ORIGINAL RESEARCH

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Maslinic acid activates mitochondria-dependent apoptotic pathway in cardiac carcinoma

Abstract

Purpose: Cardiac carcinoma is the most common subtype of gastric cancer and its incidence has increased in recent years. The current chemotherapeutic drugs exhibit limited effectiveness and significant side effects in patients. Maslinic acid (MA) exerts an anti-tumor activity on a wide range of cancers and has no significant side effect; however, the anti-tumor effect of MA on cardiac carcinoma has not yet been explored.

Methods: MTT assays, tumor xenograft animal model, immunoblotting, MMP assessment and flow cytometry were performed in this study.

Results: MA was able to suppress the viability of cardiac carcinoma cells in both a time- and dose-dependent manner. This natural compound exhibited no cytotoxicity in normal cells. Its inhibitory effect on tumor growth was further confirmed in a mouse model. Mechanistically, MA induced the activation of p38 MAPK in cardiac carcinoma cells and, in turn, changed their mitochondrial membrane potential (MMP). Finally, caspase cascades were activated by a series of cleavages, leading to apoptosis in cardiac cancer cells. Inhibition of p38 MAPK signaling was able to rescue the effect of MA on cardiac carcinoma cells.

Conclusion: Our data demonstrated that natural compound, MA, suppressed the growth of cardiac carcinoma by inducing apoptosis via the p38 MAPK/mitochondria/caspase pathway. MA and its derivatives may be promising anti-tumor agents for cardiac carcinoma treatment in the future.

Manuscript submitted 4th January, 2014

Manuscript accepted 23rd June, 2014

Clin Invest Med 2014; 37 (4): E217-E225.

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Cardiac carcinoma (also called carcinoma of the gastric cardia) is an adenocarcinoma that originates from the epithelial cells located in the connection between stomach and esophagus and is among the most common types of gastric cancer. Although surgery is still the most effective regimen for cardiac carcinoma, it is not effective for patients at the late stages of the disease, especially when metastasis has already occurred [1]. Chemotherapy exhibits some effectiveness in the treatment of metastatic cardiac carcinoma [1]; however, the effectiveness of current drugs is too limited to prolong the survival of the patients. Furthermore, current drugs also induce toxicity in normal tissues [1]. Novel compounds that exert anti-tumor activity on cardiac carcinoma cells without toxic side effects are needed.

Maslinic acid (MA) is a naturally occurring pentacyclic triterpene and is isolated from a wide range of edible and medicinal plants. MA has been shown to exert anti-tumor activity on some types of cancer [2], including melanoma [3], prostate cancer [4], hepatocellular carcinoma [5] and intestinal cancer [6, 7]. Previous studies have demonstrated that MA can induce apoptosis in cancer cells [2, 8]. In addition, MA has no cytotoxicity to normal cells, which limits side effects when used for cancer treatment [9].

The ability of MA to suppress cardiac carcinoma growth has not yet been tested. In this study, several cardiac cancer cell lines and specimens from patients were employed to study the effect of MA on cardiac carcinoma and to ascertain the mechanisms involved.

Materials and Methods

Compounds

Maslinic acid (MA) was generously provided by Dr. Zhang (Yunnan Agriculture University, Kunming, China). The molecular weight of MA is 472 Daltons. MA was dissolved with DMSO at a concentration of 10 mM and diluted to the indicated concentrations when needed.

Cell cultures

Human carcinoma of gastric cardia cell lines, SGC-7901 and BGC-823, and normal human liver cells, L-02, were purchased from Shanghai Cell Collection (Shanghai, China). Normal human lung fibroblast cell line, MRC-5, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were all cultured in the recommended media (ATCC-formulated Eagle's Minimum Essential Medium for MRC-5; RPMI-1640 for SGC-7901, BGC-823 and L-02; all media was purchased from Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Life Technolo-

gies) and 5 mM glutamine in a 5% CO₂-containing humidified atmosphere at 37°C.

Primary culture

The protocols have been approved by Human Investigations or Ethics Committee in Zhengzhou University (Zhengzhou, China). Cardiac carcinoma specimens were obtained from the patients in the Department of Gastroenterology, the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). The specimens were placed in 20% FBS-containing media, minced with scissors and digested for at least 1 h. The cells were centrifuged at 800 rpm for 15 min, resuspended in RPMI-1640 media with 20% FBS and cultured at 37°C in a humidified atmosphere of 5% CO₂ for 24 h. The supernatant was discarded and replaced with fresh RPMI-1640 media with 20% FBS then primary cancer cells were cultured following routine procedures. No mycoplasma was detected in the primary cultures.

