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Enhanced regeneration of the ligament–bone interface using a poly(L-lactide–co-ε-caprolactone) scaffold with local delivery of cells/BMP-2 using a heparin-based hydrogel

Jongman Lee [a,b], Won Il Choi [a,b], Giyoong Tae [a,b,*], Young Ha Kim [a,b], Seong Soo Kang [c], Se Eun Kim [c], Sang-Heon Kim [d], Youngmee Jung [d], Soo Hyun Kim [d]

[a] Department of Materials Science and Engineering and Department of Nanobio Materials and Electronics, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea
[b] Reasearch Center for Biomolecular Nanotechnology, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea
[c] Department of Surgery, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Republic of Korea
[d] Biomaterials Research Center, Korea Institute of Science and Technology, Seoul 130-650, Republic of Korea

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ABSTRACT

Recently, the ligament–bone (LTB) junction has been emphasized for the effective transmission of mechanical force and the reduction in stress concentration between the soft ligament and hard bone tissue. The aim of this study was to regenerate an integrated LTB interface by inoculating LTB-relevant cells, isolated from fibrocartilage (FC) or ligament (LIG), separately into each designated region in a single porous cylindrical PLCL scaffold. An injectable, heparin-based hydrogel that has proved to be effective in the culture of chondrocytes as well as the sustained release of growth factor was employed to locally deliver fibrochondrocytes and osteoinductive bone morphogenetic protein-2 (BMP-2) into the FC region, to promote FC regeneration. In in vitro experiments the hydrogel-combined FC systems produced significantly larger amounts of calcium and glycosaminoglycans (GAGs), but less collagen and DNA than FC samples without the hydrogel and all LIG samples. After in vivo subcutaneous implantation in mice for 8 weeks the secreted calcium and GAG contents of the hydrogel-containing FC samples were superior or similar to those of the in vitro hydrogel-containing FC samples at 6 weeks. As a result of the enhanced production of calcium and GAG, the in vivo hydrogel-containing FC samples produced the highest compressive modulus among all samples. Histological and immunofluorescence analysis as well as elemental analysis also confirmed a denser and more homogeneous distribution of calcium, GAG, osteocalcin and neovascularization marker in the in vitro/in vivo hydrogel-containing FC systems than those without hydrogel. These results also show the beneficial effects of BMP-2 added using the hydrogel. In summary, the use of a heparin-based hydrogel for the local delivery of fibrochondrocytes and BMP-2 could accelerate the maturation and differentiation of LTB-specific FC tissues, and it was also possible to recreate the unique stratification of calcified FC and LIG tissues in a single porous PLCL scaffold in terms of both biochemical and biomechanical properties.

1. Introduction

Tissue engineering of ligament–bone (LTB) interfaces, termed "enthesis", has the challenge of achieving the entire functional integration of soft ligament tissues with hard bone tissues, because their junction is essential to transmit mechanical load, minimize stress concentration and, in turn, prevent graft failure [1–3]. The anterior cruciate ligament (ACL), the most frequently damaged ligament in the knee [4], is responsible for stabilizing the knee joint and is connected to the subchondral bones through four gradual transition interfaces with controlled heterogeneity of cell type and matrix composition [5]: ligament, non-calcified fibrocartilage, calcified fibrocartilage and bone. The ligament zone is fibrous connective tissue containing fibroblasts embedded in a collagen type I and type III matrix. The non-calcified fibrocartilage zone is relatively avascular and is composed of ovoid-shaped fibrochondrocytes within a collagen type II and proteoglycan matrix. The calcified fibrocartilage zone consists of hypertrophic circular fibrochondrocytes in a calcified matrix with collagen type X, which only exists in this region. Subchondral bone contains osteoblasts,

* Corresponding author at: Department of Materials Science and Engineering and Department of Nanobio Materials and Electronics, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea. Tel.: +82 62 970 2305; fax: +82 62 715 2304.
E-mail address: gytae@gist.ac.kr (G. Tae).

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osteocytes and osteoclasts surrounded by a calcified collagen type I matrix. Unless a gradual fibrocartilaginous transition is present the mechanical stability of ACL reconstruction grafts might be adversely affected, with inadequate tissue integration at the graft–bone junction after reconstructive surgery [6,7]. Therefore, the development of continuous and heterogeneous transition complexes mimicking the native insertion site is required to improve the biological fixation of ACL grafts with subchondral bone.

As to the engineering of ACL–bone interfacial reconstruction, there have been a wide variety of healing strategies, as recently published in two review papers [5,8]. In general, it can be classified into two approaches: (1) building an entire heterogeneous complex with specialized transition regions as a single construct; (2) recreating the individual interface tissues to be integrated between the ACL graft and the surrounding bone through biological fixation. The former comprises a triphasic composite structure intended to form three distinct yet continuous interface regions [9], a collagen scaffold with a gradient of retrovirus encoding the osteogenic transcription factor (Runx2), which induces heterogeneous cell phenotypes and biochemical properties [10], and a poly(ethylene glycol) diacrylate (PEG-DA) hydrogel incorporating a hydroxyapatite (HA)/RGD (Arg–Gly–Asp) peptide complex, which promotes the formation of a LTB interface with increased cellular adhesion and mechanical properties [11]. The latter includes an ACL graft–bone tunnel healing model using autografts (i.e. patellar ligaments or hamstring tendons) [12], allografts [13], mechanical fixation (i.e. interface screws or pins) [14] and tissue engineered constructs with the aid of natural/synthetic biomaterials [15–17], cell sources [16,18] and growth factors [15,18,19], resulting in the recreation of fibrocartilaginous interface tissues which bridge between the ACL graft and subchondral bone.

