In vivo intervertebral disc regeneration using stem cell–derived chondroprogenitors

Laboratory investigation

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Object. There is currently no biologic therapy to repair or restore a degenerated intervertebral disc. A potential solution may rest with embryonic stem cells (ESCs), which have a potential to grow indefinitely and differentiate into a variety of cell types in vitro. Prior studies have shown that ESCs can be encouraged to differentiate toward specific cell lineages by culture in selective media and specific growth environment. Among these lineages, there are cells capable of potentially producing nucleus pulposus (NP) in vivo. In this investigation, the authors studied ESC-derived chondroprogenitors implanted into a degenerated disc in a rabbit. For this purpose, a rabbit model of disc degeneration was developed.

Methods. A percutaneous animal model of disc degeneration was developed by needle puncture of healthy intact discs in 16 New Zealand white rabbits. Series of spine MR imaging studies were obtained before disc puncture and after 2, 6, and 8 weeks. Prior to implantation, murine ESCs were cultured with cis-retinoic acid, transforming growth factor β, ascorbic acid, and insulin-like growth factor to induce differentiation toward a chondrocyte lineage. After confirmation by MR imaging, degenerated disc levels were injected with chondrogenic derivatives of ESCs expressing green fluorescent protein. At 8 weeks post-ESC implantation, the animals were killed and the intervertebral discs were harvested and analyzed using H & E staining, confocal fluorescent microscopy, and immunohistochemical analysis. Three intervertebral disc groups were analyzed in 16 rabbits, as follows: 1) Group A, control: naïve, non-punctured discs (32 discs, levels L4–5 and L5–6); 2) Group B, experimental control: punctured disc (16 discs, level L2–3); and 3) Group C, experimental: punctured disc followed by implantation of chondroprogenitor cells (16 discs, level L3–4).

Results. The MR imaging studies confirmed intervertebral disc degeneration at needle-punctured segments starting at ~2 weeks. Postmortem H & E histological analysis of Group A discs showed mature chondrocytes and no notochordal cells. Group B discs displayed an intact anulus fibrosus and generalized disorganization within fibrous tissue of NP. Group C discs showed islands of notochordal cell growth. Immunofluorescent staining for notochordal cells was negative for Groups A and B but revealed viable notochordal-type cells within experimental Group C discs, which had been implanted with ESC derivatives. Notably, no inflammatory response was noted in Group C discs.

Conclusions. This study illustrates a reproducible percutaneous model for studying disc degeneration. New notochordal cell populations were seen in degenerated discs injected with ESCs. The lack of immune response to a xenograft of mouse cells in an immunocompetent rabbit model may suggest an as yet unrecognized immunoprivileged site within the intervertebral disc space. (DOI: 10.3171/2008.12.SPINE0835)

Key Words • disc degeneration • disc regeneration • notochordal tissue • rabbit model • stem cell

Abbreviations used in this paper: AF = anulus fibrosus; AP = anteroposterior; ESC = embryonic stem cell; GFP = green fluorescent protein; MSC = mesenchymal stem cell; NP = nucleus pulposus; PAS = period acid Schiff stain; TGFβ = transforming growth factor β.
matrix that holds the water molecules that maintain the viable function of the intervertebral disc.\textsuperscript{1,2,21} The notochordal cells may represent a stem cell population within the NP, much like the mesenchymal cells within the bone marrow. Human as well as animal studies have shown that with age, the notochordal cell population disappears.\textsuperscript{2,5,11} It is believed that as notochordal cells age, they may terminally differentiate into chondrocytes within the NP in older animals.\textsuperscript{11} Because the notochordal cell is believed to produce proteoglycan, the water-holding matrix of the intervertebral disc, terminal differentiation of these cells could initiate the process of disc degeneration.\textsuperscript{10,23} The cells of the mature NP in adult humans as well as in many animal species are primarily small and terminally differentiated chondrocytes.\textsuperscript{13} Therefore, we were interested in the process of differentiation of ESC derivatives into notochordal cells as a way to regenerate the NP of the degenerated disc. A number of animal models of disc degeneration exist; however, we chose the rabbit model with modifications in the technique to preserve the NP.\textsuperscript{13,20}

