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An Experimental Study of the Regeneration of the Intervertebral Disc With an Allograft of Cultured Annulus Fibrosus Cells Using a Tissue-Engineering Method

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Study Design. Cultured annulus fibrosus cells within an atelocollagen honeycomb-shaped scaffold with a membrane seal were allografted into the lacunas of intervertebral discs of which the nucleus pulposus had been vaporized using an indocyanine green dye-enhanced laser. Regeneration of the intervertebral disc was assessed based on the viability and histologic status of the allografted annulus fibrosus cells, as well as the prevention of narrowing disc space.

Objectives. To study the regeneration of intervertebral disc after laser discectomy using tissue-engineering methods.

Summary of Background Data. Intervertebral disc is the most avascular tissue in the human body, and its ability to regenerate is as low as that of articular cartilage. When nucleotomy is carried out, little regeneration of the annulus fibrosus is observed; consequently, intervertebral disc degeneration is inevitable.

Methods. Annulus fibrosus cells isolated from 20 Japanese white rabbits were labeled with a PKH-26 fluorescent dye and seeded within an atelocollagen honeycomb-shaped scaffold with a membrane seal. Annulus fibrosus cells cultured in atelocollagen honeycomb-shaped scaffold with a membrane seal for 1 week were allografted into the lacunas of intervertebral discs of recipient rabbit of which the nucleus pulposus had been vaporized using an ICG dye-enhanced laser. Soft radiograph photographs of the lumbar spine of these anesthetized rabbits were taken, the disc space measured, and the lumbar spine extracted 2, 4, 8, and 12 weeks after the operation. The proliferation of allografted annulus fibrosus cells with 5-bromo-2'-deoxyuridine/PKH-26 fluorescent labels was assessed using consecutive frozen sections, and safranin-O staining carried out for histologic evaluation.

Results. The allografted annulus fibrosus cells were viable and showed proliferation activity with a hyaline-like cartilage being produced. The narrowing of the intervertebral disc space of the cell translation group was significantly prevented, as shown, up to 12 postoperative weeks.

Conclusion. The annulus fibrosus cells cultured in an ACHMS-scaffold were allografted into the lacunae of nucleus pulposus (obtained using laser vaporization), as well as the hole of annulus fibrosus (obtained by laser fiber insertion) of rabbit intervertebral discs. These cells were viable and showed cell proliferation in the disc tissues of recipients. [Key words: regeneration, intervertebral disc, scaffold, annulus fibrosus, tissue engineering]
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Intervertebral disc is histologically categorized as fibrocartilage. It is the most avascular tissue in the human body, and its ability for tissue regeneration is as low as that of articular cartilage.[14] When nucleotomy, a surgical treatment for intervertebral disc herniation, is carried out, almost no regeneration of the nucleus pulposus (NP) as well as the annulus fibrosus (AF) occurs; therefore, the degeneration of intervertebral disc is inevitable.[8,10]

We have previously reported a minimally invasive surgical procedure, namely, percutaneous laser disc decompression.[21] An indocyanine green (ICG) dye-enhanced laser was chosen for percutaneous laser disc decompression because of its high selectivity, enabling vaporization of the NP without causing damage to important surrounding tissue (Figure 1A). Furthermore, the safety and effectiveness of this laser have been demonstrated in animal experiments.[21] The selectivity of this method allows the preservation of the inner AF and junctional area, playing a central role in the postoperative repair mechanism.[8,23] Nevertheless, natural healing is still required for adequate repair of the intervertebral disc tissue.

Transplantation of cultured articular chondrocytes[3,16] and transplantation of cultured chondrocytes within the atelocollagen gel[7,14,24] are already clinically used to repair or regenerate articular cartilage. Cartilage tissue plays a central role in the mechanical functioning. Therefore, tissue engineering of the cartilage has been recognized as particularly important in today's aging society. Using cultured AF cells, we are studying the possibility to regenerate intervertebral disc.

