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Intervertebral Disc Cell Therapy for Regeneration: Mesenchymal Stem Cell Implantation in Rat Intervertebral Discs

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Abstract—This study explores the use of mesenchymal stem cells (MSCs) for intervertebral disc regeneration. We used an *in vivo* model to investigate the feasibility of exogenous cell delivery, retention, and survival in the pressurized disc space. MSC injection into rat coccygeal discs was performed using 15% hyaluronan gel as a carrier. Injections of gel with or without MSCs were performed. Immediately after injection, fluorescently labeled stem cells were visible on sections of cell-injected discs. Seven and 14 days after injection, stem cells were still present within the disc, but their numbers were significantly decreased. At 28 days, a return to the initial number of injected cells was observed, and viability was 100%. A trend of increased disc height compared to blank gel suggests an increase in matrix synthesis. The results indicate that MSCs can maintain viability and proliferate within the rat intervertebral disc.

Keywords—Biomechanics (molecular, cellular, and tissue)/cell and tissue engineering, Modeling and simulation in bioengineering/animal model, Biomaterials and biological interfaces/Musculoskeletal joint systems.

INTRODUCTION

Intervertebral discs are fibrocartilaginous tissues interposed between vertebral bodies in the spine. They impart mobility to the spine while transmitting forces from one vertebra to the next.[7] An intervertebral disc derives its structural properties largely through its ability to attract and retain water.[8] Proteoglycans in the nucleus pulposus, a mucoprotein gel in the approximate center, osmotically exert a "swelling pressure" that enables the disc to support spinal compressive loads.[14] Circumscribing the nucleus is a collagenous structure called the annulus fibrosus. Swelling pressure in the nucleus creates tensile stress within the collagen fibers of the annulus and ligamentous structures surrounding the spine.[1]

Degeneration of the intervertebral disc is a physiologic process that is characteristic of aging in humans. With aging, the intervertebral disc undergoes alterations in volume, structure, shape, composition, and biomechanical properties.[2] The process of disc degeneration is characterized by a loss of cellularity, degradation of the extracellular matrix, and as a result, morphological changes and alterations in biomechanical properties.[2] The most consistent chemical change observed with aging is loss of proteoglycan and concomitant loss of water and disc pressure.[16] Secondary changes due to redistribution of tissue stress include fibrocartilage production with disorganization of the annular architecture and increases in type II collagen.[22] Functional consequences of these changes include loss of the mechanical function of the intervertebral disc and a decrease in the ability of the disc to absorb and distribute loads applied to the spine.[26]

Intervertebral disc degeneration is an important and direct cause of spinal pathologies that account for most neck, mid-back, and low-back pain.[2,5,15,28] Spinal pathologies that are associated with disc degeneration include segmental instability, spondylolisthesis, spinal stenosis, disc herniation, and discogenic back pain. Together, these disorders related to degeneration of the intervertebral disc account for 80% of all elective surgeries on the spine, and a healthcare cost of over $30 billion.[13] Most patients who experience back pain are treated with rest, exercise, and anti-inflammatory medications. However, each year, an estimated 800 thousand patients in the United States and 1.1 million worldwide report feeling mostly dissatisfied, and hospitalization or surgery may become necessary.[24] For these patients, relatively few clinical options for treatment exist (discectomy, laminectomy, or fusion), each of which has significant morbidity and unreliable outcomes.[4,11,29] Clearly, a reliable method for prevention and treatment of spinal dysfunction related to disc degeneration would have significant social, economic, and healthcare implications.

The development of alternative strategies for treating degenerated discs that restore disc structure and function is a contemporary challenge in the field of bioengineering. We speculate that biologic repair strategies that increase disc cellularity, and hence matrix synthesis, can be therapeutic.

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Our proposed strategy for disc regeneration is to restore the appropriate cell and matrix contents within the nucleus pulposus with foreseen benefit to the function of the disc on whole. Mesenchymal stem cells (MSCs) may be an excellent cell type for this application since they are readily available (from bone marrow), and can differentiate into a variety of connective tissue cell types.[3,9,17,18]

The long-term success of cell therapy for the degenerated disc will depend upon many factors. One factor is whether the injected cells can be retained within the disc given the significant internal pressures generated during activities of daily living (in the range of 1 MPa in humans).[27] A second is whether these cells survive and differentiate into cells capable of producing appropriate matrix. The purpose of this study was to test the feasibility of implanting bone-marrow-derived MSCs in intervertebral discs. We accomplished this using an *in vivo* rat model and hyaluronan gel as a carrier.

