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RESEARCH ARTICLE

Caffeic Acid Induces Intrinsic Apoptotic Pathway in MG-63 Osteosarcoma Cells Through Bid Truncation and Cytochrome *c* Release

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Abstract

BACKGROUND: Caffeic acid has been reported to induce apoptosis in MG-63 osteosarcoma cells via caspases activation. However, apoptotic pathway that is involved in the caffeic acid-induced apoptosis is still unclear. Present study aimed to investigate the role of cytochrome *c* (Cyt *c*) release and BH3-interacting death (Bid) activation in caffeic acid-induced apoptosis in MG-63 osteosarcoma cells.

METHODS: MG-63 cells were cultured, pretreated with/without Z-VAD FMK and treated with/without 10 µg/mL caffeic acid. Treated MG-63 cells were then lysed, homogenized, and processed further to prepare cell lysate and mitochondrial fraction. Immunoblotting method was used to measure the amount of Bid and truncated Bid (t-Bid) as well as mitochondrial and cytosolic Cyt *c*.

RESULTS: The amount of Bid and mitochondrial Cyt *c* in MG-63 cells decreased in a time-dependent manner, while the amount of t-Bid and cytosolic Cyt *c* increased in a time-dependent manner. By pretreatment of 100 µM Z-VAD-FMK for 2 h, the amount of Bid and mitochondrial Cyt *c* was significantly higher, while the amount of t-Bid and cytosolic Cyt *c* was significantly lower after caffeic acid treatment for 6 and 12 h compared to MG-63 cells that were not pretreated.

CONCLUSION: Caffeic acid could induce Cyt *c* release through the activation of Bid in MG-63 osteosarcoma cells.

KEYWORDS: caffeic acid, osteosarcoma, MG-63 cells, Bid, t-Bid, cytochrome c, Z-VAD-FMK

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Introduction

Osteosarcoma is a primary malignant bone tumor characterized by the presence of polyhedral or spindle mesenchymal cells which produce and accumulate disorganized and immature osteoid matrix.(1) Jaw osteosarcoma commonly arises in the mandible and comprises only 6% of all osteosarcoma cases.(2) Several conventional approaches can be used to treat osteosarcoma, including surgery, radiotherapy, and chemotherapy.(3) Although the combination of standard treatments improves the 5-year overall survival rate of osteosarcoma patients (4,5), poor response to these treatment regimens still persisted.(3) Therefore, the discovery of novel therapy for treating osteosarcoma is still necessary (6) to avoid possible side effects (7) as well as improve the outcome of osteosarcoma patients.(8)

A wide variety of natural compounds and their derivatives has been reported to have an anticancer potential. Caffeic acid (3,4-dihydroxycinnamic acids) is a major natural polyphenol found in food plants, such as buckwheat (9), blueberry (10), and sweet potato (11). This compound has been known to have numerous physiological

effects, such as antimicrobial (12), antioxidant (13), and anti-inflammatory properties (14). In addition, caffeic acid has been reported to inhibit osteoclastogenesis by inhibiting NF-κB activation.(15-19) Inhibition of NF-κB plays a key role in tumor proliferation.(20) Previous studies have reported that caffeic acid induced apoptosis in MG-63 osteosarcoma cells via caspases activation.(21,22)

Crosstalk between the extrinsic and intrinsic apoptotic pathway involves the cleavage of BH3-interacting death (Bid) to form truncated Bid (t-Bid). t-Bid enter the mitochondria and cause cytochrome *c* (Cyt *c*) release from mitochondria to cytosol. Cyt *c* is involved in the formation of the apoptosome complex, which activates downstream effector caspases, such as caspase-3.(23,24) Apoptotic pathway that is involved in the caffeic acid-induced apoptosis is still unclear. Present study aimed to investigate the role of Cyt *c* release and Bid activation in caffeic acid-induced apoptosis in MG-63 osteosarcoma cells.

Methods

MG-63 Cell Culture

MG-63 cell culture was performed as previously described. (22) MG-63 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (BioSource, Camarillo, CA, USA), and antibiotic-antimycotic containing 0.5 µg/mL amphotericin, 200 units/mL penicillin, and 200 µg/mL streptomycin (Gibco). After reaching 80% confluency, MG-63 cells were dissociated with trypsin (Gibco) and sub-cultured.

