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In situ chondrogenic differentiation of human adipose tissue-derived stem cells in a TGF-β_1 loaded fibrin–poly(lactide-caprolactone) nanoparticulate complex

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ABSTRACT

When conducting cartilage tissue engineering with stem cells, it is well known that chemical and physical stimulations are very important for the induction and maintenance of chondrogenesis. In this study, we induced chondrogenic differentiation of human adipose tissue-derived stem cells (hASCs) *in situ* by effective stimulation via the continuous controlled release of TGF-β1 from a heparin-functionalized nanoparticle–fibrin–poly(lactide-*co*-caprolactone) (PLCL) complex. PLCL scaffolds were fabricated with 85% porosity and 300–500 μm pore size by a gel-pressing method. Heparin-functionalized nanoparticles were prepared by a solvent-diffusion method, composed of poly(lactide-*co*-glycolide) (PLGA), Pluronic F-127, and heparin, and then TGF-β1 was loaded to the nanoparticles. A mixture of hASCs, fibrin gels and TGF-β1 loaded nanoparticles was then seeded onto PLCL scaffolds and cultured *in vitro*, after which they were subcutaneously implanted into nude mice for up to five weeks. The results of *in vitro* and *in vivo* studies revealed that chondrogenic differentiation of the hASCs on the complex was induced and sustained by continuous stimulation by TGF-β1 from the heparin-functionalized nanoparticles. In addition, there was no significant difference between the predifferentiation condition prior to incubation in chondrogenic medium and the proliferation condition, which suggests that *in situ* chondrogenic differentiation of hASCs was induced by the TGF-β1 loaded nanoparticles. Consequently, the hybridization of fibrin and PLCL scaffolds for three-dimensional spatial organization of cells and the effective delivery of TGF-β1 using heparin-functionalized nanoparticles can induce hASCs to differentiate to a chondrogenic lineage and maintain their phenotypes.

1. Introduction

The self-healing of articular cartilage defects under normal physiological circumstances is difficult due to the histological characteristics of cartilage and the aneural and avascular tissue [1–3]. One of the treatments of cartilage defects involves the use of engineered functional cartilage produced using living cells, which requires the use of a biocompatible polymer and stimulation to induce chondrogenesis [4–6]. Although autologous chondrocytes are useful in cartilage regeneration, their wider application is limited due to the proliferative capacity of terminally differentiated chondrocytes, the formation of fibrous cartilage, and the morbidity of donor sites. Thus, there have been many studies conducted to evaluate the use of adult stem cells in cartilage tissue engineering. Recently, adipose tissue-derived stem cells (ASCs) have been regarded as good candidates for the repair and regeneration of articular hyaline cartilage due to their self renewal, long term cell viability and multilineage differentiation potential [7–14]. In addition, ASCs have advantages such as the ability to be obtained in large quantities from various body parts under local anesthesia with minimal discomfort.

It is well known that chemical and physical stimulations are very important in the induction of differentiation and the maintenance of lineage to chondrogenesis in cartilage tissue engineering using mesenchymal stem cells [15–17]. It has been shown that transforming growth factor-β_1 (TGF-β_1), which is an isoform of TGF-β, plays an important role in the regeneration of articular cartilage and promotes chondrogenic differentiation of chondroblasts and mesenchymal stem cells [18–21]. Furthermore, fibrin gel is known to help cells that have differentiated to the chondrogenic lineage to maintain their phenotype and to synthesize cartilage extracellular matrix, even though it has rapid degradability and weak mechanical properties [22,23]. Until now, the pellet culture or the hydrogel