METHODS

MSC Isolation from Rat Bone Marrow

MSCs were harvested from the bone marrow of three adult rats (Sprague-Dawley, 270–300 g). The procedure was approved by the University of California, San Francisco Committee on Animal Research. After euthanasia, the humeri, tibiae, and femora were excised and ends of the bones were removed. The lumen of each bone was flushed with Dulbecco's Modified Eagle's Medium (DMEM) using a 19-gauge needle. After lysing red blood cells in Ammonium Chloride Potassium (ACK) lysing buffer, the remaining marrow was plated in a flask in Mesencult medium with murine stimulatory supplements (Stem Cell Technologies, Vancouver, BC) and incubated at 37°C/5% CO_2 for 3 days. The medium and suspended hematopoietic cells were then discarded. Additional fractionation was not performed.

In Vivo Cell Injection into Rat Intervertebral Discs

Adherent marrow stromal cells were removed from the plate by washing with 0.25% trypsin in EDTA for 5-10 min. The trypsin was deactivated with fetal bovine serum (FBS), and cells were suspended in saline and stained with CM-DiI membrane stain (Molecular Probes, Eugene, OR). After staining, cells were suspended in DMEM, incorporated into a 15% hyaluronan gel (Genzyme, Cambridge, MA) at a density of 1×10^7 cells/ml, and loaded into a metered syringe.

Injections were made into rat coccygeal discs in accordance with procedures approved by the UCSF Committee on Animal Research. After identifying the target disc by X-ray, the needle (29 gauge) with attached syringe was inserted into the nucleus pulposus using custom fixturing and fluoroscopic guidance. A volume of 50 μl was injected. Six discs were treated per tail, each tail in the same order. Four discs were injected with gel containing cells, one was injected with gel without cells (blank), and one received needle insertion without injection (sham). A normal disc proximal to the treated discs in each tail was used as a control. Thus, each tail contained four replicates of cell-injected discs.

A total of 12 rats (Sprague-Dawley, 270–300 g) were included in the study, 3 rats per time point. Discs from two rats were used to quantify the number of injected cells in the disc. Discs ($n = 8$ cell-injected at each time) were frozen in tissue-freezing medium (Fisher Scientific, Pittsburgh, PA) and sectioned 60 μm thick in a transverse plane, totaling approximately 30 sections from each disc. The number of injected cells in each section was counted manually under fluorescence microscopy to visualize the CM-DiI stain using a Texas Red filter (Chroma, Rockingham, VT). Under 100× magnification, all injected cells within the section could be visualized. The total number of cells in the disc (nucleus and annulus) was calculated by summing the results from all sections.

Discs from the third rat at each time point ($n = 4$ cell-injected) were used to quantify the percentage of viable injected cells in the nucleus pulposus. The nucleus from each disc was excised and incubated overnight in a 0.025% collagenase solution in DMEM with 10% FBS. The digested tissue was then centrifuged, washed once in phosphate-buffered saline (PBS), and suspended for 1 h in 0.05% (v/v) calcein-AM solution (Molecular Probes), a cell-permeant reagent that reacts with intracellular esterases within live cells to produce green fluorescence. The stained cells were washed again in PBS, and placed in a chamber slide for visualization under fluorescence microscopy. Live injected cells were discerned from native cells by the presence of the red CM-DiI fluorescent membrane stain. The percentage of viable cells was calculated by dividing the number of cells with both green and red stains by the total number of cells with red stain. This calculation assumes that the dead cells have negligible levels of intracellular esterases and that all live cells are stained, as expected by the manufacturer's stated properties.

Disc heights were measured from X-rays taken before and immediately after injection (at each time, $n = 6$ for normal, sham, and blank gel discs; $n = 28$ for cell-injected discs) and at the 14- and 28-day time points ($n = 2$ for normal, sham, and blank gel discs; $n = 8$ for cell-injected discs). During each X-ray, the tail was fixed in place using custom hardware to insure reproducibility of angle and magnification. Disc height was measured as the distance between adjacent vertebral bodies using Spot image capturing software (Diagnostic Instruments, Sterling Heights, MI). For each disc, the percentage change in height from the value before injection was calculated.

