Insulin-Like Growth Factor 1 Activates PI3k/Akt Signaling to Antagonize Lumbar Disc Degeneration

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Key Words
Lumbar disc degeneration • Insulin-like growth factor 1 (IGF-1) • Akt • FoxO1 • MMP3

Abstract

Background/Aims: The pathogenesis of Lumbar disc degeneration (LDD) has been extensively studied in the past. In particular, a role of matrix metalloproteinase 3 (MMP3) in the disease initiation and progression has been recently reported. However, an involvement of Insulin-like growth factor 1 (IGF-1)-stimulated phosphatidylinositol-3 kinase (PI3k) / Akt signaling pathway-mediated control of MMP3 in LDD has not been acknowledged.

Methods: We examined the serum IGF-1 levels and activation of the receptor for IGF-1 (IGF-1R) in resected discs in patients with LDD, compared to the fractured discs from traumatized, non-LDD subjects as a control. We analyzed the effects of IGF-1 on the activation of IGF-1R, Akt and MMP3 in a human nucleus pulposus SV40 cell line (HNPSV). We transfected HNPSV cells with a constitutive nuclear FoxO1, and analyzed its effect on the activation of IGF-1R, Akt and MMP3. Results: LDD patients had significantly lower levels of serum IGF-1, and LDD discs had significantly lower levels of activated IGF-1R. IGF-1 induced phosphorylation of MMP3, and then phosphorylation of its downstream factor Akt in the HNPSV cells, resulting in significantly inhibition of MMP3. Further, FoxO1 nuclear retention completely abolished the inhibitory effects of IGF-1 on MMP3 in HNPSV cells.

Conclusion: Together, IGF-1/Akt/FoxO1/MMP3 regulatory machinery may control the development of LDD.

Introduction

Lumbar disc degeneration (LDD) is one of the most common causes of low back pain and neck pain, and also one of the most of least understood among all related diseases [1-3]. Conceptually, LDD describes the symptoms of pain and possibly radiating weakness or numbness stemming from a degenerated disc in the spine, and is a product of lifelong degradation with synchronized remodeling of discs and neighboring vertebrae, including...
concomitant adaptation of the disc structures to the alterations in physical loading and responses to the occasional injury [1-3].

Definition of LDD largely depends on the method of evaluation. Radiographic data have been used in epidemiologic studies of patients, particularly before the advent of magnetic resonance imaging [1-3]. In addition to the information gained from microscopic, histologic, or biochemical analysis, surgical and autopsy samples can provide a macroscopic measurement of degeneration [1-4]. However, qualitative methods of evaluating LDD from magnetic resonance imaging are available and preferred for screening of the disease in a large population.

The pathogenesis of LDD has been extensively studied in the past [1-3]. Especially, a role of matrix metalloproteinase 3 (MMP3) in the disease initiation and progression has been recently acknowledged [5-12]. However, the exact molecular regulation of MMP3 in the LDD setting remains largely unknown.

The insulin-like growth factors (IGFs) are highly similar to insulin [13-15]. The IGF signaling pathway initiates with binding to cell-surface receptors (IGF-1R and IGF-2R) by either ligands [Insulin-like growth factor 1 (IGF-I) and Insulin-like growth factor 2 (IGF-2)] [13-15]. Although IGF-1 binds to both IGF-1R (high affinity) and the insulin receptor (low affinity), the IGF-1R has been shown to be the major functional receptor for IGF-I, which is a receptor tyrosine kinase to activate phosphatidylinositol-3 kinase (PI3k) / Akt signaling pathway upon stimulation [13-15]. Phosphorylated Akt is a natural stimulator of cell growth and proliferation, and a potent inhibitor of programmed cell death [13-15], and Akt functions through its downstream targets, the mammalian target of rapamycin (mTOR) and forkhead box protein 01 (FoxO1) [16].

FoxO1 orchestrates many genes that regulate cell proliferation, apoptosis and transition [16], and FoxO1 itself is regulated through phosphorylation, ubiquitination and acetylation [16]. Akt-mediated phosphorylation at three conserved residues on FoxO1 results in exclusion of FoxO1 proteins from the nucleus to the cytoplasm, resulting in inhibition in FoxO1-dependent gene regulation [16]. A role of IGF-1R/Akt signaling pathway on the pathogenesis of LDD has been reported [17], whereas the detailed signal transduction is not completely understood. Especially, a role of FoxO1 in this pathway has not been acknowledged.

