SPINE Volume 28, Number 23, pp 2609–2620

Disc Chondrocyte Transplantation in a Canine Model: A Treatment for Degenerated or Damaged Intervertebral Disc

Timothy Ganey, PhD,* Jeanette Libera, PhD,† Verena Moos, PhD,† Olivera Alasevic, PhD,† Karl-Gerd Fritsch, MD, PhD,† Hans Joerg Meisel, MD, PhD,‡ and William C. Hutton, DSc¶

Study Design. Disc degeneration and osteoarthritis are diseases of the matrix. Chondrocytes that have been removed from damaged cartilaginous tissues maintain a capacity to proliferate, produce, and secrete matrix components, and respond to physical stimuli such as dynamic loading. A dog model was used to investigate the hypothesis that autologous disc chondrocytes can be used to repair damaged intervertebral disc.

Objectives. Given the capacity for the cells *in vitro* to produce matrix molecules that would be appropriate for disc chondrocytes, the focus of the experiment was to investigate whether the cells would continue to sustain metabolic function after transplantation.

Summary of the Background Data. No evidence for long-term integration exists for cell transplantation in species other than rats and rabbits. Furthermore, no controlled studies of 1-year duration have been published.

Materials and Methods. Disc chondrocytes were harvested and expanded in culture under controlled and defined conditions, returned to the same animals from which they had been sampled (autologous transplantation) via percutaneous delivery. The animals were analyzed at specific times after transplantation by several methods to examine whether disc chondrocytes integrated with the surrounding tissue, produced the appropriate intervertebral disc extracellular matrix, and might provide a formative solution to disc repair.

Results. In the context of degenerative changes in an injury model: (1) autologous disc chondrocytes were expanded in culture and returned to the disc by a minimally invasive procedure after 12 weeks; (2) disc chondrocytes remained viable after transplantation as shown by Bromodeoxyuridine incorporation and maintained a capacity for proliferation after transplantation as depicted by histology; (3) transplanted disc chondrocytes produced an extracellular matrix that displayed composition similar to normal intervertebral disc tissue. Positive evidence of proteoglycan content was supported by accepted histochemical staining techniques such as Safranin O-Fast Green; (4) both type II and type I collagens were demonstrated in the regenerated intervertebral disc matrix by immunohistochemistry after chondrocyte transplantation; and (5) when the disc heights were analyzed for variance according to treatment, a statistically significant correlation between transplanting cells and retention of disc height was achieved.

Conclusions. Autologous chondrocyte transplantation is technically feasible and biologically relevant to repairing disc damage and retarding disc degeneration. [Key words: animal model, intervertebral disc, disc degeneration, disc chondrocyte, cell-based therapeutics **Spine 2003;28:2609–2620**

Low back pain is an extremely common symptom, affecting almost three quarters of the population sometime in their life. While 90% of the population recover within 3 months, in some patients chronic back or leg pain leads to long-term physical disability and a reduced quality of life. Disc anatomy would be expected to play a pivotal role in the underlying pain, yet abnormal spine and disc morphology including disc herniation has been described as a normal component of an asymptomatic population.[7] Why is it that some patients remain asymptomatic, and is it possible to treat patients with degenerative change that become symptomatic? Given that disc herniation is thought to be an extension of progressive disc degeneration that attends the normal aging process, seeking an effective therapy that staves disc degeneration has been considered a logical attempt to reduce back pain. Previous studies have validated genetic factors[1,19,26,35] and implicated nutrition[33] as relevant to the degenerative process. However, the high prevalence across diverse populations suggests that a myriad of unidentified factors likely contribute to similar symptoms.

Because no effective therapies to retard or reverse disc degeneration have yet been devised, a variety of surgical procedures have been developed to treat disc degeneration and back pain. Unfortunately, the procedures currently available fail to offer an outcome that is prosthetic and at the same time physiologic. Surgery tends to limit motion, and fusion in particular seems to shunt excessive stresses to adjacent spinal segments. Equally concerning in selecting fusion as an option is the fact that nonunions have been reported in 5% to 35% of patients,[9,30] and that patients undergoing a repeat fusion for failed surgery in the lumbar spine may still have a clinical failure rate as high as 40%.[12,12,36,37] The advent of tissue engineering has broadened the options for considering treatments that tailor repair to distinct anatomy. In particular, the use of cell and gene therapy to endow specific

From the *Altanta Medical Center, Atlanta, Georgia, †Bergmannstrost Klinik, Halle, Germany, ‡co.don AG, Teltow, Germany, and ¶Emory Spine Center, Decatur, Georgia.
The manuscript submitted does not contain information about medical device(s)/drug(s).
Corporate/Industry funds were received in support of this work. One or more of the author(s) has/have received or will receive benefits for personal or professional use from a commercial party related directly or indirectly to the subject of this manuscript: e.g., honoraria, gifts, consultancies.
Address correspondence and reprint requests to Tim Ganey, PhD, Atlanta Medical Center, 303 Parkway Drive, NE, Atlanta, Georgia, 30312; E-mail: Timothy.Ganey@tenethealth.com

properties or repair specific tissues is widely considered an emerging modality for effecting treatment.

Numerous scientific studies have provided observations concerning the biochemistry and biomechanics of the disc, offering insights and theories into structure-function-failure relationships.[10,15,27] The most apparent cellular and biochemical changes attributable to degeneration include a decrease in cell density in the disc that is accompanied by a reduction in synthesis of cartilage-specific extracellular matrix components such as type II collagen and aggrecan. As the proteoglycan content of the disc decreases, the resulting loss of water-binding capacity by the disc matrix coupled with a subsequent reduced capacity for dissipating spinal forces are thought to lead to disc disease.[6,18,23]

Collagen plays a key load-bearing role in the disc, and changes in its extracellular matrix content have been attributed to aging as well as to the pathology of degeneration.[2] In normal intervertebral discs, at least seven different types of collagen are present (*i.e.*, types I, II, III, V, VI, IX, and XI), although types I and II are the most abundant.[4,5,11,27–29,38] The anulus fibrosus contains more type I collagen than type II, whereas the nucleus pulposus is composed mainly of type II collagen.

Calcification of the vertebral endplates is another factor thought to be relevant to disc degeneration. The passage of nutrients and waste products across the endplate depends on fluid flowing into the disc (during the night at bed rest) and flowing out during the day when we walk about.[20] Thus, shortcomings of permeability would be expected to adversely effect chondrocyte metabolism.[8,21,23]

While cells constitute only 1% of the adult disc tissue by volume, their role in matrix synthesis and metabolic turnover is vital. Most assessments of intervertebral disc failure have focused on degenerative, morphologic changes in disc tissue morphology that affect the biomechanical performance of the motion segment.[15,32] In this consideration, mechanical failure is little more than a corollary of matrix structure, which in turn depends on balanced cell metabolism for efficient maintenance of the disc matrix. Given the value of cells to the metabolic health of the disc, one therapeutic strategy would be to replace, regenerate, or augment the intervertebral disc cell population, with a goal of correcting matrix insufficiencies and restoring normal segment biomechanics.

Recent work has shown that disc aging and degeneration are accompanied by a decrease in the number of cells in the disc, a change attributable to both necrosis and apoptosis.[16] Perhaps a more important outcome of this work and that of others has been to demonstrate that disc chondrocytes retain an ability to respond to both genetic endowment and appropriate *in vivo* stimulation, and that when returned to the disc under controlled conditions integrate with the surrounding tissue.[14–16,24,31]

With this in mind, we designed a study (using the dog as our model) to investigate the hypothesis that (1) repair of the damaged disc is technically feasible; (2) autologous cells can be reproducibly cultured under defined and controlled conditions; (3) percutaneous delivery is possible; and (4) disc chondrocytes will integrate with the surrounding tissue, produce the appropriate intervertebral disc extracellular matrix, and potentially provide a functional solution to disc repair.

■ Experimental Design and Methods

The goal of this study was to test the hypothesis that restoration of intervertebral disc morphology could be achieved by transplantation of cultured autologous chondrocytes into the nucleus pulposus. As a natural model of degeneration has not been described in a large mammal, this study was fashioned after established work demonstrating that degeneration can be stimulated by damaging the outer anulus.[25] Under institutional guidelines of the Institutional Animal Care and Use Committee (IACUC), 18 purpose-bred, 2-year-old female dogs, weighing between 20 and 25 kg were studied to see whether the introduction of cultured autologous disc-derived cells would repair a damaged disc and inhibit degenerative changes. Before surgery, 125 mL of blood was obtained from each of the dogs to serve as a serum supplement for autologous cell culture. Because blood loss was insignificant during the surgical procedure, this approximate 6% to 8% loss of total blood volume was not considered an additional risk to the animals.

