Effects of daphnetin combined with Bcl2-siRNA on antiapoptotic genes in synovial fibroblasts of rats with collagen-induced arthritis

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Abstract. The aim of the present study was to investigate the effects of daphnetin combined with B cell lymphoma 2 (Bcl2)-targeted small interfering (si)RNA (si-Bcl2) on antiapoptotic genes in fibroblast-like synoviocytes (FLS) in rats with collagen II-induced arthritis (CIA). The roles of si-Bcl2 and daphnetin were determined by measuring the expression levels of Bcl2. Protein and mRNA expression levels of Bcl2 in FLS were determined by flow cytometry and reverse transcription-quantitative polymerase chain reaction. Apoptosis of FLS was also determined by flow cytometry. It was revealed that treatment with si-Bcl2 or daphnetin alone resulted in downregulation of Bcl2 mRNA and protein expression. In addition, the mRNA expression levels of the signal transducer and activator of transcription 3 (STAT3), which transcriptionally regulates the activity of mitochondria, were reduced. The combination of si-Bcl2 and daphnetin exhibited an enhanced effect on rheumatoid arthritis FLS (RAFLS), in which the apoptotic rate was significantly higher than either treatment alone. The results suggested that si-Bcl combined with daphnetin may have an enhanced effect in promoting apoptosis of RAFLS derived from CIA rats, and a possible underlying molecular mechanism may function through the downregulation of Bcl2 expression and STAT3, which is located upstream of Bcl2 in the mitochondrial apoptotic pathway. The results of the present study are expected to provide theoretical and experimental basis for the treatment of RA and the medicinal development of daphnetin combined siRNA.

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is characterized by chronic joint inflammation, synovial hyperplasia and the progressive destruction of cartilage and bone (1,2). It has been revealed that a primary manifestation in RA is the excessive proliferation of RA fibroblast-like synoviocytes (RAFLS) (3,4), which may be closely associated with impaired RAFLS apoptosis (5,6), which is believed to be a result of overexpression of the anti-apoptotic gene B cell lymphoma 2 (Bcl2) (7,8). Therefore, the downregulation of Bcl2 expression to increase RAFLS apoptosis may be a promising therapeutic option for the treatment of RA (9).

Daphnetin, an active ingredient extracted from *Daphne odora* var. *marginata*, possesses many biological and medical properties, including antiparasitic, anti-inflammatory and analgesic properties (10). Daphnetin may also kill aphids, inhibit protein kinases and improve abnormal blood rheology of type 2 diabetic rats (11). Results from our preliminary study performed in 2011 revealed that daphnetin may have therapeutic and immunomodulatory effects in rats with collagen II-induced arthritis (CIA) (12). In 2012, it was demonstrated that the apoptosis rate of RAFLS was elevated upon treatment with daphnetin (13).

RNA interference (RNAi) is a gene silencing technology. RNAi has been widely used for the anti-inflammatory treatment of RA in a number of studies worldwide (14-18). The results of these studies have revealed that RNAi is a promising therapeutic option for RA. However, RNAi targeting of anti-apoptotic genes has not yet been reported in RA.

In the present study, Bcl2-targeted small interfering (si)RNA (si-Bcl2) was synthesized and transfected into RAFLS using a liposome-mediated method. si-Bcl2 treatment was demonstrated to successfully interfere with the expression of Bcl2. Subsequently, RAFLS were treated with daphnetin, and the apoptotic rate was measured, along with the mRNA expression levels of Bcl2 and signal transducer and activator of transcription 3 (STAT3). In addition, the possible underlying mechanism by which daphnetin combined with si-Bcl2 affects antiapoptotic gene expression in the RAFLS of CIA rats was investigated. The present study is expected to make a new contribution to the treatment of RA.

Materials and methods

Materials. FLS from CIA rats and healthy rats were purchased from Shanghai Institute of Health Industry Co., Ltd. (Shanghai, China). Daphnetin, extracted from *Daphne odora* var. *marginata* by high-speed counter-current chromatography, was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Other reagents were purchased as follows: Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); siRNAs (Ribobio Co., Ltd., Guangzhou, China); Opti-MEM I (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); reverse transcription-quantitative polymerase chain reaction (RT-qPCR) primers (Sangon Biotech Co., Ltd., Shanghai, China); ReverTra Ace qPCR RT kit (Toyobo Life Science, Osaka, Japan); SYBR Green Real Time PCR Master mix (Toyobo Life Science); rabbit anti-Bcl2 antibody conjugated to fluorescein isothiocyanate (FITC; bs0032r-F; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China); annexin V/propidium iodide (PI) kit (KeyGen Biotechology Co. Ltd., Nanjing, China); diethyl pyrocarbonate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); and TransZol (Beijing TransGen Biotech Co., Ltd., Beijing, China).

