# Vascular endothelial growth factor in degenerating intervertebral discs of rat caudal vertebrae

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## Abstract:

**Introduction:** Discogenic back pain remains poorly understood with respect to etiopathogenesis, despite being a considerable burden. We sought to examine the expression of vascular endothelial growth factor in injured intervertebral discs in rat caudal vertebrae.

**Methods:** Forty-eight male Sprague Dawley rats were assigned to 2 groups according to disc puncture injury: puncture (n = 32) or non-puncture (n = 16). Disc puncture was performed percutaneously such that the incision would be in the primary plane of motion for the coccygeal discs 5-6, 6-7, and 7-8. A 26-gauge needle was used to puncture each disc 10 times. Punctured discs were examined histologically by hematoxylin and eosin staining at 1, 7, 14, and 28 days post-injury.

**Results:** Vascular endothelial growth factor was localized immunohistochemically, and determined quantitatively using an enzyme-linked immunosorbent assay. Peak inflammation occurred on the 7th day post-injury, but tissue degeneration continued until day 28. Local expression of vascular endothelial growth factor tended to be highest in the annulus fibrosus on the 7th and 14th days after puncture injury. The level of vascular endothelial growth factor was highest 1-day post-injury, and then gradually decreased thereafter. Furthermore, vascular endothelial growth factor levels in the puncture group were significantly higher than those in the non-puncture control group (p < 0.05).

**Conclusions:** We found increased expression of the inflammatory cytokine vascular endothelial growth factor in injured intervertebral discs, suggesting that vascular endothelial growth factor may be clinically important in discogenic back pain. **Keywords:** 

inflammation, cytokine, intervertebral disc, rat, vascular endothelial growth factor

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# Introduction

According to large-scale epidemiological studies in the United States, the lifetime prevalence of lower back pain is approximately 80%, with an estimated medical cost of 100 billion U.S. dollars annually<sup>1-3)</sup>. According to the Compre-

hensive Survey of Living Conditions 2013 by the Ministry of Health, Labor and Welfare in Japan, lower back pain was found in 92.2 men per 1,000 men and 118.2 women per 1,000 women, and was the most common symptom among complaints, first among males and secondly, among females in Japan. Nonspecific lower back pain without obvious or-

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ganic cause on physical examination or imaging has a frequency of 85%-90%, and can be difficult to treat<sup>4,5)</sup>. Various studies have been conducted to determine the cause of nonspecific lower back pain. Structures considered as origins of nonspecific pain include muscles/fascia, intervertebral joints, the spinal cord and cauda equina, and intervertebral discs. Intervertebral disc-related pain accounts for approximately 45% of patients with lower back pain; therefore, discogenic lower back pain has gained particular attention<sup>67</sup>.

Burke et al. reported that inflammatory cytokines, such as tumor necrosis factor  $\alpha$ , interleukin (IL)-6, nerve growth factor, and inflammatory intermediate substance, are increased in the intervertebral discs of patients with chronic discogenic lower back pain<sup>8</sup>. Shinohara et al. noted abnormal intradiscal elongation of free nerve endings in degenerative intervertebral disc disease9. Therefore, microinflammation and abnormal intradiscal neural elongation may constitute the principal causes of discogenic lower back pain, especially in the context of mutual interaction. Vascular endothelial growth factor (VEGF) is a factor that influences microinflammation and abnormal neural elongation. VEGF not only has a major role in vascularization, but is also involved in vascular permeability and survival. With respect to inflammatory disorders, VEGF binds VEGF receptor 1 in a pathway involving the transition and activation of macrophages<sup>10</sup>, and may play a role in discogenic lower back pain through this mechanism. However, information regarding the quantification of VEGF expression in discogenic back pain is scant, and the location of VEGF expression in degenerating intervertebral discs remains unknown. Therefore, we sought to study temporal changes in VEGF expression in a rat model of lower back pain based on intervertebral disc injury in the caudal vertebrae.

# **Materials and Methods**

All protocols for animal procedures were approved by the ethics committees of Chiba University following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (1996 revision).

#### Surgery

Forty-eight male Sprague Dawley rats weighing 250-300 g were assigned to 2 groups according to disc injury: puncture (n = 32) or non-puncture (n = 16). The animals were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally), and were treated using an aseptic technique throughout the experiment. Disc puncture was performed percutaneously such that the incision would be in the primary plane of motion of the coccygeal (Co) discs Co 5-6, Co 6-7, and Co 7-8. Discs were punctured 10 times with a 26-gauge needle to a depth of 2.0 mm to depressurize the nucleus. The puncture procedure was performed according to a previously reported method<sup>11)</sup>. Punctured discs were examined at 1, 7, 14, and 28 days post-injury.

