RESEARCH ARTICLE



Caffeic Acid Induced Apoptosis in MG63 Osteosarcoma Cells Through Activation of Caspases

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Background: Caffeic acid has been reported that when it is combined with all-trans retinoic acid, it can inhibit proliferation activity of SaOS-2 or OSA-01 cells. In addition, caffeic acid merely could reduce cell viability of SaOS-2 cells. However, there is not any study in caffeic acid's possible effect to induce apoptosis in osteosarcoma cell.

Materials and Methods: MG-63 cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. Cells were treated with various concentrations of caffeic acid. Apoptosis were analyzed with Sub-G1 assay and activation of caspase-8, -9, and -3 were analyzed with immunoblotting. Caffeic acid-induced percentage of apoptotic cells and cleaved-8, -9, -3 were then statistically analyzed.

Results: Sub-G1 results showed that caffeic acid significantly induced apoptosis in MG-63 osteosarcoma cells in concentration dependent manner. Immunoblotting results showed that caffeic acid induced cleavage of caspase-8, -9 and -3. Cleaved-caspase-8 and -9 were increased at 1-hour treatment of caffeic acid, while cleaved-caspase 3 was increased markedly at 6-hours treatment of caffeic acid.

Conclusion: Caffeic acid induces apoptosis significantly in concentration dependent manner through caspase-dependent intrinsic apoptotic pathway.

Keywords: caffeic acid, osteosarcoma, MG-63, apoptosis, caspase

Introduction

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents. Osteosarcoma originates from primitive mesenchymal bone forming cells and often occurs in long bones, such as proximal tibia and distal femur.^{1,2} Osteosarcoma was rarely curable even with treatment of ablative surgery.² Some clinical studies reported regime consists of the combination of surgery and intensive

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multi-agent chemotherapy improved currently 5-year overall survival rate to 60-80%. L2 However poor response to treatment regimes is still remained. Therefore novel approach is still underway to provide better response and to limit the use of various combination of chemotherapies due to possible side effects.

Nowadays several new therapeutic agents, which specifically target proliferation, cell angiogenesis, immune





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system signaling pathways in cancer cells, either as single therapeutic agent or in combination with conventional chemotherapy, have showed promising data in preclinical research and clinical trials. In addition approach in inducing apoptosis in osteosarcoma cells was also being introduced. Ursolic acid, ferulic acid, polydatin, mistletoe alkali and many others, trigger apoptosis in human MG-63 osteosarcoma cells.

Caffeic acid (3,4-dihydroxycinnamic acids), a natural resource, occurs in food mainly as an ester with quinic acid called chlorogenic acid (3-O-caffeoylquinic acid). Caffeic acid has been well reported to inhibit osteoclastogenesis. Besides osteoclastogenesis, caffeic acid was reported to induce apoptosis in SCM1 gastric and HeLa cervical cancer cells. Caffeic acid has been reported that when it is combined with all-trans retinoic acid, the inhibitory effect in proliferation activity of SaOS-2 or OSA-01 cells was significantly increased. In addition, caffeic acid merely could reduce cell viability to around 55% in SaOS-2 cells. However, to our knowledge, there is not any study in caffeic acid's possible effect to induce apoptosis in osteosarcoma cell. Therefore current study was conducted to investigate the apoptotic effect of caffeic acid in osteosarcoma cells.

Materials and methods

Cell culture

MG-63 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.*^{14,15} Briefly, MG63 cells were treated with 0-10 μg/ml caffeic acid (Wako, Osaka, Japan) or chlorogenic 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).

Immunoblotting

Stimulated 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, 1 mM p-amidinophenyl methanesulfonyl fluoride hydrochloride and protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Samples were separated by sodium dodecyl sulfate (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 (HRP)-conjugated donkey antirabbit (Amersham, Buckinghamshire, UK) or 1:2000 diluted horseradish peroxidase-conjugated sheep antimouse (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) and semi-quantified using UVIband software (UVItech).

Statistical analysis

Shapiro Wilk test was performed to detemine distribution of apoptosis induced by caffeic acid or chlorogenic acid in various concentrations with Sub-G1 assay, and $10~\mu g/mL$ caffeic acid-induced cleaved-caspase-8, -9 or -3 in serial time with immunoblotting. Independent samples T or Mann Whitney test was performed to compare apoptosis induced by caffeic acid and chlorogenic acid in various concentrations with Sub-G1 assay and $10~\mu g/mL$ caffeic acid-induced cleaved-caspase-8, -9 and -3 in serial time with immunoblotting. Statistical analyses were performed using IBM SPSS Statistics for Macintosh version 21 (SPSS IBM, Armonk, NY, USA). Statistically significance was expressed as p<0.05.

