Tissue Remodeling in the Intervertebral Disc:
Response to Dynamic Loading and Growth Factors

by

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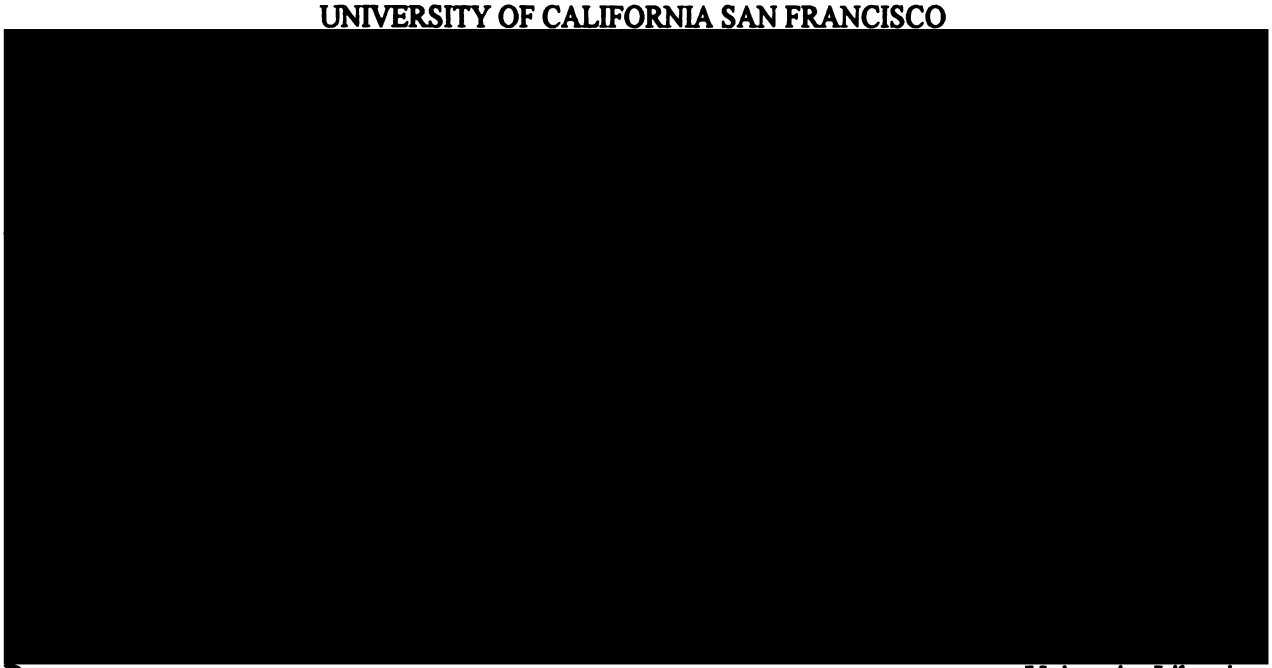

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Abstract

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Disc degeneration is a chronic remodeling process that results in alterations of matrix composition and decreased cellularity. The objective of this work was to explore two important topics related to disc degeneration: 1.) the role of mechanical loading in alterations of disc biology, and 2.) the potential to repair degenerated discs. The first study tested the hypothesis that dynamic mechanical forces are important regulators of disc cellularity and matrix synthesis. The second study hypothesized that exogenous growth factors can stimulate regenerative remodeling in the degenerated disc *in vivo.*

To study dynamic forces, a murine model of dynamic loading was developed that used an external loading device to cyclically compress a single disc in the tail. Dynamic loading induced differential effects that depended on frequency and stress. The results suggested a tolerance to loading above which evidence of both an anabolic response (increased proteoglycan content and matrix gene expression) and a degenerative response (cell death) were found. Finite element modeling suggested that the responses might be due to variation of strain environment.

The effects of exogenous growth factors on degenerated discs were studied *in vivo* using a murine model of compression-induced degeneration. Degenerated discs were given single or multiple injections of GDF-5, TGF-β, IGF-1, bFGF, or saline as control and analyzed either one week or four weeks after treatment. In some growth factor

treated discs, expansion of inner annular chondrocyte populations into the nucleus were observed. The results indicated that annular chondrocytes may be responsive to some growth factors *in vivo*, and that GDF-5 and TGF-β may be mitogens for annular chondrocytes.

In summary, this work provides new knowledge of the mechanisms of tissue remodeling *in situ* in the intervertebral disc. The results have implications in the prevention and treatment of disc degeneration. The responses to dynamic mechanical forces suggest that loading conditions may be optimized to promote maintenance of normal structure and function. The cellular response to growth factors observed in degenerated discs demonstrates the possibility of their use in new therapeutic modalities for repair.

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Chapter 3. Biological Response of the Intervertebral Disc to Dynamic Loading

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Chapter 1. Introduction

Frequent low back pain affects more than 26 million Americans between the ages of 20 and 64 [1]. It is a leading cause of workers' disability and has an economic impact of roughly $50-100 billion each year [2]. As a result, the prevention and treatment of low back pain is an active area of research. Much of this research has focused on intervertebral discs for the simple reason that aberrations of disc structure are observed clinically in many individuals with pain. Evidentially, epidemiological studies have demonstrated that pain in the lower back and lower extremities can occur in association with disc degeneration [3-6].

Although alterations to disc anatomy have long been reported [7-9], the biological mechanisms that result in degeneration are only now being understood. Disc degeneration is recognized as a chronic process that results in significant alteration, or remodeling, of tissue structure and composition over time. Changes to disc architecture derive in part from purely mechanical phenomenon, such as structural failure or fatigue of the tissue matrix [10, 11]. Tissue remodeling, however, depends upon the cellular response to various stimuli, as it does in bone [12], lung [13], blood vessels [14], and other tissues. Both mechanical and biochemical stimuli are present, and their effects can be interdependent. For example, mechanical loading can impair the nutrient supply to cells, thus altering their biochemical environment [15]. Conversely, the action of matrix degrading enzymes may change the tissue material properties and hence alter the cells' mechanical environment [16]. Thus, the pathogenesis of disc degeneration is complex. Elucidation of the mechanisms of degeneration and repair has been greatly enhanced by

coupling modern biological techniques with consideration of disc biomechanics. Despite these advances, many questions exist.

The overall objective of this work is to provide new understanding of tissue remodeling in the intervertebral disc. It consists of two separate studies that address (1.) degenerative remodeling in response to mechanical force and (2.) regenerative remodeling in response to biochemical agents, specifically growth factors. These studies seek insight into two unanswered questions related to disc degeneration. First, how does mechanical loading typical of human activity affect disc biology? Second, what approach might be used to repair degenerated discs? Answers to these questions will lead to better measures for prevention of disc degeneration and improved treatments for those suffering from low back pain.

To the first end, an *in vivo* model of cyclic compression was developed to test the cellular response to dynamic loading. To the second end, a static-load model was used to induce degeneration and then test the effects of different growth factors. The two studies share a common approach: tissue remodeling is addressed *in vivo* using an animal model. Historically, studies of the disc have used tissue in culture, cadaveric spines, or clinical cases to address the causes and treatment of degeneration. Most of the previous animal models induce alterations to the disc by surgical injury to the annulus [17-19] or by non-calibrated perturbations of the mechanical environment [20, 21]. The benefit of the current approach, compared with *in vitro* systems, is that normal structure and physiology are intact. Compared with most of the existing animal models, the models employed herein use calibrated methods in physiologic range to apply mechanical loads to the disc.

The background that motivates the current experiments is discussed in Chapter 2. Chapter 3 describes the biological response of the disc to dynamic mechanical forces. Chapter 4 addresses the effects of various growth factors injected into degenerated discs.

Chapter 2. Intervertebral Disc Background

Disc Anatomy

The intervertebral disc is a complex structure interposed between each pair of vertebral bodies. At the center of the disc lies the nucleus pulposus, a gelatinous solid that is rich with sulfated proteoglycans. At birth, the nucleus has a water content of approximately 90%, which decreases to 80% in the young adult and continues to decline with age [22]. The nucleus contains a loose network of fine, type II collagen fibers with proteoglycan, hyaluronan, and lesser amounts of other molecules dispersed throughout. Similar to cartilage, the predominant proteoglycan is aggrecan. The cells of the nucleus at birth are chondrocytes and notochordal cells. The notochordal cells are a remnant of embryonic development and are eliminated within the first decade. The cell density in the young adult is the lowest of any connective tissue [23]

The annulus fibrosus is a collagenous ring of tissue surrounding the nucleus pulposus. The annulus consists of concentric lamellae containing ordered arrays of collagen fibers. The fibers are oriented roughly 60° to the axis of the spine, and the fiber direction alternates between each layer [24]. A gradient of collagen types exists. Type I collagen is predominantly in the outer annulus and decreases toward the center; type II collagen is predominantly in the inner annulus and decreases outward. Different cell types are associated with the varying collagen types: chondrocytes in the inner annulus, fibroblasts in the outer regions. Collagen fibers in the outer annulus attach directly to the vertebral body. In the inner annulus, fibers attach to the cartilage endplates.

Cartilage endplates are layers of hyaline cartilage that cover the adjacent vertebral bodies over the nucleus and inner annulus. They are the main routes of diffusion of

nutrients into the disc. Although they are only 1 mm thick in the adult human [23], they play an important role in limiting fluid flow in and out of the disc.

Anionic charge of the nuclear matrix creates an osmotic driving force for imbibition of water into the nucleus. The uptake of water causes the nucleus to swell, and because it is spatially constrained, it exerts a swelling pressure radially on the annulus and axially on the vertebral bodies. Thus, even at rest, the collagen fibers in the annulus are placed in tension by the nuclear pressure. This state of pre-stress provides an innate mechanical stability to the disc. When compression is applied, the nucleus and inner annulus experience compressive stress while the outer annulus is in tension. The heterogeneity of annular cells and matrix reflects this variability in stress state. In the outer annulus, the cellular and matrix components are similar to tendon and ligament. The inner annulus, on the other hand, is composed of fibrocartilage.

Disc Degeneration

Alterations in disc structure and composition occur in all adults with age. In general, degeneration is regarded as a distinct process in which the extent and rate of these changes is greater [23, 25]. The distinction, however, between physiologic and pathologic is not clear, and degeneration and age-related changes are frequently described in the same light.

One of the most common changes with age and degeneration is a decrease in nuclear water content [26]. This results from a decrease in the quantity and quality of nuclear proteoglycans. The result is a decrease in swelling pressure and subsequent loss

of disc height and nuclear volume. As degeneration progresses, the concentration of viable cells declines sharply [27].

Degeneration of the annular architecture is also common. In early stages of degeneration, the organization of collagen fibers in inner annular lamellae deteriorates [28]. This can result in increased bulging of the annulus when loads are applied. Tears, clefts, or fissures may eventually form, creating a pathway for protrusion or herniation of nuclear material. As degeneration progresses, annular collagen fibers become more disorganized and the boundary between annulus and nucleus becomes indistinguishable. Metaplasia of fibrocartilage occurs with the appearance of chondrocyte-like cells in regions previously occupied by fibroblasts. By late stage degeneration, the disc is largely an amorphous mat of collagen fibers with few cells and significantly reduced water content.

With age and degeneration, cell viability in the cartilage endplate decreases. The endplates become calcified which leads eventually to ossification. Thinning and microfractures also occur. Observations of changes to the endplates that precede those in the nucleus or annulus have lead to speculation that disc degeneration on whole derives from endplate degeneration [29, 30].

Biomechanics

The disc functions mechanically to transmit forces from one vertebra to the next while enabling limited motion between vertebrae [31]. Normal activities create complex combinations of compression, bending, rotation, and shear on discs. The mechanical response depends on the properties of the nucleus and annulus, which are quite distinct

because of their different molecular compositions. The gelatinous nature of the normal nucleus endows it with the behavior of a pressurized fluid. When compression is applied to the spine, the hydrostatic pressure within the nucleus increases. The pressure approximates 1.5 times the vertical load applied per unit area [32]. Nuclear pressure creates a hoop stress in the annulus and bulging in the vertebral body endplates.

The annulus is relatively rigid when subjected to symmetric compression and resists compressive stresses directly [33]. Compressive stress creates radial bulging of the annulus. Bulging and nuclear pressure produce tensile stresses in the collagen fibers. Bending, shear, and torsion also place the annular collagen fibers in tension. The measured tensile properties of the annulus correlate well with its function. The stiffness, strength, and elastic recovery of the annulus tested under axial tension are higher in the outer region than in the inner [34, 35]. Regional variation may reflect the need for enhanced properties in the outer annulus to reduce nonuniformity of stress [36]. Differences between inner and outer annulus may also be due to degenerative changes that are usually more pronounced in the inner annulus. Stiffness, elastic recovery [34], failure stress, and strain energy density at failure [37] decrease with degeneration.

The disc possesses viscoelastic characteristics that give it time-dependent behavior [31]. This effect is due in large part to fluid flow through the cartilage endplates and annulus fibrosus. The balance of opposing forces determines the water content within the disc. The nuclear swelling pressure draws water into the disc while mechanical pressure tends to drive it out. When load is applied to the disc, the equilibrium is disturbed and water flows out until a new equilibrium is achieved. Resistance to fluid flow from the nuclear and annular matrices and the cartilage endplates

limits the rate at which water can be expelled or reabsorbed. Viscoelastic properties of the solid matrix have also been proposed as determinants of the time-dependent behavior of the disc [38].

The viscoelastic properties of the disc are important to its mechanical function. Elastic deformation of the annulus allows the disc to absorb energy transmitted down the spine. The time-dependent behavior, however, prevents rapid collapse under high impact, rapid loading. During sustained loading, disc creep occurs. An initial elastic deformation is followed by a gradual reduction in disc height as water flows out of the disc. The loss of nuclear water reduces the hydrostatic properties of the nucleus and produces stress concentration within the annulus [39]. The consequences to annular structure, cell metabolism, and pain production may be negative. After creep, the disc is stiffer in compression and has less resistance to bending. Similarly, degenerated discs, in which the water content is decreased, are less viscoelastic [40]. The ability to attenuate shocks and distribute the load uniformly over the endplates is diminished.

Measurement of compressive stress in degenerated discs shows a transfer of load from nucleus to annulus and increase in stress concentration in the posterior annulus [41]. This is a likely reason for the development of fissures, clefts, or ruptures. Attempts to produce disc prolapse through the annulus *in vitro* have been made using various types of loading conditions. The resistance to symmetric axial compression is very high, and failure occurs not in the annulus but in the vertebral body along the cartilage endplate [31]. Sudden prolapse in the annulus can occur when compression is combined with hyperflexion. Perhaps more relevant, gradual prolapse has been produced by cyclic loading using combinations of flexion, lateral flexion, and compression [42] and flexion,

compression, and rotation [43]. These findings suggest that fatigue damage can leave a disc vulnerable to structural failure.

Etiology of Degeneration

Because of the discs' structural function, mechanical forces on the spine have historically been suspected as a cause of degeneration. Accident-related trauma is a risk factor for degeneration because of acute structural damage created, for example, to the annulus or cartilage endplate [44-47]. Animal models have demonstrated that injury to the annular structure can initiate progressive degeneration [18, 19, 48]. In humans, the association is less clear because the time sequence of degeneration and back accidents is unknown. It is possible that accident-related trauma occurs because the disc has already begun the degenerative process.