The dogs were divided into two basic groups, with four animals receiving autologous cells containing a nuclear marker and the other 14 receiving autologous cells without a nuclear marker. Animals were radiographed to establish a baseline for pre-existing spine pathology. Under general anesthesia, an approach was made to the posterolateral aspect of the dog lumbar spine. Lumbar intervertebral discs at three levels (L1/L2, L2/L3, and L3/L4) were identified as study levels for the procedure and disc tissue was collected. Approximately 200 mg of tissue was collected from the lateral aspect of the anulus, 100 mg of anulus material, and 100 mg of nucleus pulposus material.

In this study, the L1–L2 intervertebral disc had tissue removed but did not receive chondrocyte transplantation, the L2–L3 disc was approached but not violated and served as a surgical control, and the L3–L4 level had disc material removed and received chondrocyte transplantation 12 weeks later. The wound sites were closed with resorbable suture and the animals returned to their holding area. None of the animals had problems related to the surgery and all regained full function.

Cell Culture Procedures. Tissue samples obtained from healthy dog discs were minced and enzymatically digested in a 50-mL Falcon tube using 20 to 25 mg collagenase type II (Biochrome, Germany) at 37C for 8 hours in a gyratory shaker (110 rev/min). Isolated cells were washed and resuspended in culture medium with the addition of 10% autologous dog serum that had been obtained before surgery. Disc chondrocytes were propagated in monolayer culture under Good Manufacturing Practice (GMP) conditions (co.don AG, Teltow, Germany) and maintained under strictly autologous conditions throughout the protocol using each dog's serum to supplement individual cultures. Such care eliminated the risk of immune response, and at the same time afforded sterile working conditions at all times within a Class S100 working area.[22]

The sampled disc cells were expanded in culture through several passages, with a goal of establishing a population of disc chondrocytes capable of producing matrix and sustaining an expanded volume within the damaged disc. The average

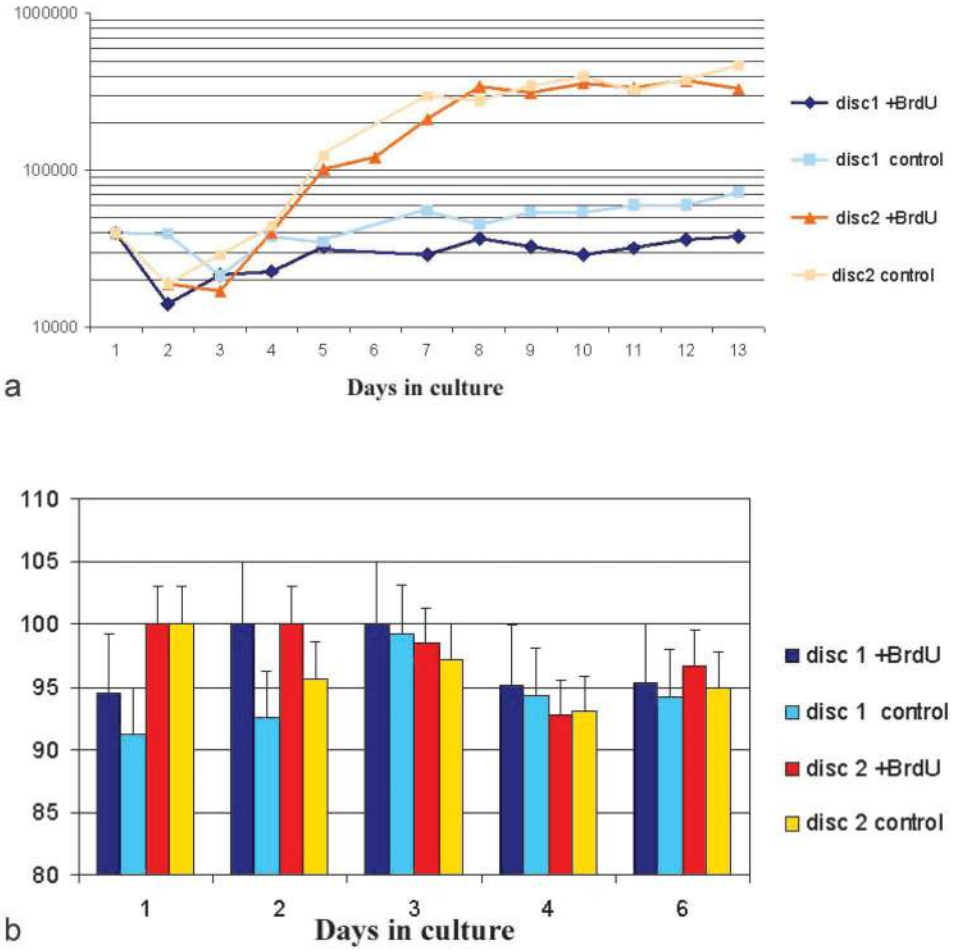

Figure 1. The proliferative capacity of BrdU-labeled cells in monolayer culture was similar to native control cells of the same dog. **A,** Comparison of cell growth of labeled versus unlabeled disc cell cultures. Graph depicts cell number graphed as days in culture. **B,** Cell viability, measured during the first passage after labeling of the cells, was between 92% and 100% in both labeled and unlabeled cultures, indicating that BrdU does not interfere with cell viability.

number of cells expanded and transplanted in each L3–L4 disc was approximately 6 million cells.

An important criterion for evaluating the success of cell transplantation in the disc repair procedure was identifying that matrix regeneration was attributable to transplanted chondrocytes rather than a result of inherent disc capacity for self-repair. BrdU, an analog nucleotide of thymidine, is incorporated into the nucleus during DNA synthesis and can later be identified by immunohistochemical techniques. As such, it is possible to analyze morphology *in situ* after repair and delineate cells that were transplanted from those already present in the host tissue. To verify the source of disc repair and matrix regeneration, BrdU was used as a cell marker in four animals.

BrdU-Incorporation Into Intervertebral Disc-Derived Chondrocytes. During the past 4 days in monolayer culture, the cells in passage two were tagged by adding a small concentration of BrdU (1:1000) to the culture medium (cell-labeling reagent from a BrdU labeling kit Amersham, Piscataway, NJ, USA). To perform growth curves, monolayer cells in passage one were cultivated in six-well plates and the cell number in each well was determined daily. Viability of the cells was assessed by staining with trypan blue.

The proliferative capacity of BrdU-labeled cells in monolayer culture was compared with unlabeled control cells of the same dog (Figure 1A). The doubling times of the cells were comparable in the BrdU-labeled and the control cell cultures, showing that BrdU did not affect the proliferation capacity of the chondrocytes *in vitro*. While differences between dogs could be distinguished, no difference between treatments could be ascertained.

The cell viability, measured during the first passage after labeling of the cells, reflected between 92% and 100% in both labeled and unlabeled cultures, indicating that BrdU does not interfere with cell viability (Figure 1B). Native as well as labeled cells continued to proliferate through seven passages. Taken together, these data indicate that incorporated BrdU did not interfere with the proliferative capacity or the viability of dog disc chondrocytes.

Disc Chondrocyte Transplantation. Twelve weeks after disc tissue had been harvested, the autologous disc chondrocyte cell cultures were transplanted at L3–L4 on each of the dogs. The intervertebral disc between L1–L2 served as the control for untreated degeneration. Cells were shipped from Teltow, Germany, overnight at 4C to 8C for transplantation. Animals were anesthetized, placed in right lateral recumbence, and the L3–L4 level was located by fluoroscopic imaging. As the previous surgeries had been performed from the right lateral side, the cultured cells were introduced through the left side of the anulus. The dog was draped, and a 25-gauge stylus was guided to the center of the intervertebral disc (Figure 2).

To assure that the cells would be placed into an intact nucleus cavity and not leak out through an anular fissure, interdiscal pressure was recorded before introducing the cells. This was accomplished with an inline pressure gauge developed spe-

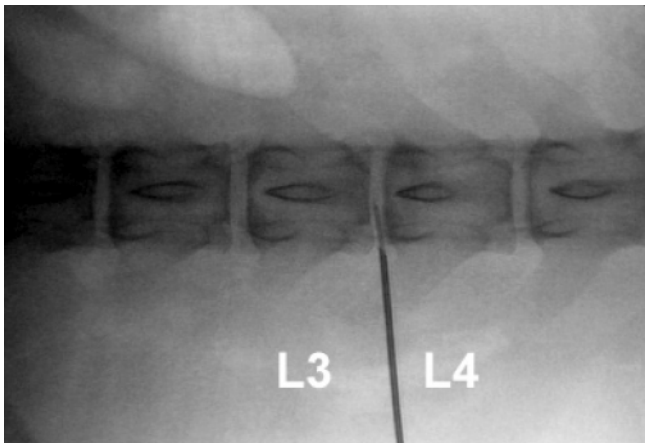

Figure 2. A 25-gauge needle was guided to the central nucleus pulposus area of the L3–L4 intervertebral disc to direct the transplantation of autologous disc chondrocytes.

cifically for this project (Rehau, Rehau, Germany). A 1.0-cm^3 bolus of saline was injected and a pressure of 600-mm Hg was sustained for 3 minutes to assure that the nucleus space was intact. After the pressure had been recorded and assurances gained that cell would be contained, the saline was withdrawn and the cultured chondrocytes were injected. The stylus was removed and the dogs were sent to recovery.

