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Human Umbilical Cord Blood–Derived Mesenchymal Stem Cells in the Cultured Rabbit Intervertebral Disc:

A Novel Cell Source for Disc Repair

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Abstract

Objective—Back pain associated with symptomatic disc degeneration is a common clinical condition. Intervertebral disc (IVD) cell apoptosis and senescence increase with aging and degeneration. Repopulating the IVD with cells that could produce and maintain extracellular matrix would be an alternative therapy to surgery. The objective of this study was to determine the potential of human umbilical cord blood–derived mesenchymal stem cells (hUCB-MSCs) as a novel cell source for disc repair. In this study, we intended to confirm the potential for hUCB-MSCs to differentiate and display a chondrocyte-like phenotype after culturing in micromass and after injection into the rabbit IVD explant culture. We also wanted to confirm hUCB-MSC survival after transplantation into the IVD explant culture.

Design—This study consisted of micromass cultures and in vitro rabbit IVD explant cultures to assess hUCB-MSC survival and differentiation to display chondrocyte-like phenotype. First, hUCB-MSCs were cultured in micromass and stained with Alcian blue dye. Second, to confirm cell survival, hUCB-MSCs were labeled with an infrared dye and a fluorescent dye before injection into whole rabbit IVD explants (host). IVD explants were then cultured for 4 wks. Cell survival was confirmed by two independent techniques: an imaging system detecting the infrared dye at the organ level and fluorescence microscopy detecting fluorescent dye at the cellular level. Cell viability was assessed by staining the explant with CellTracker green, a membrane-permeant

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Disclosures: Financial disclosure statements have been obtained, and no conflicts of interest have been reported by the authors or by any individuals in control of the content of this article. Y. Zhang, D.G. Anderson, and H.S. An are responsible for experimental design, data interpretation, and manuscript preparation. D. Markova performed experiments on micromass culture and reverse transcription polymerase chain reaction of human type II collagen gene, as well as cell survival experiments with fluorescent dye labeling. A. Chee and C. Gupta performed cell tracking experiments with the infrared dye. V. Markov and B. Saitta isolated and characterized stem cells and provided technical expertise on expanding and using the cells in the rabbit intervertebral disc explant culture system. M. Enomoto-Iwamoto helped with micromass culture.

tracer specific for live cells. Human type II collagen gene expression (from the graft) was assessed by polymerase chain reaction.

Results—We have shown that hUCB-MSCs cultured in micromass are stained blue with Alcian blue dye, which suggests that proteoglycan-rich extracellular matrix is produced. In the cultured rabbit IVD explants, hUCB-MSCs survived for at least 4 wks and expressed the human type II collagen gene, suggesting that the injected hUCB-MSCs are differentiating into a chondrocyte-like lineage.

Conclusions—This study demonstrates the ability of hUCB-MSCs to survive and assume a chondrocyte-like phenotype when injected into the rabbit IVD. These data support the potential for hUCB-MSCs as a cell source for disc repair. Further measures of the host response to the injection and studies in animal models are needed before trials in humans.

Keywords

Intervertebral Disc; Degeneration; Regeneration; Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells (hUCB-MSC); Cell Therapy

Back pain and neck pain are common clinical problems. According to the United States Bone and Joint Decade, estimated annual direct medical costs for all spine-related conditions for the years 2002–2004 were \$193.9 billion in the United States.¹ In many affected patients, degenerative disc disease has been identified as a significant contributing factor to symptoms. As an alternative to the surgical removal of the diseased disc, a cell therapy approach to repair the degenerating disc may be feasible.

The etiology of disc degeneration is complex; among the risk factors are genetic predisposition and biomechanical properties.² One salient feature of the degenerative disc is that viable disc cells decrease in number, most likely because of apoptosis.³ In addition, cellular senescence causes a decreased ability of the remaining cells to maintain the intervertebral disc (IVD) matrix.⁴ At an early stage of disc degeneration, when sufficient healthy cells exist, degenerative changes can be slowed by a variety of methods such as stimulating IVD cells with growth factors, inhibiting proinflammatory cytokines, or inhibiting proteolytic enzymes.⁵ However, at late stages of IVD degeneration, when metabolically competent cells are low in number, repopulating the IVD with cells that could produce and maintain extracellular matrix would be desirable.^{5,6}