Chronic mechanical burden associated with various occupations is a potential cause. Occupational factors such as heavy lifting, bending, twisting, and sitting have been suggested as risk factors [49]. Many studies have reported an association between degeneration and heavy physical work [46, 50-53] while others found only weak or no association [54-57]. Physical loading has been suggested as having a positive effect to the disc by increasing nutrition [58]. Additionally, heavy physical training is a common part of rehabilitation for low back pain. Thus, the role of physical factors in disc degeneration remains controversial.

Despite the conflictive results from clinical studies, *in vitro* and *in vivo* laboratory studies have demonstrated that mechanical loading can induce degenerative changes in disc tissue [59, 60]. These studies will be described in more detail in Chapter 2. Taking

the results of the clinical and laboratory studies together, mechanical loading is a likely determinant of degeneration that depends on the nature of loading (frequency, intensity, and duration) and individual response.

Other possible extrinsic factors include vehicular driving [46, 61] and smoking [62-64]. Driving is attributed to the effects of another type of physical loading, whole-body vibration [65, 66]. Vibration may have deleterious effects to disc nutrition in addition to altering the creep behavior of the disc. Smoking is also suspected of adversely affecting the disc, although one study found that smoking accounted for less than 2% of the variability in disc degeneration in adults [63].

Recent evidence also suggests that genetic factors play a role in degeneration. In a study relating magnetic resonance findings in identical twins to various risk factors, disc degeneration was explained primarily by genetic influences [57]. In support of their claim, the same group later found specific vitamin D receptor alleles associated with degeneration [67]. This area of research is likely to provide important new information as the tools for rapid genetic analysis become more readily accessible.

Sources of Pain

Back pain is the primary reason for concern with disc degeneration. It is important to note that not all degenerated discs result in pain [68, 69]. Conversely, patients who suffer from back pain may have normal appearing discs on lumbar spine radiographs or MRI [70, 71]. Identifying the cause of the pain can be difficult, and there are no clear patterns, specifically, of pain type or location that result. Still, there are a

number of proposed mechanisms by which degenerated discs can result in low back and/or lower extremity pain.

Although the disc on whole is largely without innervation, there are nerve fibers in the outer annular layers and outside the disc that may be sensitized by a bulging or ruptured annulus [72-74]. Increase in nerves toward the center of the disc has been reported as degeneration progresses [75]. Second, protrusion or herniation of the nucleus may impinge on nerve roots in the posterior of the spine [76-78]. This mechanism is controversial because nerve root impingement has been identified in both symptomatic and asymptomatic individuals [79, 80]. Third, biochemical or immunologic reactions to substances released from herniated tissue may stimulate nociceptors outside the disc [81-85]. Fourth, a loss of disc height can put additional stress on the facet joints in the posterior of the spine [86]. This can lead to degeneration and pain in these joints [87]. Taken together, these mechanisms suggest that alleviation and prevention of back pain may be improved by the maintenance or restoration of normal disc structure and function.

Current Treatments

Conservative treatment may include bed rest to reduce pressure on the aggravating disc, medication to reduce pain, and exercise to strengthen the surrounding musculature [88]. In a majority of patients, conservative treatment is effective at providing some relief from pain [89]. However, a significant percentage of these patients have persistent or recurrent pain. For patients with pain following six to twelve months of conservative treatment, surgery may be the next option.

Surgical procedures may remove herniated nuclear material or a portion of the vertebra to decompress nerve roots suspected of causing pain. Alternatively, the vertebrae may be fused together using a bone graft or osteoinductive material with or without excision of the disc. Surgery has a high rate of success at providing immediate pain relief, but it does have drawbacks. Perhaps of primary concern is that the benefit of surgery diminishes over the long-term [90]. Partial discectomy to remove the nucleus of a herniated disc reduces the disc height and increases radial bulge [91]. This can produce pain as described above. Fusion provides stability to the spine at the expense of mobility of the fused segments. Long-term studies have reported that fusion promotes degeneration at other levels [92].

Motivated to improve treatment options and patient outcomes, alternative strategies for treating degenerated discs are being explored. Artificial devices to replace the intervertebral disc have been in development for more than thirty years. The primary advantage of a prosthetic intervertebral disc would be restoration of normal biomechanics. Spinal biomechanics, however, are difficult to reproduce using synthetic materials and this has limited their success in patients [93]. More recently, synthetic replacements of the nucleus only have been developed which are placed within the existing annulus to confer mechanical stability to the disc [94]. These devices contain a hydrogel to mimic the properties of the nucleus and can be inserted using minimally invasive surgical techniques. In addition to artificial devices, allografts of intact motion segments (intervertebral disc and adjoining vertebrae) have been performed in animals, but not humans, with marginal success [94].

The concept of intervertebral disc regeneration has developed in recent years from improved understanding of the cellular and molecular events of disc aging and degeneration [95]. Addressing disc degeneration as a cell-driven biochemical process has raised the possibility of manipulating cell behavior toward a beneficial outcome. General strategies by which the degenerative process might be stopped or reversed include increasing the cell content, inhibiting programmed cell death, increasing matrix synthesis, and inhibiting matrix degradation. Cytokines, such as growth factors, will likely play a role in achieving these ends. Questions as to which growth factors may be beneficial have motivated the experiments in Chapter 3.

Gene therapy is an active area of research as a tool for providing sustained levels of beneficial cytokines. The product of a transfected gene could be a growth factor, for example, to increase matrix synthesis, or it could be an antagonist in the inflammatory or pain production cascade [96]. Transplant of cell constructs has also been proposed for disc regeneration. Transplant of autologous nucleus pulposus tissue into denucleated rat discs has been demonstrated to slow the progression of degeneration [97]. A tissue engineered nucleus pulposus containing chondrocytes was also recently developed and implanted subcutaneously in nude mice as proof of concept [98]. Still in its infancy, disc regeneration has great promise but requires tremendous work.

Chapter 3. Biological Response of the Intervertebral Disc to Dynamic Loading

Introduction

Intervertebral discs impart mobility to the spine while transmitting forces from one vertebra to the next. In a young adult, the disc is a highly organized structure enabled to perform these functions. With age, disc structure and composition are altered, often resulting in impaired function and pain. Given that structural demands are repeatedly placed on the spine, mechanical factors have been suggested as playing a primary role in remodeling the disc over time [99].

The composition and organization of macromolecules in the disc, mostly proteoglycans and collagen, are critical to its mechanical behavior. As in any tissue, the resident cell population regulates the synthesis and breakdown of these molecules. Regulation of cellular events by mechanical forces has been demonstrated in many musculoskeletal tissues [100-102]. For example, cell metabolism and differentiation in tendon [103], bone [104], and cartilage [105] have been observed to change in response to altered mechanical states. Thus, it should not be surprising to find that cellular activity within the disc also is dependent on the mechanical environment to which the cells are exposed.

In fact, static hydrostatic pressure on disc tissue in culture has been shown to effect both matrix synthesis and metalloproteinase production [59, 106]. *In vivo* models have also demonstrated alterations in matrix content following aberrant loading on the disc [107, 108]. In our lab, a mouse model that provides a predetermined static load to a single intervertebral disc in the tail has been developed [60]. Over the range of loads

studied (0.4-1.3 MPa), remodeling typical of degeneration (annular disorganization, cell death, and fibrocartilage formation) occurs in a dose-dependent way. The model demonstrates the cellular and molecular response of the disc to compression, and has been used to investigate the effects of magnitude and duration of applied force to the disc.

The static model is limited, however, because it does not allow for the study of loads applied dynamically, which are more characteristic of human *in vivo* exposure. The physical and chemical environments created by dynamic forces compared with static forces may produce different effects to the disc. For example, patterns of cell and matrix deformation, fluid flow, hydrostatic pressure gradients, water content and fixed charge density are important variables to cartilage metabolism which depend on the nature of loading [109]. It has been reported for articular cartilage that dynamic compression, in contrast to static compression, can be stimulatory for matrix synthesis [110-112]. Beyond limits, however, both dynamically and statically applied forces result in degenerative changes [113-115].

Studies of dynamic forces to the disc have been largely *in vitro* and focused on the resultant biomechanical performance and fatigue failure [42, 116, 117]. An *in vivo* model to study the viscoelastic properties of pig discs following high frequency vibration has been reported recently [118]. However, there are no *in vivo* studies in the literature of the cellular and molecular response to calibrated cyclic forces for prescribed frequencies and durations. This need has motivated the development of an *in vivo* dynamic model to study degenerative remodeling. Such a model is advantageous because disc structure and physiology are intact, and thus the natural mechanisms that influence cell activity exist.

We hypothesize that dynamic mechanical forces similar to those experienced in the range of normal, human behavior are important regulators of disc cell activity. This activity may be beneficial to maintaining tissue homeostasis, or it may lead toward disc degeneration by inducing cell death and matrix destruction. In order to understand the mechanisms and dose-response relationships of both detrimental and therapeutic spinal stress, we have developed an animal model to investigate the influence of dynamic loading conditions (magnitude, frequency, and duration) on disc composition and function. Our objective in this study was to explore the biological effects of compressive loading regimens that mimic exposures experienced during human manual material handling. We have examined the cellular and molecular responses with an eye toward both anabolic and degenerative outcomes.

Methods

Animals Groups

A total of fifty-four adult (twelve week old), male, Swiss Webster mice were used in this study. The UCSF Committee on Animal Research approved all procedures. The study consisted of six groups: five loaded and one sham (Table 1). An external device was used to compress a single intervertebral disc in the tail of each mouse in the loaded groups. Discs in Groups 1-4 were compressed cyclically at one of two frequencies (0.1 Hz or 0.01 Hz) and one of two peak stresses (0.8 MPa or 1.3 MPa). Cyclic compression was applied from 0 MPa to the peak stress at a 50% duty cycle for 6 hours/day for each of seven days. Discs in Group 5 were compressed statically at 1.3 MPa stress for 3 hours/day for each of seven days. A shorter duration was used for static than dynamic

compression to equate the load-time integral. The stresses approximate those on the human lumbar spine during lifting of up to 30 lbs [119]. Sham mice in Group 6 were fitted with the device but compression was not applied.

Table 1. Experimental Groups

Group	1	2	3	4	5	6
Frequency (Hz)	0.1	0.01	0.1	0.01	static	
Peak Stress (MPa)	0.8	0.8	1.3	1.3	1.3	
Loading duration/day (hours)	6	6	6	6	3	sham
Number of days of loading	7	7	7	7	7	
Number of animals	8	8	8	7	10	13

Animal Surgery

To install the device, each mouse was anesthetized and the tail was palpated to identify the tenth coccygeal disc. Stainless steel insect pins (0.4 mm diameter) were then inserted percutaneously through adjacent vertebral bodies using a small drill. Two pins were inserted perpendicularly in each vertebral body. Prior to drilling, a plastic washer (7/8" diameter) was placed on the proximal end of the tail. After drilling, a second washer was placed on the tail against the distal set of pins. Each washer had a groove to allow simultaneous contact on the washer by all four ends of the pins on each side. The device was then slipped onto the tail and against the distal washer. It consisted of a ring-shaped latex bladder and plastic washer within a cylindrical housing (Figure 1). The housing had a hard backing and was fixed in position on the tail by sliding plastic bars

through grooves in the housing until they abutted the proximal washer. Correct positioning of the bars ensured close tolerance of the bladder with adjacent washers without compressing the disc. The bars were then glued in place.

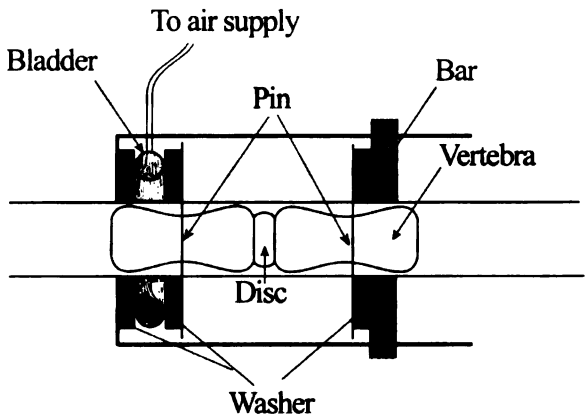

Figure 1. Loading Device Schematic

Dynamic Loading Device

Inflation of the latex bladder (California Medical Innovations, Pomona, CA) compresses the disc. When the bladder deflates, the force is removed. Prior to experiments on the mice, the force versus pressure relationship was measured for each bladder on an MTS Bionix 858 Test System (Eden Prairie, MN). An electronically controlled valve (model QB1, ProportionAir Inc., McCordsville, IN) regulated pressurization of the bladders. The valve provides a pressure waveform proportional to an analog voltage input from a standard function generator. The frequency of the pressure square-wave, and hence of disc loading, is controlled by the frequency of the cyclic voltage input. A manifold divides the air line from the valve to multiple devices. From the manifold, each air line connects to the top of a swivel above the cage. Each

bladder is connected to the underside of the swivel to provide the mouse free range of motion.

Tissue Processing

The mice were sacrificed one day after the last loading period. The pins were cut flush to the surface of the tail but not removed. After carefully removing the skin, the portion of the tail containing the treated disc and attached vertebrae was collected by cutting through the adjacent discs. The tissue was fixed in 4% paraformaldehyde (Sigma, St. Louis, MO) overnight at 4 °C and then decalcified in 19% ethylenediamine tetra-acetic acid (EDTA, Sigma, St. Louis, MO) for seven to ten days at 4 °C. The pins were removed from the tissue once the vertebrae were decalcified when they could be easily removed with a minimum of force. The tissues were processed through a graded series of ethanol and xylene, embedded in paraffin, and sectioned 6 μm thick. Processing was conducted using RNase-free solutions and instruments for subsequent *in situ* hybridization.

Histology and Image Capture

Tissue sections were rehydrated and stained by the Hall's and Brunt's Quadruple (HBQ) stain method [120]. This method uses alcian blue to stain proteoglycan, direct red to stain collagenous tissue, and Mayer's hematoxylin and celestine blue to stain cells. Sections were observed under a Nikon E800M microscope (Melville, NY). Images were captured using an Optronics digital camera (Goleta, CA) and Simple PCI imaging software (Compix, Cranberry Township, PA).

Proteoglycan Content

Mid-sagittal sections stained by the HBQ method were used to estimate relative differences in nuclear proteoglycan content between groups. Using Simple PCI software, the total area of the nucleus pulposus on the section was first measured. Then the area of the nucleus stained with alcian blue was measured. The proteoglycan content was calculated by dividing the alcian blue stained area by the total area and multiplying by 100.

Apoptosis and Cell Areal Density

Apoptotic cells in mid-sagittal sections were detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method. Sections were deparaffinized and then treated with proteinase K (Sigma, St. Louis, MO) at 20 μg/ml for 10 minutes at room temperature. A commercial TUNEL kit (Roche Diagnostics, Indianapolis, IN) was used according to the manufacturer's instructions. Sections were counterstained with Hoechst dye (Sigma, St. Louis, MO) and coversliped in aqueous ProLong Antifade medium (Molecular Probes, Eugene, OR). TUNEL-positive and counterstained cells were observed under fluorescent microscopy using either a FITC or DAPI filter, respectively. Simple PCI software was used to capture images and count the numbers of apoptotic and total cells in both annulus and nucleus. The cell areal density was calculated by dividing the total number of cells by the area of the region (annulus or nucleus) in which it was measured.

