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Original Article

Downregulated interleukin 37 expression associated with aggravation of intervertebral disc degeneration

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Abstract: Interleukin 37 (IL-37) is an anti-inflammatory cytokine which was proven to be associated with several diseases characterized with excessive-inflammation. The pathologic process of Intervertebral disc degeneration (IVDD) is also companied by uncurbed inflammation, many cytokines were reported presenting in the process. However, there is little IL-37 related knowledge in IVDD up to now. The aim of this study was to investigate whether IL-37 expression in degenerative intervertebral disc (IVD) is different from that in non-degenerative disc and to evaluate the relationship between IL-37 expression, overexpression of pro-inflammatory cytokines and development of degeneration. Human nucleus pulposus samples were obtained from patients with disc degenerative disease and vertebra fractures undergoing discectomy and fusion. Subsequently, expression of IL-37 was assessed by real-time quantitative polymerase chain reaction (RT-PCR) and western blotting. Gene expression level was measured for IL-1α, IL-1β, IL-6, IL-16, TNF-α, TGF-β1 and Smad3. Degree of degeneration was evaluated for MRI with modified Pfirrmann grading system. The results showed that IL-37 had a decreased expression in degenerative samples compared to that in normal samples both at mRNA and protein level. Instead, significant elevated gene expression of IL-1β, IL-16, TNF-α, TGF-β1 and Smad3 were detected in degenerative samples. High correlations were observed between IL-37, IL-1β, IL-16, TGF-β1, Smad3 and degeneration degree of IVD. Downregulation of IL-37 expression appeared to result in overexpression of pro-inflammatory cytokines, such as IL-1β and IL-16, in degenerative IVD and may be a contributor involved in the progression of IVDD.

Keywords: Intervertebral disc degeneration, interleukin 37, anti-inflammatory cytokine, interleukin-1 beta, interleukin 16

Introduction

IVDD is considered a major source of back pain for most individuals over the age of 50 years [1]. The pathological process of IVDD is complex, a range of alteration, including increased breakdown of matrix, cell loss through apoptosis and altered matrix synthesis was found in the process of IVDD [2-4]. Among that, the dysregulation of many cytokines in nucleus pulposus, including interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), which disrupts the local micro-environment homeostasis, is suggested playing a crucial role in the development of IVDD. IL-1α and IL-1β are also designated as IL-1F1 and IL-1F2 respectively. They are members of interleukin 1 (IL-1) gene family and important in the initiation of the inflammatory reaction. Excessive and/or dysregulated activity of IL-1α and IL-1β without an antagonistic action of IL-1 receptor antagonist (IL-1RN) was reported to be associated with disc dehydration and matrix degradation [5, 6].

There are 11 members in the IL-1 gene family, most of them are long known as pro-inflammatory cytokines, however, the biological role of the seventh member is just starting to be elucidated. Previous studies showed that in vivo expression of human IL-37 in mice reduced local and systemic inflammation in concanavalin A-induced hepatitis and lipopolysaccharide (LPS) -induced sepsis [7]. Subsequent study showed that IL-37 also reduced hepatocyte injury and liver inflammation for rats after

Table 1. study-related patients information

Patients No.	Diagnosis	Disc level	Modified Pfirrmann grade	Gender	Age
1	Lumbar disc herniation	L5-S1	6	M	51
2	Lumbar disc herniation	L3-L4	5	M	39
3	Spinal stenosis	L3-L4	4	F	62
4	Lumbar disc herniation	L5-S1	4	M	49
5	Spondylolisthesis	L4-L5	5	M	58
6	Lumbar disc herniation	L4-L5	6	F	52
7	Spinal stenosis	L3-L4	3	F	59
8	Spinal stenosis	L3-L4	4	M	53
9	Spondylolisthesis	L5-S1	5	M	56
10	Lumbar disc herniation	L4-L5	3	M	46
11	Spondylolisthesis	L4-L5	4	M	51
12	Lumbar disc herniation	L4-L5	4	M	55
13	Spinal stenosis	L5-S1	6	F	45
14	Spinal stenosis	L3-4	5	M	54
15	Vertebral fracture	L4	1	M	42
16	Vertebral fracture	L3	1	M	38
17	Vertebral fracture	L4	1	M	34
18	Vertebral fracture	L4, L5	1	F	41
19	Vertebral fracture	L2	1	M	27
20	Vertebral fracture	L1	1	F	22
21	Vertebral fracture	L4	1	M	43
22	Vertebral fracture	L5	1	M	35
23	Vertebral fracture	L2, L3	1	M	37
24	Vertebral fracture	L3	1	M	33
25	Vertebral fracture	L3	1	F	26

