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Inducing Chondrogenic Differentiation in Injectable Hydrogels Embedded with Rabbit Chondrocytes and Growth Factor for Neocartilage Formation

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A thermoreversible hydrogel of poly(NiPAAm-co-AAc) was used as an injectable cell and growth factor delivery carrier for cartilage tissue engineering. Rabbit chondrocytes were embedded in composite hydrogels coencapsulated with transforming growth factor β3 (TGFβ3). Hydrogel constructs consisting of embedded cells encapsulated by the thermoreversible hydrogel served as controls to assess the effects of TGFβ3 on chondrogenic differentiation. The hydrogel constructs were injected subcutaneously into nude mice and then monitored for up to 8 weeks after injection. After 8 weeks of implantation, the engineered cartilage acquired normal histological and biochemical properties. These results highlight the potential of growth factor in a hydrogel embedded with chondrocytes as a candidate material for neocartilage formation.

[**Key words:** cartilage, chondrocytes, thermosensitive hydrogel, transforming growth factor β3 (TGFβ3)]

Biomaterials suitable for serving as three-dimensional scaffolds for cell proliferation, delivery and therapy have become a major focus in tissue engineering (1–3). The suspension of a large number of freshly isolated cells within scaffolds may allow for their implantation to correct defects due to tissue grafting (4, 5). The ideal scaffold should be three-dimensional, highly porous with an interconnected pore network, biocompatible with a controlled degradation rate, and appropriate as a surface for enabling cell adhesion, proliferation, differentiation, and proper mechanical properties (6, 7). These scaffolds, which exist in the form of hydrogels or porous or branching networks, serve as frames upon which chondrocytes can produce new extracellular matrix (ECM) (8, 9).

In the application of cartilage formation using hydrogels, fully thermo-reversible gelling polymers have attracted considerable attention for use as scaffold materials to hold cells *in situ* (10). These polymers can revert from the solid state to the liquid state and vice versa, without losing their intrinsic properties. These polymers are fully soluble in aqueous solutions at temperatures below their lower critical solution temperatures (LCSTs), but solidify to form a hydrated gel at temperatures above their LCSTs (11, 12).

In this work, we used a thermoreversible hydrogel embedded with chondrocytes and growth factor that can offer an appropriate environment for the retention of chondrocytic phenotype and the synthesis of mechanically functional cartilage from ECM *in vivo*. The chondrocytic phenotype was assessed with a specific marker of collagen type II. Furthermore, polysaccharide and proteoglycan were detected by Alcian Blue and Safranin-O stainings. The results show that the addition of growth factor mixed with hydrogel along with the implantation of this construct can help to enhance cell differentiation. Thus, this hydrogel construct is a good candidate for cartilage tissue engineering.

MATERIALS AND METHODS

Chondrocyte isolation and cell culture Chondrocytes were isolated from the articular cartilage of the knee of a rabbit (New Zealand White) using the collagenase digestion method (13). In brief, female rabbits, 250 g, were euthanized with an overdose of Nembutal. The nonfibrillated articular cartilage of the knee was removed by sterile dissection. The cartilage was finely minced, suspended in calcium- and magnesium-free phosphate-buffered saline (CMPBS), and washed. The fragments were sequentially digested in 0.2% collagenase (Worthington Biochemical, Lakewood, NY, USA) in CMPBS for 3 h at 37°C. The cells obtained from the collagenase digests were pooled and passed through a cell strainer (70 μm, nylon; Falcon, Franklin Lake, NJ, USA) to remove the undigested matrix. The cells released in the supernatant were collected by centrifugation (1200×*g*, 15 min) and washed twice with CMPBS. The number and viability of the cells were determined using a hemocytometer and a Trypan Blue (0.25%) exclusion dye test. The collected cells were suspended in serum-free medium consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco BRL), 100 U penicillin G/ml, and streptomycin 100 μg/ml (Gibco BRL), and plated in tissue culture flasks at an initial density of 1.5×10^5 cells/cm^2. All chondrocyte cultures were performed in a humidified incubator with a 5% CO_2 atmosphere

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maintained at 37°C. The culture medium was changed every 2–3 d.