Disc Height Measurement

Disc height was measured on images of mid-sagittal, HBQ stained sections. Simple PCI software was used to measure the distance between cartilagenous endplates at three places within the nucleus pulposus. The three disc height measurements were averaged and normalized to the mean length of the two vertebral bodies in each section.

Annular Birefringence

Unstained, mid-sagittal sections were observed under plane polarized light. All sections were oriented with the longitudinal axis parallel to the plane of polarization. Crossed polarizers were used to observe birefringent light from annular collagen fibers. Images were captured and the graylevel intensity in the annulus was compared between treatment groups using Simple PCI software.

Data Analysis

Statistical analysis was performed on measures of disc height, proteoglycan content, cell death, and cell areal density. One-way analysis of variance (ANOVA) was used to detect significant differences between treatment groups. The level of significance was 0.05. When appropriate, a Fisher LSD post-hoc test was used to identify which groups were significantly different. For statistical analysis of apoptosis data, the percentage of dead cells was first transformed to the arcsine of the square root of the fraction of dead cells. The transformed values were then analyzed as described above. This transformation is commonly used for percentage data to improve compliance with the assumptions of normality and equal variance [121].

In situ hybridization

Regional expression of aggrecan and type II collagen genes within treated discs was analyzed by *in situ* hybridization. The probe used to detect aggrecan mRNA was an ^{35}S-labeled antisense RNA probe prepared by T3 polymerase transcription of ApaI-linearized bluescript plasmid-containing insert for aggrecan cDNA. The probe to collagen II mRNA was prepared by T3 polymerase transcription of EcoRI-linearized Bluescript plasmid-containing insert for mouse α1 chains of procollagen II cDNA.

Sections were deparaffinized, treated with proteinase K (20 μg/ml, 5 minutes at room temperature), and acetylated using acetic anhydride. Slides were hybridized overnight at 53°C with 33 μl of hybridization solution containing approximately 2X10^6 cpm of probe. The hybridization-probe solution was removed by washing the slides in 5XSSC containing 20 mmol/l β-mercaptoethanol (twice for 30 minutes each at 55°C), then in high stringency wash of 50% formamide, 2XSSC containing 40 mmol/l β-mercaptoethanol (twice for 30 minutes each at 60°C). The sections were subsequently equilibrated in NTE buffer (10 mmol/l Tris-HCl, pH 8, 0.5 mol/l NaCl, 5 mmol/l EDTA, twice for 15 minutes each at 37°C) before incubation with 20 μg/ml RNase (Sigma, St. Louis, MO) (30 minutes at 37°C) to digest unbound probe, then rinsed again in NTE (15 minutes). The high-stringency wash was repeated (30 minutes) before the final washes at room temperature in 2XSSC and 0.1XSSC (15 minutes each). Slides were dehydrated, coated with Kodak NBT-2 emulsion (Scientific Imaging Systems, Eastman Kodak, Rochester, NY), and exposed for several days, then developed and counterstained with Hoechst dye (Sigma, St. Louis, MO).

In situ hybridization was observed under dark field microscopy using a darklight stage (Dolan Jenner, Lawrence, MA) to illuminate silver grains on the slide. Fluorescence microscopy using a Dapi filter was used to observe cells counterstained with Hoechst dye. Cells in the adjacent growth plate were used as control.

Finite Element Model of Murine Disc

A porohyperelastic finite element model was generated from the geometry of the motion segment of the mouse tail. The model was axisymmetric about the axis of the tail. Eight-noded porous media elements were used to construct a model containing a portion of the vertebral body and one transverse plane of symmetry corresponding to the mid-height of the disc [60]. Commercial software was used for analyses (ABAQUS v6.2, HKS Inc, Pawtucket, RI).

Material properties of the annulus, nucleus, endplate, and bone were obtained from the literature for similar tissues (Table 2). The annulus was modeled as an isotropic, compressible Mooney-Rivlin material possessing the strain energy function:

$$U = \sum_{i+j=1}^{N} C_{ij}(I_1 - 3)^i (I_2 - 3)^j + \sum_{i=1}^{N} \frac{(J-1)^{2i}}{D_i}$$

where I_1 and I_2 are the deviatoric strain invariants, J is the volumetric strain, C_{ij} and D_i are strain energy coefficients, and N = 2. Values for the hyperelastic coefficients (C_{01}=C_{10}=0.1; C_{02}=C_{11}=C_{20}=0.01; D_1=D_2=35) were obtained from previous experiments in our lab [122, 123], and hydraulic permeability of human annulus was obtained from the literature [124]. Collagen fibers in the annular matrix were represented as REBARS, whose orientation with respect to the transverse plane varied with radial position to simulate the lamellae [36, 125]. Properties of REBARS were nonlinear hypoelastic with

no compressive stiffness [126]. The nucleus pulposus was also represented as a poro-hyperelastic material whose properties were based on previously validated results [60]. In addition, a novel strain-dependent swelling pressure was incorporated into the material definition of the nucleus. Swelling properties were based on extensive work by Urban and colleagues [127-129]. As the nucleus is compressed and the tissue porosity decreases, the fixed charge density increases, resulting in a greater propensity for swelling. While the relationship between porosity and swelling pressure has been shown to be non-linear [128], a linear approximation was found to be adequate for the range of porosities of interest in the model:

$$\Pi = 2.1185 - 2.5425 * P$$

where Π is the swelling pressure and P is the tissue porosity (defined as volume of fluid/total volume).

Table 2. Material Properties Used in Finite Element Model

	Elastic Modulus (MPa)	Poisson's Ratio	Permeability (mm/s)
Trabecular bone	50	0.2	1 E-8
Cartilaginous endplate (annulus)	100	0.2	1 E-10
Cartilaginous endplate (nucleus)	100	0.2	1 E-8
Annulus fibrosus with collagen fibers	Hyperelastic with REBARs	N/A	2.5 E-9
Nucleus pulposus	Hyperelastic	N/A	1 E-9

Since fluid flow through the cartilaginous endplate may be important in this analysis, a portion of the vertebral body was modeled. The cartilaginous endplate

interfacing the disc to the vertebral body was represented by an isotropic material having properties as reported in the literature [130]. Permeability in the endplate adjacent to the nucleus was enhanced to permit fluid exchange between the disc and the adjacent vertebra. Trabecular bone was modeled as transversely isotropic with properties obtained from the literature [131, 132].

Results

Histology

The structure and composition of a normal mouse disc is similar to that of a young human. The gelatinous nucleus pulposus is located centrally and contains a high water content. It has a cellular interior with proteoglycan around the perimeter. Cells in the mouse nucleus are predominantly notochordal. The annulus fibrosus forms a collar of collagenous tissue around the nucleus. It is composed of layers of ordered collagen fibers with fibrochondrocytes in the inner annulus and fibroblast-like cells in the outer annulus.

The morphology of discs loaded at 0.1 Hz and 0.8 MPa (Figure 2b) was very similar to that of shams (Figure 2a) and normals (not shown). When the frequency was decreased to 0.01 Hz, structural changes to the nucleus were observed (Figure 2c). Nuclear cells became more compacted and there was increased proteoglycan within the nucleus. This was also noted when the stress was increased to 1.3 MPa at 0.1 Hz (Figure 2d) and was most pronounced in the low frequency, high stress case (0.01 Hz 1.3 MPa, Figure 2e). The morphology of discs exposed to static load at 1.3 MPa (Figure 2f) was similar to those of low frequency, high stress.

In contrast, little or no alterations to the structure of the annulus were observed between loaded and sham discs. Lamellar structure in the annulus was generally maintained after each regimen of loading. Cellularity and cell type were also preserved in the annulus after both dynamic and static loading.

Proteoglycan Content in the Nucleus

The area of the nucleus stained with alcian blue was measured on histology sections and expressed as a percentage of the total area of the nucleus as an estimate of the proteoglycan content (Figure 3). Importantly, the total area of the nucleus did not vary significantly between groups (data not shown). Differences in the area ratios, therefore, reflect changes in proteoglycan content and not differences in total nuclear area.

Similar to qualitative observations from histology, the measured proteoglycan content in the nucleus was the same in shams and after loading at 0.1 Hz 0.8 MPa. Decreasing the frequency or increasing the stress increased the amount of nuclear proteoglycan content. One-way ANOVA followed by a Fisher LSD post-hoc test indicated that the increases for 0.01 Hz 0.8 MPa, 0.1 Hz 1.3 MPa, 0.01 Hz 1.3 MPa, and static 1.3 MPa were statistically significant compared to shams and 0.1 Hz 0.8 MPa ($p<0.05$).

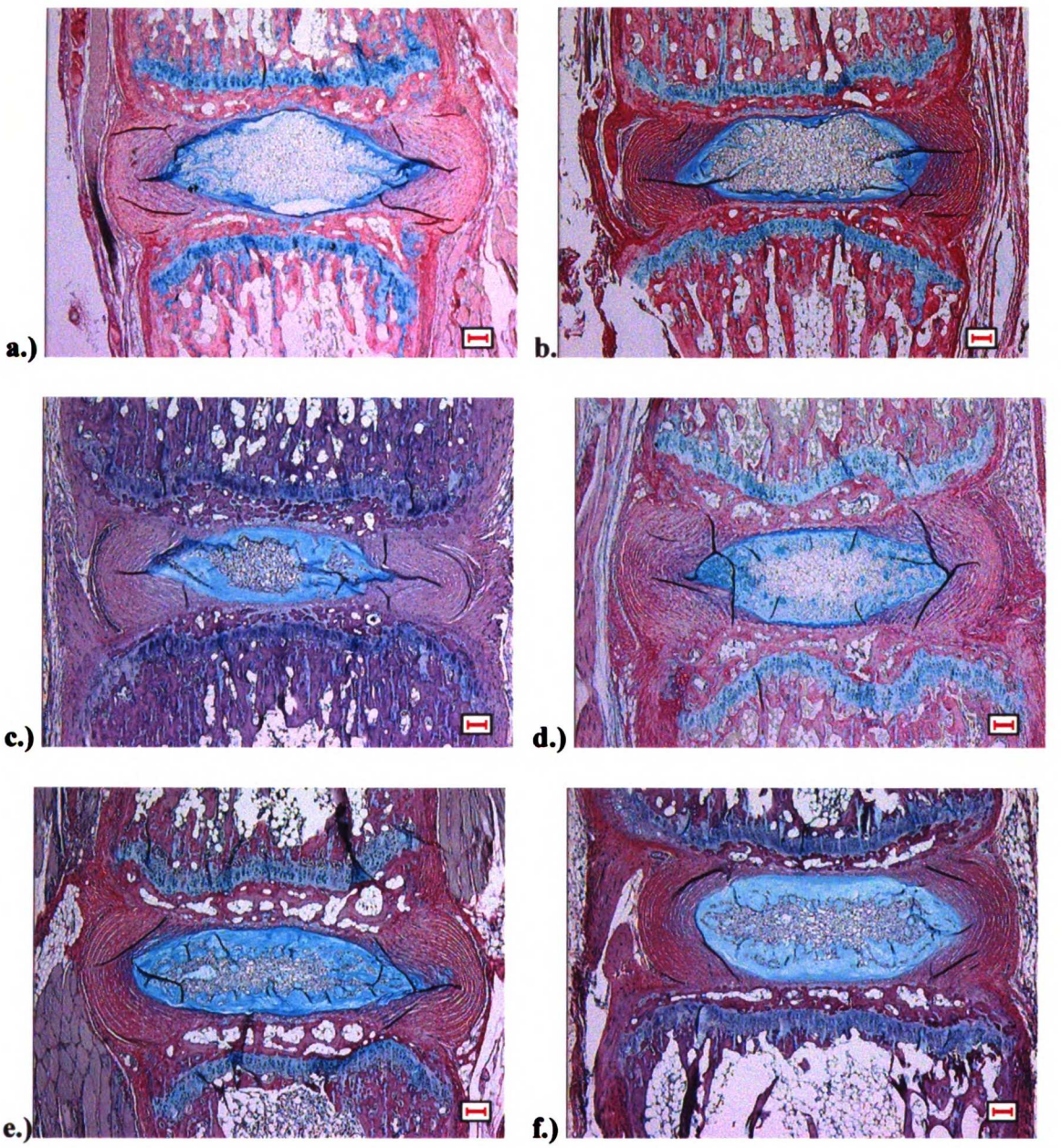

Figure 2. Mid-sagittal plane histology sections of experimental discs. Dark lines are tissue artifacts. Figure 2a.) Sham disc; 2b.) 0.1 Hz and 0.8 MPa loaded; 2c.) 0.01 Hz and 0.8 MPa; 2d.) 0.1 Hz and 1.3 MPa; 2e.) 0.01 Hz 1.3 MPa; 2f.) static load 1.3 MPa. An increase in nuclear cell compaction and proteoglycan content was observed with decreasing frequency and/or increasing stress. Red bar length = 100 μm.

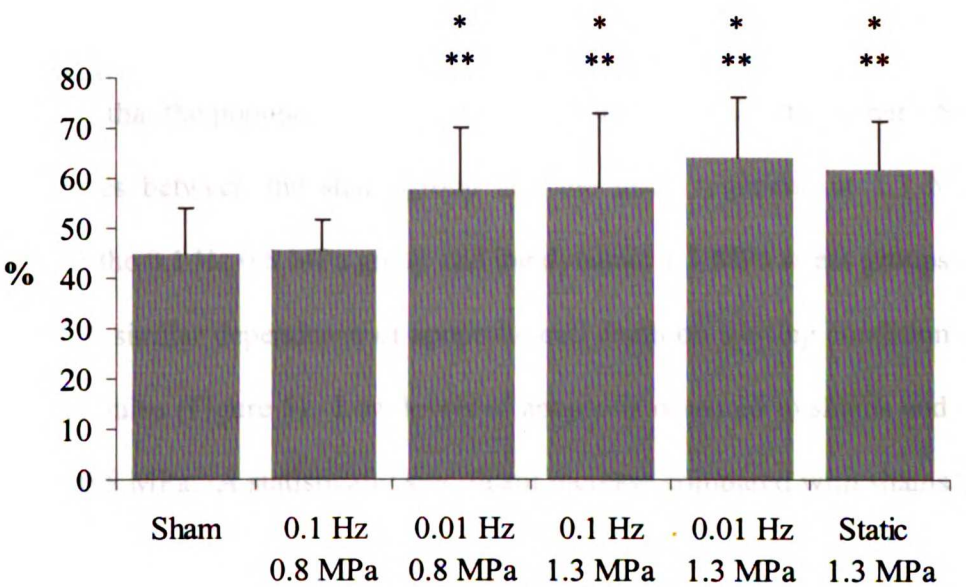

Figure 3. Proteoglycan content in the nucleus as a percentage of the area of the nucleus. * Statistically significant difference compared with sham, ** significant compared with 0.1 Hz 0.8 MPa ($p<0.05$, ANOVA and Fisher LSD test). Proteoglycan content was unchanged by loading at low stress, high frequency but increased with decreasing frequency and/or increasing stress. Data are mean ± sd.

