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Original Article

Effects of icariin on cytokine-induced ankylosing spondylitis with fibroblastic osteogenesis and its molecular mechanism

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Abstract: The aim of this study is to explore the effects of icariin on cytokine induced ankylosing spondylitis fibroblast osteogenesis type expression and its molecular mechanism. The normal fibroblasts were collected as normal control group, and the fibroblasts of hip joint capsule of AS patients were collected, which were respectively added in fetal bovine serum (group AS), fetal bovine serum and cytokines (BMP-2+TGF-beta 1) (group AS), and cell factor solution (icariin group), and observed of the osteogenic expression of fibroblast, to evaluate the impact of Icariin on it. The ALP activity, the content of collagen, osteocalcin content and cbfa1mRNA and OCmRNA of fibroblast of AS group increased compared to the normal control group and AS control group ($P < 0.01$), indicating that icariin can significantly inhibit the above changes ($P < 0.01$). Icariin can inhibit fibroblast further osteogenic differentiation through inhibiting the effect of cytokines on the fibroblast osteogenesis type markers and osteogenic gene expression and osteogenic differentiation.

Keywords: Ankylosing spondylitis, icariin, cytokine, ossification, fibroblast

Introduction

Ankylosing spondylitis (AS) is a kind of chronic inflammatory autoimmune disease, and mainly involves axial joint [1], which is lack of specific and effective treatment in western medicine [2]. The kidney-reinforcing and strengthen spine granule is a traditional Chinese medicine for treatment of AS, with the epimedium as the monarch medicine, made in China, and has good clinical effects. BMP-2 has a very strong ability of promoting osteoblast differentiation and inducing in vitro osteogensis [3], and its high expression may promote the proliferation and differentiation of fibroblast [4]. TGF-β also has the function of promoting formation of osteogenesis markers. This study took the in vitro cultured fibroblasts of AS hip joint capsular tissue as the experimental object, to observe the effect of epimedium on cytokines BMP-2 and TGF-β1 induced fibroblast osteogenic expression and its molecular mechanism.

Materials and methods

Tissue culture

A total of 10 cases of total hip arthroplasty from the Department of Orthopedic Surgery, Peking University People's Hospital, were included, males, 22 years old~43 years old, including 5 cases of AS group, and 5 cases of normal control group. The tissue adherent culture method was used to perform hip joint capsule fibroblast primary culture. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Chinese Academy of Traditional Chinese Medicine. Written informed consent was obtained from all participants.

Grouping

Icariin experiment was divided into 6 groups, which were normal control group, AS group, AS group, small, medium and large icariin dose

Table 1. Effects of Icariin on ALP activity of cytokine induced AS fibroblasts ($\bar{x}$ ± s)

Group	U/g
Normal control	18.53 ± 1.92
AS control	27.55 ± 1.51**
AS	35.96 ± 3.79**,ΔΔ
Large dose Icariin	34.07 ± 1.43**,ΔΔ
Medium dose Icariin	34.71 ± 3.45**,ΔΔ
Small dose Incariin	30.28 ± 2.67**,##

Note: Comparison with normal control group: $^{**}P < 0.01$; Comparisonwith AS control group: $^{\Delta\Delta}P < 0.01$; Comparison with AS group: $^{\#\#}P < 0.01$.

Table 2. Effects of Icariin on collagen content of cytokine induced AS fibroblast ($\bar{x}$ ± s)

Group	μg/ml
Normal control	34.536 ± 3.271
AS control	47.354 ± 5.361**
AS	59.893 ± 4.624**,ΔΔ
Large dose Icariin	58.569 ± 6.278**,ΔΔ
Medium dose Icariin	57.645 ± 3.556**,ΔΔ
Small dose Incariin	54.861 ± 4.332**,ΔΔ,#

Note: Comparison with normal control group: $^{**}P < 0.01$; Comparisonwith AS control group: $^{\Delta\Delta}P < 0.01$; Comparison with AS group: $^{\#}P < 0.01$.

group. In addition to the normal fibroblasts of normal control group, other groups were all taken AS fibroblasts as the experimental objects. In addition to the normal control group and AS control group, other 4 groups were added cytokines BMP-27 ng/ml and TGF-β 15 ng/ml, and correspondingly added in the DMEM containing 10% serum, which were packet administrated as follows: the normal control group, and AS control group and AS group were added in fetal bovine serum; the icariin small, medium, and large dose groups were respectively added sterile icariin solution in the conventional culture medium, and the final concentrations were respectively 25 μg/ml, 50 μg/ml, and 100 μg/ml.

