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An In-vitro Study on Regeneration of Human Nucleus Pulposus by Using Gelatin/Chondroitin-6-Sulfate/ Hyaluronan Tri-copolymer Scaffold

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Abstract: Tissue engineering approaches for treating degenerative intervertebral discs aim to promote tissue regeneration then retard or even reverse the degenerative process. A gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer was developed to serve as a bioactive scaffold that could help human nucleus pulposus (NP) cells to preserve their cell viability/proliferation and promote matrix synthesis. Each scaffold was seeded with 1×10^6 monolayer-expanded human NP cells and then cultured in vitro. Over a 4-week cultivation period, cell–scaffold hybrids demonstrated active cell viability/proliferation and a progressive increase in net production of glycosaminoglycans. In comparison to monolayer cells, scaffold-cultured cells showed significantly higher mRNA expression in collagen II, aggrecan, Sox9, TGFβ1, and TIMP1. Expression of mRNA was significantly suppressed in collagen I, collagen X, IL1, and Fas-associating death domain protein. Histological studies showed newly synthesized glycosaminoglycans deposits and collagen II in scaffolds. These results indicate that the tri-copolymer scaffold could be considered as a promising bioactive scaffold for regenerating human NP. **Key Words:** Gelatin—Chondroitin-6-sulfate—Hyaluronan—Human—Intervertebral disc—Tissue engineering.

The intervertebral disc has a composite structure comprised of a nucleus pulposus (NP) core, a multilayered lamina of annulus fibrosus (AF), and cartilaginous end plates. Degeneration of the intervertebral disc and associated spinal disorders are a leading cause of morbidity, resulting in substantial pain and increased health cost (1). Although the causes and pathophysiology of intervertebral disc degeneration are mostly unknown, some evidence indicates that intervertebral disc degeneration originates from the NP (2,3). The NP is a gelatinous-type tissue and contains a high concentration of proteoglycans within a loose network of collagen (4). The most notable changes in degenerative NP are a decrease in proteoglycans content and type II procollagen synthesis as well as an increase in type II collagen denaturation and type I collagen synthesis (5). As the NP loses its original osmotic properties and becomes more fibrotic, the intervertebral disc is not able to transmit intervertebral force optimally. The excessive compression load eventually leads to damage in the AF. Fragments of the NP then migrate and herniate through the defect in the AF, causing compression of nerve roots. When nucleotomy or diskectomy is carried out for eliminating back and leg pain resulting from intervertebral disc herniation, almost no regeneration of the NP or the AF will occur. Therefore, degeneration of the intervertebral disc is inevitable (6).

Regeneration of the NP tissues in the early stages of degeneration can theoretically retard or even reverse the degenerative process and possibly regain a healthy intervertebral disc. Since the approval of autologous disc chondrocyte transplantation in Germany in 1997, cell-based therapeutics has shown a potential role in the treatment of intervertebral disc degeneration (7). However, it is theoretically better to integrate cultured NP cells with bioactive scaffolds which could help in preserving the phenotype

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of NP cells and in promoting matrix synthesis. Gelatin is denatured type I collagen with preservation of many signal domains in collagen. These specific domains could play very important roles in the signal transduction and regulation of cell activity and behavior. Gelatin scaffolds could also provide a porous structure for cell penetration and attachment. Chondroitin-6-sulfate is a major component in the extracellular matrix (ECM) of the NP (8), which can up-regulate the secretion of chondroitin sulfate and hyaluronan in cartilage lineage cells (9). Hyaluronan is a hydrophilic glycosaminoglycan matrix component and is the major un-sulfated GAG in the NP (8). At low concentrations, hyaluronan stimulates chondrocytes to proliferate and to synthesize proteoglycans and chondroitin sulfate (10). The objective of this study was to assess the feasibility of using a gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer as a bioactive scaffold for culturing human NP cells in vitro with preservation of cell viability, cell proliferation, and production of crucial ECM components including GAG and type II collagen.

