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Composite scaffolds of nano-hydroxyapatite and silk fibroin enhance mesenchymal stem cell-based bone regeneration via the interleukin 1 alpha autocrine/paracrine signaling loop

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ABSTRACT

Composite scaffolds of nano-hydroxyapatite (nHAp) and silk fibroin (SF) have been reported to promote bone regeneration mainly through signaling pathways associated with cell–biomaterial interaction. However, it is unclear whether soluble factors also play a role in osteoinduction with nHAp-SF. In this study, we confirmed the biocompatibility and superior osteoinductivity of nHAp-SF scaffolds versus SF scaffolds both *in vitro* and on a calvarial defect model *in vivo*. This was followed by further analysis with microarray assay. The cDNA microarray results identified 247 differentially expressed genes in bone marrow mesenchymal stem cells (BMSCs) cultured on SF-nHAp scaffolds versus SF scaffolds. The greatest disparity in gene expression levels were observed with Il1α and Ilr2. Real-time PCR assay validated the results. The addition of IL-1α into cultures of BMSCs with SF significantly increased both Bmp2 and Ilr2 expression. However, with BMSCs alone, the Il1r2 expression increased substantially, whereas Bmp2 expression exhibited a decrease rather than increase. These data suggested that nHAp may exert osteoinductive effects on BMSCs via the secretion of IL-1α in an autocrine/paracrine fashion, and IL-1α activity could be regulated through the synthesis of IL1R2 by BMSCs upon interaction with nHAp. These results complemented our understanding of the underlying mechanisms of biomaterial osteoinductivity.

1. Introduction

As one of the three major elements of regenerative medicine, biomaterial scaffolds play an important role in orchestrating the interaction of cells with soluble bioactive factors during reconstruction of bone tissues. Among various biomaterials, hydroxyapatite (HAp) has attracted much attention for large bone defects regeneration [1], due to its excellent osteoconductivity and similarity to the mineral phase and crystalline structure of bone. However, its low compressive strength limits its application to non/low-load bearing bone repairs [2]. With the advancement of nanotechnology, much progress has been made in fabricating functional scaffolds from natural polymers, which can be incorporated together with nano-HAp (nHAp) to achieve both controllable porosity and mechanical strength [3,4]. Silk fibroin (SF) is such a natural biopolymer with remarkable mechanical strength, controllable biodegradability, excellent biocompatibility, and ease of processing [5]. Various different techniques have been utilized for SF-nHAp fabrication [6–9]. Available data from the scientific literature suggest that SF-nHAp scaffolds can be an excellent source for bone regeneration [6,10,11]. However, the underlying mechanisms of osteoinduction by SF-nHAp are not completely understood.

It was believed that the major osteoinductive component of SF-nHAp scaffolds is nHAp [11]. To date, most theories about the osteoinductivity of HAp (including nHAp) focused on interaction

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of biomaterials with the surface molecules of osteo-progenitor cells i.e. integrin superfamily [12] and focal adhesion components [13]. These interactions subsequently trigger cytoskeletal rearrangement [13] and multiple intracellular signaling cascades, such as ERK/Sox9 [12], BMP/Smad [14], Wnt [15], TGF-β, MAPK, and Notch signaling pathways [16]. Lin and colleagues found that soluble factors produced by mesenchymal stem cells (MSCs) play an important role in osteoinduction with HAp scaffolds [17]. However, the authors did not elucidate the identity of these soluble factors. In this study, we attempted to rigorously investigate differential expression of genes involved in osteoinduction by rat bone marrow MSCs (BMSCs) cultured on SF-nHAp in comparison to SF, utilizing cDNA microarray technology. It is expected that this approach could lead to the identification of novel key regulators and mechanistic pathways of the osteoinduction process.

2. Materials and methods

For *in vivo* testing, twelve male/female Sprague–Dawley (SD) rats, two months old, were purchased and housed at the Center for Experimental Animals, Zhejiang University at constant room temperature (25 °C). All animal related experimental protocols have been approved by the institutional Animal Care and Use Committee of Zhejiang University. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Inc. (St. Loius, MO, USA), and all experimental data were collected in triplicates.