Statistical analysis was performed on quantitative data for injected cell counts and disc heights using analysis of variance (ANOVA) and Student–Newman–Keuls *post hoc* tests for multiple comparisons. The level of significance was

$\alpha = 0.05$. Appropriate analyses were used to account for variability from the use of discs from multiple animals. For cell counts, animal identity was treated as a random-effects factor nested within the fixed-effects factor (time). Thus, the first null hypothesis tested was no difference existed in cell numbers owing to different animals within each group. When no difference was found, discs within each group were regarded as independent of their source. The second null hypothesis was no difference existed in cell numbers owing to time. Analysis of disc height data used two-way ANOVA to determine the effects of treatment, animal, and their interaction on disc height. Treatment (normal, sham, blank gel, cell-injected) was a fixed factor, and animal identity was a random factor.

RESULTS

In Vitro Isolation of Mesenchymal Stem Cells

After 3 days in culture, two cell phenotypes of attached marrow stromal cells were observed: large spindle-shaped cells and lesser populations of smaller round cells. This result is similar to observations reported in the literature of a mixed population of mesenchymal progenitor cells from human bone marrow stroma.[19]

In Vivo Cell Injection into Rat Intervertebral Discs

Injected Cell Counts. Injected cells were visible in the rat disc compared to normal, blank gel, and sham discs at the four time points tested. Cells were clearly visible within the nucleus pulposus, and to a lesser extent within the annulus, at 0 days [Fig. 1(b)]. At 7 and 14 days, the number of injected cells was significantly reduced (Fig. 2). A larger percentage of injected cells at 14 days was located in the annulus as opposed to the nucleus. However, injected discs at 28 days had a significantly greater population of fluorescently labeled cells within the nucleus [Fig. 1(c)]. Numbers of injected cells at 28 days were significantly different from those at 7 and 14 days, but not 0 days (Fig. 2). There was no significant difference in cell numbers among animals within each time point ($p = 0.5$).

Injected Cell Viability. Injected stem cells were 85% viable at day 0, 67% viable at 7 days, and 100% viable at 28 days after the injection. During excision, the hyaluronan gel was clearly visible in the nucleus from the 0- and 7-day injected discs. At 14 and 28 days, the gel was not apparent. Injected cells were not present at 14 days in nuclear tissue used for assessing viability. The volume of the nucleus was smaller, a defect was still visible in the annulus at the injection site, and the nucleus was filled with a clear, color-less fluid unlike native nucleus or the initial hyaluronan gel. At 28 days, the injection site was no longer apparent, and the nuclear tissue had a similar consistency to normal nucleus pulposus.