MATERIALS AND METHODS

Preparation of tri-copolymer scaffold

The proportion of each component of GAG in the human NP is 90% sulfated GAG and 10% hyaluronan (8). These proportions were used to make a scaffold mimicking the proportion of GAG in the natural NP matrix. Gelatin powder (500 mg, G-2500; Sigma Co., St. Louis, MO, U.S.A.), chondroitin-6-sulfate powder (100 mg; Sigma Co.), and hyaluronan (10 mg; Fluka Chemie AG, Buchs, Switzerland) were mixed and completely dissolved in 10 mL of double-distilled water in a 55°C water bath for 10 min. The solution was poured into a 15 mL polypropylene tube and cooled at 4°C then frozen at –20°C overnight to form a sponge. After lyophilization for 48 h, cross-linking was then undertaken by soaking the sponge in 0.1% glutaraldehyde solution (Sigma Co.) at room temperature overnight. After another lyophilization for 24 h, the dried sponge was re-crosslinked in 0.1% glutaraldehyde solution at room temperature overnight to increase the cross-linking rate. The cross-linked sponge was then soaked in 0.1 M glycine solution (Sigma Co.) at room temperature to stop cross-linking, remove residual glutaraldehyde and block the aldehyde reaction. The sponge was then lyophilized overnight. The cylindrical sponge was cut into 5-mm thick discs with a 12-mm diameter. The tri-copolymer scaffolds were sterilized by soaking in 70% ethanol (Shimakyu Chemicals Co., Osaka, Japan) overnight and were washed with phosphate-buffered saline (PBS) five times before cell seeding. Porosity was calculated by using the formula: porosity = $(D_t - D_r)/D_t$. The theoretical density, D_t, was equal to weight/volume of gelatin. The real density, D_r, was equal to weight/volume of porous gelatin sponge.

Preparation of scaffold for scanning electron microscopy (SEM)

SEM was performed for morphological observation of the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold. The tri-copolymer scaffold was dehydrated by treatment with a series of graded ethanol solutions (50% for 12 h, then 75%, 85%, and 95%, each for 2 h), and then placed overnight in a vacuum oven at 50°C before coating with gold for SEM examination. The scaffold was then examined using a JEOL JXA-804 A scanning electron microscope (JEOL-USA, Inc., Peabody, MA, U.S.A.).

Isolation and expansion of human nucleus pulposus cells

Retrieval and usage of human tissue and cells had been approved by the Research Ethical Committee at the authors' institute. Human NP tissues were obtained aseptically from six adult patients who underwent spine surgery for disorders of lumber intervertebral discs (mean age 43.2, range 24–50). Human NP cells were isolated by incubating the minced NP tissues in DMEM/F-12 medium (Sigma Co.) containing 0.2% collagenase (Sigma Co.) at 37°C overnight. The isolated human NP cells were re-suspended and washed in PBS, and their viability was determined using trypan blue dye exclusion.

Seeding and culturing human NP cells in the tri-copolymer scaffolds

Human NP cells were expanded by monolayer culture in DMEM/F-12 medium containing 10% fetal bovine serum (Biological Industries Ltd, Kibbutz Beit Haemek, Israel), 50 units/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin (Sigma Co.), and 25 μg/mL L-ascorbic acid (Sigma Co.). Appearance of monolayer-cultured NP cells was observed periodically to ensure that no fibroblastic transformation occurred. This was expected to enhance the preservation of the phenotype of these cultured NP cells. Monolayer-cultured human NP cells were trypsinized and re-suspended in the culture medium. A volume of 50 μL of cell suspension containing 1×10^6 cells was then injected into each scaffold. Cell–scaffold hybrids were then cultured in 6-well plates. Control scaffolds were not seeded with cells and were incubated under the same conditions as the

cell–scaffold hybrids. Culture medium was changed twice a week and 6 mL of culture medium was replaced for each well. A 4-week in vitro cultivation was chosen to demonstrate significant effects of tricopolymer scaffold on human NP cells. Duplicate samples from each group of cell–scaffold hybrids were removed at the end of a 2-week cultivation for water soluble tetrazolium salt (WST-1) assay to measure cell viability/proliferation and for 1,9-dimethylmethylene blue (DMMB) assay to measure sulfated GAG content. The other quadruplicate samples from each group were removed at the end of 4 weeks for DMMB assay, WST-1 assays, RNA extraction, and histological study as well as immunohistochemical study on type II collagen.

