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Production of tumor necrosis factor-α from porcine nucleus pulposus cells at various time points in cell culture under conditions of nutritional deficiency

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Abstract

Nucleus pulposus (NP) in the epidural space induces spinal nerve damage not only by mechanical but also chemical mechanism. NP has been shown to be capable of producing tumor necrosis factor-α (TNF). TNF may play key roles in the NP-induced chemical damage. One of the main pathways to reach the avascular NP is diffusion from the blood supply of the vertebral body through the cartilage endplate. On disk herniation, when NP moves to the epidural space, the distance from the endplate to the herniated NP are longer in the herniated disk than in the intact disk. That is, it seems more difficult to receive adequate nutritional supply from the endplate in the sequestrated type. However, there have been only a few reports of the appearance of TNF in NP. The present study was performed to investigate TNF production in porcine NP under conditions of nutritional deficiency. NP cells were cultured and processed for immunohistochemistry using antisera to TNF, and for ELISA to measure TNF production. The latter was compared longitudinally. The immunoreactivity increased over time. On the other hand, the results of ELISA showed a peak in TNF production 12 h, and lower amounts 1 day and 2 days after application of PBS. These observations may suggest that a nutritional deficit is a possible turn-on switch for TNF up-regulation in the NP.

Keywords: Intervertebral disk; Nucleus pulposus; Tumor necrosis factor-α

1. Introduction

Disk herniation around the lower lumbar spinal nerve roots in the epidural space often triggers sciatica. There have been many recent reports regarding the basic pathophysiology of sciatica [1–11]. These studies indicated that local application of autologous nucleus pulposus (NP) may induce axon and myelin sheath damage [2,4,7,8]. Olmarker et al. demonstrated that there was increased focal pain, seen as increased lifting of the hind paw on the operated side and increased rotation of the head toward the operated side, in rats with a combination of disk incision and displacement of the dorsal root ganglion (DRG) although disk incision and displacement per se did not induce any such behavior [9]. Therefore, it seems likely that NP in the epidural space induces spinal nerve root damage not only by mechanical but also by chemical mechanism [1,2,4,6,8]. Certain cytokines have been suggested to play key roles in the chemical damage [6,17].

Tumor necrosis factor-α (TNF) is a such cytokine that produces a wide variety of biological effects. One major action of TNF is the ability to kill certain tumor cells. TNF is also referred to as a proinflammatory cytokine, similar to interleukin-1 (IL-1), IL-6, and IL-12, as they

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have roles in initiating the cascade of other cytokines and growth factors in the immune response. Local release of TNF along the nerve trunk may be a key factor in the pathogenesis of neuropathic pain states following peripheral nerve damage [12,13]. In the uninjured rat sciatic nerve, mast cells and macrophages are scattered throughout the tissue, especially abutting the vasculature [14]. Their number increases markedly following nerve injury or inflammation of the surrounding tissue, and these inflammatory cells are sources of cytokines, including TNF. The chondrocyte-like cells in NP smears and cultured NP cells showed immunoreactivity for TNF [6,15]. Attempts have been made to compare TNF secretion in normal disk tissue with that to which lipopolysaccharide has been added to induce inflammation [16]. According to this report, no TNF secretion was detected after lipopolysaccharide stimulation. Igarashi et al. measured the amount of TNF in NP, which was homogenized in Tricine sample buffer, to compare neuropathology induced by the application of exogenous TNF to nucleus pulposus and reported that the amount determined by Western blotting was approximately 0.48 ng per lumbar disk in rats [17].

Herniated NP can be classified as protruded, extruded, or sequestrated, and the classification may be done based on surgical, magnetic resonance imaging (MRI), and computed tomography findings [18,19]. NP is located normally in the intervertebral disk. NP relies on pathways from the blood vessels of the vertebral body for the supply of nutrients and removal of metabolic waste [20,21]. One of the main pathways of nutrition, including glucose and proline, to reach the avascular NP is diffusion from the blood supply of the vertebral body through the cartilage endplate [20,22]. At disk herniation, when nucleus pulposus moves to the epidural space, the distance from the endplate to the herniated nucleus pulposus is longer in the herniated disk than in the intact disk. *It therefore seems more difficult to receive adequate nutritional supply, including glucose and proline, from the endplate in the sequestrated type*. However, there have been no studies comparing the appearance of TNF in NP under various nutritional conditions. It may be important to study the production of cytokines, such as TNF, in NP under nutritionally deficient conditions as cytokines are important factors involved in the chemical damage of spinal nerve tissue. In the present study, we examined longitudinally the production of TNF in NP under no nutritional conditions using porcine cultured disk cells.

