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The use of de-differentiated chondrocytes delivered by a heparin-based hydrogel to regenerate cartilage in partial-thickness defects

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ABSTRACT

Partial-thickness cartilage defects, with no subchondral bone injury, do not repair spontaneously, thus there is no clinically effective treatment for these lesions. Although the autologous chondrocyte transplantation (ACT) is one of the promising approaches for cartilage repair, it requires *in vitro* cell expansion to get sufficient cells, but chondrocytes lose their chondrogenic phenotype during expansion by monolayer culture, leading to de-differentiation. In this study, a heparin-based hydrogel was evaluated and optimized to induce cartilage regeneration with de-differentiated chondrocytes. First, re-differentiation of de-differentiated chondrocytes encapsulated in heparin-based hydrogels was characterized *in vitro* with various polymer concentrations (from 3 to 20 wt.%). Even under a normal cell culture condition (no growth factors or chondrogenic components), efficient re-differentiation of cells was observed with the optimum at 10 wt.% hydrogel, showing the complete re-differentiation within a week. Efficient re-differentiation and cartilage formation of de-differentiated cell/hydrogel construct were also confirmed *in vivo* by subcutaneous implantation on the back of nude mice. Finally, excellent cartilage regeneration and good integration with surrounding, similar to natural cartilage, was also observed by delivering de-differentiated chondrocytes using the heparin-based hydrogel in partial-thickness defects of rabbit knees whereas no healing was observed for the control defects. These results demonstrate that the heparin-based hydrogel is very efficient for re-differentiation of expanded chondrocytes and cartilage regeneration without using any exogenous inducing factors, thus it could serve as an injectable cell-carrier and scaffold for cartilage repair. Excellent chondrogenic nature of the heparin-based hydrogel might be associated with the hydrogel characteristic that can secure endogenous growth factors secreted from chondrocytes, which then can promote the chondrogenesis, as suggested by the detection of TGF-β1 in both *in vitro* and *in vivo* cell/hydrogel constructs.

1. Introduction

Articular cartilage is an avascular and aneural tissue with a limited capacity for self-repairs [1]. The autologous chondrocyte transplantation (ACT) is a currently available medical therapy to restore mobility and reduce pain for articular cartilage defects. Although ACT has been used clinically to improve the repair of cartilage defects with good to excellent clinical results [2,3], the recovery of articular chondrocytes leads to damage at the donor collection site [4], hypertrophy [5], or uncontrolled calcification occasionally [4]. Tissue engineering strategy focuses to improve the original ACT by combining autologous chondrogenic cells and various biomaterials that can help the healing of defect site. However, the current ACT techniques have been studied for treating full-thickness defects [6], and few reports have studied on partial-thickness defects [7]. Defects in articular cartilage are classified as either full- or partial-thickness defects according to the penetration into the subchondral bone. Partial-thickness defects seem to the discrete lesions and fissures that represent the early stages of osteoarthritis [8,9]. Those lesions become larger and deeper during the course of the disease but do not repair themselves spontaneously because they are walled off from marrow and thus have no access to macrophages, endothelial cells, and mesenchymal cells [10,11].

For ACT or tissue engineering approach to partial cartilage defect, first of all, sufficient autologous chondrogenic cells are required, and articular chondrocytes, isolated from a low load-bearing site, have been classically considered as the main cell

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source. Other cells, especially, MSCs have been also considered as an alternative cell source since they can be expanded sufficiently [12] and be differentiated into chondrocytes under the proper *in vitro* condition [13,14]. However, complications such as time consuming expansion and differentiation of MSCs with growth factors and other compounds [15,16] and the difficulty in maintaining at the regular chondrogenically differentiated state [17] make chondrocytes still as a main cell source for partial cartilage repair. To overcome the limited amount of available cells isolated from cartilage, *in vitro* expansion by monolayer culture is typically required, that leads to the de-differentiation of chondrocytes making cells to lose their ability to express articular cartilage-specific extracellular matrices (ECM) such as Type II collagen and aggrecan [18,19]. Therefore, complete re-differentiation of de-differentiated cells is essential prior to further applications.

