Inhibition of Tubulin β-chain May Play a Regulatory Role in the Development of Rheumatoid Arthritis

Zusammenfassung

Zu den pathologischen Charakteristika der rheumatoiden Arthritis (RA), einer systemischen Autoimmunerkrankung, zählen die abnorme Proliferation synovialer Fibroblasten sowie eine verstärkte Angiogenese. In dieser Studie wurde untersucht, wie sich ein Gen-Knockdown beim Tubulin-β-Ketten (TBB)-Gen auf das Verhalten der an der rheumatoiden Arthritis (RA) beteiligten fibroblastenähnlichen Synoviocyten (SF) auswirkt, welches signifikant von der Tubulinsynthese beeinflusst wird. Bei fünf RA-Patienten wurden kultivierte SF aus dem gemischten Synovialgewebe des Kniegelenks isoliert und mit gegen TBB gerichteten siRNA behandelt. Nach 24- und 36-stündiger Transfektion wurden mittels RT-PCR die Effizienz des Knockdowns und die Expression der relevanten Gene bestimmt. Mithilfe von MTT-, Transwell- und Wound Scratch-Assays sowie einer durchflusszytometrischen Analyse wurden die Zellproliferation und die Migrationskapazität nach dem TBB-Knockdown beurteilt. Außerdem wurden die TBB-Expression nach Inhibition der ERK-, STAT3- und NF-kB-Signalwege gemessen. Die Expression von IL-17, TNF-α, IL-1α, IL-1β und IL-6 nach dem TBB-Knockdown quantifiziert. Die Proliferation, Invasion und Migration von RASF waren nach dem TBB-Knockdown signifikant reduziert. Im ELISA zeigten sich eine signifikante Reduktion der Interleukin-1β (IL-1β)-Sekretion und eine nicht signifikant reduzierte Sekretion von TNF-α, IL-17 und IL-1α.
Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease caused by multiple genetic and environmental factors and results in inflammation of the joints. Pathological characteristics of RA include abnormal proliferation of synovial fibroblasts and increased angiogenesis [1–3]. These processes are significantly affected by tubulin, which plays an essential role in cell proliferation. Anti-mitotic compounds (microtubule de-stabilizers) such as vincristine and vinblastine have been widely applied in antineoplastic agents, based on the biological characteristics of microtubules [4, 5]. Proinflammatory cytokines TNF-α, IL-6, and IL-1 are able to strengthen the migration and invasion of RA synovial fibroblasts (RASFs) [6, 7]. Tubulin β-chain (TBB) has been confirmed to have significant expression in synovial membranes and synovial fluids of RA patients using two-dimensional western blotting, immunohistochemistry and ELISA [8].

The proliferation and invasion of RASFs are associated with nuclear transcription factor kappa B (NF-κB). The inflammatory response in RASFs is activated by NF-κB-dependent processes including the expression of anti-apoptotic genes as well as pro-inflammatory cytokines and effector molecules, such as cyclooxygenase (COX) and prostaglandin [9–12]. Mitogen-activated protein kinase (MAPK) is an effector molecule in highly activated signal cascade transmission. IL-1 and TNF-α induce synovial tissues to activate the expression of MAPK-3, MAPK-4, MAPK-6, and MAPK-7 [13–15]. An important role of the extracellular signal-related kinase (ERK) pathway in the development of RA has also been illustrated [41, 57]. Signal transducers and activators of transcription (STAT) is a cytoplasmic protein family taking part in the cytokine and growth factors cell response to regulate the expression of the genes related to the cell cycle and abnormal cell proliferation [16]. STAT3 expression is increased in RASFs and promotes cell proliferation and inhibits cell apoptosis [17, 18]. The effective inhibitors of ERK, NF-κB, and STAT3 are PD98059 [19], PDTC [20] and STATTIC [21, 22], respectively.

In this study, primarily cultured SFs were treated with siRNA targeting TBB to study the effect of TBB on cell proliferation, invasion, migration and related inflammatory factors to further elucidate the role of TBB in RA pathogenesis and provide an effective basis for clinical diagnosis.

