To Investigate the Roles of IL-20, and BMP-2 on Intervertebral Disc Inflammation, Chemotaxis, and Angiogenesis under a Hypoxia Condition

Introduction

After disc herniation, we can observe obvious inflammation and angiogenesis, which is the natural healing process after disc injury. Hsieh M Y, et al. (instructed by Prof Chang) at 2007 found that IL-20 can control angiogenesis in a direct or indirect pathway. In current study (Kudo from Hongo, et al. 2007), disc injury has been modeled, and we reported angiogenesis and inflammation increased with the addition of rHBMMP-2 12 weeks after disc injury. In our recent research (NSC project), we found that the expression of IL-20 and its receptors in disc cells combined with IL-1 might contribute to the pathogenesis of inflammation in herniated disc tissues by promoting immune cell infiltration, angiogenesis, and inflammation.

In 2006, Koo-Yong Ha, et al. reported that the expression of Hyposia Inducible Factor-1 (HIF-1) (βIF) and αIF in herniated discs. In 2002, Haro H, et al. reported that vascular endothelial growth factor (VEGF) expressed in injured disc tissues. Furthermore, we also reported that receptor expression is controlled by the hyposia condition. Intervertebral disc is an avascular structure after adult, and HIF-1 and VEGF are important factors associated with angiogenesis, and both factors and the receptors have been proved to expose in human disc after injury. In this study, we intend to focus on intervertebral disc inflammation, chemotaxis, and angiogenesis under a hyposia condition. Furthermore, we want to investigate the roles of IL-20, and BMP-2 in these environments. We design an in-vitro study under hyposia condition to elucidate the pathogenesis and molecular mechanisms of healing process after disc injury.

The methods for this study are immunohistochemical staining, western blot, real-time PCR, to detect the expression of proteins, and mRNA with regards to the inflammation, chemotaxis, and angiogenesis in different stages of disc injury under a hyposia condition.

Materials and Methods

Antibody

Isotype IgG1 antibody was commercially available (R&D Systems). Monoclonal antibodies against collagen type II were commercially available (Millipore). Monoclonal antibodies against IL-20 was constructed and purified as described previously.

Patients and controls

 Patients who were diagnosed with HIVD and received a discectomy in the Department of Orthopedics at National Cheng Kung University Medical Center, were included in this study. The diagnosis of HIVD was based on the clinical presentation of low back pain and sciatica, with the report of lumbar disc herniation from their nerve conduction velocity and electromyogram. In addition, all patients received an MRI examination, which revealed the disc herniated from the posterior facet joints. Conservative treatment had failed in all patients, so they agreed to receive operative discectomy. The human studies were both approved by the Human Experiment and Ethics Committee of National Cheng Kung University Medical Center, and were performed in accordance with the Guidelines of the Declaration of Helsinki.

Primary culture of human intervertebral disc cell

The human intervertebral disc cells at the levels of L4-5 and LS-1 were isolated from patients with HIVD. The primary culture of intervertebral disc cells from herniated disc was collected as previous described. 31 The disc tissues were digested with 0.05% collagenase (Sigma) in Dulbecco modified Eagle medium and Ham F-12 medium (DMEM/F12) (Gibco, Grand Island, NY) for 4 hours. After enzymatic digestion, the suspension was centrifuged and washed with medium. The isolated cells were cultured in DMEM/F12 medium with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin.

Immunohistochemical staining of human disc cell

The disc cells were fixed in 3.7% paraformaldehyde and then permeabilized using PBS with 0.1% Triton X-100. The disc cells were blocked by incubation in PBS with 0.1% bovine serum albumin/sodium azide/0.1% Tween-20, antibody (IL-20, collagen type II monoclonal antibody, and then with secondary antibody according to the same procedure that for immunohistochemical staining.

Experimental condition hyposia stimulation

Hyposia experiments were performed in both cell types using a standard 250 μl chamber (BioCo. Grade. Vineland, N.J.)

dehydrated by positive infusion of a 5% carbon dioxide/95% nitrogen gas mixture. Cells were starved for 0.5 hours in serum-free medium and then placed in the hypoxia chamber. Equal atmospheric pressure was ensured by monitoring infusion with a standardized pressure gauge. During the experiment, cell cultures were placed in a standard humidified tissue incubator at 37°C and continuous oxygen saturation was monitored and kept below 1%. Total cellular RNA was then isolated after 0, 0.5, 1, 2, 4, 8, and 12 hours.