Previous attempts to induce the re-differentiation of de-differentiated chondrocytes include the application of an environment supporting a spherical morphology of cells such as pellets [18,20] and 3-D matrices using natural/synthetic polymer [21–25]. Among them, hydrogels such as hyaluronan [24], alginate [21], and agarose [26] showed good results of re-differentiation of de-differentiated chondrocytes after culturing for 3–4 weeks because they possess highly hydrated three-dimensional networks mimicking the native cartilage. But, these studies typically employed the chondrogenic media and/or growth factors such as TGF-β1/3 during culture and sometimes inflammatory responses and foreign body giant cell reaction after *in vivo* implantation were reported in Ref. [4].

Recently, we demonstrated that an injectable heparin-based hydrogel, formed by Michael-type reaction between thiolated heparin and diacrylated poly(ethyleneglycol) (PEG-DA) [27], was an excellent matrix for the *in vitro* cultivation of articular chondrocytes close to their primary state [28]. Chondrocytes were proliferated and produced the proper GAGs in the heparin-based hydrogels while maintaining their phenotypes in a normal culture medium without growth factor or chondrogenic components. Therefore, in the present study, we characterized the ability of this heparin-based hydrogel as a 3-D matrix to induce the re-differentiation of de-differentiated chondrocytes under a normal cell culture condition (no growth factors or chondrogenic components). Then, we optimized the hydrogel for re-differentiation of cells by modulating the precursor concentration of the hydrogel because the elasticity or stiffness of matrix is an important factor influencing the cell functions [29,30]. In addition, the cartilage tissue formation of the cell/hydrogel was evaluated by subcutaneous implantation in nude mice. Finally, cartilage regeneration was evaluated by delivering the optimized cell/hydrogel constructs to partial-thickness cartilage defects in rabbits.

2. Materials and methods

2.1. Materials

Heparin (sodium salt, from porcine intestinal mucosa, Mw 12 kDa) was purchased from Cellsus Ins. (Cincinnati, OH, USA). Poly (ethylene glycol) diacrylate (PEG-DA, Mw 6 kDa, degree of substitution 98%) was purchased from Sunbio Inc. (Anyang, Korea). Sodium chloride, potassium phosphate monobasic, sodium phosphate dibasic, potassium chloride, glycine, papain, sodium phosphate, sodium–EDTA, cysteine–HCl, chondroitin sulfate, agarose, fast green, and Safranin-O were obtained from Sigma Chemical Co. (St. Louis, MO, USA). PBS (phosphate buffered saline, 0.01 mol/L PBS solution with 0.138 mol/L NaCl and 0.0027 mol/L KCl, pH = 7.4) was prepared with potassium phosphate monobasic and sodium phosphate dibasic. As a cell culture medium, Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/L of D-glucose and glutamine, fetal bovine serum (certified), penicillin G, and streptomycin were used (all from Gibco, NY, USA). Trypsin–EDTA (ethylenediaminetetraacetic acid) (0.25%) was also purchased from Gibco. Tri-reagent was obtained from Molecular Research Center Inc. (Cincinnati, OH, USA). Anti-rabbit TGF-β1 antibody was obtained from Novus International Inc. (St. Charles, MO, USA). Anti-TGF-β1 detection antibody, Avidin–HRP conjugate, and ABTS liquid substrate were purchased from PeproTech (Rocky Hill, NJ, USA). Rabbit anti-goat IgG-H&L (TR) was obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA).

2.2. Preparation and characterization of mechanical properties of the heparin-based hydrogel

Heparin-based hydrogels were prepared by a Michael-type addition reaction between thiolated heparin (Hep-SH) and diacrylated poly (ethylene glycol) (PEG-DA), as previously reported by us [27]. Heparin-based hydrogels were made by dissolving 40% Hep-SH and 6 kDa PEG-DA (1:1 molar ratio of thiol group of Hep-SH and acrylate group of PEG-DA) in a culture medium to make 3, 5, 10, 15, or 20 wt.% of gel precursor solution which was sterilized by filtration. In all cases gelation was completed within 10 min at 37 °C. More detailed information about the gelation kinetics of the heparin-based hydrogel at various concentrations was previously reported [27].

Modulus of the cultured heparin-based hydrogel was measured using a rheometer (Gemini, Malvern Instruments, UK, USA), equipped with a temperature controller at 37 °C and a solvent trap. Samples were analyzed with sandblast parallel plate geometry. An angular frequency of $\omega = 1$ rad/s and a strain of $\gamma = 0.1\%$ were selected to ensure a linear regime of oscillatory deformation.