Apoptotic Cell Death

Apoptotic cell death in the nucleus also varied with loading conditions (Figure 4). At 0.1 Hz and 0.8 MPa, levels of apoptosis in the nucleus were low and were approximately the same as in shams. The mean percentage of apoptotic cells increased after loading at 0.01 Hz 0.8 MPa. Further increase was observed after loading at 1.3 MPa, using either dynamic (0.1 or 0.01 Hz) or static loading. One-way ANOVA indicates that the population means are not equal ($p=0.03$). The Fisher LSD test detected differences between the sham group and the three regimens at 1.3 MPa stress, and between the 0.1 Hz 0.8 MPa group and the dynamic 1.3 MPa stress groups ($p<0.05$).

A similar dependence of apoptotic cell death on loading conditions was observed in the annulus (Figure 5). Low levels of apoptosis occurred in shams and discs loaded at 0.1 Hz 0.8 MPa. A statistically significant increase compared with shams and 0.1 Hz 0.8 MPa occurred when discs were loaded at 0.01 Hz 0.8 MPa or static 1.3 MPa (Fisher LSD, $p<0.05$). Annular apoptosis was also increased in discs loaded at 0.1 Hz 1.3 MPa and 0.01 Hz 1.3 MPa although not to a level that was statistically significant.

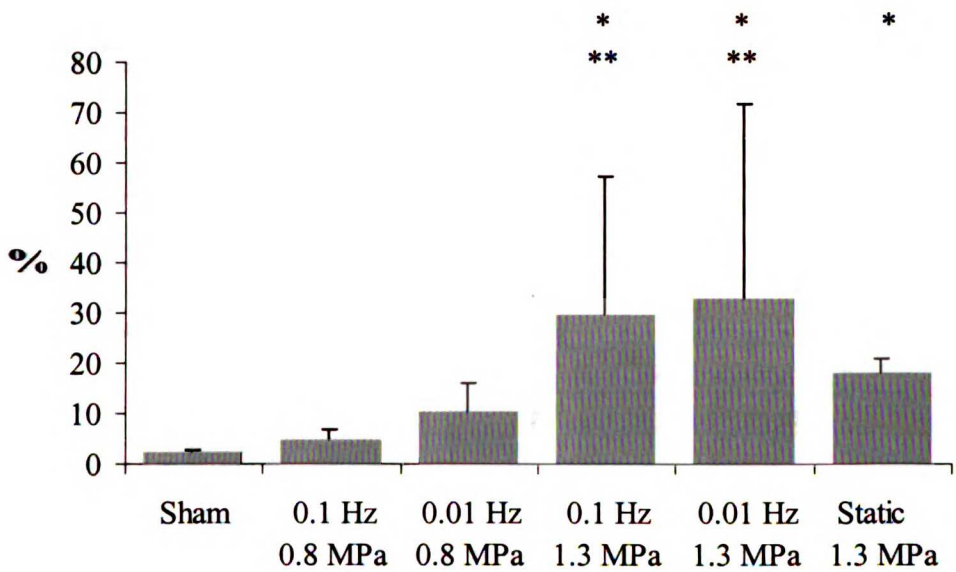

Figure 4. Percentage of apoptotic cells in the nucleus as a function of loading condition. * Statistically significant difference compared with sham, ** significant compared with 0.1 Hz 0.8 MPa ($p<0.05$, ANOVA and Fisher LSD test). Apoptosis in the nucleus was unchanged by loading at low stress, high frequency but increased with decreasing frequency and/or increasing stress. Data are mean ± sd.

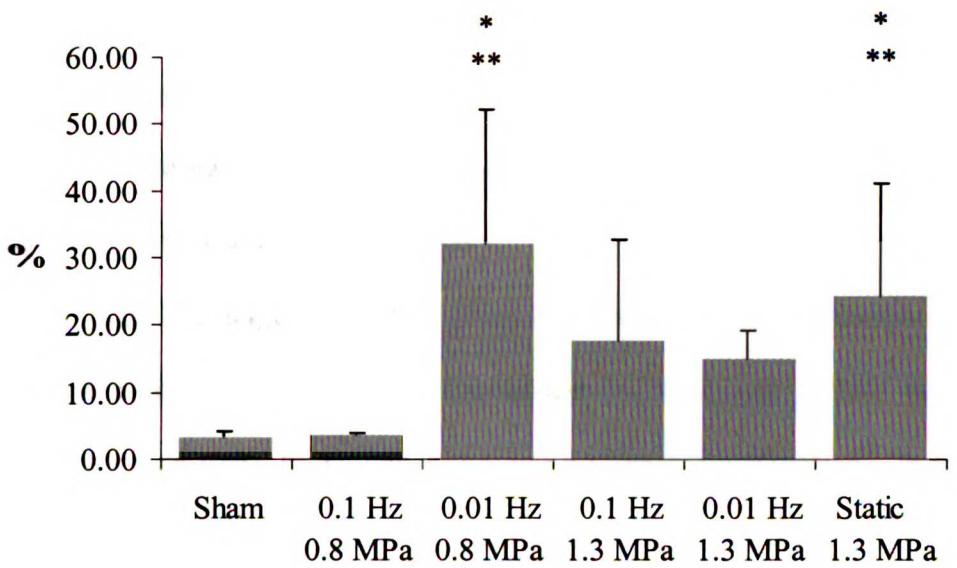

Figure 5. Percentage of apoptotic cells in the annulus as a function of loading condition. * Statistically significant difference compared with sham, ** significant compared with 0.1 Hz 0.8 MPa ($p<0.05$, ANOVA and Fisher LSD test). Apoptosis in the annulus was unchanged by loading at low stress, high frequency but increased with decreasing frequency and/or increasing stress. Data are mean ± sd.

In situ Hybridization

In situ hybridization was used to detect regional changes in aggrecan and Type II collagen gene expression. In shams, strong aggrecan expression occurred in cells of the nucleus and along the cartilage endplates (Figure 6a). Expression was usually absent in the annulus although occasionally present in some inner annular chondrocytes. A slight increase in cells containing aggrecan mRNA was observed in the annulus in discs loaded at 0.1 Hz 0.8 MPa (Figure 6b). Further increase was observed after loading at 0.01 Hz 0.8 MPa (Figure 6c) and at 1.3 MPa statically and at either frequency (data not shown). No changes to aggrecan expression within the nucleus or along the endplates of loaded discs relative to shams were observed.

Type II collagen mRNA in sham discs was detected primarily in cells along the cartilage endplate (Figure 7a). Expression in inner and middle annular cells was occasionally present. In general, there was no expression from cells in the nucleus with the exception of four of thirteen sham discs that had a few positive cells along the perimeter at the midheight. Type II collagen expression was largely unchanged after loading at 0.8 MPa stress at either frequency (data not shown). However, greater numbers of positive cells in the annulus and nucleus were detected in discs loaded at 1.3 MPa at either frequency (Figure 7b). Patterns of gene expression in the statically loaded discs at 1.3 MPa were similar to those loaded dynamically at the same stress.

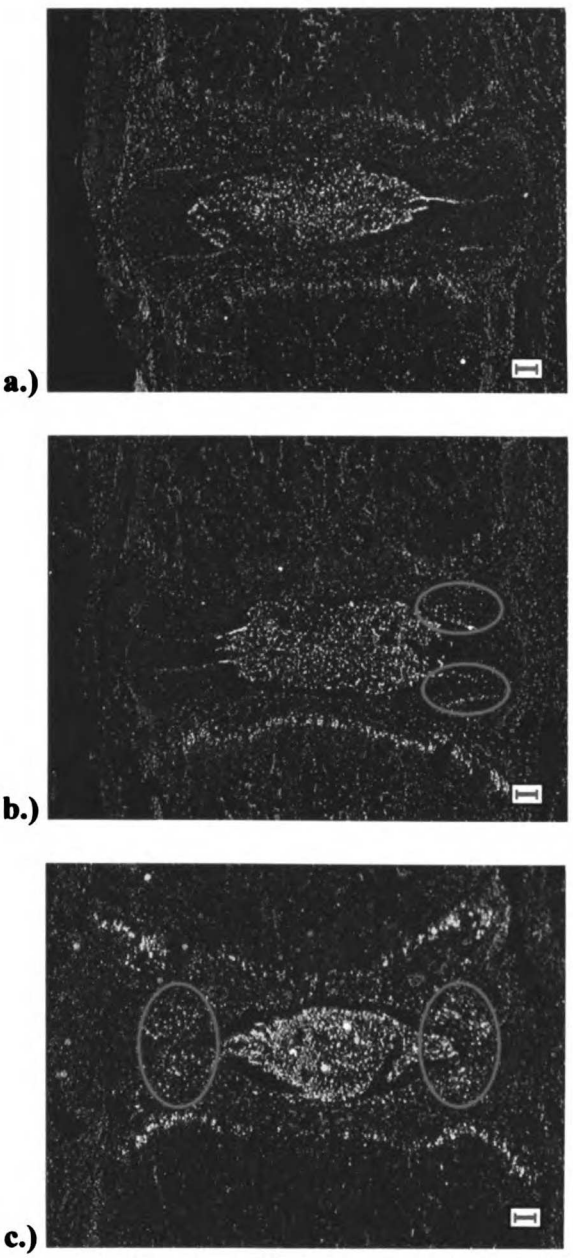

Figure 6. Aggrecan *in situ* hybridization. 6a.) sham; 6b.) 0.1 Hz and 0.8 MPa; 6c.) 0.01 Hz and 0.8 MPa. Cells positive for aggrecan mRNA appear yellow, all others appear blue. Orange rings denote areas of increased aggrecan expression. Small increases in aggrecan mRNA were observed after loading at low stress, high frequency; higher levels at all other loading conditions. Red bar length = 100 μm.

Disc height/Cell Areal Density/Annular Birefringence

In loaded discs versus sham we found no significant differences in disc height and cell areal density in the annulus or nucleus (Table 3). Under polarized light microscopy, no qualitative differences in patterns of annular birefringence were noted between loaded and sham discs.

Table 3. Disc Height and Cell Areal Density Measurements

	Normalized Disc Height† (mm/mm*1000) mean ± sd	Annular Cell Areal Density (cells/mm^2) mean ± sd	Nuclear Cell Areal Density (cells/mm^2) mean ± sd
sham	108 ± 15	1063 ± 135	1190 ± 327
0.1 Hz 0.8 MPa	107 ± 19	1163 ± 332	1345 ± 201
0.01 Hz 0.8 MPa	107 ± 19	1006 ± 175	1350 ± 418
0.1 Hz 1.3 MPa	105 ± 13	985 ± 212	1169 ± 119
0.01 Hz 1.3 MPa	100 ± 11	1066 ± 202	1113 ± 331
static 1.3 MPa	110 ± 14	821 ± 176	993 ± 280

† Resting disc height divided by mean length of adjacent vertebral bodies

Discussion

Our results indicate that dynamic loading can induce differential effects in the disc. These effects vary with the stress and frequency of loading. For example, proteoglycan content increased in the nucleus following lower frequency and/or higher stress loading. Cell death in the nucleus and annulus was also higher after loading at

lower frequency and/or higher stress. Thus, frequency, in addition to stress magnitude and duration, is an important variable to the regulation of disc cell function *in vivo*.

Interestingly, we observed effects to disc cell viability from loading in the absence of obvious structural damage to the annulus. Previous studies proposed that cell death observed in cyclically loaded chondrocytes and smooth muscle cells is an early stage event in the pathogenesis of osteoarthritis and vein graft arteriosclerosis, respectively [115, 133, 134]. In the disc, increasing levels of apoptosis have been associated with the progression of degeneration [135, 136]. In the present study, therefore, the increases in cell death under certain loading conditions portend an eventual degenerative outcome in those discs. Our results support the hypothesis that degeneration can be the result of an active cellular response to loading and not merely a reflection of acute trauma to the structural integrity of the disc.

In contrast to cell death, however, we also saw evidence of an anabolic response to loading. Increased proteoglycan content within the nucleus and increased matrix gene expression in both annulus and nucleus were observed. These changes suggest a reparative reaction to an altered mechanical environment that seeks to restore homeostasis. Such a response is not unexpected. A similar stimulation of matrix gene expression was shown in a previous experiment when mouse discs were statically loaded for short durations and then allowed to recover [137]. An *in vitro* study of cyclic tension on nucleus pulposus cells also demonstrated a significant, although transient, increase in collagen synthesis [138]. The anabolic response of cartilage explants or cell constructs *in vitro* to dynamic loading has been demonstrated by numerous studies. In general, matrix

synthesis was stimulated by cyclic compression at frequencies similar to those in this experiment [111, 112, 139, 140].

The results from our study appear to agree with the proposal of a cell tolerance to mechanical loading [100]. At the 0.8 MPa stress level, the cellular response was more vigorous at lower frequency loading. The lack of significant cell death at 0.1 Hz 0.8 MPa indicates that the stimulus for apoptosis was below some threshold. At the same time, a small increase relative to shams in aggrecan gene expression suggests that a different threshold, one for matrix synthesis, had been exceeded in some cells but not others. When the frequency was decreased or the stress increased, a greater stimulus may have been provided that exceeded the threshold for apoptosis or matrix synthesis from a greater number of cells.

The exact stimulus is unclear but may be closely related to tissue strain [141, 142]. Strain produced by mechanical loading may affect cellular activity directly by cell and matrix deformation [143, 144] or indirectly by altering features such as tissue permeability, water content, pH, and oxygen tension [59, 145, 146].

We hypothesize that combinations of frequency and stress result in unique strain environments that may explain the differential cellular responses. To test this hypothesis, we used a finite element model of the disc to predict the strain energy density generated by the various loading conditions used in this study. Strain energy density has been theorized as a remodeling stimulus in bone [147, 148] and was used in place of the strain tensor to simplify the description of the strain state. Because of the viscoelastic properties of the disc, frequency and stress combine to produce different amounts of strain energy density (Figure 8). The values in the figure are for an element along the

midheight of the inner annulus and have been normalized to the high frequency, low stress case. The lowest strain energy density (SED) occurs for the high frequency, low stress case where the biological response was the least, and the SED (and the response) increases when the frequency is lowered and/or the stress level is raised. Static load at the highest stress results in the greatest strain energy density. Similar trends were also predicted for hydrostatic strain and maximum shear strain.