Tissue Analysis. The animals were humanely killed 3 months (3 dogs), 6 months (7 dogs), 9 months (4 dogs), and 12 months (4 dogs) after the cell transplantation and the tissue analyzed. Immediately after the dogs were killed, their lumbar spines were removed, packed in ice and prepared for MRI analysis in a 1.5 T Siemens scanner (Magneton 63P/4000; Siemens, Iselin, NJ). The spine was surrounded with normal saline to maximize the field strength without adversely impacting the signal sensitivity. Images were collected in both T1- and T2-weightings.

After the MRI scans, the spines were frozen *en bloc* and

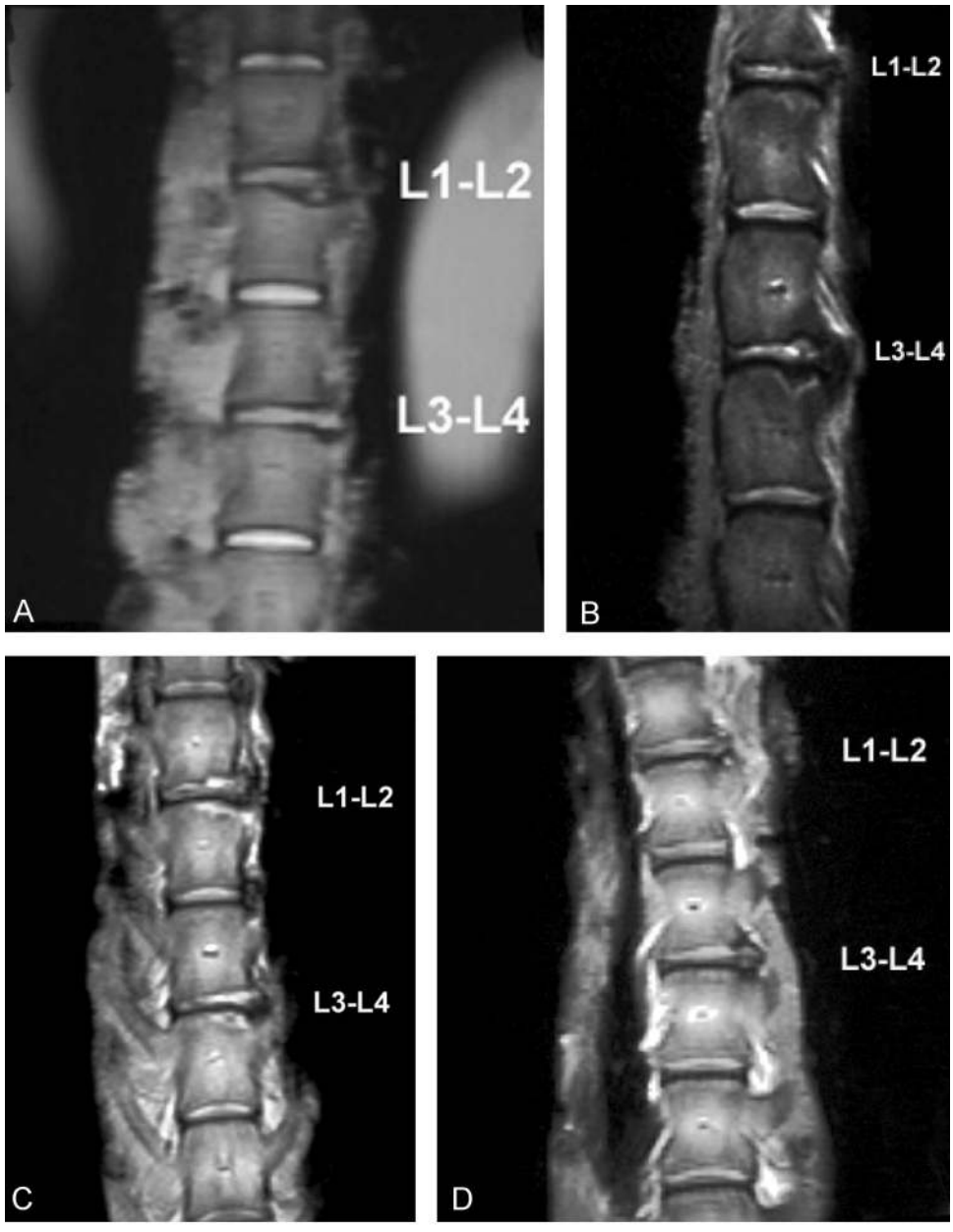

Figure 3. Over the course of study, progressive resolution of the subchondral margins of bone were achieved, clear reduction in vertebral bone edema was seen, and signal intensity within the intervertebral disc more closely approached the control levels. At each time point examined, the levels that had received the cells appeared to demonstrate better morphology. **A,** 3 months. **B,** 6 months. **C,** 9 months **D,** 12 months

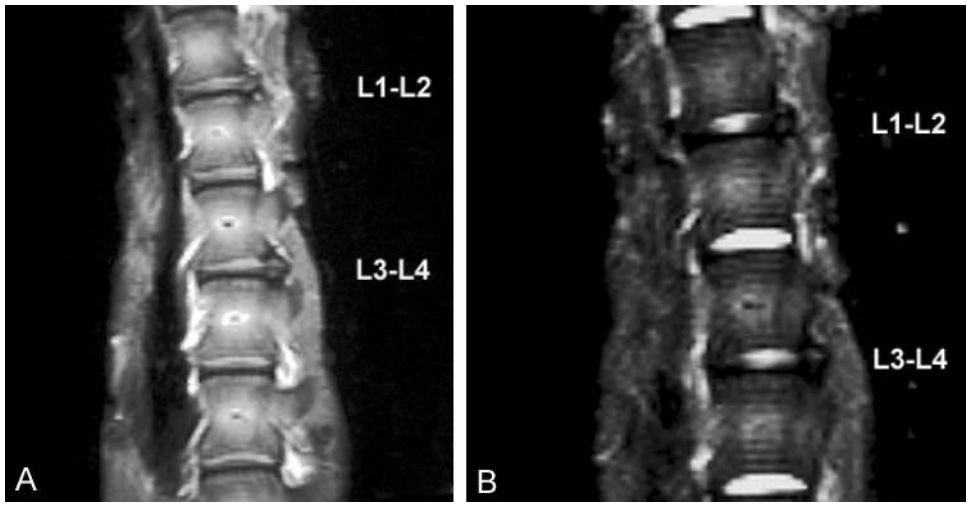

Figure 4. In the T2-weighted image of the animals at 12 months (**B**), the intensity of the signal is more diffuse in L3–L4, and in contrast to the level having not receiving cells (L1–L2), more closely silhouettes the shape of a normal disc nucleus; **(A)** shows the same animal as depicted in Figure 3D.

radiographed in both the A/P and lateral positions in a Faxitron Radiograph unit (Faxitron 38305 N Radiograph System; Hewlett Packard, Palo Alto, CA). Using a band saw, the spines were sliced into parallel coronal slabs of tissue and re-radiographed on Kodak TL-2 (Eastman Kodak, Rochester, NY) by direct x-radiation using the Faxitron. High detail radiographs directly apposed to the film eliminated magnification and allowed direct measurement of the disc space and vertebrae length to be made from the films. By interpreting the disc heights from images made of still frozen tissue, it was possible to evaluate height differences associated with the treatments (see later).

Tissue Processing. Photographs were taken of the individual slabs and the tissues were thawed into cold 10% neutral buffered formalin. Tissues were processed by two different methods. The dorsal (posterior) half of the tissue was dehydrated in rising concentrations of alcohol and embedded into methyl methacrylate for thin sectioning without decalcification. Sections were cut at 4 μm, collected on glass, stained with both toluidine blue by the method of MacNeal and also by the Goldner method for trichrome.[3] Individual sections were evaluated by light microscopy.

