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Original article

# Annexin A2, up-regulated by IL-6, promotes the ossification of ligament fibroblasts from ankylosing spondylitis patients

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ABSTRACT

*Background:* Annexin A2, a calcium-dependent phospholipid binding protein, is involved in osteogenesis. The objective of the present study was to explore the expression of Annexin A2 in spinal ligament tissues (LT) of ankylosing spondylitis (AS) patients and determine its pathological functions.
*Methods:* mRNA and protein expression of Annexin A2 was detected by real-time PCR and Western blotting, respectively. Interleukin-6 (IL-6) concentration in serum was assessed by enzyme linked immunosorbent assay. Alkaline phosphatase (ALP) activity was measured with ALP activity kit on a microplate reader.
*Results:* mRNA and protein expression of Annexin A2 in LT, and IL-6 concentration in serum were significantly increased in AS patients. Moreover, exogenous IL-6 treatment significantly up-regulated Annexin A2 expression and ALP activity. Silencing of Annexin A2 expression significantly ameliorated IL-6-induced ossification of fibroblasts from AS patients, as indicated by ALP activity, expression of proteins associated with osteogenic differentiation, including bone morphogenetic protein-2, osteocalcin and osterix, and the ratio of osteoprotegerin to receptor activator of NF-κB ligand. Further MEK inhibitor experiments suggested that Annexin A2 may exert its function through extracellular signal-related kinase pathway.
*Conclusions:* Annexin A2, up-regulated by IL-6, may promote ligament ossification of AS patients.


## 1. Introduction

Ankylosing spondylitis (AS) is a common inflammatory autoimmune disease with a prevalence of 0.2–0.5% worldwide [1]. It caused chronic spinal and extraspinal inflammation, as well as progressive spinal ankylosis, leading to irreversible structural and functional impairments and decreased life quality [2]. Fibroblasts are the most abundant connective tissue cells in ligament tissue (LT). Recent studies have demonstrated the important role of fibroblasts in LT ossification and ankylosis [3,4].

Annexin A2 belongs to Annexins family, which are known to bind acidic phospholipids with high affinity in a calcium-dependent manner [5]. Annexin A2 functions as a receptor for tissue plasminogen activator (t-PA) [6] and it is involved in DNA replication [7] and protein transportation [8]. Annexin A2 was up-regulated in various types of human cancer [9–12] and plays multiple roles in regulating cancer cell behavior, such as cell proliferation, apoptosis, cell migration and invasion [10,12–14]. It has been reported that Annexin A2 plays a critical role in regulating alkaline phosphatase (ALP) activity and facilitating the mineralization process of SaOSLM2 osteoblastic cells [15]. Annexin A2 was up-regulated during the osteogenesis of human bone marrow-derived mesenchymal stem cells (hBMSCs) [16]. However, the roles of Annexin A2 in fibroblast ossification of AS patients remain largely unknown.

Cytokines including interleukin-6 (IL-6) have been found elevated in the serum of AS patients. Previous studies have reported a correlation between IL-6 levels and the disease activity of AS patients [17–19]. IL-6 has been shown to activate extracellular signal-related kinase (ERK) signaling pathway [20], which is a member of the mitogen-activated protein kinase (MAPK) family and can stimulate the differentiation of human mesenchymal stem cells (hMSCs) into osteoblasts [21–23]. A

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previous study demonstrated that expression of Annexin 1 [24] in a lung cancer cell line was induced by IL-6, whereas other studies showed that Annexin 1 [25,26] negatively regulated IL-6 expression in lung fibroblast cell lines and macrophages. IL-6 secretion in a prostate cancer cell line was decreased concomitantly with Annexin A2 silenced [27]. However, whether IL-6 induced fibroblast ossification and whether Annexin A2 is involved in the IL-6-induced fibroblast ossification is unknown.

In this study, we hypothesized that Annexin A2 expression was regulated by IL-6, and played a role in fibroblast ossification, thus contributing to the AS pathogenesis. We measured Annexin A2 mRNA and protein level of LT, and IL-6 concentration in the serum of AS patients and controls. Moreover, we isolated primary cultured fibroblasts from AS LT to investigate the role of Annexin A2 in IL-6-induced fibroblast ossification.

## 2. Materials and methods

### 2.1. LT specimens and serum samples

Thirty patients meeting the modified New York criteria [28] for AS, and 6 patients with thoracolumbar spinal injuries admitted to Department of Orthopedics, Changhai Hospital Affiliated to the Second Military Medical University (Shanghai, China) were enrolled this study. Human specimens of spinal LT were obtained from 6 AS patients at the time of spinal surgery. Control LT were obtained from 6 patients with thoracolumbar spinal injuries during open reduction and internal fixation surgery.

Sera samples were obtained from 30 AS patients before surgery. Sera samples obtained from age matched 30 health volunteers were used as normal control. This study was approved by the independent ethics committee, Changhai Hospital. Written informed consent was obtained from all participants.