Materials and methods

Clinical Materials

The synovial tissues of patients with RA were provided by Qianfoshan Hospital of Shandong Province, China. Patients with RA (n = 5) all met the American College of Rheumatology classification criteria/ACR 2010 [23, 24] and the joints involved in osteoarthritis were excluded (distal interphalangeal [DIP] joints, first metatarsophalangeal [MTP] joint, and first carpometacarpal [CMC] joint) [24]. Demographic characteristics are presented in Table 1.

The five patients participating in the study were given written informed consent and allowed their biological samples to be genetically analyzed. The Ethical Committee of Shandong Academy of Medicinal Sciences approved this study. The synovial tissues of patients with RA were collected in 50-mL microcentrifuge tubes containing Hank’s balanced salt solution to avoid contamination during transport. All synovial tissues were processed in time to prevent the loss of bioactivity.

Primary culture and purity identification

The synovial tissues were collected from five patients with RA during operation and washed 3 times with Hank’s balanced salt solution in a sterile Petri dish. The tissues were finely chopped, mixed and incubated with 40 µL/mL type II collagenase (1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco’s modified Eagle’s medium (DMEM, HyClone, Thermo Fisher Scientific, Waltham, MA, USA) containing 1 % penicillin and streptomycin (Solabio, Beijing, China) for 6 h at 37 °C with 5 % CO2. The tissue was then treated with an

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equivalent volume of 0.25% trypsin in phosphate-buffered saline (PBS; Solabio, Beijing, China). Cells were filtered and centrifuged at 1500 rpm for 5 min. The cells were cultured overnight in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone; Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) as well as 1% penicillin (100 IU/mL) and 1% streptomycin (100 µg/mL). The cells were passaged when the cell attachment rate reached 80%. Patient RASFs were used at passage 4-6. Cells were negative for CD14, CD3, CD19, and CD56 expression as identified by flow cytometric analysis using a Coulter Epics XL flow cytometer (Beckman Coulter, Brea, CA, USA). Phycocerythrin (PE)-conjugated CD14 (cat. no. 12-0149), fluorescein isothiocyanate (FITC)-conjugated CD3 (cat. no. 11 0039), FITC-conjugated CD19 (cat. no. 11 0199) and PE-conjugated CD56 (cat. no. 12-0567) antibodies were obtained from ebioscience Inc. (San Diego, CA, USA) and used at a 1:50 dilution.

**Transient transfection**

The optimal siRNA (RiboBio, Guangzhou, China) concentration (160 nmol/L) for transient transfection was identified through siRNA concentration profiles. RASFs were incubated in a 24-well plate (1 × 10^5 cells/well) (NEST, Shanghai, China). Cultured SFs were treated with siRNA at the optimal concentration of 160 nmol/L using HiPerFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The cells were harvested for analysis 24 h after transfection. Transient transfection was performed under the guidance of the HiPerFect Transfection Reagent Handbook. Expression of TBB mRNA following the transfection of three different siTBBS (siTBBS-1, siTBBS-2, and siTBBS-3) was detected by RT-qPCR at 24 h and 36 h. siTBBS-1 sequence: 5'- GTCGAGTGTGCTAGTGAAGGAG -3'; siTBBS-2 sequence: 5'- GCACTCTCCTTATGATGTCCATGAAGGA -3'; siTBBS-3 sequence: 5'- GCAGTATCGAGCTCTCACA -3'. The siTBBS-3 sequence showed a higher transfection rate than the siTBBS-1 and siTBBS-2 sequences in the optimization experiment and the siTBBS-3 sequence showed a higher transfection rate than the siTBBS-1 and siTBBS-2 sequences in the optimization experiment and was thus used in the experimental group. A negative siRNA was used as the negative control and treatment with transfection reagent only was used as a mock treatment.