We have previously reported the preparation of an atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) as a carrier material for AF cells.[6,20] The AF cells *in vitro* were able to grow and remained phenotypically stable in the ACHMS-scaffold. In addition, the AF cells cultured in an ACHMS-scaffold

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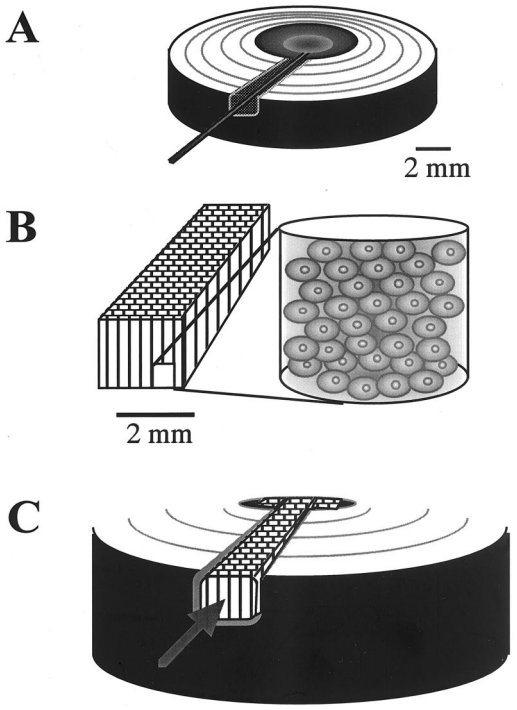

Figure 1. Experimental scheme. **A**, NP was selectively vaporized using ICG-dye-enhanced diode laser. **B**, High-density three-dimensional culturing of AF cells was carried out in the ACHMS-scaffold using tissue engineering. **C**, AF cells in the ACHMS-scaffold were allografted into the lacunas of NP and the hole of AF to regenerate intervertebral disc.

maintained the viability and ability to produce Type II collagen and proteoglycan (Figure 1B).[20] In the present study, AF cells cultured in an ACHMS-scaffold were allografted into the lacunas of intervertebral discs of which the NP had been vaporized using an ICG dye-enhanced laser (Figure 1C). Regeneration of the intervertebral disc *in vivo* was assessed based on the viability and histologic status of the allografted AF cells (Figure 1).

■ Materials and Methods

Animal experiments were carried out according to the protocol approved by the Animal Experimentation Committee of the National Defense Medical College.

AF Cell Culture in an ACHMS-Scaffold and PKH-26 Labeling. Annulus fibrosus from the intervertebral discs of 20 female Japanese White rabbits (4 weeks old; average weight, 1.0 kg) were ground and then digested for 1 hour in F12/DMEM (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 0.4% (w/v) Pronase E (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) and subsequently for 4 hours in F12/DMEM containing 0.025% (w/v) bacterial Collagenase P (Boehringer Mannheim GmbH, Mannheim, Germany). The AF cells were then isolated before labeling with PKH-26[5,19] fluorescent dye (PKH-26 Red Fluorescent Cell Linker Kit, Sigma, St. Louis, MO), seeded in an ACHMS-scaffold (diameter 11 mm, thickness 2 mm) and placed in 48 specially prepared well-plates, at a relatively high density of 2×10^6 cells per scaffold. The cells were then cultured in F12/DMEM supplemented with 10% fetal bovine serum, 50 μg/mL ascorbic acid (Sigma), and 100 U/mL streptomycin-penicillin at 37 C in an atmosphere of 5% CO_2 and 95% air. An ACHMS-scaffold is composed of a biodegradable atelocollagen matrix in which holes are aligned in one direction. This structure facilitates the supply of nutrients to the cells as well as the release of waste and other products. After AF cell culturing in an ACHMS-scaffold for 1 week, allografting was carried out.

The lipophilic dye PKH-26 is a nonradioactive substance with no known cellular toxicity, having a fluorescent half-life of >100 days in erythrocytes (manufacturer's package insert). The fluorescence of PKH-26 is not transferred to other cells but does transfer to daughter cells.[19] Labeling was performed according to the manufacturer's instructions, *i.e.*, reacting 2×10^7 cells with PKH-26 in F12/DMEM without serum at 25 C for 3 minutes.