Cultured FLS from CIA rats. FLS were cultured and passaged in Dulbecco's Modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% foetal bovine serum (FBS, Tianjin Haoyang Biological Manufacture Co., Ltd., Tianjin, China). All cultures were incubated at 37˚C in 5% humidified CO_2. Following 2 days incubation, FLS were digested using 0.25% trypsin prior to centrifugation at low speed (112 x g for 10 min at 25˚C). Nonadherent granulocytes were removed, and FLS were cultured in 24-or 6-well plates ($5x10^4$ or $1x10^5$ cells/ml) in 500 μl or 2 ml DMEM, respectively, containing 10% FBS without penicillin and streptomycin for 24 h following treatment with siRNA (various concentrations) or daphnetin (40 μg/ml, 37˚C).

siRNA synthesis and transfection. All siRNA sequences were purchased from Ribobio Co., Ltd. (Guangzhou, China). A total of three siRNA sequences specific to Bcl2 were selected: i) si-Bcl2.1, GCCUCCGACCCUACGGAAA; ii) siBcl2.2, CCUACUGACUCAUGGACUU; and iii) siBcl2.3, CCUCUU GUCCCAUACUAUU. siRNAs were chemically synthesised and annealed by Nanjing Anbo Ruila Biotechnology Co., Ltd. Three targeting siRNA was respectively used to silence the Bcl2 gene in FLS of CIA rats. siRNAs labelled with fluorescent cyanine 3 (Cy3; siN05815122149; Ribobio Co., Ltd.) were used to test whether the transfection was successful. An siRNA specific to an invalid gene (siN05815122147; Ribobio Co., Ltd.) was used as a negative silencing control. Experimental groups were used to determine the optimal siRNA concentration, including siRNA transfection negative control group (NC group), si-Bcl2.1-transfection group, si-Bcl2.2-transfected group and si-Bcl2.3-transfection group.

Transfection was conducted according to the manufacturer's protocol. Briefly, 50 μl Opti-Mem I (serum-free, penicillin-free and streptomycin-free) was used in 24-well plates (or 250 μl in 6-well plates) to dilute 5, 2.5, 1.25, 0.75, 0.5 and 0.25 μl annealed siRNA (or 20, 10, 5, 3, 2 and 1 μl siRNA in 6-well plates). Another 50 μl Opti-Mem I was used in 24-well plates (or 250 μl in 6-well plates) to dilute 1 μl Lipofectamine 2000 (or 5 μl in 6-well plates). Subsequently, the diluted siRNA-Lipofectamine 2000 complex was incubated at room temperature for 20 min. This mixture was then added to 400 μl FLS cell culture in 24-well plates ($5x10^4$ cells/well), or 1,500 μl in 6-well plates ($1x10^5$ cells/well). A concentration gradient of the siRNA was generated as follows: 200, 100, 50, 30, 20 and 10 nM. Following 6 h incubation at 37˚C, an equal volume of DMEM supplemented with 10% FBS was added to the cells. When combined with siRNA, daphnetin (final concentration 40 μg/ml) was added to the FLS cell culture as appropriate for each of the treatment groups. Following 24 or 48 h transfection at 37˚C, Cy3 fluorescence was observed under a fluorescent microscope to confirm that the transfection was successful. The transfected RAFLS were washed with DMEM and then immediately used for subsequent experiments.

Combined treatment of CIA rat FLS cells with daphnetin and si-Bcl2. Experimental groups were: i) FLS of untreated healthy Wistar rats (healthy group); ii) untreated FLS of CIA rats (CIA group); iii) daphnetin (40 μg/ml)-treated group (E003 group); iv) si-Bcl2-treated group; v) daphnetin (40 μg/ml) plus si-Bcl2 combined treatment group (E003-B group). Each group of cells was treated by the method of siRNA transfection as described above. Following treatment ($5x10^4$/$1x10^5$ cells/well at 37˚C in 5% humidified CO_2), FLS were cultured for 24, 48 and 72 h, and total RNA was extracted to measure the relative mRNA expression levels of Bcl2 and STAT3. In addition, resuspended FLS were used to measure Bcl2 protein expression levels and to assess apoptosis by flow cytometry.

