JBUON 2014; 19(2): 372-376
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Madecassic acid inhibits the mouse colon cancer growth by inducing apoptosis and immunomodulation

Hua Zhang[1], Ming Zhang[2], Yun Tao[3], Ge Wang[1], Bing Xia[1]

[1]*Department of Gastroenterology, Zhongnan Hospital of Wuhan University, Wuhan;* [2]*Department of Cardiology, Central Hospital of Hubei Xiangyang, Xiangyang;* [3]*Department of Emergency, Xiangyang Hospital, Hubei University of Medicine, Xiangyang, China*

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.

Correspondence to: Bing Xia, MD, PhD. Department of Gastroenterology, Zhongnan Hospital of Wuhan University, Donghu Road 169, Wuhan 430071, Hubei Province, China. Tel:+86 27 67813061, Fax:+86 27 67812892,
E-mail:bingxia_hb@163.com, bingxia@aliyun.com
Received: 16/02/2014; Accepted: 01/03/2014

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 u/ml penicillin, 100 μg/ml streptomycin in humidified atmosphere with 5% CO_2 at 37 °C. Culture medium was replaced daily with fresh medium until cell confluency 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 x10^7/ml to male mice 5 weeks of age, mice were administered 0.5 ml of madecassic acid in bidistilled (bd) H_2O (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 of PBS with final cell density of 1 x10^7/ml to male mice 5 weeks of age, mice were administered 0.5 ml of madecassic acid in bd H_2O (the amount of madecassic acid in bd H_2O was mouse weight-dependent, according to the criteria of 50 mg/kg) or only 0.5 ml of bd H_2O (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 H_2O, 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-NH_4Cl, 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 10^7/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 ° 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 x^2 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 3.24 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

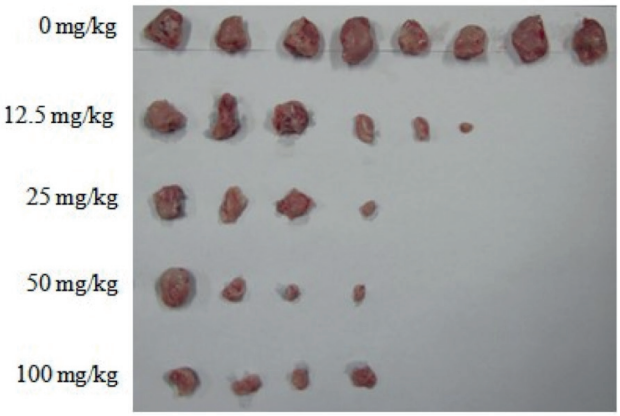

Figure 1. Madecassic acid treatment inhibits tumor growth. Tumor-bearing mice were administered with different doses of madecassic acid. Ten days after administration, mice were sacrificed and the induced tumors were excised and pictured. X axis=the number of tumors formed in the mice with tumor cell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

to final dose of 50 mg/kg madecassic acid (Figure 1, Table 1). According to the effects of tumor growth inhibition, we then used 50 mg/kg madecassic acid for the following experiments.

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

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

Madecassic acid (mg/kg)	*0*	*12.5*	*25*	*50*	*100*
Average tumor weight±SD (g)	11.3±1.23	4.15±0.75*	3.24±0.77*	2.12±0.34*	2.10±0.31*