MTT assays

Indicated cells (1×10^4 cells) were seeded in each well of 96-well plates. Overnight, compounds at the indicated concentrations were added to each well and 24 h later, 50 μ l 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 1 μ g/ml) was added to each well. After 4 h, the media was removed and 150 μ l DMSO were added to each well. Spectrophotometric absorbance of the samples was measured with Microplate Reader Model 550 (Bio-Rad Laboratories, city, Japan) at 570 nm with a reference wavelength of 655 nm. The percentage of living cells was calculated using the following formula: cell survival rate = absorbance value of infected cells / absorbance value of uninfected cells. Eight reduplicate wells were measured at each concentration and all experiments were performed at least three times.

Animal experiments

Procedures for animal experiments were approved by the Committee on the Use and Care on Animals of Zhengzhou University (Zhengzhou, China). Briefly, 5×10^6 SGC-7901 cells were subcutaneously injected into the flanks of 4-week-old male BALB/c nude mice. When tumors reached nearly 7-9 mm in diameter, 21 mice were randomly split into three groups: control group, low concentration (5 mg/kg) MA group and high concentration (20 mg/kg) MA group (n=7 for each group). MA was intraperitoneally administered every other day until experiments were finished. Tumor diameter was measured by periodic measurements with calipers and volume

FIGURE 1. Illustration of maslinic acid (MA) structure

was calculated using the following formula: tumor volume (mm^3) = maximal length (mm) $\times$ (perpendicular width (mm))²/2. Animals were sacrificed once the tumor volume exceeded 2000 mm^3 or at the end of experiments (Day 35). No mice died of tumor loading during the experiment.

Immunoblot assays

Total proteins were extracted from cells with M-PER[®] Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA). Mitochondria were isolated from cells using the Mitochondria Isolation Kit (Thermo Scientific) and mitochondrial proteins were extracted using M-PER[®] Mammalian Protein Extraction Reagent (Thermo Scientific). Proteins were separated with 10-12% polyacrylamide gel electrophoresis and transferred onto 0.45 μm nitrocellulose membranes. The membranes were blocked with 5% fat-free dry milk in PBS and incubated with primary antibodies. The membrane was incubated with corresponding secondary antibodies and visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). The involved antibodies include phospho-p38 MAPK (1:1000), p38 MAPK (1:1000), cytochrome C (1:1000), COX (1:1000), cleaved caspase 9 (1:1000), cleaved caspase 3 (1:1000), cleaved PARP (1:1000) and GAPDH (1:2000). These antibodies were all purchased from Cell Signaling Technology (Beverly, MA, USA). The quantification of blot intensity was performed using ImageJ software (Version 1.47).

MMP evaluation

The change in mitochondrial membrane potential (MMP) in the indicated cells was detected using JC-1 staining based on flow cytometry (Invitrogen Life Technologies, Grand Island, NY, USA), following the manufacturer's procedures. After 24 h incubation of MA, the cells were washed with PBS three times and then resuspended in PBS at the concentration of 1×10^6 cells/ml. The cells were stained with 4 μl JC-1 (1 mg/ml) in a dark room at 37 $^{\circ}\text{C}$ for 1 h. The JC-1 positive cells were detected by flow cytometer (Aria II, BD Biosciences, San Jose, CA, USA).

Apoptosis detection

Propidium iodide (PI) staining was used to quantify the apoptotic rates of cardiac carcinoma cells treated with MA. Cells (3.5×10^5) were cultured in each well of 6-well plates. Overnight, the cells were treated with MA and 24 h later the media was replaced with PBS and the cells were stained with PI (50 mg/ml) (BD Biosciences). The percentage of Sub G₀/G₁ population, which represents apoptotic cells, was analyzed using cytometrical analysis (Aria II, BD Biosciences).

Blocking of the p38 MAPK pathway

The cells were pretreated with SB203580 (10 μM , Cell Signaling Technology) 1 h prior to MA treatment. Subsequent experiments were performed to detect the survival, p-38 MAPK expression, MMP and apoptotic rates of cardiac carcinoma cells, following the procedures described elsewhere.

Statistical analysis

Each experiment was performed at least three times. All values were reported as means $\pm$ SD and compared at a given time point using an unpaired, two-tailed Student's t-test. Data were considered to be statistically significant when $p < 0.05$ (*) and $p < 0.01$ (**).

