Induction of apoptosis by casticin in cervical cancer cells: reactive oxygen species-dependent sustained activation of Jun N-terminal kinase

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Casticin, a polymethoxylflavone from Fructus viticis used as an anti-inflammatory agent in Chinese traditional medicine, has been reported to have anti-cancer activity. The purpose of this study was to examine the apoptotic activity of casticin on human cervical cancer cells and its molecular mechanism. We revealed a novel mechanism by which casticin-induced apoptosis occurs and showed for the first time that the apoptosis induced by casticin is mediated through generation of reactive oxygen species (ROS) and sustained activation of c-Jun N-terminal kinase (JNK) in HeLa cells. Casticin markedly increased the levels of intracellular ROS and induced the expression of phosphorylated JNK and c-Jun protein. Pre-treatment with N-acetylcysteine and SP600125 effectively attenuated induction of apoptosis by casticin in HeLa cells. Moreover, casticin induced ROS production and apoptotic cell death in other cervical cancer cell lines, such as CasKi and SiHa. Importantly, casticin did not cause generation of ROS or induction of apoptosis in normal human peripheral blood mononuclear cells and embryonic kidney epithelium 293 cells. These results suggest that ROS generation and sustained JNK activation by casticin play a role in casticin-induced apoptosis and raise the possibility that treatment with casticin might be promising as a new therapy against human cervical cancer.

Keywords human cervical cancer; casticin; apoptosis; reactive oxygen species; c-Jun N-terminal kinase

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Introduction

Cervical cancer is one of the most common malignancies for women in the world [1,2]. About 500,000 new cases are diagnosed each year, and its 5-year survival rate is only about 52% [3,4]. Of these, 11,270 new cases and 4070 deaths happened in USA in 2009, which is much lower than those in developing countries. It is a general phenomenon that the tumor suppressive factor p53 is inactive in most malignant cells and that almost half of the cancers have mutations in p53 [5]. Human papillomavirus infects the body, thus causing p53 mutation that can lead to human cervical cancer [6]. Radiotherapy and/or chemotherapy strategies are used in the clinical treatment of cervical cancer through induction of tumor cell apoptosis [7,8]. However, conventional drugs not only trigger apoptosis but also exert lethality on normal cells, causing serious toxicity [7,8]. Consequently, it is clinically important for us to find new adjuvant chemotherapy drugs that can induce apoptosis with higher selectivity and lower toxicity [7,8].

Flavonoids are found in many plants in nature. It is well documented that flavonoids can inhibit cell growth and induce cell apoptosis in various cancers [9–13]. For example, polymethoxyflavones have widespread and strong cytotoxicity (IC₅₀ between nM and low μM level), and thus may have potentials as chemopreventive and chemotherapy drugs [9,10]. It has been shown that the C-3’ and C-5 hydroxyl as well as the C-3 and C-4’ methoxyl groups are required for the significant anti-proliferative activity of flavone and that 5,3’-dihydroxy-3,6,7,4’-tetramethoxylflavone (casticin, also called vitexicarpin) also contains these groups [14]. It has been reported that casticin significantly inhibits the proliferation of HeLa cells with an IC₅₀ of 1.286 μM [14]. In addition, many studies have shown that casticin can inhibit the growth of human myelogenous leukemia cells [9] and induce cell death of leukemia cells through induction of apoptosis or mitotic catastrophe [15], even without functional p53. We previously reported that casticin-induced apoptosis of cervical cancer cells was mediated by reactive oxygen species (ROS) generation and mitochondrial signaling pathways [16]. Thus, it is worth investigating why casticin can induce cell apoptosis in p53 mutated human cervical cancer HeLa cells and its molecular mechanism. It has been shown that similar flavones, such as apigenin [17,18], jaceosidin [19], acacetin [20], luteolin, kaempferol, and quercetin [21,22], cause a higher
level of ROS in cancer cells than in normal cells, which ultimately causes cytotoxicity. Related studies have demonstrated that the activation of c-Jun N-terminal kinase (JNK), mitochondrial membrane potential collapse, cytochrome c release, caspase activation, and a series of cascade reactions that ultimately lead to cell death, are triggered by ROS generation. Thus, the purpose of this study was to investigate the molecular mechanism for regulating ROS generation and continuous JNK activation by casticin.

Materials and Methods

Drugs and chemical reagents
Casticin was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). The chemical constitution of casticin is shown in Fig. 1. Casticin has a molecular weight of 374.3, appears as yellow crystals, and has a purity of 98.0%.