WST-1 assay for cell proliferation

Water soluble tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzen disulfonate (WST-1), had been demonstrated to be a simple and rapid measurement of cell proliferation with extremely low cytotoxicity. A 10% working solution was made by mixing 1 volume of cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Mannheim, Germany) with 9 volumes of DMEM/F-12 medium. Cell viability and proliferation in cell–scaffold hybrids were tested at the end of the second and fourth weeks of cultivation. Tricopolymer scaffolds with no cell seeded served as controls, and were regulated in the same culture condition as the cell–scaffold hybrids. Each scaffold, including cell–scaffold hybrids and control scaffolds, was incubated with 1 mL of WST-1 working solution in a 12-well plate at 37°C for 2 h. Then 100 μL of reacted solution was transferred to a 96-well microplate. OD_{450} was measured using a VERSAmax microplate absorbance reader (Molecular Devices Corp., Sunnyvale, CA, U.S.A.).

DMMB assay for quantitative measurement of sulfated GAG

In DMMB assay, sulfated GAG content was quantitated by a decrease in OD_{570}. The DMMB solution was prepared by dissolving 16 mg of DMMB (Aldrich-Chemie GmbH, Steinheim, Germany) in 5 mL of 95% ethanol. The dissolved dye was diluted in 3 mL formic acid (Sigma Co.) and 25.6 mL 1 M NaOH (Sigma Co.), and the volume made up to 1 L with distilled water, which provided a formate buffer of pH 3.5. After 2 weeks and 4 weeks of cultivation, each control sample or cell–scaffold hybrid was digested in 0.1% papain solution (Sigma Co.) at 60°C for 16 h. Then 40 μL of sample digests were mixed with 250 μL DMMB reagent in a 96-well microplate at room temperature. OD_{570} was measured in a microplate absorbance reader and was compared with a linear standard curve obtained from GAG standards (0–100 μg/mL of chondroitin-4-sulfate; Biocolor Ltd, Newtownabbey, North Ireland, U.K.) to estimate the GAG content in each sample.

RNA extraction, reverse transcription (RT), and real-time polymerase-chain-reaction (PCR)

At the end of 4 weeks, one cell–scaffold hybrid from each experimental group was immersed in 2 mL RNA*later* RNA Stabilization Reagent (QIAGEN GmbH, Hilden, Germany) immediately after removal from the culture medium. RNA from human NP cells cultured in scaffolds was extracted using RNeasy Protect Mini Kit (QIAGEN GmbH). During the extraction process, RNA was treated with RNase-free DNase (QIAGEN GmbH) to eliminate the influence of DNA in the PCR. An aliquot of RNA extract was used for RNA content determination at OD_{260} by DU 7500 spectrophotometer (Beckman Instrument Inc., Fullerton, CA, U.S.A.). Complement DNA was synthesized from extracted RNA with SuperScript III First-Strand Synthesis System for RT-PCR, oligo-(dT) primer, and dNTP mix (Invitrogen Corporation, Carlsbad, CA, U.S.A.) at 50°C for 50 min. Complement DNA synthesized from 10 ng RNA was used for each primer of real-time PCR. Real-time PCR was amplified for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), type I collagen (col1a1), type II collagen (col2a1), type VI collagen (col6a1), type X collagen (col10a1), aggrecan, decorin, Sox9, matrix metalloproteinase-9 (MMP9), tissue inhibitor of metalloproteinase-1 (TIMP1), interleukin-1 (IL1), transforming growth factor-β1 (TGFβ1), and Fas-associating death domain protein (FADD). Each reaction was performed with 9 μL cDNA solution, 1 μL Assay-on-Demand Gene Expression Assay Mix (Applied Biosystems, Foster City, CA, U.S.A.), and 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems). Reactions were performed on an ABI PRISM 7700 Sequence Detection System, using ABI PRISM 7700 Sequence Detection Software 1.9.1 (Applied Biosystems) for 40 cycles. The level of mRNA expression of each target gene was normalized to GAPDH. RNA in 1×10^6 monolayer-cultured NP cells from each patient was also extracted serving as references for later comparisons between scaffold-cultured cells and monolayer-cultured cells. ΔC_T values of each target gene were calculated by subtracting the value of cycle threshold (C_T) for GAPDH from the C_T value of each target gene. The relative expression of each target gene was calculated using

the expression of $2^{-\Delta\Delta C}{}_T$ by paired comparison of the ΔC_T values obtained from cells of the same patient which had been cultivated in the tri-copolymer scaffolds and in monolayer.