*$p<0.05$ vs 0 mg/kg madecassic acid. SD: standard deviation

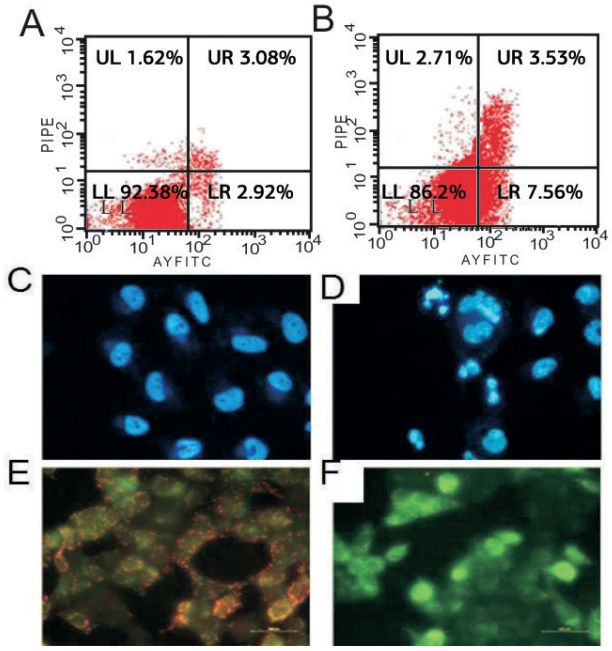

Figure 2. Madecassic acid treatment induces cancer cell apoptosis. Cells from the ascites of tumor-bearing mice without (**A**) or with (**B**) madecassic acid administration were stained by Annexin V-FITC/PI followed by flow cytometry to analyse cell apoptosis. Upper left (UL): shows the percent of dead cells; Upper right (UR): shows the percent of non-active cells; Lower left (LL): shows the percent of apoptotic cells; Lower right (LR): shows the percent of living cells. Mouse colon CT26 cells cells were treated with (**D, F)** or without (**C, E**) madecassic acid (50 μg/mL) for 48 hrs and then Hoechst 33342 and JC-1 staining were performed to examine the cell morphology. **D, F** show considerably more apoptotic cells.

Table 2. Mean percentage (±SD) of CD4+ and CD8+ T lymphocytes subpopulations

	Without madecassic acid administration	With madecassic acid administration
$CD4^+$	9.53±1.11	21.38±2.52*
$CD8^+$	6.18±0.86	10.07±1.32

*p<0.05 vs without madecassic acid. SD: standard deviation

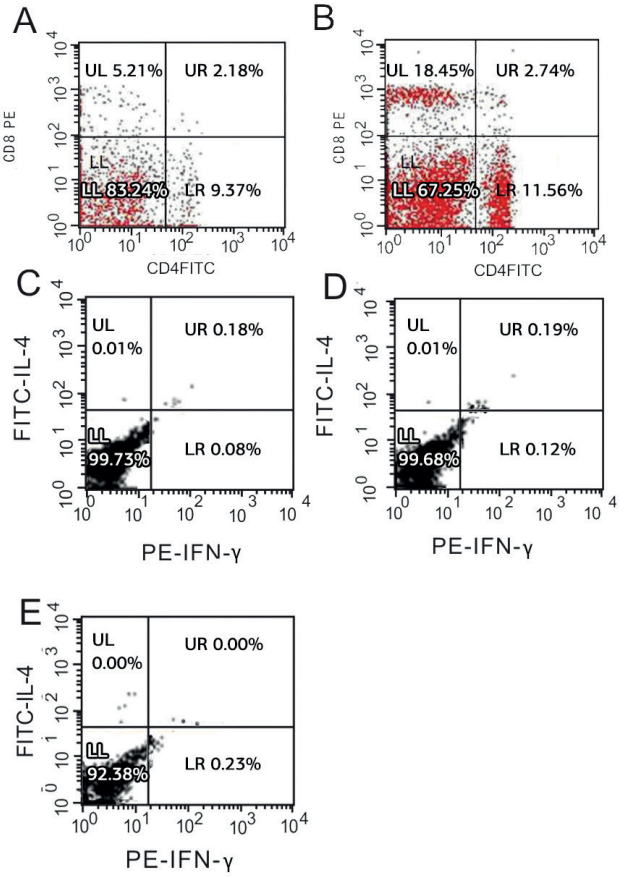

Figure 3. Madecassic acid facilitates immunomodulation of lymphocytes. Spleen lymphocytes from the tumor-bearing mice without (**A**) or with (**B**) madecassic acid administration were stained with fluorescent-labeled CD4+ or CD8+ antibody for 30 min, then flow cytometry analysis was performed to measure the percentage of CD4+ or CD8+ T-lymphocytes subpopulations. Prepared mice spleen lymphocytes from normal mice (**C**), tumor-bearing mice without madecassic acid (**D**) or tumor-bearing mice with madecassic acid administration (**E**) were fixed, stained with labeled antibodies against intracellular cytokines IL-4 and IFN-γ for 30 min followed by flow cytometry analysis to measure the percentage of cells secreting IL-4 or IFN-γ.