Laser Irradiation and Transplantation. Recipients were 40 female Japanese white rabbits (14–16 weeks of age; average weight 3.2 kg). The rabbits were classified into the following recipient groups: i) the cell-containing scaffold insertion group, in which AF cells cultured in an ACHMS-scaffold were allografted after laser vaporization; ii) the scaffold insertion group, in which only the ACHMS-scaffold was inserted after laser vaporization; iii) the noninsertion group, in which laser vaporization was performed but neither cells nor the scaffold carrier was inserted; and iv) the sham group, in which a sham operation (without vaporization or insertion) was carried out. The rabbits were anesthetized with intramuscular injections of 120 mg ketamine and 9 mg xylazine. A vertical incision (length, about 8 cm) was made dorsally, and subcutaneous ablation revealed the area from the peritoneum and the lateral muscles rectus abdomens to the intervertebral discs. A 27-gauge needle was inserted into three intervertebral discs L3–L4, L4–L5, L5–L6, and a microinjector was used to inject 20 μL of ICG (Diagnogreen, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) solution (1 mg/mL), with an absorption maximum at 805 nm in the absorption spectrum of a diode laser (DIOMED25, power output: 25 W, DIOMED Ltd., Cambridge, U.K.). A hole (diameter 2.5 mm) was made extending from the AF to the NP. Subsequently, diode laser intermittent irradiation for 15 seconds through a single silica fiber (DM-6060, Olympus, Tokyo, Japan) 600 μm in diameter delivered a total energy of 50 J to vaporize the NP. The ACHMS-scaffold, containing 1-week cultured AF cells, was cut longitudinally into four sections, two of which were inserted using a cannula consisting of a 16-gauge Surflo catheter (Terumo Co. Ltd., Tokyo, Japan), into the lacunas after vaporization of the NP as well as the hole of the AF. The other two sections were used as a control for determination of the fluorescence strength in proliferation assay of allografted AF cells.

Evaluating Disc Space. Soft radiographic photographs of the lumbar spine of the anesthetized rabbits were taken with a super soft radiograph apparatus (SOFTEX-CSM-2, SOFTEX Co. Ltd., Osaka, Japan). The disc space of two rabbits from each of the four recipient groups, using the three of the same discs (L3–L4, L4–L5, L5–L6) from each of these rabbits (n = 6, total of 24 discs), was measured at 2, 8, and 12 postoperative weeks. The disc space narrowing was determined using the Bradner disc index[2,22] (Figure 2). Each value was compared with the preoperative value.

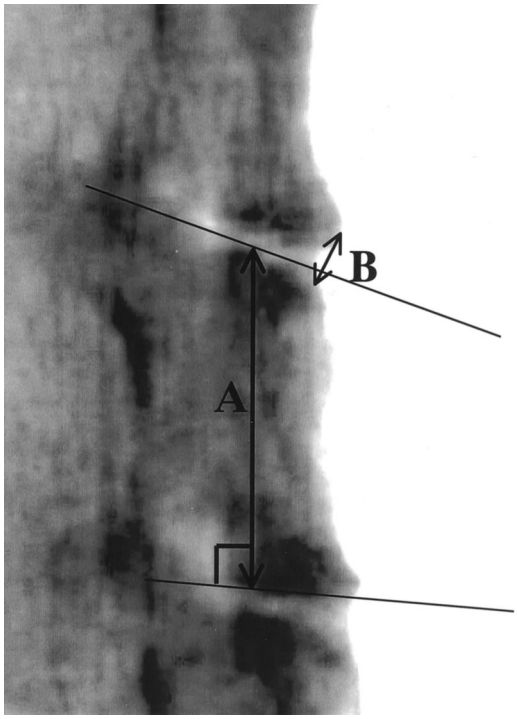

Figure 2. Bradner disc index: B/A. **A**, Maximum height of vertebral body. **B**, Disc height at its anterior portions.

Evaluation of Proliferation Activity of Allografted AF Cells. Three lumbar spines of two rabbits were chosen from each of the four recipient groups (n = 6, total of 24 lumbar spines) and were extracted at 2, 4, 8, and 12 postoperative weeks. All osseous sections as well as cartilage endplates were removed, and the intervertebral discs, consisting of NP and AF, were analyzed. To measure cell proliferation activity of the allografted cells, the extracted intervertebral discs were placed in an organ culture medium containing F12/DMEM, including anti-5-bromo-2'-deoxyuridine (BrdU)-fluoresce-F(ab')$_2$ monoclonal antibody fragments[4] (In Situ Cell Proliferation Kit, Roche Diagnostics GmbH, Tokyo, Japan) for 90 minutes at 37 C in an atmosphere of 5% CO_2 and 95% air. The antibody fragments specifically recognize BrdU and have been shown not to cross-react with other intracellular substances such as thymidine or uridine. This method enables identification of growing cells using limited fluorescent labeling to the nucleus of the cell during the synthesis phase (S-phase) of the cell cycle. After labeling, frozen sections of the organ-cultured tissues were prepared and studied under a fluorescent microscope (Nikon Ltd., ECLIPSE E 600, Tokyo, Japan).

