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Th17 lymphocyte levels are higher in patients with ruptured than non-ruptured lumbar discs, and are correlated with pain intensity

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ABSTRACT

Background: Th17 lymphocytes have important roles in inflammation and autoimmune disease. Research on relationship between Th17 lymphocytes and pain associated with lumbar disc herniation (LDH) is limited. The purpose of this study was to examine the association of pain and Th17 lymphocyte and interleukin (IL)-17 levels in patients with herniated and non-herniated lumbar discs.

Methods: Thirty-four patients with single lumbar intervertebral disc herniation (median age, 44 years), and 17 healthy adults (median age, 37 years) were enrolled. Patients were divided into 2 groups depending on their magnetic resonance imaging (MRI) results and visual observations during surgery (group P, non-ruptured disc, $n = 15$; group E, ruptured disc, $n = 19$). Patients received posterior or transforaminal lumbar interbody fusion. Preoperative pain intensity was recorded using a visual analogue scale (VAS) score. The percentage of Th17 lymphocytes and IL-17 and prostaglandin E2 (PGE2) levels in peripheral blood were determined. Disc tissue was examined by immunohistochemistry for Th17 and IL-17 expression.

Results: Preoperative VAS pain scores were significantly higher in group E than group P (8.32 ± 1.04 vs. 6.33 ± 2.68, respectively, $p = 0.009$). Similarly, PGE2 level was greater in group E than group P (3.75 ± 1.41 pg/ml vs. 2.63 ± 0.89 pg/ml, respectively, $p = 0.011$). Compared to healthy controls ($1.05 \pm 0.19\%$), the percentage of Th17 cells was significantly greater in group P ($1.52 \pm 0.62\%$, $p = 0.031$), and the percentage in group E ($2.99 \pm 1.09\%$, $p < 0.001$) was significantly greater than in group P. The IL-17 expressions were similar. VAS pain score was positively correlated with Th17 proportion ($r = 0.489$, $p = 0.003$), and IL-17 concentration ($r = 0.458$, $p = 0.007$). PGE2 was also positively correlated with Th17 proportion ($r = 0.539$, $p = 0.001$), and IL-17 concentration ($r = 0.500$, $p = 0.003$). The expression of IL-17 was higher in the cells of group E and group P compared with normal tissue ($p < 0.001$).

Conclusions: Immune system activation is responsible, at least in part, for the pain experienced by patients with LDH, and increased levels of Th17 lymphocytes and IL-17 contribute to the pain.

Introduction

Lumbar disc herniation (LDH) is a common disorder among adults, with a reported lifetime occurrence as high as 40%.[1] Patients with LDH experience low back pain, and this is one of the most costly problems in Workers' Compensation systems.[2] The overwhelming majority of back pain is associated with degeneration, especially herniation and disruption of the lumbar intervertebral disc (IVD).[3,4] Study has shown that numbness, not pain, occurs when the spinal nerves are compressed, and inflammation is the main cause of low back pain.[5]

Two new subpopulations of T cells have recently been described, T-helper (Th)17 and regulatory T (Treg) cells. Th17 cells, which produce high levels of interleukin (IL)-17, have been implicated in exacerbating the immune response to infections.[6] Th17 cells have been characterized as producers of interleukin (IL)-17A (also known as IL-17), IL-17F, IL-21, IL-22, and IL-26 in humans.[7,8] In the past few years, Th17 cells and their effector cytokines have been increasingly recognized as key players in inflammation, autoimmunity, and allergic reactions.[9–11]

In recent decades, many imaging and surgical difficulties associated with IVD disease have been addressed,[12–14] and novel diagnostic and therapeutic strategies have been developed.[15,16]

Abbreviations: LDH, lumbar disc herniation; IL, interleukin; IVD, intervertebral disc; Th, T helper lymphocytes; PBMCs, peripheral blood mononuclear cells; PGE2, prostaglandin E2; VAS, visual analogue scale.

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However, the mechanisms which underlie the pain due to LDH remain incompletely understood. Thus, the purpose of this study was to examine the association of pain, Th17 lymphocytes, and IL-17 in patients with herniated and non-herniated lumbar discs.

Materials and methods

Patients

Thirty-four patients with single disc lumbar IVD herniation (median age, 44 years; range 30–72 years), and 17 healthy adults (median age, 37 years; range, 27–59 years) were enrolled in this study. The inclusion criteria for patients were (1) typical osphyalgia or sciatica symptoms, (2) positive Lasegue's sign or Bragard test, (3) single disc lumbar IVD herniation, (4) reduction in the thickness of the degenerated disc on spinal X-ray, reduction or disappearance of the physiologic spinal curvature, reduced signal of the involved disc on magnetic resonance imaging (MRI) (T2-weighted), and no signs of calcification on computed tomography (CT). Exclusion criteria for patients and healthy controls were (1) history of osteoarthritis, spondylolysis, spondylolisthesis, acute or chronic inflammatory disease, hypertension or diabetes mellitus, (2) have taken or are currently taking immunosuppressive drugs, and (3) previous surgery for IVD disease.

Normal IVD control tissue was also obtained from 5 patients during surgical correction of scoliosis. Informed consent was obtained from all participants. Ethical approval for the study was obtained from the Medical Ethical Committee of Qilu Hospital, Shandong University.