Results

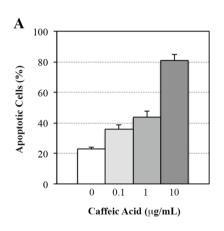
Caffeic acid induced apoptosis in MG-63 cells significantly MG-63 cells were significantly induced into apoptosis by treatment of caffeic acid (Figure 1A, Table 1 and 3). Results showed that 0, 0.1, 1 and $10 \,\mu\text{g/mL}$ caffeic acid induced 23.00

 \pm 1.00%, 35.67 \pm 3.06%, 43.67 \pm 3.79%, 81.00 \pm 3.61% of apoptotic cells, respectively (average \pm standard deviation). Percentages of apoptotic cells were significantly increased along with the increment of caffeic acid concentrations. Meanwhile, chlorogenic acid concentration-dependently induced apoptosis in MG-63 cells as well (Figure 1B), but resulted percentages of apoptotic cells were markedly lower.

Chlorogenic acid in concentration of 0, 0.1, 1 and 10 μ g/mL induced 23.00 \pm 1.00%, 26.33 \pm 2.52%, 31.00 \pm 3.46%, 36.67 \pm 3.51% of apoptotic cells, respectively.

Caffeic acid induced apoptosis through caspase-8, -9 and -3

Based on immunoblotting results (Figure 2A, Table 2 and 4), all cleaved-caspase-8, -9 and -3 were observed. Bands



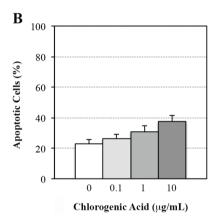
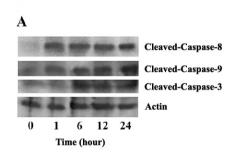
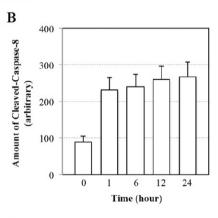
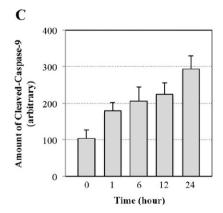


Figure 1. Caffeic acid induced apoptosis in MG-63 cells. MG-63 cells were starved for 12 hours and treated with various concentrations of caffeic acid (A) or chlorogenic acid (B) for 48 hours. Cells were collected and processed according to Sub-G1 assay. These experiments were repeated 3 times.







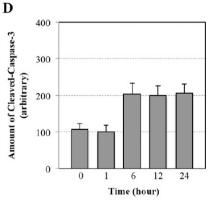


Figure 2. Caffeic acid induced cleavages of Caspase-8, -9 and -3. MG-63 cells were starved for 12 hr and treated with 10 μ g/mL caffeic acid for 0, 1, 6, 12 and 24 hours. Cells were collected, lysed and processed further for immunoblotting to detect cleaved caspase-8, -9 and -3. These experiments were repeated 3 times.

of cleaved-caspase-8 were increased markedly started from treatment of caffeic acid for 1 hour and increased slightly until 24 hours. Semi-quantification of caspase-8 (Figure 2B) confirmed the results and showed the cleavages were significantly induced by caffeic acid at 1-hour treatment. Bands of cleaved-caspase 9 were seen started from treatment of caffeic acid for 1 hour, then gradually increased. Semi-quantification of caspase-9 (Figure 2C) showed significant time-dependent of cleaved-caspase 9 formations. Bands of cleaved-caspase 3 were increased markedly started from treatment of caffeic acid for 6 hours and maintained constant

until 24 hours. Semi-quantification of caspase-3 (Figure 2D) confirmed the results and showed the cleavages were significantly induced by caffeic acid at 6 hours treatment.

Discussion

Our investigation in caffeic acid was initially started from its potential in inhibiting RANKL and TNF-induced osteclastogenesis. 8-10 Several reports showed that tumor cells could induce osteoclastogenesis, including ameloblastoma 16, oral cancer 17 and osteosarcoma 18/

Table 1. Comparative statistical analysis of apoptosis induced by caffeic acid or chlorogenic acid in various concentrations with Sub-G1 assay.

Group	One Way Analysis	Comparative Analysis			
Caffeic Acid		$0.1~\mu g/mL$	$1 \mu g/mL$	$10~\mu g/mL$	
0 μg/mL		0.002 ^a ,*	0.001 ^a ,*	0.000 ^a ,*	
$0.1~\mu g/mL$	ANOVA p=0.000*	-	$0.046^{a,*}$	$0.000^{a,*}$	
1 μg/mL	p 0.000	$0.046^{a,*}$	-	$0.000^{a,*}$	
Chlorogenic Acid		0.1 μg/mL	1 μg/mL	10 μg/mL	
0 μg/mL	Kruskal Wallis p=0.024*	0.100^{a}	$0.046^{b,*}$	$0.002^{a,*}$	
$0.1~\mu g/mL$		-	0.077^{b}	$0.010^{a,*}$	
1 μg/mL	F 3.32.	0.077^{b}	-	0.121 ^a	

^aIndependent samples T test, ^bMann Whitney, **p*<0.05.

Table 2. Comparative statistical analysis of 10 $\mu g/mL$ caffeic acid-induced cleaved-caspase-8, -9 or -3 in different time with immunoblotting.