Multiple sources of donor cells have been considered for cell therapy to repair the degenerating IVD.^{5,6} It is feasible to transplant autologous IVD chondrocytes expanded in vitro. Gruber et al.⁷ have demonstrated the long-term survival of transplanted autologous IVD cells embedded in a collagen matrix in sand rats. Transplanted autologous IVD cells survived for at least 1 yr in a canine model.⁸ Autologous human IVD cells derived from a therapeutic discectomy have also been tested in a pilot study⁹; however, it is difficult to obtain nucleus pulposus (NP) cells without contamination of other cells (e.g., fibroblasts, leukocytes) in this setting. Bone marrow stromal cells and other adult stem cells are also attractive sources for cell-based therapy.^{10–15} Recently, Leckie et al.¹⁶ have presented data showing that injecting human umbilical tissue-derived cells into the NP improved the biomechanical properties of the degenerating IVD in vivo.

In this study, we have selected human umbilical cord blood–derived mesenchymal stem cell (hUCB-MSC) as a cell source to repair the degenerative disc tissues. We have previously shown that hUCB-MSCs have full potential to differentiate into chondrogenic, adipogenic, and osteogenic lineages.¹⁷ HUCB-MSCs are taken from donated umbilical cord blood (UCB) samples and are not subjected to the ethical and political debate surrounding embryonic stem cell research. To date, hUCBs have become a widely accepted source of hematopoietic stem cells: they have been used in transplantations to treat a number of hematopoietic and malignant diseases.¹⁸ Compared with bone marrow–derived stem cell transplantation, hUCB-MSC transplantation allows for more HLA disparity, thus requiring less stringent matching between donor and recipient.^{19,20} HUCB-MSCs are less mature than adult bone marrow–derived MSCs and thus have a larger capacity for ex vivo expansion. Finally, we have previously shown that hUCB-MSCs have full potential to recapitulate basic developmental pathways when assayed at the molecular level.¹⁷ In fact, we have successfully used one of the UCB cell types as an in vitro model for somitogenesis, a basic process in musculoskeletal development.²¹

In these studies, we explored the use of hUCB-MSCs as a therapy for degenerative disc diseases by testing their ability to survive in rabbit IVD explants and to differentiate into chondrogenic lineages in both the rabbit IVD explants and in micromass cultures.

METHODS

Isolation and Characterization of MSCs from Neonatal UCB

We have isolated and characterized hUCB-MSCs.^{17,21} Briefly, UCB samples were harvested immediately after birth from term deliveries under an institutional review board–approved protocol at the New Jersey Cord Blood Bank. After removal of the placenta, blood was collected during the first 10 mins after delivery and was drained from the distal end of the umbilical vein by gravity into a plastic bag containing 25 ml of citrate phosphate dextrose anticoagulant solution (Medsep Corporation, Covina, CA). All samples (average 40 ml of blood) were donated for research after informed consent was obtained; no personal identifiers were available to the investigators. All samples were stored at room temperature and processed within 24 hrs of delivery. The UCB samples were layered carefully on Histopaque (Sigma-Aldrich Corp, St Louis, MO) and were centrifuged at 1250g for 25 mins at 20°C. The interface layer was collected, diluted with phosphate buffered saline (Invitrogen, Carlsbad, CA), and centrifuged at 500g for 10 mins. The cells were washed in phosphate buffered saline and further centrifuged at 350g for 5 mins, a method modified according to Ridings et al.²² Cell counts were performed using an automated cell analyzer (Cell-Dyn 1700, Abbott Park, IL). UCB mononuclear cells were plated at $1\text{--}2 \times 10^6$ cells/cm² in plates coated with fibronectin (5 ng/ml) in Dulbecco's modified Eagle medium (DMEM) low glucose (Invitrogen) supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA). After 5 days of incubation in a humidified atmosphere containing 5% carbon dioxide, the culture medium was replaced, and non-adherent cells were removed. After a further 10 days in culture, single colonies of adherent spindle-shaped cells were identified and isolated from individual dishes. These isolated colonies were passaged using Trypsin (0.05%) and cultured as described previously.¹⁷