hepatic ischemia/reperfusion [8]. These findings indicate that IL-37 performs a unique anti-inflammatory role similar with the IL-1RN. Subsequent studies found that several molecules and cytokines are highly effective in regulating IL-37, including LPS and transforming growth factor β1 (TGF-β1) [9, 10].

Variation of pro-inflammatory and/or anti-inflammatory activity in degeneration is suggested a important link in the chain of IVDD progression. Undermined activity of the anti-inflammatory cytokines may be resulting in a relative amplification of pro-inflammatory efficacy, leading to the runway inflammation in IVD tissue. Considering the potent anti-inflammation effect of IL-37, we supposed that an association between IL-37 expression dysregulation and overactivity of pro-inflammation exists in IVDD, whereas there is little IL-37 related knowledge in the degeneration process up to now. In light of this, the current study was carried out to determine whether the IL-37 expression is altered in degenerative disc and whether close correlations are existing between IL-37 expressions, IVDD related pro-inflammatory cytokines expression and the stages of disc degeneration.

Materials and methods

Source of human IVD tissue

Human IVD specimens were obtained with written informed consent from all participants, under the approval of the Fourth Military Medical University Ethics Committee. The degenerative disc nucleus pulposus tissues used in our study were derived from fourteen patients with lumbar disc degenerative disease. Non-degenerative specimens were obtained from eleven patients with acute burst fractures of lumbar vertebra but no signals of disc degeneration on MRI. All participants underwent posterior discectomy and fusion. The degeneration condition of IVD was evalu-

Table 2. Sequences of primers for the RT-PCR assays

Gene	Sense	Sequence 5'→3'
IL-37	F	CAAAGTCATCCATCCCTTCAGC
	R	CCGACTCCAGCATGTTCC
IL-1α	F	AGAAGAGACGGTTGAGTTTAAGCC AATCCA
	R	CTCAGGAAGCTAAAAGGTGCTGACCTA
IL-1β	F	TGTTGAAAGATGATAAGCCCACTCT
	R	CAAATCGCTTTTCCATCTTCTTC
IL-6	F	CGGGAACGAAAGAGAAGCTCTA
	R	GAGCAGCCCCAGGGAGAA
IL-16	F	ATGCCTGACCTCAACTCCACT
	R	GCCACCCAGCTGCAAGATTTC
TNF-α	F	CGAGTCTGGGCAGGTCTACTTT
	R	AAGCTGTAGGCCCCAGTGAGTT
TGF-β1	F	CTGCTACCGCTGCTGTGGCTACTG
	R	CGGTCGCGGGTGCTGTTGT
Smad3	F	ATCCTGCCTTTCACTCCCC
	R	CTGCCCCGTCTTCTTGAGTT
β-actin	F	GCAGAAGGAGATCACTGCCCT
	R	GCTGATCCACATCTGCTGGAA

ated according to the modified Pfirrmann grading system [11]. Descriptions of all specimens are shown in **Table 1**.

RT-PCR

Total RNA of all nucleus pulposus samples was prepared using TaKaRa™ MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, CN) according to the manufacturer's protocol. 1 μg of total RNA was used to synthesize cDNA using PrimeScript™ RT Master Mix Kit (TaKaRa, Dalian, CN). Real-time PCR amplifications were performed using gene specific primers and SYBR Premix Ex Taq™ II kit (Tli RNaseH Plus) according to the manufacturer's protocol. The primers sequences used for the RT-PCR analysis are shown in **Table 2**. The thermal cycling conditions were as follows: an initial denaturation at 95°C for 3 min, followed by 40 cycles of 20 seconds of denaturing at 95°C, 20 seconds of annealing at 58°C, and 20 seconds of extension at 72°C. The expression levels of the target genes were normalized to that of β-actin in cDNA samples.