Histological studies and immunohistochemistry for type II collagen

At the end of 4 weeks, one experimental sample from each group was fixed in 10% neutral buffered formaldehyde solution (Shimakyu Chemical Co.) immediately after removal from the culture medium. The specimens were embedded in paraffin and then were sectioned at a thickness of 6 μm. After deparaffinizing in xylene and graded alcohols, the sections were stained with HE staining for histological examination, alcian blue staining for GAG and antibodies against human type II collagen (Santa Cruz Biotechnology Inc., CA, U.S.A.).

Statistical analysis

A Mann–Whitney *U*-test was employed for determining the statistical significance of sulfated GAG content and OD values detected in the WST-1 assay between the cell–scaffold hybrids and the control scaffolds. The differences in mRNA expression in the cell–scaffold hybrids and monolayer-cultured NP cells were analyzed using Wilcoxon matched-pairs signed rank test. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Scanning electron microscopy

An SEM photograph of a gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold showed a highly porous structure with an average 100 μm pore size (range 50–250 μm) (Fig. 1). All the pores in the tri-copolymer scaffolds were interconnected. The copolymer scaffold had a porosity of 70%.

FIG. 1. An SEM photograph of a gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold showing a highly porous structure with an average pore size of 100 μm (range 50–200 μm) (original magnification 200×).

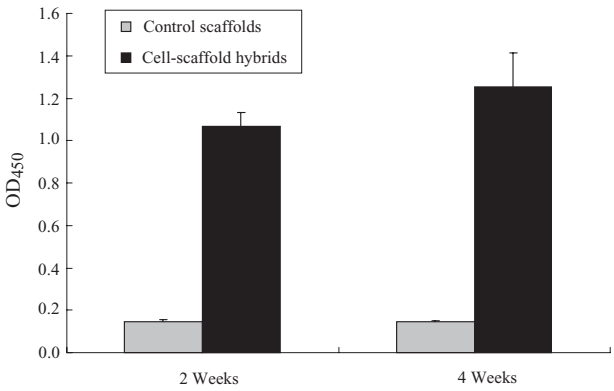

FIG. 2. In WST-1 assay, OD values from cell–scaffold hybrids were significantly higher than those from control scaffolds after 2-week and 4-week cultivations ($P = 0.004$). OD values from 4-week cultivated cell–scaffold hybrids were also significantly higher than those from 2-week hybrids ($P = 0.037$).

WST-1 assay

The OD_{450} obtained from cell–scaffold hybrids and control scaffolds are summarized in Fig. 2. Cell–scaffold hybrids showed significantly higher OD values than control scaffolds at the end of 2-week (1.068 ± 0.066 vs. 0.148 ± 0.006, $P = 0.004$) and also 4-week cultivation (1.253 ± 0.146 vs. 0.145 ± 0.007, $P = 0.004$). Moreover, the OD_{450} from 4-week cell–scaffold hybrids was significantly higher than that from 2-week hybrids ($P = 0.037$).

DMMB assay for sulfated GAG content

Average sulfated GAG content was 5.37 ± 0.43 mg (range 4.72–5.87 mg) in the freshly made gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold. It has been shown that most of the GAG originally entrapped in the scaffold substrate, as well as the GAG being synthesized by the disc cells, can not be retained by the scaffold and is lost into the culture medium in the in vitro environment (10). The average sulfated GAG content in the control scaffolds decreased to 2.12 ± 0.26 mg (range 1.85–2.50 mg) after 2 weeks and decreased to 0.43 ± 0.38 mg (range 0–0.87 mg) after 4 weeks of in vitro cultivation. Cell–scaffold hybrids contained 2.58 ± 0.21 mg (range 2.29–2.80 mg) of sulfated GAG after 2-week and 2.37 ± 0.18 mg (range 2.17–2.65 mg) after 4-week in-vitro cultivation (Fig. 3a). The sulfated GAG content in cell–scaffold hybrids was significantly higher than in the control scaffolds at both time points ($P = 0.016$