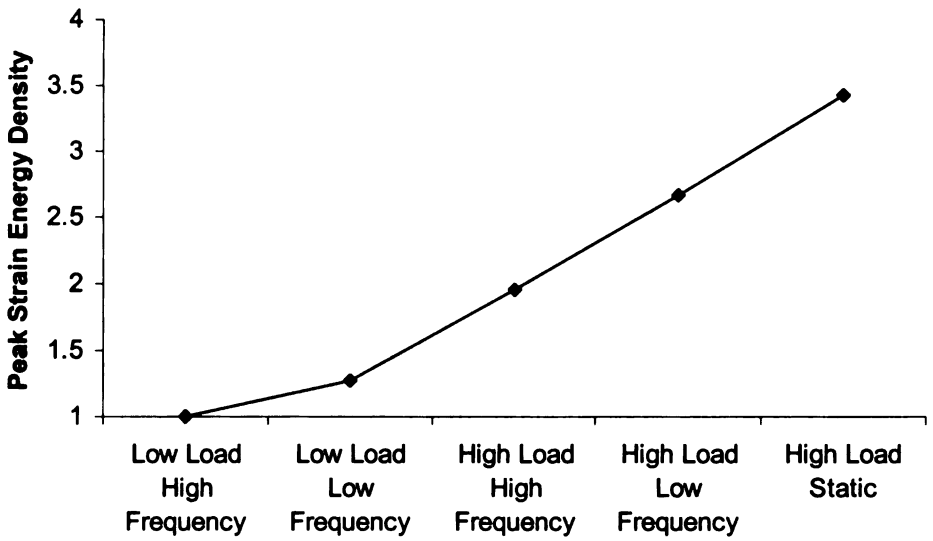

Figure 8. Finite element model predictions of the peak strain energy density in the midheight inner annulus. Results normalized to the value at high frequency, low stress.

Interestingly, the response of cells to the three loading conditions at 1.3 MPa (0.1 Hz, 0.01 Hz, and static) were statistically equivalent though the relative strain energy densities were different. In other words, the differences in response were sufficient to observe a tolerance limit but not a graded response in the sample size employed. This suggests a response limit to 1.3 MPa stress within the period of loading in this experiment. Further distinction between responses may require varying the levels of stress, frequency, or duration of the loading. Duration may be particularly significant, as

temporal effects of strain on both the matrix (fatigue) [42, 43, 149] and cells [100, 150, 151] are an important aspect of remodeling induced by cyclic loading.

A single parameter, the daily stress (or strain) stimulus, has been theorized for bone remodeling to account for the cumulative effects of cyclic loading [147, 148, 152]. We compared this theory with our observations of remodeling in the disc by calculating the daily stimulus using the peak strain energy density values from our finite element model:

$$\Psi = \left[\sum_{day} n_i U_i^{\,m} \right]^{1/m}$$

where U = peak strain energy density (N/mm^2), n = number of peak events, and *m* is a weighting term. Based on strain energy density, proposed values of *m* for bone remodeling range from 1 to 4. (The stimulus was derived using the energy effective stress or strain, which is proportional to $U^{1/2}$. Values of *m* based on effective stress or strain for bone range from 2 to 8) [147, 153]. Values of the daily stimulus in our experiment would be expected to increase for loading conditions in which an increased biological response was observed. However, the theory did not correlate with our results using values of *m* in the range of those for bone. This may reflect tissue variability in the weighting of peak mechanical events that stimulate the resident cells. A value of $m = 10$ does result in an increasing trend of daily stimulus that correlates with our data. Alternatively, theories based on peak magnitudes may be insufficient to describe disc remodeling because they do not account for the entire strain history [150, 154]. For example, the duration of loading *per cycle* may be an important temporal effect that influences cell response more than simply achieving a particular strain.

The stresses and frequencies in this study were chosen because they were within the range of human activity. Manual material handling requires that individuals lift and carry heavy loads with repetition. The compressive force at the human L5/S1 level has been estimated to be 1.2 to 1.3 times body weight, or approximately 900 N [155, 156]. At the same level, a force of approximately 1500 N is generated by picking up a 30 lb weight [119]. For a given weight in hand, the force on the disc can increase dramatically depending on posture, body weight, and lift acceleration [157]. Assuming an average cross sectional area of 1250 mm^2 for a human L5 vertebral body endplate [158], the stresses produced by these activities are in the range of 0.7 to 1.2 MPa. In this experiment, we used similar values of stress (0.8 and 1.3 MPa) applied to mice. However, there is variability in what may be considered a "high stress" between mice and humans. Although the highest level of stress in this study, 1.3 MPa, is quite high for a murine caudal disc, stresses in human discs can be much greater [31, 157]. Similarly, humans are exposed to frequencies higher and lower than those used in this study [157]. Therefore, it is important to recognize that the loading conditions used here are a small sample of human exposure.

Based on our results, the strain produced by combinations of stress and frequency may be more important to disc health than the magnitude of either alone. Importantly, it would be erroneous to hold too strongly to the exact values of *stress* and *frequency* in extrapolating these results to humans. Instead, we hypothesize that the biological events that accompany specific *strains* in the mouse may likely also occur when the same strains are produced in humans. In the future, we plan to use analytical and numerical models of

disc deformation under load to correlate murine disc strains and biological remodeling to that in humans.

There are a few limitations in our study that deserve consideration. The device is designed to apply compression symmetrically. However, some discs appeared to have been loaded asymmetrically. This was evidenced in histology, cell death, or *in situ* hybridization observations. Having looked at multiple sections, however, from all the mice in all the groups, asymmetry of loading appears to be a small, random error distributed over all the groups. There is no reason to believe that any one group had more asymmetry than another. So, it is not a significant factor in the interpretation of the results.

A second limitation was large variability in the cell death data within some groups. One explanation is variability in the function of each device, although every effort was made to assure that all devices function in the same way. The other possibility is variability in the individual tolerances of different mice. How much is attributed to each is unclear, but may be better understood in the future after refinements and improvements to the function of the device. Regardless of the cause, a larger sample size may have led some of the observed trends to become significant.

We believe the trends identified from observations of a few sections per disc are representative of the tissue whole. In this study and in previous studies, multiple sections for each disc have been analyzed, and it was found that for nearly all of the discs there was little section-to-section variability. This may be unique to the mouse because the total volume of the disc is small. In a larger animal, it may well be necessary to analyze multiple sections to get a good representation of the whole.

Generally speaking, the discs in our study were not constrained in bending. The diameter of the device housing is much larger than the diameter of the tail, and the bladder when deflated is compliant so the tail can bend. But, there is some constraint because of the rigidity of the washers and housing. This is controlled for by comparing loaded discs to shams that also wore the device. Any effect to the disc because of bending constraints is small because the shams look much like normal discs.

The adult murine nucleus pulposus contains notochordal cells. In contrast, notochordal cells are eliminated in humans during childhood [159]. The question then is how is death of notochordal cells in mouse discs relevant to remodeling in human discs? First, the mouse model may provide some understanding of the fate of notochordal cells in the young human. Second, with regards to cell death, our study demonstrates that it is possible to create an environment through mechanical loading that is deleterious to cell viability. If cell death had been limited to notochordal cells, this might suggest some property of notochordal tissue alone. However, appreciable cell death was also observed in the annulus under certain loading conditions. In essence, we have triggered apoptosis in a heterogeneous population of cells in a structurally intact disc *in vivo*. Given that the human disc has a similar structure and also a heterogeneous population of cells, our results indicate that similar effects may be produced in human discs by loading.

Conclusions

In conclusion, our study demonstrates that tissue remodeling within the disc depends upon the dynamic loading conditions to which it is exposed. The results suggest that maintenance of healthy tissue may be achieved by appropriate selection of stress,

frequency, and duration. Beyond some threshold, however, degenerative changes may occur.

In the future, we plan to expand our model to include bending and to study the response over a broad range of loading conditions. From this we hope to define the dose response of the disc and to identify the mechanisms that distinguish beneficial from degenerative outcomes. These results, in turn, will be important in developing preventative strategies for disc degeneration.

Chapter 4. *In Vivo* Growth Factor Treatment of Degenerated Intervertebral Discs

Introduction

Current modalities for alleviating discogenic pain leave the degenerated disc untreated, partially removed, or entirely eliminated. In the long-term, many patients find that the earlier benefit from conservative or surgical treatments diminishes [90, 160-162]. Lacking in the clinicians' arsenal are methods to repair damaged discs. Disc repair might be used in concert with conservative therapy to treat defects that might otherwise linger or worsen. After partial discectomy of a herniated disc, repair could provide a means for replacement of the excised tissue and improvement of spinal stability. During fusion, repair of early stage degeneration at adjacent levels might minimize the risk of subsequent complications.

Biological approaches to repair target the diminished cell and matrix contents of degenerated discs [94, 95]. The macromolecular content of the nucleus and annulus are critical to their normal structure and function. With age and degeneration, a decrease in the nuclear proteoglycan content and quality is observed [23, 24, 27]. The nuclear swelling pressure, as a result, is reduced as the nucleus is less able to imbibe water [127]. The ordered arrays of collagen fibers in the annulus become disorganized and frayed, probably due to alterations of their mechanical environment [28, 29]. One approach to biological repair is to restore the nuclear swelling pressure by increasing the synthesis of proteoglycans within the nucleus. Following this, one might expect an increase in disc height and improvement in mechanical function and stability. Increase in the nuclear

swelling pressure would restore tensile stress to the annulus. This in turn could stimulate annular cells to produce new collagen leading to repair of defects in the annular structure.

Growth factors have been proposed as a stimulus for matrix synthesis within the degenerated disc. Thompson et al. studied the effects *in vitro* of IGF-1, EGF, bFGF, and TGF-β on cultured cells from normal canine discs [163]. Cells from the annulus, transition zone, and nucleus were responsive to growth factors with regards to increased cell proliferation and proteoglycan synthesis. More recently, Takegami et al. and Matsumoto et al. [164, 165] describe increased proteoglycan synthesis of rabbit disc cells from the nucleus and annulus when treated with OP-1 (BMP-7) *in vitro*. Nishida et al. developed an *in vivo* rabbit model of adenovirus-mediated transfer of the human TGF-β encoding gene [166]. They observed an increase in TGF-β expression and a 100% increase in proteoglycan synthesis after transfection.

Studies to date have demonstrated that exogenous growth factors can regulate normal disc cell function both *in vitro* and *in vivo*. However, the *in vivo* response of cells within degenerated discs is unknown. Further, realistic expectations of the extent and nature of regeneration and the definition of a beneficial response are unclear. In this study, we used an existing murine model of disc degeneration to test the hypothesis that regenerative remodeling in degenerated discs can be stimulated by growth factor treatment. We evaluated four different growth factors: TGF-β, IGF-1, GDF-5, and bFGF. Our objective was to explore alterations of the spatial and temporal pattern of disc cellularity and matrix gene expression *in situ* following injection of growth factors into degenerated discs. These growth factors were selected for their reported potential for

chondrogenesis and matrix production either in disc or cartilage. We explore the issues related to effective growth factor treatment and the potential for a beneficial outcome.

Methods

Animals Groups

A total of seventy-eight adult (twelve week old), male Swiss Webster mice were used in this study. The UCSF Committee on Animal Research approved all procedures involving mice. Degeneration was induced in a caudal disc of each mouse by mechanical compression as described below. The mice were then divided into one of three groups (Table 4). Degenerated discs in Group 1 (n=30) were injected once with one of the growth factor solutions or saline as a control and then sacrificed after one week. Group 2 discs (n=22) were injected once and sacrificed after 4 weeks. Group 3 discs (n=25) were injected multiple times, once a week for four weeks, and then sacrificed four weeks after the first injection.

Table 4. Numbers of Animals Per Experimental Group

	Group 1 Single Injection, 1 Week Timepoint	**Group 2** Single Injection, 4 Week Timepoint	**Group 3** Multiple Injections, 4 Week Timepoint
PBS – control	6	5	5
GDF-5	5	5	6
TGF-β	7	7	5
IGF-1	6	5	5
bFGF	6	--	4

Disc Degeneration Model

Degeneration of a single, caudal disc was induced by applying static compression to the disc (Figure 9). The loading method has been previously described [135]. The procedure began with palpating the tail to identify the tenth coccygeal disc. Stainless steel insect pins (0.4 mm diameter) were inserted percutaneously through adjacent vertebral bodies using a small drill. Two pins were inserted perpendicularly in each vertebral body. An elastic loop was then placed around each of the four opposing ends of the pins to compress the disc at 1.3 MPa stress. The calibrated loops were made by cutting silicone tubing at a measured thickness to produce the desired force when stretched around the pins. A plastic cylinder to prevent the mouse from chewing the elastics covered the loaded disc. The mouse was afforded free range of motion while the loading device was in place. The discs were loaded for seven days, and then the cover was removed and the load released by cutting the elastics. The pins were cut flush to the skin but not removed. After a three week period of no loading, injection treatment was begun.

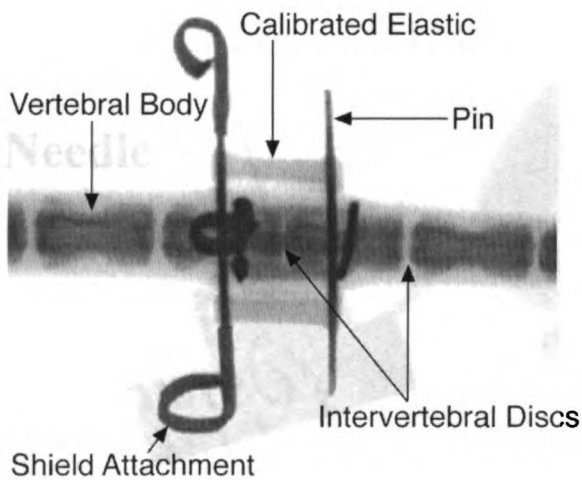

Figure 9. Static loading device to induce coccygeal disc degeneration in a mouse.

Growth Factor Injection

Each degenerated disc was injected with an equal volume of one of five solutions: 1 μg/ml GDF-5 (gift from BioPharm), 1 μg/ml IGF-1 (Sigma), 1 μg/ml bFGF (gift from Orquest), 200 ng/ml TGF-β (Sigma), and PBS as a control. All solutions contained 0.1% bovine serum albumin (Sigma) to minimize undesired protein adhesion to the walls of the tubing and needle used for injection. Injections were made by inserting a 33-gauge needle (Hamilton) directly into the center of the disc. A plastic jig was used to fix the tail in place and guide the needle into the center (Figure 10). X-ray confirmed the location of the needle prior to each injection. To overcome the inherent swelling pressure in the nucleus, an excess volume, 8 μl, was slowly injected over ten minutes to allow time for the solution to perfuse into the tissue. Preliminary injections on separate mice were conducted using FITC-labeled dextran (MW 70kD) in solution (Molecular Probes) to verify that the injection protocol results in delivery and retention of high molecular weight compounds to the center of the disc (Figure 11).

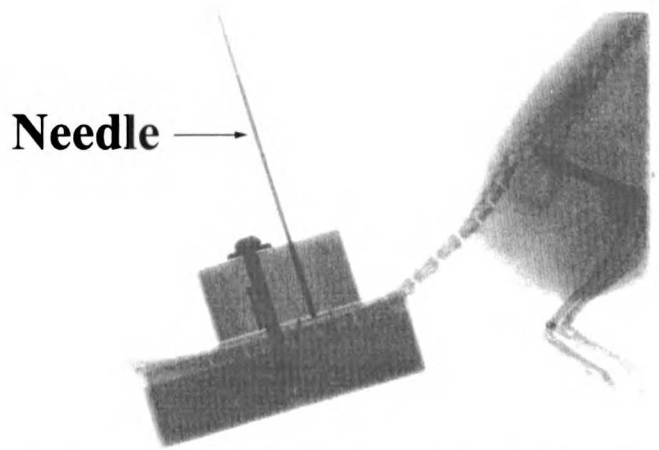

Figure 10. X-ray of injection procedure showing the tail fixed within an acrylic jig to guide the needle into the center of the disc.

