Polydatin regulates the apoptosis and autophagy of fibroblasts obtained from patients with ankylosing spondylitis

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Running title: Regulatory effects of polydatin on fibroblasts isolated from ankylosing spondylitis patients
Abstract

The development of ankylosing spondylitis (AS) occurs due to excessive proliferation of fibroblasts. Polydatin, a monomeric compound isolated from a traditional Chinese medicine polygonum cuspidatum, exhibits anti-inflammatory and anti-arthritis effects. However, the mechanisms underlying the regulatory effects of polydatin on the proliferation, apoptosis and autophagy of fibroblasts obtained from patients with AS remain unclear. The aim of this study was to investigate the therapeutic effects of polydatin on symptoms associated with AS. Multiple cellular and molecular biology experiments were performed in the present study, such as cell viability assay, western blotting, flow cytometry, monodansylcadaverine (MDC) staining and immunofluorescence assays. In the present study, the results revealed that polydatin induced the apoptosis of fibroblasts isolated from patients with AS by upregulating the expression of active caspase-3 and Bax, and downregulating the expression of Bcl-2. Meanwhile, polydatin was revealed to enhance the autophagy of fibroblasts by increasing the expression levels of LC3II, Beclin 1 and Atg5. The results of MDC and immunofluorescence assays further demonstrated that polydatin significantly induced the formation of autophagosomes in fibroblasts. Furthermore, polydatin-induced apoptosis and autophagy were markedly inhibited following treatment with the autophagy inhibitor, 3-Methyladenine (3-MA). In conclusion, the results of the present study indicated that polydatin induces the apoptosis and autophagy of fibroblasts obtained from patients suffering from AS, and that polydatin may represent a therapeutic agent for the future treatment of patients with AS.

Key words: polydatin, apoptosis, autophagy, 3-Methyladenine, ankylosing spondylitis, fibroblast.
Introduction

Ankylosing spondylitis (AS) is a chronic autoimmune disease, and is characterized by a gradual disease course and debilitation of affected individuals. During the early stages, the main symptom of AS is inflammatory back pain. Patient mobility decreases as the disease progresses, and many patients ultimately suffer from body disability (1-4). At present, there are no specific treatment options available for patients with AS, which has become a worldwide health concern due to its early onset, long disease course and high disability rate. Symptoms associated with AS include the inflammation of multiple joints, such as the sacroiliac joints, vertebrae and peripheral joints (5). Fibroblasts represent the main cell type present in the connective tissue surrounding joints. During inflammation, the proliferation and synthesis of connective tissue is enhanced following interaction with inflammatory mediators, which represents the main pathological change associated with connective tissue hyperplasia and fibrosis. Pathological changes associated with AS are related to the proliferation of fibroblasts. Symptoms of rheumatoid arthritis are attenuated by increased levels of autophagy in synovial fibroblasts (6).

Polydatin (3,4,5-trihydroxystilbene-3-β-D-glucoside; POLYDATIN) is a monomer compound isolated from Polygonum cuspidatum Sieb. et Zucc., which exhibits numerous biological functions, such as anti-inflammatory, antioxidant and antineoplastic (7,8). Previous studies have indicated that polydatin has multiple biological activities, such as suppression of inflammatory factor production and decreasing the expression of matrix-degrading protease in human osteoarthritic chondrocytes. In addition, polydatin attenuates osteoarthritis in murine models (9). Effective treatment of polydatin was revealed to attenuate symptoms associated with collagen-induced arthritis via regulation of antioxidative and anti-inflammatory effects in mice, as well as activation of MMP-9 expression (10). Polydatin significantly induces apoptosis and autophagy in multiple myeloma by mediating the expression levels of Beclin 1, Atg5 and LC3 (11). Moreover, polydatin promotes cardiac function by increasing the autophagy flux (12).

There have been numerous studies that have investigated the biological activities and pharmacological functions associated with polydatin; however, few studies have investigated
the effects of polydatin on patients with AS. In the present study, the function of polydatin associated with autophagy and apoptosis of fibroblasts obtained from patients with AS was investigated.