The ventral (anterior) half of the tissue was similarly thawed into 10% neutral buffered formalin, decalcified with Titriplex (Merck KGaA, Darmstadt, Germany) after dehydration and embedding in paraffin using standard protocols. Samples were cut using a rotary microtome (Leica, Cambridge Instruments, Deerfield, IL), collected on glass slides, and prepared for immunohistochemistry. Safranin O-Staining was performed on serial sections of dog disc samples after they had been deparaffinized to evaluate matrix proteoglycan content.

BrdU content and collagen expression was assessed from serial sections of deparaffinized sections. Immunohistochemistry for BrdU was performed according to the provider's manual by using a rabbit-anti-BrdU polyclonal antiserum and a peroxidase conjugated antirabbit secondary antibody (cell proliferation kit; Amersham, Piscataway, NJ). The staining was visualized with DAB as a peroxidase substrate and the samples counterstained by eosin. Collagen expression was assessed using the avidin biotin complex (ABC) method (Vector Labs, Burlingame, CA). Primary monoclonal antibodies were selected that recognized human and dog collagen types I and II (ICN, Costa Mesa, CA). The bound primary antibody was

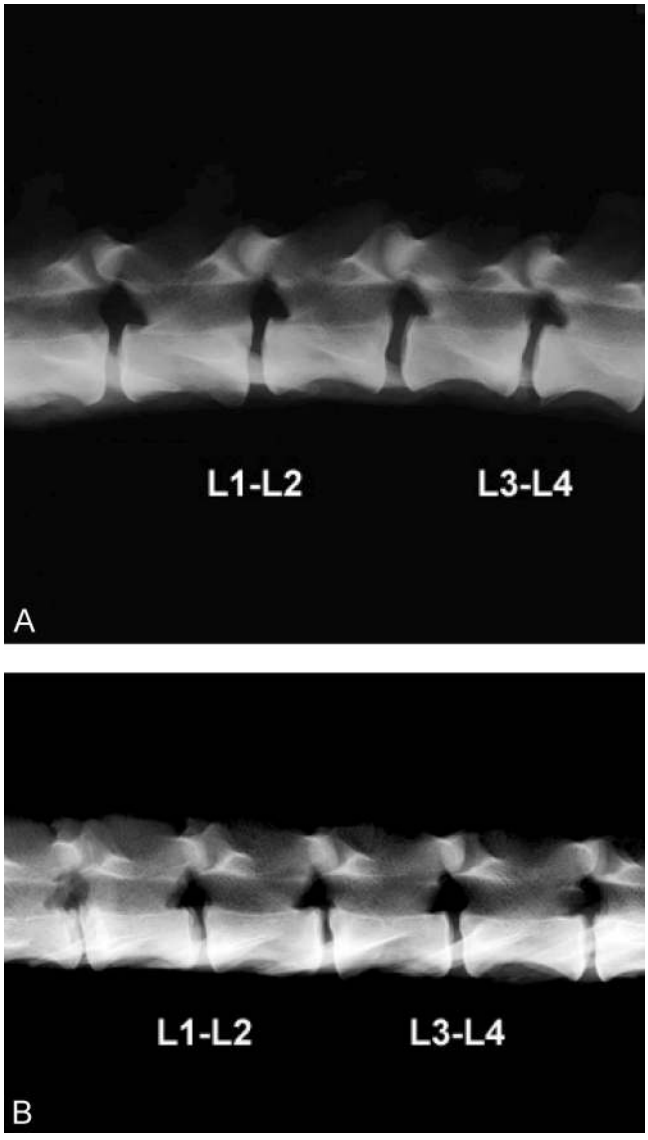

Figure 5. High-detail radiographs of intact spines did not reveal appreciable differences in morphology between 3-month **(A)** and 12-month animals **(B)**.

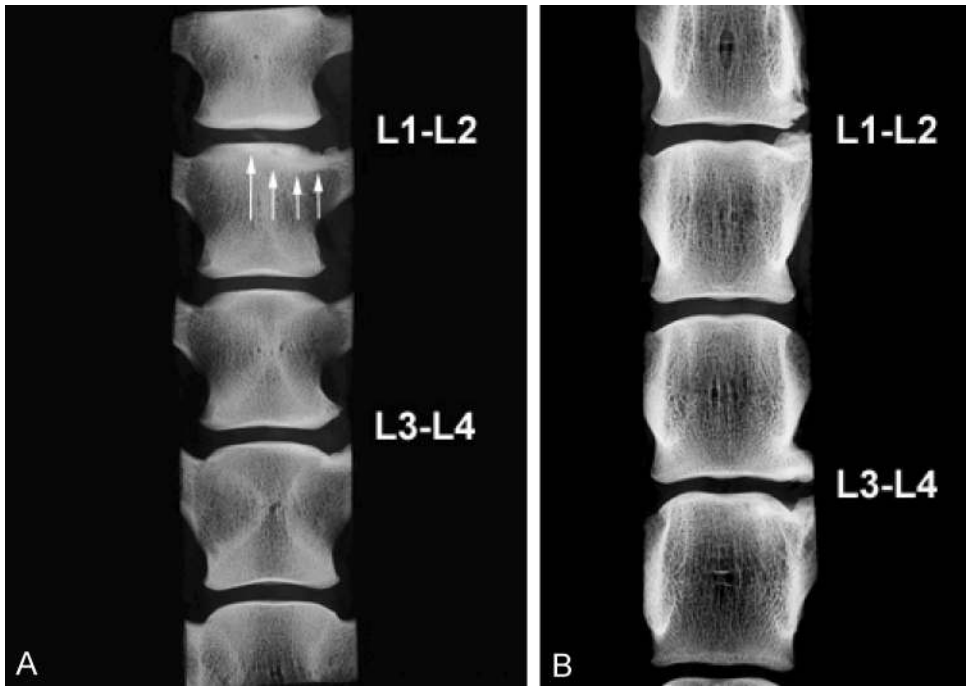

Figure 6. High-detail radiographs of the cut sections of the spine allowed us to measure the disc heights and to appreciate the trabecular architecture and fine details of the regenerative process. Differences in the **(A)** 3-month and **(B)** the 12-month spines are show in example and the early sclerotic changes in the 3-month subchondral endplate (arrows) can be appreciated by comparing it with the spine that has undergone 12 months of regenerative change.

detected using secondary biotinylated donkey-antimouse antisera (Dianova, Hamburg, Germany) and an extravidin-alkaline phosphatase conjugate (Sigma, St. Louis, MO). The stainings were visualized with Fast-Red (Sigma, St. Louis, MO) as a substrate for the alkaline phosphatase. Sections were counterstained by Hematoxylin and mounted with crystal mount. Articular cartilage bone sections were used as respective controls for collagen types I and II antibodies.

■ Results

Radiographs were made of all animals at 2-month intervals to assure that individual animals selected for tissue analysis represented the course of healing for the group rather than a disconnected singular frame of reference. The similarity of appearance across the study animals in these periodic assessments further suggested that tissue pathology and the regenerative course of healing would be both randomized and comparable.

MRI

Tissue response to surgery was evident in both sagittal and coronal views, with the right-side surgical damage apparent throughout the study. Spines that were evaluated after 3 months demonstrated areas of acute damage, active bone remodeling, and marrow edema (Figure 3A) that became less apparent 6 months (Figure 3B) and 9 months after transplantation (Figure 3C). Although all levels that had surgical treatment were identifiable 12 months after transplantation (Figure 3D), differences in appearance were evident between the untreated degenerated level (L1–L2), the normal intervertebral disc (L2–L3), and the level that had received the chondrocyte transplantation (L3–L4). Subchondral resolution of the marrow and re-establishment of the vertebral margin with the disc of the L3–L4 level was the primary positive change associated with time. After 12 months, regeneration of nucleus material was slightly better at the level having received the cell therapy (L3–L4) in both T1- and T2-weighted images (Figure 4). The differences between the two levels were interpreted from the prospective of shape of the regenerate material as well as in the central intensity of the regenerate matrix. Both experimental levels differed substantially from the L2–L3 control level.

Radiography

Independent of the posttransplantation time interval examined, postmortem views of the intact spines did not reveal appreciable differences in morphology. Small differences in tissue density were detected near the endplate in some animals but were insufficient to be used for meaningful assessment of underlying pathology (Figure 5). When the spines were sectioned and the tissue exposed to high-detail radiography, then differences in marrow, bone structure, and height became apparent (Figure 6). Sclerotic changes of the subchondral disc plate were present in all animals at 3-months. Largely dependent on postsurgical healing, by 6 months all animals had redefined the disc–vertebral interface.