Bone morphogenetic protein-2 (BMP-2), a member of the transforming growth factor β (TGF-β) superfamily [20], is the most extensively studied osteogenic promoter to induce in vitro osteoblastic differentiation in several cell types (i.e. bone marrow stromal cells [21], fibroblasts [22] and chondrocytes [23]). BMP-2 has also been shown to support mineralized tissue formation, with the gene expression of bone-specific markers both in vitro and in vivo [24,25], and to encourage bone formation in vivo through intramembranous and endochondral ossification [26]. Moreover, BMP-2 has the capability to induce the in vitro chondrogenic differentiation of various cell types (i.e. embryonic stem cells [27], bone marrow stromal cells [28] and synovium-derived progenitor cells [29]), to maintain the differentiated chondrocyte phenotype (i.e. type II collagen and aggrecan expression) in two- and three-dimensional systems [30,31] and to stimulate cartilage proteoglycan synthesis in vitro and in vivo [32,33].

Poly(L-lactide–co-ε-caprolactone) (PLCL) co-polymer has been utilized as a mechano-stimulating tissue engineering scaffold for tendons [34], blood vessels [35] and cartilage [36], due to its highly flexible and elastic properties and superior recovery. In a previous tendon study porous PLCL scaffolds seeded with tenocytes were subjected to dynamic tensile extension (10% strain) using a bioreactor, which resulted in enhanced cell proliferation and up-regulated collagen type I expression [34]. Thus, we demonstrated that this porous PLCL scaffold with excellent interconnectivity might be a suitable material for tendon/ligament tissue engineering.

Here we have investigated the regeneration of the LTB interface using a single porous cylindrical PLCL scaffold. The scaffold was divided into fibrocartilage (FC) and ligament (LIG) regions; the FC region was seeded with interface-relevant fibrochondrocytes while the LIG region was separately seeded with fibroblasts. Using an injectable, heparin-based hydrogel, a small amount of BMP-2 was locally delivered to the FC region along with the fibrochondrocytes to accelerate the formation of a calcified fibrocartilagious transition region. It is known that calcified fibrocartilage plays a very important role as an interlocking region of tissue interdigitation ensuring secure attachment to the subjacent bone, increasing the resistance to shear and force [5]. It has already been shown that heparin has BMP-2 binding domains that release BMP-2 at a sustained, controlled rate during tissue culture [37]. After cultivation with stratified interfacial FC–LIG tissues the unique reconstruction of the LTB interface was evaluated with regard to tissue-specific extracellular matrix (ECM) secretion and mechanical properties (compressive modulus), and the effects of using the hydrogel and BMP-2 were analyzed.

2. Materials and methods

2.1. Co-polymerization and characterization of PLCL

Co-polymerization of PLCL (typical composition was 60:40 mol.%) was carried out in a 250 ml glass ampule containing L-lactide (0.555 mol) and ε-caprolactone (0.37 mol), as reported previously [34]. Stannous octoate (1×10^{-4} mol) was diluted with dried toluene and added as a catalyst. The ampoules were maintained overnight under a reduced pressure to remove toluene after purging three times with nitrogen at room temperature, and then sealed by heating with a torch under vacuum. They were co-polymerized at 150 °C in a silicone oil bath for 24 h with stirring. After co-polymerization PLCL was dissolved in chloroform and filtered through a 0.4 μm pore membrane filter. The polymer was precipitated into an excess of methanol and dried under vacuum.

The mole ratio of L-lactide to ε-caprolactone in the co-polymer was analyzed by ^{1}H NMR spectroscopy (JEOL JNM-LA300WB) using $CDCl_3$ as the solvent and tetramethysilane as a reference for chemical shift. Number average (M_n) and weight average (M_w) molecular weights were determined using a gel permeation chromatograph (GPC) (VE 2001 GPC solvent/sample module, Viscotek GPC max) equipped with a series of GPC columns (PLgel 5 μm Guard 50 × 7.5 mm, PLgel 5 μm MIXED-C 300 × 7.5 mm and PLgel 10 μm MIXED-B 300 × 7.5 mm, Polymer Laboratories). Chloroform was used as the mobile phase at a flow rate of 1.0 ml min^{-1} at 25 °C.

2.2. Fabrication of PLCL scaffolds for the LTB interface

Cylindrical PLCL scaffolds with a high porosity were prepared by an extrusion–salt leaching method as described previously [34]. PLCL solution in chloroform at a concentration of 10 wt.% was mixed with NaCl particles (200–300 μm, 90 wt.% salt content) and extruded into a cylindrical shape (5 mm in diameter) using a home-made piston extrusion tool. The residual chloroform was evaporated at room temperature for 2 days and further removed under vacuum for 1 week. A possible skin layer on the outer surface of scaffolds was removed by sandpaper treatment. A cross-sectioned image of the porous PLCL scaffold was observed using scanning electron microscopy (SEM) and the porosity was determined using the equation ($n = 5$) [36]:

$$\text{Calculated porosity} = (1 - \text{density of scaffold}/\text{density of PLCL}) \times 100 \quad (1)$$

where the density of PLCL is 1.21 g cm^{-3}.

In order to study the regeneration of LTB interfaces the scaffold was divided into three parts; fibrocartilage (FC) regions at the two ends (10 mm in length) and the ligament (LIG) region in the center (20 mm in length) (Fig. 1A). The boundaries between thw FC and LIG zones were tied with Tygon tubing (2 mm in length) to prevent cells and/or hydrogel from crossing over to other parts.