The proliferation of allografted cells with BrdU/PKH-26 fluorescent labels was assessed at 2, 4, 8, and 12 postoperative weeks using 6 different microscopic views. Images on slide film were scanned into the computer, and the intensity of fluorescence was determined using NIH imaging software (version 1.62, National Institutes of Health).

Histologic Evaluation. Consecutive frozen cross-sections about 6 μm thick were prepared from the intervertebral discs of recipients. After PKH-26 fluorescence was observed and photographed, the cartilage matrix of the samples was stained with safranin-O (Merck Co. Ltd.).

Statistical Analysis. The Dunn test was used as a *post hoc* test using a one-way analysis of variance in the statistical analysis of the results.

■ Results

Disc Spacing

Progressive narrowing of the intervertebral disc space was observed in the cell-containing scaffold insertion group, the scaffold insertion group, and the noninsertion group. A greater degree of narrowing was obtained in the noninsertion group and the scaffold insertion group when compared with cell-containing scaffold insertion group at 2 postoperative weeks. And at 12 postoperative weeks, the noninsertion group and the scaffold insertion group showed a disc space narrowing of 33% and 45%, respectively, whereas for the cell-containing scaffold insertion group, the value was <20%, indicating a significant difference ($P < 0.05$, Figure 3).

Viability and Proliferation Activity of Allografted Cells

In the cell-containing scaffold insertion group, PKH-26-related fluorescence was observed at 2, 4, 8, and 12 postoperative weeks in >90% of the cells, indicating that the almost all allografted cells were viable (Figure 4A). To further show proliferative activity of the cells, a cell-labeling assay with BrdU specifically binding to nuclei of S-stage cells in the cell cycle was carried out. This assay showed also positive results at 2, 4, 8, and 12 postoperative weeks (Figure 4B). The ratio of BrdU-positive cells to PKH-26-positive cells was about 10%, showing no statistically significant difference in ratio (Figure 4C). These observations indicated that allografted AF cells possessed cell proliferative activity at each postoperative time point determined, *i.e.*, 2 to 12 weeks.

Histologic Findings

In the noninsertion group, a minor cellularity was generated on the laser-vaporization and amorphous tissue stained weakly with safranin-O in the NP (Figure 5A)

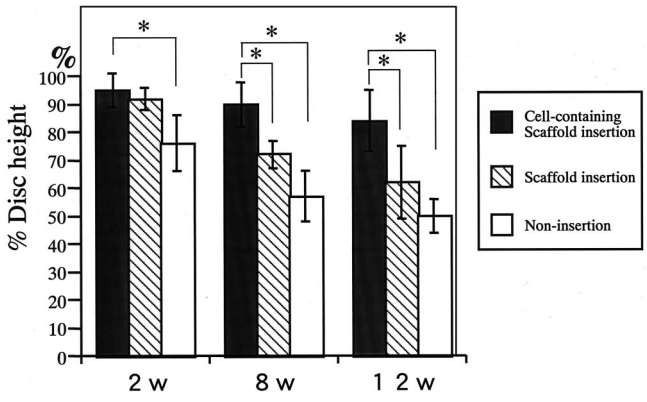

Figure 3. Progressive narrowing in intervertebral disc space. Narrowing of disc space was smaller in the cell-containing scaffold insertion group than in the noninsertion group and scaffold insertion group (*$P < 0.05$ *vs.* cell-containing scaffold insertion group, n = 6 for each group). Disc height (Bradner's index) of sham-operated animals was defined as 100%.