**Materials and methods**

**Isolation and culture of fibroblasts isolated from patients suffering from primary AS**

A total of 40 patients with AS (25 males and 15 females) treated at Beijing Traditional Chinese Medicine Hospital (Beijing, China) between April 2017 and May 2018 were included in the present study. All patients were aged between 25-45 years old. The diagnostic criteria for AS patients complied with a previously published study (13). Basic clinical and pathological data of included patients were collected following the provision of written, informed consent. The present study was approved by the Ethics Committee of Beijing Traditional Chinese Medicine Hospital. The isolation and culture methods of fibroblast cells obtained from AS patients were performed according to a previously published protocol (14). Human fibroblasts CCD-18Co were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Eagle's Minimum Essential Medium with 10% fetal bovine serum. Polydatin standard product was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany; cat. no. 15721).

**Cell Counting Kit-8 (CCK-8) assay to determine cell viability**

Cell viability was evaluated using a CCK-8 assay (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer’s protocol. Briefly, fibroblasts obtained from patients with AS were seeded into a 96-well plate at a density of $5 \times 10^3$ cells/well and then incubated overnight. Following this, 200 $\mu$L polydatin (0, 0.33, 1, 3 or 10 $\mu$M) was added into each well and cells were incubated for a further 24, 48 or 72 h. After incubation, 10 $\mu$L of CCK-8 reagent was added to each well and then incubated for 2 h. Absorbance values were measured at 450 nm using a Thermo Multiskan FC microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).
**Flow cytometric analysis of cell apoptosis**

Apoptotic cells were analyzed according to a previously described method (15). Briefly, fibroblasts isolated from patients with AS were treated with either polydatin (3 μM) or DMSO for 48 h. Following this, the two groups of fibroblast cells were isolated and then resuspended in PBS. Apoptotic cells were stained with dual-staining Annexin V-FITC and propidium iodide (PI; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All experiments were repeated in triplicate.

**Western blot analysis**

Fibroblasts isolated from patients suffering from AS were exposed to either DMSO, 3 μM polydatin or 3 μM polydatin + 5 mM 3-MA for 48 h. Following this, fibroblast cells were gathered and dissolved in RIPA buffer (Sigma-Aldrich; Merck KGaA). Equal amounts of protein were quantified using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology) and subsequently loaded onto SDS-PAGE gels. Following polyacrylamide gel electrophoresis, proteins were transferred onto PVDF membranes (Thermo Fisher Scientific, Waltham, MA, USA). PVDF membranes were subsequently blocked with 5% skimmed milk in TBST for 1 h and then incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: anti-active caspase 3 (Abcam, Cambridge, UK; cat. no. ab2302; 1:1000), anti-Bax (Abcam; cat. no. ab32503; 1:1000), anti-Bcl-2 (Abcam; cat. no. ab32124; 1:1000), anti-β-actin (Abcam; cat. no. ab8227; 1:1000), anti-Beclin 1 (Abcam; cat. no. ab207612; 1:1000), anti-LC3 (Abcam cat. no. ab128025; 1:1000), anti-Atg 5 (Abcam; cat. no. ab228668; 1:1000). Immunodetection was performed using anti-rabbit (1:5000) secondary antibodies and an enhanced chemiluminescence detection kit (16).

**Monodansylcadaverine (MDC) staining**

Fibroblasts isolated from patients with AS were treated with either DMSO, 3 μM polydatin or 3 μM polydatin + 3-MA at 37°C for 48 h, and then stained with MDC (50 μmol/L) at 37°C for 30 min. Following this, the cells were washed three times with phosphate buffer solution, and autophagic vacuoles were instantly observed using a phase contrast microscope.
(Olympus Corporation, Tokyo, Japan) and then counted.

**Immunofluorescence**

Fibroblasts were exposed to polydatin (3 μM), DMSO or polydatin combined with 3-MA at 37°C for 48 h. Immunofluorescence assays were performed according to a previously described method (17). Next, cells were incubated with primary antibodies against LC3 and stained with DAPI at 4°C overnight and subsequently incubated with secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 37°C for 1 h. Autophagosome formation was observed by fluorescence microscopy (Olympus Corporation, Tokyo, Japan).

**Statistical analysis**

All experiments were performed a minimum of three times and data are presented as the mean ± standard deviation. Comparisons between two groups was analyzed by performing the Student’s t-test. Comparisons among multiple groups were performed via one-way analysis of variance followed by the Dunnett’s test. P<0.05 and P<0.01 were considered to indicate statistically significant differences.