Preparation of poly(*N*-isopropypacrylamide-co-acrylic acid) [poly(NiPAAm-co AAc)] co-polymer The poly(*N*-isopropypacrylamide-co-acrylic acid) [p(NiPAAm-co-AAc)] copolymer was synthesized using the method reported in a previous study (11). In brief, copolymers of NiPAAm with small amounts of acrylic acid (AAc) (typically 2–5 mol%) were synthesized by free radical polymerization in benzene (10% (w/v) monomer concentration) with 2,2′-azobisisobutyronitrile (AIBN) as an initiator (7×10^{-3} mol of AIBN/mol of monomer). The monomer solution was bubbled with dry N_2 gas for 30 min. After adding AIBN, the mixture was degassed under vacuum condition for 30 min prior to polymerization. Polymerization was conducted at 60°C for 16 h.

TGFβ3 release from hydrogel Thermoreversible hydrogel (250 μl, 7 wt%) was mixed with 100 ng/ml of transforming growth factor β3 (TGFβ3) and was placed in polypropylene conical tubes with 1 ml of DMEM medium on an orbital shaker. Blanks only hydrogels were used as controls. The medium was removed, frozen, and replenished at 0 and 6 h and 1, 3, 7, 10, 14, 21 d. Enzyme-linked immunosorbent assay (ELISA) for TGFβ3 (R&D Systems, Minneapolis, MN, USA) was performed to determine the concentration of active protein released.

Chondrocyte encapsulation After chondrocytes were counted, the cell suspension was centrifuged again and the resulting supernatant was removed. Chilled aqueous solutions of p(NiPAAm-co-AAc) without growth factor (group A, n=12), or with 100 ng/ml TGFβ3 (group B, n=12) were prepared. The chondrocytes were added to the solutions to obtain suspensions having a cellular concentration of 4×10^6 cells/ml. p(NiPAAm-co-AAc) embedded with a suspension of chondrocytes (250 μl, 1×10^6 cells) was mixed by vigorous vortexing. The cell suspensions were kept below 7°C to maintain the liquid state of the copolymers and placed into a syringe for injection into nude mice.

Nude mouse implantation Female BALB/c mice (6 weeks old) were divided into four groups. In group I, the control group (n=12), 200 μl of p(NiPAAm-co-AAc) solution was injected into the back subcutis of the mice. In group II (n=12), 200 μl of p(NiPAAm-co-AAc) solution (with TGFβ3 and cells) was injected into the back subcutis of the mice. At 1, 4, and 8 weeks posttreatment, the mice were sacrificed (n=4 for each time point) with an overdose injection of anesthetic, and the skin, including the injected site (2×2 cm^2), was carefully taken off for subsequent biological examinations. Photographs of the skin flaps were taken to record the tissue appearance around the treated site.

Biochemical assays for chondrocyte proliferation and GAG production At each time point, samples and negative controls from each time point were extracted from the sacrificed nude mice, rinsed in 2.5 ml of PBS, homogenized with a pellet grinder (Fisher Scientific), and digested in 500 ml of a proteinase K solution (1 mg/ml proteinase K, 10 mg/ml pepstatin A, and 185 mg/ml iodoacetamide) in PBE buffer (6.055 mg/ml Tris(hydroxymethyl aminomethane, 0.372 mg/ml EDTA, pH 7.6, adjusted with HCl) at 60°C for 16 h. After the collection and digestion of all the samples and controls, specimens underwent three repetitions of a freeze/thaw/sonication cycle (30 s at 80°C, 30 min at room temperature, 30 s of sonication) to extract total DNA from the cell cytoplasm. DNA and GAG assays were then run in triplicate for each experimental and control group at each time point. The cell number was calculated by measuring double-stranded DNA content using the PicoGreen assay (Molecular Probes, Eugene, OR, USA) according to the instructions of the manufacturer. The fluorescence of negative, cell-free controls was subtracted from the fluorescence values of experimental groups to adjust for the fluorescence of the material. The PicoGreen assay was also used to determine DNA content. GAG content was determined using a biochemical assay, the dimethylmethylene blue dye (DMMB) assay. When DMMB binds to GAG, a red color is produced, allowing for the quantification of GAG by measuring absorbance at 520 nm. The GAG content in the hydrogels was calculated by comparing the obtained fluorescence values with those of a curve generated from standards of known amounts of chondroitin sulfate. A microplate reader (BIO-TEK Instrument, Winooski, VT, USA) was utilized for both absorbance and fluorescence measurements.

