Summary

Purpose: To investigate the antitumor effects of madecassic acid and to investigate the mechanism by which madecassic acid treatment functions in malignancies.

Methods: Mouse colon CT26 cancer cells injected in mice subcutaneously and intraperitoneally were used to evaluate the tumor growth inhibition by madecassic acid administration. The immunomodulation, cell apoptosis and mitochondrial membrane potential change were evaluated by flow cytometry, cell immunostaining and JC-1 staining, respectively.

Results: Madecassic acid inhibited tumor growth in tumor-bearing mice. CT26 cell apoptosis rate and of the cells from ascites was increased after madecassic acid treatment. Mitochondrial membrane potential in CT26 cells also decreased after madecassic acid treatment. CD4+ and CD8+ T-lymphocytes subpopulations increased, while the ratio of CD4+/CD8+ decreased in after madecassic acid administration.

Conclusions: Madecassic acid inhibits in vivo CT26 cell-induced tumor growth by facilitating cell apoptosis and increasing immune defense mechanisms.

Key words: antitumor effects, cell apoptosis, immunomodulation, madecassic acid, tumor growth

Introduction

Malignant tumors represent one of the leading causes of death. With the advancement of understanding of tumorigenesis in the past 5 decades, the conventional medical treatments of cancers, including chemotherapy, radiotherapy, and surgery significantly improved the survival of cancer patients. Nevertheless, the overall therapeutic outcomes are still far from being satisfactory [1], since accumulating evidence showed that the current approaches to cancer therapy are accompanied with adverse effects, such as the disruption of body’s natural defense mechanisms [2,3]. Therefore, new strategies to cancer treatment are being developed to combat the disease. Among them, immunotherapeutics is viewed as the most promising approach against cancer [4].

Recently, plant-derived polysaccharides have been paid more and more attention due to their anticancer properties and the capability to improve the body’s immunomodulation. Oriental medicine has a long history of using herbs to prevent and treat diseases, including cancer, by modulating the body’s natural immune defense system. Madecassic acid is the active extract of the commonly used centella asiatica [4]. A previous study [5] showed that madecassic acid possesses antiinflammatory properties. However, it is unknown whether madecassic acid possesses antitumor properties, and the underlying antitumor mechanism is poorly understood.

In this study we investigated the role of madecassic acid in a cancer CT26 cells-bearing mouse model.
**Methods**

**Cells, cell culture and treatment**

Mouse CT26 cells were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 μ/ml penicillin, 100 μg/ml streptomycin in humidified atmosphere with 5% CO₂ at 37°C. Culture medium was replaced daily with fresh medium until cell confluence reached 80-90%. To examine the apoptotic effects induced by madecassic acid, CT26 cells were treated with madecassic acid at concentrations of 2, 10, 50 or 250 μg/mL for 48 hrs; then JC-1 staining and Hoechst staining were performed. For JC-1 staining, stock solution of JC-1 (Invitrogen, Carlsbad, CA, USA) in DMSO (5 mg/ml) was diluted in 2 μg/ml with RPMI 1640 medium, and then cells were incubated in the JC-1 solution for 30 min. Afterwards, cells were rinsed with PBS for three times and then were inspected under microscope. For Hoechst staining, cells were firstly fixed with methanol/glacial acetic acid (3:1) for 15 min. After washing twice with PBS, cells were incubated in Hoechst staining solution (Invitrogen, Carlsbad, CA, USA) for 5 min at 37°C. After two washings with PBS, cells were inspected under microscope.

**Subcutaneous injection, measurement of tumor weight and cell apoptosis analysis**

CT26 cells were harvested and pre-washed three times with sterilized PBS. One day after subcutaneous injection of 100 μl PBS with final cell density of 1 x 10⁷/ml to male mice 5 weeks of age, mice were administered 0.5 ml of madecassic acid in bidistilled (bd) H₂O (the content of madecassic acid was 12.5, 25, 50 and 100 mg/kg, respectively). Ten days after administration, mice were sacrificed and the induced tumor was weighted.