Western blotting analysis

All specimens were weighted, lysed and homogenized, then underwent a centrifugation at 12000 rpm for 15 minute in 4°C. Specimens protein was assayed using the bicinchoninic acid protein concentration determination for the supernate. After ensuring linearity of band density, samples (10 μg total protein) were applied to 10% polyacrylamide gels, proteins were separated using standard SDS-PAGE protocols [12] and transferred to polyvinylidene difluoride membranes (BioRad, Hercules, CA). Subsequently, the membrane was blocked with 5% bovine serum albumin (BSA) and incubated at room temperature for 1 hour. This was followed by a 3-h incubation in 5% BSA containing the primary antibody (rabbit anti-human IL-37 antibody, Abcam, Cambridge, UK) at a 1:100 dilution. After washing, the membrane was incubated in PBS, containing the secondary antibody (goat anti-rabbit conjugated with horseradish peroxidase, Boster, Wuhai, CN) for 1 hour at room temperature. Visualized bands were detected using the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Little Chalfont, UK). The densitometric analysis of the protein band was performed using Bio-Rad Quantity One software (BioRad, Hercules, USA). The IL-37 protein content was expressed relative to β-actin in arbitrary units.

Statistical analysis

The results are presented as means ± standard deviation. Difference of cytokines expression levels between degenerative and non-degenerative samples was determined using one-way analysis of variance (ANOVA). The degree of association between variables was calculated using Pearson correlation coefficient or Spearman's nonparametric correlation. All statistical tests were performed with SPSS version 17.0 (SPSS Inc., Chicago, USA). *P* values were two-tailed and a significance level of 5% was used throughout.

Results

RT-PCR and western blotting revealed the differential expression of IL-37 between non-degenerative and degenerative discs

Changes of IL-37 expression in nucleus pulposus were determined by RT-PCR and western blotting assays. The expression profile of IL-37 was detected both in non-degenerative and degenerative samples. Nevertheless, significantly decreased expression was confirmed in degenerative tissues both at mRNA (**Figure 1**,

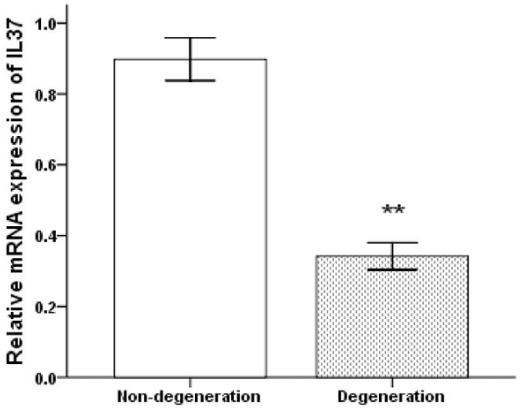

Figure 1. mRNA expression level of IL-37 relative to β-actin was significantly decreased in degenerative samples compared to that in the normal samples. **p<0.01 vs. non-degenerative groups.

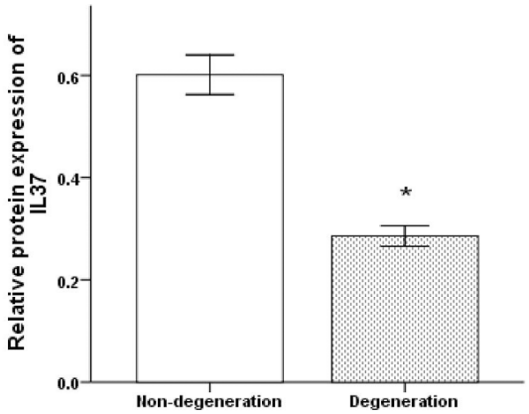

Figure 2. Protein expression level of IL-37 relative to β-actin was significantly decreased in degenerative samples compared to that in the normal samples. *p<0.05 vs. non-degenerative groups.

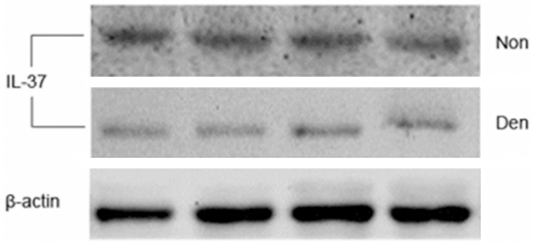

Figure 3. Photograph of the IL-37 western blotting results of normal and degenerative samples. Non, non-degeneration, Den, degeneration.