Results

MA reduced the viability of cardiac carcinoma cells

The stereochemical structure of maslinic acid (MA) was shown in Fig. 1. First, the effect of MA on the survival of cardiac carcinoma cells was examined. The viability of the cell lines derived from carcinoma of gastric cardia, SGC-7901 and BGC-823, as well as the primary cancer cells from cardiac carcinoma patients, was found to be reduced by MA in a dose-dependent fashion (Fig. 2A). In addition, DMSO exhibited some cytotoxicity to SGC-7901 cells at high concentrations (Fig. 2B). In contrast, the survival of normal cells, and Het-1A, MRC-5 and L-02 cells, was not affected until the dose of MA exceeded 100 μM (Fig. 2C). The IC₅₀ values were listed in Table 1. The above data showed that MA's cytotoxicity exhibited a relative selectivity for cardiac carcinoma cells.

MA reduced the growth of cardiac carcinoma xenografts in vivo

The effect of MA on the growth of cardiac carcinoma was evaluated in mice. SGC-7901 cells were subcutaneously injected into the flanks of nude mice to establish a tumor model.

FIGURE 2. *MA reduced the viability of cardiac carcinoma cells.* (A) The viability of SGC-7901, BGC-823 and two primary cardiac carcinoma cells treated with MA of indicated concentrations for 24 h, was detected with MTT assays. The bar represented means $\pm$ SD of three independent experiments. (B) The viability of cells was examined when treated with indicated concentration of DMSO. (C) The viability of normal cell lines, Het-1A, MRC-5 and L-02 cells were also detected under the same treatments.

TABLE 1. IC₅₀ value for maslinic acid and DMSO on cell lines

Cell lines	compound	IC ₅₀ (μ M)* means $\pm$ SD
SGC7901	DMSO	>10%
SGC7901	Maslinic acid	33.09 $\pm$ 3.15
BGC-823	Maslinic acid	23.85 $\pm$ 4.02
Primary 1	Maslinic acid	80.65 $\pm$ 7.94
Primary 2	Maslinic acid	45.71 $\pm$ 6.48
Het-1A	Maslinic acid	298.1 $\pm$ 26.04
MRC-5	Maslinic acid	867.6 $\pm$ 124.77
L-02	Maslinic acid	359 $\pm$ 54.12

* IC₅₀ value for DMSO on SGC7901 cells was expressed as %.

A

FIGURE 3. *MA inhibited the growth of cardiac carcinoma xenograft.* 5×10^6 SGC-7901 cells were used to establish cardiac carcinoma xenograft in BALB/c nude mice. MA was intraperitoneally administrated at the concentrations of 5 mg/kg (low concentration) ($n=7$) or 20 mg/kg (high concentration) ($n=7$). (A) The diameters of tumors were periodically measured and the volumes were calculated following the formula described in Materials and Methods. (B) After sacrificing, the tumors were weighted and shown as means $\pm$ SD.

MA was intraperitoneally injected at two different doses (5 mg/kg and 20 mg/kg) every other day. The results indicated that MA administration reduced the growth of tumor xenografts in a dose-dependent way, and high concentration of MA was able to suppress the growth of cancer xenograft by approximately 50% (Fig. 3A). The measurement of tumor weights also confirmed the inhibitory effect of MA on SGC-7901 xenograft growth (Fig. 3B).

p38 MAPK signaling was activated in cardiac carcinoma cells treated with MA

The mechanisms by which MA exerted its anti-tumor activity on cardiac carcinoma were investigated. Immunoblot analysis revealed that the phosphorylation of p38 MAPK was greatly increased in cancer cells treated with MA in both a dose- and time-dependent manner, suggesting that p38 MAPK signaling was activated by this stimulation (Fig. 4A and 4B).

Mitochondria-dependent apoptosis was triggered by MA in cardiac carcinoma cells

Considering that p38 MAPK is a major inducer of apoptotic pathway in cancer cells, MA was studied to determine if it induced apoptosis in carcinoma of gastric cardia. The evaluation of mitochondria membrane potential (MMP) in SGC-7901 cells revealed that MA treatment reduced MMP in cardiac car-

cinoma cells (Fig. 5A). Consistently, immunoblot assays revealed that pro-apoptotic protein, cytochrome C, was released into the cytosol from mitochondria under MA treatment (Fig. 5B).

Subsequent experiments indicated that the cleavages of caspase 3 and PARP also occurred in SGC-7901 cells treated with MA (Fig. 6A). Finally, MA-triggered apoptosis was confirmed in cardiac carcinoma cells by flow cytometrical analysis of the sub-G₀/G₁ population (Fig. 6B).

The activation of the p38 MAPK pathway mediated the effect of MA on carcinoma of gastric cardia

To confirm the importance of p38 MAPK activation for MA's anti-tumor activity on SGC-7901 cells, SB203580 was used to inhibit the activation of this signaling (Fig. 7A). SB203580 greatly increased both the viability and MMP of SGC-7901 cells under the treatment of MA (Fig. 7B and 7C). Consistent with this observation, apoptosis was found to be inhibited by SB203580 suppression of p38 MAPK signaling in cardiac carcinoma cells treated with MA (Fig. 7D). Collectively, these data suggest that p38 MAPK-mediated activation of the endogenous apoptotic pathway is indispensable for the anti-tumor activity of MA.