Disc Height Analysis

Individual discs were compared for differences and examined for statistical relationships in disc height. The measurements were made using the high detail radiographs as shown in (Figure 6). The height of each disc was measured at the center and at a position 25% of the disc width on either side of the center. Each disc was measured twice, and the six measurements were averaged to generate an average disc height. The animals at 12-months were analyzed both within the group and separately to assess the effect of longer duration on difference in tissue morphology. A significant difference be-

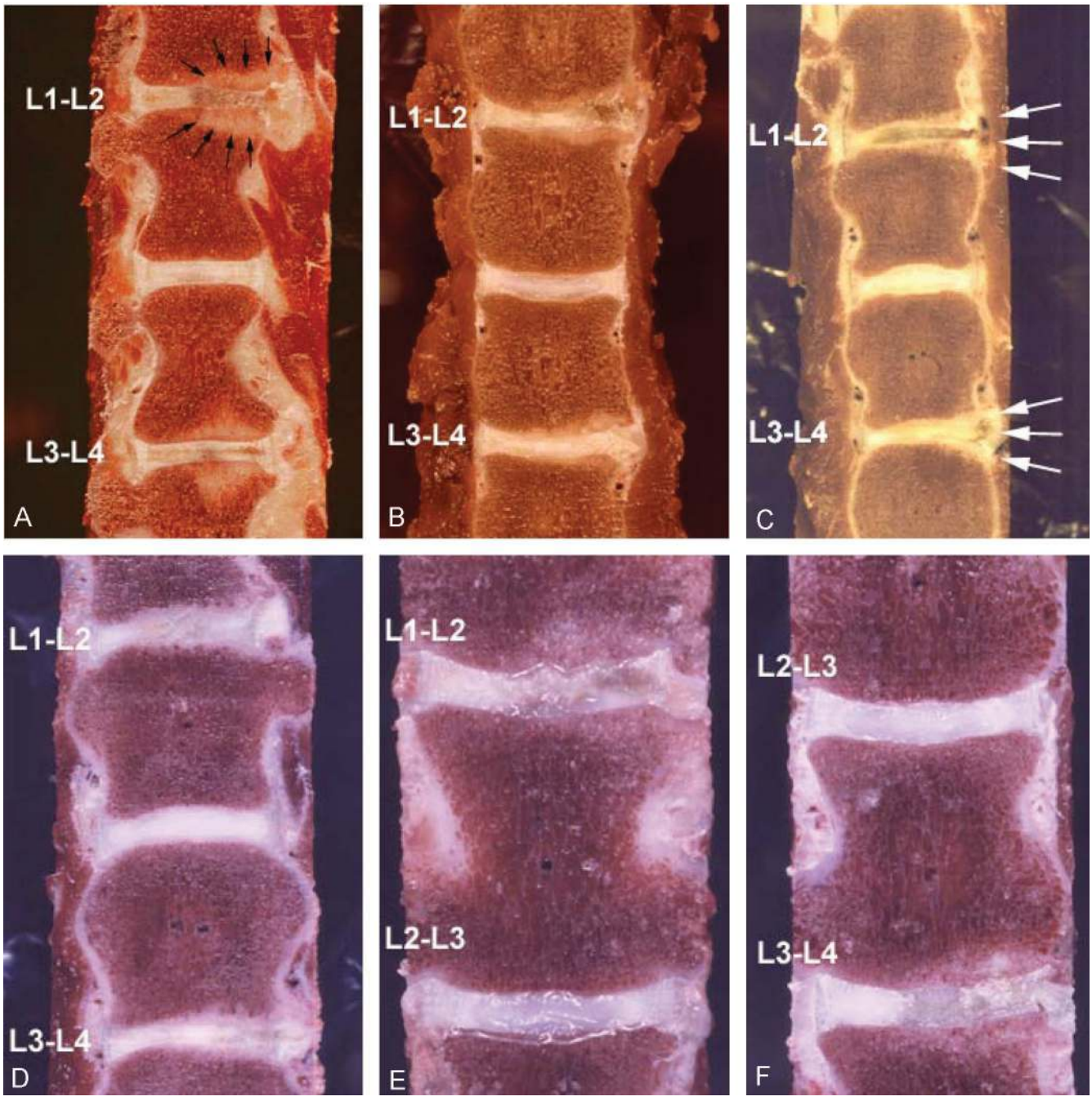

Figure 7. The hallmark of healing was seen in the reduction of inflammation, the margination of the intervertebral disc at the levels treated, and in the formation of scar tissue in the untreated levels (L1–L2). All healing was progressive and separation of the intervertebral disc a key positive feature of the longer time intervals. **A,** Animal at 3 months; black arrows delineate the surgical injury site. **B,** Animal at 6 months. **C,** At 9 months; white arrows show the periosteal lateral margin involvement of the vertebral body. **D,** Animal at 12 months. Closer analysis comparing the control (L2–L3) with the surgical untreated (L1–L2) **(E)** and the level having received the cells (L3–L4) **(F)** demonstrates that the intervertebral disc that had received the cells attained a more lucent matrix centrally, suggesting a richer proteoglycan content.

tween L1–L2 and L2–L3 was present at 6 months ($P = 0.03$) and at 12 months ($P = 0.025$). When only the four 12-month dogs were analyzed, comparisons between L1–L2 and L2–L3, and between L1–L2 and L3–L4 achieved significance, but not L2–L3 compared with L3–L4, as might be expected. This finding suggested that differences again were greater between dogs, and that four dogs were insufficient to establish a definite result.

ANOVA, excluding the four animals at 12-months, analyzed by time across levels remained nonsignificant ($P = 0.42$). However, when the analysis was expanded to include the 12-month data, the variation in disc height achieved significance ($P = 0.04$). This suggests that the change in mean height was time dependent and that the dogs at 12-months were driving the change. The suspected trend of a longitudinal, evolving distinction be-

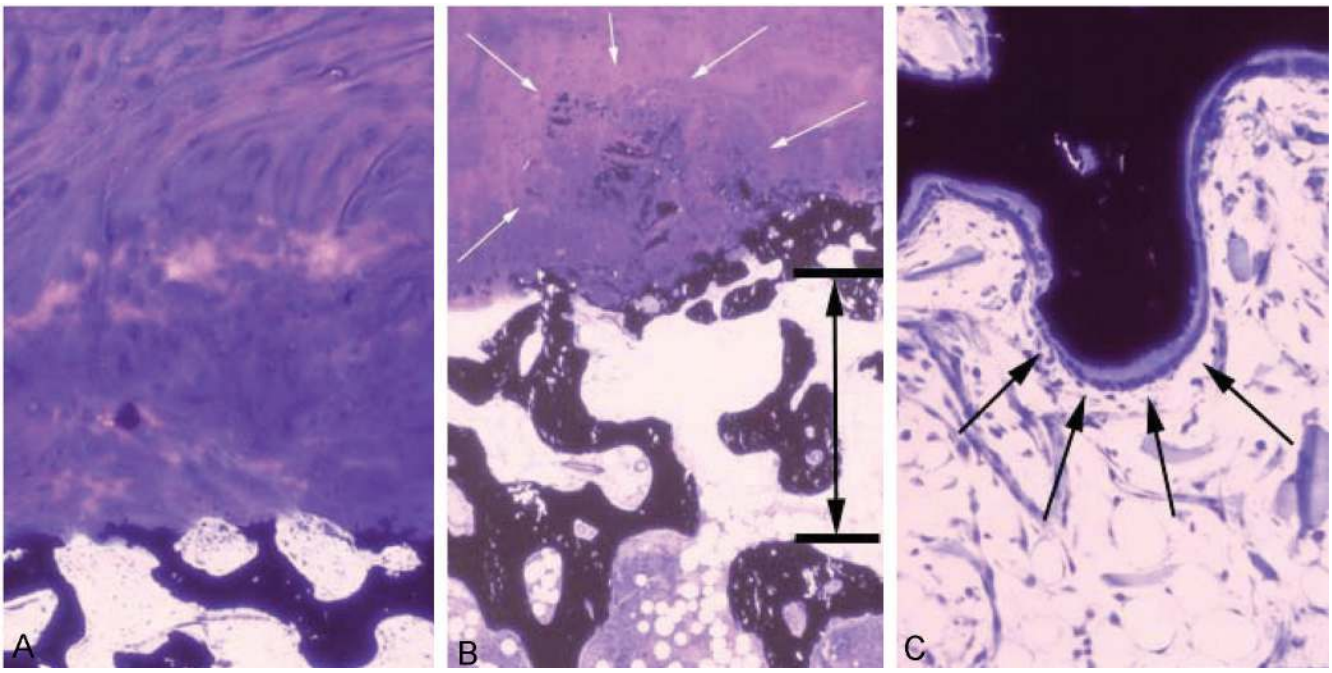

Figure 8. The vertebral margin with the disc was an active site of remodeling in the level that had received disc chondrocyte transplantation **(A)** (200x). The level that had not received cells was also establishing a margin between the disc and the vertebral bone, although calcification was apparent and indicated by the white arrows **(B)** (100x). Stratification of the cellular and acellular marrow is indicated by the bracketed area **(B)**. Active remodeling of the trabecular surfaces could be seen in both the levels receiving transplanted cells and those untreated after surgery **(C)** (400x).

tween treatment levels was confirmed with the addition of the four dogs at 12-months. The between-dog differences remained significant relevant to the interdog differences, supporting the hypothesis that more animals over longer intervals would likely yield statistically relevant differences based on the treatment.