**Results**

**Polydatin suppressed the proliferation of fibroblasts isolated from patients with AS**

The structure of polydatin is presented in Fig. 1A. CCK-8 assays were used to determine the effect on the viability of fibroblasts isolated from patients with AS following treatment with polydatin (0, 0.33, 1, 3 and 10 μM) for 24, 48 or 72 h. As presented in Fig. 1B, 3 μM and 10 μM polydatin markedly decreased the cell viability of fibroblasts. However, the number of cells was significantly decreased by >50% following treatment with 10 μM polydatin. Therefore, 3 μM polydatin was chosen for use in subsequent experiments. In addition, polydatin had very limited effect on normal human fibroblasts CCD-18Co (Fig. 1C). These results suggested that polydatin may suppress the proliferation of fibroblasts obtained from
patients with AS.

**Polydatin induced the apoptosis of fibroblasts isolated from patients with AS**

To further demonstrate the effect of polydatin on the apoptosis of fibroblasts obtained from patients with AS, fibroblasts were treated with 3 μM polydatin for 48 h. Following this, the apoptosis of fibroblasts was detected by flow cytometry. As indicated in Fig. 2A and B, the apoptosis levels of fibroblasts were significantly increased following treatment with polydatin compared with fibroblasts treated with DMSO. In addition, the expression levels of active caspase 3, Bax and Bcl-2 apoptosis-associated proteins in fibroblasts were detected by western blotting. In the polydatin-treated group compared with the DMSO group, the expression levels of active caspase 3 and Bax were markedly increased, while the expression of Bcl-2 was significantly decreased (Fig. 3C-E). These results suggested that polydatin induced the apoptosis of fibroblasts obtained from patients with AS.

**Polydatin induced the autophagy of fibroblasts isolated from patients with AS**

Whether polydatin induces autophagy in fibroblasts isolated from patients with AS was investigated. Western blotting was performed to determine the expression levels of the following autophagy-associated proteins: LC3I/II, Beclin 1 and ATG 5. As presented in Fig. 3A-D, levels of LC3II, Beclin 1 and ATG 5 were markedly increased in polydatin-treated fibroblasts compared with the DMSO group (P<0.01). Moreover, fibroblasts were treated with autophagy inhibitor 3-MA to further determine the effect of polydatin on the cells. As indicated in Fig. 3, polydatin-induced increases in LC3II, Beclin 1 and ATG 5 protein expression levels were significantly inhibited following treatment with 3-MA compared with cells treated with polydatin alone (P<0.01). These results suggested that polydatin induced the autophagy of fibroblasts, and this effect was reversed by 3-MA.

**Polydatin induced the formation of autophagic vacuoles in fibroblasts obtained from patients with AS**

MDC and immunofluorescence assays were performed to further investigate the effect of
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Polydatin on the formation of autophagic vacuoles in fibroblasts isolated from patients with AS. The results of MDC staining indicated that the numbers of autophagic vacuoles and autophagosomes were markedly increased in fibroblasts treated with polydatin compared with fibroblasts treated with DMSO (Fig. 4A and B). In addition, the numbers of autophagic vacuoles and autophagosomes were significantly decreased following treatment with 3-MA compared with fibroblasts treated with polydatin alone (P<0.01; Fig. 4A and B). Furthermore, immunofluorescence data revealed that expression levels of LC3-II were increased following treatment with polydatin, which was associated with increased autophagosome formation due to its association with the autophagosome membrane (Fig. 4C and D). These results further suggested that polydatin increased autophagy in fibroblasts obtained from patients with AS.

Inhibition of autophagy reversed polydatin-induced apoptosis of fibroblasts isolated from patients suffering from AS

The effect of polydatin on the proliferation of fibroblasts obtained from patients with AS following incubation in the presence or absence of 3-MA was investigated. CCK-8 and Annexin V/PI assays were performed to detect cell proliferation and apoptosis, respectively. The results demonstrated that levels of cell viability and Bcl-2 were markedly decreased, while protein expression of active caspase 3 and Bax were markedly increased following treatment with polydatin compared with fibroblasts treated with DMSO; however, these effects were reversed following treatment with 3-MA (Fig. 5A-E). Additionally, this was further confirmed by the results of the Annexin V/PI assay (Fig. 5F and G). These results demonstrated that polydatin-induced cell autophagy and apoptosis were markedly inhibited following treatment with the autophagy inhibitor, 3-MA.

