Original Article

Interleukin-1β induces metabolic and reactive oxygen species changes and apoptosis in annulus fibrosus cells

Xing Yang1,2,3*, Lei Wang1,2*, Zhangqin Yuan1,2, Pinghui Zhou1,2, Genglei Chu1,2, Bin Li1,2, Junying Sun1

1Department of Orthopaedics, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China; 2Orthopedic Institute, Soochow University, Suzhou, Jiangsu, China; 3Changshu No.1 People’s Hospital, Changshu, Jiangsu, China. *Equal contributors.

Received May 21, 2017; Accepted November 7, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: Degeneration of the annulus fibrosus (AF), a critical load-bearing component of the intervertebral disc (IVD), commonly leads to degenerative disc disease (DDD). To investigate the potential effects of interleukin (IL)-1β on AF cells, we evaluated metabolism, intracellular reactive oxygen species (ROS), and apoptosis of these cells in vitro. Four experimental groups, one control group and three treatment groups treated with 1, 10 or 100 ng/ml IL-1β, were examined in this study. After 24 or 48 h, the proliferation and cell cycling of AF cells were not apparently affected by IL-1β treatment. However, significant upregulation of ADAMTS4 and downregulation of aggrecan were seen in all treatment groups. Depending on the treatment time, IL-6, TIMP-1, MMP3, and ADAMTS5 expression was upregulated significantly, while Col-I, Col-II, and TIMP2 expression was significantly downregulated in certain groups (mostly in 10 and 100 ng/ml treatment groups). In addition, IL-1β significantly increased intracellular ROS levels in AF cells. Importantly, apoptosis of AF cells was increased significantly by IL-1β treatment. Taken together, our results demonstrated that IL-1β induced significant inflammatory reactions in AF cells, leading to metabolic and ROS level changes and increased apoptosis, which suggest the involvement of inflammation in DDD development.

Keywords: Annulus fibrosus cells, intervertebral disc, inflammation, IL-1β, apoptosis

Introduction

Low back pain is a common musculoskeletal disorder affecting the quality of life in adults with a tremendous socioeconomic impact [1]. The intervertebral disc (IVD) plays an important role in maintaining mechanical functions, and IVD degeneration is the main reason for low back pain [2]. Numerous studies have suggested a correlation between degenerative disc disease (DDD) and loss of disc extracellular matrix (ECM) caused by perturbed ECM homeostasis [3, 4]. In addition, enzymes mediating ECM degradation, including matrix metalloproteinases (MMPs), are upregulated during IVD degeneration and aging, which may lead to increased ECM degradation [5-7]. Inflammation is regarded as a response to tissue injury and plays a vital role in the physiopathology of tissues including IVDs [8]. Indeed, IVD diseases are usually correlated to increased levels of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α), within the disc. For example, a study from LeMaitre et al. showed that degenerated discs from clinical patients with chronic back pain had higher IL-1β and TNF-α expression than the non-degenerated discs [9]. Surgical samples from DDD patients also showed higher levels of TNF-α positive cell than those from the normal group [10]. Therefore, inflammatory mediators appear to play a critical role in DDD and possibly DDD-related back pain.

Apart from the changes in inflammation and metabolism involved in DDD development, excessive apoptosis of disc cells is also pivotal in DDD, which frequently contributes to neck or low back pain [11]. Numerous factors, including abnormal mechanical stresses [12, 13], IL-1β [14], and serum withdrawal [15], can lead to apoptosis of IVD cells both in vivo and in vitro.