Chondrogenic Differentiation in a Pellet Culture System

Two different clones of hUCB-MSCs derived from two separate donors were used for this study. The pellet culture was repeated two times with each clone ($n = 4$). The population doubling time is estimated to be 45 hrs when cultured in monolayer. Chondrogenic differentiation was induced using a pellet culture technique described by several other groups,^{23–25} with some modifications. Approximately 6×10^5 hUCB-MSCs (passage 3) were centrifuged at 450g for 10 mins in a 15-ml polypropylene tube (Corning Inc), and the pellets were cultured in complete chondrogenic medium DMEM high-glucose (GIBCO, Invitrogen) containing sodium pyruvate (110 $\mu\text{g/ml}$), dexamethasone (100 nM), ascorbic acid phosphate (25 $\mu\text{g/ml}$), L-proline (40 $\mu\text{g/ml}$), and 0.1% insulin-transferrin-selenium (ITS) (Cellgro) and in the presence or absence of transforming growth factor (TGF)- β_3 (10 ng/ml; Sigma). The medium was replaced twice weekly for 14 days. After the 2-wk period, cell pellets were fixed with 4% paraformaldehyde and were embedded in paraffin. The sections were then stained with Alcian blue at pH2.5 and counterstained with hematoxylin and eosin. The sections of the pellets were also subjected to immunohistochemical staining for type II collagen. The slides were incubated with 0.1% pepsin in 0.02 N HCl for 10 mins at 37°C. After blocking with 10% goat serum in phosphate buffered saline containing 0.1% bovine serum albumin (BSA) for 1 hr at room temperature, the slides were incubated with anti-human type II collagen rabbit polyclonal antibody (1:400, SL-LB-1297; Cosmo Bio, Tokyo, Japan) or nonimmune rabbit immunoglobulin G for 1 hr at room temperature, followed by antibody visualization using SuperPicture Polymer detection system (Invitrogen). The slides were then counterstained with Methyl Green.

IVD Explant Culture

New Zealand white rabbits (2.5–3.0 kg, mixed male and female) were used to prepare rabbit IVDs under the protocol approved by the Thomas Jefferson University Institutional Animal Care and Use Committee (approval 795C). Detailed methodology has been described previously.²⁶ Briefly, rabbits were anesthetized and infused with heparin intravenously to prevent blood clots from blocking the nutrient diffusion through endplate pores.^{27,28} The rabbits were then euthanized. Whole lumbar IVDs with the surrounding endplates were isolated ($n = 6$ discs/animal) and maintained in organ culture in a tissue culture plate with a surface area of 3.8 cm^2 /well (Corning) in DMEM with 20% fetal bovine serum supplemented with ascorbic acid; the medium was changed daily.

Evaluation of the Implanted hUCB-MSC Survival by Infrared Fluorescence

To track hUCB-MSCs at the organ level, cells were labeled with infrared (IR) dye (CellVue NIR815 dye; LI-COR Biosciences) according to manufacturer's instructions. Before injection, the lipid regions of the cell membrane of hUCB-MSCs were stably incorporated with the IR dye bound to long aliphatic tails. The IR dye used here emits light of 815-nm wavelength and could be detected through the IVD explant tissues. The labeled hUCB-MSCs were resuspended at a concentration of 1×10^7 cells/ml in serum-free media. Eight microliters of the cell suspension (1×10^7 cells/ml) was then injected into the central region of each rabbit disc (RD) explant using a Hamilton syringe with a 26-gauge needle.

The IVDs were cultured for 4 wks. After cell injection, the IVD explants injected with IR dye-labeled cells were scanned weekly and analyzed using the dual-channel Odyssey IR Imaging System (LI-COR Biosciences). IR fluorescence intensity counts per microliter of the IVD explants were determined using LI-COR imaging software.