Detection of gene expression in disc cells cultured conditions hyposia stimulation using reverse transcription-PCR (RT-PCR)

Primary intervertebral disc cells (1 x 106 cells/well) were cultured in serum free DMEM/F12 medium and hypoxia stimulation. After treatment, total RNA was extracted using Total Direct (Life Science, CA) and underwent reverse transcription according to the manufacturer's instructions. The expression of mRNA of IL-1β, IL-6, IL-8, BMP-2, BMP-4, VEGF, MMP-3, MIF, and MCP-1 was analyzed using RT-PCR with gene-specific primers (Table 1). β-actin was also amplified as an internal control. Amplified products were visualized in 1.5% agarose gels containing ethidium bromide, and photographed.

Real-time PCR

Detection of amplified template was accomplished with SYBR Green 1 (Bio-Rad, Hercules, CA, USA) chemistry using an MJ Research DNA Engine Opticon 2 fluorescence detection system (MJ Research Inc., South San Francisco, CA, USA). The specific primers used in real-time PCR were the same as those used in semi-quantitative reverse transcription-PCR. Individual PCR reaction contained 10 μl of cDNA (dilute 1: 50), 1.25 μl of 10 M forward primers, 1.25 μl of 10 M reverse primers, and 1.25 M of SYBR Green 1 in a final volume of 25 μl. Individual PCR products were analyzed using melt-point analysis. Samples were heated from 50°C to 95°C, and the decline in fluorescent signals of each individual sample was assessed. The fluorescence/temperature dependent generation of signals was assumed using the manufacturer's software program. Maxima (m) GAPDH was used as an internal control gene to normalize for Cnt amounts. Real-time PCR data were analyzed using the comparative threshold cycle (Ct) method according to the manufacturer's instructions.

Statistical Analysis

The significance of differences in the means between the two groups was assessed using Student's t-test. Statistical significance was set at p<0.05.

Table 1: Primer pairs used for amplifying human transcripts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>5'-ACCTTCTGGGTTCCTCTGTC-3'</td>
<td>5'-AGGCAGTGGAAGAGGAGGTC-3'</td>
<td>121</td>
</tr>
<tr>
<td>IL-20</td>
<td>5'-ACCTTCTGGGTTCCTCTGTC-3'</td>
<td>5'-AGGCAGTGGAAGAGGAGGTC-3'</td>
<td>137</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-ACCTTCTGGGTTCCTCTGTC-3'</td>
<td>5'-AGGCAGTGGAAGAGGAGGTC-3'</td>
<td>104</td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-ACCTTCTGGGTTCCTCTGTC-3'</td>
<td>5'-AGGCAGTGGAAGAGGAGGTC-3'</td>
<td>106</td>
</tr>
<tr>
<td>MIF</td>
<td>5'-ACCTTCTGGGTTCCTCTGTC-3'</td>
<td>5'-AGGCAGTGGAAGAGGAGGTC-3'</td>
<td>188</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5'-ACCTTCTGGGTTCCTCTGTC-3'</td>
<td>5'-AGGCAGTGGAAGAGGAGGTC-3'</td>
<td>106</td>
</tr>
<tr>
<td>VEGF</td>
<td>5'-ACCTTCTGGGTTCCTCTGTC-3'</td>
<td>5'-AGGCAGTGGAAGAGGAGGTC-3'</td>
<td>112</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-ACCTTCTGGGTTCCTCTGTC-3'</td>
<td>5'-AGGCAGTGGAAGAGGAGGTC-3'</td>
<td>121</td>
</tr>
</tbody>
</table>

*The primer sets were used as internal reference

Discussion

Intervertebral disc is a unique structure in that it is avascular with its nutrition and oxygen supply being dependent on the diffusion of solutes through the vertebral endplate and to and from the vertebral body. The oxygen levels in intervertebral disc, therefore, are assumed to be low. Oxygen is an important modulator of gene expression; however, little is known about how intervertebral disc regulates genes in response to O2 tension and whether this regulation occurs through IL-20 or IL-1β.

Results

Figure 1. Immunoblot detection of pro-inflammatory cytokines in human disc (a) IL-1β, (b) IL-6, (c) IL-8, (d) BMP-2

Figure 2. Effect of hypoxia stimulation on gene expression in cells. Primary cultured disc cells (1 x 10^6) (IVD) were exposed to 0, 2, 4, 6 hours. Real-time PCR analysis was performed using comparative threshold cycle (Ct) method according to the manufacturer's instructions.

Conclusions

To sum up the results, we suggested hypoxia and IL-20 may be the important mechanisms for the inflammation and chemotaxis after disc herniation.