All patient received MRI studies for diagnosis of IVD disease. The determination of the disc degeneration grade was according to Pfirrmann grading system based on MR signal intensity, disc structure, distinction between nucleus and anulus, and disc height. Patients were divided into 2 groups depending on their MRI results and visual observations during surgery. Patients in whom the annulus fibrosus was intact and nucleus pulposus was completely sealed by the annulus fibrosus were categorized as group P. Patients in whom the annulus fibrosus was ruptured and nucleus pulposus was not completely sealed by the annulus fibrosus, and thus the nucleus pulposus was exposed to circulation were categorized as group E. For a diagnosis of rupture, both MRI evidence of rupture and visual observation of rupture during surgery had to be present.

All patients with IVD disease received conservative therapy for a minimum of 3 months before surgery. Patients were treated with non-steroidal anti-inflammatory drugs (NSAIDs) and/or hormones prior to surgery; however, all medications were discontinued 1 week before surgery. Patients received either posterior lumbar interbody fusion (PLIF) or transforaminal lumbar interbody fusion (TLIF). During surgery, the condition of the intervertebral disc herniation and the intactness of the fibres was examined by exposing the lesion. After removal of the herniated intervertebral disc, it was immediately rinsed with PBS and placed in a cryopreservation tube under sterile conditions. The sample was preserved at −80 °C until further analysis.

Before surgery, 4 ml of fasting blood was drawn with a heparin sodium anticoagulant tube in the early morning, and 4 ml of fasting blood was drawn from health volunteers as a control. Preoperative pain intensity was recorded using a visual analogue scale score (VAS) that was obtained 1 day before surgery (0 = no pain, 10 = worst imaginable pain). The characteristics of study subjects are presented in Table 1.

Table 1
Patient characteristics.

	Group P	Group E	Scoliosis	Healthy controls
Number	15	19	5	17
Male/female	10/5	12/7	2/3	11/6
Age (years)	45 (31–72)	43 (30–64)	31 (19–49)	37 (27–59)

Flow cytometry analysis of Th17

We analysed percentage of Th17 in peripheral blood mononuclear cells (PBMCs) of patients with flow cytometry. Flow cytometry was used to evaluate intracellular IL-17 production, which identifies the Th17 cytokine-producing cells, in both groups of IVD patients and healthy control patients. In brief, heparinized peripheral whole blood (100 μl) with an equal volume of Roswell Park Memorial Institute 1640 medium was incubated for 5 h at 37 °C, 5% CO_2 in the presence of 25 ng/ml of phorbol myristate acetate (PMA) (ENZO, BML-PE160-0001), 1 μg/ml of ionomycin (ENZO, ALX-450-007-M001), and 1.7 μg/ml monensin (ENZO, 380-026-M100). PMA and ionomycin are pharmacological T-cell-activating agents that mimic signals generated by the T-cell receptor (TCR) complex, and have the advantage of stimulating T cells of any antigen specificity.[17] Monensin was used to block intracellular transport mechanisms, thereby leading to an accumulation of cytokines in the cells. The aforementioned make IL-17 producing cells more observable.[11]

After incubation, the cells were stained with PE-Cy5-conjugated anti-human CD3 (eBioscience, 15-0038-42) and FITC-conjugated anti-human CD8 monoclonal antibodies (eBioscience, 11-0088-42) at room temperature in the dark for 15 min to delimitate CD4+ T cells, because CD4 is downmodulated when cells are activated by PMA. Next, 100 μl of Reagent A was added to all samples to fixate the cells for 15 min. After washing the cells with 3 ml PBS, 100 μl of Reagent B (permeabilization buffer) and the manufacturer recommended dose of PE-conjugated anti-IL-17 monoclonal antibody were added to each sample for 15 min to permeabilize and stain IL-17 antigen. Isotype controls were used to enable correct compensation, and confirm antibody specificity. Stained cells were analysed by flow cytometry using a FACScan cytometer equipped with CellQuest software (BD Bioscience Pharmingen). CD3+ T subsets were gated by flow cytometry, and the proportions of Th17 to CD3+CD8− T (CD4+) subsets were determined.

Serum concentrations of IL-17 and prostaglandin E2 (PGE2)

The serum concentrations of IL-17 and PGE2 of IVD disease patients were measured by ELISA, following the manufacturer's instructions (IL-17: eBioscience, BMS2017; PGE2: Cayman, 514531). All samples were measured in duplicate.

Immunohistochemical staining

IVD tissue specimens from LDH patients and scoliosis patients were flash-frozen in liquid nitrogen following retrieval during surgery, and were subsequently cryosectioned into 4–8 μm sections. The sections were rewarmed at room temperature for 30 min followed by fixation with acetone at 4 °C for 10 min The sections were incubated with H_2O_2 for 10 min to eliminate the activity of endogenous peroxidase, followed by incubation for 2.5 h with human IL-17 affinity purified polyclonal antibody (R&D, AF-317-NA) diluted 1:100 in blocking buffer. The samples were washed with PBS, and incubated for 30 min with secondary antibodies in blocking buffer. The sections were washed with phosphate buffered saline (PBS) after fixation, and after each incubation. Tissue sections were counterstained with haematoxylin at room temperature to stain the cell nuclei. Samples were imaged using an IX71 inverted phase-contrast microscope (Olympus, Japan) with 20× and 40× objective lenses.