**Detection of the inhibition efficiency of siRNA by RT-qPCR and Western blotting**

Total RNA was extracted from cultured cells and human tissue using a Total RNA kit (R6834; Omega BioTek, Norcross, GA, USA) and reverse transcribed using a ReverTra Ace qPCR RT kit (FSQ-101; Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The primers for the amplification of TBB were as follows: forward, 5'- CAGCTCTTCTCCCTGACCCC -3' and reverse, 5'- CTCAAGCCCGTGTTTCTAGGGA -3'. Denaturation was at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s. The comparative threshold cycle (Ct) method was used to determine the relative expression of mRNA. Relative target gene expression was normalized to the mRNA and protein level of GAPDH. Western blotting was used to identify the protein-level expression of TBB. The cells were collected and washed 3 times using PBS to collect cell sediment, which was lysed in lysis buffer (RIPA:PMSF = 100:1) for 30 min at 4°C. Protein was collected from the supernatant after centrifugation (12000 rpm for 30 min) at 4°C and quantified with the Enhanced BCA Protein Assay Kit (P0009, Beyotime Biotechnology, China). The protein samples (50 mg/well) were denatured in 5 × SDS-PAGE buffer (protein volume: 5 × SDS-PAGE buffer volume × 4:1) for 5 min at 100°C. The protein samples were electrophoresed on a 10% SDS-polyacrylamide gel (S1:60 V, 5 min; S2:100 V, 10 min; S3:150 V) and transferred to polyvinylidine fluoride membranes. The membrane was blocked with 3% skim milk for 30 min and then incubated with primary antibodies for 12 h at 4°C and secondary specific antibodies for 1 h at room temperature, each followed by treatment with tris-buffered saline Tween. The results were visualized by chemiluminescence using ECL in ImageQuant LAS MINI 4000 (GE Healthcare Life Sciences, USA).

**Cell proliferation assay**

SFs were incubated in a 96-well plate (2-4 × 10^4 cells/well; NEST, Shanghai, China) and treated with siRNA as described above. After incubation for 24, 48, or 72 h, 20 µL of 5 mg/mL MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide] in PBS was added to each well, and cultures were incubated for 1 h at 37°C. The absorbance was measured in triplicate at 490 nm using a spectrophotometer (DNM-9602 G; Prolong Group, Beijing, China).

**SFs migration**

SFs were incubated in 24-well plates (NEST, Shanghai, China) at a density of (8-12) × 10^4 cells/well and treated with TBB siRNA as above. The SFs were incubated at 37 °C until reaching 80% confluence. The cell monolayers were scratched linearly in three areas with the tip of a 10-µl pipette and washed 3 times with PBS. The growth conditions of the SFs were observed and photographed with an inverted microscope at 0 and 24 h following transfection. The number of cells that had migrated into the scratched area was quantified in 5 random fields at 100x magnification and the average number was calculated 24 h after the transfection.

**Cell invasiveness**

SFs were seeded into the upper chamber of a Transwell plate at a density of (2-4) × 10^4 cells/well and treated with siRNA as described above. The upper and lower chambers were then filled with medium without FBS, containing 1% antibiotics, followed by incubation at 37 °C for 6-8 h. Subsequently, the lower chamber was filled with 20% FBS and 1% Penicillin-Streptomycin Solution in DMEM, followed by further incubation for 24 h. The non-invaded cells at the upper surface of the membrane were removed with cotton swabs and the invaded cells on the lower side were fixed in 4% paraformaldehyde, followed by incubation at 4 °C overnight, and stained with Giemsa (Solabio) for 1 h. The number of cells that had transgressed through the filter was quantified in 5 random fields at 100x magnification and the average number was calculated. Cells were observed using an XDS-1B microscope.