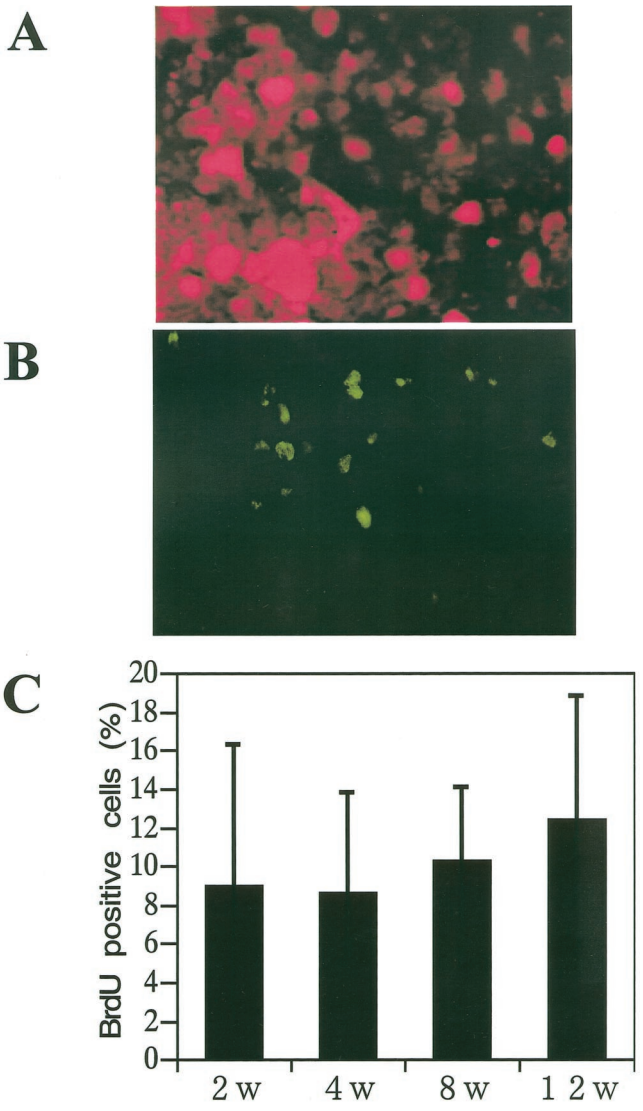

Figure 4. Viability and proliferation activity of allografted AF cells. **A**, PKH-26 fluorescence in cultured cells 12 weeks after allografting (original magnification ×400). **B**, BrdU fluorescence in nuclei of allografted AF cells during culturing period 12 weeks after allografting (original magnification ×400). **C**, Ratio of BrdU-positive cells. Ratio of BrdU-positive cells to PKH-26-positive cells was approximately 10%, not changing over observed time period.

and in the AF (Figure 5D). In the scaffold insertion group, a small number of endogenous cells in the NP migrated into the scaffold, resulting in a weak staining. However, the migrated cells and produced matrix did not completely occupy the available space of the scaffold (Figure 5B). In the AF of the scaffold insertion group, the scaffold was surrounded by cicatricial tissue with a minor cellularity and weak staining (Figure 5E). In the cell-containing scaffold insertion group, AF cells were able to proliferate in the scaffold, resulting in a strong safranin-O staining in the NP (Figure 5C). In the AF of the cell-containing scaffold insertion group, a marked accumulation of cartilage-like matrix with a strong staining was observed inside and around the scaffold, resulting in hyaline-like cartilage and a strong safranin-O staining, which is histologically very similar to normal AF tissue (Figure 5F).

■ Discussion

Recent widespread use of magnetic resonance imaging has shown that intervertebral disc degeneration progresses asymptomatically with age and is irreversible.[8,10] In addition, the disc space narrows and the amount of water contained in intervertebral discs decreases with age. Herniation of the intervertebral disc results from the NP protruding from fissures of the degenerated AF, and nucleotomy actually exacerbates intervertebral disc degeneration.[8,10] Nucleotomy or minimally invasive surgical procedures, such as percutaneous nucleotomy or percutaneous laser disc decompression, inevitably exacerbate intervertebral disc degeneration,[10,22] although it may favor the occurrence of a new disc herniation.[18]

Mochida *et al*[10] reported that the progress of intervertebral disc degeneration following percutaneous nucleotomy is related to the amount of NP extracted. Extraction of a small amount (1.0 g) was found to result in a slower progression of degeneration and better results over the long-term, when compared with extraction of a larger amount. These results have drawn attention to the issue of preventing disc degeneration after nucleotomy. It is thought that severely degenerated intervertebral discs, as often seen after nucleotomy, are a cause of back pain suffering. Any treatment promoting disc tissue repair after nucleotomy as well as preventing the progression of intervertebral disc degeneration would be valuable in a clinical setting.

