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The restoration of full-thickness cartilage defects with BMSCs and TGF-beta 1 loaded PLGA/fibrin gel constructs

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ABSTRACT

Poly(lactide-co-glycolide) (PLGA) sponge was filled with fibrin gel, bone marrow mesenchymal stem cells (BMSCs) and transforming growth factor-β1 (TGF-β1) to obtain a construct for cartilage restoration in vivo. The PLGA sponge lost its weight steadily in vitro, but degraded much faster in the construct of PLGA/fibrin gel/BMSCs implanted in the full-thickness cartilage defects. The in vivo degradation of the fibrin gel inside the construct was prolonged to 12 wk too. The CM-DiI labeled allogenic BMSCs were detectable after transplantation (implantation) into the defects for 12 wk by small animal in vivo fluorescence imaging and confocal laser scanning microscopy. In vivo repair experiments were firstly performed by implantation of the PLGA/fibrin gel/BMSCs and PLGA/BMSCs constructs into full-thickness cartilage defects (3 mm in diameter and 4 mm in depth) of New Zealand white rabbits for 12 wk. The defects implanted with the PLGA/fibrin gel/BMSCs constructs were filled with cartilage-like tissue containing collagen type II and glycosaminoglycans (GAGs), while those by the PLGA/BMSCs constructs were filled with fibrous-like tissues. To repair the defects of larger size (4 mm in diameter), addition of growth factors was mandatory as exemplified here by further loading of TGF-β1. Implantation of the PLGA/fibrin gel/BMSCs/TGF-β1 constructs into the full-thickness cartilage defects for 12 wk resulted in full restoration of the osteochondral tissue. The neo-cartilage integrated well with its surrounding cartilage and subchondral bone. Immunohistochemical and GAGs staining confirmed the similar distribution of collagen type II and GAGs in the regenerated cartilage as that of hyaline cartilage. The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) revealed that the cartilage special genes were significantly up-regulated compared with those of the TGF-β1 absent constructs.

1. Introduction

The arthritis is mostly caused by cartilage deficiency. The injured cartilage can hardly repair automatically and lead to further degeneration. Some conventional methods such as debridement, microfracture, osteochondral grafting and autologous chondrocytes implantation (ACI) are thus developed and used in clinic [1]. As an alternative treatment, tissue engineering has been demonstrated a promising approach to restore the cartilage defects too. The success of this technique relies critically on the seed cells and scaffolds and thereby the structure and functions of the regenerated cartilage.

Among the various scaffolds used, the hybrid scaffold prepared by filling soft hydrogel into hard sponge is very promising for the cartilage regeneration since their advantages can be maintained while the shortcomings can be avoided [2–6]. Apart from the more even distribution of cells and maintenance of the cell phenotype, bioactive factors such as functional genes and growth factors are conveniently loaded into the filled hydrogel with preserved bioactivity. These factors are known to regulate the proliferation and differentiation of the seed cells, which is particularly important when stem cells are used. Actually, the use of stem cells, in particular the bone mesenchymal marrow stem cells (BMSCs) has a lot of advantages over the autologous chondrocytes, and has achieved great success in cartilage and bone repair [7–10]. Particularly, both the cartilage and bone can be simultaneously repaired when the BMSCs are used, and result in better remodelling and integration with the host surface zone [11,12].

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It is known that differentiation of the BMSCs requires suitable stimuli, which can be achieved with a large variety of different growth factors. Transforming growth factor-β1 (TGF-β1) is one of the most powerful growth factors and is routinely used to induce BMSCs to chondrocytes in vitro. There are two ways to construct the hybrid scaffold which has the ability to regulate the stem cell differentiation in vivo: direct loading of the growth factors, and loading of functional genes encoding the growth factors. In a previous study, plasmid DNA encoding TGF-β1 was loaded into the fibrin gel filled poly(lactide-co-glycolide) (PLGA) sponge. In vivo experiment demonstrated that the cartilage defects were successfully restored in the rabbit knees [13]. However, the safety of the gene therapy still remains a big concern, especially for a long term application. In contrast, the biofunctions and biosafety of the growth factors are more definite with a limited function time.

In this study, recombinant protein TGF-β1 is encapsulated into the fibrin gel and then filled into the PLGA sponges to obtain a composite construct for articular cartilage repair in vivo. Attention is paid to the in vivo degradation of the PLGA and fibrin gel, the function of the fibrin gel on the cartilage repair, and the overall repair effect by the composite constructs.

2. Materials and methods

2.1. Materials

PLGA with a copolymer ratio of 75/25 (lactide/glycolide) was purchased from China Textile Academy. Its weight-average (*Mw*) and number-average (*Mn*) molecular weights were 154 kDa and 76 kDa respectively. The fibrinogen was isolated from fresh human plasma (the Blood Centre of Zhejiang Province of China) by a freezing–thawing cycle [14].

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco. Millipore water was used throughout the study. All other reagents were of analytical grade and used as received.