Because ESCs have the ability to differentiate along different cell lineages, they represent a possible source of notochordal cells. The use of ESCs as a possible therapy for disc degeneration is a recent development. Investigators have studied the use of MSCs for intervertebral disc regeneration.\textsuperscript{17} However, stem cells have not been used to investigate intervertebral disc regeneration in an in vivo model of NP degeneration. Researchers have shown that ESCs can be induced to differentiate toward an NP-like phenotype by creating a growth environment (that is, hypoxia) similar to that found in the intervertebral disc.\textsuperscript{18} Studies have shown that ESCs can differentiate toward a chondrogenic lineage in the presence of selective culture media supplemented with TGFβ, dexamethasone, and ascorbate.\textsuperscript{6,14,15} We hypothesized that, if placed in a degenerated disc environment, the pretreated ESCs, which were encouraged to differentiate toward a chondrogenic lineage, could differentiate to produce new disc material. Because it is believed that the notochordal cell is the first cell line seen during the embryogenesis of the NP, we also hypothesized that the notochordal cell type would indeed be the first cell line seen histologically. Later in the developmental process of the disc, these notochordal cells may terminally differentiate into chondrocytes. This study represents an initial investigation into the implantation of ESCs into a degenerated intervertebral disc model and the production of cells similar to those seen in the embryogenesis of the disc, namely notochordal cells.

Methods

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC #113–07) at Providence Medical Center, Southfield, Michigan.

Establishing an In Vivo Animal Model for Disc Degeneration

Experimental Animals. A total of 16 skeletally mature female New Zealand white rabbits, aged 6–12 months, weighing between 3.2 and 3.5 kg, were used. An initial MR imaging study was performed on all rabbits under sedation to confirm that no previous disc degeneration was present, either in the control or experimental discs.

Percutaneous Technique for Inducing Disc Degeneration. Each rabbit was initially anesthetized by intramuscular injection of xylazine (3 mg/kg) and ketamine (40 mg/kg), and then placed on supplemental oxygen. The hair was shaved from the sacroiliac portion of the midback of the animal. After induction of general anesthesia, using inhalation of 2% isoflurane, the field was prepared with betadine, and draped in a sterile fashion. A mini-fluoroscopic unit was then used to identify the levels in the lumbar spine. A lateral approach from the rabbit’s right flank was taken to enter the disc segment of interest. The overlying skin was first anesthetized with 0.75% bupivacaine, and a 16-gauge needle was then advanced into the disc space through the Kambin triangle, starting ~3 cm off the midline at the level of interest. To guide needle placement, AP and lateral fluoroscopic imaging were used (Fig. 1). The needle was advanced until fluoroscopic confirmation showed the needle tip to be in the center of the disc. Needle punctures of 2 adjacent discs at L2–3 (Group B) and L3–4 (Group C) experimental groups were performed to induce disc degeneration. The L4–5 and L5–6 (Group A) disc levels served as controls. Following the procedure, rabbits were returned to their respective cages (4000 cm²), with food and water provided ad libitum for 8 weeks.

Evaluation of Disc Regeneration Using MR Imaging

Rabbits were tranquilized with an intramuscular injection of ketamine (40 mg/kg) and xylazine (3 mg/ kg), and placed supine within the MR imager coil (General Electric Medical Systems). A localizing midsagittal T2-weighted image (TR 2500 msec, TE 100 msec) was obtained to view the L1–2 through L5–6 intervertebral levels. Next, 3-mm-thick midsagittal sections were taken using T2-weighted imaging sequences (TR 2500 msec, TE 100 msec) to evaluate signal characteristics within the intervertebral disc. The T2-weighted imaging sequences (TR 5200 msec, TE 100 msec) were taken through each lumbar intervertebral disc. The MR imaging evaluations were performed initially and then postoperatively at 2, 6, and 8 weeks (Fig. 2).