Total RNA extraction and reverse transcription. Total RNA was isolated from FLS ($5x10^4$ cells) following gene silencing using TRIzol (All-in gold Biological Co., Ltd.), according to the manufacturer's protocol. cDNA was synthesized using the ReverTra Ace qPCR RT kit (Toyobo Life Science, Osaka, Japan) was used. Briefly, extracted RNA was heat denatured at 65˚C for 5 min and immediately cooled on ice. Subsequently, 1 μl RT Enzyme mix, 1 μl Primer mix and 4 μl 5X RT buffer were incubated with 1 μg DNA-free total RNA for 15 min at 37˚C. The mixture was incubated for 5 min at 98˚C to inactivate the RT enzyme, and the first-strand cDNA was stored at 4˚C until use in qPCR.

RT-qPCR. For qPCR, reactions were performed in a volume of 20 μl SYBR-Green Real Time PCR Master mix (Toyobo Life Science). The primers used in this study were as follows: Bcl2, forward 5'-GGGATGCCTTTGTGGAACTAT-3, reverse 5'-AGGTATGCACCCAGAGTGATG-3' (124 bp); STAT3, forward 5'-GGGCACAAACACAAAAGTGAT-3', reverse 5'-CAGTCACAATCAGGGAAGCAT-3' (140 bp); β-actin, forward 5'-TGACAGGATGCAGAAGGAGA-3', reverse 5'-TAGAGCCACCAATCCACACA-3' (106 bp). Thermocycling conditions included initial denaturation at 95˚C for 1 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 15 sec and extension at 72˚C for 45 sec. qPCR was performed on an ABI 7500 PCR instrument. A relative quantitative assay was adopted (19),

