Gallic acid induces apoptosis and inhibits cell migration by upregulating miR-518b in SW1353 human chondrosarcoma cells

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Abstract. Gallic acid (GA), a natural agent, is widely distributed in plants with a range of biological effects and has been of potential interest as an anticancer agent. However, its effects on chondrosarcoma cell apoptosis are still undefined. In the present study, the possible mechanisms of GA-induced apoptosis were explored in SW1353 cells, a human chondrosarcoma cell line. Our results showed that GA inhibited cell viability dose- and time-dependently. Morphological examination of GA-treated cells exhibited the typical features of cell death, such as rounding up of the cells and cell shrinkage. Wound-healing assay indicated that GA inhibited the migratory abilities of SW1353 cells. Hoechst 33258 staining assay and Annexin V/PI staining assay exhibited apoptosis induction by GA. To determine the molecular mechanism of GA-induced apoptosis, the expression levels Bcl-2, Bax, caspase-3 and caspase-9 were determined in SW1353 cells treated with GA. We found that GA downregulated the expression of the anti-apoptotic protein Bcl-2, and upregulated the expression of the pro-apoptotic protein Bax, and the activation of caspase-3 and caspase-9. To identify the possible mechanisms, the changes of microRNA expression were tested using the miRCURY™ LNA expression array. It was observed that the miR-518b gene was upregulated in treated cells. Taken together, these data show that GA induces apoptosis and inhibits cell migration by upregulating miR-518b in SW1353 cells.

Introduction

Chondrosarcoma, the production of cartilage-like matrix by tumor cells, is the second most common type of primary malignant bone tumor after osteosarcoma and a common form of tumor in patients aged more than 20 years (1,2). Chondrosarcoma has been found to be relatively chemo- and radio-therapy resistant for their extracellular matrix, low percentage of dividing cells, and poor vascularity (3,4). Chemo- and radio-therapy have not been tested for efficacy, but in clinical routine they are not considered as active for the therapy of this tumor and surgical resection still prevails as the primary mode of therapy for chondrosarcoma. Since chondrosarcoma is a type of highly malignant tumor with a potent capacity for distant metastasis and local invasion (5), the 10-year survival rate of this tumor being unchanged over the past 40 years and ranging from 29% to 83% depends on the chondrosarcoma subtype and grade (6,7).

Development of better strategies of improving chondrosarcoma clinical management is therefore a challenging problem, and novel therapeutic approaches are needed. Recently, an increasing number of reports have described a new class of small regulatory RNA molecules termed microRNAs (miRNAs) that are implicated in chondrosarcoma progression (8).

miRNAs are a class of small non-coding RNAs that have been identified as post-transcriptional regulators of gene and play important roles in maintaining normal cellular functions. The miRNAs mainly bind to the 3' untranslated regions (UTRs) of target messenger RNAs (mRNAs), leading to the blockade of mRNA translation or mRNA degradation. Increasing evidence shows that miRNAs have significant roles in diverse biological changes and processes. Deregulation of miRNAs expression leads to diverse human disease types, including cancer (13). In human cancer, miRNAs can function as oncogenes or tumor suppressor genes during tumor progression and development.

Recently, multiple new chemotherapeutic agents have been developed and some are in clinical trials. Although some of them have produced promising results, their therapeutic spectrum is narrow along with toxicity. This toxicity problem at therapeutic concentration has led to search for anticancer compounds derived from nature. Gallic acid (GA; 3,4,5-trihydroxylbenzoic acid, Fig. 1), a natural polyhydroxyphenolic compound, is widely distributed in various plants and fruits (15,16). GA possesses various pharmacological activities including anti-inflammatory, antimicrobial, antioxidant and anticancer activities in various cancer cells (17,18), and the toxicity is reported as an LD₅₀ dose of 5 g/kg body weight in rats (19).
In the present study, the aim was to explore the anticancer property of GA on SW1353 human chondrosarcoma cells. We investigated the changes of cell viability, morphology, wound healing, apoptosis, Bcl-2, Bax, caspase-3, caspase-9, and miRNAs expression in SW1353 cells treatment with GA. The results show that GA induces apoptosis and inhibits cell migration by upregulating miR-518b in SW1353 cells. It is of great importance to further explore the biological functions, clinical significance, and target genes of miRNAs in human chondrosarcoma.