The IVD is mainly composed of two distinct components, the inner nucleus pulposus (NP) and outer annulus fibrosus (AF). The integrity of
AF is critical for maintenance of intradiscal pressure and confining the NP, a fundamental requirement to maintain the structure and physiological functions of IVDs [16, 17]. AF defects may induce mechanical strain concentration, cell death, and proinflammatory cytokine production [18, 19]. Previous studies have suggested that IL-1β is a significant catabolic cytokine in IVDs, which not only increases matrix-degrading enzyme activity, but also enhances apoptosis in NP and AF tissues [20, 21]. IL-1β also increases the sensitivity of AF cells to mechanical stress [22]. In a recently published study, Oh and his colleagues used a ROCK inhibitor to immortalize rat AF cells and successfully established an AF cell line [23]. This cell line is useful to understand the mechanisms of DDD. To understand the potential effects of IL-1β on AF cell biology, we investigated the responses of AF cells cultured in an in vitro inflammatory environment that partially mimicked the diseased disc microenvironment. The effects of inflammation on AF cell metabolism, intracellular reactive oxygen species (ROS) levels, and apoptosis were examined in this study.

Materials and methods

**AF cell line and culture conditions**

The rat AF cell line was a generous gift from Prof. Di Chen (Rush University) [23]. The cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1) (Hyclone, GE, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco, Thermo, USA), and 1% penicillin and streptomycin at 37°C with 5% CO₂.

**Treatment of cells with IL-1β**

At 80-90% confluence, cells were trypsinized, seeded into appropriate culture plates, and cultured as described previously. Recombinant human IL-1β was from Prosvec-TanyTechnogene Ltd (Suzhou Ketone Bio-Pharma Co., Ltd). Four experimental groups were established, one control and three treatment groups. In the three treatment groups, 1, 10 or 100 ng/ml IL-1β was added to the medium. The cells were then incubated for 24 or 48 h. Cultures without added IL-1β served as the control group. Cells from all groups were incubated at 37°C with 5% CO₂.

Cell viability assays

Cells were seeded at 2,000 per well in 96-well plates. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China) following the manufacturer’s instructions. Three independent experiments were performed.

Colony formation assay

Cells were seeded in six-well plates at 1,000 cells/well and cultured for 14 days to allow colony formation. After incubation, cells were washed with PBS and fixed with methanol. After further rinsing with PBS twice, cells were stained with 0.1% crystal violet. Visible colonies were then counted manually.

Expression analysis and quantitative real-time PCR

Expression of ECM and metabolic genes (Col-I, Col-II, aggrecan, MMP-3, TIMP1, TIMP2, ADAMTS4, and ADAMTS5) and the inflammatory gene IL-6 was analyzed by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from cells with an RNA Simple Total RNA Kit (Tiangen, Beijing, China). cDNA was prepared from the mRNA using a Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative qPCR was performed using primers, cDNA, and a QuantNova™ SYBR Green PCR Kit (Qiagen). Real-time PCR amplifications were performed with a CFX96 system according to the following protocol: 95°C for 15 min, followed by 40 cycles of amplification, each consisting of a denaturation step at 94°C for 15 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 30 s. GAPDH was used as an internal control. Values for all target genes were normalized to internal control values. The fold changes were calculated according to the relative quantification method (RQ = 2−ΔΔCT). Primer sequences are shown in Table 1.

Western blot analysis

For western blot analyses, total proteins were extracted from cultured cells and then quantitated using a bicinchoninic acid assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. Proteins were fractionat-
Interleukin-1β induces metabolic changes

Table 1. Primer sequences used for genes analyzed in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer (5’→3’)</th>
<th>Reverse Primer (3’→5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-I</td>
<td>TTCTGAAACCCCTCCCTCTTT</td>
<td>CCACCCCCAGGATAAATAACT</td>
</tr>
<tr>
<td>Col-II</td>
<td>CGAGGGGACCGAAAGGAGAAC</td>
<td>AGGCGGCAAGGTACCCTGAT</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>AGACACCCCTACCCCTGTCT</td>
<td>AAGGTGCAAGGGGATCACC</td>
</tr>
<tr>
<td>MMP-3</td>
<td>GGAAGCGTAGGGAATGAAGA</td>
<td>ATGCAATGGTTAGGTAGGAC</td>
</tr>
<tr>
<td>TIMP1</td>
<td>TGCAACTGGGACTGCTTAT</td>
<td>ACAACGTCCGAATCTCTTGAG</td>
</tr>
<tr>
<td>TIMP2</td>
<td>GCATCACCAGGAAGAAGGC</td>
<td>GTCCATCCAGGGGACTCAT</td>
</tr>
<tr>
<td>ADAMTS4</td>
<td>GCGCGATTCTACATCGACT</td>
<td>GCGGTCACGATCATATGCTT</td>
</tr>
<tr>
<td>ADAMTS5</td>
<td>CTCAGGGCCAGGATCAGGAA</td>
<td>CTGTCGTCAGGATGCGCTT</td>
</tr>
<tr>
<td>IL-6</td>
<td>CACAGGAGTACCCGACCA</td>
<td>CAGAATGGCCATGCCAACAC</td>
</tr>
</tbody>
</table>