Evaluation of the Implanted hUCB-MSC Survival by Fluorescent Microscopy

For tracking implanted cells at the cellular level, hUCB-MSCs were labeled with fluorescent dye (DiI). Cell membranes were stained with a lipophilic dye and detected by their red fluorescence. Specifically, CM-DiI (Molecular Probes) was added to the culture media at 2 μ M concentration for 1 hr 1 day before injection and was incubated with media free of dye overnight. On the day of cell transplantation, the MSCs were trypsinized and resuspended in serum-free culture media at 1×10^7 /ml, and 8 μ l of cells was injected into the RD explant. The IVDs were cultured for 4 wks. At the end of the 4-wk culture period, the discs, with their adjacent end-plates, were incubated overnight with fresh medium containing 0.25 μ g/ml CellTracker Green chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Eugene, OR), which stains only live cells. CellTracker Green is a dye that is sensitive to the mitochondrial membrane potential, thus indicating that cells are viable.¹¹ A decrease in membrane potential would signal that there is a drop in the energy status of the cells possibly linked to the induction of apoptosis.²⁹ Subsequently, the discs were incubated overnight with medium without dye to remove unbound dye. The NP tissues then were scooped out with a scalpel and mounted on glass slides. Live cells from the discs stained with Cell-Tracker Green were examined by the Fluoview Laser Confocal system equipped with an Olympus microscope (Olympus 1X70; excitation at 492 nm, emission at 517 nm) and detected by their green fluorescence. Transplanted cells, which have been stained with DiI, were also examined and detected by their red fluorescence. The two images were superimposed to demonstrate colocalization of DiI with CellTracker green, indicating live transplanted cells.

Documentation of Graft (Human) Chondrocyte Marker Gene Expression by Reverse Transcription Polymerase Chain Reaction

To confirm that human type II collagen (hCOL2) gene was expressed within the disc, semi-quantitative reverse transcription (RT) polymerase chain reaction (PCR) was performed. hUCB-MSCs cultured in monolayer were trypsinized and resuspended in serum-free culture media at 1×10^7 /ml, and 8 μ l of cells was injected into the RD explant. The discs were cultured for 4 wks. After the 4-wk post-implantation time point, the discs were separated from the adjacent endplates with a scalpel. Total cellular RNA was isolated using Trizol reagent (Invitrogen) and tissues were homogenized using a rotor-stator device (Omni International, Marietta, GA). Next, 0.75 μ g of total RNA from each sample was reverse transcribed to generate first-strand cDNA, using Superscript III RT and oligo-dT as a primer (Invitrogen). PCR was performed with the Platinum Taq DNA Polymerase (Invitrogen), as previously described.³⁰ hCOL2 α 1 subunit (referred to as hCOL2) was amplified using a pair of primers (GenBank accession number NM_033150.2; forward primer, 5'-TCC CAA AGG TGC TCG AGG AGA-3'; reverse primer, 5'-CAC GAT CAC CCT TGA CTC C-3'; expected product length, 395 bp). Rabbit glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control for RNA loading (GenBank accession

number L-23961; forward primer, 5'-AGG TCA TCC ACG ACC ACT TC-3'; reverse primer, 5'-GTG AGT TTC CCG TTC AGC TC-3'; expected product length, 202 bp). Human GAPDH gene was amplified with a pair of human-specific primers (GenBank accession number NM_002046.3; forward primer, 5'-ATG GGG AAG GTG AAG GTC GG-3'; reverse primer, 5'-GCC AGT GGA CTC CAC GAC GT-3'; expected product length, 300 bp). Each PCR reaction consisted of an initial denaturation at 95°C for 5 mins; followed by 30 cycles of denaturing at 94°C for 30 secs, annealing at 60°C for 30 secs, and extension at 72°C for 30 secs; and finishing with an extension step of 7 mins at 72°C. PCR products were separated in 2% agarose gel. The density of individual band was analyzed with Quantity One-4.5.0 Basic 1-D Analysis Software (Bio-Rad).

RESULTS

HUCB-MSCs Form Micropellets Contain Proteoglycans Stained with Alcian Blue

Chondrogenic differentiation was induced using pellet culture technique in the absence (Figs. 1A, C) or presence (Figs. 1B, D) of TGF- β_3 for 2 wks.

Figures 1A and B show microscopic images of the sections that were prepared from the micro-pellets and stained with Alcian blue, followed by counterstaining with hematoxylin and eosin (at $\times 20$ magnification). Figures 1A' and B' show images of the pellets (areas within the boxes) at $\times 40$ magnification. Both pellets cultured with or without TGF- β_3 were positive for Alcian blue staining, indicating accumulation of highly sulfated proteoglycan. Figure 1C and D show microscopic images of the sections that were prepared from the micropellets stained with hematoxylin and eosin. Figures 1C' and D' show images of the pellets (areas within the boxes) at $\times 40$ magnification. Both pellets cultured with or without TGF- β_3 contained round chondrocyte-like cells. Thus, hUCB-MSCs can produce and accumulate highly sulfated proteoglycan under the high-density culture condition both in the presence and absence of TGF- β_3 , suggesting that the hUCB-MSCs have chondrogenic potential.