P=0.008) and protein (**Figures 2**, **3**, P<0.05) level.

Increased expression of pro-inflammatory cytokines and IL-37 regulated cytokines was observed in degenerative samples

The mRNA expression changes of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, interleukin 16 (IL-16) and TNF-α) and cytokines related to the IL-37 expression (TGF-β1 and Smad3) were assessed by RT-PCR in non-degenerative and degenerative samples. The mRNA expression levels of IL-1β (P<0.05), IL-16 (P=0.006), TNF-α (P<0.05), TGF-β1 (P=0.003) and Smad3 (P=0.002) were significantly higher in degenerative samples compared to them in normal samples (**Figure 4**). But no expression changes were observed at IL-1α (P>0.05) and IL-6 (P>0.05) between two groups. The association degree of mRNA expression between IL-37, IL-1β, IL-16, TNF-α, TGF-β1 and Smad3 was calculated using Pearson correlation coefficient. Significant negative correlations were observed between IL-37, IL-1β (r=-0.853, P<0.05), IL-16 (r=-0.931, P=0.008), TNF-α (r=-0.391, P<0.05), TGF-β1 (r=-0.953, P=0.005) and Smad3 (r=-0.965, P=0.002).

The expression of IL-37 was correlated with the degenerative stages of IVD

The MRI of fourteen and eleven lumbar discs derived from patients with disc degenerative diseases and patients with vertebra fractures respectively was assessed with modified Pfirrmann grading system. According to the grading criterion, 11, 2, 5, 4 and 3 discs were graded as 1, 3, 4, 5 and 6 degree respectively. Correlation between expression level of IL-37 mRNA and degeneration degree of IVD was evaluated using Spearman's nonparametric correlation. Significant negative correlation was found between IL-37 expression level and degeneration degree (r_s=0.826, P<0.05).

Discussion

Alterations in local tissue cytokine biology, particularly the pro- and anti-inflammatory cytokines dysregulation, were confirmed a important part involved in the process of IVDD. In normal discs, pro- and anti-inflammation activity appear to make a balance, however, the scale tilts to the former with an upregulation of pro-inflammatory cytokines expression in the degeneration of disc. Maitre et al [5] reported that normal IVD cells express both the IL-1α and IL-1β, such expression is matched by their

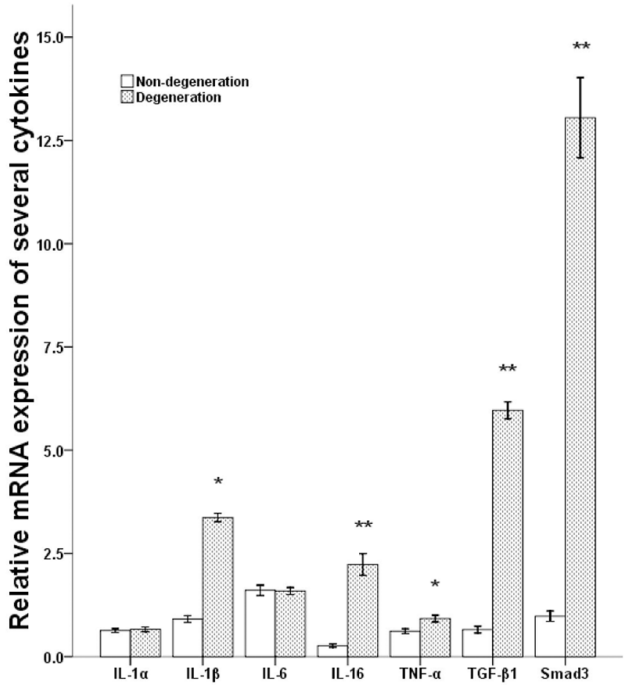

Figure 4. mRNA expression of IL-1α, IL-1β, IL-6, IL-16, TNF-α, TGF-β1 and smad3 was normalized to β-actin. Expression levels of IL-1β, IL-16, TNF-α and TGF-β1 were significantly increased in degenerative samples compared to them in normal samples. *p<0.05 vs. non-degenerative groups. **p<0.01 vs. non-degenerative groups.