**Determination of inflammatory cytokines by ELISA**

SFs were incubated in a 6-well plate at a density of (12-16) × 10^6 cells/well without FBS. SFs were treated with siRNA as described above. The culture medium was collected and centrifuged at 3000 rpm at 4°C for 5 min. An ELISA assay was then performed according to the manufacturer’s instructions. 100 µl medium was...
added to a 96 well microplate (Corning-Costar, Corning, NY, USA), which was stored overnight at 4 °C. After gently washing with PBS containing Tween 20 (Solabio, Beijing, China), samples were blocked in 1 % bovine serum albumin (Solabio) plus 5 % sucrose (Solabio) for 1 h at 37 °C. Following three washes with PBS, antibodies against IL-1α, IL-1β, IL-17, IL-6 and TNF-α (all from Abcam, Cambridge, MA, USA; dilution, 1:1,000) were applied to the plate for overnight incubation. The plate was washed, blocked and incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (ProteinTech, Chicago, IL, USA) for 3 h at 37 °C. Staining was developed using a TMB kit (CW0050; CWBIO, China). The absorbance at 450 nm was measured using a plate reader (Synergy HT; BioTek, Winooski, VT, USA). SFs treated with transfection reagent only were used as a control.

**Signaling pathway analysis**

SFs were incubated in a 12-well plate (2 × 10^5 cells/well) at 37 °C with 5 % CO2 until the cell attachment rate reached 80 %. The SFs were transferred to DMEM supplemented with 2 % fetal bovine serum and 1 % antibiotic and cultured for 24 h. The SFs were treated with ERK inhibitor (PD98059), STAT3 inhibitor (Stattic), or NF-κB inhibitor (PDTC) in different concentrations (0, 0.1, 1, 10 µmol/L) for 24 h. The total RNA of the SFs was extracted using Trizol reagent. The expression of TBB was detected by RT-qPCR.

![Flow cytometry analysis of RASFs.](image)

**Fig. 1** Flow cytometry analysis of RASFs. a The cellular morphology of RASFs detected by microscopy after being cultured to the third generation (100x magnification) b Flow cytometry analysis plot. X-axis and Y-axis indicate the fluorescence intensity of CD3 (immature T cell), CD56 (NK cell), CD19 (B cell) and CD14 (mononuclear cell). c Original flow cytometry analysis data.
Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Measurement data were expressed as the mean ± standard deviation and analysed by t-test. Count data were expressed as percentage and analysed by chi-square test. P < 0.05 was considered to indicate a statistically significant difference between groups.

Results

Cell purity identification of RASFs

After the RASFs were cultured to the third generation, their cellular morphology was detected by microscopy (Fig. 1a). More than 99% purity of RASFs was determined based on the less than 1% content of cells labelled by CD3 (immature T cell), CD56 (NK cell), CD19 (B cell) and CD14 (mononuclear cell) as detected by flow cytometer (Fig. 1b, c).

siTBB specifically silenced the expression of TBB in RASFs

The inhibitory effect of siTBB on the mRNA-level expression of TBB in RASFs compared with NC and MOCK groups was determined using real-time PCR after treatment with three siTBBs (P < 0.001) (Fig. 2a). The optimized siTBB and transfection time causing the highest silencing efficiency were identified as siTBB-3 (P > 0.05) and 24 h (P < 0.05) (Fig. 2b). The silencing effect of siTBB-3 transfection for 24 h was confirmed by Western blotting (Fig. 2b). The optimized transfection concentration was identified as 160 nmol/L with an MTT assay, in which the optimized transfection time of 24 h was further confirmed (Fig. 3a).

Migration and invasion decrease after TBB knockdown

MTT, wound healing and Transwell assays were performed after TBB knockdown to assess RASF migration and invasion and to evaluate the roles of TBB in the proliferation of RASFs. Transfection with siTBB did not reduce the proliferation of RASFs compared with NC and MOCK groups (P > 0.05, Fig. 3a). In the cell migration assay, monolayers of SFs were scratched and subsequently incubated with siTBB, control siRNA or transfection reagent for 24 h. Significantly fewer SFs were present in the wounded area after TBB knockdown (*P < 0.01) (Fig. 3b). Furthermore, the invasion assay showed that significantly fewer SFs transgressed through the Transwell filter after TBB knockdown (*P < 0.01) (Fig. 3c). Knockdown of TBB insignificantly reduced the secretion of TNF-α, IL-17, and IL-1α in RASFs. The secretion of IL-1β was significantly decreased and the secretion of IL-6 was significantly increased compared with the control group (*P < 0.01) (Fig. 3d).