Gross Pathology

Healing correlated with the interval of time over which the intervertebral discs underwent self-repair or repair with the transplanted chondrocytes (Figures 7A–D). In the 3-month animals, profound reduction of color of the vertebral bone and marrow were attributable to inflammation and wound healing. The "footprint" of the surgical site was notable at both experimental levels (Figure 7A).

In the animals at 6-months, the most noticeable change was in the healing of the disc, the loss of marrow blanching, the reduction in inflammation, and the widening difference in appearance between the level receiving the cells and the level left to heal on its own accord. While disc heights were not substantially different at this time point, the level having not received cells had generated scar tissue rather than regenerating normal disc material. Only traces of involvement of the vertebral bone were apparent, although the most lateral margin of the endplate was still remodeling (Figure 7B).

In the animals at 9-months, differences in healing between the intervertebral disc receiving cells (L3–L4) and the untreated level (L1–L2) was more obvious (Figure 7C). Dense scar tissue had developed in place of the more lucent disc material that would be expected in normal nucleus pulposus. Although the level receiving cells did not demonstrate morphology mirroring nucleus pulposus, a bursa-like separation could be seen that contained an opaque white matrix. Most concerning in this 9-month group of animals was lateral involvement of the vertebral margins. The general concept of anulus injury as sufficient to cause disc degeneration has been known for some time. A potential for lateral osteophyte formation resulting from surgical injury of the anulus was apparent in the flaring of the disc margin on the side at which surgery had been performed both at the L1–L2 and at the L3–L4 levels.

Differences in morphology reflected the selective organization of the tissues, and the interface between disc and the subchondral interface of the vertebral body (Figure 7D). Intervertebral discs receiving cells (L3–L4) achieved a distinction from the bone margins, while the untreated L1–L2 levels developed a "fuzzy" interface with the bone. The lateral margins of the vertebral bone were involved irrespective of the treatment arm of the study (Figure 7E,F).

Tissue Morphology

Tissue morphology correlated with the imaging data and reflected inflammatory processes and cell activity responsible for re-establishing disc anatomy. The vertebral bone–intervertebral disc was a site of tissue flux at 3 months (Figure 8), with calcification of cartilage and marrow fibrosis chief signs of remodeling activity. Within the vertebral body, activated osteoblasts and thick osteoid material were visible on the trabecular sur-

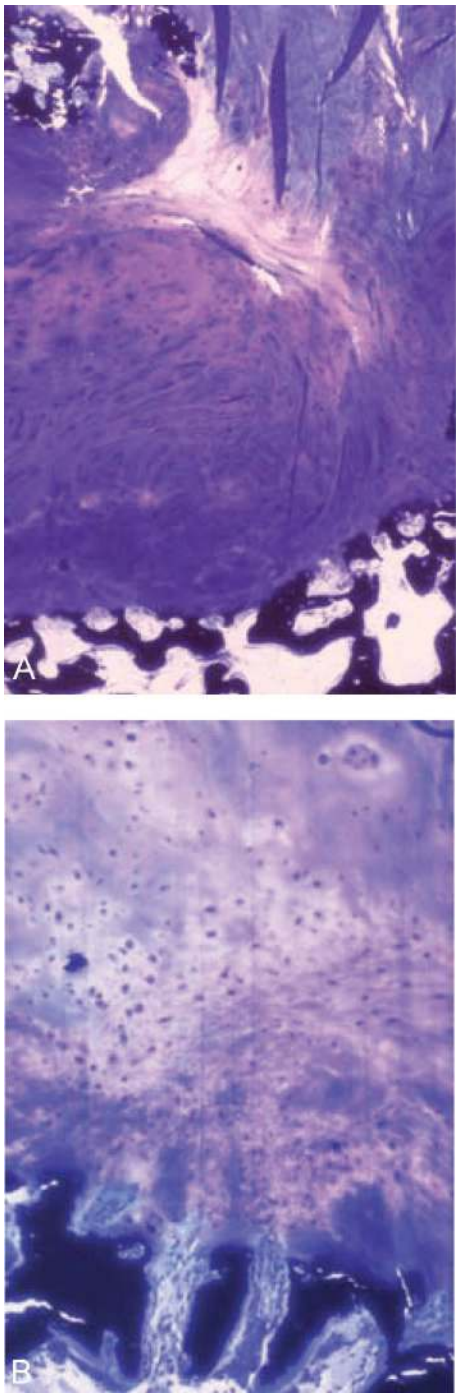

Figure 9. In evaluating the animals at 3 months at the interface of the disc and the vertebral body, the matrix within the disc that had received cells (L3–L4) **(B)** demonstrated abundant matrix formation and a higher matrix-to-cell ratio than the level not receiving cells (L1–L2) **(A)** (200x).

faces (Figure 8). Such remodeling was not unexpected and present in both the control discs and at the levels that had received cells (Figure 9).

The longer that the cells were allowed to integrate (*i.e.*, 6 months, 9 months, 12 months), the more complete the demarcation between the subchondral vertebral bone and the intervertebral disc. Progressive consolidation and restoration of the disc morphology was evident in the 6-month, 9-month, and 12-month tissues (Figure 10). Matrix staining with Safranin O-Fast Green to evaluate proteoglycan content further supported the advantage of cell transplantation in regenerative therapy (Figure 11). Staining intensity was more intense within the vertebral bone/intervertebral disc interface of the treated disc. Safranin O positive-pericellular matrices within the nucleus of the treated disc indicated matrix regeneration after cell transplantation.

Immunohistology

Tissue staining for both type I and type II collagen could be demonstrated after disc chondrocyte transplantation (Figure 12). Generation of type II collagen at the vertebral bone-intervertebral disc cartilage interface was encouraging. While the study was not designed to test whether functional restitution was achieved, the lack of such an essential matrix component as collagen would offer little confidence that expected norms for cartilage physiology might be achieved.

BrdU Validation

In addition to demonstrating components of a tissue-specific matrix, it was also possible to verify that the transplanted cells were present as an integral part of the new tissue (Figure 13). Both at high and low magnification, evidence of the BrdU-labeled cells could be seen. No cells with label were seen accept in the levels that had received transplanted chondrocytes. In some cases the label could be seen within individual cells within a chondrocyte clone, and in other sections of the tissue, individual chondrocytes with matrix domains were observed. Based on the number of cells transplanted and the fact that the cells were expanded under monolayer culture conditions before injection, spacing between labeled cells was interpreted as proof that the transplanted chondrocytes had undergone the anticipated shift in morphology. The flattened, fibroblastic shape of the chondrocyte that was essential to successful proliferation *in vitro* was found *in situ* to have transformed into a spherical chondrocyte morphology, and the matrix synthesis and disc tissue regeneration that was assessed by microscopy was attributed to those transplanted cells.

■ Discussion

This study evaluated whether autologous disc chondrocyte transplantation might be an appropriate therapeutic treatment to repair disc damage and inhibit degeneration. In this context, several important observations emerged. Autologous disc chondrocytes were expanded in culture and returned to the disc by a minimally invasive procedure after 12 weeks. Under defined conditions, it was possible to assure phenotype and assess metabolic capacity of the cells before transplantation. Disc chondrocytes remained viable after transplantation as shown by BrdU incorporation and maintained a capacity for proliferation after transplantation as depicted by histology. Transplanted disc chondrocytes produced an extracellular matrix that contained components similar to