#### Histology

Discs and adjacent vertebrae were harvested together, fixed in 4% paraformaldehyde, dehydrated with ethanol, decalcified in ethylenediaminetetraacetic acid, and embedded in paraffin wax. Specimens were sliced sagittally at 5  $\mu$ m thickness and stained with hematoxylin and eosin (HE). HEstained specimens in the puncture group included those at 1, 7, 14, and 28 days post-injury (n = 4, each time point). Eight uninjured discs (the non-puncture group) were used as controls. Disc architecture was examined at 40 x magnification. The cellular aspects of the nucleus, annulus, and extraannulus were examined at ×200 magnification. Administration of an overdose of pentobarbiturate (100-120 mg/kg) was used for euthanasia.

#### Enzyme-linked immunosorbent assay

Discs were harvested, frozen in liquid nitrogen, pulverized or homogenized, and digested in a liquid buffer. VEGF expression was determined using an enzyme-linked immunosorbent assay (ELISA) according to the protocol stipulated by the manufacturer (R&D Systems). Sample-included discs harvested at 1, 7, 14, and 28 days postoperatively (n = 4, each time point). Eight uninjured discs were used as controls (n = 2, each time point).

#### *Immunohistochemistry*

Sections were prepared as previously described for histology. Immunohistochemical staining was performed using a Histofine Simple Stain Rat Max-Po kit (Nichirei) according to the protocol stipulated by the manufacturer. The primary antibody used was mouse monoclonal anti-VEGF antibody (1:100, overnight at 4°C; Santa Cruz Biotechnology). Following visualization of the labeled polymer, prepared by combining amino acid polymers with peroxidase and secondary antibody Fab fragment, using chromogenic diaminobenzidine, sections were counterstained with hematoxylin. VEGF expression was examined.

# Statistical analyses

The Kruskal-Wallis test was used to assess temporal changes in VEGF expression. VEGF expression in the puncture and non-puncture groups was compared at corresponding time points using the Mann-Whitney U-test. Data are represented as the arithmetic mean with standard error. Differences were considered significant at p < 0.05.

## Results

# Histology

Fig. 1 and 2 show HE staining for each group and the changes in VEGF expression over time, respectively. Fig. 1 shows the architecture of intervertebral discs. Fig. 2 shows the cellular components of the nucleus, annulus, and extraannulus. Compared with the non-puncture group, VEGF in



# Annulus fibrosus

Figure 1. Temporal changes in histology of injured caudal vertebrae discs. 214

(A) Non-puncture group. Days after puncture: (B) 1 day, (C) 7 days, (D) 14 days, and (E) 28 days. Hematoxylin and eosin staining ( $\times$ 40). On the 14th day, edema and degeneration produced tortuosity in the annulus fibrosus, and the cavity of the nucleus pulposus was closed.







**Figure 3.** Enzyme-linked immunosorbent assay of vascular endothelial growth factor (VEGF) in caudal vertebrae discs. The level of VEGF peaked 1 day post-injury, and then gradually decreased thereafter; however, VEGF remained significantly greater in the puncture group.

degenerating intervertebral discs there was an inflammatory cell infiltrate noted at the distal end of the annulus fibrosus in the puncture group. There was a decrease and slight degeneration of nuclei in the puncture group on the first day after puncture. On the 7th day after injury, an increase in inflammatory cells and edema was noted, the annulus fibrosus was particularly prominent, and the cavity of the nucleus pulposus was almost completely filled with inflammatory infiltrate. There was obvious fibroblastic proliferation. On the 14th day after injury, edema and degeneration of the annulus fibrosus had progressed considerably, and the cavity of the nucleus pulposus was closed. However, inflammatory cell infiltration of the annulus fibrosus and nucleus pulposus abated, as did proliferation of fibroblasts. On the 28th day after injury, we observed a loss of the nucleus pulposus cavity; the tortuosity of the annulus fibrosus and the number of inflammatory cells were similar to those seen on the 14th day after injury (Fig. 1, 2).

## **VEGF ELISA**

The results of quantitative determination of VEGF using ELISA are shown in Fig. 3. In the puncture group, the VEGF level was greatest 1 day after puncture, after which a gradual decline was noted; however, at all time points, the non-puncture group exhibited lower VEGF levels. The VEGF levels remained significantly higher than those observed in the non-puncture group at 28 days (Fig. 3).

# Immunohistochemistry

VEGF immunoreactivity for each group and the changes in VEGF expression over time are shown in Fig. 4. VEGF expression was recognized in the annulus fibrosus and nucleus pulposus of each group with the respective time course. Compared with the non-puncture group, the puncture group tended to have a higher expression of VEGF in the region between the endplate and annulus fibrosus on the 7th day after puncture and thereafter. Cells strongly immunoreactive for VEGF were located in the transient lesion of the annulus and the endplate.