Materials and methods

Cell culture. SW1353 cells, a human chondrosarcoma cell line, were obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone), 100 µg/ml streptomycin and 100 U/ml penicillin. The cells were maintained under standard culture conditions of 37°C, 5% CO₂ and 95% humidified air. Stock solutions were prepared by dissolving the GA (Sigma Chemical Co., MO, USA) powder in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml, and the final concentration of DMSO in the medium was no more than 0.5%.

Cell viability by MTT analysis. The cells were seeded at a density of 2000 cells per well of 96-well plate for 24 h, and subsequently treated with various concentration of GA (0, 5, 10, 20, 30, 40, 60 µg/ml) for 48 h or with 30 µg/ml of GA for 6, 12, 24, 36, 48 and 72 h. At the end of treatment times, 20 µl of MTT stock solution (5 mg/ml) was added to each well, and cells were incubated at 37°C for 4 h. Thereafter, media were aspirated from the wells, followed by addition of 200 µl of DMSO, and the cells were shaken for 10 min. The color formed was determined by measuring the OD at 550 nm using an ELISA plate reader (BioTek, Model EXL 800, USA).

Observation of morphologic changes. The cells were cultured in 35 mm Petri dish at a concentration of 5x10⁴ cells for 24 h, and continuously treated with different concentration of GA for 48 h. The cell morphology was observed using a phase-contrast microscope (Olympus, Japan).

Wound healing analysis. SW1353 cells were plated into 6-well plate, and grow to confluence, and then made a straight scratch (stimulating a wound) with a pipette tip. The cells treated with or without GA were allowed to migrate for 48 h. After scratching, images were taken under the inverted microscope to assess the ability of the cells to migrate into the wound area. The distance of wound closure (compared with untreated at 48 h) was measured in three-independent wound sites per group.

Observation of apoptosis by fluorescent microscopy with Hoechst 33258 staining. After treatment with or without GA, the SW1353 cells were fixed in 4% neutral formaldehyde and stained with 10 µM Hoechst 33258 (Sigma) at 37°C for 30 min in the dark. The photographs of cells were taken using a fluorescent microscope (Olympus).

Detection of apoptosis by flow cytometry analysis with Annexin V/PI staining. SW1353 cells were cultured in 35 mm Petri dish at a concentration of 5x10⁴ cells for 24 h, and then treated with or without GA for 48 h. The apoptosis of SW1353 cells was tested by flow cytometry analysis using a fluorescence-activated cell sorting (FACS) caliber (Becton-Dickinson, CA, USA) with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. Staining was performed according to the manufacturer's instructions.

Real-time polymerase chain reaction (PCR) analysis. Total RNA was isolated from SW1353 cells treated with GA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using random primers and Superscript™ III (Invitrogen). PCR reactions were carried out in total volumes of 25 µl using SYBR Florescence Quantization kit (Invitrogen) in the ABI PRISM 7700 Sequence Detection System. The forward and reverse primers for the amplifications are as follows: Bcl-2 forward 5'-ATG TGT GTG GAG AGC GTC AA-3' and reverse 5'-ACA GTT CCA CAA AGG CAT GTC AA-3'; Bax forward 5'-GGG GAC GAA CTT GAC AGT AA-3' and reverse 5'-CAG TTT AAG TGT CCC TCA GA-3'; 122 bp. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5'-CAG CCT CCA GAT CAT CAG CA-3' and reverse 5'-TGT GGT CAT GAT TCC TTC CA-3', 106 bp) was used to normalize each reaction.

Western blot analysis. Total cellular protein was extracted from SW1353 cells treated with or without GA using TRIzol reagent, and protein concentrations were examined by Bio-Rad protein assay. An equal amount of protein was separated on SDS-PAGE, and then transferred onto PVDF membranes (Invitrogen). Blots were incubated with anti-Bcl-2, Bax and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) followed by an HRP-conjugated secondary antibody. Immunoreactive proteins were visualized by western blotting chemiluminescence luminal reagent (Santa Cruz Biotechnology). Protein concentrations were quantitated using the Tocan 190 protein assay system (Bio-Rad, USA) and normalized to β-actin in the sample.

