RESEARCH ARTICLE

Caspase Inhibitor Diminishes Caffeic Acid-induced Apoptosis in Osteosarcoma Cells

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Received date:Jun 13, 2017; Revised date: Aug 2, 2017; Accepted date: Aug 3, 2017

Abstract

BACKGROUND: Caffeic acid has been shown to induce apoptosis in MG63 osteosarcoma cells. Along with the apoptotic induction, caffeic acid was shown to activate caspase-8, -9 and -3. However, the role of caspase in mediating caffeic acid-induced apoptosis in MG63 cells are not clear yet. In this study, caspase role was further investigated by inhibiting caspase activity in the caffeic acid-induced apoptosis system in the MG63 cells.

METHODS: MG63 cells were cultured, starved, pretreated with/without Z-VAD FMK and treated with/without 10 µg/mL caffeic acid. To quantify the number of apoptotic MG63 cells, Sub-G1 method was performed. The caffeic acid-induced apoptotic morphology was confirmed with 4',6-diamidino-2-phenylindole (DAPI) staining and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Meanwhile, to detect apoptotic underlying mechanism, immunoblotting was performed to detect caspase-8, -9 and -3.

RESULTS: The MG63 cells were significantly induced into apoptosis with the treatment of 10 µg/mL caffeic acid for 48 hours. However, pretreatment of 100 µM Z-VAD-FMK, a pan caspase inhibitor, for 2 hours, the percentage of apoptotic MG63 cells was significantly diminished. The apoptotic phenomenon induced by caffeic acid as well as the inhibition of Z-VAD-FMK were confirmed by DAPI staining and TUNEL assay. Cleaved caspase-8, -9 and -3 were formed markedly upon the treatment of caffeic acid. Pretreatment of 100 µM Z-VAD-FMK could inhibit the cleaved caspase-8, -9 and -3.

CONCLUSION: Taken together, caffeic acid has the potential to induce apoptosis in MG63 cells, specifically through the caspase signaling pathway.

KEYWORDS: caffeic acid, apoptosis, MG63, caspase, Z-VAD FMK

Indones Biomed J. 2017; 9(3): 160-4

Introduction

Osteosarcoma is common in children or young adults. Osteosarcoma is reported to be the second highest cause of cancer-related deaths in this age group, mainly because of the frequent fatal metastases to the lungs.(1) Treatment using various chemotherapy regimens in the group of patients with different subtypes, did not significantly affect the survival of the patient. Therefore, new alternatives and other forms of therapy are needed.(1,2)

Various studies have been done to achieve the key to successful treatment of osteosarcoma. Approach through the mechanism of cancer cell apoptosis and the empowerment of natural materials to be one promising alternative, such as Ayamurasaki and Simon extract.(3,4) Two varieties of sweet potato (*Ipomoea batatas L.*) are reported to contain caffeic acid compounds that proved to have the ability to inhibit

osteoclastogenesis.(4-6) Caffeic acid significantly reduces the activity of nuclear factor κB (NFκB), a cell signaling pathway involved in osteoclastogenesis (5), in which NFκB inhibition can cause tumor cells to stop proliferating or becoming more sensitive to the actions of anti-tumor agents.(7)

Caffeic acid and related polyphenolic compounds today attract much special attention because of its ability to protect the human body from oxidative stress that can lead to cancer (8), aging (9), and cardiovascular disease (10). A previous study has shown that caffeic acid can induce apoptosis in MG63 osteosarcoma cells and show the presence of caspase-8, -9 and -3 in the induction of apoptosis.(11) However, the role of caspase in mediating caffeic acid-induced apoptosis in MG63 cells are not clear yet. Therefore, in this study, caspase role was further investigated by inhibiting caspase activity in the caffeic acid-induced apoptosis system in the MG63 cells.

Methods

Cell Culture

MG63 cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (Gibco) and antibiotic-antimycotic containing 200 units/mL penicillin, 200 µg/mL streptomycin and 0.5 µg/mL amphotericin (Gibco) in a humidified, 37°C, 5% CO2 incubator. Upon reaching 80% confluence, cells were sub-cultured.

Sub-G1 Assay

Sub-G1 assay of apoptotic cells was performed as described by Sandra, *et al.*(12,13) Briefly, MG63 cells were treated with 0-10 µg/ml caffeic acid (Wako, Osaka, Japan), harvested and suspended in 1 mL of hypotonic fluorochrome solution (50 µg/mL propidium iodide in 0.1 % sodium citrate plus 0.1 % Triton X-100). Cell suspension was placed at 4°C in the dark for 2 hours before the flow cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured with a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

4',6-diamidino-2-phenylindole (DAPI) Staining

DAPI staining was performed as described by Sandra, *et al.*(14,15) Cells were seeded onto coverslips, stimulated, rinsed in phosphate buffered saline (PBS) and fixed with ice-cold tricholoroacetic acid and gradient ethanol. Then cells were permeabilized with 0.1% BSA and 0.1% Triton X-100 for 3 minutes, and stained with 1 µg/ml 4',6-Diamidino-2-phenylindole (Calbiochem) for 3 minutes. Then, the coverslips were rinsed with PBS, mounted with glycerol on glass slides, and evaluated under fluorescence microscope.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