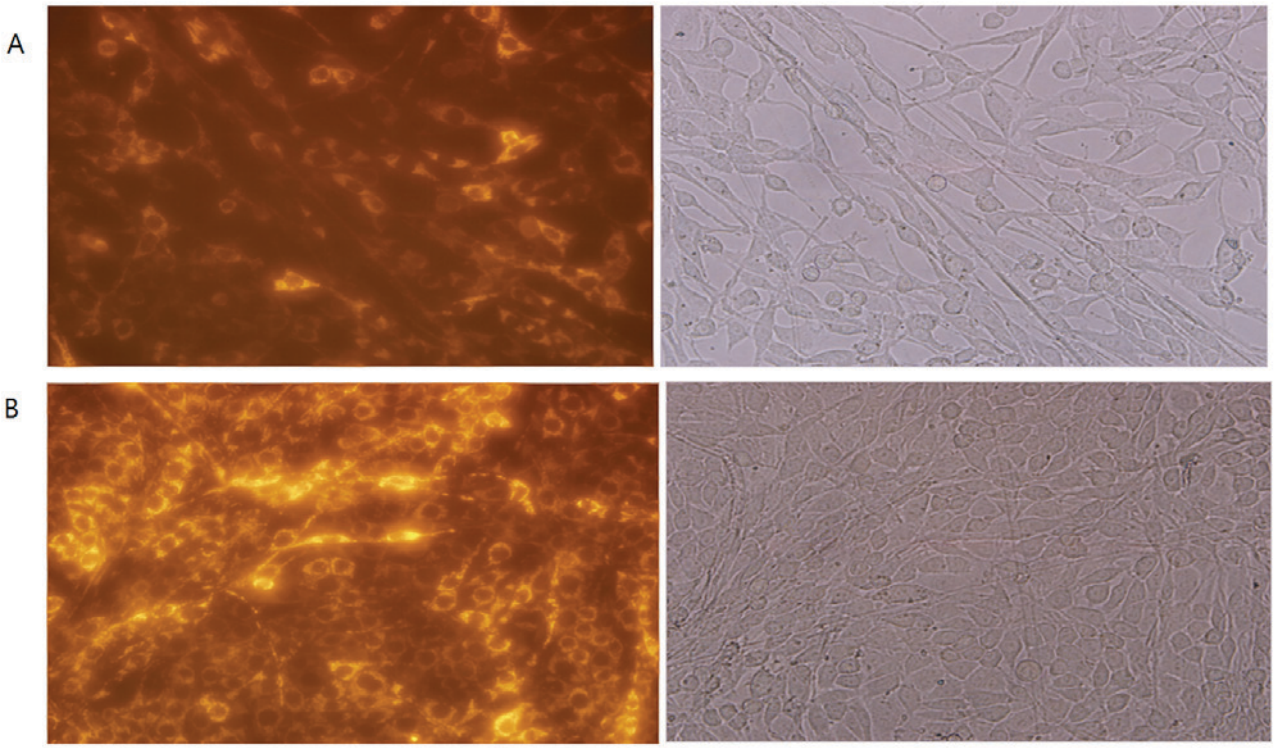

Figure 1. Observation of fluorescence. The optimal siRNA was selected, and FLS were transfected with Cy3-labelled siRNA to test whether the transfection was successful. Magnification, x400 (A) Following 24 h incubation, orange fluorescence was observed by an inverted fluorescence microscope. (B) Orange fluorescence from FLS cells transfected with Cy3-labeled siRNA following 48 h incubation. Right hand images are non-fluorescent for comparison. Bcl2, B cell lymphoma 2; Cy3, cyanine 3; FLS, fibroblast-like synoviocytes; siRNA, short interfering RNA.

and the melting curve increased following amplification. The reference gene β-actin, and Bcl2 and STAT3 were simultaneously amplified in the samples of each experimental group. Each sample was tested in triplicate. Samples were obtained from ≥3 independent experiments to calculate the mean and standard deviation. The formula $2^{-\Delta\Delta Cq}$ was used to calculate the relative expression of Bcl-2, STAT3mRNA (19).

Flow cytometry. Bcl2 protein expression analysis and the apoptotic rate of FLS from CIA rats were performed on a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For the Bcl2 protein, a FITC-conjugated rabbit anti-rat Bcl2 antibody (Biosynthesis Corporation, Beijing, China) was used. FLS cells ($1x10^5$ cells/well) were treated as aforementioned, collected by trypsinization following 72 h incubation, fixed by 1% paraformaldehyde at room temperature for 15 min, and resuspended with 100 μl or 500 μl PBS. Following collection, cells were stained with (FITC)-labeled rabbit anti-rat Bcl2 antibody (1:100) and 70% ethanol added in the dark for 15 min at room temperature for permeabilisation. Bcl2 protein expression was assessed by determining the average fluorescence intensity. Apoptosis of FLS was examined using a FITC-labeled Annexin V/propidium iodide (PI) Apoptosis Detection kit (Nanjing KeyGen Biotechology Co. Ltd., Nanjing, China) according to the manufacture's protocol. Cells ($1x10^5$ cells/well) treated as aforementioned for 72 h were collected by trypsinization, fixed by 70% ethanol in the dark for 15 min at 4˚C and washed twice with PBS. The cells were resuspended in 500 μl binding buffer. Then 5 μl Annexin-V-FITC and 5 μl PI-FITC were added and incubated in the dark for 30 min at room temperature. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using Cell Quest software version 6.1.2 (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis. All data are expressed as the mean ± standard deviation, and were analysed by one-way analysis of variance followed by LSD post hoc multiple comparison test using SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). A Student's t-test was used to determine the significances of differences in multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cy3-labeled siRNA transfection efficiency of FLS. Cy3-labeled si-Bcl2 was transfected into FLS. To determine whether the transfection was successful, the fluorescence was observed under a microscope at 24 and 48 h post-transfection (Fig. 1A and B, respectively).

Optimisation of experimental conditions. Identification of the optimal siRNA concentration and incubation time was examined, as well as determining the optimal treatment time for daphnetin. All experimental conditions were designed to assess the mRNA expression levels of the antiapoptotic gene Bcl2, which was determined using RT-qPCR. The results revealed that Bcl2 mRNA expression in the si-Bcl2.1 group was significantly lower compared with the NC, si-Bcl2.2, and si-Bcl2.3 groups (Fig. 2A). As demonstrated in Fig. 2B, 100 nM

si-Bcl2.1 inhibited Bcl2 mRNA expression most following transfection for 48 h compared with the NC group (Fig. 2B). In a previous study, it was determined that 40 μg/ml daphnetin significantly promoted FLS apoptosis (13). Therefore, the present study focused on determining the most suitable length of time required to downregulate Bcl2 mRNA expression. The results demonstrated that Bcl2 mRNA expression in FLS cells following daphnetin treatment for 48 or 72 h was significantly lower compared with cells treated for 24 h and untreated CIA FLS cells (Fig. 2C). However, daphnetin treatment for 48 h was not statistically significant (P>0.05) compared with for 72 h, so cells were treated for 48 and 72 h to observe Bcl2 mRNA expression in the later combined experiment.