Analysis of caspase-3 and caspase-9 activation. The activities of caspase-3 and caspase-9 were tested by a colorimetric assay using the caspase-3 and caspase-9 activation kits (Invitrogen), according to the manufacturer's instructions. The treated cells were lysed with provided lysis buffer for 30 min on ice, and extracts were quantified using the Bio-Rad protein assay. The
protein (100 µg) was incubated with the colorimetric tetrapeptides (50 µl), Asp-Glu-Val-Asp (DEAD)-pNA (specific substrate of caspase-3) or Leu-Glu-His-Asp (LEHD)-p-nitroaniline (pNA) (specific substrate of caspase-9) at 37˚C for 2 h, and then estimated the caspase-3 and caspase-9 activation as instructed by the manufacturer in a 96-well microtiter plate.

MiRNA microarray hybridization. Three samples of total RNA were obtained from the SW1353 cells treated with or without GA, and labeled using the miRCURY Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY LNA Array (version 16.0) (KangChen Bio-tech, Shanghai, China). Following the washing steps the slides were scanned using the Axon GenePix 4000B microarray scanner. Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction.

Statistical analysis. All data were analyzed using the SPSS package for Windows (version 13.0). Statistical analysis of the data was performed with Student’s t-test and ANOVA. P-values <0.05 were considered statistically significant.

Results

GA inhibits the viability of SW1353 cells. The viability of SW1353 cells treated with GA was determined by MTT assay. As shown in Fig. 2A, the cells were treated with final GA concentrations of 5 µg/ml (96.19±1.43%), 10 µg/ml (86.38±3.11%), 20 µg/ml (70.94±3.16%), 30 µg/ml (52.22±4.76%), 40 µg/ml (37.67±3.55%), and 60 µg/ml (18.13±2.29%) for 48 h dose-dependently reduced cell viability compared to untreated cells (100±0.00%) (P<0.05, P<0.01), with an estimated half-maximal inhibitory concentration (IC₅₀) value of 30 µg/ml in this study. Treatment with 30 µg/ml of GA tested the effect of cell viability on SW1353 cells for different periods of time. As shown in Fig. 2B, GA gradually decreased cell viability with the increase of exposure time, suggesting that GA decreases cell viability in a dose- and time-dependent manner.

GA has antagonistic effects on the migration of SW1353 cells. The wound healing assay showed the migratory abilities of tumor cells, cell migration was decreased in SW1353 cells treated with GA. Treated cells closed the wound by 20 µg/ml (63.47±4.90%), 30 µg/ml (18.61±2.78%) and 40 µg/ml (9.78±2.07%) after 48 h, whereas the untreated cells closed 92.49±1.98% of the wound during the same period (P<0.01) (Fig. 4), indicating that GA may inhibit cell migration by inducing apoptosis.

GA mediates apoptosis of SW1353 cells. To determine whether the cell growth and cell-migration suppressive effect of GA is due to apoptosis, we analyzed the cells in the presence of...
Hoechst 33258 staining, by fluorescence microscopy. Untreated cells exhibited distribution of the stain and round homogeneous nuclei features, whereas apoptosis in treated cells increased gradually in a dose-dependent manner and showed changes of typical apoptosis, including reduction of cellular volume, staining bright and condensed or fragmented nucleus (Fig. 5A).

To further verify that apoptosis was induced by GA, SW1353 cells were analyzed by exposure to phosphatidylserine on the cell surface by Annexin V/PI staining followed by FACS analysis. As shown in Fig. 5B, (LL) (Annexin V/PI double-negative population) represents viable cells; indicated as LR or UR (Annexin V-positive/PI-negative or Annexin V/PI double-positive population) indicates cells undergoing early or late apoptosis, respectively. The percentage of cells undergoing apoptosis (including the early and late apoptotic cells) with GA treatment was significantly higher than that in untreated cells (P<0.05, P<0.01) (Fig. 5C and D). These data suggest that GA induces SW1353 cell apoptosis in a dose-dependent manner.