Flow cytometry analyses of apoptosis, cell cycle, and ROS levels

The apoptotic rate was measured by flow cytometry using an Annexin V-FITC/PI kit (Beyotime, Shanghai, China). After treatment with or without IL-1β (1, 10 or 100 ng/ml) for 24 or 48 h, cells were trypsinized, centrifuged, and washed in PBS at 4°C. The washed cells were resuspended in binding buffer at a density of 1×10⁶ cells/ml. The cells were then stained with 5 μl Annexin V-FITC and 10 μl propidium iodide (PI) in the dark at room temperature for 15 min. A 400 μl aliquot of the buffer was added to the resuspended AF cells that were then analyzed by flow cytometry to detect the apoptotic fraction in each sample. Cell cycle profiles were determined by analyzing DNA content using PI staining. To measure intracellular ROS levels, AF cells were loaded with the indicator dye from the Reactive Oxygen Species Assay kit (TIANDZ, Beijing, China) and incubated on a shaker at 37°C for 30 min. Intracellular ROS levels were then estimated by flow cytometry, following the manufacturer’s protocol.

Statistical analysis

Statistical analyses were performed using SPSS 17.0. All data are presented as the means ± standard error of the mean (SEM). Comparisons were made by one-way analysis of variance (ANOVA) followed with independent sample t test. P-values of less than 0.05 were considered to be statistically significant.

Results

IL-1β partly inhibits AF cell proliferation and colony formation, but does not affect cell cycling

We first examined the effects of IL-1β on AF cell proliferation using the CCK-8 assay. Compared with the control group, cell proliferation was not significantly affected by all concentrations of IL-1β treatment during the first 4 days (Figure 1A). On day 5, the proliferation of cells treated with 100 ng/ml IL-1β was significantly decreased compared with that of the control group. However, there were no significant differences between the other groups and the control group. After treatment with IL-1β, the number of colonies in 10 and 100 ng/ml groups was much smaller than that in the control group (Figure 1B, 1C). To test whether IL-1β affected the cell cycle, we used flow cytometry to analyze AF cells before and after IL-1β treatment for 24 or 48 h. As a result, there was no significant difference in the percentage of cells in G1 and G2 phases among all groups, indicating that IL-1β treatment did not apparently affect AF cell cycling (Figure 1D-G).

IL-1β induces apoptosis of AF cells

Cell apoptosis was detected by flow cytometry using double staining with annexin V and PI. There was an increase in the percentage of apoptotic cells treated with all doses of IL-1β for 24 h (Figure 2A, 2B). The apoptotic rate in the control group was 2.3 ± 0.5%, which increased to 6.5 ± 0.2%, 10.4 ± 0.7%, and 11.1 ± 0.4% after IL-1β treatments at 1, 10, and 100 ng/ml for 24 h, respectively. After 48 h of treatment, cell apoptosis showed a similar trend with apoptotic rates of 3.8 ± 0.5%, 11.1 ± 0.4%, 21.3 ± 0.7%, and 24.4 ± 0.6%, respectively (Figure 2C, 2D).