HUCB-MSCs Form Micropellets Contain Type II Collagen Confirmed by Immunostaining

In addition, we immunostained the micropellets with an anti-hCOL2 antibody (Fig. 2). Figures 2A and A' show hUCB-MSC micropellets cultured with media containing TGF- β_3 , whereas Figures 2B and B' show pellets cultured without TGF- β_3 . Both pellets were stained positive for type II collagen (Figs. 2A', B'); TGF- β_3 did not enhance type II collagen production.

HUCB-MSCs Survive in Cultured Rabbit IVD Explants for at least 4 wks

We next tested whether hUCB-MSCs could maintain their viability when transplanted into the IVD for cell therapy. We used an IR dye to track hUCB-MSCs. IR dye stains lipophilic membranes and does not transfer from one cell to another. When the cells divide, the dye is expected to divide equally between the daughter cells. Approximately 8×10^4 IR dye-labeled hUCB-MSCs were injected into cultured rabbit IVD explants ($n = 2$). The rabbit IVD explants were cultured for 4 wks. IR dye was examined at weekly intervals (Fig. 3). In Figure 3, the panels on the left show the IVD explants scanned by an IR imager. The

injected cells were detected using an 800-nm-wavelength channel, 1 to 4 wks after injection, and is represented in green (Figs. 3A1–A4). The RD contour was detected using a 700-nm-wavelength channel, 1 to 4 wks after injection, and is represented in red (Figs. 3B1–B4). Figures 3C1–C4 show superimposed images of panels A1–A4 and B1–B4, showing the injected cells with the disc contour. Colocalization of the injected cells (green) and disc contour (red) is represented in yellow. In Figure 3, the right panel shows the integrated fluorescence intensity of the IR dye in the whole disc (counts per microliter) detected in the 800-nm channel. The fluorescence intensity of each disc had decreased by about 40% at the end of the 4-wk culture period compared with the 1-wk time point ($P = 0.0120$). The IR dye is light sensitive but is expected to be stable in the cultured IVD explants because the endplates are opaque. Once the cells break down, the dye is thought to diffuse out into the media, although this has not been demonstrated by experiments. Thus, in this study, the decrease in fluorescence intensity is likely caused by cell death.

To confirm at the cellular level that the hUCB-MSCs were indeed viable, we stained the MSCs with DiI and CellTracker Green (Fig. 4). Before injection into the IVD explant, hUCB-MSCs were labeled with a lipophilic dye, and DiI, which stained the cell membranes red, were injected into the rabbit IVD explants. DiI stains the lipophilic membranes and is not transferred from the cells stained with this dye to neighboring cells. The IVDs were cultured for 4 wks. At the end of the 4-wk culture period, the IVD explants were stained with CellTracker Green, which stain only live cells. The NP tissues were scooped out and examined with a confocal fluorescence microscope. hUCB-MSCs stained red with DiI were detected within the NP tissue (indicated by arrows in Fig. 4A). Live cells, including both the host and transplanted cells, were stained green with CellTracker Green. Figure 4B shows that both the host and graft cells were labeled green with Cell-Tracker Green, suggesting that these cells are still viable after 4 wks in culture. Arrows in Figure 4B indicate cells corresponding to the DiI-labeled cells in Figure 4A. Finally, Figure 4C shows the images in Figures 4A and B superimposed. Arrows point to orange cells that demonstrate a portion of those transplanted cells surviving at least 4 wks.