GA regulates the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax. Bcl-2 family proteins such as anti-apoptotic Bcl-2 and pro-apoptotic Bax, central in mitochondrion-mediated apoptosis regulation, also determines the fate of cells. To further study the mechanism of GA, the mRNA and protein expression of Bcl-2 and Bax in treated cells were examined by real-time PCR and western blot analysis, respectively. The results of real-time PCR assay showed that GA treatment profoundly decreased Bcl-2 mRNA and increased Bax mRNA expression in SW1353 cells compared to that in untreated cells (P<0.01) (Fig. 6A and B), and the protein levels of Bcl-2 and Bax were similar to their respective mRNA expressions (Fig. 6C-E), suggesting GA induces mitochondrion-dependent apoptosis in SW1353 cells by the regulation of Bcl-2 family proteins.

GA enhances the activation of caspase-3 and caspase-9. To investigate the downstream effectors in the mitochondrion-dependent apoptotic pathway, the activation of caspase-3 and caspase-9 was detected by colorimetric assay. As shown in Fig. 7, GA treatment significantly promoted the activation of caspase-3 and caspase-9 in SW1353 cells compared to that in untreated cells (P<0.01). Taken together, these results suggest that GA enhances cell apoptosis by the mitochondrion-dependent pathway.

GA upregulates miR-518b in SW1353 cells. miRNAs, small (<22 nt) and non-coding RNA molecules, regulate gene expression post-transcription through base pairing with mRNAs to mediate their degradation and translational repression that play an important role in many biological processes. To explore the mechanisms of GA on apoptosis in SW1353 cells, we used the miRCURY LNA expression array to analyze the changes of microRNA expression. In order to select the most significant candidates, miRNAs altered by at least 1.5-fold in all three pairs of the samples were selected. Under these strict criteria, there were 7 statistically significant miRNAs between treated and untreated cells, 6 genes were downregulated in treated cells, while 1 gene was upregulated in treated cells (P<0.05, P<0.01) (Fig. 8 and Table I). miR-518b has been identified to suppress cell proliferation by inducing apoptosis in tumor cells (20). Our results imply that GA induces apoptosis and inhibits cell migration by upregulating miR-518b in SW1353 cells.

**Discussion**

The characteristics of the tumor cells are a reduction in cell apoptosis and/or an unregulated increase in cell proliferation (21). Moreover, disrupted apoptosis play a crucial role in drug-resistance of tumor cells, and it has become a significant obstacle for the successful management of patients with chondrosarcoma (22). Since current chemotherapy regimens have limited success in improving metastasis-free survival and limited by the
Severe toxicity of conventional agents, the therapeutic bottleneck of chondrosarcoma still remains unconquered (23, 24). Natural products are important to discover new drugs. These compounds can be used as antioxidants and in tumor therapy drugs or prevention. Therefore, plant-derived natural products are worthy of further exploration. GA, one of natural products, has been associated with selective induction of cell death and antiproliferative effects, predominantly through an apoptotic mechanism, in many tumor cell lines (25, 26). However, the molecular mechanism of GA inducing apoptosis of tumor cells remains unclear. Our results showed that GA inhibited cell viability and the migratory abilities of SW1353 cells dose- and time-dependently. GA induced apoptosis by downregulating the expression of anti-apoptotic protein Bcl-2, and upregulating the expression of pro-apoptotic protein Bax, and the activation of caspase-3 and caspase-9. It was also observed that miR-518b gene was upregulated in treated cells, suggesting GA was able to induce apoptosis and inhibit cell migration by upregulating miR-518b in SW1353 cells.

Table I. Up and downregulated miRNAs in SW1353 treated with GA.

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To determine the inhibitory concentration of GA on SW1353 cells, cell viability was examined. It was tested that exposure to GA for 48 h, cell viability was inhibited as shown by MTT.

