Review Article
Grape seed procyanidins induces apoptosis and inhibits proliferation of osteosarcomas cells in vitro

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Abstract: Objective: Grape seed procyanidins (GSPs) have attracted more attention for its anti-tumor activity. In this study, we aimed to explore the effect of GSPs on the growth of osteosarcoma cells and the potential mechanisms. Methods: Human MG63 osteosarcoma cells were treated with different concentrations of GSPs (0, 10, 50, 100 μg/ml) to find an optimal stimulating dose. MTT and TUNEL assay were performed for measurement of cell variability and apoptosis. Based on the result of dose-dependent study, cells were treated with GSPs (0 and 100 μg/ml). As Bax, Bcl-2 and caspase family exert key roles in regulation of apoptosis, and Cdk's and cyclins play a critical role in the regulation of cell cycle progression, we performed western blot assay for measurement of their expressions stimulated by GSPs. ERK1/2, PI3K and Akt were also detected by western blot as the important signalling mediating cell proliferation and survival. In addition, RT-PCR was used to obtain the mRNA levels of caspase-3 and caspase-8. Results: GSPs inhibited cell variability and induced apoptosis in a dose-dependent manner. GSPs downregulated anti-apoptotic factor bcl-2 and bcl-xl expression, up-regulated pro-apoptotic factor Bax expression and increased the activity of caspase-3 and caspase-8. GSPs also significantly decreased the expression of Cdk2, Cdk4, cyclins D1 and Cyclins D2. In addition, GSPs suppressed the expression of ERK1/2 and PI3K/Akt signaling pathway. Conclusion: GSPs inhibited cell variability and induced apoptosis which might be regulated by ERK1/2 and PI3K/Akt signaling pathway. GSPs might represent a promising and effective therapeutic strategy in osteosarcoma management.

Keywords: Grape seed procyanidins, osteosarcoma, viability, apoptosis

Introduction

In recent years, advanced medical therapy develops rapidly, but tumor is still a major health burden. Osteosarcoma is a primary bone malignant tumor, which represents approximately 15% of all bone tumors [1]. As the second of most malignant bone tumors, this disease afflicted more men than women (ratio 1.5:1), and most patients are children and adolescents. Osteosarcomas predominantly occur in long bones, with characteristics of direct formation of immune bone or osteoid tissue by tumor cells [1]. They can metastasize to critical organs including brain, liver and lung. Mortality rate is associated with the potent migration capacity of osteosarcomas. To date, surgery and neoadjuvant chemotherapy are still crucial therapeutic methods of osteosarcoma. Although the 5-year survival rate was improved significantly, cure rates remain unacceptably low for high-risk patients [2]. Furthermore, the patients with metastatic diseases represent dismal prognosis, and the long-term survival rate was only 30%-35% [3]. Therefore, an effective therapy is needed to improve the survival of osteosarcoma, as many patients experience rapid distant migration.

Now, bioactive dietary phytochemicals are being new options for many human disease therapy. Grapes are a rich source of many bioactive compounds, with approximately 60% to 70% of grape polyphenols being found in seeds. Grape seed proanthocyanidins (GSPs) is extracted from grape seeds, which is a by-product of
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Grape juice and wine industries. It has been reported that GSPs possess a variety of potent pharmacological effects, including antioxidative stress and anti-inflammation [4]. In the past, GSPs had attracted more attention because it possessed a therapeutic properties against tumor and exhibited no apparent toxicity and genotoxic potential [5-7]. In a mouse model, GSPs induced the apoptosis of colon cancer cells and suppressed the colon carcino genesis [7]. Recent years, the property of anti-tumor has been tested in human study. GSPs can prevent the invasion of human head and neck cutaneous squamous cell carcinoma cells [6]. In a vitro experiment by Luan's et al, GSPs inhibited the development of vasculogenic mimicry and tumor growth in human triple-negative breast cancer cells [8]. GSPs are being considered as an alternative treatment for the management of tumor.

To the best of our knowledge, little is known about the effect of GSPs on osteosarcomas and the underlying mechanism remains unclear. Therefore, the present study aimed to assess the effect of GSPs on osteosarcomas. We designed experiments to test our hypothesis that GSPs exert an antitumor effect on osteosarcomas using human MG63 osteosarcoma cell lines as an in vitro model.

Materials and methods

GSPs acquisition

GSPs were purchased from Kikkoman Corporation (Noda, Japan). GSPs was freshly dissolved in dimethylsulfoxide (DMSO) for used.