This experiment was carried out using Apoptag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA). Briefly, cells were fixed with 1% paraformaldehyde in PBS (pH 7.4) and incubate in pre-cooled ethanol : acetic acid (2:1). Fixed cells were quenched in 3% hydrogen peroxide in PBS. Later, equilibration buffer was applied on the cells, followed by incubation of TdT enzyme in a humidified chamber for 1 hour. To stop the reaction, cells were washed with stop buffer and anti-digoxigenin peroxidase conjugate was applied on the cells in a humidified chamber for 30 minutes. For visualization, peroxidase substrate was applied and color development was monitored under microscope. Cells were later counterstained in methyl green. Negative control was done by replacing TdT enzyme with PBS, while positive control was provided along with the kit.

Immunoblotting

Immunoblotting was performed as described by Sandra, *et al.*(16,17) Treated cells were harvested and incubated with lysis buffer containing 20 mM Tris buffer (pH 7.4), 5 mM EDTA, 1% Triton-X, 50 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1mM p-amidinophenyl methanesulfonyl fluoride hydrochloride and protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to a pure nitrocellulose membrane. After blocking with 5% skim milk in phosphate buffer saline (pH 7.4), the sheets were probed with the following 1:1000 diluted antibodies: mouse monoclonal anti-cleaved caspase-8 (Asp384) (Cell Signaling, Beverly, MA, USA), rabbit polyclonal anti-cleaved caspase-9 (Asp315) (Cell Signaling) and rabbit polyclonal anti-cleaved caspase-3 (Asp175) (Cell Signaling). The secondary antibody was 1:1000 diluted horseradish peroxidase-conjugated donkey anti-rabbit (Amersham, Buckinghamshire, UK) or 1:2000 diluted horseradish peroxidase-conjugated sheep anti-mouse (Amersham) antibody. The bound antibodies were visualized using Immun Star HRP Chemiluminescent Kit (BioRad, Hercules, CA, USA). All visualized bands were captured using Alliance 4.7 (UVItech, Cambridge, UK).

Statistical Analysis

Shapiro Wilk test was performed to determine distribution of apoptosis with Sub-G1 assay. Independent samples

T test was performed to compare apoptosis pretreated with/without Z-VAD-FMK and treated with caffeic acid and. Statistical analysis was performed using IBM SPSS Statistics for Macintosh ver. 21 (SPSS IBM, Armonk, NY, USA). Statistically significance was expressed as $p<0.05$.

Results

Caspase Inhibitor Diminished Caffeic Acid-induced Apoptotic Cells

MG63 cells were significantly ($p=0.000$) induced into apoptosis with the treatment of 10 µg/mL caffeic acid for 48 hours (Figure 1). However, pretreatment of 100 µM Z-VAD-FMK, a pan caspase inhibitor, for 2 hours, the percentage of apoptotic MG63 cells was significantly ($p=0.000$) diminished.

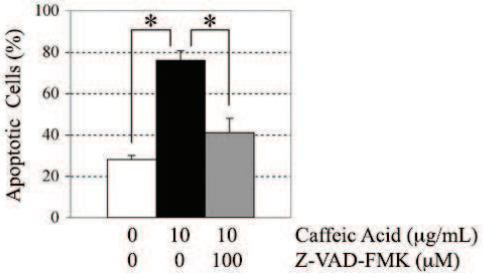

Figure 1. Caspase inhibitor diminished caffeic acid-induced apoptotic cells. MG63 cells were starved for 12 hours, pretreated with/without 100 µM Z-VAD FMK for 2 hours, before addition of 10 µg/mL caffeic acid for 48 hours. Then cells were processed for Sub-G1 assay. These experiments were repeated 3 times. Results were statistically analyzed with Independent T test. *$p<0.05$.

Caspase Inhibitor Diminished Caffeic Acid-induced Nuclear Fragmentation

With DAPI staining, treatment of 10 µg/mL caffeic acid for 48 hours clearly showed nuclear fragmentation in MG63 cells (Figure 2). Meanwhile, pretreatment of 100 µM Z-VAD-FMK for 2 hours, the nuclear fragmentation in MG63 cells was abolished.

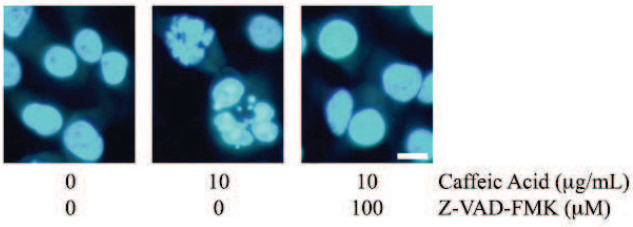

Figure 2. Caspase inhibitor diminished caffeic acid-induced nuclear fragmentation. MG63 cells were starved for 12 hours, pretreated with/without 100 µM Z-VAD FMK for 2 hours, before addition of 10 µg/mL caffeic acid for 48 hours. Then cells were processed for DAPI staining. These experiments were repeated 3 times.

