RESEARCH ARTICLE

Caffeic Acid Inhibits Tumour Mass Formation in MG-63 Cells-induced Nude Mice

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Abstract

BACKGROUND: Formation of tumour mass is one symptom of osteosarcoma development. Caffeic acid has been known to provide effective treatment but has less side effect for some cancer therapy. Studies reported that caffeic acid might promote apoptosis in MG-63 osteosarcoma cells, however, the effect of caffeic acid treatment in preventing tumour mass formation has not been well elucidated, especially in MG-63 cells-induced nude mice *in vivo*.

METHODS: MG-63 cells were pre-treated with 0, 1, or 10 µg/mL caffeic acid, and 6 hours after pre-treatment, MG-63 cells were injected into subcutaneous space of mice to induce osteosarcoma. Another model was also created by subcutaneously injecting MG-63 cells to the back of mice, and after 48 days, the visible tumour mass was injected intra-tumour with 0 or 10 µg/mL caffeic acid every 7 days for 6 times. After 90 days, mice were anaesthetised, and the nodule pictures were taken for observation and measurement.

RESULTS: In pre-treated MG-63 cells-induced mice, volumes of the mass decreased in reverse with the dose of caffeic acid given. Ten µg/mL caffeic acid pre-treatment was able to significantly lower the mass volume compared to the untreated (p<0.05). Meanwhile, the intra-tumour treatment of 10 µg/mL caffeic acid, even though not significant, was able to inhibit tumour mass formation.

CONCLUSION: Results of caffeic acid pre-treatment and caffeic acid treatment in tumour mass of mice show that caffeic acid is able to inhibit the MG-63 cells formation. This suggests that caffeic acid can be a potential anti-cancer agent.

KEYWORDS: caffeic acid, osteosarcoma, MG-63 cells, tumour mass

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Introduction

A formation of tumour mass, especially in the region of long bones, such as femur, tibia and humerus, is one of the symptoms of osteosarcoma development.(1,2) Osteosarcoma is a highly malignant bone tumour that typically composed of spindle cells producing disordered and immature osteoid.(3) An osteosarcoma grows in a radial manner and forms a ball-like mass. It compresses the surrounding muscles into a pseudo-capsular layer that later creates tumour nodules portraying the micro-extensions of the central mass. The tumour might metastasize regionally or systemically to other organs.(4)

Current standard treatment for osteosarcoma includes a combination of chemotherapy and surgery.(5) Chemotherapy allows induction of cell necrosis and improvement of outcome, meanwhile surgery removes the tumour, preserves best function of the operated part and prevents recurrence and metastasis.(6) Though earlier studies have shown that these therapies indeed refine overall survival rate of osteosarcoma patients, however, a possibility of high multi-drug resistance (MDR) risk and poor response to the treatments are still remain.(6,7) Besides, osteosarcoma cases with metastases also show a poor survival rate, which is lower than 20%.(8) Hence, it is necessary to find a natural resource that can provide an optimal effectiveness and better response for osteosarcoma treatment, as well as to reduce the formation of tumour mass because of osteosarcoma.

As natural resources, polyphenols are well known to be able to hamper cell proliferation and promote cell death in human cancer cells, as well as having antioxidant properties.(9,10) Caffeic acid (3,4-dihydroxycinnamic acids), one of phenolic acids, recently gains a lot of recognition for its potential in protecting human body from oxidative stress that might causes cancer (11,12), including osteosarcoma. Caffeic acid is commonly found in fruits and coffee in the form of ester with quinic acid called chlorogenic acid.(13,14) Caffeic acid is an antioxidant that might inhibit mutagenic and carcinogenic generation of *N*-nitroso compounds, since it is an inhibitor of the *N*-nitrosation reaction which has been proven *in vitro*.(15) Prior study reveals that caffeic acid can instigate apoptosis in MG-63 osteosarcoma cells and exhibited the presence of caspase-8, -9 and -3 in the initiation of cell death.(7) However, the effect of caffeic acid treatment in preventing the formation of tumour mass has not been well elucidated before, especially in MG-63 cells-induced nude mice *in vivo*. Tumour-bearing nude mice is the most favoured model to study cancer biology, and the effects of this experimental model are usually assessed by the measurement of tumour size and volume.(16) Hence, in this current study, the effect of caffeic acid supplementation on MG-63 cells-induced mice was assessed.

Methods

MG-63 Cell Culture

MG-63 osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (BioSource, Camarillo, CA, USA) and antibiotic-antimycotic containing 200 µg/mL streptomycin, 200 U/mL penicillin, and 0.5 µg/mL amphotericin (Gibco). MG-63 cells were maintained in a humidified condition with 5% CO_2 incubator at 37°C until the cells reached 80% confluency before being sub-cultured.

