RESEARCH COMMUNICATION

Jaceosidin Induces p53-dependent G2/M Phase Arrest in U87 Glioblastoma Cells

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Abstract

Flavonoid compounds have been shown to trigger cell cycle arrest at G0/G1, S and G2/M checkpoints, allowing cells to repair DNA damage before entry into mitosis. Jaceosidin, a flavonoid compound has been reported to induce apoptosis in various cancer cell lines. In our previous study, we have shown that jaceosidin induces apoptosis in U87 glioblastoma cells through G2/M phase arrest. However, the molecular mechanisms of jaceosidin-induced cell cycle arrest remained unclear. In the present study, mRNA and protein expression levels of major cell cycle regulatory genes were analyzed by semi-quantitative RT-PCR and Western blot studies respectively. The results demonstrated that jaceosidin-induced G2/M phase arrest in U87 cells is associated with DNA fragmentation, up-regulation of p53 and p21 and subsequent down-regulation of cyclin B1 and CDK1 expression at mRNA as well as at protein level. These findings provide insights into jaceosidin-induced G2/M phase arrest in U87 glioblastoma cells.

Keywords: Jaceosidin - U87 cells - G2/M arrest - p53 - cyclin B1-CDK1

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Introduction

Flavonoids constitute the largest and most important group of polyphenolic compounds in plants. Flavonoids are present in fruits, vegetables and beverages of plant origin and exhibit diverse biological, pharmacological and medicinal activities including anti-inflammatory, anti-allergic, anti-viral, anti-thrombotic, anti-mutagenic, anti-oxidant and anti-proliferative (Kim et al., 2008). Over the past one decade, flavonoids earned enormous attention as key elements in signal transduction pathways related to cellular proliferation, differentiation, cell cycle progression, apoptosis, angiogenesis and metastasis (Das et al., 2010). Recent studies have shown that flavonoids induce cell growth inhibition in various cancer cell lines through multiple ways. Cell cycle arrest is one of the major cell growth regulatory mechanisms (Gamet-Payrastre et al., 2000; Murray, 2004). Flavonoids have been shown to arrest cell cycle at G0/G1, S and G2/M phase (Lee et al., 2005; Li et al., 2007; Vidya Priyadarsini et al., 2010).

Jaceosidin is a flavonoid, present in plants of genus Artemisia. Up to now, jaceosidin have been reported to exert only a few chemoprevention related pharmacological activities such as inhibition of COX-2 and MMP-9 in human mammary epithelial cells, suppression of E6 and E7 oncoproteins of HPV 16, induction of apoptosis in ras-transformed human breast epithelial cells, human ovary cancer cells and U87 glioblastoma cells (Lee et al., 2005; Jeong et al., 2007; Kim et al., 2007; Lv et al., 2008; khan et al., 2011). In our previous study, we have shown that jaceosidin induces apoptosis in U87 glioblastoma cells by arresting the cell cycle at G2/M phase (Khan et al., 2011). However the mechanism underlying jaceosidin-induced G2/M phase arrest was not studied.

The present study was undertaken to determine the molecular mechanism of jaceosidin-induced cell cycle arrest by investigating some major cell cycle regulatory proteins; p53, p21, cyclin B1 and CDK1 in U87 glioblastoma cells.

Materials and Methods

Chemicals

DMEM culture medium, fatal bovine serum (FBS), Propidium iodide (PI), RNase A and DMSO were purchased from Sigma (Beijing, China). Antibodies against cyclin B1, CDK1, p21 and β-actin were purchased from cell signalling (China) and p53 was purchased from Beyotime (China). Anti-mouse and anti-rabbit Horse raddish peroxidase-conjugated secondary antibodies were purchased from Sigma (Beijing, China). ECL Western blotting detection kit was obtained from Milipore (Billerica, USA).

Cell culture and treatment

U87 glioblastoma cell line was purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and maintained at 37°C with 5% CO_2 in humidified atmosphere. Jaceosidin was dissolved in dimethyl sulfoxide (DMSO). Cells were treated with or without 100 μM jaceosidin for 24 and 48

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Table 1. The Sequences of Primers

p53 :	
Forward primer:	5'–CCG GAG GCC CAT CCT CAC CA–3'
Reverse primer:	5'–TGG CTG AGA TGA GCC CTG CT–3'
Cyclin B :	
Forward primer:	5'–AGA TGC TGC AGC TGG TTG GTG TC–3'
Reverse primer:	5'–AGG CCG ACC CAG ACC AAA GTT T–3'
Cdk1 :	
Forward primer:	5'–GGG TTC CTA GTA CTG CAA TTC GGG–3'
Reverse primer:	5'–GCT CTG GCA AGG CCA AAA TCA GC–3'
GAPDH :	
Forward primer:	5'–ATG ACA TCA AGA AGG TGG TG–3'
Reverse primer:	5'–CAT ACC AGG AAA TGA GCT TG–3'

h.