Discussion

AS is a chronic disease that is commonly associated with a variety of joint complications, which have a marked effect on patient mobility and quality of life. Therefore, the discovery of novel drugs for the treatment of patients with AS is important (18).

Previous studies have found that polydatin could induce the apoptosis of hepatocellular
carcinoma cells via inhibition of cell proliferation, invasion and migration (19). Li demonstrated that polydatin attenuates symptoms associated with arthritis in mice via suppression of inflammation and activation of MMP-9 (10). However, the effect of polydatin on the apoptosis and autophagy of fibroblasts isolated from patients with AS remain unclear. The results of the present study revealed that polydatin inhibited and promoted the proliferation and apoptosis of fibroblasts obtained from patients with AS, respectively. In addition, polydatin was demonstrated to promote the apoptosis of fibroblasts by increasing the expression levels of caspase 3 and Bax and decreasing the expression levels of Bcl-2. Therefore, the results suggested that polydatin inhibited the apoptosis of fibroblasts. In addition, polydatin has a therapeutic effect on acute monocytic leukemia via inhibition of cell growth in the S phase, induction of THP-1 apoptosis, significantly decreasing Bcl-2 expression and increasing Bax expression (20). Moreover, it has been previously demonstrated that polydatin promotes cell apoptosis in different cancer cell lines, including colorectal cancer cells, osteosarcoma cells and lung cancer cells (19,21-23). The results of the present study were consistent with these findings.

Autophagy predominantly functions as a cytoprotective mechanism, which is necessary for the survival of cells (24). LC3 has been well established to represent an indicator of autophagy. Beclin1 and ATG are important autophagy inducers (25,27). Previous studies revealed that polydatin increases autophagy in cardiac cells, multiple myeloma and pancreatic cancer (28,30). In this study, the expression levels of autophagy-associated proteins in fibroblasts obtained from patients with AS were investigated, including LC3II, Beclin 1 and Atg5. The results demonstrated that treatment with polydatin significantly increased the expression levels of LC3II, Beclin 1 and Atg5 in fibroblasts. In addition, the expression levels of LC3II, Beclin 1 and Atg5 were significantly decreased in fibroblasts following treatment with 3-MA compared with cells treated with polydatin alone, which was further confirmed by MDC and immunofluorescence assay results. Autophagy associated with apoptosis may attenuate either cell survival of cell death (31). The results of the present study revealed that polydatin-induced apoptosis and autophagy were markedly decreased following treatment with the autophagy inhibitor, 3-MA. This finding is consistent with a previous
study that demonstrated that 3-MA suppresses the apoptosis of osteoblasts in particle-induced peri-implant osteolysis models (32). Autophagy inhibitors have also been reported to inhibit the expression of Bax (33). However, the exact associations between polydatin-induced apoptosis and autophagy in fibroblasts obtained from patients suffering from AS remain unclear and require further investigation.

In conclusion, the results of the present study demonstrated that polydatin induced apoptosis and autophagy in fibroblasts isolated from patients with AS. These results indicated that polydatin may represent a potential therapeutic agent for the treatment of patients with AS.

Conflicts of interest
The authors declare no conflict of interest.

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References


(11) Yang B, Zhao S. Polydatin regulates proliferation, apoptosis and autophagy in multiple


Figure legends

**Figure 1. Polydatin suppressed the proliferation of fibroblasts obtained from patients with AS.** (A) Structure of polydatin. (B) The viability of fibroblasts obtained from patients with AS treated with polydatin (0, 0.33, 1, 3 or 10 μM) for 24, 48 or 72 h was investigated by performing CCK-8 assays. (C) The viability of CCD-18Co treated with indicated concentration of polydatin for 24, 48 or 72 h was evaluated with CCK-8 assays **P<0.01 vs. DMSO group.** (Color figure can be accessed in the online version.)

**Figure 2. Polydatin induced the apoptosis of fibroblasts obtained from patients suffering from AS.** (A) Fibroblasts were treated with 3 μM polydatin for 48 h. Apoptotic cells were observed by performing Annexin V and PI double staining. (B) Apoptosis cell rates were calculated. (C) Expression levels of active caspase-3, Bax and Bcl-2 in fibroblasts following treatment with polydatin for 48 h were investigated via western blotting. β-actin was used as an internal control. (D) The relative expression levels of active caspase 3 were quantified via normalization against β-actin. (E) The relative expression levels of Bax were quantified via normalization against β-actin. (F) The relative expression levels of Bcl-2 were quantified via normalization against β-actin. **P<0.01 vs. DMSO group; ##P<0.01 vs. cells treated with polydatin alone.** (Color figure can be accessed in the online version.)