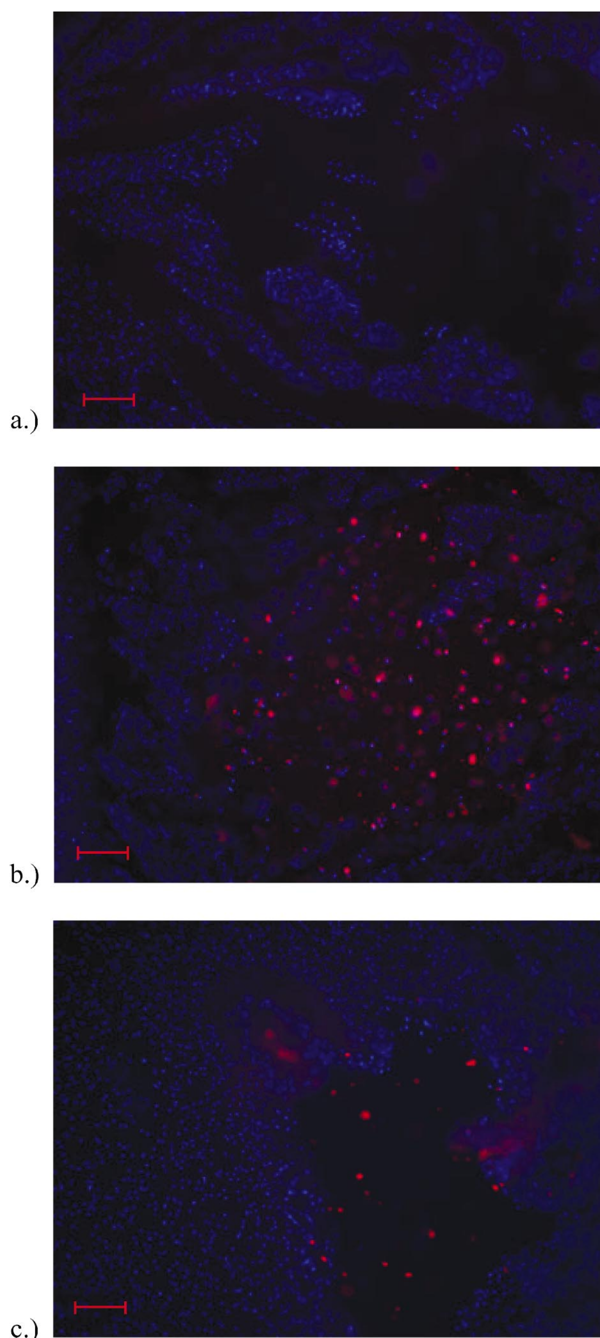

FIGURE 1. Portions of the nucleus pulposus of injected discs. Injected stem cells appear red (CM-DiI stain), endogenous cells blue (DAPI stain). (a) Hyaluronan gel alone, 0 days; (b) gel + stem cells, 0 days; (c) gel + stem cells, 28 days. Red bar length = 100 μm.

Disc Height. Immediately after injection, disc height increased significantly relative to its initial state before injection (Fig. 3). A similar change was observed from injection of gel with or without cells. At 14 days after injection, disc height of injected and sham discs were less than normal although the differences were not statistically significant ($p = 0.84$). A slight increase in disc height was observed

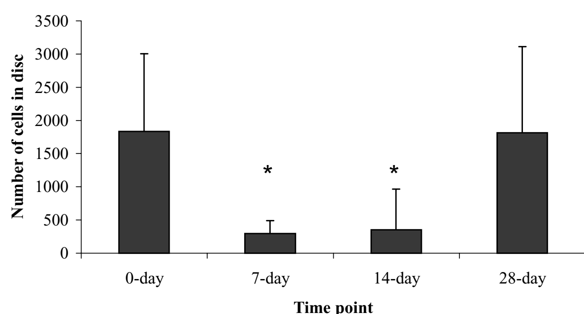

FIGURE 2. Numbers of injected cells in the disc. Data are mean ± standard deviation of $n = 8$ discs at each time point. *Significantly different than 0- and 28-day time points, $p < 0.05$.

in cell-injected discs at 28 days in contrast to a continued decline in sham- and gel-alone-treated discs. The differences at 28 days were nearly significant ($p = 0.053$). Differences between animals were not significant at 14 days ($p = 0.23$) but there was a significant interaction between treatment and animal. Conversely, a significant difference existed between animals at 28 days, but no significant interaction ($p = 0.96$)

DISCUSSION

This study demonstrates that injected MSCs in a viscous hyaluronan gel are retained and remain viable in the nucleus within 1 day after injection. At 7 and 14 days the number of injected cells was significantly reduced. Yet, a subsequent increase in labeled MSCs at 28 days demonstrates that these cells can survive the potentially hostile disc environment and proliferate. Importantly, the trend of an increased disc height for the cell-treated discs suggests that the cell proliferation was associated with an increase in matrix synthesis and disc pressure, a potentially therapeutic effect.

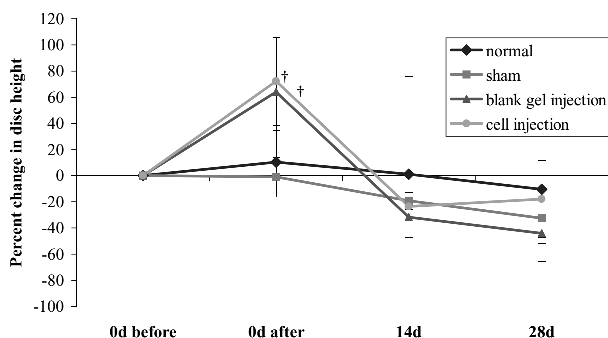

FIGURE 3. Disc height measurements in subject discs. Height was measured at the axial centerline on X-rays taken prior to cell counting and viability assays. Data are mean ± standard deviation of the % change in height = (height − height before injection)/height before ×100. Various sample sizes were used as reported in the text. †Denotes a statistically significant difference compared to normal and sham discs ($p < 0.05$).