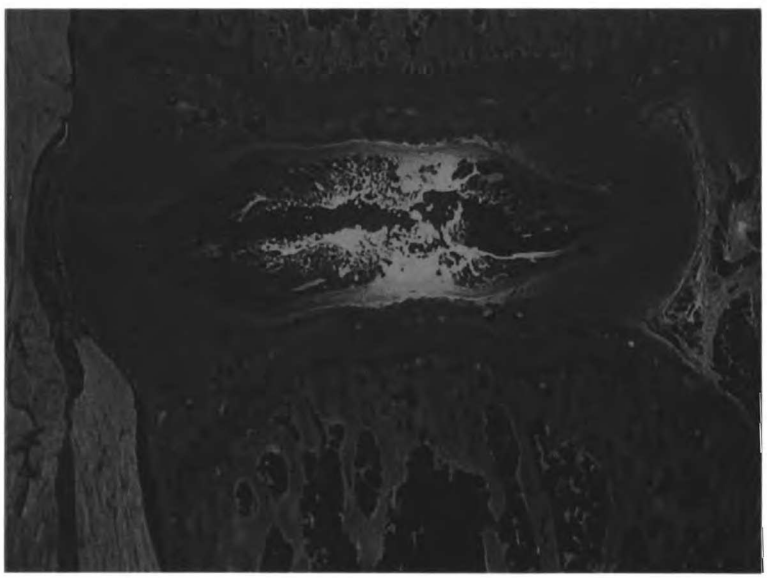

Figure 3. Localization of FITC-labeled dextran in the center of the disc to validate injection method.

Tissue Processing

After carefully removing the skin, the motion segment containing the treated disc was removed by cutting through adjacent levels. The tissue was fixed in 4% paraformaldehyde (Sigma, St. Louis, MO) overnight at 4 °C and then decalcified in 19% ethylenediamine tetra-acetic acid (EDTA, Sigma, St. Louis, MO) for seven to ten days at 4 °C. The pins were removed from the tissue once the vertebrae were decalcified when they could be easily removed with a minimum of force. The tissues were processed through a graded series of ethanol and xylene, embedded in paraffin, and sectioned 6 μm thick. The tissue was processed using RNase-free solutions and instruments for subsequent *in situ* hybridization.

Histology and Image Capture

Tissue sections were rehydrated and stained by the Hall's and Brunt's Quadruple (HBQ) stain method [120]. This method uses alcian blue to stain proteoglycan, direct red to stain collagenous tissue, and Mayer's hematoxylin and celestine blue to stain cells. Sections were observed under a Nikon E800M microscope (Melville, NY). Images were captured using an Optronics digital camera (Goleta, CA) and Simple PCI imaging software (Compix, Cranberry Township, PA).

Disc Height Measurement

Disc height was measured on images of mid-sagittal, HBQ stained sections. Simple PCI software was used to measure the distance between cartilaginous endplates at three places in the nucleus pulposus. The three disc height measurements were averaged

and normalized to the mean length of the two vertebral bodies in each section. One-way ANOVA followed by a Fisher LSD post-hoc test at α=0.05 was used to detect significant differences between groups.

Immunostaining

Proliferating cells were identified by immunostaining for proliferating cell nuclear antigen (PCNA) [167, 168]. Mid-sagittal plane sections were deparaffinized, rehydrated and blocked in 3% H_2O_2. After washing, sections were blocked using Mouse-On-Mouse kit blocking reagent (Vector, Burlingame, CA). The tissue was incubated with a mouse monoclonal antibody to PCNA (Santa Cruz Biotech, Santa Cruz, CA) at room temperature for 45 minutes. Washing five minutes in PBS was repeated three times followed by application of a biotinylated anti-mouse secondary antibody (Mouse-on-Mouse kit, Vector, Burlingame, CA) for 30 minutes at room temperature. The sections were again washed and then treated with avidin-biotin complex (ABC) reagent (Vector, Burlingame, CA) for thirty minutes followed by DAB reagent (Vector, Burlingame, CA). Sections were counterstained with hematoxylin (Vector, Burlingame, CA).

Images of PCNA immunostained sections were captured and analyzed under brightfield microscopy using Simple PCI software. Starting at a defined distance (150 μm) from the outer edge of the annulus, a rectangular region of interest (ROI) was drawn over the middle annulus, inner annulus, and transition zone. The height of the ROI was the same on each image, 150 μm above and below the midheight of the disc. The width of the ROI varied on each image with the size of the inner annulus and transition zone. The total number of cells, the number of PCNA positive cells, and the area of the ROI

were measured. Total cell density was calculated by dividing the cell count by the area of the ROI. The percentage of proliferating cells was calculated by dividing the number of PCNA positive cells by the total number of cells. One-way ANOVA at α=0.05 was used to detect significant differences between groups.

In situ hybridization

Regional expression of aggrecan and type II collagen genes within treated discs was analyzed by *in situ* hybridization. The probe used to detect aggrecan mRNA was a ^{35}S-labeled antisense RNA probe prepared by T3 polymerase transcription of ApaI-linearized bluescript plasmid-containing insert for aggrecan cDNA. The probe to collagen II mRNA was prepared by T3 polymerase transcription of EcoRI-linearized Bluescript plasmid-containing insert for mouse α1 chains of procollagen II cDNA.

Sections were deparaffinized, treated with proteinase K (20 µg/ml, 5 minutes at room temperature), and acetylated using acetic anhydride. Slides were hybridized overnight at 53°C with 33 µl of hybridization solution containing approximately 2X10^6 cpm of probe. The hybridization-probe solution was removed by washing the slides in 5XSSC containing 20 mmol/l β-mercaptoethanol (twice for 30 minutes each at 55°C), then in high stringency wash of 50% formamide, 2XSSC containing 40 mmol/l β-mercaptoethanol (twice for 30 minutes each at 60°C). The sections were subsequently equilibrated in NTE buffer (10 mmol/l Tris-HCl, pH 8, 0.5 mol/l NaCl, 5 mmol/l EDTA, twice for 15 minutes each at 37°C) before incubation with 20 µg/ml RNase (Sigma, St. Louis, MO) (30 minutes at 37°C) to digest unbound probe, then rinsed again in NTE (15 minutes). The high-stringency wash was repeated (30 minutes) before the final washes at

room temperature in 2XSSC and 0.1XSSC (15 minutes each). Slides were dehydrated, coated with Kodak NBT-2 emulsion (Scientific Imaging Systems, Eastman Kodak, Rochester, NY), and exposed for several days, then developed and counterstained with Hoechst dye (Sigma, St. Louis, MO).

In situ hybridization was observed under dark field microscopy using a darklight stage (Dolan Jenner, Lawrence, MA) to illuminate silver grains on the slide. Fluorescence microscopy using a Dapi filter was used to observe cells counterstained with Hoechst dye. Cells in the adjacent growth plate were used as control.

Results

Disc Degeneration Model

The structure and composition of a normal mouse disc is similar to that of a young human (Figure 12). The gelatinous nucleus pulposus is located centrally and contains high water content. It has a cellular interior with proteoglycan around the perimeter. Cells in the mouse nucleus are predominantly notochordal. The annulus fibrosus forms a collar of collagenous tissue around the nucleus. It is composed of layers of ordered collagen fibers with fibrochondrocytes in the inner annulus and fibroblast-like cells in the outer annulus.

The protocol for static loading on the disc results in a degenerated phenotype from which the disc does not spontaneously recover (Figure 13). Nuclear volume and disc height are reduced after loading. The nucleus is changed to a largely acellular region as a result of cell death and matrix compaction. Outward bulge of inner- and middle-annular lamellae is eliminated, and disorganization of collagen fibers occurs. Cellularity

occurs. Cellularity in the annulus is also diminished, and the cells that remain are typically larger chondrocytic cells. Metaplasia of fibrous tissue to fibrocartilage is observed in middle and outer regions of the annulus.

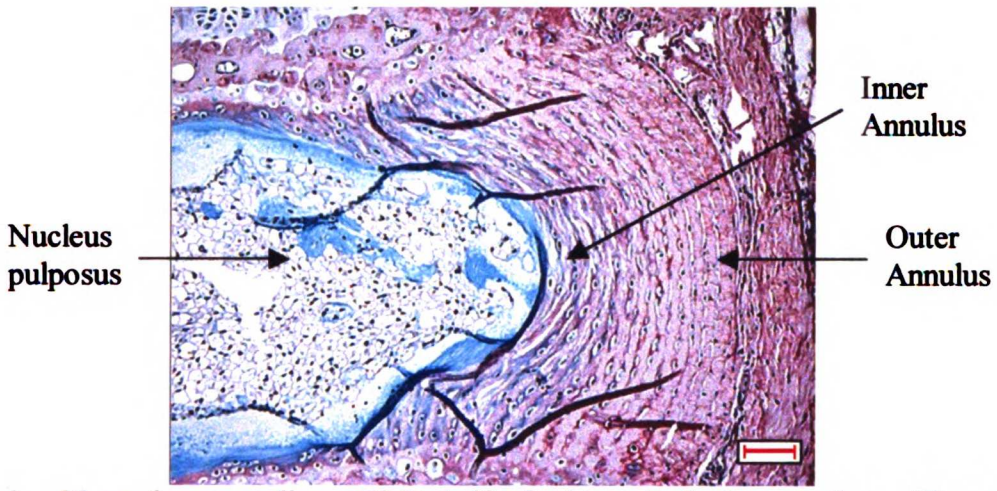

Figure 4. Normal mouse disc. Right half of mid-sagittal plane section. The nucleus contains proteoglycan and notochordal cells. The annulus is composed of ordered lamellae of collagen fibers and cells. Red bar length = 100 μm.

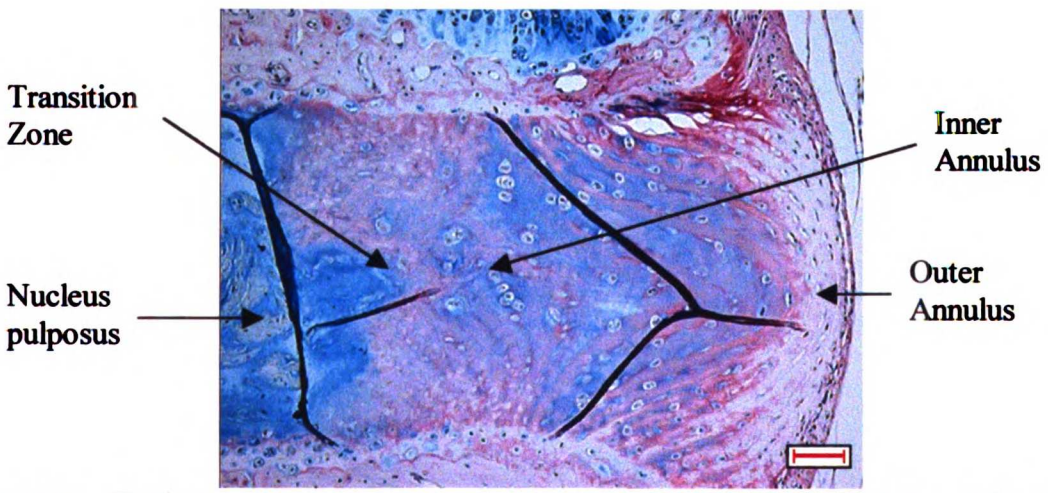

Figure 5. Typical compression-induced degenerated disc. Nuclear and annular cellularity is decreased, nuclear volume is decreased, and collagen lamellae in annulus are disorganized. Red bar length = 100 μm.

One Week Response to Single Growth Factor Injection

Overall, histologic examination revealed no significant differences between the five treatments one week after injection. Discs within each group displayed variable responses. Some regions of the inner annulus from each treatment (GDF-5, TGF-β, IGF-1, bFGF, or saline controls) contained clusters of chondrocytic cells while others were relatively acellular. No significant alterations to the cellularity of the nucleus pulposus were noted.

The mean cell density in the middle and inner annulus and transition zone was higher in bFGF and IGF-1 treated discs, although no significant differences between groups were detected (ANOVA, p=0.356) (Figure 14). The mean percentage of proliferating cells in the same region was larger relative to saline controls in discs treated with TGF-β or IGF-1 (Figure 15). However, the differences between groups were not significant (ANOVA, p=0.116). Note that larger standard deviations for growth factor treated discs reflect higher variability in the response to the growth factors compared with saline controls.

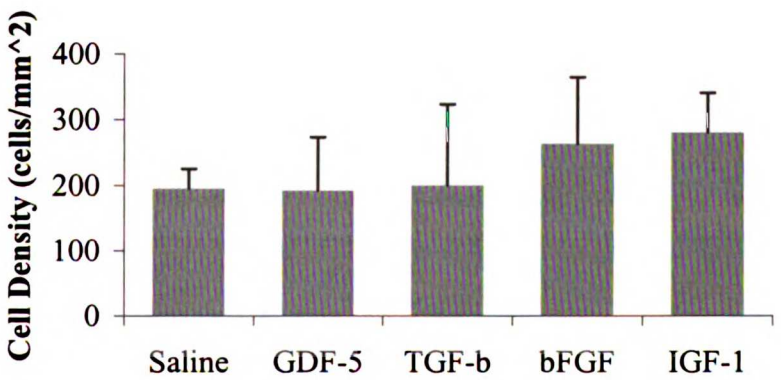

Figure 14. Mean cell density in the middle and inner annulus and transition zone one week after single injection. Higher cell density for bFGF and IGF-1 treatments compared with saline treatment. Data is mean ± sd. Differences between groups were not significant (ANOVA, p=0.356, n6 per group).

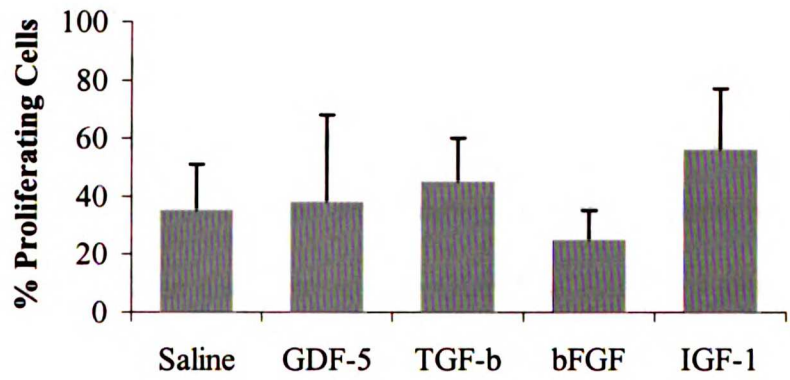

Figure 15. Mean percentage of proliferating cells in the middle and inner annulus and transition zone one week after single injection. Higher percentages following TGF-β and IGF-1 treatments compared with saline treatment. Measured by PCNA immunohistochemistry. Data is mean ± sd. Differences between groups were not significant (ANOVA, p=0.116, n6 per group).