**Figure 5.** GA promoted the apoptosis of SW1353 cells. (A) Determination of apoptosis in SW1353 cells was tested using Hoechst 33258 staining after GA treatment. Arrows indicate typical apoptotic cell (x200). (B) Treated SW1353 cells were stained with Annexin V/PI and followed by FACS analysis. Four different cell populations display representative FACS analysis scatter-grams of Annexin V/PI staining, including LL (lower left), double-negative stained cells showing live cell population; LR (lower right), Annexin V-positive/PI-negative stained cells displaying early apoptosis; UR (upper right), Annexin V/PI double-positive stained cells representing late apoptosis; UL (upper left), Annexin V-negative and PI-positive stained cells indicating dead cells. (C and D) Quantification of the early and late apoptosis in SW1353 cells by FACS analysis. Data are averages ± SD (vertical bars), *P<0.05, **P<0.01, compared to untreated cells.
Our results show that GA dose- and time-dependently inhibited the cell viability as compared to the untreated cells. The morphological changes of cells imply that cells undergo apoptosis at 48 h after incubation with the concentration of GA chosen based on the MTT assays. To study the migration of SW1353 cells further, wound healing assay was carried out. This indicated that GA may inhibit cell migration by inducing apoptosis and thereby acts as an anticancer drug. This finding corroborated well with the change of cell viability. Further Hoechst 33258 staining assay and Annexin V/PI staining assay
were performed to study apoptosis induction by GA. We found that GA induced SW1353 cells apoptosis.

Apoptosis (programmed cell death), a pathway of cell death characterized by many biochemical and morphological events (27), is initiated by two different signals, the intracellular and extracellular, and by two main pathways, the death receptor- and mitochondria-mediated pathways (28). Mitochondria-mediated apoptosis is commonly involved in death stimuli by the intrinsic pathway, which is the main mechanism of apoptosis in various mammalian cells. This pathway of apoptosis results in an increase of mitochondrial permeability, and the release of pro-apoptotic molecules from the intermembrane space of the mitochondria into the cytosol, such as cytochrome c, Smac/DIABLO and apoptosis-inducing factor, and then activating the caspase-cascade system (29,30). The members of Bcl-2 family regulate the mitochondrion-dependent apoptosis, such as Bax, one of pro-apoptotic Bcl-2 family proteins, control the formation of pores in the mitochondria, whereas Bcl-2, one of anti-apoptotic Bcl-2 family proteins, can prevent cell death by interfering with the activation of Bax (31,32). Therefore, the ratio of Bax to Bcl-2 is critical for determining the release of mitochondrial cytochrome c which activates caspase-9, and then subsequently induces the activation of effector caspases, such as caspase-3 (33,34).

To further explore the molecular mechanism involved in GA-induced apoptosis, the expressions of Bcl-2, Bax, caspase-3 and caspase-9 were assessed in SW1353 cells. Our results showed that GA could upregulate Bax expression and downregulate Bcl-2 expression in SW1353 cells, suggesting GA induces apoptosis by affecting the ratio of Bax/Bcl-2. Caspase activity was quantified by colorimetric assay. We evaluated both caspase-3 and caspase-9, and our results have shown a clear increase in the caspase-3 and caspase-9 activities, indicating that GA induced apoptosis in SW1353 cells is by the activation of the intrinsic pathway.

To gain insight into the molecular mechanism involved in GA-induced apoptosis by mitochondria mediated pathway, the expression of miRNA was assessed in SW1353 cells. There were 7 statistically significant miRNAs between treated and untreated cells, including 6 genes downregulated in treated cells, while 1 gene was upregulated in treated cells. Considering...
the function of miR-518b in invasion and metastasis, it will be interesting to explore molecular mechanisms mediating miR-518b indirectly or directly affecting cell progression in chondrosarcoma. However, there are opposite observations in the expression of miR-518b in other tumors, such as miR-518b upregulated in extranodal marginal zone lymphomas compared to gastritis and in hepatocellular carcinoma compared to non-cancerous tissue (20, 35). As each miRNA can control the expression of hundreds of different target genes containing tumor suppressor genes and oncogenes, the function for the pro-tumor or antitumor roles of a miRNA was determined by the competition among its target genes in specific tumor types (36-38). Our results showed that miR-518b markedly increased in SW1353 cells treated with GA. In the future experiments, it is very important to validate targets of miR-518b by further functional assays.

In conclusion, our data demonstrate that GA induces mitochondrion-dependent apoptosis and inhibits cell migration by upregulating miR-518b in SW1353 cells. These results indicate that GA may be a potential novel antitumor agent for the treatment of chondrosarcoma. Further study on GA treatment of tumors, is required especially given the potential for cross-reactivity and unintended consequences when taken with other antitumor agents.

Acknowledgements

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References