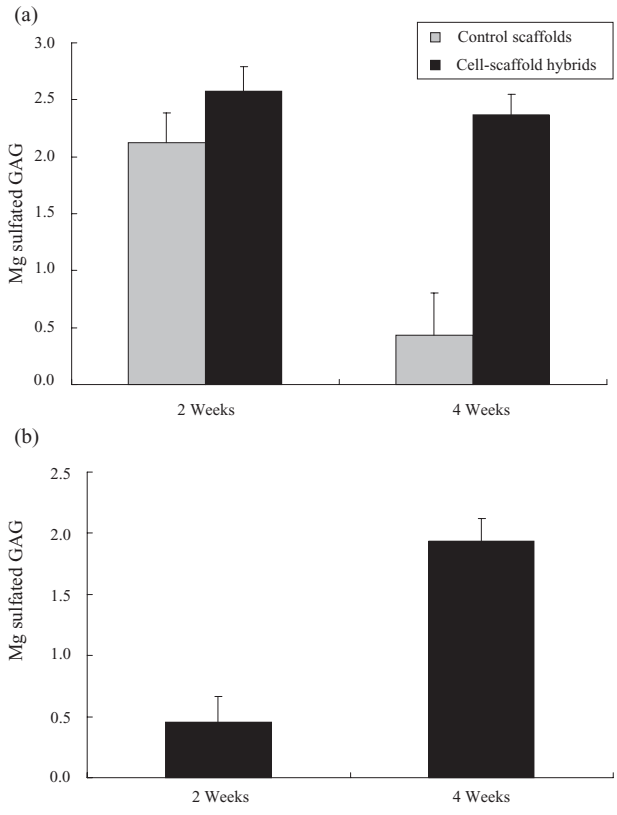

FIG. 3. (a) After 2-week cultivation, the cell–scaffold hybrids contained significantly higher sulfated GAG content than control scaffolds (2.58 ± 0.21 mg vs. 2.12 ± 0.26 mg, $P = 0.016$). The cell–scaffold hybrids also contained significantly higher sulfated GAG content than control scaffolds after 4 weeks of in vitro cultivation (2.37 ± 0.18 mg vs. 0.43 ± 0.38, $P = 0.004$). (b) The differences of sulfated GAG content between cell–scaffold hybrids and control scaffolds were significantly larger in 4-week cell–scaffold hybrids (1.94 ± 0.18 mg) than that in 2-week samples (0.45 ± 0.21 mg, $P = 0.004$).

and $P = 0.004$, respectively). Furthermore, the differences in sulfated GAG content between the cell–scaffold hybrids and control scaffolds were significantly larger in 4-week cell–scaffold hybrids (average 1.94 ± 0.18 mg, range 1.47–2.22 mg) than that in 2-week hybrids (average 0.45 ± 0.21 mg, range 0.16–0.68 mg, $P = 0.004$) (Fig. 3).

MRNA expression in real-time RT-PCR

Relative expressions of each target gene between cell–scaffold hybrids and monolayer-cultured cells are summarized in Fig. 4. Cells cultured in tri-copolymer scaffolds showed a 5.5-fold increase in mRNA expression of type II collagen (average $546 \pm 66\%$, range 496–677%, $P = 0.028$) in comparison with monolayer cells. In cell–scaffold hybrids, the mRNA expressions were significantly suppressed in type I collagen (average $41 \pm 21\%$, range 21–78%, $P = 0.028$) and type X collagen (average $46 \pm 23\%$, range 12–72%, $P = 0.028$). The mRNA expression of type VI collagen was not significantly changed (average $76 \pm 35\%$, range 38–137%, $P = 0.17$). For proteoglycans in cell–scaffold hybrids, significant increase of mRNA expression was found in aggrecan (average $161 \pm 38\%$, range 108–197%, $P = 0.028$) but not in decorin (average $92 \pm 41\%$, range 40–135%, $P = 0.75$). The mRNA expression of Sox9 in scaffold-cultured NP cells was significantly enhanced (average $168 \pm 56\%$, range 113–230%, $P = 0.028$). Even though the mRNA expression of MMP9 was not significantly affected (average $90 \pm 45\%$, range 10–138%, $P = 0.75$), the mRNA expression of TIMP1 was significantly promoted (average $259 \pm 70\%$, range 175–357%, $P = 0.028$) in the cell–scaffold

