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Repair of rabbit radial bone defects using true bone ceramics combined with BMP-2-related peptide and type I collagen

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ABSTRACT

An ideal bone graft material is the one characterized with good biocompatibility, biodegradation, osteoconductivity and osteoinductivity. In this study, a novel synthetic BMP-2-related peptide (designated P24) corresponding to residues of the knuckle epitope of BMP-2 was introduced into a biomimetic scaffold based on sintered bovine bone or true bone ceramics (TBC) and type I collagen (TBC/collagen I) using a simulated body fluid (SBF). Hydroxylapatite crystal mineralization with a Ca/P molar ratio of 1.63 was observed on the surface of P24/TBC/collagen I composite by scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDX) and X-ray diffraction (XRD) techniques. Cell adhesion rate evaluation of bone marrow stromal cells (BMSCs) seeded on materials *in vitro* showed that the percentage of cells attached to P24/TBC/collagen I composite was significantly higher than that of the TBC/collagen I composite. A 10 mm unilateral segmental bone defect was created in the radius of New Zealand white rabbits and randomly implanted with three groups of biomaterials (Group A: P24/TBC/collagen I composite; Group B: TBC/collagen I composite and Group C: TBC alone). Based on radiographic evaluation and histological examination, the implants of P24/TBC/collagen I composite significantly stimulated bone growth, thereby confirming the enhanced rate of bone healing compared with that of TBC/collagen I composite and TBC alone. It was concluded that BMP-2-related peptide P24 could induce nucleation of calcium phosphate crystals on the surface of TBC/collagen I composite. The TBC/collagen I composite loaded with the synthetic BMP-2-related peptide is a promising scaffold biomaterial for bone tissue engineering.

1. Introduction

Bone tissue engineering is a research area that combines two or more of the following: harvested cells, recombinant signaling molecules, and three-dimensional (3D) matrices. Various clinical applications in bone replacement may provide solutions for generating a new bone tissue with good functional and mechanical qualities, reducing the risks and expenses of using autografts, and allografts [1].

During the last decade, different biomaterials have been designed for new bone formation, such as bone-derived collagen, decalcified bone matrix (DBM), fibrin, calcium phosphate, hydroxyapatite–collagen composite, synthetic poly(glycolic-co-lactic acid) polymer, titanium and so on [2–5]. True bone ceramics (TBC) or sintered bovine bone is an osteoconductive bioactive bone substitute material which has excellent biocompatibility with hard tissues, and high osteoconductivity and bioactivity despite its low degradation rate, mechanical strength and osteoinductive potential [6,7]. It has neither antigenicity nor cytotoxicity [1,8,9]. The type I collagen is the main component of extracellular matrix of bone tissue that can serve as an excellent delivery system for bone morphogenetic proteins (BMPs) and help the migration and penetration of osteoblasts and vessels [10]. When collagen was combined with TBC particles, it can prevent TBC dispersion in implants, resulting in an easy model biomaterial, which showed promising application in bone tissue engineering [11].

BMPs are members of the transforming growth factor β superfamily which have various biological activities including the induction of cartilage and bone formation, organogenesis, cell differentiation, cell proliferation, apoptosis and so on [12,13]. BMP-2 has already been clinically applied to accelerate bone regeneration in both fracture healing and spinal fusion [14,15]. Research shows that BMP-2 has two epitopes referred to as the "wrist epitope" and the "knuckle epitope". The wrist epitope is considered to bind to BMP receptor IA and the knuckle epitope to BMP receptor type II [16–18]. Recombinant DNA technologies have simplified BMP production, the high dose requirement for therapies is not only costly, but also produces other effects such as bony overgrowth and immunological reactions [19]. To overcome its drawbacks, we designed and synthesized a short peptide (designated P24) according to the residues of the knuckle epitope of BMP-2 using the solid-phase synthesis method. Our previous study demonstrated that the P24 peptide could enhance the osteoblastic differentiation of bone marrow stromal cells [20–23].

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In this study, we combined the prepared TBC/collagen I composite with the BMP-2-related peptide to produce a newly biomimetic composite biomaterial (P24/TBC/collagen I composite) that combines osteoconductivity with osteoinductivity, and are postulating that P24/TBC/collagen I composite could prove to be a superior bone substitute material, both structurally and functionally, which could be used in the repair of bone defects.