2.1. Preparation of SF scaffolds

SF solution at 60 mg/mL (6%, w/v) was prepared in a 100,000 ultra-clean room and was obtained as a gift from Zhejiang Xingyue Biotechnology Co. Ltd. (Hangzhou, China). Briefly, cocoons were cut and boiled for 1 hour (h) in an aqueous solution of 0.02 M Na_2CO_3, then boiled in water for 1 h and washed thrice with distilled water. The degummed silk fibroin was then dissolved in an aqueous solution of 9 M LiBr at 60 °C for 45 minutes (min) to yield a 20% (wt/v) solution. This solution was filtered through a 5 mL syringe filter, followed by dialysis (MWCO 3500 g/mol; Pierce, Woburn, MA) against milliQ water (18.2 MΩ) for 2 days (d) with 4 complete water changes in between. Finally, the purified SF solution was dialyzed against 10% (wt/v) polyethylene glycol (PEG; Mw: 10,000; Sigma-Aldrich) solution to obtain a concentration of 6% (wt/v). The salt leaching solution was prepared according to the method described by Kaplan and colleagues [18]. Briefly, a 10 mL syringe with a sealed needle-end was used as the mold and 8 holes with a diameter of 0.5–1.0 mm were made near the needle-end. A 3.5 g aliquot of granular NaCl (size 300–500 μL) was added slowly into the mold and 2.5 mL of silk solution was subsequently added in slowly. Then the syringe was pressed quickly to discharge the water completely. Finally, the syringes were placed at 4 °C for 36 h, room temperature for 6 h and 60 °C for 1 h, and the solid SF were then released into 2 L ddH_2O refreshed every 12 h for 48 h to remove the NaCl. Porous SF scaffolds can be obtained by freezing the samples at −70 °C and freeze-drying at −4 °C.

2.2. Fabrication of SF-nHAp composite scaffolds

SF-nHAp composite scaffolds were fabricated by a modified two-step mineralization process. Briefly, the process included three steps: (1) SF scaffolds were cut into pieces about 2 mm in thickness and 15 mm in diameter. (2) ten pieces of SF sheets were statically immersed into 50 mL of 0.1 M $CaCl_2$ ethanol solution for 10 min and then shaken in 50 mL water-free ethanol (the volume ratio between ethanol and SFs = 20:1) for 15 min at 150 rpm. (3) The SF sheets obtained from step 2 were directly immersed into 50 mL of 0.1 M K_2HPO_4 aqueous solution for 10 min and then shaken in ddH_2O (the volume ratio between water and SF = 20:1) for 15 min at 150 rpm. After repeating steps (2), (3) and (2) with fresh solution, the SF were placed into 10 L of fresh simulated body fluid (SBF) for 4 d at 37 °C statically. Finally, the scaffolds were thoroughly washed with ddH_2O for 24 h in a shaker at 250 rpm, and the water was changed every 8 h. The dry scaffolds can be obtained after freeze-drying at −4 °C for 48 h. The SBF was prepared as previously reported [19,20]. In brief, NaCl (8.035 g), $NaHCO_3$ (0.355 g), KCl (0.225 g), $K_2HPO_4 \cdot 3H_2O$ (0.231 g), $MgCl_2 \cdot 6H_2O$ (0.311 g), 1 M HCL (38 mL), $CaCl_2 \cdot 2H_2O$ (0.3675 g), $NaSO_4 \cdot 10H_2O$ (0.071 g), and $NH_2C(CH_2OH)_3$ (*Tris* buffer, 6.118 g) were dissolved in 1 L SBF solution. Millipore water was used as the solvent to dissolve the following chemicals, one at a time, and the pH was adjusted to 7.4 using 1 M HCl.

2.3. Structural and morphological characterization of scaffolds

X-ray power diffraction (XRD) data were recorded on a Japan Burker D8/advance X-ray diffractometer system with graphite mono-chromatized CuKα irradiation (λ = 0.15418 nm), together with a diffractometer scan step size of 2θ = 0.02°, and dwell time of 2 s/step, over a 2θ range of 10–70°. The structure of the scaffolds was analyzed directly by attenuated total reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy with a NICOLET AVA TAR370 Spectrometer (Thermo Scientific, FL, USA). For each measurement, 16 scans were made with a resolution of 1 cm^{-1}, with the wavelengths ranging from 450 to 4000 cm^{-1}. Scanning electron microscopy (SEM; Hitachi, S-4800) was used to characterize the morphology of the pure SF and the SF-HAp scaffolds at an accelerating voltage of 10 kV. SF samples were initially cut into sections approximately 1–1.5 mm in thickness and then immersed into milliQ water for 30 min. These were then frozen at −70 °C and freeze-dried at −4 °C for 48 h. Samples were coated with gold for 45 seconds by using a gold sputter coating equipment (Hitachi E-1010 ion sputter, JEOL, Japan). Images of cells on the surface of scaffolds were captured at magnifications of 50, 500 and 10k×.