Cell cultures

Human MG63 osteosarcoma cell lines were obtained from cell bank of Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, USA), L-glutamine (2 mM), 100 U/ml penicillin and streptomycin at 37°C in a 5% CO₂ atmosphere. Cells were passaged when they reach 80% confluence.

Cell viability analysis

Briefly, cells were seeded at a density of 5×10⁴ cells per well into 96-well plates and incubated overnight prior to expose. Then, these cells were randomly divided into 4 groups, GSPs was added into the medium to achieve different concentration (0, 10, 50, 100 μg/ml) for 48 h. Cells viability were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previous reported [9]. Finally, we measured the absorbance according to the manufacturer’s protocol. Each treatment concentration was replicated at least in 5-wells.

TUNEL assays

MG63 cells were incubated with or without GSPs (0, 50, 100 μg/ml) for 48 h. Then, Terminal deoxynucleotidyl Transferase-mediated dUTP nick end labeling (TUNEL) apoptosis detection kit (Roche Diagnostic, Indianapolis, IN) was used to assess the apoptosis of cells based on the manufacturer protocol. TUNEL-positive cells were recorded and apoptosis rate was determined. Experiment was carried out in triplicate.

Western blot

The cells were harvested and cell lysates were prepared. The total proteins of cells were extracted and equal amounts of proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). After a block for 2 h in 5% skim milk at room temperature, the membranes were incubated overnight at 4°C with primary antibodies for Bax antibody (1:1000, Abcam, UK), Bcl-2 antibody (1:1000, Abcam, UK), Bcl-x1 antibody (1:1000, Abcam, UK), pro-caspase-3 antibody (1:1000, Cell Signaling Technology, USA), cleaved-caspase-3 antibody (1:1000, Cell Signaling Technology, USA), pro-caspase-8 antibody (1:1000, Cell Signaling Technology, USA), cleaved-caspase-8 antibody (1:1000, Cell Signaling Technology, USA), cyclin-dependent kinase 2 antibody (Cdk2, 1:1000, Cell Signaling Technology, USA), cyclin-dependent kinase 2 antibody (Cdk4, 1:1000, Cell Signaling Technology, USA), Cyclin D1 antibody (1:1000, Cell Signaling Technology, USA), Cyclin D2 antibody (1:1000, Cell Signaling Technology, USA), phosphorylated phosphatidylinositol 3 kinase 3 kinase antibody (P-Pi3K, 1:1000, Abcam, UK), total phosphatidylinositol 3 kinase 3 kinase antibody (T-Pi3K, 1:1000, Abcam, UK), phosphorylated protein kinase B (P-Akt, 1:1000, Abcam, UK), total protein kinase B (T-Akt, 1:1000, Abcam, UK), phosphorylated extracellular signal-regulated kinase 1/2 antibody (P-ERK1/2, 1:1000, Cell Signaling...
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RNA isolation and real time PCR

Total RNA was extracted from the harvest cells of different treatment groups using Trizol reagent (Invitrogen, Carlsband, CA). RNA was synthesized to cDNA, and real time PCR was performed for evaluation of caspase-3 and -8 expression, respectively. The primers for GAPDH, caspase-3 and -8 as follows: GAPDH, forward, 5'-ACAACCTTGGTATCGTGGAAGG-3', reverse, 5'-GCCATCACGCCAACAGTTTC-3'; caspase-3, forward, 5'-TGCTACTCCACAGCACTGGTTA-3', reverse, 5'-CATGGCACAAAGCAGATGGAGA-3'; caspase-8, forward: 5'-TTTCACTGTGTTAGGGTGGTA-3', reverse, 5'-CCTGTAATCCCAAGC A CTTTGGGAG-3'. GAPDH was used as an internal reference transcript and relative expression of the target genes was calculated.

Statistical analysis

The data are presented as means ± SD. SPSS 13.0 software (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. One-way ANOVA with LSD post-hoc analysis and Student two-tailed t test were conducted for statistical comparison when appropriate. A P value less than 0.05 was considered to indicate a statistically significant difference.

Results

Inhibiting effect of GSPs on the viability of MG63 cells

To determine the cytotoxic effect of GSPs on MG63 osteosarcoma cells, MTT assay was performed. We found incubation of MG63 cells with 3 concentrations of GSPs all caused a significant decrease in cells viability compared with non-GSPs-treated control cells (Figure 1). Furthermore, the decreased viability of MG63 cells induced by GSPs was in a dose-dependent manner (Figure 1). The results showed that GSPs could inhibit MG63 osteosarcoma cells viability.