Alkaline phosphatase (ALP) activity determination

The third generation fibroblasts of normal cells and AS were prepared of single cell suspension, using alkaline phosphatase assay kit, and the activity of ALP (595 nm) was determined by using spectrophotometric method: ALP (U/g) = determined value × (determined tube absorbance/standard tube absorbance) × phenol content of standard pipe ÷ samples containing protein grams. The spectrophotometer was 722 spectrophotometer, provided by Shanghai Metash Instruments Co., Ltd (Shanghai).

Table 3. Effects of Icariin on osteocalcin content of cytokine induced AS fibroblast ($\bar{x}$ ± s)

Group	OD value
Normal control	1.53 ± 0.14
AS control	2.79 ± 0.07**
AS	3.49 ± 0.17**,ΔΔ
Large dose Icariin	2.86 ± 0.31**,ΔΔ
Medium dose Icariin	2.95 ± 0.24**,##
Small dose Incariin	3.29 ± 0.13**,##

Note: Comparison with normal control group: $^{**}P < 0.01$; Comparisonwith AS control group: $^{\Delta\Delta}P < 0.01$; Comparison with AS group: $^{\#\#}P < 0.01$.

Determination of collagen content

The third generation fibroblasts of normal and AS were prepared of single cell suspension, using spectrophotometric method (wavelength 540 mm), the collagen content in the supernatant was determined. The sample HPR concentration = standard HPR concentration × the determined sample absorbance/standard HPR absorbance value of this concentration; sample (1 ml hydrolysate) collagen content percentage = sample HPR concentration/tissue volume × D × 100 (D as the calculation constant = 7.46).

Determination of the supernatant osteocalcin (OC) content

The third generation fibroblasts of normal and AS were prepared of single cell suspension; OC content was determined by radioimmunoassay [GC1200 γ counter radioimmunoassay (automatic), USTC Holdings co., ltd, Hefei].

Real time quantitative PCR

The third generation fibroblasts of normal and AS were prepared of single cell suspension, RNA was extracted by one-step Trizol extraction, and the expression Cbfa1 and OC mRNA were determined by reverse transcription, the primer was as the follows [5, 6] (Promega, Madison, Wisconsin, USA). The reaction condition was 42°C water bath 60 min, and 95°C 5 min for termination reaction.

Table 4. Effects of Icariin on cbfa1mRNA expression of cytokine induced ASfibroblast ($\bar{x}$ ± s)

Group	Cbfa1/β-action (ct)
Normal control	1.87 ± 0.15
AS control	1.57 ± 0.12**
AS	1.27 ± 0.18**,ΔΔ
Large dose Icariin	1.38 ± 0.15**,Δ
Medium dose Icariin	1.40 ± 0.14**
Small dose Incariin	1.45 ± 0.23**,#

Note: Comparison with normal control group: $^{**}P < 0.01$; Comparisonwith AS control group: $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$; Comparison with AS group: $^{\#}P < 0.05$.

Table 5. Effects of Icariin on expression of OCmRNA of cytokine induced ASfibroblast ($\bar{x}$ ± s)

Group	OC/β-action (ct)
Normal control	1.58 ± 0.14
AS control	1.28 ± 0.11**
AS	0.96 ± 0.09**,ΔΔ
Large dose Icariin	1.02 ± 0.21**,Δ
Medium dose Icariin	1.01 ± 0.17**
Small dose Incariin	1.12 ± 0.19**,#

Note: Comparison with normal control group: $^{**}P < 0.01$; Comparisonwith AS control group: $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$; Comparison with AS group: $^{\#}P < 0.05$.