Cell Cycle Analysis

Cell cycle analysis was performed as described previously (Rasul et al., 2011). Briefly U87 glioblastoma cells were treated with or without 100 μM of jaceosidin for 24 and 48 h. The cells were then washed with PBS and fixed with 70% ice cold ethanol at 4°C for overnight. After washing twice with PBS, cells were stained with PBS solution containing 50 μg/mL of PI and 100 μg/mL RNase A for 30 min in the dark at room temperature. The stained cells were analyzed for cell cycle phase distribution and DNA contents by flow cytometry (Beckman Coulter, Epics XL).

Flourescent Microscopy

Treated and untreated U87 glioblastoma cells were collected by centrifugation at 300×g for 5 min, washed twice with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. After centrifugation, cells were washed with PBS, stained with PI (50 μg/mL) and incubated at 37°C in dark for 30 min. At the end of incubation, the cells were washed and resuspended in PBS for the observation of nuclear morphology under fluorescence microscope (Olympus 1x71).

RNA isolation and semi-quantitative RT-PCR

Total RNA was extracted from U87 glioblastoma cells after treatment with 100 μM jaceosidin for 0, 24 and 48 h, using Trizol (Invitrogen), following the product protocol. The purity of RNA was checked by OD260/280 of RNA samples (<1.8) using NanoDrop 1000 (Thermo Scientific) spectrophotometer. 1μg of total RNA was subjected for reverse transcription using M-MLV reverse transcriptase and oligo (dT) primer (Fermentus) to synthesize cDNA in a total volume of 20 μL at 42°C for one hour followed by enzyme inactivation at 70°C for 5 minutes. This was followed by 30 cycles of (94°C: 1 min; 58°C: 30 sec; 72°C: 1min) and a final extension of 72°C for 10 minutes. PCR product was visualized on a 1% agarose gel containing ethidium bromide. The sequences of forward and reverse primers are given in Table 1.

Western blot analysis

U87 glioblastoma cells were treated with or without 100 μM jaceosidin for indicated time period, rinsed twice with PBS and lysed on ice with WIP cell lysis reagent (BIOSS, Beijing Biosynthesis Biotechnology Co. LTD) supplemented with 1% PMSF for 30 min. The insoluble protein lysate was removed by centrifugation at 12000 rpm for 15 min at 4°C. The protein concentrations were determined using NanoDrop 1000 (Thermo Scientific) spectrophotometer. 70 µg of proteins were resolved on 12% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% (w/v) non-fat milk and washing with Tris-buffered saline-Tween solution (TBST), membranes were incubated with respective primary antibodies at 4°C for overnight and washed three times with TBST. The blots were then incubated with anti-rabbit or anti-mouse horse raddish peroxidase conjugated secondary antibodies for 1 h at room temperature. After washing with TBST three times, signals were detected using ECL plus chemiluminescence kit on X-ray film (Millipore Corporation, Billerica, USA).

Statistical Analysis

The results were expressed as the mean ± SEM and statistically compared with the control group or compared within the groups using one- way ANOVA follwed by "Tukey's Multiple Comparison Test" and $P < 0.05$ was considered statistically significant

Results

Jaceosidin induces G2/M phase arrest in U87 glioblastoma cells

Cell cycle phase distribution was analyzed using PI staining and flow cytometry analysis. The data showed that jaceosidin induced cell cycle arrest at G2/M phase in a time-dependent manner. Treatment with jaceosidin at 100 µM showed a statistically significant increase in G2/M phase from 14.6% to 55.5% and 78.6% with a concomitant decrease in G0/G1 phase from 64.3% to 28.4% and 7.4% after 24 and 48 h respectively. However S phase experienced significant reduction only after 48 h treatment compared to control group.

Jaceosidin induces DNA fragmentation in U87 glioblastoma cells

DNA damage may lead to growth arrest at both G1 and G2 phases of the cell cycle. The effect of jaceosidin on DNA damage was detected using PI staining and fluorescence microscopy. After 48 h treatment with 100 μM jaceosidin, an obvious change in nuclear morphology including nuclear shrinkage and DNA fragmentation was observed (Figure 2).