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culture with fibrin or alginate has been used for cartilage tissue engineering using hASCs. In these cases, there were several drawbacks such as mechanical deformability, undue elasticity, and low volume stability [11,22,24]. For overcoming the mechanical weaknesses, several groups have been studied for cartilage tissue formation using PLGA scaffolds and hASCs [24,25]. However, in order to induce the chondrogenic differentiation of hASCs, the *in vitro* induction periods with chondrogenic media, gene transductions, or the addition of growth factors to scaffolds would be needed. Previously, we generated cell-complex constructs with PLCL scaffolds that had elastic mechanical properties similar to articular cartilage and fibrin gel to mimic the native cartilage microenvironment and guide the three-dimensional spatial organization of cells and extracellular matrix (not published). The PLCL scaffolds degraded very slowly, which would compensate the weakness of hydrogels. *In vivo*, the mass of PLCL scaffolds decreased gradually to about 20% of the initial mass after 4 months [26]. Also, the PLCL scaffolds have very elastic mechanical properties and that was maintained under continuous stimulations [26–28]. The PLCL scaffolds with 85% porosity showed almost complete recovery, applied up to 500% tensile strain [11,22,28]. The use of elastic PLCL scaffolds to enable the physiological stimulation of specific cells and guide tissue growth would complement systems that only use fibrin gels [29,30]. Furthermore, it has been reported that the delivery of growth factor using a heparin-functionalized nanoparticles-fibrin gel complex effectively induced *in vivo* tissue regeneration via the biological activities of the delivered growth factor [31,32].

To successfully guide cartilage repair and regeneration using functional engineered cartilage with stem cells requires particular growth factors that induce chondrogenesis as well as a stable and bioactive scaffold similar to native cartilage. Therefore, in this study, we fabricated a functional nanoparticle–fibrin–PLCL complex that induced chondrogenic differentiation of human adipose tissue-derived stem cells (hASCs) *in situ* via effective growth factor stimulation that was induced through the continuous controlled release of TGF-β_1. Specifically, we fabricated a highly-elastic PLCL scaffold using a gel-pressing method and TGF-β_1 loaded PLGA nanoparticles via a solvent-diffusion method. We then seeded a mixture of hASCs, fibrin gels and nanoparticles onto PLCL scaffolds, after which we evaluated their abilities to promote chondrogenic differentiation and to sustain their lineage in complex *in vitro* and *in vivo*.

2. Materials and methods

2.1. Preparation of PLCL scaffolds and TGF-β_1 loaded nanoparticles

PLCL was synthesized and used in a gel-pressing method to fabricate sheet-form scaffolds using a previously described method [29]. Briefly, PLCL synthesized in our laboratory was dissolved in chloroform (5% w/v) and then mixed with NaCl particles (300–500 μm, 85% w/w). The chloroform was then evaporated in air to form the PLCL gels, which were subsequently pressed in a sheet-forming mold. Next, the residual chloroform was evaporated for 48 h at room temperature, after which it was completely removed under vacuum for 24 h. The salts were then leached out by placing the gels in distilled, deionized water with constant shaking for 3 days. The resulting scaffolds were then freeze-dried for 24 h, after which they were sterilized with ethylene oxide gas.

The heparin-functionalized nanoparticles were prepared using a spontaneous emulsion solvent-diffusion method that has been described elsewhere [31,32]. Briefly, 20 mg of PLGA (RESOMER®RG 756; 75 mol% of lactide, M_W 90,000; Boehringer Ingelheim, Germany) were dissolved in 1 ml of DMSO (Sigma) and then slowly added to a 5% (w/v) Pluronic aqueous solution that contained 60 mg of heparin sodium (189 IU/mg, M_W 12,500; Celsus Laboratories, Cincinnati, OH, USA) to prepare the heparin-functionalized nanoparticles. The nanoparticles were then sterile filtered (0.45 μm, Whatman, UK), after which they were collected by centrifugation (Supra 22K, Hanil Science Industrial Co., Korea) at 16,000*g* for 1 h and then resuspended in 20 μl of phosphate buffered saline (PBS). Next, 5 μl of the resuspended nanoparticles were mixed with 1 μg of TGF-β_1 (R&D Systems, Minneapolis, MN, USA) and the mixture was then incubated at 4 °C overnight with gentle rotation. The TGF-β_1 loaded functional nanoparticles were prepared immediately prior to seeding the cells onto the scaffolds.

Table 1
List of primers used in the real time PCR analysis of *in vitro* samples.