Casticin was prepared in dimethyl-sulfoxide (DMSO) as a 10-mM stock solution and diluted in a medium to the indicated concentration before use. Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, USA), RPMI 1640 medium (Invitrogen), fetal bovine serum (FBS; Invitrogen), cell apoptosis enzyme-linked immunosorbent assay (ELISA) detection kit (Roche, Palo Alto, USA), propidium iodide (PI; Sigma, St Louis, USA), ethidium bromide (Sigma), 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes Inc, Eugene, USA), rabbit anti-human JNK polyclonal antibody (Cell Signaling, Beverly, USA), mouse anti-human p-JNK, c-Jun, and p-c-Jun antibodies (Santa Cruz Biotechnology, Santa Cruz, USA), and rabbit anti-caspase-3 and anti-PARP polyclonal antibodies (Cell Signaling) were purchased.

Cell lines and cell culture
The human cervical cancer cell lines (HeLa, CasKi, and SiHa) and human embryonic kidney epithelium 293 cell line were purchased from the China Centre for Type Culture Collection (Wuhan, China), maintained in DMEM supplemented with 10% FBS, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37°C in a humidified atmosphere of 5% CO2. Normal human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density-gradient centrifugation and cultured in RPMI 1640 medium supplemented with 20% FBS. PBMCs were acquired from a healthy volunteer after obtaining informed consent.

Histone/DNA ELISA for detecting apoptosis
The cell apoptosis ELISA detection kit was used to detect apoptosis in cells treated with casticin according to the manufacturer’s protocol. Briefly, cells were seeded in a 96-well plate at a density of 1 × 10⁴ cells/well for 24 h, and the testing agents were then added to the culture medium containing 10% FBS. After 24 h, we transferred the cytoplasm of the control and treatment groups to the 96-well plate pre-coated with streptavidin that had been previously incubated with the biotinylated histone antibody and peroxidase-tagged mouse anti-human DNA for 2 h at room temperature. The absorbance was measured at 405 nm, with the EXL-800-type enzyme-linked immunosorbent apparatus (Bio-Tek, Winchester, USA).

Flow cytometry analysis using PI staining
Cells were seeded at a density of 4 × 10⁶ cells/well in 250 ml culture flasks for 24 h and then treated with the medium containing various concentrations of the testing agents and 10% FBS for 24 h or 2.0 μM casticin for the indicated time. PI staining for DNA content analysis was performed, as previously described [23].

Determination of ROS
Intracellular ROS accumulation was measured by flow cytometry (FCM) using the fluorescent probe DCFH-DA as previously described [23]. Cells (2 × 10⁶) were collected after treatment with various concentrations of test agents and washed with serum-free medium and then incubated with 1 ml DCFH-DA for 30 min at 37°C in the dark. After incubation, the cells were washed with serum-free medium three times and analyzed within 30 min by FCM (EPICS-XL, Beckman, Pasadena, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Western blot analysis
Total cell extracts were obtained as previously described [23]. Cell lysate containing 50 μg of protein was separated on a 7.5%–12.5% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis and then blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, USA). Anti-p-JNK, -total JNK, -p-c-Jun, -total c-Jun, -caspase-3, -PARP, and -β-actin (1:1000 dilution for each) were used.
as primary antibodies. Signals were detected using an ECL kit (Amersham Pharmacia Biotech, Piscataway, USA). Images were scanned followed by densitometry analysis with Alphazmager 2200 software (Silk Scientific, Orem, USA). The ratios of p-JNK/total-JNK and p-c-Jun/total-c-Jun were determined for the phosphorylation level of JNK or c-Jun protein.

Statistical analysis
The data are presented as the mean values from three separate experiments ± SD. Data were statistically analyzed by a Dunnet’s test after a one-way analysis of variance. $P < 0.05$ was considered significant difference.

Results

Effect of casticin on apoptosis in HeLa cells
Exposure of HeLa cells to 0.5, 1.0, 2.0, 4.0 μM casticin for 24 h significantly induced histone/DNA fragmentation in a concentration-dependent manner [Fig. 2(A)]. As shown in Fig. 2(B,C), casticin treatment resulted in a dose- and time-dependent increase of the sub-G1 population in

![Figure 2 Casticin induced apoptosis of HeLa cells](image-url)
HeLa cells \((P < 0.05)\). After treatment with casticin at concentrations of 2.0 and 4.0 \(\mu M\), respectively, 23.97\% \(\pm\) 1.33\% and 46.38\% \(\pm\) 3.64\% of HeLa cells underwent apoptosis [Fig. 2(B)]. In addition, casticin (4.0 \(\mu M\)) activated caspase-3 and induced cleavages of its substrate PARP in HeLa cells. These cleavages were indicated by a decrease in the uncleaved forms of caspases and its substrate, and/or the appearance of their cleaved forms [Fig. 2(D), lane 2]. Taken together, the results above demonstrate that casticin, in a dose-dependent manner, induces apoptotic cell death of HeLa cells.