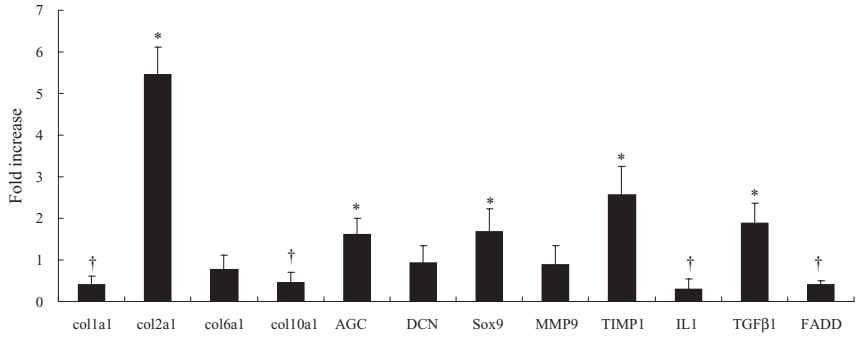

FIG. 4. Relative expressions of each target gene between the cell–scaffold hybrids and monolayer-cultured cells were obtained from real-time RT-PCR. After monolayer human NP cells being cultured in tri-copolymer scaffolds, significant enhancements (*) of mRNA expressions were found in type II collagen (col2a1, $546 \pm 66\%$), aggrecan (AGC, $161 \pm 38\%$), Sox9 ($168 \pm 56\%$), TIMP1 ($259 \pm 70\%$), and TGFβ1 ($189 \pm 48\%$). Significant suppressions (†) were observed in mRNA expression of type I collagen (col1a1, $41 \pm 21\%$), type X collagen (col10a1, $46 \pm 23\%$), IL1 ($29 \pm 26\%$), and FADD ($42 \pm 9\%$). The mRNA expressions were not significantly changed in type VI collagen (col6a1, $76 \pm 35\%$), decorin (DCN, $92 \pm 41\%$), and MMP9 ($90 \pm 45\%$).

hybrids. For mRNA expression in cytokines, IL1 was significantly suppressed (average 29 ± 26%, range 2–58%, $P = 0.028$) but TGFβ1 was significantly enhanced (average 189 ± 48%, range 122–246%, $P = 0.028$). The mRNA expression of FADD was significantly suppressed (42 ± 9%, range 31–56%, $P = 0.028$) in human NP cells after being cultured in the tri-copolymer scaffold.

Histological studies and immunohistochemistry for type II collagen

For sections obtained from the cell–scaffold hybrids cultured for 4 weeks, HE staining revealed a cluster of cells along with abundant matrix deposition between scaffold substrate (Fig. 5a). Alcian blue staining showed blue-stained GAG spots on the surface of the scaffold substrate of the 4-week cultivated cell–scaffold hybrid. In the immunohistochemical staining for type II collagen, many dark-stained type II collagen fibrils could also be observed between scaffold substrates in the 4-week cell–scaffold hybrid (Fig. 5b) but not in the control scaffold.