Embryonic Stem Cell Culture and Differentiation

Culture and Labeling of ESCs. Mouse ESCs (7AC5), originally obtained from the American Type Culture Collection, were maintained using a previously described protocol.\textsuperscript{3} These cells were further grown in Dulbecco modified Eagle medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), and 5.6% of the supplement (0.1% gentamicin, 0.2% streptomycin, and 0.12% penicillin). To maintain the cells in an undifferentiated state, the medium was supplemented with 1000 U/ml of leukemia inhibitory factor (Chemicon International, Inc.) and mouse embryonic fibroblasts. The mouse embryonic fibroblasts
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were developed from embryos of mice (CF strain) and were maintained and grown following standard culturing techniques. The ESCs were cultured on 0.1% gelatin-coated tissue culture plates as previously described. Also, GFP was used to identify the ESCs once they were injected into the intervertebral disc. Fluorescent microscopy (Olympus) and confocal microscopy were used to confirm fluorescent labeling of the cells prior to implantation as well as to identify GFP-expressing cells after their implantation into the rabbit NP (Figs. 3B, 3C, and 4H).

Differentiation of ESCs to Chondrogenic Progenitors Prior to Implantation. The ESCs were incubated on gelatin-coated tissue culture plates in a selective medium, supplemented with a mixture of TGFβ (5 ng/ml), ascorbic-acid phosphate (50 µg/ml), and insulin-like growth factor (10 µg/ml) and lacking leukemia inhibitory factor. The medium was changed every 2 days. To direct the transformation of these cells along a chondrocyte lineage, the ESCs were treated with cis-retinoic acid (10⁻⁷ M). The differentiated ESCs were monitored by light microscopy on a daily basis. After 12–14 days of incubation in the medium, chondroprogenitor cells were detected. At the end of 4 weeks, the differentiated chondrogenic cells were removed and injected into the degenerated intervertebral discs of the Group C rabbits. The experimental Group C rabbits’ discs had previously been punctured to induce disc degeneration, which was confirmed using MR imaging.

Identification of Chondroprogenitors Using Alcian Blue Staining. Alcian blue (Sigma-Aldrich), a proteoglycan and glycoprotein stain, was used to detect and distinguish chondroprogenitor ESCs. Both of these components are also major constituents of the extracellular gelatinous proteoglycan matrix, which is found within the NP of the intervertebral discs. Samples were washed with phosphate-buffered saline and fixed with freshly made 4% paraformaldehyde for 30 minutes. The samples were then incubated with 1% Alcian blue in 3% acetic acid for 1 hour, rinsed in distilled water, and observed for blue staining (Fig. 3A).

Implantation of ESC Derivatives Into Degenerated Rabbit Intervertebral Discs

Rabbits were tranquilized, general anesthesia was induced, and the animals were surgically prepared as previously described. The GFP-expressing ESCs at a concen-
etration of $10^6$ cells in 20 µl of Dulbecco modified Eagle medium solution were prepared for injection. Guided by AP and lateral fluoroscopy, the cells were then injected with a 32 gauge 25 µl Hamilton syringe into the L3–4 degenerated disc segments (Group C), which was confirmed by MR imaging.

**Immunohistochemical Analysis of Intervertebral Disc Material**

At 8 weeks postimplantation, rabbits were killed using intramuscular ketamine (25 mg/kg) followed by intravenous injection of sodium pentobarbital (1.2 g/kg). Spines were subsequently harvested for intervertebral disc processing. Three separate groups (A, B, and C) of intervertebral discs were analyzed.

The intervertebral discs were fixed in 10% neutral buffered formalin for 1 week and decalcified with ethylenediaminetetraacetic acid (0.75 M, pH 7.8). The intervertebral discs were embedded into paraffin and cut into axial sections (5 µm thickness) by using a microtome. The sections were stained with H & E and analyzed qualitatively under light microscopy (Olympus model; magnifications × 100 and × 400). Immunofluorescent analysis of both control and experimental group intervertebral discs was performed using a fluorescent lamp objective at low- and high-power magnification. Confocal microscopy was performed at Wayne State University, Detroit, Michigan. A board-certified pathologist performed independent and blinded review of tissues.

**Results**

**In Vivo Model of Intervertebral Disc Degeneration**

The MR imaging studies confirmed that disc degeneration occurred by 2 weeks postoperatively in all intervertebral discs punctured (Groups B and C; Fig. 2). Also, as evidenced by MR imaging studies, the process of disc degeneration progressed until animals were killed, and resulted in disc height loss and diminished signal intensity as well as a progressive decrease in the NP surface area over the 8-week postoperative period. These results are consistent with observations reported by others.20 However, unlike other techniques of disc degeneration, the percutaneous technique maintained the NP within the center of the intervertebral disc, and no extruded or herniated disc fragments were observed. Conversely, in the control group, the appearance of the intact discs re-
mained unchanged over the same time period. The height and MR imaging signal intensity remained unchanged in the healthy control discs. There were no operative complications or deaths associated with this model. Rabbits tolerated the procedure well without any postoperative behavioral problems or neurological signs.