Jaceosidin arrests U87 cells at G2/M phase via p53 upregulation

Since p53 is crucial for the induction of growth arrest by numerous stress signals, at different cell cycle checkpoints,

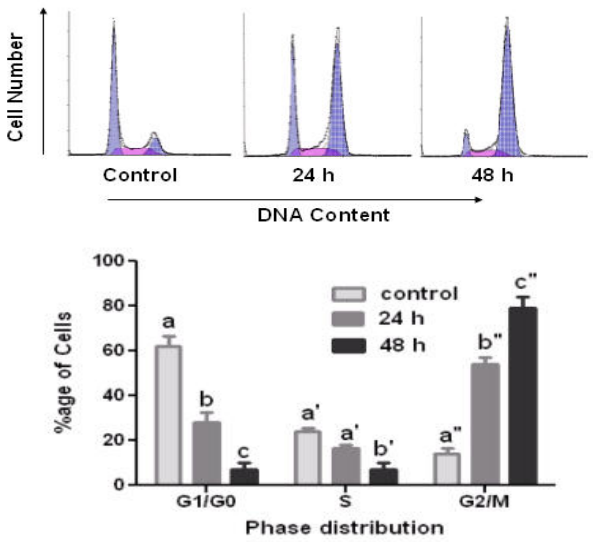

Figure 1. (1A): Flow cytometric analysis of the cell cycle distribution in U87 glioblastoma cells treated with 100 μM jaceosidin for various time intervals. (1B): Data are expressed as Mean ± SEM. of three independent experiments. Columns not sharing the same superscript letter differ significantly (P< 0.05)

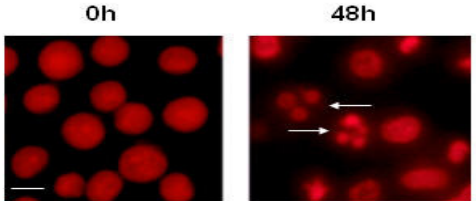

Figure 2. Nuclear Morphological Changes of U87 Glioblastoma Cells Using PI Staining and Fluorescence Microscopy. Cells were treated with 100 μM jaceosidin for 0 h and 48 h respectively. Arrows indicate the condensed and fragmented nuclei. Scale bar = 50 μm

we analyzed the mRNA level and protein level in U87 glioblastoma cells treated with 100 μM jaceosidin for various time intervals, by semi-quantitative RT-PCR and western blots respectively. As shown in Figure 3, p53 mRNA level was up-regulated in U87 glioblastoma cells in a time-dependent manner. Meanwhile, there was significant increase in protein level of p53 (Figure 4), which was consistent with transcriptional data, suggesting that jaceosidin is involved in cell cycle arrest via up-regulating p53 at both transcriptional and translational levels.

Jaceosidin induces expression of p21

Activation of p21 has been shown to participate in G0/G1 as well as G2/M phase arrest of cell cycle (Brehm et al., 1998; Flatt et al., 2000). The results showed that jaceosidin up-regulated p21 at mRNA as well as at protein level in U87 glioblastoma cells of treatment groups in a time-dependent manner (Figure 3 and 4).

Jaceosidin induced G2/M phase arrest in U87 cells via down-regulation of Cyclin B1 and CDK1

Mammalian cell cycle is regulated at different checkpoints by the interaction of a variety of cyclins and cyclin-dependent kinases (CDKs). One of the checkpoints, the G2/M, is regulated by cyclin B1 and CDK1 complex. Next we checked the expression of cyclin B1 and CDK1

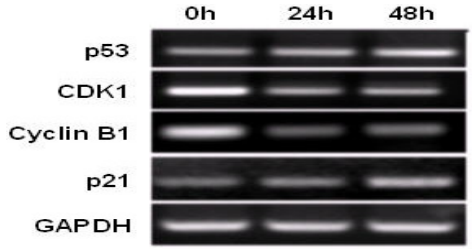

Figure 3. Time-dependent Expression of p53, p21, Cyclin B1 and CDK1 in mRNA Level. U87 glioblastoma cells were treated with 100 μM jaceosidin for indicated time intervals. mRNA expression was determined by RT-PCR

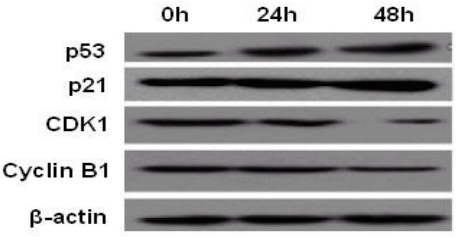

Figure 4. Time-dependent Eexpression of p53, p21, CDK1 and Cyclin B1 in Protein Levels. U87 glioblastoma cells were treated with 100 μM jaceosidin for indicated time intervals. The expression of p53, p21, CDK1 and Cyclin B1 was determined by Western blot analysis

at both mRNA and protein level. Our results showed that jaceosidin treatment down-regulated mRNA level of cyclin B1 and CDK1 in U87 cells as shown in Figure 3. These results were further confirmed by western blot studies. As shown in Figure 4, jaceosidin treatment markedly reduced the protein level of cyclin B1 and CDK1 in a time-dependent manner.