2.4. Compressive modulus assay of scaffolds

The strength–strain curves and compressive modulus of the scaffolds were measured using a universal testing machine equipped with a 10 N maximum load cell (model 5543, Instron, Canton, MA). The scaffold samples were cut into cylinders of 4.5 mm in diameter and 4 mm in height, and were then completely immersed in 0.1 M PBS for 24 h. The protocol was referred to as Mikos's method [21], and was carried out at 0.5 mm/min crosshead speed with up to 30% strain. The compressive modulus of each scaffold was defined by the slope of the initial linear section of the stress–strain curve. The compressive strength was determined by drawing a line parallel to this, starting at 1% strain. The point at which this line crossed the stress–strain curve was defined as the compressive strength of the scaffold. Five samples of each scaffold group were analyzed and the results were presented as mean ± standard deviation (sd).

2.5. Rat BMSCs derivation and characterization

Rat bone marrow was explanted from the femur and BMSCs were isolated by rapid plastic adhesion of bone marrow-derived cells. The multi-potency of these cells was evaluated by tri-lineage differentiation. Approximate $1–2 \times 10^5$ cells were cultured in osteo-, chondro-, and adipo-inductive media respectively for 3 weeks. The protocols were the same as described in our previous publications [22,23]. Osteogenesis was confirmed by Alizarin Red staining, chondrogenesis was evaluated by Safranin O staining (SO), and adipogenesis was assayed by Oil red staining. The self-renewal capacity of BMSCs was evaluated by the Colony-Forming Units (CFU) assay. Approximately 100 cells were cultured in complete media in a 35 mm dish for 2 weeks. The surface molecular profile of BMSCs was analyzed by flow cytometry assay [6], with CD29 (561796, BD Pharmingen), CD44 (Cat# 550974, BD Pharmingen), CD45 (Cat# 561867, BD Pharmingen), CD90 (Cat# 561973, BD Pharmingen) and CD146 (Cat# Ab75769, Abcam) monoclonal antibodies.

2.6. Cell morphology and proliferation on scaffolds

The SF and SF-nHAp scaffolds were placed in 96-well plastic culture plates and incubated in Dulbecco's Modified Eagle Medium-Low Glucose (DMEM-LG) medium overnight. The medium was then removed and a suspension of 5×10^4 rat BMSCs in 50 μL of culture medium was placed on each scaffold. Three hours later, 150 μL of culture medium was added onto the cell-scaffold complexes. Cell viability and proliferation was determined after 1 d, 3 d, 5 d and 7 d of culture, utilizing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution and absorbance measurements at a wavelength of 490 nm. On 1 d and 7 d of culture, the scaffolds were fixed in 2.5% glutaraldehyde, and then dried by the CO_2 critical-point drying method for SEM imaging.

2.7. Assessment of the alkaline phosphatase activity of BMSCs

Osteogenic differentiation was assessed by measuring the alkaline phosphatase (ALP) activity of rat BMSCs grown on the SF and SF-nHAp scaffolds. Scaffolds of cylinder shape with a diameter of 12 mm were cut into pieces of 1.5–2.0 mm in thickness and transferred into 24-well plastic culture plates and a total of 2×10^5 BMSCs were seeded onto each scaffold. After 1 d of culture, the medium was changed to osteogenic differentiation medium, which was the same as the protocol in the Section 2.5 and changed every 3 d. On d 7 and 14, the samples were removed and ALP activity was measured using the BCIP/NBT ALP Color Development Kit (Beyotime Institute of Biotechnology, Shanghai, China) and AKP/ALP Determination Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.8. Transplantation into calvarial defects

The *in vivo* osteogenic potential of the scaffolds was evaluated in a calvarial defect model in SD rats. The SF and SF-nHAp scaffolds were cut into pieces of 5 mm × 5 mm × 1 mm for implantation. Under general anesthesia, the cranium was exposed through a medial incision. Bilateral full-thickness quadrate defects of 5 mm × 5 mm in symmetry to the sagittal suture were made by an electric dental drill. The left defect was implanted with a SF-nHAp scaffold, and the right defect was implanted with a SF scaffold. In four rats, defects on both sides were left untreated to serve as blank controls. The whole calvarias were harvested for evaluation after 8 and 16 weeks post-surgery.