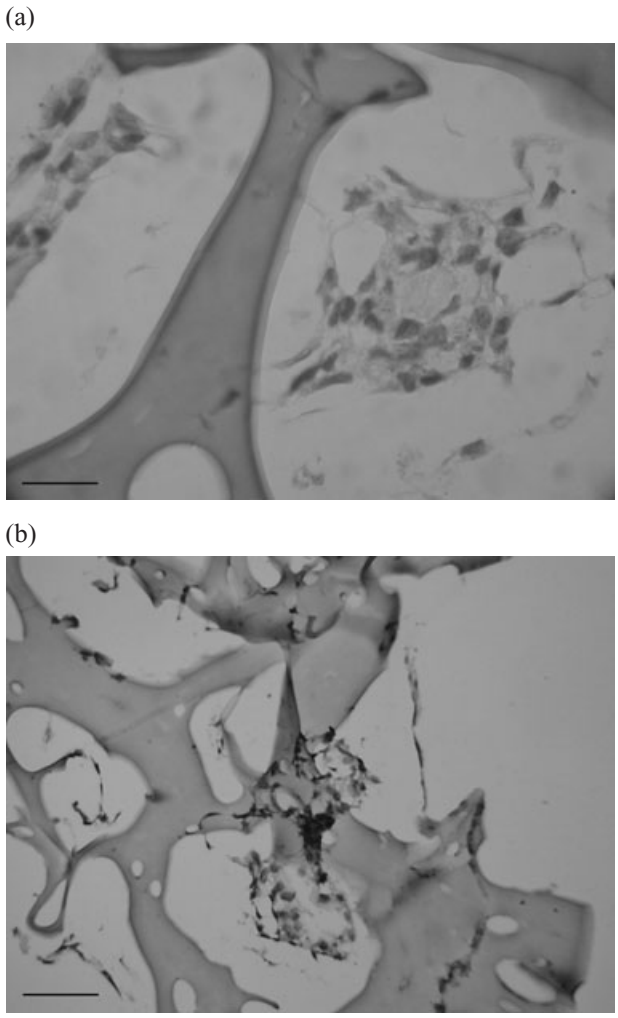

FIG. 5. (a) For sections obtained from 4-week cultivated cell–scaffold hybrids, HE staining revealed clustering of cells along with abundant matrix deposition between the scaffold substrate (original magnification 400×, bar = 25 μm). (b) Immunohistochemical staining for type II collagen disclosed many dark-stained type II collagen fibrils between scaffold substrates (original magnification 100×, bar = 100 μm, counterstained with hematoxylin).

DISCUSSION

There have been a few studies that have cultured human or animal intervertebral disc cells on various bioactive scaffolds in vitro (11–14). But searching for ideal compositions of bioactive scaffold for a 3-dimensional culture of intervertebral disc cells with persistent manifestation of cell viability/proliferation as well as production of ECM is an ongoing task and the answer is still ambivalent. The gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold developed in this study comprises three biological molecules (gelatin, chondroitin-6-sulfate, and hyaluronan) which can interact with the cultured cells and living tissues. Since collagen and gelatin degrade rapidly, the degradation of gelatin should be slowed down after cross-linking with glutaraldehyde (15). The gelatin, chondroitin-6-sulfate, and hyaluronan molecules would gradually be released from the tri-copolymer scaffolds and would stimulate proliferation and matrix production of intervertebral disc cells. Moreover, the optimal pore size and sufficient porosity observed in the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffolds should be beneficial for cell penetration, growth, proliferation, and subsequent matrix deposition. The gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold could be considered as a promising bioactive scaffold for regeneration of intervertebral discs and theoretically would be superior to most of the biomaterials used for culturing intervertebral disc cells as cited in the literature.

The toxicity of glutaraldehyde is derived from (1): residual glutaraldehyde in the gelatin/chondrotin-6-sulfate/hyaluronan tri-copolymer scaffold, and (2) reversal reaction of cross-linked molecules. In preparing the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold, 0.1 M glycine has been used to remove the residual glutaraldehyde and to block the aldehyde reaction. The concentration of glutaraldehyde solution applied in the study was 0.1%. This level was much lower than the general use in bio-prostheses such as porcine heart valve or

pericardium for cardio-patch. These bio-prostheses are generally accepted and tolerated in clinical experience. The toxicity of glutaraldehyde in the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold should be unremarkable.

The NP and the AF each consists of unique cells and matrix depositions. It is logical to conclude that regeneration should be achieved using cells derived from the lost tissue. Studies based on the AF cells and/or animal cells may not be able to provide crucial information regarding human NP cells. For the purpose of regenerating human NP tissues in the early stage of intervertebral disc degeneration, the effect and benefit of bioactive scaffolds on culturing human NP cells should be especially emphasized.

WST-1 assay is a simple and rapid measurement to detect cell viability/proliferation without disruption of scaffolds. As the cell–scaffold hybrids generated a significantly higher OD value in the WST-1 assay after 2-week and 4-week in vitro cultivation, it indicates that there was persistent activity in cell proliferation, viability and metabolism in human NP cells cultured in the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold. In addition, as Fas-mediated apoptosis has previously been demonstrated in articular chondrocytes and intervertebral disc cells (16), significant suppression in mRNA expression of FADD suggests that the Fas-mediated apoptosis was down-regulated in human NP cells after being cultured in the tri-copolymer scaffold. These results indicate that the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold offers a good environment for human NP cells to manifest active cell viability/proliferation as well as lower the tendency of apoptosis.