Influence of daphnetin combined with si-Bcl2 on mRNA and protein expression of Bcl2 and mRNA expression of STAT3 in RAFLS derived from CIA rats. In the optimal conditions, si-Bcl2.1 was transfected into FLS and daphnetin was added to FLS cultures 6 h following gene silencing. Bcl2 and STAT3 mRNA expression levels were determined by RT-qPCR (Fig. 3A and B, respectively). Bcl2 protein expression was assessed by determining the average fluorescence intensity using flow cytometry (Fig. 3C). The results demonstrated that Bcl2 mRNA expression in the E003-B group was significantly lower than that in the other treatment groups. In addition, Bcl2 mRNA expression in the E003-B group treated for 48 h was significantly lower compared with that in the same group treated for 72 h (P<0.05), and no significant difference was observed in expression between the E003-B group treated for 48 h and the healthy group treated for 48 h (P>0.05; Fig. 3A). It suggested that daphnetin combined with si-Bcl2 for the treatment of RA was effective, particularly as in the treatment for 48 h, Bcl2 mRNA was downregulated most. Consistent with Bcl2 mRNA expression, STAT3 mRNA expression in the E003-B group was also decreased compared with the other treatment groups (P<0.05; Fig. 3B). Bcl2 protein expression in the E003-B group was significantly lower compared with expression in the other treatment groups and the CIA group (P<0.05; Fig. 3C).

Effects of daphnetin combined with si-Bcl2 on apoptosis of RAFLS derived from CIA rats. The apoptotic rate of RAFLS derived from CIA rats was also determined by flow cytometry. The results demonstrated that the apoptotic rate of FLS in the E003-B group was significantly higher than that of other groups (P<0.05; Fig. 4).

Discussion

RA is a common systemic autoimmune disease that is characterised by chronic joint inflammation, synovial hyperplasia and progressive destruction of cartilage and bone. This process is sustained and recurs so that the affected joints swell and lose functionality. If RA is not treated in a timely manner, it may result in severe joint deformity, leading to multiple joint dysfunction and other organ diseases. RA and complications caused by RA, such as cancer (20,21), are a major cause of disability or lost labour in China. However, the pathogenesis of RA remains to be elucidated. Recently, studies have revealed that RA may be associated with an abnormal autoimmune