Basic research on the prevention of intervertebral disc degeneration has been conducted by Nishimura and Mochida.[13] Using a rat model in which a herniation was induced in the caudal vertebrae of rats, the reinsertion of either fresh or cryopreserved NP was found to prevent the progression of intervertebral disc degeneration. Okuma *et al*[15] reported a similar success in controlling the progress of intervertebral disc degeneration in a rabbits by reinsertion of the NP. In addition, Nishida *et al*[11,12] reported the possibility of treating degenerated intervertebral discs by introducing the TGF-β1 gene into NP cells of rabbits using an adenovirus as vector. However, intervertebral disc herniation also involves degeneration of the AF, and even minimally invasive surgical procedures cause additional damage to the AF, accelerating further degeneration. To our best knowledge, there are no reports describing the application of a scaffold seeded with AF cells to prevent intervertebral disc degeneration.

The intervertebral disc has a high degree of heterogeneity: containing both the NP and the AF as well as having an irregular cell distribution. The NP and the AF each consists of unique cells, and it is logical to conclude that regeneration could be achieved using cells derived from the lost tissue. The AF cells are chondrocyte-like cells within a dense, mechanically strong matrix. The NP

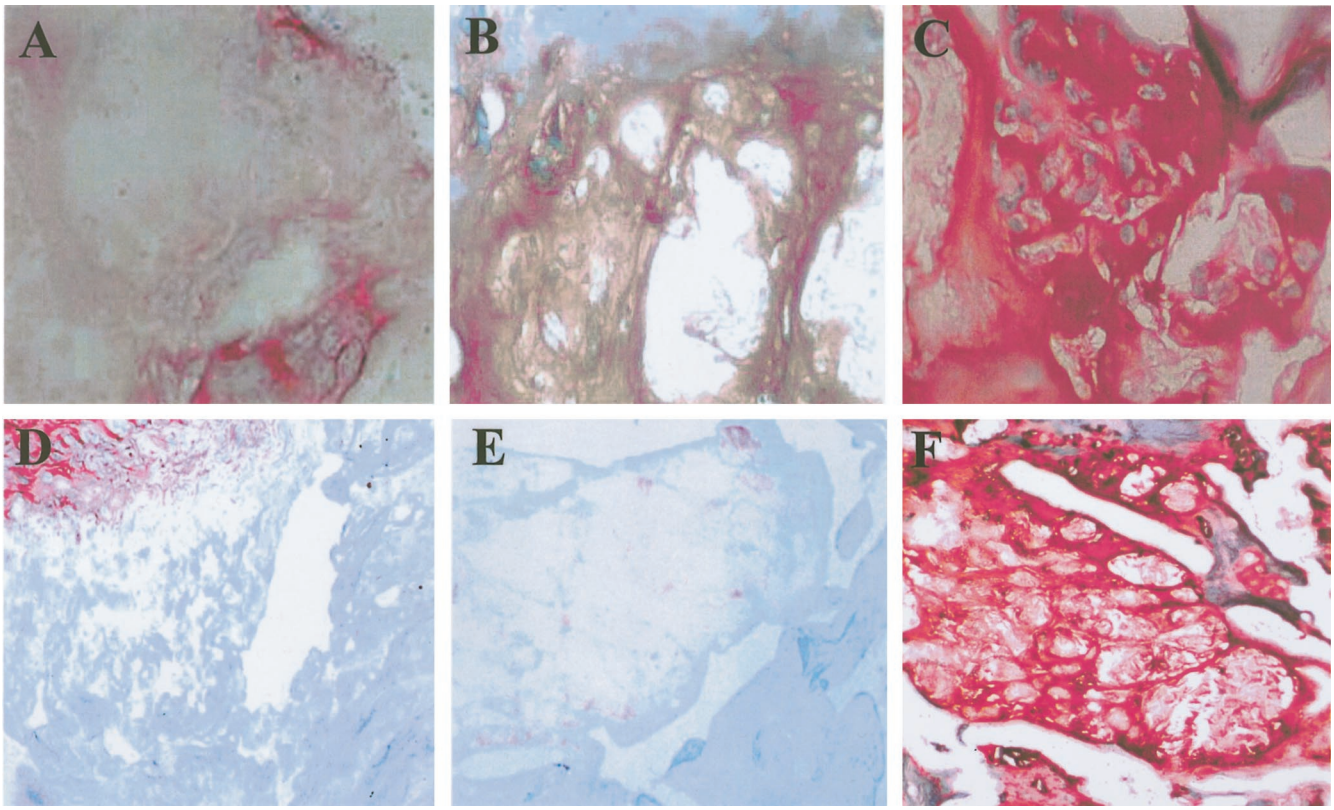

Figure 5. Observations of allografted AF cells in the intervertebral disc 12 weeks after allografting (safranin-O staining, original magnification ×100). **A**, Tissue repair with minor cellularity in NP of the noninsertion group. **B**, Cell migration into the scaffold (inside the NP) in the scaffold insertion group, with minor cellularity and staining. **C**, Allografted AF cells in NP of cell-containing scaffold insertion group, with intense staining. **D**, No staining in damaged AF of the noninsertion group. **E**, Carrier with minor cellularity in AF of the scaffold insertion group. **F**, Allografted cultured AF cells and accumulated matrix intensely stained in the AF of the cell-containing scaffold insertion group.

contains a large volume of water, with matrix being mechanically weak. The NP cells of young rabbits are mainly large vacuolated cells and a minor amount of smaller cells.[17] This morphologic heterogeneity was also reported by Maldonado and Oegema for adult dog intervertebral discs.[9] The small NP cells behave much the same as AF cells in a primary culture, but the function of the large NP cells is still unclear, *e.g.*, speculating to be residual notochordal cells or chondrocytic cells. Differentiating markers of notochordal cells are required for the definitive characterization of the phenotype of postnatal NP cells.[17]

