Original Article

Antitumor effects of crocin on human breast cancer cells

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Abstract: Crocin is a chemical extracted from saffron and it is the most important kind of pigment of saffron. It has been proposed as a promising candidate for cancer prevention. In this study, we investigate the growth inhibition and the apoptosis of MCF-7 cells induced by Crocin, and explore the underlying molecular mechanism. We found that Crocin can significantly inhibit the proliferation of MCF-7 cells, and induce their apoptosis through mitochondrial signaling pathways including the activation of Caspase-8, upregulation of Bax, the disruption of mitochondrial membrane potential (MMP), and the release of cytochrome c. The studies showed that Crocin induced apoptosis of MCF-7 cells partially through caspase-8 mediated mitochondrial pathway. Therefore, we postulate that Crocin might have cancer-preventive and cancer-therapeutic benefit for human breast cancer.

Keywords: Crocin, breast cancer, apoptosis

Introduction

Breast cancer is the most common malignancy and one of the leading causes of cancer-related mortality amongst female worldwide. The incidence of breast cancer in the world, especially in most big cities, is dramatically increasing nowadays. During the period of 1980-2001, the incidence of breast cancer in various areas is 3.1% per year in growth, by 2010, the global incidence of breast cancer is as high as 1.6 million [1]. Many women ultimately develop metastatic breast carcinoma which is essentially an incurable disease, and the prognosis has changed little over the past decade. The majority of patients succumb to their disease within 2 years of diagnosis [2]. Based on the cancer stage, current treatment options include surgery, then perhaps radiation, hormonal therapy, and/or chemotherapy. Chemotherapy is an effective clinic treatment to resist cancer [3]. However, their clinical utility tends to be limited by the frequent development of drug resistance or other side effects [4]. Therefore, the search for novel chemotherapeutic agents has kindled great interest of scientists from diversified disciplines.

Crocin is a chemical extraction from saffron and it is the most important kind of pigment of saffron, formed from saffron acid glycosylation, water-soluble carotenoid are extremely rare. Its special solubility in water and its strong inhibitory effect on cancer cells, making it more expected to be used in cancer treatment. Many studies had shown that Crocin could inhibit proliferation and induce apoptosis in many kinds of cancerous cells [5]. So, we try to do some research about the effect of Crocin on breast cancer.

Apoptosis is a developmental phenomenon and process including deleting unneeded structures, sculpting structures and eliminating abnormal, damaged, redundant or harmful cells [6]. Apoptosis is a complicated process. The key molecular mechanism includes the death receptor pathway and mitochondrial pathway [7]. The mitochondria are the main site of action for members of apoptosis-regulating protein family, the Bcl-2 family, which play a critical role in the mitochondrial pathway of apoptosis [8, 9]. The antiapoptotic Bcl-2 family proteins like Bcl-2 and Bcl-xL inhibit the release of certain pro-apoptotic factors from mitochondria. In contrast, pro-apoptotic Bcl-2 family molecules like Bax and Bak induce the release of mitochondrial apoptogenic molecules into the cytosol [10]. Apoptotic signals induce a conformational change in the Bax protein, which is inti-
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In this study, we demonstrate that Crocin-induced apoptosis was related to the activation of caspase-8, 9, Bax upregulation, mitochondrial membrane potential disruption in MCF-7 cells. We concluded that Crocin induced MCF-7 cells apoptosis through activating caspase-8 and then the significant changes of Bax conformation, which resulted in increased loss of mitochondrial membrane potential, increased release of cytochrome c and activation of caspase-9 and caspase-3.

Materials and methods

Materials

Crocin (purity > 99%) was purchased from Sigma (USA), dissolved in distilled water (DW), and stored at -20°C. Human p53 ELISA Kit and Human cytochrome c ELISA Kit were purchased from Boster Biological Technology Inc. (Wuhan, China). Caspase-8 and caspase-9 colorimetric substrates, IETD-pNA, LEHD-pNA, MitoCapture Mitochondrial Apoptosis Detection Kit were purchased from BioVision Research Products (CA, USA). The mouse monoclonal antibody Bax (B-9) was purchased from Santa Cruz Biotechnology, Inc. (CA, USA).

Cell culture

MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), and maintained in a humidified atmosphere of 5% CO₂ in air at 37°C, two passages weekly.

Cell viability assay (MTT assay)

Cell viability was measured by the MTT method [13]. Briefly, cells were incubated in 96-well plates at a density of 4 × 10⁴ cells/cm² and incubated under various conditions as indicated. 20 µL of MTT tetrazolium salt (Sigma) dissolved in Hanks’ balanced solution at a concentration of 5 mg/mL was added to each well with the indicated treatment and incubated in CO₂ incubator for 4 h. Finally, the medium was aspirated from each well, and 150 µL of DMSO (Sigma) was added to dissolve formazan crystals, and the absorbance of each well was obtained using a Dynatech MR5000 plate reader at a test wave-length of 490 nm with a reference wavelength of 630 nm. The following formula was used to calculate cell viability: percentage cell viability = (absorbance of the experiment samples/absorbance of the control) × 100%.