Caffeic Acid Treatment

MG-63 cells were seeded into a 24-well plate containing DMEM supplemented with antibiotic-antimycotic and pretreated with/without 100 µM Z-VAD-FMK for 2 h. MG-63 cells were then treated with 10 µg/mL caffeic acid (Wako, Osaka, Japan) and incubated for 1, 6, and 12 h.

Cell Lysate and Mitochondrial Fraction Preparation

Cell lysate and mitochondrial fraction were prepared as previously described.(25) MG-63 cells were homogenized in 200 µL of ice-cold solution containing protease inhibitors cocktail, 0.3 M sucrose, and 10 mM Tris–HCl (pH 7.5). MG-63 cells were then centrifuged for 60 min at 100,000 g, 4°C. The resulting supernatant was collected as the cytosolic fraction, while the resulting pellet was resuspended in 200 µL ice-cold solution containing 150 mM NaCl, 1% Triton X-100, 10 mM Tris–HCl (pH 7.5), and mixture of protease inhibitor. The pellet was then sonicated and centrifuged for 30 min at 10,000 g, 4°C. The supernatant was collected as the mitochondrial fraction.

Immunoblotting

The amount of Bid and t-Bid as well as mitochondrial and cytosolic Cyt *c* were measured using immunoblotting method as previously described (21) with modification. Briefly, treated MG-63 cells were harvested and lysed 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, 1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride and protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Samples from each experimental group were separated using sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Upon blocking with 5% skim milk solution, the membrane was incubated with rabbit polyclonal anti-Bid (Cell Signaling, Beverly, MA, USA) or mouse monoclonal anti-cytochrome *c* antibody (Becton Dickinson, Franklin Lakes, NJ, USA). The secondary antibody was horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (Amersham, Buckinghamshire, UK) or HRP-conjugated sheep anti-mouse antibody (Amersham). The membrane was then immersed in HRP color development solution to visualize the bound antibodies. All bands were documented and semi-quantified using Alliance 4.7 (UVITech, Cambridge, UK) and UVIband software (UVItech), respectively.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 20 (SPSS IBM, Armonk, NY, USA). Shapiro-Wilk test was used as a normality test. Independent sample t-test or Mann-Whitney test was performed to compare the amount of Bid and t-Bid, as well as the amount of mitochondrial and cytosolic Cyt *c* in each experimental group. A $p<0.05$ was considered as statistically significant.

Results

Caffeic Acid Induced the Activation of Bid in MG-63 Cells

The amount of Bid in MG-63 cells was significantly lower in a time-dependent manner compared to the negative control group after caffeic acid treatment for 1, 6, and 12

h. By pretreatment of 100 μM Z-VAD-FMK for 2 h, the amount of Bid after caffeic acid treatment for 6 and 12 h was significantly higher compared to MG-63 cells that were not pretreated (Figure 1). In contrast, the amount of t-Bid in MG-63 cells was significantly higher in a time-dependent manner compared to the negative control group after caffeic acid treatment for 1, 6, and 12 h. However, pretreatment of Z-VAD-FMK significantly decreased the amount of t-Bid after caffeic acid treatment for 6 and 12 h (Figure 2).

Caffeic Acid Induced the Cyt *c* Release to Cytosol of MG-63 Cells

Upon caffeic acid treatment, the amount of mitochondrial Cyt *c* in MG-63 cells decreased in a time-dependent manner. The amount of mitochondrial Cyt *c* after caffeic acid treatment for 1 h was not significantly different compared to the negative control group. Meanwhile, the amount of mitochondrial Cyt *c* after caffeic acid treatment for 6 and 12 h was significantly lower than the negative control group. By pretreatment of 100 μM Z-VAD-FMK for 2 h, the amount of mitochondrial Cyt *c* after caffeic acid treatment for 6 and 12 h was significantly higher compared to MG-63 cells that were not pretreated (Figure 3). Inversely, the amount of cytosolic Cyt *c* in MG-63 cells increased in a time-dependent manner. The amount of cytosolic Cyt *c* after caffeic acid treatment for 1 h was not significantly different, compared to the negative control group. Meanwhile, the amount of cytosolic Cyt *c* after caffeic acid treatment for 6 and 12 h was significantly higher than the negative control group. Pretreatment of Z-VAD-FMK significantly decreased the amount of cytosolic Cyt *c* after caffeic acid treatment for 6 and 12 h (Figure 4).

