Insulin-like growth factor binding protein 2 regulates cell proliferation and migration in the adjuvant arthritis synovial cells through ERK signaling pathway

Xinliang Wang¹,³, Yanan Li², Lin Chen³, Zhiqi Hou³

¹Department of Orthopedic Surgery, The First Affiliated Hospital of Jinan University, Guangzhou 510632, China; ²Department of Biochemistry Laboratory, Guangzhou Medical University, Guangzhou 510180, China; ³Department of Orthopedic, Guangzhou First People’s Hospital, Guangzhou Medical University, Guangzhou 510180, China

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Abstract: To probe the role of Insulin-like growth factor binding protein 2 (IGFBP2) in regulating cell proliferation, invasion, migration and apoptosis of fibroblast-like synoviocytes (FLS) from rheumatoid arthritis (RA) patients, the mRNA and protein expression were measured by qRT-PCR, Western blot and immunofluorescence assay first. Then, siRNA against IGFBP2 was used to inhibit IGFBP2 expression in RA-FLS. The proliferation of RA-FLS was detected by CCK8 assay, invasion and migration in vitro were detected by transwell chamber assay and wound healing assay, the apoptosis of RA-FLS was observed by flow cytometry. Increased IGFBP2 was discovered in RA-FLS compared to FLS. In RA-FLS, the level of IGFBP2 was down-regulated by siRNA, siRNA IGFBP2 decreased the proliferation, invasion and migration of RA-FLS, and increased the apoptosis rate. Furthermore, siRNA IGFBP2 restrained the phosphorylation of ERK. IGFBP2 regulated the cell proliferation, invasion, migration and apoptosis of RA-FLS, which was mediated by the ERK signaling pathway. Our research suggested that targeting IGFBP2 might be a promising therapy for RA.

Keywords: Rheumatoid arthritis, fibroblast-like synoviocytes, IGFBP2, ERK

Introduction

Rheumatoid arthritis (RA) is considered an autoimmune disease characterized by chronic synovial joint inflammation, and closely related to the immune response of lymphocytes, plasma cells, macrophage infiltration and lymphoid follicles [1, 2]. RA is a painful disease that caused the infiltration of inflammation cells, fibrin deposition, synovial hyperplasia and progressive destruction of cartilage and bone, osteoporosis or the systemic bone loss associated with RA increases the risk for fragility fractures, which can affect quality of life dramatically in RA patients [3]. The prevalence of this disease is approximately 1% of the population worldwide [4]. Current treatments for RA including drug therapy, immunotherapy, surgical treatment and psychotherapy, although they can reduce pain, they also have side effects such as increased risk of infection and not work for everyone [5].

Due to the diversity of pathogenic pathways in RA, the therapy of RA remains unmet medical need. Fibroblast-like synoviocytes (FLS) are a main constituent of the synovial hyperplasia, play a critical role in the immunopathogenesis of RA [6]. It has been widely accept that RA-FLS keep their ability of increased proliferation, anchorage-independent growth and invasion. In addition, RA-FLS could produce substantial levels of pro-inflammatory cytokines and chemokines, such as IL-6, IL-1β, IL-17, TNF-a and MCP-1 [7-9]. It was reported that RA-FLS also expresses a variety of matrix metalloproteinases (MMPs), small ubiquitin-related modifier-1 (SUMO), vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which leading to promote cell proliferation and apoptotic resistance [10, 11].

The insulin-like growth factor (IGF) signaling pathway has been proved to be involved in the
RA progression. Human insulin-like growth factors binding proteins (IGFBPs) are indirectly binding to IGF also produced in IGF-1 stimulated osteoblast proliferation and differentiation [12, 13]. Previous studies showed that overexpression of IGFBP2 might be involved in the progression and development of different kinds of cancer, for instance, gliomas, ovarian cancer, prostatic cancer and gastric cancer [14, 15]. According to the result of DNA microarray, IGFBP2 is up-regulated in the RA patients when compared with the healthy people [16, 17].

However, there is very little data on the role and expression of IGFBP2 plays during the RA progression. In the present study, we have detect the expression and activity of IGFBP2 in RA-FLS cells, the role of IGFBP2 was predicted with the proliferation and migration of RA-FLS cells in the development of RA.

Materials and methods

Cell lines and culture

RA-FLS cells (NO. 408RA-05a) and FLS cells (NO. 408-05a) were purchased from Cell Applications (California, USA). All the cells were cultured routinely in DMEM high glucose medium (Gibco, New York, USA) supplemented with 10% FBS (Gibco, New York, USA), streptomycin (50 units/mL) and penicillin (100 units/mL) at 37°C in a humidified incubator containing v/v 5% CO2.

CCK-8 assays

First, FLS cells and RA-FLS cells were transfected with negative control siRNA (NC siRNA) or IGFBP2 siRNA, respectively. Second, 2000 cells/well were seeded in triplicate in 96-well plates. Cell viability was measured by the Cell Counting kit-8 (CCK-8, Beyotime, China) kit, 10 µl of CCk-8 stock solution were added to each well for 3 hours incubation at 37°C in a humidified incubator containing v/v 5% CO2.