2.3. Culture of chondrocyte inside the heparin-based hydrogel

Rabbit articular chondrocytes were isolated from the cartilage of rabbits (2-week-old New Zealand White rabbit, Samtako Bio Korea, Osan, Korea) as described previously in Ref. [31]. Isolated cells were cultured on culture dishes in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 50 μg/mL streptomycin, and 50 units/mL penicillin at 37 °C in a standard cell culture condition (humidified atmosphere of 5% CO_2). After passaging 4 times (P4), chondrocytes were encapsulated into the hydrogel by adding cells to the precursor gel solution during gelation. The gel precursor solution (50 μL) containing chondrocytes (1×10^7 cells/mL) was poured into a 96 well plate with an ultralow attachment surface (Corning, NY, USA), and incubated for 30 min at 37 °C in a humidified 5% CO_2 atmosphere. In the next step, the cell-containing gel was immersed in 200 μL/well of cell culture medium and then maintained in a standard cell culture condition up to 4 weeks. The medium was replaced with the fresh one for every 2–3 days.

2.4. RNA isolation and real-time PCR

Total RNA was isolated from the cell/hydrogel constructs using Tri-reagent. Purified RNA was quantified with a spectrophotometer and also checked by the electrophoresis in 1% agarose gel. One μg/μL of RNA was reverse-transcribed with ImProm-IITM reverse transcriptase (Promega, WI, USA) according to the manufacturer's protocol [32], using iQ™5 real-time PCR detection system (Bio-Rad, CA, USA) for 5 min at 25 °C for annealing and for 60 min at 42 °C for extending the first strand, as described previously [33]. The final cDNAs were then subjected to real-time PCR to determine the expression of genes for Type I collagen, Type II collagen, and aggrecan. PCR reaction was carried out with specific primers and iQ SYBR Green Supermix (Bio-Rad) containing dNTPs, iTaq DNA polymerase, $MgCl_2$, and SYBR Green I fluorescein for an initial denaturation at 95 °C for 5 min, followed by 40 cycles of PCR. Each cycle was proceeded at 95 °C for 20 s, 62 °C for 20 s, and 72 °C for 40 s, in accordance with the manufacturer's recommendations. Relative quantification was calculated by the $2-(\Delta\Delta Ct)$ method and all gene expressions were normalized to the expression of glyceraldehyde-3-phosphatase dehydrogenase (GAPDH), a house-keeping gene. The sequence of primers for GAPDH, Type I collagen, Type II collagen, and aggrecan were as follows: GAPDH: 5-TCACCATCTTCCAGGAGCGA-3, 5-CACAATGCCGAAGTGGTCGT-3; Type I collagen: 5-GGCTTTCCTGGAGAGAAAGG-3, 5-ATAGAACCAGCAGGGCCAGG-3; Type II collagen: 5-AACACTGCCAACGTCCAGAT-3, 5-CTGCAGCACGGTATAGGTGA-3; aggrecan: 5-CCTTGGAGGTCGTGGTGAAAGG-3, 5-AGGTGAACTTCTCTGGCGACGT-3. PCR products were electrophoresed in 1% agarose gels and were visualized after ethidium bromide staining.

2.5. Quantification of sulfated-glycosaminoglycans (GAGs)

The amounts of sulfated-glcosaminoglycans (GAGs) were estimated using dimethylmethylene blue (DMMB) metachromatic assay (Sigma) [34]. In brief, the color reagent was prepared by dissolving 16 mg of DMMB in a solution (1000 mL, pH 3.0) containing 3.04 g of glycine, 2.37 g of NaCl, and 95 mL of 0.1 mol/L HCl. The chondrocyte-cultured hydrogels were immersed in a digestion solution with 500 μg/mL papain, 5 mM Na_2EDTA, and 5 mM cysteine–HCl in 0.1 M sodium phosphate (pH 6.2). After overnight incubation at 60 °C for digestion, they were centrifuged at 13,000 *g* for 15 min. The supernatant was collected and stored at −20 °C until the GAG assay was performed. The total amounts of GAGs were determined by measuring an absorbance at 525 nm and comparing with a standard curve of shark chondroitin sulfate in the range of 0–50 μg/mL. Signal from the heparin-based hydrogel itself without cells was considered as a baseline, so the GAG amount was calculated using the additional intensity from the deposited GAGs in the hydrogel.