Group	One Way Analysis	Comparative Analysis			
Cleaved-Caspase 8		1 hour	6 hour	12 hours	24 hours
0 hour	Kruskal Wallis p=0.071	0.003 ^a ,*	0.046 ^b ,*	0.002 ^a ,*	0.002 ^a ,*
1 hour		-	0.268^{b}	0.374^{a}	0.321^{a}
6 hours		0.268^{b}	-	0.268^{b}	0.268^{b}
12 hours		0.374^{a}	0.268^{b}	-	0.845^{a}
Cleaved-Caspase 9		1 hour	6 hour	12 hours	24 hours
0 hour	ANOVA p=0.001*	0.063 ^a	0.016 ^a ,*	0.005 ^a ,*	0.002 ^a ,*
1 hour		-	0.450^{a}	0.210^{a}	$0.026^{a,*}$
6 hours		0.450^{a}	-	0.549^{a}	$0.046^{a,*}$
12 hours		$0.002^{a,*}$	0.549^{a}	-	0.066^{a}
Cleaved-Caspase 3		1 hour	6 hour	12 hours	24 hours
0 hour	ANOVA p=0.000*	0.649 ^a	0.006 ^a ,*	0.007 ^a ,*	0.004 ^a ,*
1 hour		-	$0.006^{a,*}$	$0.007^{a,*}$	$0.004^{a,*}$
6 hours		$0.006^{a,*}$	-	0.794^{a}	0.942^{a}
12 hours		$0.004^{a,*}$	0.794 ^a	-	0.728 ^a

^aIndependent samples T test, ^bMann Whitney, *p<0.05.

Table 3. Distribution analysis of apoptosis induced by caffeic acid or chlorogenic acid in various concentrations with Sub-G1 assay.

Group	Shapiro-Wilk	Distribution
Caffeic Acid		
0 μg/mL	1.000	normal
$0.1~\mu g/mL$	0.637	normal
1 μg/mL	0.253	normal
$10 \ \mu g/mL$	0.537	normal
Chlorogenic Acid	i	
0 μg/mL	1.000	normal
$0.1~\mu g/mL$	0.780	normal
1 μg/mL	0.000	abnormal
10 μg/mL	0.843	normal

Table 4. Distribution analysis of 10 $\mu g/mL$ caffeic acid-induced cleaved-caspase-8, -9 or -3 in different time with immunoblotting.

Group	Shapiro-Wilk	Distribution		
Cleaved-Caspase 8				
0 hour	0.058	normal		
1 hour	0.144	normal		
6 hours	0.000	abnormal		
12 hours	0.537	normal		
24 hours	0.114	normal		
Cleaved-Caspase 9				
0 hour	0.497	normal		
1 hour	0.107	normal		
6 hours	0.253	normal		
12 hours	0.157	normal		
24 hours	0.132	normal		
Cleaved-Caspase 3				
0 hour	0.637	normal		
1 hour	0.148	normal		
6 hours	0.702	normal		
12 hours	0.416	normal		
24 hours	0.194	normal		

Ewing sarcoma¹⁹ cells. Later on, caffeic acid with/without combination of retinoic acid was found out to inhibit proliferation of osteosarcoma cells.¹³ Current results added more information that caffeic acid could induce apoptosis in osteosarcoma cells significantly and specifically, apoptosis induced by chlorogenic acid was much lower than caffeic acid. The apoptotic induction potential of caffeic acid has been reported in different cancer cells, SCM1 gastric

and HeLa cervical cancer cells as well.^{11,12} In addition, current results showed percentages of apoptotic cells were significantly increased along with the increment of caffeic acid concentrations, suggesting that caffeic acid induced apoptosis in MG-63 cells in a concentration dependent manner.

Current results showed that caffeic acid induced apoptosis through caspase-8, -9 and -3, suggesting that mitochondrial or intrinsic apoptotic signaling pathway could also be involved. These results are in accordance to previous report, caffeic acid induced translocation of cytochrome c from mitochondria to cytoplasm in HeLa cells. ¹² Caspase activation has been recognized as the key to apoptosis, in which the presence of caspase which has been cleaved or activated marking the cells undergo apoptosis. ²⁰ Apoptosis involves diverse cell proteases and response to various apoptotic signals. Cleavage and degradation of the protease would result in cell death. Cleaved-caspase 3 was reported to push the cell into apoptosis and the action was irreversible. Thus, caspase 3 is an apoptotic executor, which is an important target for regulating apoptosis. ²¹

Caspase-8 and -9 are essential proteases for intrinsic apoptotic pathways, which caspase-3 will act as a downstream effector in the pathway. 22,23 Although the intrinsic and extrinsic pathways involve some different molecules, but both of these pathways can be interconnected. 23 Activation of caspase-8 can directly lead to the activation of extrinsic pathway and/or activation of Bid, which then will affect mitochondrial membrane potential and cytochrome c release will be initiated. Taken together, current results suggested that caffeic acid induces apoptosis significantly in concentration dependent manner through caspase-dependent intrinsic apoptotic pathway. Further investigation on extrinsic apoptotic pathway should be conducted.

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