The mechanical properties and physiologic functions of the intervertebral disc mainly depend on the composition and specific molecular interactions among components of its ECM including proteoglycans and collagens (17). In the in vitro culture environment, most of the GAG would be continuously lost from the scaffolds into culture medium (10). The GAG newly synthesized by cultured cells would also be lost into culture medium throughout the culture period. Therefore, measuring the total amount of GAG which was synthesized by the cultured human NP cells was not possible. However, this study still showed significantly higher GAG contents in the cell–scaffold hybrids than that in the control scaffolds after 2-week and 4-week cultivations. Differences between cell–scaffold hybrids and control scaffolds were also larger in 4-week cell–scaffold hybrids when compared with 2-week hybrids. After 4 weeks of cultivation, alcian blue staining showed blue-stained GAG spots on the surface of the scaffold substrate. These findings indicate that human NP cells cultured in the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold can synthesize significant amount of GAG instead of keeping the originally entrapped GAG inside the scaffold substrate. Because large aggregating proteoglycans provide higher compression stiffness than nonaggregating proteoglycans (18), increased mRNA expression of aggrecan in the cell–scaffold hybrids suggests a higher possibility of proteoglycans aggregation and subsequent higher compression resistance in the tissue-engineered NP tissues.

Collagens are the other important components in the ECM of the NP. Normal human NP tissue contains about 80% of type II collagen and 10–20% of type VI collagen (19,20). As the NP degenerates, more fibrotic and immature collagens including type I and X collagens appear (20,21). Since various extents of degeneration already existed in the human NP tissues obtained for this study, an ideal tissue-engineered NP tissue should be expected to have a higher production of type II collagen and lower production of type I and X collagens. The mRNA expression of human NP cells cultured in the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffolds showed a 5.5-fold increase in mRNA expression of type II collagen, a 59% decrease in type I collagen and a 54% decrease in type X collagen. The possibility and severity of fibrotic transformation could theoretically be reduced in the tissue-engineered NP tissues. Furthermore, as the expressions of type II collagen and proteoglycans in normal intervertebral disc cells are regulated by the "master chondroregulatory gene" Sox9 (22), the up-regulation of Sox9 expression suggested an improved chondrogenesis process in the cell–scaffold hybrids. In addition to the up-regulation in mRNA expression, immunohistochemical study revealed cell–scaffold hybrids contained abundant type II collagen fibrils which were logically considered to be synthesized by the human NP cells cultured in the tri-copolymer scaffold. These results indicate that the human NP cells cultured in the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffolds were functionally active in producing essential components of the NP including GAG and type II collagen, as well as minimizing the risk of fibrotic transformation in cell–scaffold hybrids.

The production of cytokines, MMPs, and TIMPs were also altered when human NP cells were cultured in the tri-copolymer scaffold. IL1 was implicated to increase the production of MMPs and proteoglycans degradation in the intervertebral discs

(23). TGF-β1 was shown to enhance the intervertebral disc cells in mitogenesis and proteoglycan synthesis (24). MMPs are a family of enzymes that are capable of degrading major structural components of the intervertebral disc (25). TIMPs bind to MMPs and result in an inability of MMPs to cleave macromolecules (26). After monolayer-cultured human NP cells were seeded onto the tri-copolymer scaffold, the mRNA expression of IL1 was prominently suppressed and the mRNA expression of TGF-β1 was significantly promoted. Even though the mRNA expression of MMP9 was not affected, the mRNA expression of TIMP1 was markedly enhanced. These findings suggest that the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold can provide a favorable environment for human NP cells to enhance matrix synthesis, as well as lessen matrix degradation through the mechanism of modifying production of cytokines and tissue inhibitors of MMPs.

In degenerative and nucleotomized intervertebral discs, the matrix in the NP is always insufficient and could be sometimes empty. Development of a tissue-engineered prosthesis to replace whole intervertebral disc or NP is an attractive way to treat degenerative intervertebral discs. However, the combination of cultured human NP cells and bioactive scaffolds, such as gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer investigated in this study, is another promising approach to regenerate NP tissues. Because it is difficult to define what is a sufficient amount of collagen and GAG for NP, any extent of increase in the NP matrix through a regenerative treatment could provide some additional strength and may be helpful for retarding or even reversing the degenerative process of the intervertebral disc. After a continuous regenerative process, the biomechanical properties of the regenerated NP tissue should gradually improve.

CONCLUSIONS

In this study, it was demonstrated that the gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold was able to preserve the viability/proliferation of human NP cells and able to stimulate the production of important ECM components in an in vitro culture environment. The gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold could be considered to be a promising bioactive scaffold, and it warrants further investigation into its application in regeneration of nucleus pulposus.

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