Flow-cytometric analysis of cell apoptosis

Firstly, RA-FLS cells were transfected NC siRNA or IGFBP2 siRNA as described above and plated in six well plates. After 48 h cultivation, cells were collected and washed with PBS. Then, cells were incubated with Annexin V-fluorescein isothiocyanate and propidium iodide (Sigma-Aldrich) for 20 min in the dark, respectively. Finally, according to the manufacturer’s instructions, the cells were analyzed by flow cytometry (Beckman Coulter, USA).

QRT-PCR analyses

Total RNAs was extracted from cultured cells with TRizol reagent (Invitrogen) according to the manufacturer’s protocol. The extracted RNA samples were determined by the 260/280 nm ratio. PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa) was used for qRT-PCR. The primers for IGFBP2: 5'-TGTTCGTGACTGGTGTAAC-3', 5'-ATGGCATGGACGTGGGTACAT-3' and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-TGTTCGTGACTGGTGTAAC-3', 5'-ATGGCATGGACGTGGGTACAT-3'. The relative expression of IGFBP2 was normalized to the expression of GAPDH by using the 2-ΔΔCt method. All samples were performed in triplicate.

Cell invasion assay

According to the manufacturer’s instructions [18], the cell invasion assay was performed in membrane invasion 24-well culture system chambers (Coster) with a matrigel layer (BD Bioscience). RA-FLS cells transfected with NC siRNA or IGFBP2 siRNA were seeded in each upper chamber, and incubated for 48 h at 37°C in DMEM with 10% FBS. The cells that invaded the matrigel layer were fixed with 4% paraformaldehyde, stained with crystallized purple, and counted with Upright Metallurgical Microscope (OLYMPUS CX41).

Wound healing assay

Cell migration ability was measured by using scratch wound-healing assay, after the RA-FLS cells transfected with NC siRNA or IGFBP2 siRNA were seeded in a 6-well plate, a micro pipette tip was used to scratch a wound. The cellular debris was washed with serum-free medium for three times. The wound area was photographed under Upright Metallurgical Microscope (OLYMPUS CX41) at 0 h, 48 h.

Western blot analysis

Western blot analysis of cultured RA-FLS cells was performed as previously described [19]. Whole cell lysates were extracted from cultured
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RA-FLS cells in the ice-cold RIPA buffer (Beyotime Biotechnology, China), the total protein concentration were determined by BCA Protein Quantitation Kit (Beyotime Biotechnology, China). Briefly, 30 µg protein/lane of the cell lysates were separated on 10% SDS-PAGE and electrophoresed to PVDF membrane (Bio-Rad). The membranes were blocked with 5% nonfat milk for 1 hour at 4°C. Then, the membranes were incubated with Rabbit polyclonal to IGFBP2 (1:100, NO. ab91404, Abcam) on ice overnight. Next day, mouse monoclonal [6C5] to GAPDH (1:500, NO. ab8245, Abcam) was used to incubate the membranes for 2 hours.

The protein bands were visualized by enhanced-chemiluminescence (ECL kit) reagents (Pierce), and imaged using ChemiDoc XRS (BIO-RAD, USA). GAPDH used as an internal control to confirm equal protein loading.

**Immunofluorescence**

Immunofluorescence analysis was performed as described previously [20]. Firstly, the transfected RA-FLS cells were plated in six-well tissue culture plate for 24 h. Next, the cells were fixed with 4% paraformaldehyde for 30 min and 0.1% Triton X-100 for 10 min. After washing, the...
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cells were blocked with 3% BSA at 37°C. The cells were stained with the anti-IGFBP2 antibody (ab91404; Abcam) in a wet box for overnight at 4°C. Followed by, incubated with Goat
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Statistical analysis

Data were expressed as mean ± standard deviation (SD). The findings were performed using one-way analysis for statistical analysis. P value of <0.05 was considered statistically significant. All the experiments in this study were performed in three or more trials.

Results

The expression of IGFBP2 in FLS cells and RA-FLS cells

In order to investigate the function of IGFBP2 in rheumatoid arthritis, firstly, we evaluated the expression levels of IGFBP2 in FLS cells and RA-FLS cells. As shown in Figure 1A and 1B, the results of qRT-PCR and western blot showed that the mRNA and protein expression of IGFBP2 remarkably increased in RA-FLS cells when compared with FLS cells. Furthermore, the results of immunofluorescence also proved that the IGFBP2 expression level increased in RA-FLS cells than in FLS cells (Figure 1C). Therefore, these results indicated that IGFBP2 may play an important role in rheumatoid arthritis.

Effects of IGFBP2 on proliferation, invasion and apoptosis in vitro

In order to study the mechanisms underlying the effects of IGFBP2 on proliferation, invasion and apoptosis of RA-FLS cells, IGFBP2 siRNA was transfected into RA-FLS cells to down-regulate the IGFBP2 expression, NC siRNA was served as negative control. As shown in Figure 2A, efficiency of transfection was verified by significantly decrease IGFBP2 expression which was determined by qRT-PCR. We found exogenous inhibition of IGFBP2 could inhibit the proliferation and invasion of RA-FLS cells by CCK-8 assay (Figure 2B). In addition, IGFBP2 siRNA could alleviate the invasive capacity and migratory ability of RA-FLS cells when compared to NC (Figure 2C and 2D). We also found RA-FLS cells transfected with IGFBP2 siRNA had a significant higher rate of apoptosis by flow cytometry (Figure 2E).