GSPs induced the apoptosis of MG63 cells

Tumor cells apoptotic response was assayed using TUNEL assay. The percentage of TUNEL-positive cells among 3 groups were as follows: 4.0% in control group, 16.3% in small-dose GSPs group, 36.9% in moderate-dose GSPs group, 47.7% in large-dose GSPs group as summarized in Figure 2. Importantly, GSPs-induced...
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Figure 3. The effects of GSPs on apoptotic pathway proteins in MG63 cells. (A) Representative western blot analysis of Bax, Bcl-2 and Bcl-xl; (B) Quantitative analysis of (A); (C) The ratio of the protein expression levels between Bax and Bcl-2; (D) The mRNA expression levels of Caspase-3 and Caspase-8 by RT-PCR; (E) Representative western blot analysis of pro-caspases and cleaved caspases; (F) Quantitative analysis of the protein levels of pro-caspases in (E); (G) Quantitative analysis of the ratios of cleaved caspases and pro-caspases in (E). *P<0.05.
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apoptosis was dose-dependent. Considering large-dose GSPs achieved the best effect compared to the other groups, we used the large dose (100 μg/ml) of GSPs for subsequent experiment. The results indicated that GSPs caused a strong programmed cell death in MG63 cells.

GSPs promoted pro-apoptotic signaling pathways

Apoptosis is a complex progress, which is considered as critical protection against tumor progression. Pro-apoptotic proteins and anti-apoptotic proteins were involved in the apoptotic progress of tumor cells. The proteins of Bcl-2 family exert a key role in regulation of cell apoptosis or survival by acting as a promoter or inhibitor of cell death [4]. In the present study, we further assessed the protein expression of Bax, Bcl-2 and Bcl-xl. Western blot analysis revealed that 100 μg/ml GSPs treatment caused an enhancement in the levels of pro-apoptotic proteins Bax and a downregulation in the levels of anti-apoptotic protein Bcl-2 and Bcl-xl, as compared with expression in the control group (Figure 3A, p<0.05). Meanwhile, 100 μg/ml GSPs also increased the ratio of the protein expression levels between Bax and Bcl-2 (Figure 3B, p<0.05).

In addition, RT-PCR and Western blot analysis revealed that treatment MG63 cells with GSPs induced more mRNA and protein expression of caspase-3 and caspase-8 than those in control group (Figure 3E and 3F, p<0.05). Meanwhile, we also detected the levels of cleaved caspase-3 and caspase-8 and found that cleaved caspase-3 and caspase-8 were increased in GSPs-induced MG63 cells (Figure 3E and 3G, p<0.05). These findings suggested that GSPs triggered the intrinsic apoptotic signaling pathway.

GSPs treatment decreased the expression of Cdks and cyclins regulating cell cycle progression

Suppression of tumor cells cycle progress has been documented as an effective strategy for the control of tumor growth [10]. In vitro experiment, GSPs treatment resulted in an arrest of cell cycle progress in head and neck squamous cell carcinoma (HNSCC) cells [10]. In human non-small cell lung cancer cells, GSPs treatment caused a significant higher number of cells in G1 phase [11]. Considering Cdks and cyclins play critical roles in the regulation of cell cycle progression [12], we focused on the assessment of the protein expression of Cdks and cyclins. As shown in Figure 4, treatment of...
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MG63 cells with 100 μg/ml GSPs for 48 h markedly decreased the expression levels of Cdk2, Cdk4, Cyclin D1 and Cyclin D2 assessed by western blot (Figure 4A and 4B, p<0.05).

GSPs suppressed the activation of ERK1/2 and PI3K/Akt signaling pathway playing pivotal roles in cell proliferation and survival

Extracellular signal-regulated kinase1/2 (ERK-1/2) and phosphatidylinositol 3-kinase (PI3K) are important signaling pathways that mediate proliferation, growth, motility and invasion of osteosarcoma cells [13]. We targeting ERK1/2 and PI3K/Akt pathways and determined their expression levels with western blot. As shown in Figure 5, GSPs treatment inhibited the phosphorylation of ERK1/2, PI3K and Akt. Therefore, it suggested that GSPs could suppress the activation of ERK1/2 and PI3K/Akt signaling pathways.

Discussion

Natural plant products are promising treatments for tumor growth and metastasis. GSPs showed an anti-carcinogenic activity both in vivo and in vitro experiment and accumulated experimental evidence had proved the safety of GSPs [7]. In the present study, we therefore assessed the therapeutic effect of GSPs on osteosarcoma and explored the mechanisms using MG63 osteosarcoma cells. Our results showed that GSPs significantly decreased the viability and induced apoptosis of MG63 cells associated with suppression of ERK1/2 and PI3K/Akt signaling pathways.