The goal of regenerating the intervertebral disc tissue is not only achieving anatomic morphology but also restoring its functioning. Thus, the NP and AF tissue should first be regenerated in order for both histologic and mechanical functions to be regenerated. For this purpose, we created an ACHMS-scaffold as a novel carrier, enabling us to culture a high density of AF cells in three dimensions. The phenotype of chondrocyte-like cells *in vitro* was maintained during at least 3 weeks, as shown by continuous Type II collagen mRNA expression in Northern blot analysis as well as Type II collagen and proteoglycan expression in Western blot analysis.[20] These results strongly indicate the applicability of ACHMS-scaffolds as cell carrier.

In the present study, we examined intervertebral disc regeneration using AF cells, based on findings in basic research using intervertebral disc degeneration models[8] and chemonucleolysis.[1,23] The findings indicated that chondrocyte-like cells in the inner AF, or the so-called junctional area, which constitutes the boundary area among the inner AF, NP, and cartilage endplate, play a critical role in the tissue repair mechanism.[23] Allografted AF cells exhibited a proliferation activity, resulting in the production of hyaline-like cartilage, and the regenerated AF tissue was found histologically similar to the original. The mechanical functioning of regenerated intervertebral discs remains to be investigated. However, the finding that the narrowing of the intervertebral disc space could be significantly prevented in the cell-containing scaffold insertion group suggests that both prevention of intervertebral disc degeneration and improvement of mechanical strength may be achieved.

In this study, we used tissue-engineering methods to carry out allografting to regenerate damaged AF and NP. After laser vaporization of the NP of rabbit intervertebral discs, we allografted AF cells cultured in the ACHMS-scaffold into the lacuna of the disc. Results showed that allografted AF cells were viable *in vivo* and exhibited proliferation activity. Although certain problems remain to be solved, these initial results suggest that

it seems possible to achieve regeneration of intervertebral discs through allografting of AF cells.

■ Conclusion

Using tissue-engineering methods, cultured AF cells were allografted into the NP lacunas and the damaged AF of rabbit intervertebral discs, resulting from laser vaporization. They exhibited cell proliferations and productions of hyalinelike matrix in a long period.

■ Key Points

- Tissue engineering methods were used to carry out allografting and regenerate the lacunas produced using laser vaporization and the damage AF.
- The postoperative narrowing of the intervertebral disc space was significantly prevented in the cell-containing scaffold insertion group.
- Allografted AF cells were viable and showed proliferating activity with the hyaline-like cartilage being produced.