Here, we studied the molecular mechanisms underlying IGF-1/Akt/FoxO1/MMP3 regulatory axis on the pathogenesis of LDD.

Materials and Methods

Specimens from patients

A total of 45 subjects (23 with resected LDD discs and 22 traumatized, non-LDD subjects with fractured discs as controls) were included in this study for serum IGF-1 levels, and the activation of IGF-1R in the discs. All the subjects had no accompanying diseases (e.g. diabetes) that may affect serum IGF-1 levels. All specimens had been histologically and clinically diagnosed at Qingpu Branch of Zhongshan Hospital Affiliated to Fudan University from 2008 to 2014. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained (NO: GDR452). Serum IGF-1 was measured by using Coated Tube Immunoradiometric Assays (Diagnostic Systems Laboratories, Webster, TX, USA), according to the instructions of the manufacturer. Phosphorylated IGF-1R (pIGF-1R) levels were measured by Western blot and normalized to total IGF-1R levels.

Human disc cell line, reagents and transfection

A human disc cell line, nucleus pulposus SV40 (HNPSV) has been described before [18]. The HNPSV was a gift from Prof. Sakai, and was cultured in Dulbecco's modified Eagle medium (DMEM, Gibco; Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Sigma-Aldrich, St Louis, MO, USA), penicillin (100μg/ml) and streptomycin (250ng/ml) at 37°C, in a 5% CO₂ atmosphere. Recombinant human
IGF-1 (100 ng/ml) and Akt inhibitor LY294002 (20 µmol/l) were also purchased from Sigma-Aldrich. HNPSV cells were transfected with a plasmid carrying both a constitutively nuclear form for FoxO1 and a GFP reporter that were connected with a 2A sequence, as has been previously described [19-21]. The small 2A peptide sequences, when cloned between genes, allow for efficient, stoichiometric production of discrete protein products within a single vector through a novel "cleavage" event within the 2A peptide sequence [22-25]. The transfected cells were termed as HNPSV-nFoxO1. HNPSV-nFoxO1 expressed high levels of nuclear FoxO1, which cannot be phosphorylated by phosphorylated Akt (pAkt) due to a defect on phosphorylation site.

**Immunocytochemistry**

The cultured cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 6 hours, and then used for immunocytochemistry. Primary antibody used in immunohistochemistry is rabbit anti-FoxO1 (Cell Signaling, San Jose, CA, USA). GFP was detected by direct fluorescence. Secondary antibodies were Cy3-conjugated anti-rabbit antibody (Jackson ImmunoResearch Labs, West Grove, PA, USA).

**Western blot**

Protein was extracted from the cultured cells by RIPA buffer (Sigma-Aldrich) for Western Blot. The supernatants were collected after centrifugation at 12000×g at 4°C for 20 min. Protein concentration was determined using BCA protein assay, and whole lysates were mixed with 4×SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100 °C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are anti-IGF-1R, anti-phosphorylated-IGF-1R (pIGF-1R), anti-Akt, anti-phosphorylated-Akt (pAkt), anti-MMP3 and anti-α-tubulin (all purchased from Cell Signaling). Secondary antibody is HRP-conjugated anti-rabbit antibody (Jackson ImmunoResearch Labs). Images shown in the figure were representative from 5 repeats. Densitometry of Western blots was quantified with NIH ImageJ software. The phosphorylated protein levels were first normalized to total protein, and then normalized to controls. MMP3 were first normalized to α-tubulin, and then normalized to controls.

**Quantitative PCR (RT-qPCR)**

Total RNAs were extracted from the cultured cells with RNeasy kit (Qiagen, Hilden, Germany), and used for cDNA synthesis. Quantitative PCR was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were analyzed with 2-ΔΔCt method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to controls.