2.2. Preparation and characterization of PLGA sponge

The PLGA sponges with a pore size ranging from 280 μm to 450 μm were fabricated by a gelatin porogen leaching method as reported previously [15]. The PLGA sponges were cut into 4 × 4 × (8–10) mm in dimension with a razor blade. Each sample with a weight (W_0) of ~25 mg was immerged into 5 mL of phosphate-buffered saline (PBS) (pH 7.4) at 37 °C for up to 24 wk. The buffer was replaced with fresh PBS every week. At a predetermined time interval, 5 samples were taken out and rinsed with Millipore water gently, and were subsequently freeze-dried for characterizations of mass and molecular weight. The weight loss were defined as $(W_0-W_t)/W_0\times100\%$, where W_t is the weight at t time. Each value was averaged from 5 parallel samples, and expressed as mean ± standard deviation (SD). The molecular weight was determined by gel permeation chromatography (GPC, Waters 515).

2.3. Fabrication and characterization of the hybrid scaffold

The fibrinogen (40 mg/mL, 0.9% NaCl solution) and thrombin (Sigma, 5 U/mL in 40 mM $CaCl_2$ solution) solutions were sterilized by filtering through syringe filters, respectively. The final fibrinogen concentration used in all the experiments was 20 mg/mL, which was similar to the optimal concentration reported for BMSCs culture (18 mg/mL [16]). The fibrinogen solution was filled into the pores of PLGA sponges under reduced pressure. Then the composite scaffolds were taken out and immersed into the thrombin solution at 37 °C for 30 min to form strong fibrin gel. The hybrid scaffolds were freeze dried for SEM evaluation.

2.4. Surgical grafting procedures and tissue retrieval

The committee on animal experimentation of Zhejiang University approved all the animal experiments. The rabbit BMSCs were isolated by short-term adherence to plastic as described previously [17]. In this research, a defect model with two different sizes was employed. In the first experiment, a smaller size (3 mm in diameter and 4 mm in depth) defect was made to compare the performance of PLGA/fibrin gel/BMSCs and PLGA/BMSCs constructs. The PLGA/fibrin gel/BMSCs constructs were prepared as follows. 1×10^7 BMSCs in 1 mL fibrinogen solution were loaded into the PLGA sponges under reduced pressure. After taken out, the composite scaffolds were immersed into the thrombin solution at 37 °C for 30 min to gel the fibrinogen. The PLGA/BMSCs group was prepared by direct loading of the BMSCs. Then the composite constructs were implanted into the full-thickness cartilage defects of 5 rabbits as shown in Fig. S1. The same procedures of surgical grafting and tissue retrieval were used as described previously [13].

Then the defects of a bigger size (4 mm in diameter and 4 mm in depth) were made to evaluate the cartilage restoration by the constructs either loaded with TGF-β1 or not. The PLGA/fibrin gel/BMSCs/TGF-β1 constructs were prepared as follows. 1×10^7 BMSCs in 1 mL fibrinogen/TGF-β1 (1 μg, human recombinant protein from Peprotech) solution were loaded into the PLGA sponges under reduced pressure. After taken out, the composite scaffolds were immersed into the thrombin solution at 37 °C for 30 min to gel the fibrinogen. The PLGA/fibrin gel/BMSCs constructs were similarly prepared except that the TGF-β1 was absent. Then the composite constructs were implanted into the full-thickness cartilage defects of 8 rabbits.

In vivo degradation of the PLGA sponge and fibrin gel was carried out by implantation of the PLGA/fibrin gel/BMSCs constructs into full-thickness cartilage defects. At pre-determined time interval, the constructs were drilled out. The residue fibrin gel was characterized by immunohistochemical staining of fibrinogen. Molecular weight of the PLGA sponges was measured as follows. After the tissue chips were digested by papain at 60 °C for 12 h, the supernatant was discarded by centrifugation to obtain the precipitate, which was freeze dried and then dissolved by tetrahydrofuran (THF) for GPC analysis.

In order to track the implanted BMSCs, the cells were stained by chloromethyl-benzamidodialkylcarbocyanine (CM-DiI, Molecular Probes) following the method described previously [18]. This fluorescent marker intercalates within the cell membrane lipid bilayer and is useful to track the live cells in vivo [19,20]. The dye does not influence the cell metabolism and viability for a number of cell types [21]. The samples were observed by small animal in vivo fluorescence imaging system (Maestro). For a confocal laser scanning microscopy (CLSM, LSM 510, Zeiss) observation, the samples were fixed in neutral buffered formalin and decalcified before frozen sectioning. The images were taken after the sections were stained by 4′,6-diamidino-2-phenylindole(DAPI, Sigma).

2.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

For qRT-PCR, total RNA was extracted using the RNeasy Mini Kit (Qiagen) and ~1 μg of total RNA was used for reverse-transcribed into complementary DNA (cDNA) with the Omniscript RT Kit (Qiagen). The primer sequences specific for the target gene and the internal control gene (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) used for qRT-PCR are listed in Table 1. Here the genes for