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

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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 (hMSCs) [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].
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 listed as follows: Homo sapiens Annexin A2 (NM_001002857.1), 5'-TTCGACTGTTGATGACACAC-3' and 5'- GTGACCTCATCCACCCATTTG-3'; Homo sapiens GAPDH (NM_0012567991), 5'- AACCCATCACCATCTC -3' and 5'- AGGCTTGTTCATATATC-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, Bedford, MA, USA). Antibodies against BMP-2 (bone morphogenetic protein-2, Ab82511; Abcam, Cambridge, MA, USA), OCN (osteocalcin, Ab3876; Abcam), OSX (ostexir, Sc-22538; Santa Cruz Biotech., Santa Cruz, CA, USA), OPG (osteoprotegerin, Ab183910; Abcam), RANKL (receptor activator of NF-κB ligand, Ab45039; Abcam), ERK1/2/#4965; 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 chemiluminescent 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 1 (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) supplemented 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% CO2 atmosphere.

2.6. Lentivirus production

shRNA targeting human Annexin A2 mRNA (GTCTGTCAAAGCCTATACT, shAnnexin A2) and a non-specific scramble shRNA sequence (CTTACGTAAAGCGGCTTG, 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/m Polybrene (Sigma, St. Louis, MO, USA).

2.7. Cell treatment

Fibroblasts isolated from AS patients were plated in 6-well plates (3.0 × 105 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), PD89059 (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).
2.9. Statistical analysis

All statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA). All data were presented as the mean value ± SD. Statistical significance was determined by two-sided Student’s t test. Pearson correlation analysis was performed to evaluate a possible correlation between Annexin A2 and IL-6. Difference was considered significant with a value of P < 0.05.

3. Results

3.1. Higher levels of annexin A2 and IL-6 in AS patients than in controls

We first investigated the expression of Annexin A2 in AS and control LT via real-time PCR and Western blot. Real-time PCR analysis revealed significantly increased Annexin A2 mRNA expression (Fig. 1A) in AS compared to control LT. The Western blot results further validated the higher expression of Annexin A2 in the AS group than the normal group (Fig. 1B). IL-6 has been found elevated in the serum of AS patients [17–19]. We then detected serum levels of IL-6 by ELISA assay. Comparing with normal group, serum concentration of IL-6 in AS patients was increased by 78.1% (Fig. 1C, n = 30, P < 0.001). In order to examine a possible relationship between Annexin A2 and IL-6 in AS patients, Pearson correlation analysis was performed. The analysis results revealed that Annexin A2 mRNA level was correlated with IL-6 serum levels in AS patients (Fig. 1D, n = 6, r = 0.8727, P < 0.05). These data may indicate an association of Annexin A2 and IL-6 during the pathogenesis of AS.

3.2. Annexin A2 expression and ALP activity was enhanced by IL-6 treatment

Fibroblasts are the most abundant connective tissue cells in LT. In order to explore the effect of IL-6 on Annexin A2 expression, we stimulated primary fibroblast of AS patients with IL-6 (0–20 ng/mL). IL-6 treatment (10 and 20 ng/mL) for 24 h led to a significant increase in Annexin A2 expression at both mRNA (Fig. 2C) and protein (Fig. 2D) levels. ALP is a well-known early bone marker and its activity can be stimulated by IL-6 [29]. We found that IL-6 dose-dependently up-regulated ALP activity in primary fibroblasts (Fig. 2E).

3.3. Silencing of annexin A2 ameliorated IL-6-induced fibroblast ossification

Fibroblast ossification is implicated in the pathogenesis of AS. In order to study the function of Annexin A2 on IL-6-induced fibroblast ossification, Annexin A2 shRNA (shAnnexin A2) lentivirus was produced and infected primary fibroblasts of AS patients. Lenti viral transduction efficiency was about 80% and no obvious cell death was observed at 48 h after viral transduction (Fig. S1). Primary fibroblasts were divided into four groups: Group 1, Control cells treated with DMSO; Group 2, cells treated with 20 ng/mL of IL-6 for 24 h; Group 3, cells infected with control virus (shNC) for 48 h and then treated with 20 ng/mL of IL-6 for 24 h; and Group 4, cells infected with shAnnexin A2 virus for 48 h and then treated with 20 ng/mL of IL-6 for 24 h. As shown in Fig. 3A and B, shAnnexin A2 lentivirus infection caused a notable decrease of Annexin A2 expression in primary fibroblasts treated with IL-6.

Treatment with 20 ng/mL of IL-6 for 24 h significantly elevated ALP activity, which was notably decreased by knocking down of Annexin A2 expression (Fig. 3C).

Proteins associated with osteogenic differentiation, including BMP-2, OCN and OSX were detected by Western blot. IL-6 exposure significantly elevated the expression of BMP-2, OCN and OSX. The effects of IL-6 on the detected proteins were reversed by Annexin A2 knockdown (Fig. 3D). The ratio of OPG to RANKL is considered to be one of the determining factors in osteoclastogenesis [30]. IL-6 treatment resulted in a significant reduction in RANKL expression with a concomitant increase in OPG expression compared with the control cells. Such effects were rescued by Annexin A2 knockdown. Our data revealed that IL-6-induced fibroblast ossification through Annexin A2.

3.4. Annexin A2 induces activation of MAPK/ERK pathway

ERK is one of the components of osteogenic signaling pathways [21–23]. We found that IL-6 treatment induced the phosphorylation of ERK, which was partially suppressed by Annexin A2 knockdown (Fig. 3D).