**ELISA**

The concentration of secreted MMP3 in the conditioned media was determined by ELISA Kit (R&D System, Los Angeles, CA, USA). ELISAs were performed according to the instructions of the manufacturer. Briefly, the collected condition media was added to a well coated with primary antibody, and then immunosorbent by biotinylated primary antibody at room temperature for 2 hours. The color development catalyzed by horseradish peroxidase was terminated with 2.5 mol/l sulfuric acid and the absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

**Statistical analysis**

All statistical analyses were carried out using the SPSS 18.0 statistical software package. All values are depicted as mean ± standard deviation and are considered significant if p < 0.05. All data were statistically analyzed using one-way ANOVA with a Bonferoni correction, followed by Fisher’s exact test to compare 2 groups.
Results

Lower levels of IGF-1 were detected in the serum of LDD patients

We analyzed serum IGF-1 levels in a total of 45 subjects (23 with resected LDD discs and 22 traumatized, non-LDD subjects (CTL)), and found that the serum IGF-1 levels in LDD patients were significantly lower than CTL (Fig. 1A).

Deactivation of IGF-1R signaling was detected in degenerated discs

We further analyzed the activation of IGF-1R (phosphorylation of IGF-1R compared to total IGF-1R) in the resected discs from these subjects by Western blot. We detected significant deactivation of IGF-1R in LDD discs, compared to CTL (Fig. 1B). These data imply a possible involvement of IGF-1 signaling in the pathogenesis of LDD.

IGF-1 induces Akt phosphorylation to suppress MMP3 in disc cells

Since MMP3 has been recently shown to promote development of LDD, we were prompted to analyze the relationship between IGF-1R signaling and MMP3 levels in the disc cells. It is well-known that IGF-1/IGF-1R signaling activates PI3k/Akt, which further activates downstream factors mTor or FoxO1. Thus, we gave IGF-1 to cultured human disc cells HNPSV.

Fig. 1. IGF-1 and IGF-1R in LDD patients. (A) We analyzed serum IGF-1 levels in a total of 45 subjects (23 with resected LDD discs and 22 traumatized, non-LDD subjects (CTL)), and found that the serum IGF-1 levels in LDD patients were significantly lower than CTL. (B) We analyzed the activation of IGF-1R (phosphorylation of IGF-1R compared to total IGF-1R) in the resected discs from these subjects by Western blot. We detected significant deactivation of IGF-1R in LDD discs, compared to CTL. *p<0.05. Statistics: one-way ANOVA with a Bonferroni correction, followed by Fisher’s exact test to compare 2 groups.

Fig. 2. Effect of IGF-1 on protein of IGF-1R, Akt and MMP3 in disc cells. IGF-1 was given to cultured human disc cells HNPSV, with or without a specific Akt phosphorylation inhibitor LY294002. Western blot was performed on IGF-1R, pIGF-1R, Akt, pAkt, MMP3, shown by representative immunoblots.
We found that IGF-1 significantly increased phosphorylation of IGF-1R, by representative images (Fig. 2), and by quantification (Fig. 3A). Activation of IGF-1R further significantly increased the phosphorylation of Akt, by representative images (Fig. 2), and by quantification (Fig. 3B). Moreover, activation of PI3k/Akt significantly suppressed the expression of MMP3, by Western blot (Fig. 2, 3C), by RT-qPCR (Fig. 3D), and by ELISA on the secreted protein (Fig. 3E). Further, a specific Akt inhibitor LY294002 completely abolished the effects of IGF-1 on the phosphorylation of Akt and the suppression on MMP3 (Fig. 2, 3B-E), without affect IGF-

Fig. 3. IGF-1-induced Akt phosphorylation to suppress MMP3 in disc cells. IGF-1 was given to cultured human disc cells HNPSV, with or without a specific Akt phosphorylation inhibitor LY294002. (A-E) Effects of IGF-1 with or without LY294002 on phosphorylation of IGF-1R (A), the phosphorylation of Akt (B), and the expression of MMP3, by Western blot (C), by RT-qPCR (D), and by ELISA on the secreted protein (E). *p<0.05. N=5. Statistics: one-way ANOVA with a Bonferoni correction, followed by Fisher’s exact test to compare 2 groups.