Collagen content assay Collagen content was measured using the hybrid Tullberg-Reinert method (14). Briefly, a dye reagent, sirius red (100 μg/ml), was made by adding picric acid (3 ml) and 0.1 M NaOH (2.56 ml, pH 3.5) to a total volume of 1 *l* of distilled water. The harvested samples were located under wetting conditions and sample weight was measured to test the water content. The collagen solution (100 μl) was extracted using an extraction solution, dried in a 96 microwell plate in a 37°C dry oven for 24–48 h, and received 100 μl of a dye solution after undergoing shaking for 1 h. After a fixing step was carried out for 10 min, the samples were washed five times with a 0.01 N HCl solution. The washed samples were resolved with 0.1 N NaOH. The absorbance was measured at a wavelength of 550 nm using a Biorad microplate reader, 3550. The collagen concentration was calculated by comparing the values with the optical densities of standard solutions of bovine collagen (0–500 g/ml tracheal cartilage; Sigma Chemical, St. Louis, MO, USA). The standard values were 0, 20, 40, 60, 80, and 100 g. The amount of collagen in the tissue was extrapolated using a graph of these values. This method of quantifying collagen in cell cultures offers a number of advantages over other methods based either on the chemical analysis of hydroxyproline or on proline-labeling assays.

Immunohistochemistry of implanted constructs The injection site was completely excised and processed for classical histology. In brief, samples from each time point were embedded in optimum cutting temperature (OCT) compound (Tissue-Teks 4583; Sakura Finetek USA) and frozen. The specimens were cut into 10 μm thick sections at −20°C, and were stained with hematoxylin and eosin (H&E) to allow an observation of the nuclei and cytoplasm. The stained sections were examined using optical microscopy to determine the level of cell proliferation and were photographed using a digital camera. The embedded sections were stained with Safranin-O and Alcian Blue for a histological evaluation. The sections were also immunolabeled for the presence of type II collagen, and were visualized using a horseradish peroxidase conjugated secondary antibody. Images were taken of the stained sections, and randomized and graded by three readers in terms of their cell arrangement, proteoglycan, collagen staining (blind study), and collagen-specific staining for the immunolabeled sections.

RESULTS AND DISCUSSION

***In vitro* testing of release patterns of heparin-bound TGFβ3 and free TGFβ3** In this study, we examined the effect of the long-term release of TGFβ3 on cartilage formation using a three-dimensional system *in vitro*. To confirm the long-term release profile of TGFβ3, cumulative release was examined (Fig. 1). The release curves for TGFβ3 exhibited initial bursts during the first 3 d. The TGFβ3 curve suggested a release plateau after 2 weeks. The presence of TGFβ3 in p(NiPAAm-co-AAc) was detected after 3 weeks, the point at which nearly all the TGFβ3 in the p(NiPAAm-co-AAc) polymer had been released. This indicates that the TGFβ3 remained in the hydrogels for a long period of time.

Nude mouse implantation of polymer The gross mass of the transplanted cell constructs showed that new tissues had formed in the presence of TGFβ3 until 8 weeks after the

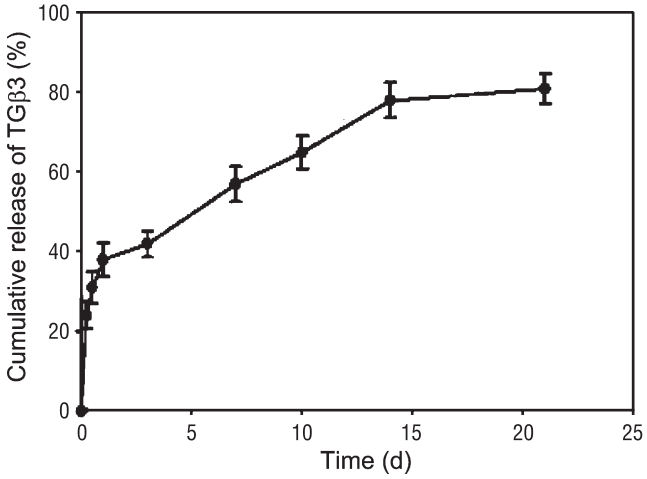

FIG. 1. TGFβ3 release profiles from hydrogel constructs.