Four Week Response to Single Growth Factor Injection

At four weeks, clusters of inner annular fibrochondrocytes were qualitatively more abundant in the GDF-5 (Figure 17) and IGF-1 (Figure 18) discs than the saline controls (Figure 16). In several cases, these cell populations extended into the nuclear space (Figure 17, yellow oval). Discs treated with TGF-β did not appear significantly different from saline controls. Single injection of bFGF at four weeks was not tested.

In GDF-5 treated discs at four weeks, the mean cell density in the middle and inner annulus and transition zone was higher compared to saline injected controls. However, the differences between groups were not significant (ANOVA, $p=0.333$) (Figure 19). The mean percentage of proliferating cells in the same region was larger relative to saline controls in discs treated with GDF-5 or TGF-β. Again, the differences between groups were not significant (ANOVA, $p=0.257$) (Figure 20).

Disc height measurements between treatment groups four weeks after a single injection were significantly different (ANOVA, $p<0.001$). Multiple comparison testing suggests that disc height was significantly increased following GDF-5 injection compared with saline (Fisher LSD test, $p<0.05$) (Figure 21). Disc height was also greater in IGF-1 injected discs compared with saline injected although not to a significant level. No significant difference was observed for TGF-β injected discs compared with saline. By comparison, all treatments were significantly less than sham discs that were neither loaded nor injected (Fisher LSD test, $p<0.05$).

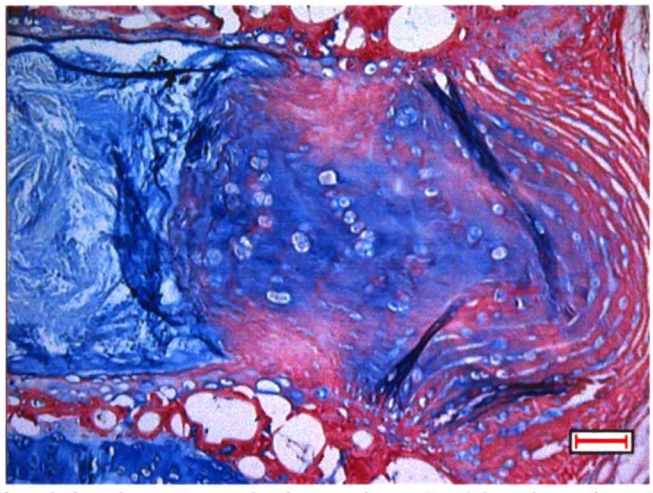

Figure 8. Single saline injection, 4 week timepoint. Red bar length = 100 μm.

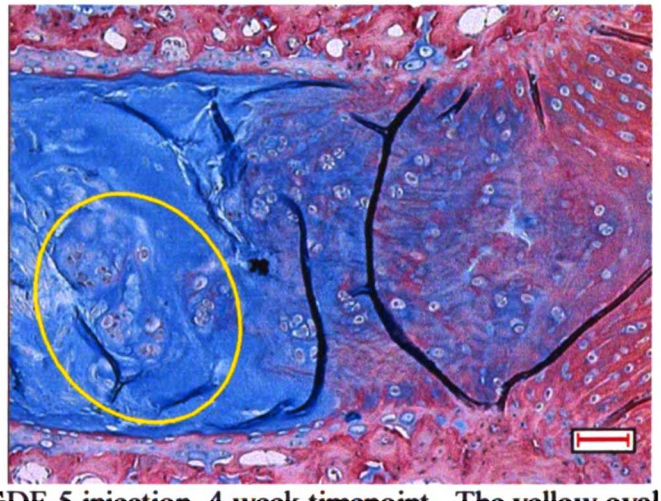

Figure 9. Single GDF-5 injection, 4 week timepoint. The yellow oval indicates annular-derived chondrocytic cells within the degenerated nucleus. Red bar length = 100 μm.

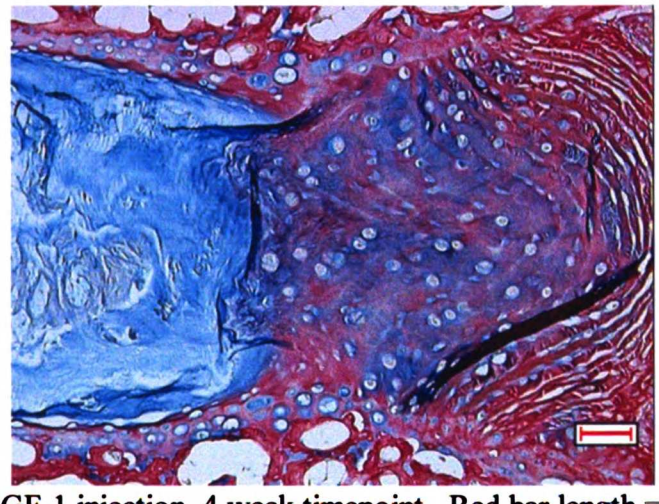

Figure 10. Single IGF-1 injection, 4 week timepoint. Red bar length = 100 μm.

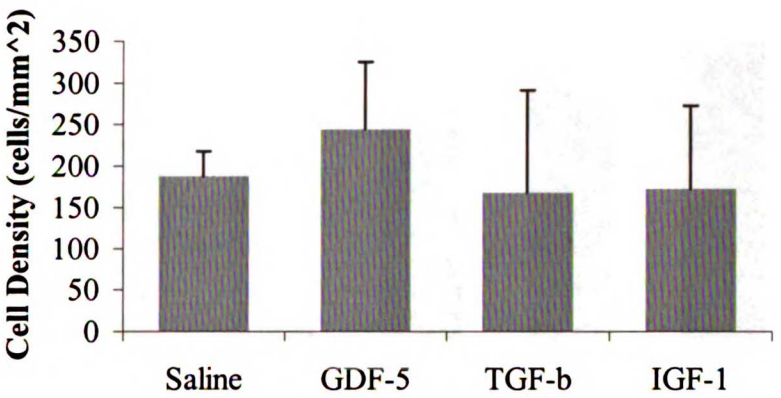

Figure 19. Mean cell density in the middle and inner annulus and transition zone four weeks after single injection. Higher cell density for GDF-5 treatment compared with saline treatment. Data is mean ± sd. Differences between groups were not significant (ANOVA, p=0.333, n5 per group).

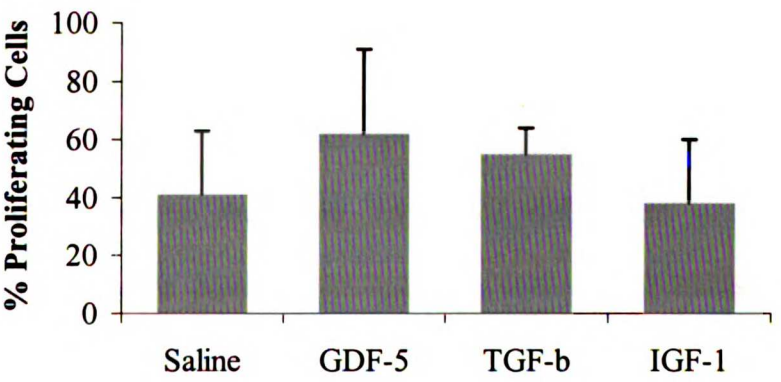

Figure 20. Mean percentage of proliferating cells in the middle and inner annulus and transition zone four weeks after single injection. Higher percentages following GDF-5 and TGF-β treatments compared with saline treatment. Measured by PCNA immunohistochemistry. Data is mean ± sd. Differences between groups were not significant (ANOVA, p=0.257, n5 per group).

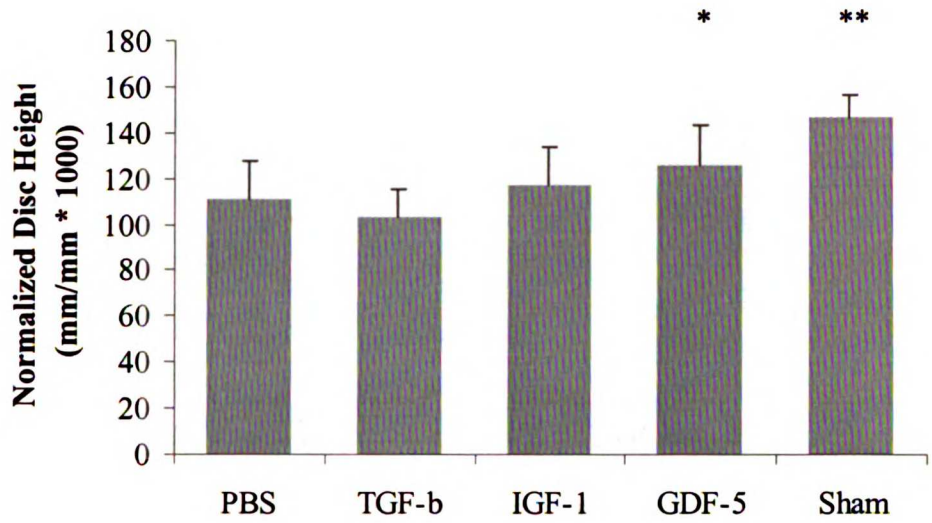

Figure 21. Disc height at four week timepoint. Data is mean ± sd. * Statistically significant difference from saline injected discs (Fisher LSD test, $p<0.05$, n5 per group). ** Statistically significant difference from all treatment groups.

In situ hybridization for aggrecan and type II collagen mRNA was performed to identify cells with a chondrocytic phenotype. In general, cells in the inner and middle annulus from all groups were positive for aggrecan and type II collagen mRNA. Aggrecan and type II collagen co-localized. A qualitative difference was noted following GDF-5 treatment. In GDF-5 treated discs containing expanded cell aggregates, aggrecan and type II collagen expression were also observed within the nucleus (Figure 22 and 23, respectively).

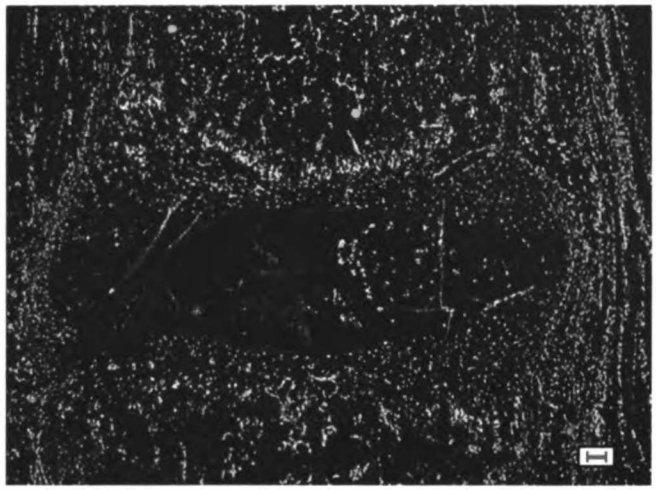

Figure 14. Aggrecan mRNA expression in GDF-5 single injection disc, 4 week timepoint. Mid-sagittal section showing entire disc and portions of adjacent vertebral bodies. Positive cells appear yellow, all other cells blue. Cells in the nucleus and annulus are positive for aggrecan mRNA. The line of positive cells outside the disc are chondrocytes in the growth plate. Red bar length = 100 μm.

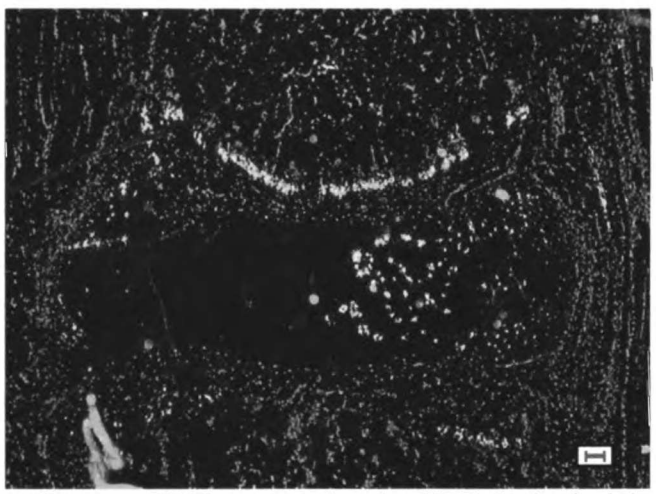

Figure 15. Type 2 collagen mRNA expression in GDF-5 single injection discs, 4 week timepoint. Positive cells appear yellow, all other cells blue. Cells in the nucleus and annulus are positive for type 2 collagen mRNA. The line of positive cells outside the disc are chondrocytes in the growth plate. Red bar length = 100 μm.

Four Week Response to Multiple Growth Factor Injections

When given in multiple injections (once per week for four weeks), TGF-β (Figure 25) appeared to have a stimulatory effect on annular fibrochondrocytes compared with saline controls (Figure 24). The yellow oval (Figure 25) identifies aggregates of chondrocytic cells in the degenerated nucleus after treatment with TGF-β. Based on examination of serial sections, these cells appear to be derived from the annulus. Aggrecan and type II collagen gene expression were detected in these cells by *in situ* hybridization (Figures 26 and 27, respectively).

Increased populations of chondrocytes in the annulus or nucleus were not observed in discs given multiple injections of GDF-5, IGF-1, or bFGF. To the contrary, several GDF-5 and IGF-1 discs had an inflammatory reaction in the adjacent vertebrae and connective tissue that infiltrated the nucleus and collapsed the disc. This was also noted in one of the saline injected discs and may be an effect of multiple insertions of the needle. For this reason, cell density and proliferation were not measured. Aside from the discs with inflammatory reactions, no significant alterations to disc height were observed between groups.

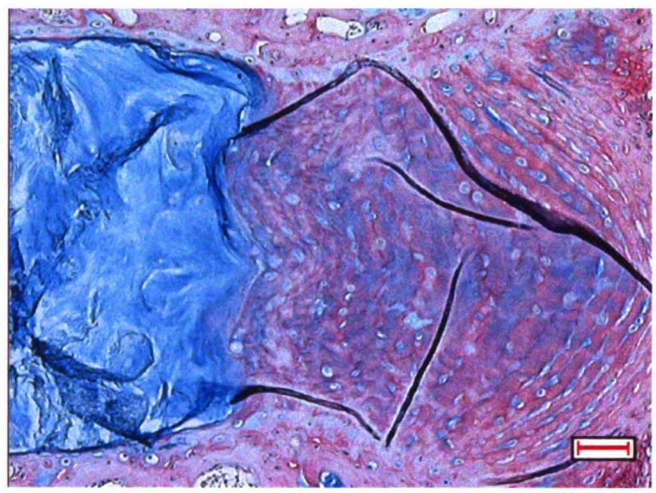

Figure 16. Multiple saline injections, 4 week timepoint. Red bar length = 100 μm.