**Figure 3. Polydatin induced the autophagy of fibroblasts isolated from patients suffering from AS.** (A) Fibroblasts were treated with either 3 μM polydatin or polydatin + 5 mM 3-MA for 48 h. Expression levels of LC3I, LC3II, beclin 1 and Atg5 in cells were analyzed via western blotting following 48 h of culture. (B) The relative expression levels of LC3-II were quantified via normalization against β-actin. (C) Relative expression levels of beclin I were quantified via normalization against β-actin. (D) Relative expression levels of ATG5 were quantified via normalization against β-actin. **P<0.01 vs. DMSO group; ##P<0.01 vs. cells treated with polydatin alone.
Figure 4. Polydatin-induced formation of autophagic vacuoles in fibroblasts obtained from patients with AS. (A) Fibroblasts were treated with either 3 μM polydatin or polydatin + 5 mM 3-MA for 48 h, and MDC staining was subsequently performed to observe the formation of autophagosomes (×200 magnification). (B) The number of autophagosomes in fibroblasts was determined. (C) Images of fibroblasts stained with LC3 and DAPI following treatment with 3 μM polydatin or polydatin + 5 mM 3-MA for 48 h. (D) Relative fluorescence expression levels were quantified by LC3 and DAPI staining. **P<0.01 vs. DMSO group; ##P<0.01 vs. cells treated with polydatin alone. (Color figure can be accessed in the online version.)

Figure 5. Inhibition of autophagy reversed polydatin-induced apoptosis of fibroblasts isolated from patients with AS. Fibroblasts were treated for 48 h with either 3 μM polydatin or polydatin + 5 mM 3-MA. (A) Cell viability was determined via performance of CCK-8 assays. (B) Expression levels of active caspase-3, Bax and Bcl-2 in fibroblasts were analyzed by western blotting following treatment with polydatin for 48 h. (C-E) The relative expression levels of active caspase 3, Bax and Bcl-2 were quantified via normalization against β-actin. (F) Cell apoptosis was detected via Annexin V/PI assays. (G) Apoptosis cell rates were calculated. **P<0.01 vs. DMSO group; ##P<0.01 vs. cells treated with polydatin alone. (Color figure can be accessed in the online version.)
Fig. 1

A. The structure of polydatin

B. Fibroblasts obtained from patients

C. CCD-18Co
Fig. 2
Fig. 3

A

LC3 I
LC3 II
Beclin 1
ATG5
β-actin

DMSO  Polydatin 3 μM  Polydatin + 3MA

B

Relative protein expression

DMSO  Polydatin 3 μM  Polydatin + 3MA

LC3 II

**

C

Relative protein expression

DMSO  Polydatin 3 μM  Polydatin + 3MA

Beclin 1

**

**

D

Relative protein expression

DMSO  Polydatin 3 μM  Polydatin + 3MA

ATG5

**

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# # # #
Fig. 4

A

DMSO
Polydatin_3 μM
Polydatin + 3MA

B

MDC positive cells

DMSO
Polydatin_3 μM
Polydatin + 3MA

C

LC3
DAPI
Merge

D

Relative fluorescence activity

DMSO
Polydatin_3 μM
Polydatin + 3MA
Fig. 5

A. OD value (400 nm) for DMSO, Polydatin, and Polydatin + 3MA.

B. Western blot analysis of active caspase 3, Bax, Bcl-2, and β-actin for DMSO, Polydatin, and Polydatin + 3MA.

C. Caspase 3 relative protein expression for DMSO, Polydatin, and Polydatin + 3MA.

D. Bax relative protein expression for DMSO, Polydatin, and Polydatin + 3MA.

E. Bcl-2 relative protein expression for DMSO, Polydatin, and Polydatin + 3MA.

F. Flow cytometry analysis of Annexin V positive cells for DMSO, Polydatin, and Polydatin + 3MA.

G. Annexin V positive cell count (%) for DMSO, Polydatin, and Polydatin + 3MA.