### 2.2. RNA extraction and real-time PCR

Total RNA was extracted from tissue samples or cultured cells using TRIzol Reagent (Invitrogen, Carlsbad, California, USA) and reverse transcribed with cDNA synthesis kit (Thermo Fisher Scientific, Rockford, IL, USA) following the manufacturers' instructions. The cDNAs were stored at −80 °C until further use. Real-time PCR was performed to detect mRNA levels of Annexin A2 on an ABI 7300 real-time PCR machine (Applied Biosystems, Foster City, CA, USA) with GAPDH as an internal control. Cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 45 s. The primers used were list as follows: Homo sapiens Annexin A2 (NM_001002857.1), 5′-TTCCCGCTTGGTTGAACAC-3′ and 5′- GTGACCTCATCCACACCTTTG-3′; Homo sapiens GAPDH (NM_001256799.1), 5′- AATCCCATCACCATCTTC −3′ and 5′-AGGCTGTTGTCATACTTC-3′.

### 2.3. Western blot analysis

Protein was extracted from tissue samples or cultured cells by using radioimmunoprecipitation assay (RIPA) buffer. After protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membranes (Millipore, Bredford, MA, USA). Antibodies against BMP-2 (bone morphogenetic protein-2, Ab82511; Abcam, Cambridge, MA, USA), OCN (osteocalcin, Ab3876; Abcam), OSX (osterix, Sc-22538; Santa Cruz Biotech., Santa Cruz, CA, USA), OPG (osteoprotegerin, Ab183910; Abcam), RANKL (receptor activator of NF-κB ligand, Ab45039; Abcam), ERK1/2(#4695; Cell Signaling Technology, Danvers, MA, USA), p-ERK1/2 (#4376; Cell Signaling Technology) and GAPDH (#5174; Cell Signaling Technology) were used in Western blot analysis following the manufacturer's protocol. Signals were detected with enhanced chemiluminescenct substrate (ECL, BioRad, Richmond, CA, USA). Densitometric analysis was performed with Image J software (http://rsb.info.nih.gov/ij/, Bethesda, MD, USA).

### 2.4. Enzyme linked immunosorbent (ELISA) assay

Secretions of IL-6 were assessed with ELISA assay kit (Bio-Swamp life science, Shanghai, China) in accordance with the instructions of the manufacturer. Absorbance was read at a wavelength of 450 nm on a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

### 2.5. Cell culture

Spinal ligaments were obtained with the informed consent of patients with AS. After removal of the ossified tissue, fibrous tissues of the ligaments were washed in cold PBS, minced into small pieces and digested with collagenase I (Sigma, St. Louis, MO, USA) for 5 h at 37 °C. Isolated fibroblasts were cultured in Dulbecco' Modification of Eagle's Medium/Ham's F-12 medium (DMEM/F12) (HyClone, Logan, UT, USA) supplemental with 10% Fetal Bovine Serum (FBS, GIBCO, Carlsbad, California, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. The fibroblasts were maintained at 37 °C in 5% $CO_2$ atmosphere.

### 2.6. Lentivirus production

shRNA targeting human Annexin A2 mRNA (GTCTGTCAAAGCCTATACT, shAnnexin A2) and a non-specific scramble shRNA sequence (CCTAAGGTTAAGTCGCCCTCG, shNC) were cloned into a lentiviral vector pLKO.1-EGFP (Biovector, Beijing, China). The full-length human Annexin A2 were cloned into the expression vector pLVX-puro (Clontech, Palo Alto, CA, USA).

Lentiviral constructs of shAnnexin A2, shNC, pLVX-puro empty vector or pLVX-puro-Annexin A2 were cotransfected with viral packaging plasmids into HEK293T cells by using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per the manufacture's instruction. After 48 h, viral supernatant was collected and then filtered through a 0.45 μm filter. Target cells were infected with indicated virus in the presence of 8 μg/mL Polybrene (Sigma, St. Louis, MO, USA).

### 2.7. Cell treatment

Fibroblasts isolated from AS patients were plated in 6-well plates ($3.0 \times 10^5$ cells/well), infected with shNC virus, shAnnexin A2 virus, Vector virus or Annexin A2 virus as indicated and cultured for another 48 h. Fibroblasts were then stimulated with IL-6 (PeproTech, Rocky Hill, NJ, USA; 20 ng/mL), PD98059 (Sigma; 10 μM) or DMSO. After 24 h, the cultured media was collected to detect ALP activity. Annexin A2 mRNA and protein levels were detected by Western blot and real-time PCR.

### 2.8. Alkaline phosphatase (ALP) activity assay

ALP activity in the cultured medium was measured using ALP activity kit (Jiancheng, Nanjing, China). The integrated absorbance was measured at 520 nm on the microplate reader (Bio-Rad Laboratories Inc.). Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific). ALP activity was given as units per milligram of protein (U/mg protein).