Apoptosis assay

Apoptotic rates were determined by flow cytometry analysis using commercially available annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (KeyGEN, Nanjing, China). MCF-7 cells were treated with 10, 25, 50 µg/ml Crocin for 24 h and washed with PBS buffer, then fixed in ice-cold 70% ethanol for 15 min. A suspension of 100 µL was taken and incubated with 5 µL propidium iodine (20 µg/ml) and 5 µL annexin V-FITC in the dark for 30 min at room temperature. Finally, 400 µL cold PBS was added to each tube and analyzed by flow cytometry (Epicsxl, BECKMAN, USA).

Caspase activity assay

Caspase-8, caspase-9 protease activities were determined using a commercially available kit according to the manufacturer’s instructions. Briefly, MCF-7 cells (1 × 10⁵) treated with 50 µg/ml Crocin for 24 h were resuspended in 50 µL of chilled cell lysis buffer; cells were then incubated on ice for 10 min and centrifuged for 1 min in a microcentrifuge (10,000 g); then supernatants were transferred (cytosolic extract) to a fresh tube and put on ice for immediate assay or aliquot and stored at -80°C for future use. Next, diluted 50-200 µg protein was added to 50 µL cell lysis buffer for each assay, then 50 µL of 2 × reaction buffer and 5 µl of the 4 mM IETD-pNA (caspase-8)/LEHD-pNA (caspase-9) were added and incubated at 37°C for 1-2 h. Samples were read at 405 nm in a micro-liter plate reader.

Measurement of mitochondrial membrane potential (MMP)

MMP was measured by flow cytometry using the cationic lipophilic green fluorochrome Rh-123 [14]. Cells were harvested, washed twice with PBS, incubated with 1 µM Rh123 at 37°C for 30 min, and washed twice with PBS. Fluorescence was determined by flow cytometer with an excitation wavelength of 480 nm at FL-1 filter.
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Western blot analysis

Cells at 1 × 10⁷ cells/ml were treated with DMSO, Crocin and harvested after the indicated time. After the lysis procedure, the lysates were centrifuged at 12000 g for 10 min at 4°C. The determination of the protein concentration of supernatants using the BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL, USA), equal amounts of protein (50 mg) from each sample were separated by electrophoresis through SDS-PAGE gels (4-12% Tris-HCl, Nu, Invitrogen, Merelbeke, Belgium) and transferred to Hybond-C Super membrane (Amersham Pharmacia Biotech, Piscataway, NJ).

ELISA assay

Cytochrome c release was detected with commercially available ELISA kit using microtiter plates coated with human cytochrome c protein. In brief, 1:100 diluted cell lysates (including cytochrome c) and cell culture supernatant (including p53) were added to designated wells, then 50 µL of diluted biotin-conjugate was added to each well and incubated for 2 h at room temperature. Thereafter, microplates were emptied and washed three times with wash buffer, then 100 µL of diluted streptavidin-HRP was added to each well and the incubation continued for another hour at room temperature. Microplates were emptied and washed three times with wash buffer, 100 µL of TMB substrate solution was added to all wells, then incubated in the dark for 15-20 min. Then, add stop solution in 100 µL/well, samples were read at 450 nm in a microtiter plate reader within 1 h.

Statistical analysis

The data were expressed as means ± SD. Statistical analysis was performed by using Student’s t-test (two-tailed). The criterion for statistical significance was taken as P < 0.05.

Results

Crocin inhibits cell growth of MCF-7

Using MTT assay, we first examined whether Crocin could inhibit the proliferation of MCF-7 cells. As shown in Figure 1, the growth of MCF-7 cells was suppressed by the presence of various concentrations of Crocin in a time- and dose-dependent manner. And at 24 h, the IC₅₀ of Crocin on MCF7 cells is 60 µg/ml, while at 48 h, the IC₅₀ is 12.5 µg/ml, which shows the powerful growth inhibition of Crocin.

Crocin induces apoptosis of MCF-7 cells

To determine the apoptotic sensitivity of Crocin on MCF-7 cells, fluorescence staining was performed to detect the apoptotic cells. Treatment of MCF-7 cells with 10 µg/ml, 25 µg/ml, 50 µg/ml Crocin resulted in increasing number of apoptotic cells (Figure 2A). The quantification of apoptotic cells induced by Crocin was further calculated by flow cytometry analysis. At the dose of 50 µg/ml, the total percentage of apoptotic cells was 55.9%, which is significantly higher than the control group (Figure 2B).

Crocin enhances Bax conformation, caspase-8 activity and p53 expression

In this study, we examined the expression of Bax and bcl-2. After treatment with Crocin, the expression of Bax increased significantly than the control group and bcl-2 expression decreased with the accumulation of Crocin (Figure

Figure 1. Antiproliferative effects of various doses Crocin on MCF-7 cells. Cells were incubated with various concentration of Crocin and cell growth was determined by MTT after 24 h and 48 h treatment. Mean (points) ± SE (bars).
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Figure 2. Effect of Crocin on apoptosis of MCF-7 cells. A. Crocin caused strong apoptosis in MCF-7 cells after treated with various concentrations of Crocin for 24 h. The cells were collected and stained with annexin V-FITC/PI. B. The quantitative results of flow cytometry analysis. *P < 0.05 vs control group, n = 3.

