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Effects of proinflammatory cytokines on axonal outgrowth from adult rat lumbar dorsal root ganglia using a novel three-dimensional culture system

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Abstract

BACKGROUND CONTEXT: Degeneration of the intervertebral disc is often associated with low back pain and increased infiltration of nerve fibers originating from dorsal root ganglia (DRG). The degenerated disc is also characterized by the presence of proinflammatory cytokines, which may influence axonal outgrowth. Toward an improved understanding of the growth of DRG neurons into compliant extracellular matrices, we developed a novel experimental system to measure axonal outgrowth of adult rat lumbar DRG neurons within three-dimensional (3D) collagen hydrogels and used this system to examine the effects of interleukin 1β (IL-1β) and tumor necrosis factor (TNF)-α treatment.

PURPOSE: The aim was to investigate the effects of proinflammatory cytokines on 3D neuronal growth into collagen matrices.

STUDY DESIGN: This was an in vitro study of neurite outgrowth from adult rat lumbar DRG into collagen gels in response to IL-1β and TNF-α.

METHODS: Lumbar DRG were obtained from adult Sprague Dawley rats, bisected to expose cell bodies and placed onto collagen gel constructs prepared in 24-well Transwell inserts. Dorsal root ganglia were then treated with nerve growth factor (NGF)–free Neurobasal media (negative control) or NGF-supplemented media containing 0, 1, and 10 ng/mL of IL-1β and TNF-α. After 7 days, collagen gel–DRG constructs were immunostained for phosphorylated neurofilament, an axonal marker. Simple Neurite Tracer (Fiji/ImageJ) was used to quantify 3D axonal outgrowth from confocal image stacks. Data were analyzed using one-way analysis of variance, with Tukey HSD post hoc correction at a level of $p<.05$.

RESULTS: Immunostaining showed robust axonal outgrowth into collagen gels from all NGF-treated DRG. The negative control demonstrated very few and short neurites. Tumor necrosis factor-α (1 and 10 ng/mL) significantly inhibited axonal outgrowth compared with NGF-only media ($p<.026$ and $p<.02$, respectively). After IL-1β treatment, average axon length was 10% lower at 1 ng/mL and 7.5% higher at 10 ng/mL, but these differences were not statistically significant. Among cytokine treatments, however, average axon length in the IL-1β (10 ng/mL) group was significantly higher than that in the other groups ($p<.05$).

CONCLUSIONS: A novel 3D collagen gel culture system was used to investigate factors modulating neuronal ingrowth. Our results showed that NGF was necessary to promote neurite growth

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into collagen gels. In the presence of proinflammatory cytokines, high concentrations of IL-1β induced significantly higher axonal outgrowth than TNF-α and low levels of IL-1β.

Keywords: Dorsal root ganglia; Explant culture; IL-1β; TNF-α; Neurite extension; Collagen gel; Axonal outgrowth

Introduction

Discogenic low back pain (LBP) is a major health concern because of its debilitating effects on quality of life and its socioeconomic impact. Effective therapeutic approaches to relieving LBP are limited in part by an imperfect understanding of pathogenic pain pathways. In the normal intervertebral disc (IVD), sensory nerve fibers originating from dorsal root ganglia (DRG) are localized around the periphery of the annulus fibrosus (AF) [1–3]. In contrast, painful discs associated with degenerative disc disease have been shown in patients and animal models to possess greater numbers of nerve fibers that penetrate deeper into the AF and sometimes into the nucleus pulposus (NP) [4–6]. At the same time, there is increased presence of nociceptive transmitter molecules such as the pain-associated neuropeptides substance P and calcitonin gene–related peptide in most axons and their cell bodies in the DRG [7,8]. These findings suggest that nerve ingrowth into the degenerated disc could be one important factor in the complex etiology of discogenic pain.

Although the precise mechanisms are not well understood, various factors have been posited to regulate nerve ingrowth into the lumbar IVD [9–14]. In terms of biochemical stimulation, it is widely held that causal links between proinflammatory cytokines and neurotrophic factors, particularly nerve growth factor (NGF), influence the growth potential of DRG neurons into the IVDs [15,16]. Increased levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α have been observed in degenerated IVDs [17–20], and several studies have demonstrated that these cytokines are capable of upregulating neurotrophin expression in neuronal and nonneuronal cells, including AF and NP cells [11,15,21–23]. The level of NGF secreted by NP cells has been shown to be sufficient for promoting axon growth in dissociated DRG neurons [24], and innervation of the IVD by DRG neurons appears to be NGF dependent in a rat model of disc degeneration [25]. This is consistent with studies that find significantly elevated levels of NGF in degenerated IVDs from patients with discogenic pain [21,26]. However, whether IL-1β and TNF-α, themselves, promote or inhibit neurite outgrowth has not yet been clearly determined.

The mechanical environment of axons, including extracellular matrix (ECM) microstructural features and cell-matrix interactions, has also been demonstrated to be significant in regulating outgrowth. Chondroitin sulfate proteoglycans are known to be potent inhibitors of neurite outgrowth [27,28]. During development, chondroitin sulfate proteoglycans play an important role in the notochord's ability to inhibit axonal growth [29]. Furthermore, aggrecan from human IVDs is capable of repelling neurites from chick DRG [10], but the presence of disc cells enables some neurites to overcome the inhibition [30]. This is consistent with the notion that the balance between proteins that are repulsive (ie, aggrecan) and conducive (ie, laminin, collagen, and fibronectin) to neurite extension dictates growth into tissues [28]. Loss of the notochordal cell population from the NP and the progressive degradation of aggrecan from the disc during aging and degeneration would lower the barrier to nerve ingrowth. Matrix stiffness and porosity are also key modulating factors. For DRG neurons, substrates with moduli of approximately 1 kPa are optimal for neurite growth [31], and matrices of decreasing porosity tend to inhibit growth [32].

Although reported in vitro axon growth models have been useful for assessing the effects of modulating factors, interpretation of results can potentially be confounded by certain experimental variables. Studies have typically been performed using dissociated DRG neurons, DRG explants, or the SH-SY5Y neuroblastoma cell line [22,24,33,34]. In recent years, DRG explants have been more widely used because of their physiological relevance in vivo. However, even among these studies, most have been performed using neonatal [33,35] rather than adult DRG, despite well-established differences in neurotrophic profiles of maturing neurons and association of aging with LBP [36–38]. One intriguing exception was a study by Aoki et al. [16] who examined the effects of TNF-α on the growth potential of axotomized adult DRG neurons by observing immunoreactivity of activating transcription factor 3 and growth-associated protein 43 (GAP43). Finally, most in vitro studies have been performed using matrix proteins adsorbed onto stiff two-dimensional (2D) substrates [33,35,39,40], which do not accurately represent the in vivo micromechanical and microstructural environment of growing neurons.

Toward the development of a more comprehensive understanding of neurotrophic influences on axonal outgrowth in a context that is relevant to the aging spine, we developed a novel three-dimensional (3D) culture system that would enable us to dissect, in a controlled environment, the complex web of interacting factors that potentially include soluble signaling, matrix composition, biophysical stimuli, and cell-cell interactions. This in vitro experimental system allows us to characterize the unconstrained 3D extension of neurites from adult rat DRG into native collagen hydrogels. In this study, we implement and validate the system by examining the effects of IL-1β and TNF-α on neurite outgrowth in the presence