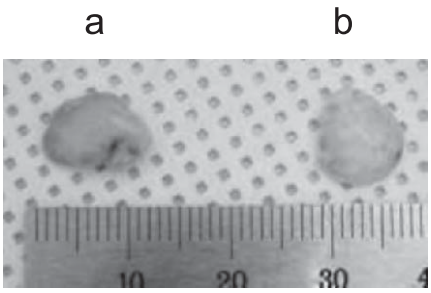

FIG. 2. Macroscopic picture of constructs for inducing chondrocyte grown subcutaneously in nude mice directly after explanation. (a) Thermoreversible hydrogel without TGFβ3; (b) thermoreversible hydrogel mixed with TGFβ3. The scale is in millimeters.

injection (Fig. 2). When the gel mixture with TGFβ3 was injected into the mice subcutaneously, the volume of the hydrogel increased gradually during the injection period. The volume of the hydrogel in the test group (with the treatment of growth factors) had increased by approximately 1.2-fold at 8 weeks, but the volume in the control group did not increase compared with the experimental groups (Fig. 2a). The volume of the test group may have differed from that of the control group because of the proliferated chondrocytes and cartilage ECM secreted as a result of TGFβ3 activity. In the case of the differentiated chondrocytes in hydrogels, cartilage matrix proteins such as collagens and GAG were produced at sufficient levels in the presence of growth factors. The accumulation of these ECM products increased the volume of the injected hydrogel containing TGFβ3. In the control group without growth factors, chondrocytes did not proliferate well and might have undergone apoptosis. However, the hydrogel constructs involving the growth factor have the potential ability to induce the proliferation in three-dimensional systems, especially *in vivo*.

Measurement of GAG/DNA content Figure 3 shows the GAG contents of the thermoreversible hydrogels with or without TGFβ3 as GAG/DNA content. As shown in Fig. 3, all of the groups demonstrated an increase in GAG production per construct over the seven-week period. The hydrogel constructs with TGFβ3 showed a significantly higher GAG

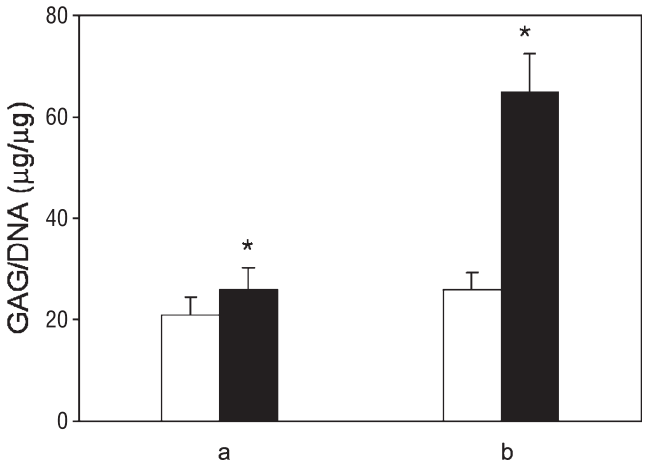

FIG. 3. GAG/DNA contents of different. chondrocytes cultured in 3-D hydrogels for one or four weeks in basal medium. Open bars, 1 week; closed bars, 8 week. (a) Thermoreversible hydrogel; (b) thermoreversible hydrogel mixed with TGFβ3. Error bars represent standard error of the mean (*$p<0.05$).

content at 8 weeks than at one week. In the 8-week culture, the GAG content per sample was significantly higher in constructs containing TGFβ3 than in those without TGFβ3.