natural inhibitor, IL-1RN. In degeneration, IL-1α and IL1-β are up-regulated without increased IL-1RN. Similarly, previous studies showed that expression of IL-6 and TNF-α was significantly increased in degenerative discs and statistically associated with disc degeneration [13, 14]. Consistent with the results of former studies, expression levels of IL-1β and TNF-α in our study were significantly higher in degenerative samples than them in the normal ones. Overexpression of these cytokines breaks the balance in the pro- and anti-inflammation, which induces a lot of tissue changes associated with degeneration, such as matrix degradation and angiogenesis [15]. However, no statistical expression changes of IL-1α and IL-6 were detected between the degenerative and normal specimens in the current study, partially attributed to the properties of objects studied in our experiments.

In the current study, another pro-inflammatory cytokine IL-16 which was shown to affect the levels of many inflammatory mediators has been discovered within the degenerative IVD with a higher content compared to the normal discs [16]. IL-16 was also characterized as an immune modulatory cytokine and described in association with several autoimmune inflammatory disorders, including atopic dermatitis and irritable bowel syndrome [17, 18]. The immune modulation role of IL-16 functions in various aspects such as chemotaxis and activation of the immune-cells [19]. As is known to all, breakdown of the immune-privilege barrier, infiltration and activation of immune cells and immune injury form an immune cascade happening in IVDD [20]. Upregulated IL-16 may be involved in the cascade, contributing to the degeneration of IVD, not only by induction of inflammatory mediators, but also participating in the autoimmune hurt.

Similar anti-inflammatory function of IL-1RN which diminishes pro-inflammation effect induced by IL-1α and IL-1β [21] was also observed at IL-37 which exhibits anti-inflammatory function by directly inhibiting the production of pro-inflammatory cytokines. Nold's study [9] showed that mouse macrophages transfected with IL-37 presented marked reduction of LPS-stimulated IL-1α, IL-1β, IL-6, IL-27 and TNF-α expression compared with the mock-transfected cells. Immunohistochemistry of synovial tissue from patients with active rheumatoid arthritis (RA) showed significantly higher amounts of IL-37 compared to the normal tissues [9]. In our study, we detected a significant decrease of IL-37 expression in degenerative discs and high correlations between the expression levels of IL-37 and IL-1β, IL-16, TNF-α. Similar results found in former study showed that siRNA treatment targeting endogenous IL-37 resulted in significant increase of several pro-inflammatory cytokines in macrophages and epithelial cells, including IL-1α, IL-1β, IL-6 and TNF-α [9]. Downregulation of IL-37 expression attenuated the potent anti-inflammatory effect of IL-37 targeting to the pro-inflammatory cytokines in above cells, which may be also the primary reason for the increased expression of the IL-1β and IL-16 in the current study.

TGF-β1 is an immune regulator which performs a prominent role in immune system by controlling several aspects of inflammatory respons-

es. Low concentration TGF-β1 is highly effective in inducing endogenous IL-37, which is relevant to the interaction of Smad3 [9], whereas our data showed a increased concentration of TGF-β1 which was negatively correlated with the IL-37 expression and may be an antecedent for the downregulated expression of IL-37.

In the current study, our data showed a significant negative correlation between IL-37 and degeneration degree, which implied that the weakened anti-inflammatory activity of IL-37 is a potential factor for the onset and progression of IVDD and illuminate a breakthrough point for the treatment of IVDD. Previous studies suggested that IL-37 which expresses in plenty of organs, such as tonsils, skin and esophagus, was an important anti-inflammatory cytokine involved in the development of many diseases [22-24]. Although peripheral monocytes and infiltrated macrophages seem to be major producer of the IL-37, it is possible that the IL-37 is also yielded by the local cells in organs. Up to now, there is no evidence that the IL-37 is sourced from the nucleus pulposus cells and annulus fibrous cells. Whether the infiltrated macrophages or local nucleus pulposus cells participated in the decreased expression of IL-37 in the current study is anticipated to be elucidated.

This is the first time the downregulated expression of IL-37 in IVDD and negative correlation between expression of IL-37 and stages of disc degeneration were demonstrated. Targeting the anti-inflammatory cytokine IL-37 might reveal an attractive treatment concept in IVDD.

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Disclosure of conflict of interest

None.

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