Fig. 4. Preparation of disc cells expressing sustained nuclear FoxO1. (A) HNPSV cells were transfected with a plasmid carrying both a constitutively nuclear form for FoxO1 and a GFP reporter that were connected with a 2A sequence. (B) The transfected cells were termed as HNPSV-nFoxO1, which expressed high levels of nuclear FoxO1, by representative immunocytochemistry images. Scar bar is 20µm.

We found that IGF-1 significantly increased phosphorylation of IGF-1R, by representative images (Fig. 2), and by quantification (Fig. 3A). Activation of IGF-1R further significantly increased the phosphorylation of Akt, by representative images (Fig. 2), and by quantification (Fig. 3B). Moreover, activation of PI3k/Akt significantly suppressed the expression of MMP3, by Western blot (Fig. 2, 3C), by RT-qPCR (Fig. 3D), and by ELISA on the secreted protein (Fig. 3E). Further, a specific Akt inhibitor LY294002 completely abolished the effects of IGF-1 on the phosphorylation of Akt and the suppression on MMP3 (Fig. 2, 3B-E), without affect IGF-
IGF-1 antagonizes LDD

1R phosphorylation (Fig. 2, 3A). These data suggest that IGF-1 suppressed MMP3 in disc cells through PI3k/Akt signaling.

Next, we aimed to find out whether IGF-1-induced Akt activation may suppress MMP3 through downstream FoxO1 signaling. Activated Akt phosphorylates FoxO1 and induces its nuclear exclusion. To examine whether FoxO1 nuclear exclusion may be required for MMP3 suppression, or FoxO1 nuclear retention may activate MMP3, we transfected HNPSV cells with a sustained nuclear FoxO1 expressing plasmid (Fig. 4A), as has been previously
described [19-21]. The transfected cells were termed as HNPSV-nFoxO1. HNPSV-nFoxO1 cells expressed high levels of nuclear FoxO1 (Fig. 4B), which cannot be phosphorylated by pAkt due to a defect on phosphorylation site. We found that sustained nuclear FoxO1 did not affect phosphorylation of IGF-1R and Akt (Fig. 5A-C), but completely abolished the inhibitory effects of IGF-1 on MMP3, by Western blot (Fig. 5A, D), by RT-qPCR (Fig. 5E), and by ELISA on the secreted protein (Fig. 5F). Together, our data suggest that IGF-1 may suppress MMP3-mediated development of LDD through Akt-induced nuclear exclusion of FoxO1. Decreased IGF-1 levels thus promote the LDD pathogenesis (Fig. 6).

Discussion

LDD hampers the health of many people worldwide [1-3]. Although the molecular basis underlying the development of LDD has been extensively studied [26, 27], a role of matrix metalloproteinase 3 (MMP3) in the disease initiation and progression has only been recently reported [5-11]. Nevertheless, the exact molecular mechanisms by which MMP3 regulates LDD have not been clarified.

Here, we reported that LDD patients had significantly lower levels of serum IGF-1, and LDD discs had significantly lower levels of activated IGF-1R. These clinical data provide a clinical relevance of our current study addressing the role of IGF-1R signaling in LDD development. The IGF-1R signaling has been well-studied in other cell types and tissue, showing a regulatory axis through receptor, PI3k, Akt and its downstream targets including mTor and FoxO1. Here, we found that IGF-1 induced phosphorylation of IGF-1R and then phosphorylation of its downstream factor Akt, which further suppressed MMP3 in the human disc cells. Moreover, FoxO1 nuclear retention in human disc cells completely abolished the effects of IGF-1 on MMP3, suggesting that Akt-induced phosphorylation and nuclear exclusion FoxO1 is responsible for the IGF-1-induced MMP3 suppression. The regulation of MMP3 by IGF-1/Akt/FoxO1 should be at transcription level, since the mRNA levels of MMP3 is adapted to IGF-1 stimulation.

These data highlight a specific molecular regulation of MMP3 during LDD development. Our findings in this model are consistent with previous studies in other system. Thus, our findings highlight IGF-1R/Akt/FoxO1 axis as a novel therapeutic target for inhibiting the development of LDD.

Disclosure Statement

The authors have declared that no competing interests exist.

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