FIGURE 4. MA triggered the activation of p38 MAPK pathway in cardiac cancer cells. (A) The expression of phosphorylated p38 MAPK was detected in SGC-7901 cells treated with MA for 24 h of 10, 25 and 50 μM by immunoblot assays. Total p38 MAPK and GAPDH were also visualized as endogenous references. The densities of blots were quantified with ImageJ software, and shown as values relative to the control group. (B) The expression of phosphorylated p38 MAPK was also investigated in SGC-7901 cells at the indicated time points (6 and 24 h). Total p38 MAPK and GAPDH were also detected as endogenous references. The densities of blots were quantified with ImageJ software, and shown as values relative to the control group.

FIGURE 5. MA reduced MMP and triggered the release of cytochrome C in cancer cells. (A) After 24 h MA treatment, SGC-7901 cells were tested for MMP using JC-1 staining. The bars represented means $\pm$ SD of three independent experiments. (B) The levels of cytochrome C in the mitochondria and cytosol in SGC-7901 cells treated with MA for 24 h were detected by immunoblot assays. COX IV and GAPDH were selected as endogenous reference of mitochondrial compartment and cytosol, respectively.

FIGURE 6. *MA triggered apoptotic events in cancer cells.* (A) MA was added to the culture of SGC-7901 cells, and 24 h later, the expressions of cleaved caspase 9, caspase 3, and PARP were examined by immunoblot assays. GAPDH were selected as endogenous reference. The densities of blots were quantified with ImageJ software, and shown as relative values to control group. (B) Under the same conditions, the apoptotic rates in SGC-7901 cells were quantified by flow cytometrical analysis of the percentages of sub-G₀/G₁ population. The bars represented means $\pm$ SD of three independent experiments.

Discussion

Although the anti-tumor activity of MA has been verified in some types of cancer [10], it is still unknown if this natural compound can induce apoptosis in cardiac carcinoma. In this study, the effect of MA on the survival of cardiac carcinoma cells was investigated through multiple approaches. Our results revealed that MA induced apoptosis in cardiac carcinoma cells by activating p38 MAPK signaling. Actually, the induction of apoptosis has been documented to be one of the major mechanisms underlying the anti-tumor activity of MA. The apoptotic pathway can be triggered by MA, both in mitochondria-dependent and -independent ways [8, 11]. The association of p38 MAPK signaling with MA's anti-tumor activity has also been indicated in previous studies. Wu *et al.* have shown that MA elicits apoptosis in salivary gland cancer cells by activating the Ca²⁺/p38 MAPK pathway [12]. Furthermore, some other compounds have also been reported to induce apoptosis in gastric cancers by activating the p38 MAPK pathway, such as calebin-A [13], and phenethyl isothiocyanate [14].

Although our studies showed that MA induces apoptosis by activating the p38 MAPK pathway, the possibility cannot be excluded that some other pathways may be also involved with the pro-apoptotic activity of MA. Some studies demonstrated that MA can also trigger the apoptotic pathway in cancer cells by JNK and NF- κ B pathways [11, 15].

In conclusion, MA was shown to suppress the growth of cardiac carcinoma by inducing p38 MAPK-dependent apoptosis. This natural compound, and its derivatives, may be promising anti-tumor agents for cardiac carcinoma treatment in future.

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FIGURE 7. The suppression of p38 MAPK activation rescued the effect of MA on SGC-7901 cells. Small molecule inhibitor, SB203580 (10 μ M), was used to suppress the activation of p38 MAPK signaling prior to the subsequent experiments. (A) Immunoblot analysis of phosphorylated p38 MAPK expression was used to confirm the effectiveness of SB203580. The expression of phosphorylated p38 MAPK was detected in SGC-7901 cells treated with MA (50 μ M) or/and SB203580 (10 μ M) for 24 h. Total p38 MAPK and GAPDH were also detected as endogenous references. (B) MTT assays were performed to determine the viability of SGC-7901 cells treated with or without MA (50 μ M). The bars represented means $\pm$ SD of three independent experiments. (C) MMP was assessed in SGC-7901 cell with or without MA treatment. The bars represented means $\pm$ SD of three independent experiments. (D) Apoptotic rates in MA-treated and control SGC-7901 cells were evaluated by determining the percentage of sub-G₀/G₁ population. The bars represented means $\pm$ SD of three independent experiments.

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