2. Materials and methods

All animal procedures were carried out in accordance with the "Principles of Laboratory Animal Care" (NIH publication No. 86–28, 1985 rev.) and were approved by our local animal ethics committee. Five pigs (Yorkshire, Hampshire and Swedish Landrace, three-months old, body weight 25 kg) received an intramuscular injection of 20 mg/kg body weight of ketamine at a dose of 50 mg/mL (Ketalar; Parke-Davis, Morris Plains, NJ), an intravenous (IV) injection of 4 mg/kg body weight of metomidate chloride at a dose of 50 mg/mL (Hypnodil; AB Leo, Helsingborg, Sweden), and 0.1 mg/kg body weight of azaperon at a dose of 2 mg/mL (Stresnil; Janssen Pharmaceutica, Beerse, Belgium). NP from the lumbar disks was obtained, and the animals were then sacrificed.

2.1. Experiment 1: TNF immunoreactivity in cultured NP cells

NP was washed once in Ham's F12 medium (Gibco BRL, Paisley, UK), then centrifuged and suspended in 5 mL of collagenase solution in Ham's F12 medium (0.8 mg/mL, Sigma Chemical Co., St. Louis, MO) for 40 min at 37 °C in 75 cm^2 tissue culture flasks. The separated NP cell pellets, *which consist of notochordal cells*, were suspended in DMEM/F12 1:1 medium (Gibco BRL) supplemented with 1% L-glutamine 200 mmol/L (Gibco BRL), 50 μg/mL gentamicin sulfate (Gibco BRL), and 10% fetal calf serum (Gibco BRL). *The cells were seeded as primary culture in three 75 cm^2 culture flasks at density of 1×10^4 cells/cm^2, and cultured at 37 °C in an atmosphere of 5% CO_2 in air for 3 weeks*. The cells were removed and cultured directly on tissue culture treated glass slides (Becton Dickinson & Company Labware, Franklin Lakes, NJ). After 5 days on the glass slides, the cells were treated with 0.01 M phosphate buffer saline (PBS) instead of the medium at 37 °C in an atmosphere of 5% CO_2 in air for 6 h (Group 6H), 1 day (Group 1D), or 2 days (Group 2D). The cultured cells were also examined without changing to PBS as controls (Group 0H). The glass slides were fixed with acetone for 15 min when the cell numbers were around 30 in square fields of 520 × 680 μm. Endogenous tissue peroxidase activity was quenched by soaking the materials for 30 min in 0.3% hydrogen peroxide solution (Sigma) in PBS. The materials were treated for 60 min in blocking solution, consisting of 0.01 M PBS containing 0.3% Triton X-100 and 1% bovine serum albumin (BSA, Intergen, New York, NY) at room temperature. Slides were processed for immunohistochemistry by the avidin-biotinylated peroxidase complex technique using mouse antisera to porcine tumor necrosis factor-α monoclonal purified antibody (TNF, 1:100, clone 4F4, Endogen, Cambridge, MA) diluted with blocking solution, biotinylated rabbit anti-mouse IgG (1:300, Dako Cytomation Denmark A/S, Glostrup, Denmark), avidin biotin reagent (Dako), and 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma). After each step, the materials were rinsed three times in 0.01 M PBS. Details of this method have been published elsewhere [6]. The TNF products were observed by light microscopy. As a negative control, 1% BSA (Intergen) suspended in PBS was applied instead of the primary antibody in the same manner. To determine the degree of TNF immunoreactivity, 10 images of five slides were chosen at random, photographed using a digital camera (DXC-950P; Sony, Tokyo, Japan), and assessed with a computer-assisted imaging