High Mechanical Strain of Intervertebral Disc Cells Promotes Secretion of Inflammatory Factors Associated with Disc Degeneration and Pain

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Introduction: Treatment of chronic illnesses such as osteoarthritis and low back pain presently consumes a significant amount of healthcare budgets worldwide. The current healthcare focus is shifting towards a better understanding of underlying causes and pathological mechanisms of such diseases in order to generate better treatment strategies, decrease morbidity and reduce the overall economic impact. Low back pain is a debilitating disorder requiring a significant amount of resources for treatment and is currently ranked 6th amongst costliest treatable chronic illnesses (1). Low back pain is often attributed to intervertebral disc (IVD) degeneration, synonymously referred to as degenerative disc disease (DDD). Commonly implicated factors associated with degeneration include up-regulated inflammatory cytokines, loss of resident cell populations, altered biomechanics, uneven loading of the spine and excessive mechanical stretch of tissue and cells (2). However, due to the complexity of disc degeneration, the mechanical and molecular mechanisms underlying this process remain poorly understood. Excessive mechanical loading of IVDs is thought to alter matrix properties and influence disc cell metabolism, contributing to degenerative disc disease and development of discogenic pain. Very little is known, however, about how mechanical strain induces these changes. This study investigated the cellular and molecular changes as well as which inflammatory receptors and cytokines were up-regulated in human intervertebral disc cells exposed to high mechanical strain (HMS). The impact of these metabolic changes on neuronal differentiation was also explored to determine a role in the development of disc degeneration and discogenic pain.

Methods: Cell Isolation: With approval of the institutional review board, and in collaboration with the provincially run organ donation program: Transplant Quebec, three human lumbar spines were harvested. A total of 5 discs each from human donors were obtained (age range 19-22, mean 20.5). Human IVDs were separated from the adjoining vertebral body with a scalpel and divided into nucleus pulposus (NP) and the inner annulus fibrosus (AF). Cells were enzymatically isolated from the separated regions as previously described (3) and were cultured at 37º C, 5% CO2 in DMEM containing 4.5g/L of glucose and supplemented with 10% fetal calf serum (FCS), 25 mmol/L HEPES, 0.25 μg/mL fungizone, 50 μg/mL L-ascorbate, and 2 mmol/L GlutaMAX. 50 μg/mL of gentamicin sulphate. All reagents were from Invitrogen/Gibco.

High Mechanical Strain: Culture medium was replaced with serum-free media, and isolated human IVD cells were exposed to HMS (20% cyclical stretch at 0.0001 Hz for 8 hours/day over 2 days) on high-extension silicone rubber dishes coupled to a mechanical stretching apparatus (4) and compared to static control cultures. Conditioned media was collected and RNA was extracted from cells using TRizol, and cDNA was generated.

Quantitative Real-Time PCR: Gene expression of toll-like receptors (TLR2 and TLR4), neuronal growth factor (NGF) and tumor necrosis factor alpha (TNFα) was assessed using Taqman array probes and a 7500 Fast Real Time system (Applied Biosystems) and the ΔΔCt method. 18s ribosomal RNA was used as the endogenous control.

Cytokine Array Blots: Collected conditioned media was analyzed for 23 common inflammatory cytokines using commercially available array blots according to manufacturer’s instruction (RayBiotech Inc, Norcross GA). Chemiluminescence detection was performed using the ECL reagent provided in the array kit and visualized with ImageQuant LAS4000 (GE Healthcare).

ImageQuant TL software was used for pixel quantification.

Neurite Sprouting Assay: Rat pheochromocytoma cell lines express receptors for NGF and can be induced to undergo neuronal differentiation and sprout axon-like neurites. 2x105 PC12 cells/well (ATCC Manassas, VA) were seeded on 6-well culture dishes coated with 50 μg/mL of collagen type I and 0.1% Poly-L-Lysine (70-150 kD; Sigma). Cells were allowed to attach to culture surfaces for 24 hours (Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1% Antibiotic-Antimycotic solution, 5% FBS and 10% Horse serum - all from Gibco/Invitrogen). After cell attachment, control wells were changed to 0.1% DMSO vehicle. Remaining wells were subjected to 1.5 mL of conditioned media collected from experiments above (static and HMS NP cultures). After 4 days three random phase images per sample were taken from each individual experiment (n=3) and the number of neurites per cell body were counted. Phase images were captured using a Zeiss Axiosvert 40C microscope equipped with a Canon Powershot A640 digital camera attached to a Zeiss MC80DX 1.0× tube adapter. LIVE/DEAD (Invitrogen) assays images were captured using an Olympus IX81 inverted fluorescence microscope.

Results: Culture of human IVD cells in modified silicone surfaces did not alter cell viability or metabolic processes. HMS caused
up-regulation of TLR2, TLR4, NGF and TNFα gene expression in IVD cells (Figure 1). Medium from HMS cultures contained significantly elevated levels of GRO, IL-6, IL-8, IL-15, MCP-1, MCP-3, MIG, TGFβ-1, TNFα and NGF. Exposure of PC12 cells to HMS-conditioned media resulted in both significantly increased neurite sprouting and cell death (Figure 2).

**Discussion:** Excessive mechanical loads can promote inflammatory and cytokine responses, factors implicated in spinal disc degeneration and low back pain. To address the molecular and cellular affects of adverse mechanical strain, isolated human AF and NP disc cells were subjected to high-magnitude strains using a novel dynamic culture device coupled to a high-extension silicone rubber culture dish. Gene expression, inflammatory and cytokine responses, and conditioned media were analyzed revealing up-regulation of TLR2, TLR4, NGF and TNF gene expression, and higher levels of secreted GRO, IL-6, IL-8, IL-15, MCP-1, MCP-3, MIG, TGFβ-1, and TNFα. Furthermore, conditioned media from high mechanical strain cultured AF and NP cells was able to promote significant neurite outgrowth in PC12 cells. The conditioned media, however, also caused a significant increase in cell death compared to static, NGF- and DMSO-treated controls. These data indicate that adverse mechanical strain can directly cause NP and AF secretion of factors associated with disc degeneration and discogenic pain, and may have neurotoxic effects.

**Significance:** HMS culture of IVD cells in vitro drives cytokine and inflammatory responses associated with degenerative disc disease and low back pain. This ground-breaking work provides evidence for a direct link between cellular strain, secretory factors, neo-innervation and potential degeneration and discogenic pain in vivo.

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**References:**

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![Figure 1: Gene expression of IVDs vs. static cultured AF and NP cells.](image1)

![Figure 2: Media from NP and AF cells cultured under high mechanical strain promotes neurite outgrowth of PC12 cells.](image2)