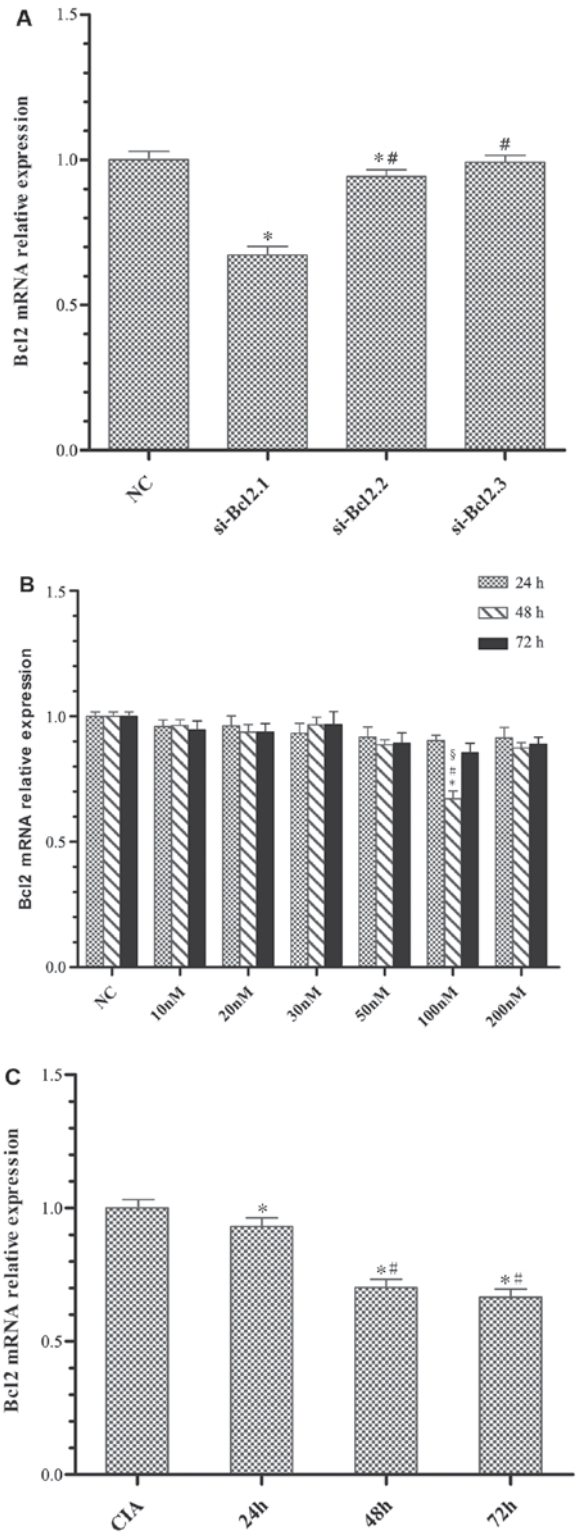

Figure 2. Optimization of experimental conditions. (A) si-Bcl2.1, si-Bcl2.2 or si-Bcl2.3 (100 nM) was transfected into FLS. Reverse transcription-quantitative polymerase chain reaction was performed using primers specific to Bcl2 and β-actin, and relative quantification of gene expression was performed by the $2^{-\Delta\Delta Cq}$ method. *P<0.05 vs. NC group; #P<0.05 vs. si-Bcl2.1 group; (B) Dilution curve of si-Bcl2.1 to identify the optimal siRNA concentration and incubation duration that significantly reduced mRNA expression levels of Bcl2. *P<0.05 vs. NC group; #P<0.05 vs. 24 h group at the same concentration; §P<0.05 vs. 72 h group at the same concentration. (C) FLS were treated with E003 (40 μg/ml) for 24, 48 and 72 h. The effects of E003 were assessed by determining Bcl2 mRNA expression levels. *P<0.05 vs. CIA group; #P<0.05 vs. 24 h group. Bcl2, B cell lymphoma 2; CIA, collagen II-induced arthritis; E003, daphnetin; FLS, fibroblast-like synoviocytes; NC, negative control; si-Bcl2, short interfering RNA targeting Bcl2.