In order to further study the function of Annexin A2, Annexin A2 lentivirus was produced and infected primary fibroblasts of AS patients. Primary fibroblasts were divided into four groups: Group 1, cells infected with Vector virus (Vector) for 48 h and then treated with DMSO for 24 h; Group 2, cells treated with 10 μM PD98059 for 24 h; Group 3, cells infected with Annexin A2 virus for 48 h then treated with DMSO for 24 h; and Group 4, cells infected with Annexin A2 virus for 48 h and then treated with PD98059 for 48 h. Annexin A2 lentivirus infection resulted in a notable increase of Annexin A2 expression, while PD98059 had no effects on Annexin A2 expression. PD98059 significantly inhibited ERK phosphorylation (Fig. 4A), ALP activity (Fig. 4B), as well as the expression of proteins associated with osteogenic differentiation, but remarkably enhanced the ratio of OPG to RANKL (Fig. 4B). Such effects were partially reversed by Annexin A2 overexpression. Thus, these data suggested that Annexin A2 may promote fibroblast ossification by activating MAPK/ERK pathways.

4. Discussion

Annexin A2 plays a critical role during osteogenesis process of osteoblastic cells [15] and hBMSCs [16]. Increasing evidence has
showed that IL-6 was increased in AS patients and associated with the activity of this disease [17–19]. The association of IL-6 and Annexin expression has been revealed in various cell types [24–27]. Previous studies indicated that IL-6 may exert its functions by activating multiple signaling pathways, such as JAK/STAT and ERK. ERK can stimulate the differentiation of hMSCs into osteoblasts [21–23]. The current study showed that Annexin A2 expression was induced by IL-6 and might play a role during AS pathogenesis through activating ERK signaling.

Firstly, real-time PCR and Western blot analysis demonstrated that Annexin A2 was increased in AS LT compared with control LT although the sample size was relative small. IL-6 concentrations in the serum was higher in AS patients than in normal controls (Fig. 1). We then isolated fibroblasts from LT and the increased

Fig. 2. Annexin A2 expression was significantly increased by IL-6 treatment. Primary cultured fibroblasts were treated with IL-6 (0, 10 or 20 ng/ml) for 24 h. Annexin A2 mRNA and protein levels were detected by real-time PCR (A) and Western blot (B), respectively. (C) ALP activity in the cultured medium was determined using ALP activity kit (Jiancheng, Nanjing, China). ***P < 0.001 versus control cells. ###P < 0.01 versus 10 ng/ml IL-6-treated cells.

Fig. 3. Annexin A2 ameliorated IL-6-induced fibroblast ossification. Primary fibroblasts were divided into four groups: Group 1, Control cells treated with DMSO; Group 2, cells treated with 20 ng/ml of IL-6; Group 3, cells treated with 20 ng/ml of IL-6 and Control virus (shNC); and Group 4, cells treated with 20 ng/ml of IL-6 and shAnnexin A2 virus. At 24 h after viral infection, cells were collected. (A, B) Annexin A2 mRNA and protein expression was detected by real-time PCR (A) and Western blot (B), respectively. (C) ALP activity in the cultured medium was determined. (D) Western blot analysis of osteogenic differentiation, osteoclastogenesis associated proteins, p-ERK1/2 and ERK1/2. ***P < 0.001 versus control cells. ###P < 0.01, ####P < 0.001 versus 20 ng/ml of IL-6 and shNC treated cells.
expression of Annexin A2 was observed in fibroblasts from AS patients (Fig. 2A and B). To explore the effects of IL-6 on Annexin A2 expression, fibroblasts from AS patients were treated with IL-6. As shown in Fig. 2C and D, IL-6 exposure dose-dependently increased Annexin A2 expression at both mRNA and protein levels, as well as ALP activity. Furthermore, decreased expression of Annexin A2 by RNAi significantly suppressed IL-6-induced fibroblast ossification as indicated by ALP activity, expression of proteins associated with osteogenic differentiation, and the ratio of OPG to RANKL (Fig. 3). Our study suggest a critical role of Annexin A2 in IL-6-induced fibroblast ossification.

MAPK/ERK singling is one component of osteogenic signaling pathways [21–23]. Cells with increased expression of Annexin A1 showed constitutive activation of MAPK/ERK [31,32]. In the present study, IL-6 and ectopic expression of Annexin A2 remarkably induced ERK activity, while silencing of Annexin A2 remarkably inhibited ERK activity (Figs. 3 D and 4 A). Further, the inhibitory effects of MEK inhibitor, PD98059 on ERK activity and fibroblast ossification was reversed by Annexin A2 overexpression (Fig. 4), which suggested Annexin A2 might promote fibroblast ossification partially by activating ERK pathway. Our data demonstrated that Annexin A2 expression may be enhanced by IL-6, thus leading to the activation of ERK and fibroblast ossification during the pathogenesis of AS. It has been reported that Annexin A1 could regulate the ERK signaling cascade by activating ERK-regulating kinase MEK or associating with the adapter protein Grb-2 [31,32]. The detailed mechanisms how Annexin A2 inhibited ERK activity required further investigation.

In conclusion, exposure with IL-6 in LT fibroblasts significantly enhanced Annexin A2 expression, activated ERK signaling and induced fibroblast ossification. Silencing of Annexin A2 partially suppressed the effects of IL-6 in ERK signaling and fibroblast ossification. Our findings suggested that an inhibitory strategy against Annexin A2 might be of great help for the treatment of AS.

Conflict of interest

The authors declare that they have no competing interests.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2016.09.091.

References

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