IL-1β increases intracellular ROS levels

To further investigate whether IL-1β affects the AF microenvironment, we used flow cytometry to measure intracellular ROS levels. Flow cytometry results confirmed that intracellular
Interleukin-1β induces metabolic changes

Figure 1. Effects of various IL-1β concentrations on proliferation and cell cycling of AF cells. Various concentrations of IL-1β did not significantly affect cell proliferation (A). The relative number of colonies was calculated at 14 days after seeding (B). Representative images of cell colonies (C). Percentages of AF cells at G2 phase after treatment with various concentrations of IL-1β and representative cell cycle images at 24 h (D, E) and 48 h (F, G).

Lar ROS levels were significantly increased in all treatment groups, except for the 1 ng/ml IL-1β, 24 h group. Compared with control cells (250.3 ± 38.0), ROS signals were significantly increased by IL-1β to 344.5 ± 32.5 (10 ng/ml) and 465.3 ± 29.3% (100 ng/ml) after IL-1β.
Interleukin-1β induces metabolic changes

Treatment for 24 h (Figure 3A, 3B). After 48 h of treatment, the ROS intensity was increased significantly with intensities of 470.3 ± 27.1 (1 ng/ml), 707.7 ± 38.5 (10 ng/ml), and 914.7 ± 65.3 (100 ng/ml), respectively (Figure 3C, 3D).

**IL-1β induces the gene expression of proinflammatory cytokine IL-6**

Compared with the control group, IL-6 was significantly upregulated in groups treated with 10 or 100 ng/ml IL-1β for 24 or 48 h. IL-6 expression was also significantly elevated in cells receiving 1 ng/ml IL-1β for 48 h. However, this effect was not observed in cells treated with 1 ng/ml IL-1β for 24 h (Figure 4A).

**IL-1β treatment down-regulates ECM gene expression**

qRT-PCR results demonstrated significant downregulation of aggrecan in all treatment groups after both 24 and 48 h (Figure 4B). In addition, western blotting showed a similar trend in expression of aggrecan after treatment with IL-1β for 48 h (Figure 4C, 4D). Compared with the control group, Col-I was significantly downregulated in groups treated with 10 or 100 ng/ml IL-1β at both time points. In contrast, after treatment with 1 ng/ml IL-1β, no significant changes were found in Col-I expression at either time point (Figure 4E). Compared with the control group, Col-II was significantly downregulated at both time points by 10 or 100 ng/ml IL-1β. A similar trend was also observed in groups treated with 1 ng/ml IL-1β for 48 h, but not in those treated for 24 h (Figure 4F).

**IL-1β regulates the expression of metabolism-related genes**

To evaluate the effects of IL-1β on expression of some key genes involved in DDD, we examined expression of MMP-3, TIMP-1, TIMP-2, ADAMTS4, and ADAMTS5. qRT-PCR results
Interleukin-1β induces metabolic changes

showed significant upregulation of ADAMTS4 in all treatment groups at both time points (Figure 5A). Compared with the control group, only 10 and 100 ng/ml IL-1β caused significant upregulation of TIMP-1 after treatment for 24 or 48 h (Figure 5B). In addition, MMP-3 was significantly upregulated in all treatment groups except the 1 ng/ml group after 24 h of IL-1β treatment (Figure 5C). We also performed western blotting, and the results showed that the expression of MMP-3 was significantly increased after 1L-1β treatment for 48 h (Figure 5D, 5E). There were no significant differences in TIMP-2 expression among all groups at the 24 h time point. However, there was a significant decrease in TIMP-2 expression after IL-1β treatment for 48 h (Figure 5F). Regarding ADAMTS5, there was upregulation in the 10 and 100 ng/ml treated groups at 48 h (Figure 5G).

Discussion

Although chronic low back pain is a common debilitating disorder with serious socioeconomic consequences, the causes of disc degeneration, especially its molecular events and cellular changes, are poorly understood. In this study, we investigated whether IL-1β induced any potentially relevant effects in AF cells in vitro, including inflammatory reactions, metabolic and microenvironmental changes, and finally apoptosis.