However, there was no obvious difference in the overall VEGF expression in the nucleus pulposus (Fig. 4).

# Discussion

Histology suggested that the inflammatory peak occurred on the 7th day post-injury, but that tissue degeneration continued until the 14th day. Therefore, we suggest that tissue degeneration continued even after the inflammatory peak in this model of lower back pain using intervertebral disc injury. Generally, acute inflammatory processes in injured tissue, such as vasodilatation, hemorrhage, and neutrophilic infiltration, begin as a vascular response on days 1-3 postinjury.

However, macrophage migration, fibroblast cell growth, and the formation of new collagenous material occur during the initial granulation tissue formation on days 3-7, as the tissue begins the repair process. Scarring develops as a product of decreasing fibroblast cell numbers and increased collagen deposition from day 10 onwards<sup>12,13)</sup>. In a rat model of lower back pain using puncture of caudal vertebrae, a decrease in tissue of the nucleus pulposus and an increase in collagenous material have previously been observed after 14 days, with the nucleus pulposus replaced by fibrous tissue after 28 days<sup>14)</sup>. In the current study, temporal changes in the acute inflammatory response and tissue repair process were similar to those previously reported. Local expression of VEGF, as observed immunohistochemically, tended to localize to the annulus fibrosus on the 7th and 14th days after puncture when the puncture and non-puncture groups were compared. ELISA showed that VEGF expression was maximally increased on the 1st day after injury, gradually decreasing thereafter; however, VEGF expression remained significantly greater in the puncture group at 14 and 28 days post-injury. Similarly, IL-6 expression has been shown to increase in the intervertebral discs of rats on the 4th day after injury, with gradual decreases thereafter<sup>15)</sup>. IL-6 can elevate VEGF expression<sup>16</sup>; thus, IL-6 may modulate VEGF levels on the 1st and 7th days after injury. Macrophages, which play an important role in the tissue repair process, increase the expression of VEGF, potentially via interaction with the cells of the annulus fibrosus<sup>17,18</sup>). In the present study, a peak in VEGF expression was noted on the 7th day after injury, and prominent infiltration of the annulus fibrosus by macrophages was recognized with HE staining. Macrophage infiltration may be responsible for a persistent increase in the expression of VEGF. A limitation of our study is the validity of our model of lower back pain using degenerated intervertebral discs, which may be inadequate. We used puncture of intervertebral discs in the caudal vertebrae as a model for degenerative intervertebral disc injury. An acute local re-



**Figure 4.** Immunohistochemistry of VEGF in caudal vertebrae discs. (A-E) Annulus fibrosus, and (a-e) nucleus pulposus. Diaminobenzidine chromogen staining (×200). Compared with the non-puncture group, the puncture group tended to have a higher expression of VEGF in the region between the endplate and annulus fibrosus on the 7th day after puncture and thereafter.

sponse, possibly similar to the response in humans, can be reproduced in the intervertebral disc with puncture injury. However, the model does not accurately reflect a continuous load, such as that associated with standing in humans; thus, the model may be insufficient to evaluate chronic lower back pain or chronic intervertebral disc degeneration. To our knowledge, a model appropriate for chronic lower back pain or chronic intervertebral disc degeneration has not yet been established. The present study is also limited by a lack of precise quantification of localized VEGF expression. Instead, we used immunohistochemistry to localize expression of VEGF qualitatively. To quantitate localized expression of VEGF, ELISA of isolated tissue from the annulus fibrosus and nucleus pulposus should be conducted. In immunostaining, VEGF was not accurately quantitatively evaluated, and we only confirmed where VEGF was expressed. Therefore, it cannot be said that the expression of VEGF was high; it can only be said that it had a high tendency. VEGF expression may increase for tissue repair in the acute phase and may promote abnormal nerve elongation into the intervertebral disc by prolonging during the recovery period. However, this study did not evaluate nerve elongation. Evaluation of immunostaining and nerve elongation is future task.

In future research, ELISA may be able to clarify the relationship between inflammatory cytokines and VEGF by measuring not only VEGF but also IL-6 and TNF- $\alpha$  in the same study.

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In this study, we did not examine what kind of macrophages increase, and we have not examined whether VEGF binds to VEGFR-1 expressed in macrophages. However, expression of VEGF mediated by macrophages has been reported from several literatures, and it is necessary to confirm this point in the future as well.

The present findings suggest that local tissue degeneration continues even after a peak inflammatory response to intervertebral disc injury. In particular, there were increases in VEGF expression during the acute injury and subsequent tissue repair phases. This suggests that VEGF may play a role in the pathogenesis of discogenic lower back pain.

Conflicts of Interest: None

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