HUCB-MSC Express Type II Collagen, a Cartilage Marker Gene, in Cultured Rabbit IVD Explants After a 4-wk Culture Period

To detect human IVD-like phenotype marker genes, the rabbit IVD explant cultures injected with hUCB-MSCs were examined by RT-PCR. Approximately 8×10^4 cells were injected into each disc explant (8 μ l of 1×10^7 /ml cells). At the end of the 4-wk culture period, IVD tissues were separated from the adjacent endplates, and total cellular RNA was extracted. Quantitative RT-PCR was performed with custom-designed primers for hCOL2 and human GAPDH and rabbit GAPDH as internal controls (Fig. 5, left panel). hUCB-MSCs cultured in monolayer did not express hCOL2 mRNA (Fig. 5, left panel, lane 1). Lane 2 of this panel shows that RD not injected with human cells did not express hCOL2 or human GAPDH, confirming that the human primers are not cross reacting with rabbit genes. The hCOL2 gene was expressed only in RD explants injected with hUCB-MSC (Fig. 5, left panel, lane 3). The densities of individual bands were analyzed with Quantity One 4.5.0 software (Bio-Rad). The ratio of hCOL2 collagen bands to human GAPDH bands was calculated and is presented in the right panel.

DISCUSSION

As an alternative to the surgical removal of the diseased disc, cell therapy may be a promising option to help reduce disc degeneration, especially in more advanced stages of disc degeneration. Multiple sources of donor cells, including articular chondrocytes, IVD cells, and MSCs, have been considered for cell therapy to repair the degenerating disc. Because the ongoing political and ethical battles have limited embryonic stem cell use, other cells lines, such as adult MSCs, UCB-MSC,¹⁷ and, more recently, placenta or umbilical tissue-derived stem cells,¹⁶ are attracting more attention.

In this set of experiments, three lines of UCB-MSC isolated from three different individuals were used. Using flow cytometry, we found that all these cell lines expressed the MSC surface markers CD29, CD44, CD73, CD90, and CD105, and neither of the UCB-derived cells stained for the hematopoietic markers CD11b, CD34, and CD45, nor for the endothelial marker CD31. We also were able to differentiate them in vitro into adipocytes, chondrocytes, and osteocytes. Moreover, the colony forming unit–fibroblasts assay was positive.¹⁷ These data were accepted as proof of the presence of UCB-MSCs. We did not perform the extensive testing and analysis that were described in the Markov et al.¹⁷ article. We did, however, follow the Markov et al.¹⁷ approach for one cell line where we had a special interest. It was possible to perform microarray analysis for each one of the obtained cell lines. However, we were able to confirm that the cell lines did indeed contain MSCs according to the MSC definition of phenotype: the self-renewing capacity (i.e., the ability to form colony forming unit–fibroblasts and the potential to differentiate into multiple cell types).

In this study, we have shown that hUCB-MSCs formed proteoglycan-rich micromass when cultured in pellet, a capacity similar to that of bone marrow–derived MSCs.³¹ Culture media described by multiple groups to induce bone marrow–derived MSCs into chondrogenic pathway all include dexamethasone, ascorbic acid, and TGF- β_1 or TGF- β_3 .^{23,24,32} The high-density culture condition such as pellet culture system is also conducive to chondrogenesis. Markov et al.¹⁷ were the first to describe chondrogenic differentiation of the hUCB-MSCs. To determine whether TGF- β enhances chondrogenesis, in this study, the pellets were cultured with or without TGF- β_3 . The addition of TGF- β_3 seemed to enhance proteoglycan by Alcian blue staining in the cultured pellet but did not enhance type II collagen by immunostaining. This suggests that hUCB-MSCs have considerable differences compared with other stem cells. Therefore, TGF- β_3 was not used to pre-treat cells before injecting into the cultured rabbit IVD explants.

The cell tracking experiment with the IR dye shows that hUCB-MSCs injected into the IVD explants persisted for at least 4 wks, although the total IR dye intensity decreased by about 40% (Fig. 3). Figure 4 shows that cells labeled with DiI were detectable at the 4-wk time point in the IVD explants and were stained with CellTracker green, indicating that they were still viable. Finally, Figure 4 shows that the injected hUCB-MSCs are distributed throughout the NP, suggesting that these cells were integrated into the host tissue. We have extended the IVD culture period beyond the 1-wk time frame described by Risbud et al.¹² The extended

cell survival is likely facilitated by the heparin infusion done before killing the animals, which may enhance the disc cell nutrition by maintaining patent end-plate pores.^{28,33}