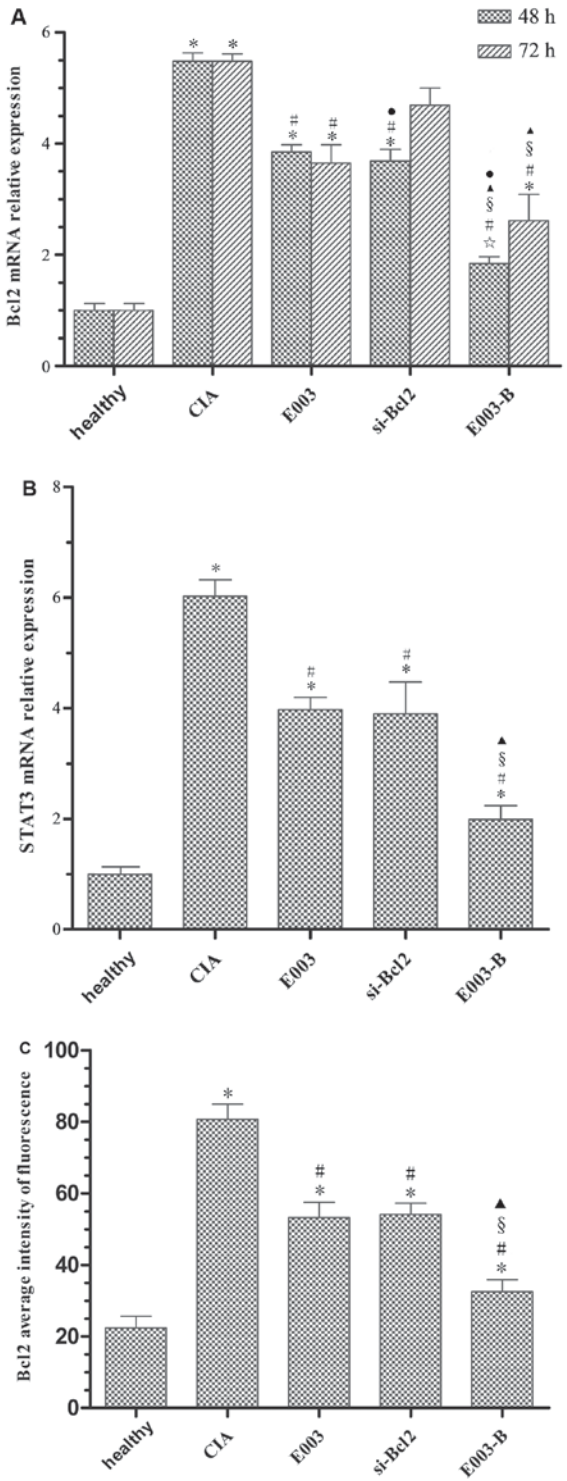

Figure 3. Influence of E300 combined with si-Bcl2 on the gene encoding antiapoptotic Bcl2 and the signalling protein STAT3. (A) mRNA expression levels of Bcl2 in healthy FLS, CIA FLS or CIA FLS treated with E003, si-Bcl2 or E003-B. 100 nM si-Bcl2.1 was transfected into FLS for 48 h and 40 μg/ml E003 was cultured together with FLS for 72 h. Owing to the different periods of time required for si-Bcl2 and E003 to affect Bcl2 mRNA expression in FLS, mRNA expression of Bcl2 was measured after the cells had been incubated with both agents for 48 and 72 h. (B) STAT3 mRNA expression was determined following treatment of FLS for 72 h. (C) Bcl2 protein expression was detected by flow cytometry, also following treatment of FLS for 72 h. Cells in all groups were treated as in section 2 for 72 h. *P>0.05 vs. healthy group; *P<0.05 vs. healthy group $^{\#}$P<0.05 vs. CIA group; §P<0.05 vs. E003 group; $^{\blacktriangle}$P<0.05 vs. si-Bcl2 group; $^{\bullet}$P<0.05 vs. 72 h group. Bcl2, B cell lymphoma 2; CIA, collagen II-induced arthritis; E003, daphnetin; FLS, fibroblast-like synoviocytes; si-Bcl2, short interfering RNA targeting Bcl2; STAT3, signal transducer and activator of transcription 3.

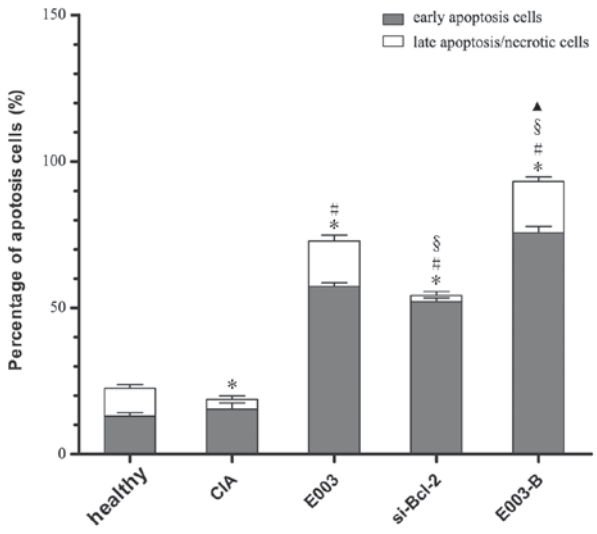

Figure 4. The influence of E300 combined with si-Bcl2 on apoptosis of FLS. si-Bcl2.1 (100 nM) was transfected into healthy FLS, CIA FLS or in CIA FLS treated with 40 μg/ml E003, si-Bcl2 or E003 and si-Bcl2 combined for 72 h. *P<0.05 vs. healthy group; $^{\#}$P<0.05 vs. CIA group; §P<0.05 vs. E003 group; $^{\blacktriangle}$P<0.05 vs. si-Bcl2 group. Bcl2, B cell lymphoma 2; CIA, collagen II-induced arthritis; FLS, fibroblast-like synoviocytes; si-Bcl2, short interfering RNA targeting Bcl2.

process mediated by T cells and/or macrophages and the abnormal proliferation of synovial cells (22,23). A previous study demonstrated that synovial cells produce a variety of pro-inflammatory cytokines following activation and a number of matrix-destructing enzymes through inflammatory signals, causing persistent inflammation and progressive synovial tissue destruction in RA patients (24). It is now considered that FLS cells do not only fill space, but they are also directly responsible for cartilage destruction and drive both inflammation and autoimmunity. Active RAFLS cells stimulate the expression of proinflammatory cytokines, such as interleukin (IL)-8 and IL-6, and also form a complex that constantly changes the expression levels of various molecules without external stimulation by altering the expression of regulatory factors, signalling molecules and apoptosis-associated molecules (25,26). RAFLS cells, which are characterised by incomplete conversion, exhibit tumour-like proliferation with sustained activation, although they may escape from the inflammatory environment and have strong invasive properties (27), and this is closely associated with impaired apoptosis of RAFLS. Impaired apoptosis prolongs the life of RAFLS, causing joint cartilage and bone destruction (28).

