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CLINICAL STUDY

Effect of Icariin on apoptosis and expression of Fas, Fas ligand, B cell lymphoma, and Bcl-2-associated X protein in CD4+ T lymphocytes from patients with ankylosing spondylitis

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Abstract

OBJECTIVE: To investigate the effects of icariin on apoptosis and the expression of Fas, Fas ligand (FasL), B cell lymphoma (Bcl-2), and Bcl-2-associated X protein (Bax) in CD4 + T lymphocytes from patients with ankylosing spondylitis.

METHODS: Primary cultures of peripheral blood CD4+ T lymphocytes were established and treated with icariin at high, medium, and low doses (0.5, 0.25, and 0.125 mg/mL). Sulfasalazine treated and helthy cells were used as controls. Apoptosis of treated cells was determined by flow cytometry. Reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assays were used to determine the effects of icariin on the expression of Fas, FasL, Bcl-2, and Bax. The activity of caspase 8 and caspase 3 was determined by a colorimetric assay.

RESULTS: The mRNA and protein expression of Fas, and activity of caspase 8 and caspase 3 in CD4+ T lymphocytes were increased by icariin ($P < 0.05$). Conversely, the mRNA and protein expression of Bcl-2 was decreased ($P < 0.05$). The expression of FasL and Bax were not significantly different between groups. The proapoptotic effects of icariin were dose-dependent.

CONCLUSION: Icariin induces the apoptosis of CD4+ T cells from patients with AS comparing to normal control. Therefore, the induction of apoptosis may be the likely mechanism of action of icariin's antirheumatics activities.

Keywords: Spondylitis, ankylosing; Fas; Fas Ligand protein; Lymphoma, B-Cell; Bcl-2-associated X protein; CD4-positive T-lymphocytes; Apoptosis; Icariin

INTRODUCTION

Ankylosing spondylitis (AS) presents with chronic systemic inflammation which primarily affects the axial skeleton (sacroiliac joints and spine).[1] Studies have suggested that CD4+ T cells play an important role in the morbidity of AS. Notably, in the peripheral blood, the

frequencies of CD4 + T cells were higher in patients with AS than in healthy individuals.[2,3] However, thus far, there have been few scientific reports on apoptosis of CD4+ T cells and the expression of apoptosis-related genes in AS.

Chinese herbal medicine has been widely used for thousands of years in the treatment of fracture and joint diseases. Yinyanghuo (*Herba epimedii*), which is recorded as the Traditional Chinese Medicine, is used as an antirheumatic. Bushen Qiangji granule, which contains Yinyanghuo (*Herba epimedii*) as a major component, increases the expression of Fas and FasL by CD4 + T cells.[4] Icariin ($C_{33}H_{40}O_{15}$; molecular weight: 676.67) is the main active flavonoid glucoside isolated from Yinyanghuo (*Herba epimedii*) and displays anti-inflammatory potential.[5] It was found to have a therapeutic effect on osteoporosis due to ovariectomy in rat models and also in postmenopausal women,[6] and may be one of the active constituents affecting the differentiation of osteoblasts.[7]

The aim of this study is to explore the mechanism of action of icariin on the Fas signaling pathway in CD4+ T lymphocytes from patients with AS.

MATERIALS AND METHODS

Drugs and reagents

Icariin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The powder was dissolved at a concentration of 500 μg/mL in RPMI 1640 (Gibco, New York, NY, USA) for in vitro assays and stored at − 20 ℃ until use. The icariin was diluted into high, medium, and low doses (0.5, 0.25, and 0.125 mg/mL). Sulfasalazine (SASP) (625 μM) treated and normal contral cells were used as control groups.

Collection, isolation and culture of CD4+ T lymphocytes

After gaining approval from the ethics committee of Guang An Men Hospital affiliated to China Academy of Traditional Chinese Medicine and patients' written informed consent, patients with AS who fulfilled the modified New York criteria[8] were included in the study. All patients were over 18 years of age. Samples of peripheral blood CD4 + T lymphocytes were obtained from five patients with AS and three age- and gender-matched healthy controls. The characteristics of each group are summarized in Table 1.

The RosetteSep Human CD4 + T Cell Enrichment cocktail (StemCell Technologies, Vancouver, Canada) was added to anticoagulated (preferably heparinized) blood of AS patients (50 μL cocktail/mL blood) with gentle mixing and incubated at room temperature for 20 min. After dilution with Phosphate Buffered Saline (PBS) followed by Ficoll density gradient centrifugation (1200 × *g*, 20 min), the interface was harvested, cells pelleted (1200 × *g*, 20 min, room temperature), and suspended in 2% FBS staining buffer. The cells were maintained at 37℃ and 5% CO_2 in RPMI 1640 media supplemented with 10% fetal calf serum (FCS). CD4 + T lymphocytes were subsequently incubated with 1 mL of RPMI 1640 medium supplemented with 10% FCS plus various concentrations of drugs as indicated in a 6-well-plate (2×10^5 cells/well) for 48 h.

Flow cytometric analysis of Annexin/PI stained CD4+ T lymphocytes was used to determine the effects of icariin on apoptosis after treatment with icariin.

Enzyme-linked immunosorbent assay (ELISA)

$CD4^+$ T cells were harvested and lysed in lysis buffer (Beyotime, Shanghai, People's Republic of China). Fas, FasL, Bcl-2, and Bax were measured by commercially available ELISA kits (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

The mRNA levels of icariin treated cells were investigated by RT-PCR. The primers were designed using

Table 1 Characterization of the studied population

Item	AS patients (n = 5)	Healthy (n = 3)	P value
Age (years)	31.4 (23-44)	36.7 (28-47)	0.197
Male (%)	75	66.7	0.371
Symptoms duration (years)	11 (8-21)	NA	-
ESR (mm/h)	23 (11-38)	NA	-
CRP (mg/dL)	0.8 (0.2-1.7)	NA	-
ASDAS-CRP	3.8 (2.4-4)	NA	-
BASDAI	5.8 (3.7-7.3)	NA	-
BASFI	5.7 (2.1-7.5)	NA	-
HLA-B27 (%)	75	NA	-

Notes: NA: not applicable; AS: ankylosing spondylitis; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; ASDAS: ankylosing spondylitis disease activity score; BASDAI: bath ankylosing spondylitis disease activity index; BASFI: bath ankylosing spondylitis functional index; HLA: human leukocyte antigen.