The primer sequence: β-actin: The forward primer: 5'-GAG ACC TTA AAC ACC CCA GCC3', the reverse primer: 5'-AAT GTC ACG CAC GAT TTC CC3', the amplification fragment was 263 bp. cbfa1: the forward primer: 5'-CAG CCA CCT TTA CTT ACA CCC3', the reverse primer: 5'-CAG CGT CAA CAC CAT CAT TC3', the amplification fragment was 306 bp. OC: the forward primer: 5'-AGG GCA GCG AGG TAG TGA3', the reverse primer: 5'-CCT GAA AGC CGA TGT GGT3', the amplification fragment was 150 bp.

Western blotting

The third generation fibroblasts of normal and AS were prepared of single cell suspension, and the supernatant was taken to determine the protein concentration with Coomassie brilliant blue method. Coomassie Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute).

Statistical analysis

All data are expressed as $\bar{x}$ ± s. SPSS13.0 statistical software package was used for statistical analysis, and the differences between the two groups was tested by using the two independent samples t test.

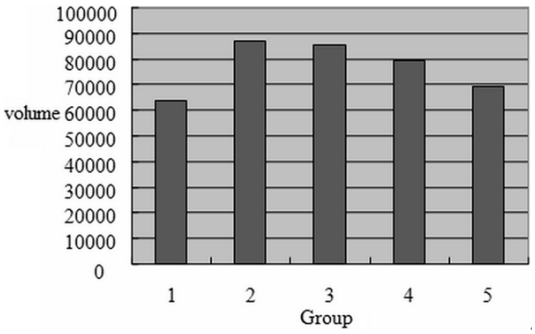

Figure 1. Expression of cbfa1 protein. 1: AS control group, 2: AS group, 3, 4, 5: large, medium, small dose icariin group, respectively.

Results

ALP activity

The results show that, after administration, large dose group of icariin had significant inhibition effect on the ALP activity of cytokine induced AS fibroblast ($P < 0.01$); and medium dose and small dose icariin group had no significant effect ($P > 0.05$, **Table 1**).

Collagen content

The results show that, after administration, the large dose of containing icariin serum had significant inhibition effect on supernatant collagen content of cytokine induced AS fibroblast ($P < 0.05$); and medium and small dose of icariin had no significant effect ($P > 0.05$, **Table 2**).

Osteocalcin content

The results show that, after administration, the large and medium dose of incarrin group had significant inhibition effect on supernatant osteocalcin content of cytokine induced AS fibroblast ($P < 0.05$); and small dose of icariin had no significant effect ($P > 0.05$, **Table 3**).

Cbfa1mRNA expression

The results show that, after administration, the large dose icariin had significant inhibition effect on expression of cbfa1mRNA of cytokine induced AS fibroblast ($P < 0.05$); and medium and small dose of icariin had no significant effect ($P > 0.05$, **Table 4**).

OCmRNA expression

The results show that, after administration, the large dose of icariin had significant inhibition effect on expression of OCmRNA of cytokine induced AS fibroblast ($P < 0.05$); and medium and small dose of icariin had no significant effect ($P > 0.05$, **Table 5**).

Expression of cbfa1 protein

The results show that the medium and large dose of icariin had a certain inhibition effect on expression of cbfa1 protein of cytokine induced AS fibroblasts; and the small dose of icariin had no such effect (**Figure 1**).

Discussion

Ankylosing spondylitis (AS) is a kind of chronic inflammatory disease [7], mainly invades of sacroiliac joint, spine apophysis (synovial joint), paravertebral soft tissues and peripheral joints, which can be associated with extra articular manifestations, and belong to the category of seronegative spondyloarthropathy [8]. Its characteristics are almost involving all sacroiliac joints, and its characteristic pathological change is enthesitis [9]. The common symptoms were low back stiffness or pain, which may alleviate after the activity, and in later period, which may appear spine ankylosis, deformities, and even serious impairment of function [10]. The disease has onset occult, lingering illness, high disability rate, and seriously affects the patient's life, and labor [11]. The modern medicine has no specific therapy [12]. In recent years, traditional Chinese medicine has good curative effect in the treatment of AS. The kidney- reinforcing and strengthen spine granule is traditional Chinese medicine for treatment of AS, developed by Department of Rheumatology Chinese Academy of Traditional Chinese Medicine, and has good clinical effect. Epimedium, as the main drug of kidney- reinforcing and strengthen spine granule, plays a decisive role, so this study is intended to explore the mechanism of icariin in the treatment of AS, in order to seek more effective treatment for the AS.