Histological Analysis of Intervertebral Disc Material

Trypan blue test confirmed 87% viability of injected cells. Alcian blue staining confirmed production of proteoglycan and glycoprotein (Fig. 3A). Fluorescence microscopy confirmed that the pre-implantation ESC derivatives expressed GFP prior to implantation (Fig. 3B, 3C).

Microscopic Findings and Immunohistochemical Analysis of Intervertebral Disc Material

Postmortem H & E staining and histological analysis of Group A intervertebral disc showed aged chondrocytes and the absence of notochordal cells. The chondrocytes appeared as small cells with dark-staining cytoplasm and a shrunken, relatively dense nucleus. Group B discs displayed some fissuring of the AF and generalized disorganization of fibrous tissue within the NP (Fig. 4C and D). Group C discs showed viable new cartilage forming as well as notochordal cell growth.

The H & E–stained discs of Group C showed that the NP was focally infiltrated by hypercellular groups, principally arranged in cords and occasionally admixed with large lobulated (physaliphorous-type) cells, diffusely separated by pale, loose myxoid stroma. The cell groups were generally composed of 3–6 small, round-to-ovoid nuclei, surrounded by eosinophilic cytoplasm in the corded structures and bubbly, foamy cytoplasm in the lobulated cells of physaliphorous type. Both forms exhibited strong pankeratin activity and did not exhibit significant...
nuclear pleomorphism or mitoses. There was neither associated necrosis nor inflammatory cells, as evidenced by the absence of macrophages or leukocytes. The patterns described strongly resembled those of chordomas, which was consistent with notochordal tissue (Fig. 4A and B). This cell histological finding was not noted in the healthy control discs (Group A) nor in the degenerated discs that were not implanted with ESC derivatives (Group B). Additionally, cell histological findings differed from the initial preimplantation chondrogenic cells, for which the histological features have been previously described as spherical fibroblastic morphology.6 This implied that the chondroprogenitors did differentiate into notochordal tissue after implantation into the degenerated disc. Morphometric analysis to quantify the amount of cells that did differentiate was not done in this study. Confocal fluorescent analysis was negative for Groups A and B but revealed viable fluorescing notochordal cells within experimental Group C discs implanted with ESCs (Fig. 4H). Sixty-micrometer sections were scanned and the fluorescent pattern of the notochordal tissue was seen through the entire thickness of the cell, indicating that these cells originated from our implanted GFP-labeled ESC derivatives. Fluorescence would not have occurred if these cells were not viable and expressing GFP. Of note, no inflammatory response, which would be counted as evidence of cell-mediated immune response, was noted in any of the 3 groups. Additionally, there was no evidence of the teratoma or “tumor” formation that had been noted in similar chondroprogenitors injected into the mouse subcutaneous tissue, as was seen in our previous studies.6

Discussion

The process of intervertebral disc degeneration occurs in all of us as we age, and its treatment in symptomatic patients has significant socioeconomic impact.5,13,24 Many studies have shown that notochordal cells, the precursors of the disc, are no longer present after 10 years of age.11 During embryogenesis, notochordal cells are believed to be responsible for the formation of spine and intervertebral disc, as well as for maintenance and metabolic control of the NP later in life. The relationship between loss of notochordal cells with age and the onset of disc degeneration can perhaps best be understood by the changes in biomechanics of the discs as a consequence of proteoglycan loss. The proteoglycans are the hydrophilic moiety of the intervertebral disc. These molecules are uniquely structured to hold water and therefore provide the cushioning quality of the intervertebral disc. It has been shown in recent studies that notochordal cells produce 1.5-fold more proteoglycans and extracellular matrix than do terminally differentiated chondrocytes.12 As the notochordal cells differentiate to chondrocytes in the NP, less water-holding proteoglycan matrix is available. A cascade of events ensues, resulting in disc degeneration, desiccation, and collapse. Consequently, the AF begins to fissure and crack, contributing to a vicious cycle of disc degeneration potentially resulting in chronic low-back pain.