**Effect of casticin on ROS generation in HeLa cells**

After treatment of HeLa cells with 0.5, 1.0, 2.0, 4.0 \(\mu M\) casticin for 6 h, the level of ROS increased in a dose-dependent manner and was abrogated by pre-treatment with the thiol-containing anti-oxidant, 10 mM \(N\)-acetylcysteine (NAC) [Fig. 3(A)]. Time course experiments revealed that the levels of ROS increased initially at 1 h, reached a peak at 6 h, and persisted for up to 24 h after treatment with 2.0 \(\mu M\) casticin [Fig. 3(B)].

**Effect of casticin-induced ROS generation on apoptosis in HeLa cells**

To determine a link between elevation of the intracellular ROS level and apoptotic cell death in casticin-treated cells, HeLa cells were pre-incubated with NAC before treatment with casticin. The increase of histone/DNA fragmentation induced by casticin was attenuated by pre-treatment with 10.0 mM NAC \((P < 0.05)\) [Fig. 4(A)]. Meanwhile, the results of FCM analysis indicated that the sub-G1 population was reduced with the combination of casticin and NAC [Fig. 4(B)]. In the presence of NAC, the ability of casticin to activate the caspases was suppressed [Fig. 2(D), lane 3]. These findings provide evidence that the apoptosis induced by casticin in HeLa cells is dependent on ROS generation.

**Effect of casticin on JNK activation in HeLa cells**

JNK activation was measured by western blot analysis of phosphorylated JNK and its downstream target c-Jun. In HeLa cells treated with casticin (0.5, 1.0, 2.0 \(\mu M\)) for 12 h, JNK activation in a dose-dependent manner was observed [Fig. 5(A)]. Time course experiments revealed initial JNK activation at 6 h post-treatment, peak JNK activation at 12 h, and persistent activation for up to 24 h [Fig. 5(B)].

**Effect of JNK activation by casticin on apoptosis of HeLa cells**

Figure 6(A) showed that the expression of phosphorylated JNK protein after 12 h in casticin-treated cells was inhibited by pre-treatment with NAC and SP600125 (a JNK inhibitor). To examine the effects of casticin-stimulated JNK activation on induction of apoptosis in HeLa cells, we used SP600125. SP600125 substantially reduced casticin-induced apoptosis of HeLa cells [Fig. 6(B,C)]. These results suggest that ROS production and JNK activation are required for casticin-induced apoptosis in HeLa cells.

**Effects of casticin on apoptosis and ROS generation in other human cervical cancer cells and normal human PBMCs**

We next investigated whether casticin induced apoptosis and ROS generation in other cervical cancer cells using the
Casticin induces apoptosis by ROS and activation of JNK

Discussion

The present study has demonstrated that casticin (i) dose-dependently induced apoptosis in the human cervical cancer cell lines HeLa, CasKi, and SiHa; (ii) led to a concentration-dependent generation of ROS; (iii) induced apoptosis of HeLa cells through generation of ROS; (iv) triggered the activation of JNK in a ROS-dependent manner; (v) induced JNK activation-dependent apoptosis of HeLa cells; and (vi) did not induce apoptosis or ROS production in PBMCs and 293 cells.

Casticin has been reported to inhibit the proliferation of HeLa cells with an IC_{50} of 1.286 μM [14]. Recently, we found that casticin significantly induced apoptosis of cervical cancer cells by ROS generation and mitochondrial signaling pathways [16]. Shen et al. [15] showed that casticin induced cell death of leukemia cells through induction of apoptosis or mitotic catastrophe. We demonstrated that casticin significantly induced apoptotic cell death in the human cervical cancer cell lines HeLa, CasKi, and SiHa (Figs. 2 and 7) and that this effect was not specific to human cervical cancer cell types with respect to p53 mutation or loss.