**Intraperitoneal administration**

CT26 cells were harvested and pre-washed three times with sterilized PBS. One day after intraperitoneal injection of 500 μl PBS with final cell density of 1 x 10⁷/ml to male mice 5 weeks of age, mice were administered 0.5 ml of madecassic acid in bd H₂O (the amount of madecassic acid in bd H₂O was mouse weight-dependent, according to the criteria of 50 mg/kg) or only 0.5 ml of bd H₂O (as control group). Seven days after administration, ascites was drained and cells were prepared from the ascitic fluid. After three washings with PBS, cell apoptosis was analyzed by Annexin V-FITC/PI (Invitrogen, Carlsbad, CA, USA) double staining, followed by flow cytometry analysis.

**Preparation of mice spleen lymphocytes**

Mice with 50 mg/kg madecassic acid or bd H₂O, as described previously, were sacrificed and freshly extracted mice spleens were incubated in RPMI 1640 medium supplemented with 10% FCS. Spleens were milled on 200-nylon net with sterilized handle of syringe followed by centrifugation with 500 rpm for 15 min. Spleen lymphocytes were transferred to 5ml of Tris-NH₄Cl, pH 7.2. After two washings with RPMI 1640 medium, cells were maintained in complete RPMI 1640 medium supplemented with 10% FCS with final cell density of 1 x 10⁷/ml.

**Mice spleen lymphocytes staining and CD4⁺/CD8⁺ analysis**

Blood samples from mice eyeball and prepared mice spleen lymphocytes in Eppendorf tube were centrifuged at 500 rpm for 15 min. After removal of the supernatant, 500 μl rpm 4% paraformaldehyde (Sigma, St. Louis, MO, USA) in PBS were added to each tube to suspend cells for 5 min at room temperature. Three ml of ice-cold staining buffer were then added and incubated for 5 min at 4°C followed by 200 rpm centrifugation for 10 min to remove the supernatant. One μl of fluorescent-labeled CD4⁺ or CD8⁺ antibodies (BD Pharmingen; San Diego, CA, USA) diluted in 50 μl of PBS was added in each tube and incubated for 30 min in the dark. Afterwards, cells were washed three times with PBS and suspended in 200 μl of PBS for flow cytometry analysis.

**Statistics**

The Pearson x² test and the Independent-Sample-t-test were used for analyses. A p-value of <0.05 was considered as statistically significant. All statistical analyses were performed using the SPSS 13.0 program (SPSS Inc, Chicago, IL, USA).

**Results**

**Madecassic acid treatment inhibits tumor growth**

To investigate the antitumor effects of madecassic acid, mice were injected subcutaneously with CT26 cells followed by administration of different doses of madecassic acid. Tumors induced by CT26 cell injection were then extracted and weighted. In control mice without madecassic acid administration, the average weight of induced tumor was 11.3 g. When administering the final dose of 12.5 mg/kg madecassic acid, the average weight of induced tumors was significantly reduced to 4.15 g (p<0.05); what’s more, the effects of tumor growth inhibition was dose-dependent, from 5.24 g in response to final dose of 25 mg/kg madecassic acid, 2.12 g in response to final dose of 50 mg/kg madecassic acid and 2.10 g in response
Madecassic acid induces apoptosis and immunomodulation in colon cancer

Madecassic acid treatment induces cancer cell apoptosis

To demonstrate whether the inhibited tumor growth was due to cell apoptosis induced by madecassic acid administration, CT26 cells were injected intraperitoneally and then 0.5 ml of madecassic acid solution (the amount of madecassic acid in solution was mouse weight-dependent, according to the criteria of 50 mg/kg) was administered to the mice. The results showed that, compared with the control group (Figure 2A), madecassic acid administration markedly increased the cell apoptosis rate (p<0.05; Figure 2B).