Measurement of collagen content The total collagen content of the chondrocytes cultivated in the thermoreversible hydrogel mixed with TGFβ3 was measured as a function of time (Fig. 2). The collagen contents of the cells encapsulated in the thermoreversible hydrogel with growth factor showed marked differences after 1, 4, and 8 week incubation times. This can be attributed to the effects of the growth factors in the hydrogel on proliferation. However, the level of release and densities of collagen by chondrocytes in the hydrogel without growth factors did not change during the experimental period (data not shown).

Immunohistochemistry of implanted construct To conform the specific marker of collagen type II for chondrogenesis, the immunohistological characteristics and collagen type II phenotype expression of cartilage tissues from the hydrogels with or without TGFβ3 were evaluated by immunostaining with a collagen type II antibody and staining with hematoxylin (Fig. 3). The formation of cartilage tissue within the hydrogels was observed in both cases 8 months after seeding. Collagen type II, which was produced by freshly isolated chondrocytes, was predominantly expressed by the chondrocytes cultivated in the hydrogel containing TGFβ3. This immunohistological result is consistent with the findings of *in situ* hybridization, which show the retention of phenotypes to a greater extent. The expression level of mRNA in cell constructs containing TGFβ3 was higher than that in the control group. In general, chondrocytes cultured on TCPS lose their cartilage tissue phenotypes. To prevent the dedifferentiation, considerable effort has been made to utilize three-dimensional cultures of chondrocytes. Although cell-to-cell interactions among chondrocytes are important in preventing dedifferentiation, the poor growth rate in hydrogels is one of the major problems related to the *in vitro* cultivation of chondrocytes, and often results in dedifferentiation.

In cartilage regeneration, a distinct cartilage-specific mor-

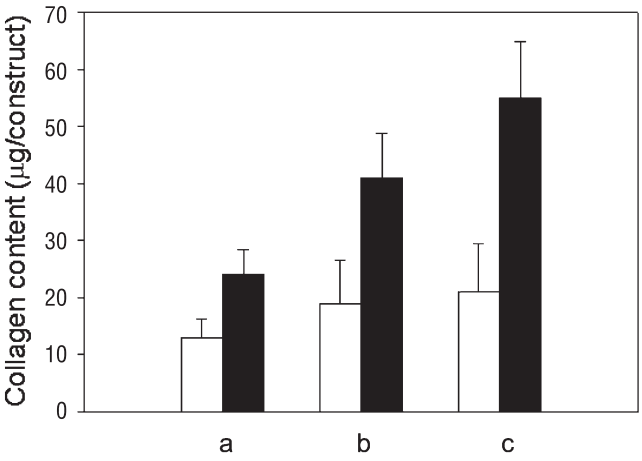

FIG. 4. Total collagen content of constructs of nude mouse transplanted with hydrogel constructs containing rabbit chondrocytes after 56 d. Open bars, Thermoreversible hydrogel; closed bars, thermoreversible hydrogel mixed with TGFβ3. a, 1 week; b, 4 weeks; and c, 8 weeks.

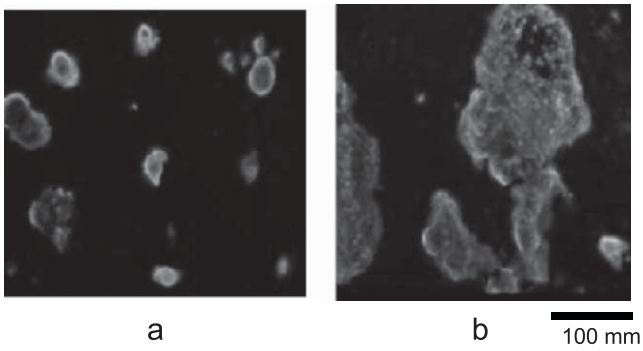

FIG. 5. Collagen type II staining of chondrocytes embedded in thermoreversible hydrogels with and without TGFβ3 after eight week incubation. (a) Thermoreversible hydrogel without TGFβ3 and (b) thermoreversible hydrogel mixed with TGFβ3. The length of the inserted bar is 100 μm.