Figure 4 Casticin induced ROS-dependent apoptosis of HeLa cells (A) HeLa cells were treated with different concentrations (0.5, 1.0, and 2.0 μM) of casticin in the presence or absence of 10 mM NAC for 24 h, and the histone/DNA fragments were analyzed by ELISA. Columns represent the mean from three independent experiments and bars represent standard deviation. *P < 0.05; **P < 0.01; ***P < 0.001 vs. 0.1% DMSO; #P < 0.05; ##P < 0.01; ###P < 0.001 vs. treatment with 10 mM NAC plus the same concentration casticin. (B) HeLa cells were treated the same as in (A), and the sub-G1 cell population was analyzed by FCM using PI staining. Columns represent the mean from three independent experiments and bars represent standard deviation. *P < 0.05; **P < 0.01; ***P < 0.001 vs. 0.1% DMSO; #P < 0.05; ##P < 0.01; ###P < 0.001 vs. treatment with 10 mM NAC plus the same concentration casticin.

Figure 5 Casticin induced JNK activation in HeLa cells (A) Cells were treated with 0.5, 1.0, and 2.0 μM casticin for 24 h. The phosphorylation level of JNK was analyzed by immunoblotting. The relative ratio of p-JNK/JNK and p-c-Jun/c-Jun are shown as the average results of three independent experiments. One experiment representative is shown. (B) The phosphorylation levels of JNK were analyzed by immunoblotting after 2.0 μM casticin treatment for 6, 12, and 24 h, and then the relative ratios of p-JNK/JNK and p-c-Jun/c-Jun were calculated. One experiment representative is shown.
ROS, active, transitory, and oxygenic compounds, including H₂O₂, O₂, and hydroxyl radicals, are metabolites of biochemical processes in the body. There is an integrated system of clearing ROS in the body to keep balance. Oxidation of cell membrane phospholipids, enzyme, and DNA [24–27] by high levels of ROS can alter function of signal transduction, platelet aggregation, immune control, and the regulation of cell growth, and they can also cause necrosis or apoptosis [28–31]. As the generation of ROS is the result of disordered functioning of mitochondria and metabolite augmentation, there may be ways to regulate ROS selectively in cancer cells [12,32]. We made use of FCM using a DCFH-DA probe to measure ROS after casticin treatment in human cervical cancer cells, and it was found that casticin generated ROS in human cervical cancer cells in a dose-dependent manner (Figs. 3 and 7). Furthermore, NAC suppressed the apoptosis of HeLa cells by casticin, suggesting that the apoptotic effect was dependent on ROS generation. These results are coincident with that in our previous study [16]. It has been shown that ROS generation is upstream of JNK activation [33], so Mitogen-Activated Protein Kinase Kinase Kinases might be positively activated by ROS to further activate JNK. However, it is possible that JNK activation is triggered through caspase cascades induced by ROS [34–36].

In spite of contradictory evidence regarding JNK regulation of apoptosis, significant evidence exists showing that sustained JNK activation causes apoptosis through the mitochondria-dependent pathway [34,37]. It was found that the phosphorylation levels of JNK and c-Jun increased in a dose-dependent manner after various concentrations of casticin treatment for 24 h, and this could be inhibited significantly by pre-treatment with NAC or SP600125. This suggested that an ROS-dependent effect of sustained JNK activation was caused by casticin. Further, ELISA and FCM experiments showed that SP600125 attenuated the apoptosis of HeLa cells induced by casticin, indicating that this may be a key mechanism for sustained activation of JNK resulting in casticin-induced apoptosis in HeLa cells (Fig. 6). We recently reported that casticin-induced apoptosis of human hepatocellular carcinoma cells is involved in DR5 upregulation [38]. However, whether casticin induces increase of DR5 protein in human cervical cancer cells remains be elucidated.

Kobayakawa et al. [11] reported that casticin markedly inhibited the growth of KB cells, but did not inhibit the proliferation of A431 cells, which is similar to the normal cell lines 3T3 Swiss Albino and TIG-103. In the present study, we showed that casticin specifically induced apoptosis in human cervical cancer cells but not in PBMCs and
293 cells (Figs. 2 and 7), although the mechanism of selective induction of apoptosis has not been determined. Our findings suggest that casticin may be a specific anti-tumor agent with low toxicity.

In summary, the present study has for the first time demonstrated that casticin induced dose-dependent apoptosis in p53 mutated human cervical HeLa cells and highlighted the molecular mechanism of ROS generation and sustained JNK activation that resulted in apoptosis. The results of this article raise the possibility that casticin might be a promising candidate for human cervical cancer therapy.

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**References**

Casticin induces apoptosis by ROS and activation of JNK