In the clinic, many diseases are associated with impaired apoptosis of lesion cells. FLS cells in RA are tumour-like proliferation (27). The induction of cell apoptosis is an efficient strategy with which to inhibit proliferation. Inducing the apoptotic pathway with active ingredients in Chinese medicine has become a popular target for inhibition of tumour or tumour-like growth. The present study used daphnetin extracted from natural *D. odora,* which has many pharmacological applications, such as anti-inflammatory, analgesic, antitumoural and immune regulatory properties (29-32). A previous study by the authors of the present study demonstrated that daphnetin not only inhibited immunised foot swelling and inflammation

but also relieved the degeneration of articular chondrocytes, increased the number of forkhead box $P3^+$ regulatory T cells and regulated T helper 17 cells to achieve immune balance in CIA (33). In 2012, it was also demonstrated that daphnetin treatment demethylated the apoptosis genes death receptor 3, programmed cell death 5, Fas ligand and the promoter region of p53 in CIA (13).

RNAi-associated RNA silencing pathways constitute a group of small-RNA-based silencing mechanisms that is conserved in eukaryotic cells and affect their whole existence (34). RNA silencing requires a number of enzymes, and different species require different enzymes, generally including argonaute (which binds to siRNA), and P-element induced wimpy testis protein (35). RNAi is an efficient sequence-specific gene knockout technology that has been rapidly developed for applications in infectious diseases and gene therapy areas of cancer. It may be a novel idea that an efficient siRNA may be used to treat RA alone or in combination with biological agents or other medicines. However, studies have focused on using RNAi for anti-inflammation-based treatments of RA (36,37). The use of RNAi to remove or turn off specific genes highly expressed in RAFLS, such as the antiapoptosis protein Bcl2, has not been previously reported. The present study investigated the ability of combined treatment with a Bcl2-specific RNAi with daphnetin, an active ingredient of traditional Chinese medicine, to downregulate antiapoptosis gene expression, aiming to promote RAFLS apoptosis and reduce the over-proliferation of these cells.

In the present study, daphnetin was combined with si-Bcl2 to explore the effect of this combined treatment on antiapoptotic gene expression and the underlying molecular mechanism of RAFLS of CIA rats. Bcl2 exerts an antiapoptotic effect by blocking common signalling pathways of apoptosis. Previous studies have demonstrated that Bcl2 gene expression is significantly higher in the synovial tissue of RA patients and that it inhibits apoptosis by preventing cytochrome C (Cyto C) release from mitochondria and also by regulating caspase activity following Cyto C release (38). A report by Ferdek *et al* (39) revealed that Bcl2 serves important roles in Ca^{2+} signalling by influencing inositol triphosphate receptors and regulating Ca^{2+}-induced Ca^{2+} release. In the present study, it was demonstrated that Bcl2 was highly expressed in synovial cells in CIA rats, and treatment with daphnetin or si-Bcl2 alone resulted in mRNA and protein expression levels that were significantly lower than those in the CIA group. There was clear synergy between daphnetin and si-Bcl2 in downregulating Bcl2 mRNA and protein expression levels.

Apoptosis is initiated by environmental stimuli and induces a variety of other gene regulation mechanisms. It serves an important role in eliminating cells that have aged in the body or have the potential for uncontrolled growth, and also maintains homeostasis of organs. The antiapoptotic gene Bcl2 is primarily involved in the mitochondrial pathway and endoplasmic reticulum pathway. STAT3, an important member of the STAT family, is commonly expressed in human tumours and is widely involved in many processes, such as tumour invasion and metastasis, angiogenesis, apoptosis resistance and immune evasion. STAT3, located upstream of Bcl2 gene regulation in many apoptotic signalling pathways, may regulate the expression of Bcl2 to block apoptosis. Li *et al* (40) reported that downregulation of STAT3 expression using RNAi significantly reduced Bcl2 and cyclin D expression and thereby induced glioblastoma stem cell apoptosis and inhibited cell growth. Lee *et al* (41) demonstrated that STAT3-mediated IL-17 expression induces the upregulation of Bcl2 expression, and IL-17 promotes proliferation of RAFLS through the STAT3 apoptosis pathway. In the present study, it was revealed that the apoptosis of FLS cells increased with daphnetin treatment alone or with si-Bcl2 treatment alone. In the mitochondrial apoptotic signalling pathway, there was a corresponding decline in STAT3 expression, and there was also an enhanced association between daphnetin and si-Bcl2 in downregulating STAT3 expression.

The results of the present study suggested that there may be an interaction between daphnetin and si-Bcl2 in reducing the expression levels of Bcl2 and STAT3, which promoted FLS apoptosis in CIA rats. The effects of daphnetin combined with sibcl-2 in the present study may provide a new direction for the treatment of RA. However, the mechanism of these effects on FLS in RA remains to be elucidated.

Acknowledgements

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