Effects of IGFBP2 on ERK signal pathway in RA-FLS cells

ERK (extracellular signal-regulated kinase) pathway is associated with cell growth and apoptosis of RA-FLS cells. To investigate the contribution of IGFBP2 siRNA in regulating cellular signaling, we detected the signaling of ERK pathway with Western blot. As shown in Figure 3A and 3B, the phosphor-ERK expression was

Anti-Mouse IgG H&L (FITC) (1:1000, abcam) for 1 h in the dark at 37°C. Nuclei were counterstained with DAPI (1 μg/mL) for 15 min. Images of the cells were obtained using an Olympus confocal microscope (Olympus America, USA).

Figure 3. Effects of IGFBP2 siRNA on ERK signaling pathway. A. Western blot detected the effects of IGFBP2 siRNA on ERK activation in RA-FLS. B. The protein expression of IGFBP2 and ERK were quantified. ***P<0.001 vs NC siRNA.

***P<0.001 vs NC siRNA.
downregulated by IGFBP2 siRNA, and no significant changes were observed in total-ERK.

Discussion

RA is a chronic disease, and a special cell population are more susceptible to rheumatoid synovium which known as activated RA-FLS. Ample evidence indicated that synovial hyperplasia is a symbol of RA. We focused on FLS for a host of reasons, most notably because they contribute a key regulatory cell in RA and produce major pro-inflammatory and joint damage [21].

In the present study, we first confirmed IGFBP2 highly expressed in RA-FLS when compared to FLS in the mRNA and protein expression level (Figure 1). According to the previous studies, IGFBP2 displayed an increased expression in case of lung epithelial damage, IGFBP2 gene knockout mice significantly decreased cell transfer and the invasiveness of tumor angiogenesis [22, 23]. The researches demonstrate that increasing IGFBP2 plays an important role in the growth of breast cancer tumor cells, apoptosis and the angiogenesis of tumor, through the IGFBP2/IGF-1/IGF1R signaling pathway both in vitro and in vivo [24]. Activity of IGFBP2 is associated with metabolic dysfunction including diabetes, diabetes and insulin resistance, it was proposed that IGFBP2 could modulate the hepatic metabolic dysfunction through regulating IGF-1 homeostasis. In this study, IGFBP2 siRNA was used to down-regulate IGFBP2 expression on RA-FLS, and we demonstrate that IGFBP2 siRNA could conduct an inhibition of proliferation, induction of apoptosis, anti-invasion and anti-migration role in RA-FLS (Figure 2).

According to the research, IGFBP2 has an Arg-Gly-Asp (RGD) cell adhesion element that can attach to integrin receptors, ultimately, activate extracellular signal-regulated kinases (ERKs) in order [25]. Increasing IGFBP2 levels has been shown to activate the ERK signaling pathway, could stimulate proliferation in glioblastoma cells, breast cancer cells and ovarian cancer cells [26-28]. On the contrary, PD98059 and U1026 as the inhibitors of the ERK pathway abrogated IGFBP2-induced activation [26]. Silencing IGFBP2 with lentivirus-based shRNA inhibit IGF1-induced phosphorylation of ERK in PC-3 cells, decreased activation of AKT and ERK pathway in human leukemia cells [29]. ERK signaling is closely related to cellular growth, differentiation and proliferation in cancer-related cellular processes. In this study, silencing IGFBP2 with siRNA was shown to inhibit the phosphorylation of ERK in RA-FLS (Figure 3), and it was essential for IGFBP2-induced proliferation, invasion, migration and apoptosis in RA-FLS. Therefore, IGFBP2 were mainly exerted its functions through the ERK signaling pathway.

Conclusions

In summary, IGFBP2 showed a higher mRNA and protein expression level in RA-FLS than that in FLS. Silencing IGFBP2 was shown to decrease proliferation, invasion, migration and apoptosis via ERK signaling pathway in RA-FLS. Therefore, silencing IGFBP2 levers may represent a new targeted therapy for the treatment of rheumatoid arthritis.

Disclosure of conflict of interest

None.

Authors’ contribution

Xinliang Wang performed the experimental work. Yanan Li participated in experimental design and coordination. Lin Chen and Zhiqi Hou contributed to analysis and interpretation of data. The article was written by Xinliang Wang. All authors have read and approved the final manuscript.

Address correspondence to: Dr. Xinliang Wang, Department of Orthopedic Surgery, The First Affiliated Hospital of Jinan University, No. 613 Huangpu Road, Guangzhou 510632, China; Department of Orthopedic, Guangzhou First People’s Hospital, Guangzhou Medical University, Guangzhou 510180, China. Tel: (86) 13580415009; E-mail: wxl_37543582@qq.com

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