Madecassic acid treatment facilitates immunomodulation in lymphocytes

To evaluate the immunomodulatory properties of madecassic acid, we firstly examined the CD4+ and CD8+ T-lymphocyte subpopulations in prepared tumor-bearing mice spleen lymphocytes by flow cytometry. Compared with the control tumor-bearing mice group without madecassic acid administration (Figure 3A), the treatment with madecassic acid to tumor-bearing mice caused a significant increment in the number of CD4+ T lymphocytes and an increase in the number of CD8+ T lymphocytes (p<0.05; Figure 3B). By calculating the ratio of CD4+/CD8+ T lymphocytes, we also found that this ratio in mice administered madecassic acid was significantly higher than that in the control mice (p<0.05; Figure 3, Table 2), suggesting madecassic acid treatment effectively facilitated the immunodulation in tumor-bearing mice.

Discussion

In anticancer therapy, besides the conventional chemotherapy, radiotherapy and surgery, immunotherapy with active compounds in natural products represents a promising strategy, since this strategy is with much less negative effects compared with conventional therapies, improving at the same time the body’s immune defense system. Among these active compounds, multiple plant-derived and microorganism-isolated polysaccharides have been reported to exert anticancer and immunostimulating activities [6-9,13].

In this study, using mouse colon cancer cells and a cancer-bearing mice model, we comprehensively investigated the impact of madecassic acid on cell apoptosis and immunomodulation. We found that treatment with madecassic acid significantly induced cell apoptosis and changed the mitochondrial membrane potential. Furthermore, in cancer-bearing mice, madecassic acid administration markedly reduced the size of solid tumor and decreased the T-lymphocytes subpopulation ratio of CD4+/CD8+; however, IFN-γ and IL-4 secretion increased in spleen lymphocytes. Collectively, these results clearly showed that madecassic acid exerts anticancer activity by inducing cell apoptosis and stimulating immunomodulation.

At cellular level, tumorigenesis is a disorder of cell proliferation and cell apoptosis, namely unlimited cell proliferation and excessively low rate of cell apoptosis. In anticancer therapy it is

Figure 1. Madecassic acid treatment inhibits tumor growth. Tumor-bearing mice were administered with different doses of madecassic acid. Ten days after administration, mice were sacrificed and the induced tumors were excised and pictured. X axis=the number of tumors formed in the mice with tumor cell injection. Y axis=the different doses of madecassic acid administration to tumor-bearing mice.

Table 1. Average weight of induced tumor in response to madecassic acid administration

<table>
<thead>
<tr>
<th>Madecassic acid (mg/kg)</th>
<th>0</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
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<tr>
<td>Average tumor weight±SD (g)</td>
<td>11.3±1.23</td>
<td>4.15±0.75*</td>
<td>3.24±0.77*</td>
<td>2.12±0.34*</td>
<td>2.10±0.31*</td>
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*p<0.05 vs 0 mg/kg madecassic acid. SD: standard deviation
accepted that inhibition of cancer cell proliferation only delays the progression of tumorigenesis but cannot eliminate tumor, while induction of cancer cell apoptosis is a strategy capable to reduce the tumor size or even lead to tumor elimination [11]. Therefore, screening of reagents that can induce cancer cell apoptosis is of great clinical significance. In this study, after administration of madecassic acid, the induced tumor growth in mice progressively decreased in a dose-dependent manner of madecassic acid administration (Figure 1, Table 1). Cell apoptosis analysis showed that after madecassic acid administration, cell apoptosis rate in ascites of tumor-bearing mice was markedly elevated (Figure 2, B and D). Therefore, this observation would imply that the shrinkage of tumor size could be possibly due to the madecassic acid-induced cancer cell apoptosis. Though we did not comprehensively explore the mechanism by which cancer cell apoptosis was induced by madecassic acid, our immunostaining experiments suggested that madecassic acid treatment decreased the mitochondrial membrane potential (Figure 2F), which at least partly contributed to the cancer cell apoptosis.

Essentially, tumorigenesis is due to the at-
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References