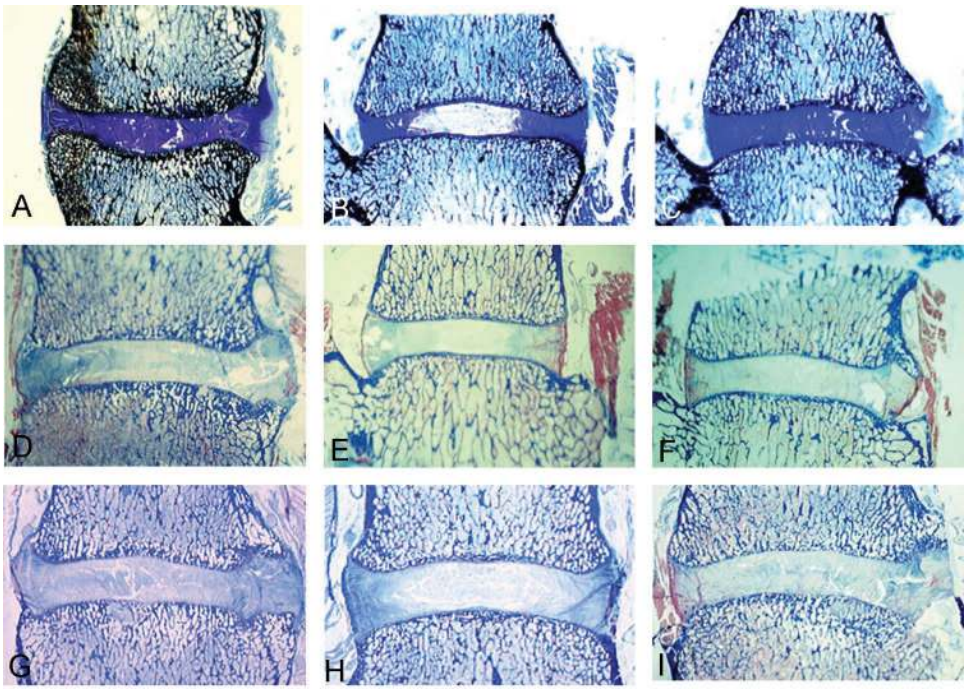

Figure 10. Comparisons are made between the levels receiving cell transplantation (L3–L4), the normal control (L2–L3), and the intervertebral disc that did not receive cells (L1–L2). Pictures (**A**, **B**, and **C**) represent the 6-month evaluation, (**D**, **E**, and **F**) the 9-month evaluation, and (**G**, **H**, and **I**) demonstrate the results 12-months after transplantation. **A, D, G:** The L1–L2 intervertebral disc. **B, E, H:** The L2–L3 levels. **C, F, I:** The L3–L4 levels. Images are 1x magnification and stained by either MacNeal's Toluidine blue or Goldner Trichrome.

normal intervertebral disc tissue. Positive evidence of proteoglycan content was supported by accepted histochemical staining techniques such as Safranin O-Fast Green. Both type II and type I collagens were demonstrated in the regenerated intervertebral disc matrix by immunohistochemistry following chondrocyte transplantation. There was a statistically significant correlation between transplanting cells and retention of disc height that was demonstrated at longer intervals after transplantation.

Although a morphotypic nucleus pulposus was not generated, cells that could appropriately be considered disc chondrocytes were identified in the intervertebral discs that had received disc chondrocyte transplantation. The observed matrix-to-cell ratio suggested strongly that these cells were elaborating a cartilage specific matrix that was appropriate with respect to both collagen and proteoglycan components. No evidence of necrotic change was present, nor were there any active signs of tissue vascularization. Absence of bone in the intervertebral space, and the productive matrix synthesis suggested that active remodeling and expression were guided by the demands of the anatomy, and that cell response after transplantation was dependent on both phenotypic identity of the cells and the biomechanical cues of the anatomy.

Cell viability and their capacity for matrix synthesis were particularly encouraging outcomes of this study. In the light of a 12-week interval between disc tissue sampling and cell transplantation, cells were placed into an environment that had fundamentally changed in both composition and function. Under the provision of central delivery and pressurized containment, the transplanted cells were primed in the vein of the nucleus pulposus. The

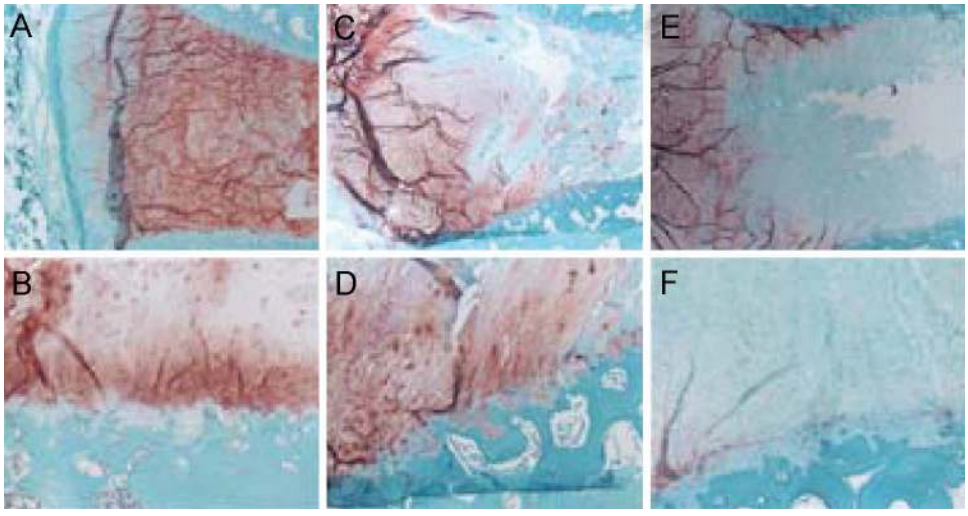

Figure 11. Safranin O staining of intervertebral discs. Hyaline cartilage-specific proteoglycans stain red; bone and damaged cartilage stain green. Untreated healthy disc (L2–L3) **(A, B)**; disc that received cells after transplantation (L3–L4) **(C, D)**; damaged untreated disc **(E, F)**; are all from the same animal at the same 6-month time point. Lateral aspect macroscopic overview with anulus fibrosus and nucleus (25x) **(A, C, E)**; bone/disc interface (200x) **(B, D, F)**.

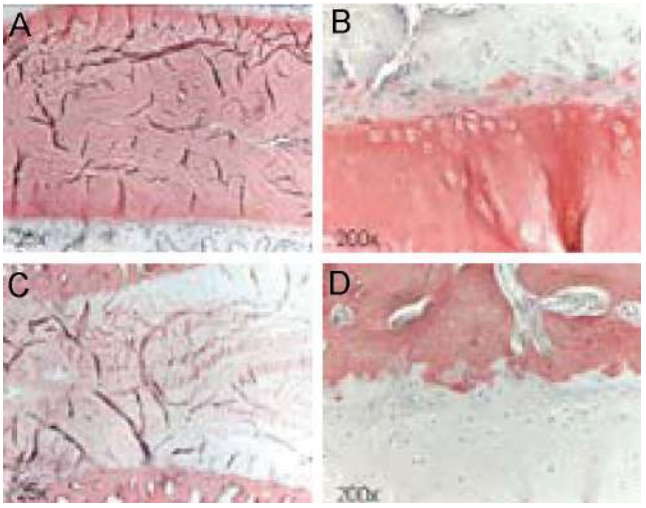

Figure 12. Immunohistochemical staining for collagen types I and II at 6 months in a disc that received chondrocyte transplantation (L3–L4). Areas expressing the respective collagen are stained red; nuclei are counterstained by hematoxylin. **A, B:** strong staining for collagen type II in the matrix of the regenerated disc. **C, D:** Slight staining for collagen type I in the newly formed matrix.

high cell to volume ratio of the transplanted cells, the deformable nature of the regional anatomy, and the inherent capacity of the cells to respond to new loading regimens all supported the vitality of the transplant conditions.

Extracellular matrix change, biomechanical variation, altered morphology, and cell viability are acknowledged steps leading to intervertebral disc degeneration. In invigorating the population of vital disc chondrocytes and achieving matrix transformation, positive action in addressing the morphology of the disc has been demonstrated. The ability to control cell conditions, potentially to imbue the cells with additional genetic capacity, and the availability of autologous tissue from discectomy procedures make this a technology that is available, effective and attractive.

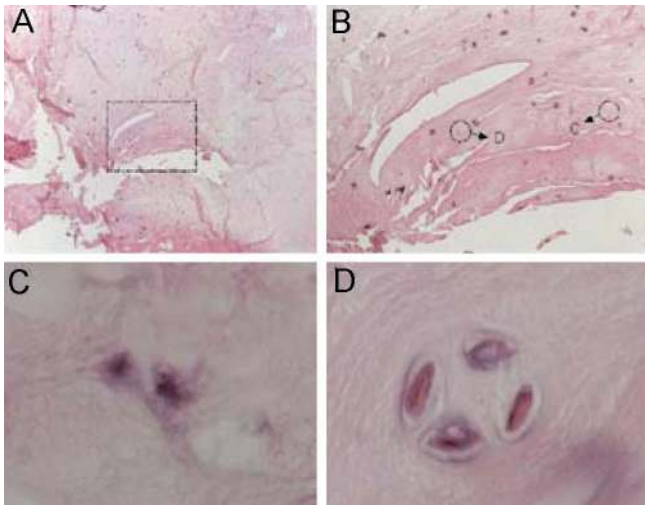

Figure 13. Staining of paraffin sections of the regenerated intervertebral disc 6 months after cell transplantation. BrdU containing chondrocytes were detected and stained by immunohistochemical procedures using DAB as the chromogen. Sections were counterstained by Eosin. BrdU positive cells are colored black. **A:** Nucleus regenerate overview (25x). **B:** BrdU-stained transplanted cells (200x). **C, D:** Single BrdU-stained transplanted chondrocytes, pericellular *de novo* synthesis of nucleus matrix (1,000x).