Figure 3. Effects of Crocin on the Bax expression, caspase-8 activity and p53 expression in MCF-7. Cells were treated with 10 µg/ml, 25 µg/ml, 50 µg/ml Crocin for 24 h. Treatment of Crocin enhances the activity of caspase-8 (C) and p53 (A) expression and Bax, bcl-2 (B) expression. *P < 0.05, statistically significant in comparison with control.

3B). Clearly, these results indicated that Bax is very important for Crocin induced MCF-7 apoptosis. It has been shown that caspase-8 can trigger Bax activation [15]. So, we also determined the activation of caspase-8 in Crocin induced apoptosis. As shown in Figure 3C, the activity of caspase-8 was significantly enhanced in cells after treatment (Figure 3C). In addition, p53 could directly regulate Bax conformation [16]. For this reason, we also determined the expression of p53 by WB. The results showed that the expression of p53 was significantly enhanced after the treatment with Crocin (Figure 3A), which indicated that p53 was also involved in the process of Crocin induced MCF-7 apoptosis.
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Crocin impairs mitochondrial membrane potential (MMP) of MCF-7 cells

Bax protein is closely related to mitochondrial membrane permeability [17]. Since Crocin could induce Bax conformation in MCF-7 cells, thus it is necessary to detect mitochondrial membrane potential. We measured MMP by flow cytometer using cationic lipophilic green fluorochrome Rh123. After the treatment with 10 µg/ml, 25 µg/ml, 50 µg/ml Crocin, mitochondrial activity was decreased significantly compared with the control (Figure 4). It suggested that Crocin can significantly induce the mitochondrial activity decline.

Crocin induces apoptosis of MCF-7 cells through the cytochrome c-mediated caspase-9 activated apoptotic pathway

Cytochrome c mediated caspase 9 apoptotic pathway is a classic mitochondrial apoptotic pathways [18]. Cytochrome c was released from mitochondria into cytoplasm accompanying with the disruption of mitochondrial membrane potential, thus activating downstream Caspase-9, resulting in cell apoptosis. In this study, the expression level of cytosolic cytochrome c was enhanced after treated with Crocin (Figure 5A). Besides, we also found that the caspase-9 activity was significantly enhanced (Figure 5B).

Discussion

Recently, traditional Chinese medicines have been researched as a new source of anticancer drugs [18]. So far, much effort has been devoted to search for agents that can significantly induce the apoptosis of cancer cells. Maximizing efficacy with minimizing side effects has become a major goal in the discovery of apoptosis inducers.

Apoptosis, or programmed cell death, is a fundamental process essential for both development and maintenance of tissue homeostasis [19]. Crocin acted as a chemical extraction from saffron and it is the most important kind of pigment of saffron. In this study, we firstly investigated Crocin induced apoptosis in MCF-7 cells. It was demonstrated that Crocin could time- and dose-dependently inhibit cell proliferation. Flow cytometry analysis indicated Crocin could induce cell apoptosis effectively in a time- and dose-dependent manner. These results suggested that Crocin could induce MCF-7 cells apoptosis.

Apoptosis induced by caspase are mainly through two pathways, mitochondrial (endogenous) pathway and death receptor (exogenous) pathway [20]. The mitochondrial pathway generally involves an induction of mitochondrial permeability transition and the subsequent release of cytochrome c, Apaf-1, and procaspase-9 assembly in the cytosol into the apoptosome, leading to caspase-9 activation, which in turn activates effector caspases such as caspase-3. The death receptor pathway involves the engagement of the death receptors on the cell membrane and recruitment of the adaptor protein FADD and procaspase-8 to form a complex known as the death-inducing signaling complex. Active caspase-8 can directly activate caspase-3. Moreover, efficient apoptotic signaling often requires a cross-talk between the two pathways. Bid, a BH3-only pro-apoptotic member of Bcl-2 family is cleaved by caspase-8. Truncated Bid translocates into the mitochondria through the activation of the pro-apoptotic
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Bcl-2 family members Bax and Bak, permeabilizes these organelles, leading to the release of cytochrome c and SMAC/Diablo. SMAC binds the inhibitors of apoptosis, relieving caspase-3 for a full activation [21]. In our study, Crocin (50 µg/ml) induced apoptosis of MCF-7 cells through inducing the release of cytochrome c and activating caspase-9, upregulating p53 and also leading to Bax conformation. It clearly indicated that Crocin induced apoptosis through a mitochondrial pathway but required p53/Bax participation.

In our study, we proposed the possible pathway of Crocin induced MCF-7 cells apoptosis. Further studies are needed to support this mechanism. In conclusion, the results of present study indicate that Crocin could induce apoptosis in MCF-7 cells through a caspase-8-dependent mitochondrial pathway, involving p53 expression, Bax conformation and mitochondrial membrane potential loss. Importantly, this study could offer a molecular basis for the further development of Crocin as a novel and pharmacologically safe chemotherapeutic agent for breast cancer.

Disclosure of conflict of interest

None.

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References

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