Discussion

Present study demonstrated that caffeic acid reduced the amount of Bid and increased the amount of t-Bid in a time-dependent manner, indicating the truncation of Bid to t-Bid. Z-VAD-FMK pretreatment markedly increased the amount of Bid, as well as diminished the amount of t-Bid, highlighting the importance of Bid activation in caffeic acid-treated MG-63 cells. This result was in accordance with a previous study that caffeic acid reduced Bid protein level in Paclitaxel-induced apoptosis of lung cancer cells.(26) In hepatocellular carcinoma cells, caffeic acid phenethyl ester (CAPE), a caffeic acid derivative, did not decrease the amount of Bid. However, a combination of CAPE and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), markedly reduced the amount of Bid.(27,28) The activation of Bid plays an important role in apoptotic signaling. Bid is cleaved to t-Bid by caspase-8 and translocated into the mitochondrial membrane. Translocation of t-Bid stimulates the release of various apoptogenic factors from mitochondria, such as Cyt *c*.(23)

The release of Cyt *c* into the cytosol plays a major role in executing apoptosis.(23) In the present study, caffeic acid reduced the amount of mitochondrial Cyt *c* and increased

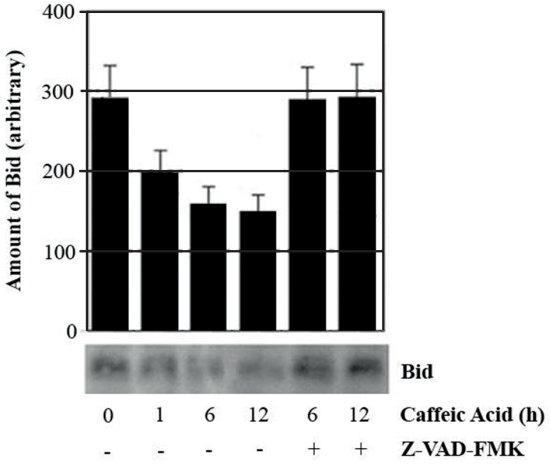

Figure 1. Caffeic acid decreased the amount of Bid in a time-dependent manner. MG-63 cells were pretreated with/without 100 μM Z-VAD-FMK for 2 h, treated with 10 μg/mL caffeic acid, and incubated for 1, 6, and 12 h as indicated in the panel. MG-63 cells were lysed, homogenized, and processed further for immunoblotting to detect Bid as described in Methods.

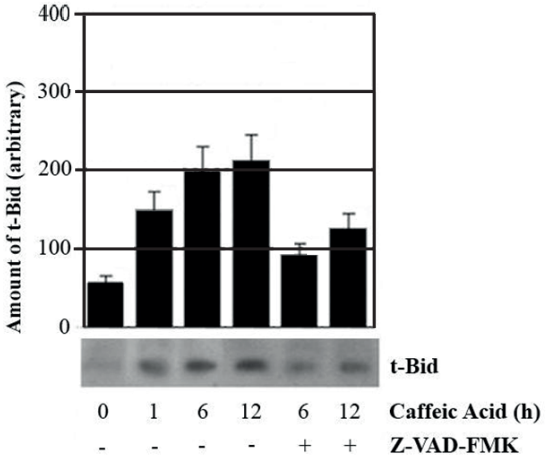

Figure 2. Caffeic acid increased the amount of t-Bid in a time-dependent manner. MG-63 cells were pretreated with/without 100 μM Z-VAD-FMK for 2 h, treated with 10 μg/mL caffeic acid, and incubated for 1, 6, and 12 h as indicated in the panel. MG-63 cells were lysed, homogenized, and processed further for immunoblotting to detect t-Bid as described in Methods.