Primer name	Forward sequence	Reverse sequence	Product size (bp)
Aggrecan	TCGAGGACAGC-GAGGCC	TCGAGGGTGTAGCGTGTAGAGA	85
SOX-9	TACGACTACACCG-ACCACCA	CTCCTCAAGGTCGAGTAGGC	217
Type II collagen	GGCAATAGCAGGT-TCACGTACA	CGATAACAGTCTTGCCCCACTT	70
Type I collagen	CAGCCGCTTCAC-CTACAGC	TTTTGTATTCAATCACTGTCTTGCC	100
Type X collagen	CAAGGCACCATCT-CCAGGAA	AAAGGGTATTTGTGGCAGCATATT	70
GAPDH	ATGGGGAAGGTG-AAGGTCG	TAAAAGCAGCCCTGGTGACC	119

For *in vitro* release, two systems containing TGF-β_1 were prepared: (1) a fibrin gel that contained heparin-functionalized nanoparticles (TGF-β_1–NP–Fibrin), and (2) a fibrin gel (TGF-β_1–Fibrin). Briefly, 100 ng of TGF-β_1 were mixed with 5 μl of the resuspended nanoparticles or the same volume of PBS without nanoparticles, after which each solution was dispersed in 35 μl of fibrinogen solution (90 mg of fibrinogen, 60 U of factor XIII, and 1000 KIU aprotinin/ml) from a fibrin glue kit (Greenplast®; Yongin, Korea). Next, 35 μl of thrombin solution (20 IU/ml of 0.6% (w/v) calcium chloride solution) were added, and the mixture was then transferred to a microtube with an inner diameter of 1.3 cm and allowed to stabilize for 1 h. Both fibrin gel-based systems were then placed into 35 ml of PBS that contained 2.0 mM sodium azide and 0.01% (w/v) BSA and incubated at 37 °C with shaking at 100 rpm. At pre-determined time intervals, aliquots (200 μl) of the release buffer were collected and stored at −70 °C until they were analyzed. To maintain a sink condition during the release experiment, 200 μl of fresh buffer was added to the release buffer after sampling and the buffer was completely replaced with an equal volume of fresh buffer once a week. The amount of total TGF-β_1 released was assessed using a Human TGF-β_1 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.2. Isolation and culture of hASCs

Subcutaneous and omental tissue specimens of white human fat were obtained from normal or slightly overweight human subjects at Catholic University ($n = 3$, mean age 53 ± 6, Female). Informed consent was obtained from all patients included in this study. hASCs were isolated using a modification of a technique that has been previously described [7]. Briefly, adipose tissue was weighed, extensively washed with PBS, minced for 10 min with fine scissors, and then enzymatically digested at 37 °C for 4 h with 0.05% type IA collagenase (Worthington Biochemical, Lakewood, NJ, USA) in PBS supplemented with penicillin–streptomycin (P–S, 100 units/ml penicillin, 100 μg/ml streptomycin; Gibco BRL; Grand Island, NY, USA). The digested adipose tissue was then centrifuged at 400*g* for 5 min to obtain a cell pellet, which was subsequently resuspended and passed through a 100 μm filter (BD Biosciences, San Jose, CA, USA) to remove debris. The isolated cells were then washed with PBS and cultured in cell culture flasks (Nunc) in culture medium Dulbecco's Modified Eagle's Medium/Nutrient Mixture Ham-12 (DMEM/F12; WelGene, Korea) that contained 10% fetal bovine serum (FBS; WelGene) and P–S at 37 °C in a humidified atmosphere under 5% CO_2.

2.3. In vitro and in vivo studies

The cultured hASCs (passage number = 2) were collected by trypsin (Gibco BRL) treatment and then resuspended in culture medium (3.3×10^8 cells/ml). The suspension of hASCs (1×10^6 cells/scaffold) and TGF-β_1 (1 μg/scaffold) loaded nanoparticles was dispersed in 35 μl of fibrinogen solution (90 mg of fibrinogen, 60 U of factor XIII, and 1000 KIU aprotinin/ml) from a fibrin glue kit, after which they were mixed well. Next, 35 μl of thrombin solution (20 IU/ml of 0.6% (w/v) calcium chloride solution) were added, and the mixture was then inoculated

Table 2
List of primers used in the RT-PCR analysis of *in vivo* samples.

Primer name	Forward sequence	Reverse sequence	Product size (bp)
SOX-9	GAACGCACATCAAGACGGAG	TATCGTTGATTTCGCTGCTC	631
Aggrecan	GCAGAGACGCATCTAATTG	GGTAATTGCGAACATCATT	504
Link protein	CCTATGATGAAGGCGGTGC	GGTAACTTGGAGTTC	342
Type II collagen	CTGCTCGTCGCCGTCCTT	AAGGGTCCCAGGTTCTCCATC	432
Beta-actin	GCCCCTCCATCGTCCACCGA	GGGCACGGCTCATCATT	493