We injected 8 μ l of cells suspended at 1×10^7 cells/ml (a total of 8×10^4 cells) into each disc. This cell number was based on nutritional needs of the cells in vitro and on the thickness of RDs, as reported by Stairmand et al.³⁴ Cells were injected by applying steady pressure on the Hamilton syringe with a 26-gauge needle. We have tested larger gauge needles and larger volumes of injected material, which resulted in leakage around the needle. Although we did not test the shear force inside the needle, we injected the cells over about a 10-sec period to reduce to shear force and improve cell survival. In the future, a range of cell numbers will be examined to further confirm the optimal number of cells for cell therapy. Our future work will also be conducted under low oxygen culture conditions, which has been shown to direct MSCs toward the IVD-like phenotype.³⁵ Finally, cell density –dependent effects of autologous canine MSCs injected into the canine discs has been shown recently.³⁶ In the future, the number of cells needed for the RDs will be determined in the RD injury model in vivo.

Whereas hUCB-MSCs cultured in monolayer do not express the type II collagen gene, they do express this gene after injection into the disc explant (Fig. 5). This suggests that the microenvironment in the rabbit IVD induced the hUCB-MSCs into a chondrogenic pathway. This is consistent with the findings by Strassburg et al.³⁷ that cellular interactions between MSCs and degenerate NP cells may stimulate MSC differentiation to an NP-like phenotype.

Future studies include determining the effects of the transplanting hUCB-MSCs on the host extracellular matrix gene expression and protein production in the rabbit organ culture system. Finally, studies in the RD injury model in vivo are needed. We hope that these studies will lay the groundwork to design hUCB-MSCs as a promising treatment option for patients with severe disc degeneration and back pain.

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FIGURE 1.

Human umbilical cord blood–derived mesenchymal stem cells cultured as micropellets stained with Alcian blue for proteoglycans. A and B, micropellets stained with Alcian blue and counterstained with hematoxylin and eosin (H&E) and examined by stereomicroscopy. C and D, paraffin sections of the micropellets stained with H&E. A and C, micropellets cultured without transforming growth factor (TGF)- β_3 . B and D, micropellets cultured in the presence of TGF- β_3 . Panels A'–D' are high-magnification images of boxed areas in panels A–D, respectively.

FIGURE 2.

Human umbilical cord blood–derived mesenchymal stem cells cultured as micropellets immunostained for type II collagen. A and A', pellet cultured with transforming growth factor (TGF)-β. B and B', pellet cultured without TGF-β. A and B, nonimmune rabbit immunoglobulin G as negative control; A' and B', immunostained with anti–type II collagen primary antibody.

FIGURE 3.

Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) persisted for up to 4 wks after injection into cultured rabbit intervertebral disc (IVD) explants. A, rabbit IVDs injected with infrared dye-labeled hUCB-MSCs were cultured and scanned weekly by a LI-COR scanner. The injected cells were detected in the 800-nm-wavelength channel and is represented in green. The rabbit disc contour was detected in the 700-nm-wavelength channel and is represented in red. Colocalization of the injected cells (green) and disc contour (red) was represented in yellow. B, integrated intensity of the fluorescence in the 800-nm-wavelength in the IVD. Error bar: standard error of the mean. * $P < 0.05$.

FIGURE 4.

Human umbilical cord blood–derived mesenchymal stem cells (hUCB-MSCs) were viable 4 wks after injection into cultured rabbit intervertebral disc explants. Nucleus pulposus (NP) tissues were scooped out and examined by confocal fluorescent microscopy. A, hUCB-MSCs stained with DiI were detected by their red fluorescence. B, live cells stained with CellTracker Green were detected by their green fluorescence. C, the overlay of panels A and B illustrating the integration of the transplanted cells (orange) into the viable native NP tissue (green). Arrows in all three panels point to injected cells.

FIGURE 5.

hCOL2 gene expression by RT-PCR 4 wks after hUCB-MSC injection into the RD. A, polymerase chain reaction products separated on agarose gel. Lane 1, hUCB-MSC cultured in monolayer; lane 2, RD; lane 3, rabbit disc injected with hUCB-MSC (RD + MSC). B, ratio of hCOL2 to hGAPDH density. hCOL2 indicates human type II collagen; RT, PCR, reverse transcription polymerase chain reaction; hUCB-MSC, human umbilical cord blood-derived mesenchymal stem cell; RD, rabbit disc; hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase; rGAPDH, rabbit glyceraldehyde-3-phosphate dehydrogenase.