References

1. Bradford DS, Cooper KM, Oegema TR Jr. Chymopapain, chemonucleolysis, and nucleus pulposus regeneration. J Bone Joint Surg Am 1983;65:1220–31.
2. Brandner ME. Normal values of the vertebral body and intervertebral disk index in adults. Am J Roentgenol Radium Ther Nucl Med 1972;114:411–4.
3. Brtberg M, Lindahl A, Nilsson A, et al. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994; 331:889–95.
4. El Ouahabi A, Thiry M, Schiffmann S, et al. Intracellular visualization of BrdU-labeled plasmid DNA/cationic liposome complexes. J Histochem Cytochem 1999;47:1159–66.
5. Horan PK, Slezak SE. Stable cell membrane labelling. Nature 1989;340:167.
6. Itoh H, Aso Y, Furuse M, et al. A honeycomb collagen carrier for cell culture as a tissue engineering scaffold. Artif Organs 2001;25:213–7.
7. Katsube K, Ochi M, Uchio Y, et al. Repair of articular cartilage defects with cultured chondrocytes in Atelocollagen gel: comparison with cultured chondrocytes in suspension. Arch Orthop Trauma Surg 2000;120:121–7.
8. Lipson SJ, Muir H. Experimental intervertebral disc degeneration: morphologic and proteoglycan changes over time. Arthritis Rheum 1981;24:12–21.
9. Maldonado BA, Oegema TR Jr. Initial characterization of the metabolism of intervertebral disc cells encapsulated in microspheres. J Orthop Res 1992; 10:677–90.
10. Mochida J, Nishimura K, Nomura T, et al. The importance of preserving disc structure in surgical approaches to lumbar disc herniation. Spine 1996;21: 1556–63.
11. Nishida K, Kang JD, Suh JK, et al. Adenovirus-mediated gene transfer to nucleus pulposus cells: implications for the treatment of intervertebral disc degeneration. Spine 1998;23:2437–42.
12. Nishida K, Gilbertson LG, Robbins PD, et al. Potential applications of gene therapy to the treatment of intervertebral disc disorders. Clin Orthop 2000; 379:234–41.
13. Nishimura K, Mochida J. Percutaneous reinsertion of the nucleus pulposus: an experimental study. Spine 1998;23:1531–8.
14. Ochi M, Uchio Y, Tobita M, et al. Current concepts in tissue engineering technique for repair of cartilage defect. Artif Organs 2001;25:172–9.
15. 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;18:988–97.
16. Peterson L, Minas T, Brittberg M, et al. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. Clin Orthop 2000;374: 212–34.
17. Poiraudeau S, Monteiro I, Anract P, et al. Phenotypic characteristics of rabbit intervertebral disc cells: comparison with cartilage cells from the same animals. Spine 1999;24:837–44.
18. Revel M, Payan C, Vallee C, et al. Automated percutaneous lumbar discectomy versus chemonucleolysis in the treatment of sciatica: a randomized multicenter trial. Spine 1993;18:1–7.
19. Samlowski WE, Robertson BA, Draper BK, et al. Effects of supravital fluorochromes used to analyze the in vivo homing of murine lymphocytes on cellular function. J Immunol Methods 1991;144:101–15.
20. Sato M, Asazuma T, Ishihara M, et al. An atelocollagen honeycomb-shaped scaffold with membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus cells from an intervertebral disc. J Biomed Mater Res 2003;64A: 248–56.
21. Sato M, Ishihara M, Arai T, et al. Use of a new ICG-dye-enhanced diode laser for percutaneous laser disc decompression. Lasers Surg Med 2001;29:282–7.
22. Sumida K, Sato K, Aoki M, et al. Serial changes in the rate of proteoglycan synthesis after chemonucleolysis of rabbit intervertebral discs. Spine 1999; 24:1066–70.
23. Tsuchida T. A pathological study of experimental chemonucleolysis with collagenase. J Jpn Orthop Assoc 1987;61:1237–49.
24. Uchio Y, Ochi M, Matsusaki M, et al. Human chondrocyte proliferation and matrix synthesis cultured in Atelocollagen gel. J Biomed Mater Res 2000;50: 138–43.