To induce disc regeneration, ESCS were pretreated prior to implantation to encourage differentiation along a chondrogenic cell lineage. In previous reports, mouse ESCs were capable of differentiating into chondrocytes via embryoid bodies when treated with TGFβ3, 5,10,16,25 Therefore, we used a similar technique to treat ESCs before implantation. Additionally, the differentiation of ESCs toward a chondrogenic cell lineage has also been found to be influenced by the hypoxic environment in which the cells were cultured. Under hypoxic conditions in vitro, MSCs were found to differentiate along a phenotype consistent with that of the NP.19 Therefore, we hypothesized that implanting ESC derivatives into the hypoxic environment of a degenerated disc could potentially encourage differentiation of these cells into viable new disc material.

A number of in vivo animal models for disc degeneration have been investigated.13,20 The model of Lipson and Muir involved the process of creating a full-thickness, ventral stab incision in the AF of the rabbit spine by using a No. 11 scalpel blade. However, when done in an open surgical fashion, this model had its limitations in that the NP herniated out of the stab incision. To overcome the extrusion of the NP while initiating disc degeneration, Sobajima et al.20 modified this model by surgically exposing the intervertebral disc via a ventral approach and stabbing the disc with a 16-gauge needle instead of a scalpel blade. Using this model, they were successful in showing a sequential process of disc degeneration that occurred over time and was documented with serial MR imaging and histological analysis. In this study, further modification of the model of Sobajima et al. was created. We performed a percutaneous disc puncture through the Kambin triangle by using AP and lateral fluoroscopy to guide needle placement (Fig. 1). Sequential MR images of the rabbit spine confirmed that reproduction of the degenerative disc process occurred at 2 weeks postprocedure (Fig. 2). This model was successful in limiting animal morbidity and mortality, initiating disc degeneration, and preserving the NP, which was the target tissue for regeneration using ESCs. However, species variations make direct correlation of this model of disc degeneration with the process that occurs in humans difficult. Namely, the rapidity with which degeneration occurs in the rabbit model after AF puncture does not reflect that seen in humans. Nevertheless, this model is cost-effective, does not appear to harm the animal, and is reproducible, making it a much more viable model than using larger animals (for example, pigs or sheep).

In this study, we also demonstrated that implanted ESC-derived chondroprogenitors could potentially differentiate into notochordal cells. Harvested Group C discs confirmed that 8 weeks postimplantation, fluorescence-labeled cells appeared to resemble notochordal tissue (that is, cells with large vacuolated cytoplasm; physaliphorous-type cells) (Fig. 4A and B). These cell types were not observed in Groups A and B. Additionally, the preimplanted chondrogenic derivatives of ESCs have spherical fibroblastic morphological features in culture, which differed from differentiated ESCs implanted in Group C, which had the appearance of notochordal-type cells.6 Histologically these cells stained as PAS-positive cords and had reduced PAS activity following glycogenase exposure.
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It might be hypothesized that over time, perhaps in months to years, the notochordal cells seen in experimental Group C would continue to differentiate toward terminal chondrocytes. Furthermore, because it is believed that the notochordal cells are largely responsible for the production of the disc proteoglycan matrix, one might suspect a relative increase in proteoglycan content within Group C disc compared with Groups A and B. This is currently under investigation in our lab. However, the exact function of the notochordal cells in forming the NP and proteoglycans is largely unknown.12

Thus, this study illustrates a reproducible model for the investigation of disc degeneration as well as potential disc regeneration by using ESC derivatives. New notochordal cell populations were seen in ESC-injected degenerated discs. The lack of immune response to xenotransplanted mouse cells in an immunocompetent rabbit model could point to a previously unrecognized immunoprivileld site within the intervertebral disc. However, we recognize that this is a preliminary investigation and that we could not provide definite proof that disc regeneration was indeed taking place. Nonetheless, this study does offer interesting insight into the potential for disc regeneration by using ESCs, and further investigations are warranted.

References


Manuscript submitted March 6, 2008. Accepted December 2, 2008.
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