Implantation of Caffeic Acid Pre-treated MG-63 Cells in Nude Mice

In this *in vivo* study, MG-63 cells were pre-treated with 0, 1, or 10 µg/mL caffeic acid (Wako, Osaka, Japan). The doses were determined based on previous study.(11) Six hours following the caffeic acid pre-treatment, $3x10^6$ MG-63 cells were then injected into subcutaneous space in the back of BALB/cAnNCrj-nu/nu nude mice to induce osteosarcoma. After 90 days, to observe the formation of visible tumour mass, the mice were anaesthetised, and the picture of the subcutaneous area was taken for observation and measurement. The mass nodule volume was measured according to previous study (17) to obtain the tumour mass volume presented in mm^3. In each group, the experiments were repeated 3 times.

Caffeic Acid Treatment for MG-63 Cells-injected Nude Mice

Meanwhile to assess the effect of caffeic acid treatment, $1x10^7$ MG-63 cells were initially injected to subcutaneous area in the back of BALB/cAnNCrj-nu/nu nude mice to induced osteosarcoma. After 48 days, the visible tumour mass was then injected intra-tumour with 0 or 10 µg/mL caffeic acid (Wako) every 7 days. After 6 times of caffeic acid injection, mice were anaesthetised, and the picture of visible tumour mass were taken for observation and measurement. The tumour mass nodule volume was also measured according to previous study (17) and presented in mm^3. In each group, the experiments were repeated 3 times.

All experiments above were performed based on the guiding principles of the "Care and Use of Animals" of Kyushu University, which was according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education that was issued by the New York Academy of Science's Ad Hoc Committee on Animal Research.

Statistical Analysis

Statistical analyses were performed using StatView-J5.0 (SAS Institute Inc, Cary, NC, USA). Results were presented in mean/average, and student's t-test was used to determine the statistical differences between the means of each experiment. A value of $p<0.05$ was considered as statistically significant.

Results

Caffeic Acid-pre-treatment Inhibits Tumour Nodule Formation

To test the potential of caffeic acid *in vivo*, caffeic acid-pre-treated $3x10^6$ MG-63 cells were injected into the subcutaneous space of the mice. Ninety days after the injection, tumour mass was developed in the injection area. However, the volumes of the mass were decreasing in reverse with the dose of caffeic acid given (Figure 1A). Average volume of visible tumour mass in mice injected with 0 µg/mL caffeic acid pre-treated MG-63 cells was 18.05 mm^3. Meanwhile, in the mice injected with 10 µg/mL caffeic acid pre-treated MG-63 cells, the volumes of visible mass were significantly lower ($p<0.05$), showing that pre-treating MG-63 cells with 10 µg/mL caffeic acid hampered the tumour growth (Figure 1B).

Caffeic Acid Intra-tumour Injection Inhibits Tumour Nodule Formation

To test the potential of intra-tumour injection of caffeic acid, $1x10^7$ MG-63 cells-injected nude mice were given 0 or 10 µg/mL caffeic acid injection after 48 days. Ninety days after the initial MG-63 cells injection, large tumour nodule was observed in the subcutaneous area of nude mice injected with vehicle only (Figure 2A). However, in nude mice which were intra-tumour injected with 10 µg/mL caffeic acid, even though not significant, yet obvious inhibition of tumour nodule formation was observed, which was shown by a smaller visible mass (with average of 12.71 mm^3) (Figure 2B).

Discussion

Successful plantation of MG-63 cells in nude mice have been reported previously, which resulted in the establishment of metastasis mice models for osteosarcoma tumour types. (18,19) Tumour growth are also present after the injection of MG-63 tumour cell line to the subcutaneous space of shaved severe combined immunodeficiency (SCID) mice (18), which was similar to current study. Ninety days after the injection of MG-63 cells, tumour mass was observed in the subcutaneous area of nude mice, forming obvious nodules in the area that were not treated with caffeic acid. In this study, the effectiveness of caffeic acid in *in vivo* model was observed by the bigger tumour mass that was formed by the untreated MG-63 cells compared to the ones formed by pre-treated caffeic acid MG-63 cells. This result suggested that caffeic acid pre-treatment promote the cell death of MG-63 population.(3)

Bigger mass nodule in the subcutaneous area was also observed in the untreated MG-63 cells-induced mice compared to the one that received intra-tumour injection of caffeic acid. This proved that the effectiveness of caffeic acid