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 shrinkange 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-

tenuation of the body's immune defense system, which makes the cancer cells escape the attack by the T cells. Therefore, stimulating the immunomodulation by reagents will contribute to the elimination of cancer cells in cancer immunotherapy [12]. With madecassic acid administration to tumor-bearing mice we showed that not only $CD4^+$ and $CD8^+$ T- lymphocytes subpopulations increased (the ratio of $CD4^+/CD8^+$ increased too), but also the secretion of IFN-γ and IL-4 increased as well (Figure 3). The results suggested that treatment with madecassic acid brought out two-step effects to mice immunomodulation. Firstly, treatment with madecassic acid to tumor-bearing mice caused a significantly increase of $CD4^+$ and $CD8^+$ T- lymphocytes subpopulation (Table 2). It was documented that $CD4^+$ T cells themselves not only attack cancer cells but they also can help $CD8^+$ T cells to attack cancer cells [13,14]. Thus, the increased $CD4^+$ and $CD8^+$ T-lymphocytes subpopulations in response to madecassic acid administration directly enhanced the body's immune defense system to antagonize tumorigenesis. Secondly, since $CD4^+$ T cells or $CD8^+$ T cells secret multiple cytokines including IFN–γ [14,15], then - following the increase of $CD4^+$ and $CD8^+$ T-lymphocytes subpopulations - the amount of secreted IFN-γ and IL-4 was further increased. Given the fact that the production of IFN-γ and IL-4 stimulates the Th1- and Th2-mediated immune response, respectively, then the increased cell percentage with IFN-γ and IL-4 secretion suggests the differential immunostimulation pathway in response to madecassic acid treatment. Altogether, the anticancer activity of madecassic acid comes possibly from the combined effects of different immunomodulation pathways activated by madecassic acid.

In conclusion, this study demonstrated that madecassic acid exerts antitumor activity by inducing cancer cell apoptosis and improving the body's immunomodulation. Our results suggest that madecassic acid is with potential clinical significance in cancer therapeutics.

References

1. Mushiake H, Tsunoda T, Nukatsuka M et al. Dendritic cells might be one of key factors for eliciting antitumor effect by chemoimmunotherapy *in vivo*. Cancer Immunol Immun 2005;54:120-128.
2. Salgaller ML, Lodge PA. Use of cellular and cytokine adjuvants in the immunotherapy of cancer. J Surg Oncol 1998;68:122-138.
3. Chabner BA, Roberts TG Jr. Timeline: Chemotherapy and the war on cancer. Nat Rev Cancer 2005;5:65-72.
4. Bonte F, Dumas M, Chaudagne C et al. Influence of asiatic acid, madecassic acid, and asiaticoside on human collagen I synthesis. Planta Med 1994;60:133-135.
5. Won JH, Shin JS, Park HJ et al. Anti-inflammatory Effects of Madecassic Acid via the Suppression of NF-κB Pathway in LPS-Induced RAW 264.7 Macrophage Cells. Planta Med 2010;76:251-257.
6. Harada T, Miura NN, Adachi Y et al. IFN-gamma induction by SCG, 1,3-beta-D-glucan from *Sparassis crispa*, in DBA/2 mice in vitro. J Interf Cytok Res 2002;22:1227-1239.
7. Yoon YD, Han SB, Kang JS et al. Toll-like receptor 4-dependent activation of macrophages by polysaccharide isolated from the radix of *Platycodon grandiflorum*. Int Immunopharmacol 2003;3:1873-1882.
8. Nergard CS, Diallo D, Michaelsen TE et al. Isolation, partial characterization and immunomodulating activities of polysacchrides from *Vernonia kotschyana* Sch. Bip. ex Walp. J Ethnopharmacol 2004;91:141-152.
9. Nam KS, Shon YH. Chemopreventive effects of polysaccharides extract from *Asterina pectinifera* on HT-29 human colon adenocarcinoma cells. BMB Rep 2009;42:277-280.
10. Won DP, Lee JS, Kwon DS et al. Immunostimulating activity by polysaccharides isolated from fruiting body of *Inonotus obliquus*. Mol Cells 2011;31:165-173.
11. Schulze-Bergkamen H, Krammer PH. Apoptosis in cancer-implications for therapy. Semin Oncol 2004;31:90-119.
12. Han SK, Yee SC, Taeseok K et al. Enhancement of antitumor immunity of dendritic cells pulsed with heat-treated tumor lysate in murine pancreatic cancer. Immunol Lett 2006;103:142-148.
13. Levitsky HI, Lazenby A, Hayashi RJ et al. In vivo priming of two distinct antitumor effector populations: the role of MHC Class I expression. J Exp Med 1994;179:1215-1224.
14. Mumberg D, Monach PA, Wanderling S et al. CD4+T cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFN-g. Immunology 1999;96:8633-8638.
15. Schüler T, Qin Z, Ibe S, Noben-Trauth N et al. T Helper Cell Type 1-associated and Cytotoxic T Lymphocyte-mediated Tumor Immunity Is Impaired in Interleukin 4-deficient Mice. J Exp Med 1999;189:803-810.