At the initial stage of AS, the patients appear progressive inflammation, and early pathological changes occur in the axial skeleton, which is characterized as inflammation and ossification of the spinal joints and ligaments and sacroiliac joints. The earliest lesions appear sacroiliac joint, then along the spine to development. In the process of cells ossification, the cytokine plays a critical role [13]. BMP has the ability of inducing osteoblast precursor cell differentiation and inducing in vitro osteogenesis [14]. Research thinks that BMP-2 mainly has the recruitment and differentiation effect on undifferentiated mesenchymal cells and bone cells [15]. At the early stage of the early bone formation, BMP-2 can not only recruit the undifferentiated mesenchymal cells to bone formation centre, and differentiate into bone cells, and can make the fibroblast fibrocyte, myoblast, and base cell of bone marrow reverse and differentiation into to bone cell line [16]. In the in vitro experiment, the results indicates that TGF-β can induce fibroblast synthesis of collagen I, II, III, IV, V, fibronectin, osteonectin, glycoprotein and ALP, inhibit of MMP activity, block extracellular matrix digestion, promote the synthesis of cell adhesion molecule receptors and bindin [17] and increase the amount of bone and cartilage tissue [18]. Research confirms that TGF-β can promote bone formation and cartilage local membrane formation, suggesting that TGF-β possess respectively strong osteogenic and chondrogenic effect, which can promote synthesis of bone matrix, such as collagen, osteonectin, and fibronectin, and involve in synthesis of bone and cartilage [19]. Experiments show that TGF-β can enhance rhBMP-2 inhibiting the expression of mRNA of collage type I, II in osteogenesis and alkaline phosphatase, suggesting that TGF-β have the effect of enhancing the rhBMP-2 inducing the osteogenesis, namely have synergistic effect with BMP in inducing osteogenesis [20].

The experimental study showed that, under the effect of cytokines BMP-2 and TGF-β1, there were greatly increased activity of alkaline phosphatase-the necessity for osteogenesis, collagen synthesis, and large amount of OC secretion in AS fibroblasts, indicating that, under the induction of cytokines, AS fibroblasts has been successfully induced expression of osteogenesic phenotype. The fibroblast that have begun to differentiation, were significantly inhibited, under the action of Chinese medicine serum, in a dose-dependent manner, indicating that icariin can inhibit the effect of cytokines on fibroblasts osteogenesic markers, so as to inhibit fibroblast further differentiate into osteogenesic phenotype. Research shows that cytokine BMP can promote the induction of expression

of osteoblast specific transcription factor, Cbfa1, and Cbfa1 is the indispensable transcription factor for osteogenic differentiation, which can participate into the development process of bone and osteogenic cell differentiation by regulating gene expression of growth factor and bone specific extracellular matrix protein, at the same time, Cbfa1 has the regulation function on expression of OC gene [21]. The experiment results show that, after the action of cytokines, gene expression of fibroblast Cbfa1 and OC enhanced, which fully confirmed the above theory. After application of Icariin, it can reduce this expression, indicating that icariin can inhibit the promotion effect of cytokine BMP-2 on the expression of AS fibroblast specific transcription factor Cbfa1 or Osx, so as to achieve the purpose of inhibiting fibroblast ossification. In conclusion, this study showed that icariin can inhibit the effect of cytokines on fibroblasts osteogenesic markers, so as to inhibit fibroblast further differentiate into osteogenesic phenotype, which open up a new way for the treatment of AS. This research studied by means of in vitro cell culture, rather than human tissue, so the study has some limitations, but the results still have certain scientific significance and reference for the treatment of AS.

Disclosure of conflict of interest

None.

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