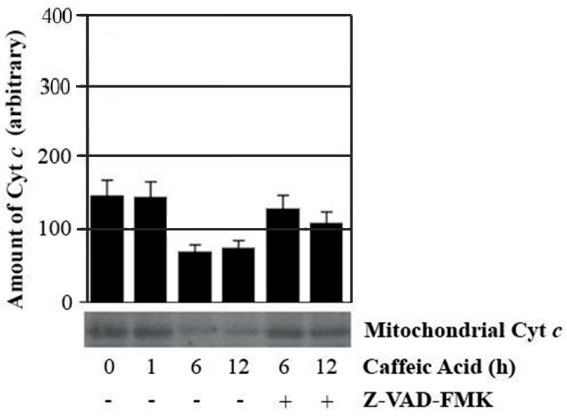

Figure 3. Caffeic acid decreased the amount of mitochondrial Cyt *c* in a time-dependent manner. MG-63 cells were pretreated with/without 100 µM Z-VAD-FMK for 2 h, treated with 10 µg/mL caffeic acid, and incubated for 1, 6, and 12 h as indicated in the panel. MG-63 cells were lysed, homogenized, and processed further for immunoblotting to detect mitochondrial Cyt *c* as described in Methods.

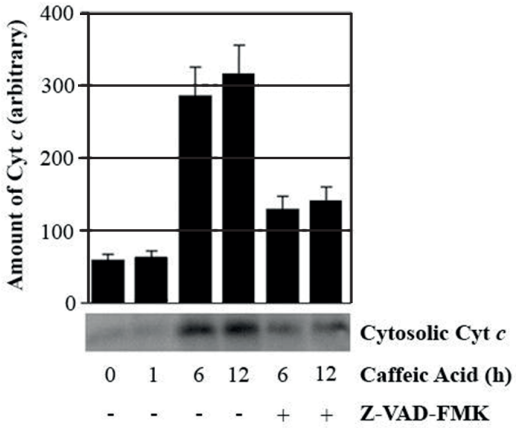

Figure 4. Caffeic acid increased the amount of cytosolic Cyt *c* in a time-dependent manner. MG-63 cells were pretreated with/without 100 µM Z-VAD-FMK for 2 h, treated with 10 µg/mL caffeic acid, and incubated for 1, 6, and 12 h as indicated in the panel. MG-63 cells were lysed, homogenized, and processed further for immunoblotting to detect cytosolic Cyt *c* as described in Methods.

the amount of cytosolic Cyt *c* in a time dependent manner, suggesting Cyt *c* release from mitochondria to cytosol. Pretreatment of Z-VAD-FMK notably increased the amount of mitochondrial Cyt *c* and reduced the amount of cytosolic Cyt *c*. This result was in line with previous studies that caffeic acid and its derivatives induced Cyt *c* release in several cancer cell lines.(29-31) Cyt *c* is one of the signaling components involved in the intrinsic apoptotic pathway. This molecule activates caspase-3 via Cyt *c*/apoptotic protease activating factor-1 (Apaf-1)/caspase-9 apoptosome complex formation. Apoptosome-activated caspase-3 then inhibits or activates target proteins, leading to biochemical and cellular events of apoptosis.(23) There are several compounds that have been reported to induce apoptosis by stimulating Bid activation and Cyt *c* release in osteosarcoma cell lines, including 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (32), triptolide (33), and tetrandrine (34).

Previous study has shown that caffeic acid triggered apoptosis via caspase-dependent intrinsic apoptotic pathway by activating caspase-3, -8, and -9.(21) This result was confirmed by another study demonstrating that Z-VAD-FMK, a pan caspase inhibitor, diminished the amount of cleaved caspase-3, -8, and -9 in caffeic acid-treated MG63 cells.(22) Present study added more information regarding possible signaling mechanisms that might be involved in the caffeic acid-induced MG-63 cell apoptosis. Caffeic acid may affect extrinsic apoptotic pathway by stimulating caspase-8 cleavage. Activated caspase-8 could directly cleave caspase-3 or trigger the truncation of Bid to t-Bid, which interconnects extrinsic and intrinsic apoptotic pathways. t-Bid induces Cyt *c* release into the cytosol, leading to the formation of apoptosome complex, which in turn activates caspase-3 and causes cell death (Figure 5). Thus, caffeic acid may affect not only the intrinsic apoptotic pathway, but also the extrinsic apoptotic pathway in MG-63 cells. There are possibilities that caffeic acid may also affect another pathway, such as caspase-independent cell death (CICD), which happens when there is a failure of caspase activation by an apoptotic signal.(35)

