 min and incubated with different primary antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. The signal was visualized using an enhanced chemiluminescence.

Measurement of mitochondrial membrane potential

The mitochondrial transmembrane potential was measured with JC-1 which is incorporated into cells depending upon the mitochondrial membrane potential. The fluorescence intensity was analyzed with a FACScan flow cytometer.

Statistical analysis

The data are expressed as mean ± SEM, and the difference between two groups was evaluated using Student’s t-test. A probability level of 0.05 was used to establish significance.

Results

Embelin induces loss of cell viability and cell apoptosis

To evaluate the effect of embelin on HL 60 cells viability, the cells were exposed to various concentrations of embelin (0–300 ug/ml) for 12–48 h. As shown in Fig. 1a, embelin decreased the cell viability in a time- and dose-dependent manner. When cells were exposed to embelin over concentrations of 3, 10, 30, 100 and 300 ug/ml for 48 h, the cell viability was (90.57 ± 3.02), (89.16 ± 2.71), (66.61 ± 2.93), (55.51 ± 3.16) and (43.86 ± 2.84)%, respectively (Fig. 1b).

To determine whether a decrease in cell viability and proliferation induced by embelin was attributed to cell apoptosis, the cell apoptosis was estimated. The cells were exposed to various concentrations of embelin for 48 h, and cell apoptosis was evaluated by Annexin V FITC- propidium iodide double staining. The results of Fig. 2 indicated that embelin induced cell apoptosis in a dose-dependent fashion with a similar pattern to that of inhibition of cell viability and cell proliferation. In subsequent experiments, cells were exposed to 30 ug/ml embelin for 48 h.

Embelin induces disruption of mitochondrial membrane potential

To evaluate the role of mitochondria in loss of cell viability by embelin, changes in mitochondrial membrane potential were examined in cells exposed to embelin. Mitochondrial membrane potential was measured using the JC-1 fluorescence dye in cells exposed to 30 ug/ml embelin for 12 and 24 h. Embelin caused disruption of mitochondrial membrane potential as evidenced by an increase in the proportion of cells with green fluorescence (Fig. 3).
Mitochondrial membrane potential decreased after 6 h of embelin treatment, and the depolarization showed a time-dependent fashion (Fig. 4).

Down-regulation of XIAP releases caspase-9 and activates caspase-3 by embelin

XIAP, as a member of anti-apoptotic proteins, was expressed in many cancers and associated with tumor progression [10, 11]. Although the BIR2 and BIR3 are necessary for XIAP to bind to and inhibit caspases [12], the BIR1 of XIAP has been shown to interact with TAK1 binding protein 1 (TAB 1), an interaction that is essential for the recruitment of TAK1 and the subsequent activation of NF-κB [13]. Embelin-induced cell death is due to down-regulation of XIAP and releases of caspase-9 followed by activation of caspase-3 which leads to cell apoptosis (Fig. 5). Cells were exposed to embelin in the presence of the caspase-3 inhibitor DEVD-CHO and the general caspase inhibitor z-DEVD-FMK. The embelin-induced cell death was prevented by these inhibitors (Fig. 6). These data indicate that embelin induces cell death through a caspase-dependent mechanisms.

We analyzed the expression of survivin and bcl-2 that are also known as anti-apoptotic proteins and showed similar changes to XIAP.

Discussion

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone, C_{17}H_{26}O_{2}, molecular weight: 294.39) is a kind of extract from Japanese Ardisia Herb, and its traditional use in Chinese herbal medicine is to dispel intestinal parasites. Soon after it was identified as a novel cell permeable inhibitor of XIAP [1], multiple trials were carried out to
explore its application in solid tumors such as hepatic cancer and pancreas cancer [4, 8]. However, it is short of studies on embelin in human leukemia cells.

The present study demonstrated that embelin caused loss of cell viability in a dose- and time-dependent manner (Fig. 1). Similarly, embelin induced cell apoptosis in a dose-dependent manner (Fig. 2). These results suggest that reduction in cell viability and proliferation was attributed to apoptosis. In subsequent experiments, we explored the molecular mechanisms of embelin-induced apoptosis.

A decrease in mitochondrial membrane potential plays an important role in apoptosis [14]. We determined whether embelin causes depolarization of mitochondrial membrane potential. Exposure of cells to embelin caused a significant reduction in mitochondrial membrane potential (Fig. 4), suggesting involvement of mitochondria in the embelin-induced cell apoptosis. Inhibitors of apoptosis proteins (IAP) are a family of related proteins that suppress apoptosis induced by a variety of pro-apoptotic stimuli. The IAP family includes XIAP, c-IAP1, c-IAP2 and survivin. Down-regulation of IAP proteins has been shown to induce apoptosis [15]. XIAP, as a member of anti-apoptotic proteins, has attracted considerable interest. Their expression has been reported to be associated with poor prognosis and increased tumor recurrence in many cancers [10].

We observed here that XIAP was strongly expressed in HL60 cells and that its expression was decreased by embelin (Fig. 5). These results suggest that embelin induces apoptosis through inhibition of XIAP expression in HL60 cells.

Growth inhibition and cell death induced by embelin are associated with release of caspase-9 and activation of caspase-3 in prostate cancer cells [16]. Consistent with these data, we observed here that embelin induces a caspase-dependent apoptosis, as evidenced by activation of caspases by embelin and protection from apoptosis by caspase inhibitors (Fig. 6).

According to what we know, there are still no reports on toxicities of embelin in humans and present animal experiments show that its major side effect is reversible reproductive inhibition and renal dysfunction occasionally. Next, we plan to carry out further experiments to test its pharmacology effect in animals.

In conclusion, the present study demonstrated that embelin resulted in human leukemia cells apoptosis through caspase-dependent mechanisms involving down-regulation of XIAP. Induction of cell death may be a promising therapeutic approach in cancer therapy. Our results suggest that embelin may be considered a potential candidate for both acute leukemia prevention and treatment. Further investigation is needed to validate the contribution of embelin to tumor therapy in vivo.

References