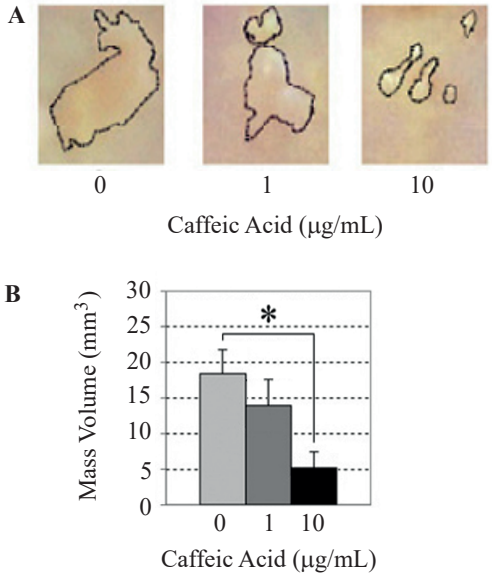

Figure 1. Caffeic acid inhibited MG-63 cells in forming a visible mass in caffeic acid-pre-treated MG-63 cells-injected nude mice. A: Visible mass was observed in nude mice injected with caffeic acid-pre-treated $3x10^6$ MG-63 cells. B: Volume of the visible mass, *$p<0.05$. These experiments were repeated 3 times.

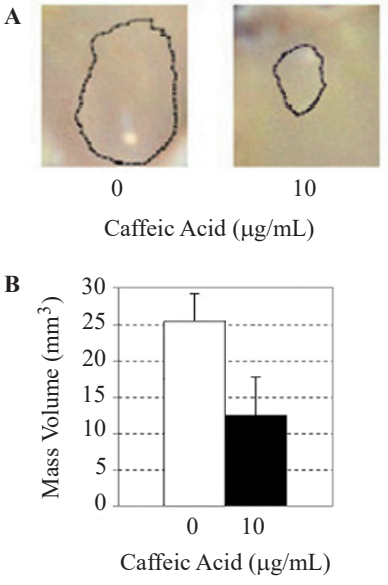

Figure 2. Intra-tumour injection of caffeic acid inhibited MG-63 cells in forming a visible mass in nude mice. A: Visible mass was observed in nude mice which were subcutaneously injected with $1x10^7$ MG-63 cells. B: Volume of the visible mass. These experiments were repeated 3 times.

in vivo also affected the formation of tumour mass induced by direct intra-tumour injection of MG-63 cells. This result suggested that caffeic acid might induces MG-63 cells into apoptosis.(3,11)

Only few studies regarding this was conducted on MG-63 cells-induced mice, however the result of this current study is in line with a study on B7-P815 cells-induced mice which also showed that ingested caffeic acid significantly hinders tumour growth in the terms of size.(20) Previous study on multiple myeloma cells, ARH-77, also shows that a derivative of caffeic acid, namely the caffeic acid phenethyl ester, is able to inhibit the tumour growth and induces cell death in a dose- and time-dependent manner. (21) Meanwhile, in this study, caffeic acid in the dose of 10 µg/mL also showed significant difference in inhibiting the tumour growth ($p<0.05$) compare to the vehicle only MG-63 cells-induced mice.

Caffeic acid as well as its derivatives are known to have properties against cancers, and are cytotoxic to tumour cells but not to the normal cells.(22,23) Caffeic acid and caffeic acid phenethyl ester significantly reduce the amount of tumour nodules in animals whose lung nodules were initiated by viable tumour cells injection. This suggests that in *in vitro* studies, these substance reveals strong cytotoxicity to cancer cells.(24) MG-63 cells treated with drug-loaded calcium phosphate nanocomposites, including caffeic acid, showed decreasing live cells due to cell death after the drug administration.(12)

Caffeic acid inhibits the tumour growth by preventing the development of tumour mass, since it induces intrinsic apoptosis pathway in osteosarcoma cells. Therefore, caffeic acid treatment can be considered as an alternative treatment for osteosarcoma besides the standard treatment regimens that are usually used. This study only observed the effect of caffeic acid supplementation in MG-63 cells. However it is also necessary to conduct further study in other osteosarcoma cell lines, such as Saos-2, U2OS, HOS, or OSA (25), since different osteosarcoma cell lines might have different attributes and response toward caffeic acid treatments.

Conclusion

Taken together, the results of caffeic acid pre-treatment as well as caffeic acid treatment in tumour mass of mice show that caffeic acid can inhibit MG-63 cells formation, which has been proven *in vivo*. This suggests that caffeic acid can be a potential anti-cancer agent.

Authors Contribution

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

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