Previous studies have suggested a correlation between increases in cell death and degenera-
Interleukin-1β induces metabolic changes

The supply of nutrients has been found to deteriorate during DDD [26], while IL-1β levels increase [27, 28]. In a study by Zhao et al., IL-1β enhanced the effects of serum deprivation on apoptosis in rat annular cells [20]. Another study showed that treatment with 75 ng/ml IL-1β induced apoptosis [29]. Consistent with these results, our data also showed that IL-1β induced apoptosis of AF cells in vitro.
Interleukin-1β induces metabolic changes

There is growing evidence that IL-1β induces synthesis of ECM-degrading enzymes and reduces synthesis of proteoglycans, Col-I, and Col-II [27, 30-32]. Le Maître et al. [27] demonstrated that IL-1β produced by native disc cells induces both ECM and cellular changes in IVD degeneration. Another study from Hoyland et al. [21] indicated that IL-1β increased enzymatic activity and an IL-1β inhibitor decreased enzymatic activity, suggesting a key regulatory role of IL-1β in ECM degradation. Consistent with these studies, our study also indicated changes in the expression of metabolism-related genes induced by IL-1β. Compared with the control group, IL-1β stimulated expression of MMP-3 and TIMP-1, and suppressed that of Col-I, Col-II, and aggrecan.

ROS are oxygen-containing molecules, including superoxide anion (O$_2^-$), hydroxyl radical (OH), hydrogen peroxide (H$_2$O$_2$), and nitric oxide (NO), which are produced during cellular metabolism and can diffuse through membranes. Overproduction of ROS may perturb the normal balance between prooxidants and antioxidants in cells, and damage DNA, proteins, and lipids [33]. Several previous studies have indicated a relationship between bone biology and redox balance regulation, implying a major role of ROS in osteoporosis [34, 35]. Other studies suggested that ROS induces chondrocyte death and matrix degradation, contributing to the progression of osteoarthritis [36]. In degenerative IVD, excessive ROS may exert a catabolic effect on AF cells through mitogen-activated protein kinase signaling [33]. In our present study, we also showed that intracellular ROS levels were increased in AF cells treated with IL-1β. These observations indicated that ROS might play an important role in the development of musculoskeletal degenerative diseases.

A major limitation of this study is the use of a rat AF cell line in vitro. This approach was chosen to ensure homogeneous effects of IL-1β without influences of other clinical characteristics, such as gender, age, and medications, which are related to the use of patient samples. Nevertheless, our findings should be confirmed in such patient samples to determine their clinical relevance. In addition, we focused our experiments on the effect of inflammation on AF cell proliferation, apoptosis, and metabolism. Thus, we did not study the effects of mechanical stress, which can also play a vital role in regulation of AF cells.

Conclusions

Our study showed that IL-1β induced an inflammatory reaction in AF cells, which provides an in vitro model for IVD degeneration, resulting in changes in metabolism and ROS levels. Furthermore, IL-1β at certain concentrations promoted apoptosis in this system. These findings clearly indicate a potential role of inflammation in the development of DDD.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant numbers 31530024, 81672213, and 81472060), National Key Research and Development Program (grant number 2016YFC1100203), Jiangsu Provincial Special Program of Medical Science (grant number BL2012004), Jiangsu Provincial Clinical Orthopedic Center, and the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

Disclosure of conflict of interest

None.

Address correspondence to: Junying Sun, Department of Orthopaedics, The First Affiliated Hospital of Soochow University, 188 Shizi St, Rm 103 Bldg 8, Suzhou 215000, Jiangsu, China. Tel: +86-512-67780101; Fax: +86-512-6778-1163; E-mail: sjsuzhou@126.com; Bin Li, Orthopedic Institute, Soochow University, 708 Renmin Rd, Rm 308 Bldg 1, Soochow University (South Campus), Suzhou 215007, Jiangsu, China. Tel: +86-512-67781163; Fax: +86-512-6778-1163; E-mail: binli@suda.edu.cn

References

Interleukin-1β induces metabolic changes


Interleukin-1β induces metabolic changes