Chemoresistance in malignant tumors, including osteosarcoma, is usually caused by disruption of the intrinsic apoptotic pathway since most chemotherapeutic agents target this pathway. Furthermore, aberrant extrinsic apoptotic pathway can also prevent osteosarcoma cell death in some cases.(36) Therefore, novel therapeutic agents that target both the extrinsic and intrinsic apoptotic pathways are needed to be explored.(37) Since caffeic acid may alter both the extrinsic and intrinsic apoptotic pathways in MG-63 cells, this compound can be used as a potential candidate of therapeutic agent for treating osteosarcoma.

The results of the present study are expected to give an insight for the development of novel molecular targeted therapy for osteosarcoma. Different osteosarcoma cell lines may have different responses to caffeic acid treatment, since the apoptotic pathway is complex and different genetic alterations in apoptotic death receptors have been identified in several osteosarcoma cell lines.(38,39) Further study should be conducted to assess caffeic acid-induced apoptosis and its signaling pathway in different osteosarcoma cell lines.

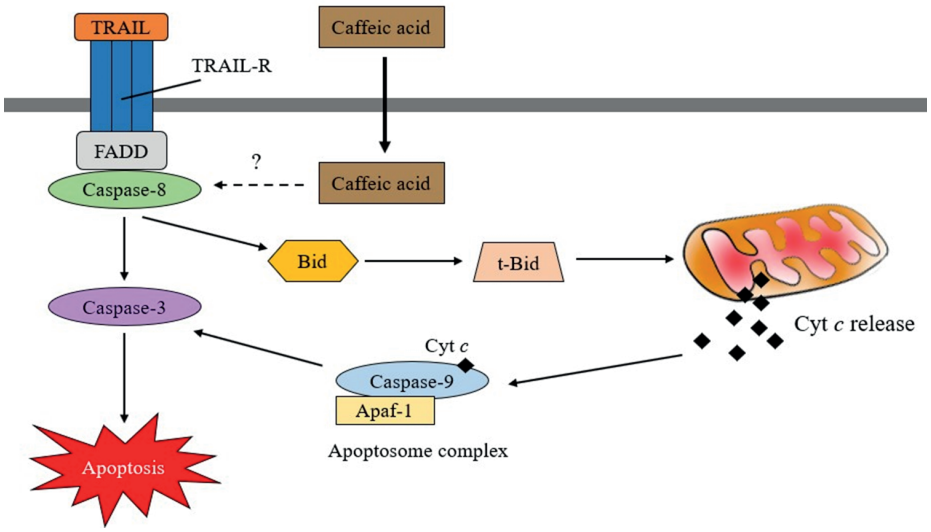

Figure 5. Possible signaling mechanisms involved in caffeic acid-induced MG-63 cell apoptosis. Caffeic acid may activate caspase-8, which could directly cleave caspase-3 or trigger the truncation of Bid to t-Bid. t-Bid induces Cyt *c* release from mitochondria, leading to the formation of apoptosome complex, which in turn activates caspase-3 and causes apoptosis. TRAIL-R: TRAIL receptor; FADD: Fas-associated death domain.

Conclusion

In conclusion, caffeic acid could induce Cyt *c* release through activation of Bid in MG-63 osteosarcoma cells. Taken together, caffeic acid could be a promising anti-osteosarcoma agent, since this compound may affect both extrinsic and intrinsic apoptotic pathway.

Authors Contribution

FS and MIR prepared study concept and design. FS, AHAW, and MA performed processing and acquisition of data. FS, MC, and MIR performed analysis and interpretation of results. MIR, AHAW and MA prepared the draft of the manuscript. FS and MIR made critical revisions of the manuscript. AHAW, MA and MC assisted in administrative, technical, and material support. FS and MIR performed supervision of the study. All authors read and approved the final manuscript.

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