MTT assay were carried out to determine the cytotoxicity of GSPs to cells and we found GSPs significantly inhibited MG63 osteosarcoma cells viability. This may be a possible mechanism of GSPs protecting against osteosarcoma.

Apoptosis is the major cause of tumor cell death and apoptosis-inducing has been regarded as a central mechanism of prevention tumor growth. Apoptosis eliminates genetically damaged cells and the cells that inappropriately proliferate induced by carcinogens, then protects against neoplastic development [12]. Many therapies aimed at the induction of apoptosis. TUNEL staining showed GSPs increased the apoptotic populations of MG63 cells in a dose-dependent manner. The results indicated that GSPs contributed to the apoptosis of osteosarcoma cells. As well known, apoptosis progress is mediated by several pathways and many regulatory molecules are involved. Bax and Bcl-2 family exert a key role in regulation of this intrinsic pathway [14]. Prior study documented that the apoptosis of tumor cells was associated with upregulation of Bax expression and downregulation of Bcl-2 expression [15]. In the present study, GSPs induced the change of protein expression levels of key members in Bcl-2 family, which contained raised pro-apoptotic protein (Bax) expression and decreased anti-apoptotic protein (Bcl-2 and Bcl-xl) expression. Moreover, treatment with MG63 osteosarcoma cells, GSPs favored an enhancement in the value of Bax/Bcl-2, which contributed to the susceptibility of tumor cells to GSPs-induced apoptosis [11].

Figure 5. The effects of GSPs on the activation of ERK1/2 and PI3K/Akt signaling pathway in MG63 cells. A. The phosphorylation levels of ERK1/2 by western blot; B. The phosphorylation levels of PI3K by western blot; C. The phosphorylation levels of Akt by western blot. *P<0.05.
In cells, the intrinsic pathway of apoptosis is mediated by mitochondria. The ratio change of Bax/Bcl-2 always enhances the permeability of mitochondrial membrane, while damages of mitochondrial membrane potential associated with the release of apoptogenetic factors [16]. The release of cytochrome C from mitochondria into cytosol initiates caspase cascade reaction, and results in the initiation and activation of apoptotic progression [17]. Caspase activation subsequently leads to apoptotic cells death. It has been reported that GSPs induce a loss of mitochondrial membrane potential and promote the activation of caspase-3 in human non-small cell lung cancer cells, while GSPs-induced apoptosis was blocked by the pretreatment with the inhibitor of caspase-3 [11]. Therefore, GSPs-induced apoptosis is correlated with the caspase cascade reaction. Our findings showed that GSPs promoted the activation of caspase-3 and caspase-8. GSPs promote the apoptotic signal transduction cascade.

It has been proved an effective strategy to suppress the tumor growth by controlling the cell cycle progress of tumor cells [10]. In most malignancies, it revealed that cell cycle regulators are always deregulated [18]. Prior study and our data all showed that GSPs treatment induced arrest of cell cycle at G1 phase [10, 11]. Cdks and cyclins play a critical role in the regulation of cell cycle progression [19]. Cdk kinase activity is the central cause of tumor progression. Our study further demonstrated a significantly decrease of Cdk2, Cdk4, Cyclin D1 and Cyclin D2 protein expression levels. The results showed that GSPs affected cell cycle regulator, which indicated that it may be one of mechanisms of GSPs suppressing tumor cells proliferation.

We further explored potential mechanisms of the effect of GSPs on osteosarcoma. ERK1/2 belongs to the mitogen-activated protein kinases (MAPKs) family and modulates cell proliferation, differentiation and survival, which exerts an important role in tumor suppression [20]. Whereas, PI3K/Akt pathway is a central signaling cascade whose activation leads to cellular proliferation, anti-apoptotic responses, enhanced metabolism, and inflammatory response [21]. Suppression of PI3k/Akt signaling was directly associated with the activation of apoptotic cells death pathway. It has been documented that ERK1/2 and PI3k/Akt mediated proliferation, growth, motility and invasion of osteosarcoma cells [13]. In this study, the results showed a reduction in activation of ERK and PI3k/Akt exposing to GSPs, which suggested that GSPs-induced apoptosis was mediated by suppressing the activation of ERK and PI3k/Akt.

Osteosarcoma is a challenging clinical problem and GSPs is safe and devoid of toxicity. In the current study, the results showed that GSPs had crucial effect on osteosarcoma. GSPs might represent a promising and effective therapeutic strategy in osteosarcoma management. Therefore, it indicates that GSPs may serve as a single agent or as an adjuvant setting for surgery or conventional cytotoxic therapy in patients with osteosarcoma.

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Disclosure of conflict of interest

None.

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