There are several potential explanations for the observed decrease in the population of injected cells at 7 and 14 days. First, the fluorescent membrane stain may have faded and could no longer be detected. This seems unlikely given the observation of greater numbers of stained cells in other discs at 28 days. Second, the injected cells may have died and been cleared or degraded. The larger cell population at 28 days indicates that cell death may not be attributed to the disc environment itself. Rather, the high initial concentration of hyaluronan may have been cytotoxic since nuclear tissue obtained from 7- and 14-day discs also contained fewer native cells. Alternatively, the decrease in native and injected cells may be due to expulsion of nuclear material through the needle tract. This third explanation suggests that injection tract healing and hyaluronan carrier viscosity were insufficient to prevent expulsion of a majority of the injected cells. At 7 and 14 days, the point of injection was observed on the exterior annulus, evidence that the injection pathway had not healed. Because the hyaluronan was not crosslinked, the carrier viscosity may have decreased over time due to dilution of the hyaluronan or enzymatic degradation, allowing the nuclear contents to be more easily extruded. This is supported by the observation of inviscid fluid within the nucleus of gel injected discs (with or without cells) at 14 days.

The results from this study help to define criteria on which to select a carrier for disc cell therapy. Although successful in the short-term, uncrosslinked hyaluronan alone appears to have limited applicability to the long-term retention of injected cells. Hyaluronan was selected because it is a natural biopolymer present in the disc matrix.[10] In concentrated solution, it forms a viscous gel that can be dispensed through a small needle.[20] As a point of reference, the measured viscosity of the initial 15% hyaluronan gel was approximately 120 Pa · s at 37°C. In the future, the time-dependent decrease in carrier viscosity may be eliminated by use of an *in situ* cross-linking gel. In light of the current findings, we believe the optimum carrier should (1) be biocompatible with therapeutic cells and host tissue; (2) be capable of delivery by injection through a small needle; (3) possess sufficient viscosity or stiffness to prevent extrusion while the needle tract heals; and (4) allow for functional adaptation of injected cells and incorporation of new matrix into tissue.

Beyond the issue of cell delivery and retention, future studies will address *in vivo* the differentiation and synthetic activity of injected MSCs. Repair of articular cartilage defects treated with MSCs has been demonstrated in rabbits.[25] Recently, Sakai *et al.* implanted lacZ-transfected MSCs in a rabbit model of disc degeneration using Atelocollagen gel as a carrier.[23] They report that degeneration was decelerated up to 8 weeks postinjection. We plan to use a similar approach using a rat model of disc degeneration, first to optimize the carrier for cell retention, viability, and differentiation and subsequently to explore the efficacy of MSC therapy to treat disc degeneration.

Several factors limit the potential extrapolation of this technique to humans. Because of gravity loading, the internal disc pressure in humans may be several times greater than that in rodent tails.[12,27] Consequently, biomechanical studies need to be performed to investigate the ability of injected materials to be retained in the disc under physiological loading conditions. Perhaps more importantly, disc cell nutrition is diffusion-dependent, and consequently the viable cell density is disc height (and hence diffusion distance) limited.[6] The diffusion distance for rodent discs is much less than human and consequently the rodent model may reflect an ideal condition. Also, the process of disc degeneration in humans may be accompanied by endplate calcification that would further decrease permeability and hence cell nutrition.[21] Whether the nuclear environment in degenerated human discs can support MSC survival and proliferation to the extent that the treatment has any measurable therapeutic benefit is yet to be determined.

CONCLUSION

The results of this study suggest that MSCs can survive injection into the disc and can proliferate *in situ*. Hyaluronan gel, however, may not be an ideal carrier for this application since a marked decrease in cell numbers occurred over the first 7 days after injection. The decrease may be related to cell death or expulsion of some injected cells before the injection tract could heal. Nonetheless, the remaining cells did proliferate over the next 3 weeks leading to a trend toward increased disc height.

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