Caspase Inhibitor Diminished Caffeic Acid-induced DNA Fragmentation

TUNEL assay which can label the 3'-OH termini in the double-strand showed that treatment of 10 µg/mL caffeic acid for 48 hours induced DNA fragmentation in MG63 cells (Figure 3). By pretreatment of 100 µM Z-VAD-FMK for 2 hours, the DNA fragmentation in MG63 cells was diminished.

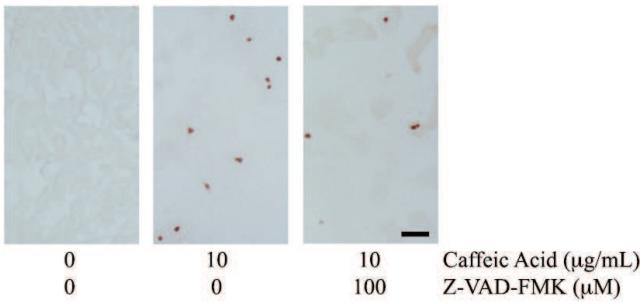

Figure 3. Caspase inhibitor diminished caffeic acid-induced DNA fragmentation. MG63 cells were starved for 12 hours, pretreated with/without 100 µM Z-VAD FMK for 2 hours, before addition of 10 µg/mL caffeic acid for 48 hours. Then cells were processed for TUNEL assay. These experiments were repeated 3 times.

Caspase Inhibitor Diminished Caffeic Acid-induced Cleavage of Caspase-8, -9 and -3

Under treatment of 10 µg/mL caffeic acid for 48 hours, cleaved caspase-8, -9 and -3 were formed markedly (Figure 4). However, pretreatment of 100 µM Z-VAD-FMK for 2 hours could inhibit the cleaved caspase-8, -9 and -3.

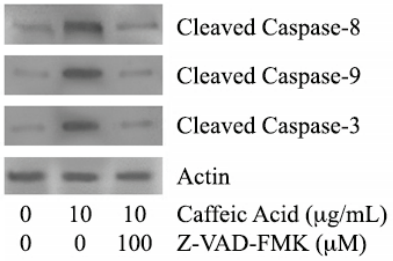

Figure 4. Caspase inhibitor diminished caffeic acid-induced activation of Caspase-8, -9 and -3. MG63 cells were starved for 12 hours, pretreated with/without 100 µM Z-VAD FMK for 2 hours, before addition of 10 µg/mL caffeic acid for 48 hours. Cells were collected, lysed and processed further for immunoblotting detection by using anti-cleaved caspase-8, -9 and -3 antibodies. These experiments were repeated 3 times.

Discussion

Caffeic acids are examples of natural ingredients that are currently being studied and have demonstrated their multi-

pharmacological potential. The results of this study confirm the potential of caffeic acid as an anti-cancer agent by inducing apoptosis in MG63 cells. This was demonstrated by a significant difference in the number of apoptotic cells between the control group and the caffeic acid-treated group ($p<0.05$), in which the percentage of apoptotic cells was higher in the caffeic acid-treated group. The results also support other previously reported studies of the potential of caffeic in inducing apoptosis in cancer cells.(8,11)

In the complex mechanism of apoptosis, caspase plays a very crucial role. In the present study, we found a significant difference in the number of apoptotic cells between caffeic acid-treated group and Z-VAD-pretreated group ($p<0.05$), in which the percentage of apoptotic cells was lower in the Z-VAD pretreated group. This suggest that after the caspase was inhibited by Z-VAD FMK, most MG63 cells did not turn into apoptosis, highlighting the importance of the caspase signaling pathway for MG63 cells to turn into apoptosis. This phenomenon was confirmed by DAPI staining as well as TUNEL assay.

The involvement of the caspase signaling pathway was also shown in previous studies using natural Indonesian plants, including Brucea javanica (17,18) and Artocarpus heterophyllus (19,20). Both of these natural plants are reported to decrease expression of pro-caspase-3. In present study, the pretreatment of Z-VAD FMK, a pan caspase inhibitor, caused a decrease of apoptotic cell number. Accordingly cleaved or activated Caspase-8, -9 and -3 by caffeic acid were markedly diminished by Z-VAD FMK. However, although it has been shown that caffeic acid can induce apoptosis in MG63 cells through the caspase signaling pathway, there are also possibility of alternative pathways or caspase-independent pathway.

The results of this study are expected to be the basis for the development of pharmacological design of a new generation of drugs for osteosarcoma. However, the complexity of apoptotic mechanisms requires further investigation, particularly to determine the caspase-independent pathway involved in the process of MG63 cell apoptosis by caffeic acid and its effect on other osteosarcoma cell types. Taken together, caffeic acid has the potential to induce apoptosis in MG63 cells, specifically through the caspase signaling pathway.

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