Discussion

Cell cycle control is the major regulatory process of cell growth. Cell cycle is regulated at various check-points and these check-points ensure that processes at each stage of the cell cycle have been accurately completed before progression into next phase (Gamet-Payrastre et al., 2000; Murray et al., 2004). Flavonoids have been reported to arrest cell cycle at G0/G1, S or G2/M phase (Lee et al., 2005; Li et al., 2007; Vidya Priyadarsini et al., 2010). Jaceosidin is a flavonoid isolated from *Artemisia argyi*. In our previous study, we have shown that jaceosidin induces G2/M phase arrest in U87 glioblastoma cells in a time-dependent manner. However the molecular mechanism underlying jaceosidin-induced G2/M phase arrest remained unclear. The purpose of the present study was to elucidate the molecular mechanism of jaceosidin-induced G2/M phase arrest in U87 glioblastoma cells.

p53, a tumor suppressor protein, has been shown to play a key role in the regulation of cell cycle and cell death. Activation of p53 by stress such as DNA damage may lead to arrest cell cycle at G0/G1, S or G2/M phase by activating its target genes especially p21 or by inhibiting cyclins and CDKs directly (Agarwal et al., 1998; Lakin et al., 1999; Flatt et al., 2000). Since U87 glioblastoma cells express wild type p53, we wished to check the possible alterations in expression of p53. The data showed that

jaceosidin increased the expression of p53 at mRNA as well as at protein level in a time-dependent manner. These data are in line with a previous report (Kim et al., 2007).

Cyclin-dependent kinase inhibitors (CDKIs) play a key part in controlling cell cycle progression by negatively regulating the cyclin-dependent kinases (CDKs) activities at a proper time in cell cycle (Murray, 2004). The p53-target gene, p21 (waf-1/cip-1), is one of the major CDKIs which has a broad spectrum of specificity in the cell cycle proteins and is able to arrest cell cycle at G0/G1 checkpoint by inhibiting all the G1 cyclin-CDK complexes as well as at G2/M checkpoint by inhibiting cyclin B1-CDK1 complex (Murray, 2004; Lu et al., 2006). In the present study, an increased expression of p21 have been observed both at mRNA and protein level in U87 glioblastoma cells after exposure to 100 μM jaceosidin in a time-dependent manner. Our data is in agreement with previous study where jaceosidin have been shown to increase the expression level of p21 in ras-transformed human breast epithelial cells (Kim et al., 2007).

Cyclins and cyclin-dependent kinases (CDKs) are the two key classes of molecules that determine the progress of cell through cell cycle. Cyclins are positive regulatory units for CDKs. On the other hand, the activity of CDKs is negatively regulated by a variety of CDKs inhibitors, of which p21 have been shown to play a key role in transition from G2 to M phase of cell cycle (Lu et al., 2006). Among the CDKs, CDK1 is activated in association with cyclin B1 in the G2/M phase progression (Su et al., 2010). In this study, we found that mRNA expression level of cyclin B1 and CDK1 is down-regulated in U87 glioblastoma cells, after treating with 100 μM jaceosidin. These results were further confirmed by Western blot studies, expression of cyclin B1 and CDK1 being markedly reduced in a time-dependent manner.

In our previous study, eupatilin another flavonoid isolated from Artemisia argyi induced G2/M phase arrest in A375 melanoma cancer cells (Al-Shawi et al., 2011). Jaceosidin's chemical structure is quite similar to eupatilin except one hydroxyl group (-OH) which is replaced by methoxy group (-CH3) in eupatilin. Eupatilin have been shown to reduce the expression of cyclin B1 and CDK1 in ras-transformed human mammary epithelial cells (Kim et al., 2004). In the present study, jaceosidin has inhibited the expression of cyclin B1 and CDK1 both at mRNA as well as at protein level which is in agreement with the results of eupatilin. Taken together, our data showed for the first time that jaceosidin induces G2/M phase arrest in U87 glioblsatoma cells via upregulation of p53 and p21 and downregulation of cyclin B1 and CDK1.

Acknowledgements

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