Applying Immunohistochemistry & Reverse Transcription PCR to Intervertebral Disc Degeneration in an Animal Model

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Sprague Dawley Rat (*Rattus norvegicus*)
Motivation

• Back pain: the number-one cause of disability in workers under age 45*
• $50 billion annually is spent in direct connection to back pain*
• Little is known about the causes
  – Disc degeneration a possible suspect
  – More information = better treatment
• Model degeneration in the rat
• Objective: develop preliminary study by applying IHC and RT-PCR

* American Chiropractic Society
Background

- Intervertebral disc composed of annulus fibrosis and nucleus pulposus
- Extracellular matrix controls disc function: components of interest
  - Nucleus: proteoglycan
  - Annulus: collagen
  - Various other proteins, enzymes, inhibitors...
- This matrix changes with degeneration

Normal (left) and Degenerate (right) disc
Immunohistochemistry

- **Definition**
  - Microscopic localization of specific antigens in tissues by staining with antibodies labeled with visible material

- **Current Objective**
  - Developing procedures
  - Creating baseline data on healthy discs

- **Future Plan**
  - Understand changes as disc degenerates in: various types of collagen, proteoglycan, and enzymes and their inhibitors
Basic Histology: Hematoxylin & Eosin

- Sagittal section
- Stains nuclei blue and tissues red
- Structure of disc apparent
- Break-up of nucleus a problem

- Higher magnification of nucleus
- Cells are sparse
Basic Histology: Alcian Blue & Picrosirius Red

- Stains proteoglycan blue and collagen red
- Distribution of the two apparent

- Transition from nucleus to annulus is clear
  - Nucleus: no visible collagen, even stain
  - Inner Annulus: some collagen, disorganized fibers
  - Outer Annulus: more collagen, organized fibers
IHC Results: Collagen I

- Most problematic stain
- Literature suggests light staining in outer annulus, none in inner annulus, nucleus
- Background effects significant
  - Reagents becoming trapped in tissue- thickness?
  - Possible cross-reactivity
IHC Results: Collagen II

- Significantly more successful stain
- Literature suggests Collagen II concentrated in inner annulus, some in nucleus
- No staining in nucleus, likely due to low overall concentration
IHC Results: Aggrecan

- Corresponds to Alcian Blue stain
- Literature also suggests aggrecan concentrated in nucleus, inner annulus, endplates
- Staining highly vivid, again, likely background effects
  - More color = less detail
  - Thick tissue sample traps reagents
IHC: Recommendations

• Goal to localize at cellular level, background must be minimized
• Possible changes:
  – Concentration
  – Time of exposure/wash
  – Thickness of section
    – Currently: 7 μm
    – Want: 5 μm
• Next step: back to histology
Gene Expression by RT-PCR

• Definition
  - the process by which a gene's coded information is translated into the structures present and operating in the cell (either proteins or RNAs)

• Current Objective
  - Develop a set of protocols to extract information from a sample
  - Apply to baseline discs

• Future Plan
  - Apply to both further baseline and degenerate disc material
PCR: Interpreting Results

- Gel electrophoresis separates DNA fragments by size (number of base pairs in gene)
  - Potential is induced over the length of the gel, DNA is charged
  - Larger segments (higher molecular weight) do not travel as far as smaller fragments
  - Allows identification of DNA present

- Leftmost column is ladder
  - Specifies size of product

http://www.cgeservice.com
RT-PCR Results

- The two columns to the right are products from L2 and L3 levels of spine, respectively:

  - Collagen I (599 bp)
  - Aggrecan (322 bp)
  - Fibronectin (481 bp)

- Collagen I: several bands- different primer needed, overamplified
- Aggrecan: overamplified
- Fibronectin (adhesion protein increases with degeneration): expression in healthy disc significant
RT-PCR: Recommendations

• Split the nucleus from the annulus
• Apply this protocol to more proteins and enzymes
• PCR should be quantified
  – Include a control sample for each sample of interest when doing PCR
  – Control will amplify a gene always present in the disc + not affected by degeneration
  – Measure the intensity of the band for the desired gene, normalize it by control
Conclusions

Work conducted this summer has shown that immunohistochemistry and RT-PCR are powerful tools which can be applied to understanding the intervertebral disc. There is, however, still a significant amount of background work which must be done before an actual study can be devised.

A deeper understanding of the intervertebral disc gained through such a study will hopefully lead to better back pain treatments and relief.