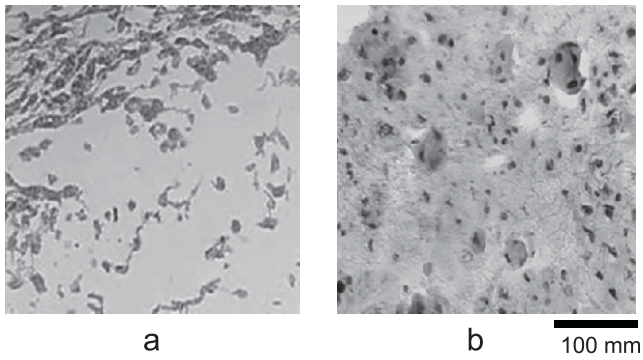

FIG. 6. Safranin-O staining of chondrocytes embedded in thermoreversible hydrogels with and without TGFβ3 after 8-week incubation. (a) Thermoreversible hydrogel without TGFβ3 and (b) thermoreversible hydrogel mixed with TGFβ3. The length of the inserted bar is 100 μm.

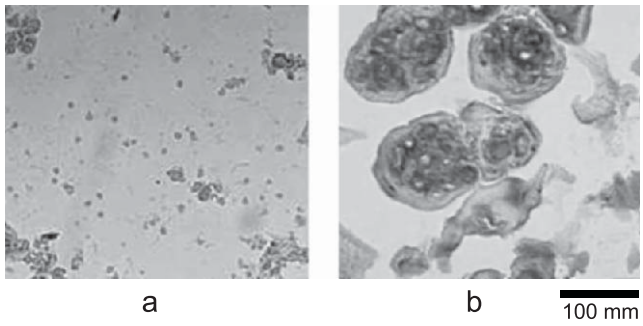

FIG. 7. Alcian Blue staining of chondrocytes embedded in thermoreversible hydrogels with and without TGFβ3 after 8-week incubation. (a) Thermoreversible hydrogel without TGFβ3 and (b) thermoreversible hydrogel mixed with TGFβ3. The length of the inserted bar is 100 μm.

phology and structural characteristics such as lacunae and collagen type II expression are typically observed. Accordingly, in the implanted constructs, the presence of a specific morphological characteristic of chondrocytes, lacunae, was tested in chondrogenic differentiation. The implanted constructs were tested using Safranin-O and Alcian Blue staining to confirm the specific changes in morphological appearance. These methods indicated that the chondrocytes encapsulated in the hydrogels with the growth factor accumulated an extensive extracellular matrix that was rich in proteoglycans and polysaccharides (Figs. 4 and 5, respectively). In contrast, cells encapsulated in the hydrogels without the growth factor produced an extracellular matrix only in the immediate vicinity of each cell. After 8 weeks of culture, the difference between the hydrogels with TGFβ3 and those with no growth factors was more significant, and lacunae formation was observed in the former. The accumulation of proteoglycans and polysaccharides in the hydrogels including the growth factor was significant and spread throughout the entire construct. This study demonstrates that the chondrogenesis of rabbit chondrocytes in a thermoreversible scaffold is well organized when 100 ng/ml TGFβ3 is included and mixed with the hydrogel. It was shown that the chondrogenic differentiation of chondrocytes requires the presence of TGFβ3 as an inducer of chondrogenesis. The results of this study suggest that this factor has significant direct effects on the growth of cells in hydrogels. This factor induces a sequence of molecular and cellular events, which are more similar to the events that occur during *in vivo* chondrogenesis at a high dose (100 ng/ml).

In summary, this study demonstrates that the chondrogenesis of rabbit chondrocytes in thermoreversible constructs is well organized when TGFβ3 is included. It was shown that the chondrogenic differentiation of chondrocytes requires the presence of TGFβ3 as an inducer of chondrogenesis. The three-dimensional cell culture *in vivo* test including TGFβ3 not only increased the level of cellular proliferation at all the time points tested (from 7 to 56 d), but also increased cell differentiation level. This result suggests that this factor has significant and direct effects on the growth of cells in hydrogels. The factor induces a sequence of molecular and cellular events that are quite similar to the events that occur during *in vivo* chondrogenesis.

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