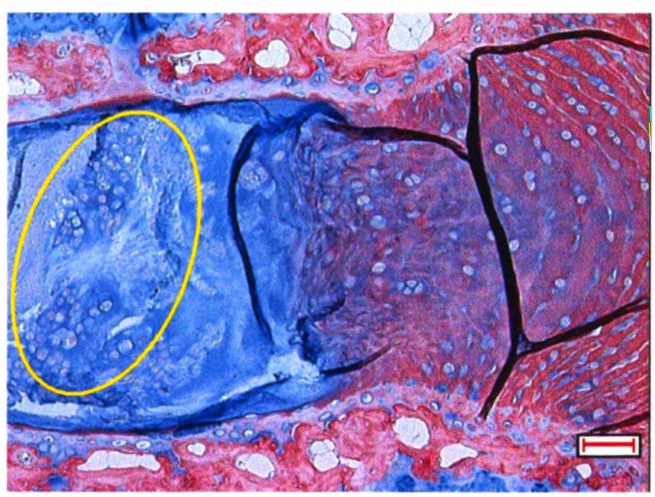

Figure 17. Multiple TGF-β injections, 4 week timepoint. The yellow oval indicates annular-derived chondrocytic cells within the degenerated nucleus. Red bar length = 100 μm.

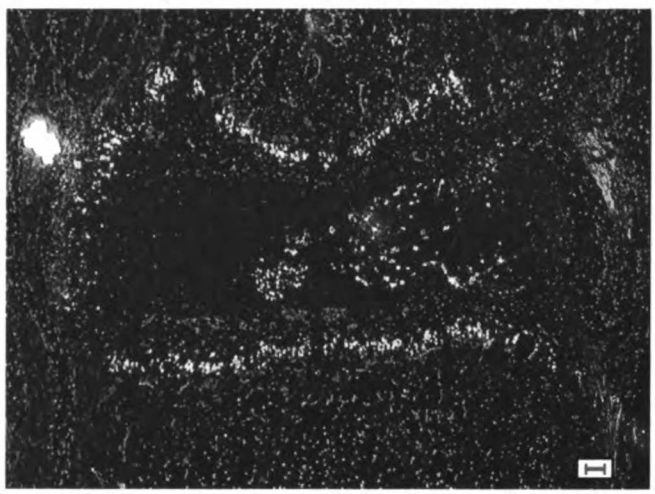

Figure 18. Aggrecan mRNA expression in TGF-β multiple injection disc, 4 week timepoint. Positive cells appear yellow, all other cells blue. Cells in the nucleus and annulus are positive for aggrecan mRNA. The line of positive cells outside the disc are chondrocytes in the growth plate. Red bar length = 100 μm.

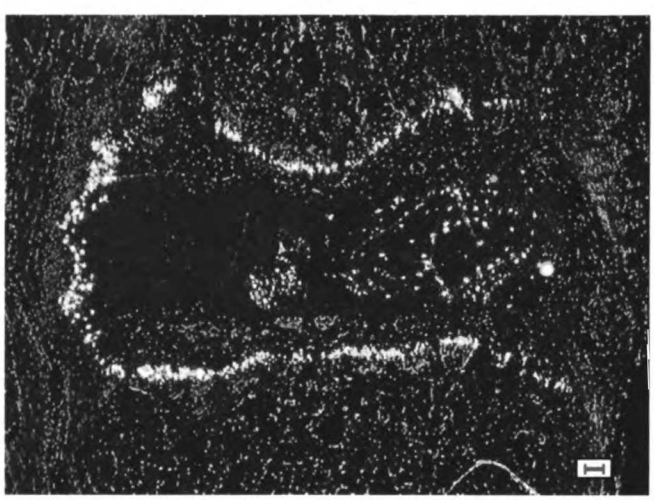

Figure 19. Type II collagen mRNA expression in TGF-β multiple injection disc, 4 week timepoint. Positive cells appear yellow, all other cells blue. Cells in the nucleus and annulus are positive for type 2 collagen mRNA. The line of positive cells outside the disc are chondrocytes in the growth plate. Red bar length = 100 μm.

Discussion

Our aim was to evaluate whether cells in degenerated discs were responsive to various growth factors. Because spontaneous regeneration is innately limited in the degenerated phenotype, we believe it is most relevant to test the factors in degenerated, as opposed to normal, discs. We confined our analysis to the short term (one week and four week after injection) to gauge which factors initiate a cellular response. Results from this initial response will be useful in directing long-term studies.

Our results indicate that annular chondrocytes in degenerated discs may be responsive to some exogenous growth factors *in vivo*. One week after injection, cellular response was insufficient to distinguish saline injected discs from those that received a growth factor. By four weeks, however, we observed an increase in isogenic groups of chondrocytes in the inner annulus and nucleus of GDF-5 treated discs. Similarly, chondrocyte aggregates were observed at four weeks in the nucleus of discs that received multiple injections of TGF-β. In both cases, these aggregates represent an expansion of annular chondrocytes into the degenerated, hypocellular nucleus. *In situ* hybridization demonstrated that these cells expressed aggrecan and type II collagen genes, suggesting increased matrix synthesis within the nucleus and inner annulus. At one week and four weeks, there was a trend of greater variability of the mean cell density and percentage of proliferating cells in growth factor treated discs compared to saline controls. The larger standard deviations suggest that cell numbers and proliferation may have been enhanced in some of these discs while in others there was little effect. This may be due to either too little cellularity prior to treatment or technical differences between injections,

discussed below. Interestingly, we saw little evidence of proliferation and migration of endplate chondrocytes.

Our results suggest that GDF-5 may be a mitogen for annular chondrocytes. This function agrees with its activity described in previous studies. Administration of exogenous GDF-5 in chick embryos has demonstrated its ability to stimulate chondrogenesis and influence chondrocyte differentiation [169]. In the developing joint, GDF-5 modulates chondrogenesis by increasing cell adhesion and chondrocyte proliferation in cell condensations [170]. We previously reported an increase in annular cellularity one week after injection of a high concentration of GDF-5 (3.5 mg/ml) [171]. Based on this result and the observations in this study from a single injection at four weeks, we expected, but did not observe, an enhanced effect from multiple injections. This may be due to the limitations of our study described below.

TGF-β also appeared to stimulate chondrocyte proliferation as evidenced by expansion of annular chondrocytes into the nucleus. This effect was observed in TGF-β treated discs only when given in multiple injections (once per week for four weeks). Our observations seem to agree with previous studies that demonstrate an increase in disc cell proliferation after exposure to TGF-β in culture [163, 172]. The reason why multiple injections of TGF-β were required versus a single injection of GDF-5 is unclear. The five-fold greater concentration of GDF-5 may be one explanation. Other possibilities are growth factor dependent variability in either cellular reactivity [173] or growth factor availability due to extracellular matrix binding [174-176].

The response to IGF-1 was less striking. Qualitatively, increased numbers of annular chondrocytes were observed in some discs relative to controls at four weeks, but

alteration to the nucleus cellularity was not evident. The measured cell density and percentage of proliferating cells were greater than controls and all other treatments at one week after a single injection, although not to a statistically significant level. Four weeks after a single treatment, cell density and proliferating cell values were the same as for control discs. This suggests that IGF-1 may have elicited an early, transient response. A greater response might be expected from multiple injections, although this was not observed. In previous studies, IGF-1 has been shown to stimulate cell division and proteoglycan synthesis in articular chondrocytes [177-179]. Canine disc cells in culture, however, had only a modest response to IGF-1 compared to a greater response to TGF-β [163]. A dose-dependent effect of IGF-1 on proteoglycan synthesis in bovine nucleus pulposus cells has been shown [180]. IGF-1 mRNA and its receptor were more prevalent in fetal discs than adult, suggesting an age dependence in activity [180]. These findings may explain the limited response to IGF-1 suggested by our results.

bFGF treatment had little effect in our model of degeneration. The measured cell density was higher, although not significantly, at one week relative to controls, but the percentage of proliferating cells was slightly less. Morphologically, there was little or no difference in any of the bFGF treated discs compared with controls at one week post-injection. For this reason, a single injection at four weeks was not evaluated. Discs that received multiple injections of bFGF over four weeks also lacked a response. We had expected that bFGF might strongly stimulate proliferation of murine disc cells based in part on the increased proliferative response of canine cells from the annulus and transition zone [163]. In articular cartilage, bFGF stimulates chondrocyte proliferation and acts to maintain a differentiated phenotype in culture [181-183]. Reactivity to bFGF

by annular cells in degenerated rat discs has also been demonstrated, and it was proposed that bFGF stimulation of cell proliferation might play a role in the progression of degeneration [184]. Our results, however, do not support an ability of bFGF to significantly affect disc cellularity.

If regeneration of the disc is the goal, one must ask, what is the desired outcome ultimately? Perhaps the outcome of primary importance clinically is elimination of pain. At present, the relation between disc structure and pain production is unclear [185]. Many patients with low back pain have degenerated or prolapsed discs, but not all [70, 71]. And some patients with degenerated discs are asymptomatic [68, 69]. However, given the current understanding of the mechanisms of discogenic pain, one might assume that restoration of normal disc height and mechanical stability may reduce pain and improve spine health. We propose, as do others, that a biological means to restore disc height and normal mechanics should focus on restoring the nuclear swelling pressure and repairing annular defects. To this end, replenishing the appropriate cell and matrix contents in the nucleus and annulus by growth factor stimulation is desired.

The responses seen in this study may be indicative of that outcome. The expansion of inner annular chondrocytes observed in GDF-5 and TGF-β treated discs may be a first step toward restoration of disc height and stability. The measured increase in disc height in GDF-5 treated discs at four weeks is a favorable outcome that supports this. Our observations of newly created fibrocartilage in the nucleus suggests that proteoglycan aggregates may be replenished in time. Such an outcome would be similar to that described after chemonucleolysis in canine discs. Bradford et al. reported an increase in disc height three months after chymopapain injection [186, 187].

Proteoglycan aggregates approaching normal levels indicated that nuclear regeneration had occurred.

Alternatively, expansion of annular fibrochondrocytes may ultimately accelerate the production of fibrous tissue throughout the disc with no improvement to mechanical function. Lipson analyzed herniated tissue and discovered that it was not pre-existing nucleus material but rather newly created fibrocartilage derived from the annulus [188]. This implies that stimulation of annular cell proliferation may be deleterious. If the structural integrity of the annulus is significantly compromised, the production of new tissue may increase the risk of prolapse. The stimulation of annular chondrocytes oberved in our study could be an early step toward this type of outcome.

Because of this dichotomy of outcomes, elucidaton of the long-term effects of growth factors in degenerated discs and the appropriate timing of growth factor treatment in the degenerative process is required. The results of this study help to define the expectations of growth factor treatment. Namely, the responses observed to GDF-5 and TGF-β indicate that exogenous growth factors in degenerated discs may act to expand the population of annular chondrocytes. This, in turn, could slow or reverse the decline of cellularity and proteoglycan content that occur during degeneration [27]. Whether improvement or even maintenance of disc height and mechanical stability are realized by such a response will likely depend on the stage of degeneration when growth factors are administered. We hypothesize that growth factor-induced proliferation similar to that in this study would be beneficial in treating discs identified early in the degenerative process. Such discs might have sufficient cellularity to respond to growth factors and sufficient structural integrity to expect a return toward normal function. In later stage

discs, the cellularity and annular structure may be too deteriorated to expect a benefit from growth factor treatment.

Importantly, we observed varied responses both within treatments and between single and multiple injections. In large part, we attribute this to variability in the cellularity and matrix architecture that results from degeneration. Our model uses mechanical compression to induce a phenotype typical of stage II or III degeneration. The loading regimen of seven days at 1.3 MPa stress followed by a three week unloaded period reproducibly creates degenerative remodeling from which the tissue does not spontaneously recover. Differences in the number of viable cells remaining in the nucleus and annulus and the degree of matrix disorganization do occur between different mice. This is a limitation of the murine model that results from variability of the compressive stress applied and the individual tolerance to loading.

This limitation, however, provides an important insight into the expectations of growth factor treatment in humans. Given that the viable cell content also varies in human degeneration, our results suggest that limited exposures to growth factors are unlikely to reproducibly create significant tissue remodeling.

Variability in response may also be related to dosing. The small volume of the mouse disc limits the fluid volume that can be injected at any one time. For example, the volume of the mouse disc is roughly 8000 times smaller than the human disc. Exposure of the cells to each growth factor is a function of the dose administered and the clearance rate. As a starting point, we chose concentrations which were approximately 10-times greater than those typically used in cell culture. In the future, refinement of doses will be

considered, as many growth factors have an optimum dose range to achieve a desired response [173, 189-191].

The rate of clearance of growth factors from the disc is an important concern. TGF-β, for example, is cleared from circulation within minutes after intravenous injection [192]. Interaction between growth factors and extracellular matrix molecules results in release from ECM over days [175]. Because we injected directly into avascular matrix, we expect the rate of clearance was more on the order of days than minutes. The rate, however, is unknown, so we included a group that received multiple injections (four injections, once per week) for comparison with singly injected discs. Response to TGF-β was observed only after given in multiple injections. This suggests that either higher doses or a method of sustained delivery are necessary. Repeated dosing did not enhance the effects of the other growth factors due in large part to inflammatory cell infiltrations created in some discs from multiple insertions of the needle. This is a limitation of our study that may be corrected by a method of sustained delivery.

Other limitations of our study are the sample size and variability in response due to technical difficulties of scale. The small size of the mouse disc makes it difficult to reproducibly insert the needle into the center of the disc. This was addressed by using a jig to fix the tail in place during injection and to direct the needle into the center of the disc. The needle was also modified so that it penetrated to the same depth in each mouse. Although every effort was made to standardize the injection procedure, some variability may be attributed to differences in the placement of the needle. Initial experiments to develop the technique, however, suggest it is a small source of error.

Finally, these experiments provided insight into some of the technical issues of treating degenerated discs. The intrinsic swelling pressure of the nucleus pulposus presents a hurdle to injecting growth factor or other solutions into the disc. Preliminary studies demonstrated that inviscid fluid, when injected rapidly, was expelled from the disc space through the needle tract. To combat this, we used a slow injection method in which the needle was inserted and left in place for ten minutes while an excess volume was slowly injected. Using this technique, solution was introduced primarily into the nucleus, secondarily into the annulus, but also outside the disc. This was beneficial in this study in which the response from any or all cells within and on the periphery of the disc was of interest. For example, a previous experiment using a high concentration of bFGF (8 mg/ml) resulted in an undersired hypercellular response outside of the disc at the one-week timepoint. In development of treatment modalities, however, localized delivery to specific regions will be important.

We expect the benefit of growth factors to come from early intervention in the degenerative process using sustained delivery or from coupling with a mechanical or cell-based device for local effects at appropriate levels. Sustained delivery may involve incorporating growth factor into a carrier material, such as a hydrogel, that is then placed into the nucleus. A number of natural and synthetic materials containing growth factors have been devised for drug delivery [176, 193, 194]. Gene therapy has been touted as a possible tool to create sustained levels of growth factors in the disc [166, 195, 196]. Additionally, artificial implants in development for replacing the nucleus pulposus may benefit from growth factors on their surface to promote annular regeneration around the device. The stimulation of annular chondrocytes observed here is especially promising

for repairing defects created during surgical implantation of synthetic, mechanical devices.

Conclusions

GDF-5 and TGF-β treatment in degenerated discs resulted in expansion of annular chondrocytes. The cells actively expessed aggrecan and type II collagen mRNA. A lesser effect was observed for IGF-1 and little or no effect from bFGF. This study indicates that annular chondrocytes in degenerated discs are responsive to some growth factors *in vivo*. The results have implications in the early intervention of disc degeneration to arrest or slow the degenerative process.

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