■ Summary

After transplantation, cultured intervertebral disc cells were viable, retained a capacity for proliferation *in situ*, demonstrated an ability to make appropriate matrix, and underwent expression consistent with the phenotypic demands of the anatomy. At the current time, autologous transplant in controlled conditions might represent the least hurdle to the clinic. It affords the least manipulation of a cell line, imposes little chance of immune rejection, and as a terminally differentiated lineage underscores the emphasis on integrating chondrocytes with the intention of repairing the intervertebral disc.

■ Key Points

- Autologous disc chondrocytes can be expanded in culture and returned to the disc by a minimally invasive procedure after 12 weeks. Under defined conditions, it was possible to assure phenotype and assess metabolic capacity of the cells before transplantation.
- Disc chondrocytes remained viable after transplantation as shown by BrdU incorporation and maintained a capacity for proliferation after transplantation as depicted by histology.
- Transplanted disc chondrocytes produced an extracellular matrix that contained components similar to normal intervertebral disc tissue. Positive evidence of proteoglycan content was supported by accepted histochemical staining techniques such as Safranin O-Fast Green.
- Both type II and type I collagens were demonstrated in the regenerated intervertebral disc matrix by immunohistochemistry after chondrocyte transplantation.
- There was a statistically significant correlation between transplanting cells and retention of disc height that was demonstrated at longer intervals after transplantation.

References

1. Annunen S, Paassilta P, Lohiniva J, et al. An allele of COL9A2 associated with intervertebral disc disease. *Science* 1999;285:409–412.
2. Antoniou J, Steffen T, Nelson F, et al. The human lumbar intervertebral disc: Evidence for changes in the biosynthesis and denaturation of the extracellular matrix with growth, maturation, aging, and degeneration. *J Clin Invest* 1996;98:996–1003.
3. Bancroft JD, Stevens A. *Theory and practice of histological techniques*. New York: Churchill Livingstone; 1982.
4. Beard HK, Ryvar R, Brown R, et al. Immunochemical localization of colla-

gen types and proteoglycan in pig intervertebral discs. *Immunology* 1980; 41:491–501.
5. Beard HK, Roberts S, O'Brien JP. Immunofluorescent staining for collagen and proteoglycan in normal and scoliotic intervertebral discs. *J Bone Joint Surg* 1981;63B:529–534.
6. Bibby SRS, Jones DA, Lee RB, et al. The pathophysiology of the intervertebral disc. *Joint Bone Spine* 2001;68:537–542.
7. Boden SD, McCowin PR, Davis DO, et al. Abnormal magnetic-resonance scans of the cervial spine in asymptomatic subjects. A prospective investigation. J Bone Joint Surg (AM) 1990;72:1178–84.
8. Buckwalter JA. Aging and degeneration of the human intervertebral disc. *Spine* 1995;20:1307–1314.
9. DePalma AF, Rothman RH. The nature of pseudarthrosis. *Clin Orthop* 1968;59:113–118.
10. Doers TM, Kang JD. The biomechanics and biochemistry of disc degeneration. *Curr Opin Orthop* 1999;10:117–121.
11. Eyre DR. Collagens of the Disc. In: Ghosh P, ed. *The Biology of the Intervertebral Disc*. Boca Raton, Florida: CRC Press; 1988:171–188.
12. Flynn JC, Hoque A. Anterior fusion of the lumbar spine: End result study with long-term follow-up. *J Bone Joint Surg* 1979;61A:1143–1150.
13. Galante JO. Tensile properties of the human lumbar annulus fibrosus. *Acta Orthop Scand* 1967;(S100):1–91.
14. Ganey TM, Meisel HJ. A potential role A potential role for cell-based therapeutics in the treatment of intervertebral disc herniation. *Eur Spine J* 2002; 2:S206–S214
15. Gruber HE, Hanley EN Jr. Analysis of aging and degeneration of the human intervertebral disc: Comparison of surgical specimens with normal controls. *Spine* 1998;23:751–757.
16. Gruber HE, Johnson TL, Leslie K, et al. Autologous intervertebral disc cell implantation. *Spine* 2002;27:1626–1633.
17. Lauerman WC, Bradford, BS, Ogilvie JW, et al. Results of lumbar pseudarthrosis repair. *J Spinal Disord* 1992;5:139–157.
18. Lipson SJ, Muir H. 1980 Volvo Award in Basic Science. Proteoglycans in experimental intervertebral disc degeneration. *Spine* 1981;6:194–210.
19. Kawaguchi Y, Osada R, Kanamori M, et al. Association between an aggrecan gene polymorphism and lumbar disc degeneration. *Spine* 1999;24: 2456–2460
20. Malko JA, Hutton WC, Fajman WA. An in vivo MRI study of the changes in volume (and fluid content) of the lumbar intervertebral disc after overnight bed rest and during an 8-hour walking protocol. *J Spinal Disord Tech* 2002; 15:157–163.
21. Maroudas A, Stockwell RA, Nachemson A, et al. Factors involved in the nutrition of the human lumbar intervertebral disc: cellularity and diffusion of glucose in vitro. *J Anatomy* 1975;120:113–130.
22. Norwig J, Josimovic-Alasevic O, Fritsch KG, et al. Integrated isolator technology-based sterile production of cell-based drugs. *Pharm Ind* 2000;63: 780–784.
23. Nerlich AG, Schleicher ED, Boos N. Volvo Award Winner in Basic Science Studies. Immunohistologic Markers for Age-Related Changes of Human Lumbar Intervertebral Discs. *Spine* 1997;22(24):2781–2795.
24. Okuma M, Mochida J, Nishimura K, et al. Reinsertion of stimulated nucleus pulposus cells retards intervertebral disc degeneration: An in vitro and in vivo experimental study. *J Orthop Res* 2000;18:988–997.
25. Osti OL, Vernon-Roberts B, Fraser RD. Anulus tears and intervertebral disc degeneration. An experimental study using an animal model. *Spine* 1990;15: 762–767.
26. Paassilta P, Lohiniva J, Goring HH, et al. Identification of a common risk factor for lumbar disk disease. *JAMA* 2000;285:1843–1849
27. Roberts S, Ayad S, Menage PJ. Immunolocalization of type VI collagen in the intervertebral disc. *Ann Rheumatol Dis* 1991;50:787–791.
28. Roberts S, Menage J, Duance V, et al. Collagen types around the cells of the intervertebral disc and cartilage end plate: An immunolocalization study. *Spine* 1991;16:1030–1038.
29. Roberts S, Menage J, Duance V, et al. Type III collagen in the intervertebral disc. Histochem J 1991;23:503–508.
30. Steinmann JC, Herkowitz HN. Pseudarthrosis of the spine. *Clin Orthop* 1992;284:80–90.
31. Sun Y, Hurtig M, Pilliar RM, et al. Characterization of nucleus pulposus-like tissue formed in vitro. *J Orthop Res* 2001;19:1078–1084.
32. Thompson JP, Pearce RH, Schechter MT, et al. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. *Spine* 1990;15:411–415.
33. Urban JP, Holm S, Maroudas A, et al. Nutrition of the intervertebral disk. An in vivo study of solute transport. *Clin Orthop* 1977;129:101–114.
34. Urban JPG, Holm S, Maroudas A, et al. The nutrition of the intervertebral disc. Effect of fluid flow on solute transport. *Clin Orthop* 1978;170:296–306.
35. Videman T, Leppavuori J, Kaprio J, et al. Intragenic polymorphisms of the vitamin D receptor gene associated with intervertebral disc degeneration. *Spine* 1998;23:2477–2485.
36. Waddell G, Kummel EG, Lotto WN, et al. Failed lumbar disc surgery and repeat surgery following industrial injuries. *J Bone Joint Surg (Am)* 1979;61: 201–206.
37. West JL, Bradford, BS, Ogilvie JW. Result of spinal arthrodesis with pedicle screw-plate fixation. *J Bone Joint Surg (Am)* 1991;73:1179–1184.
38. Wu JJ, Eyre Dr, Slayter HS. Type VI collagen of the intervertebral disc. *Biochem J* 1987;248:373–381.