The expression of TBB changes after treatment with ERK and STAT3 pathway inhibitors

RASFs were treated with PDTC (NF-κB pathway inhibitor), PD98059 (ERK pathway inhibitor), and STAT3 (STAT3 pathway inhibitor) to explore the effect of ERK, STAT3, and NF-κB p65 signal pathways on the proliferation, migration, and invasion of RASFs. The expression of TBB varied after PD98059 transfection with different concentrations (Fig. 4). The expression of TBB significantly decreased as STAT3 concentration was increased 10-fold, from 0 to 0.1 µmol/L, 1 and 10 µmol/L (Fig. 4).

Discussion

RA is a systemic autoimmune disease, which results in abnormal synovial proliferation, cartilage injury and even joint disability. RASFs can release matrix-degrading enzymes (such as MMPs and cathepsins) [25–27] and acidic components [28] causing pericellular acidification to degrade cartilage and bone. In addition, RASFs can produce receptor activator of NFκB and Dickkopf-1 to promote the differentiation and activation of osteoclasts to accelerate bone degradation [29, 30]. Microtubules, major components of the eukaryotic cytoskeleton [31, 32], are made up of polymerised α- and β-tubulin heterodimers [33, 34]. The overexpression of TBB may cause the instability of microtubules [35–40], which are found in the synovial tissue and synovial fluid in RA patients [5]. The proliferation, invasion and migration of RASFs, which are signs of bone destruction [3], were remarkably reduced after TBB knockdown (Fig. 3), suggesting the progression of RA is inhibited after TBB knockdown.

Monoclonal antibodies, recombinant protein receptors and signaling pathway inhibitors directed against inflammatory factors have been applied in clinical practice [41, 42]. The inflammatory factors TNF-α [43, 44], IL-1α and IL-17 [45] are important pro-in-
**Fig. 3** RASF proliferation, migration, invasion and cytokines expression following TBB knockdown. 

- **a** Cell proliferation was assessed using an MTT assay.
- **b** A wound healing assay was used to assess cell migration (magnification, 100x).
- **c** Invasive ability was assessed using a Transwell assay and the average number of cells invaded through the filter following 24 h of incubation was quantified (magnification, 100x).
- **d** Cytokine levels of IL-6, IL-1α, IL-1β, IL-17 and TNF-α were assessed using ELISA. Values are expressed as the mean ± standard deviation. Three independent experiments were performed for all of the above assays. *P < 0.01. NC, negative control; MOCK, control treated with transfection reagent only; IL, interleukin; siRNA, small interfering RNA; TBB, tubulin β-chain; TNF, tumor necrosis factor; NC, negative control.
Interestingly, the contribution of TBB in the inhibition on the expression of IL-6 (▶ Fig. 3d), which suggests the inhibition of TBB suppresses RA progression. IL-6 is an important cytokine contributing to the pathogenesis of various human diseases [54–56]. Interestingly, the contribution of TBB inhibition on the expression of IL-6 (▶ Fig. 3d) indicates that other factors besides TBB may influence cytokine expression and RA progression. These factors remain to be illuminated by further study.

Abnormal cellular signaling is considered to be the main source of pathological change. The STAT3, NF-kB, and ERK signaling pathways play important roles in RA [41, 57]. Previous studies have validated the anti-inflammatory effect of an inhibitor of the NF-kB signaling pathway [58], which regulates the expression of 150 genes that participate in inflammation, immune responses, and signaling pathways [58].

In conclusion, we used RNA interference technology [60] to demonstrate the important roles of TBB in the proliferation, differentiation, invasion and revascularization of RASFs. Considering the significant effect of TBB on the ERK signal pathway, TBB could be a potential target for RA immunomodulatory therapy, drug development and pathogenesis research.

Acknowledgements

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Conflict of Interest

No.

References

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