2. Materials and methods

2.1. Main agents and instruments

Muffle furnace (Shanghai Tulin Meter Company, Shanghai, China); type I collagen (Sigma C9879, U.S.A.); vacuum drying oven (Shanghai Suopu Instrument Company, Shanghai, China); lyophilizer (Beijing Boyikang Instrument Company, Beijing, China); BMP-2-related peptide (designed and prepared by our group); scanning electron microscope (Quanta200, FEI Company, Holland); and fluorescence microscope (Leica DMLA, Germany).

2.2. Synthesis of BMP-2-related peptide

BMP-2-related peptide P24 (see Fig. 1) was derived from the "knuckle epitope" of BMP-2 and prepared by the FMOC/tBu solid-phase peptide synthesis (chemosynthesis). Crude peptide produced from this method was then preliminarily purified by gel filtration. The purity of the peptide was 96.8% as determined by high performance, liquid chromatography (HPLC; on a YMC PACK ODS-AM column and a Zorbax 300SB-C18 column, with a 10–60% acetonitrile gradient (25 min) in 0.1% trifluoroacetic acid (TFA)–water, at a flow rate of 1 ml min^{-1}, with detection at a wavelength of 220 nm). Finally, the peptide sequences were confirmed by an ESI-QTOF mass spectrometer (Micromass, Manchester, UK) using the first quadrupole as a broadband filter and a TOF detector. The m/z values of molecular $[M+nH]^{+n}$ ions produced from peptide components in the fractions analyzed were correlated to the theoretical masses of the peptide fragments to identify their origin and the cleavage site.

2.3. Preparation of true bone ceramics

Fresh cancellous bovine bone was cleaned and cut into strips. The material then was boiled and immersed in a 1% sodium hydroxide and 1% hydrogen peroxide for 1 h to remove the organic components. Sintering was accomplished in two steps. Initially, the bone was placed in a furnace, and the temperature was increased to 800 °C at a heating rate of 5 °C/min and maintained for 6 h, and then cooling with the furnace. The bone was then immersed in 0.09 mol/l sodium pyrophosphate and 70 °C water baths for 72 h. The material then was sintered and temperature was increased to 1200 °C for an additional 1 h. True bone ceramics were further sawed into the different cubes, and then were sterilized by 70% ethanol for 2 h and washed with PBS solution for three times (see Fig. 2).

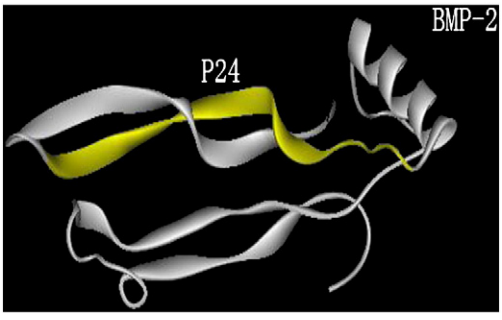

Fig. 1. BMP-2-related peptide P24: the subunit and P24 peptide were shown as grey and yellow colors respectively.

2.4. Preparation of composite materials

Collagen I was dissolved in a simulated body fluid (SBF containing ions at the following concentrations: 145.2 mM Na^+, 2.5 mM Ca^{2+}, 5.0 mM K^+, 1.5 mM Mg^{2+}, 152.0 mM Cl^-, 0.5 mM SO_4^{2-}, 4.2 mM HCO_3^-, 1.0 mM $H_2PO_4^-$, pH = 7.4, 37.0 °C.) and collagen/SBF proportion was 1 (w/v) %. Then 10 mg BMP-2-related peptide was dissolved in 1 ml collagen I solution. True bone ceramics was added to the collagen I solution with a ratio of 10:1. The solution was put in a vacuum drying oven for vacuum aspiration for 24 h, then frozen at −55 °C for 48 h, and sterilized. The TBC/collagen I was fabricated in a similar way except the addition of BMP-2-related peptide. All materials were sterilized by ethanol evaporation and frozen at −20 °C until use.

2.5. Scanning electron microscopy, energy dispersive X-ray spectroscopy and X-ray diffraction technique

Two groups of composites (P24/TBC/collagen I composite; TBC/collagen I composite) were randomly selected and coated with gold using a sputter coater. The samples were sputter coated with a layer of gold about 10 nm thick for SEM. Energy dispersive X-ray spectroscopy (EDX) (Cu was used as a standard to calibrate the equipment) was carried out with a sapphire detector (EDAX, Mahwah, NJ, USA) and X-ray diffraction (XRD) patterns (operating conditions (40 kV and 40 mA), 2θ range (20°–60°), step angle (0.017°) and step time (0.073 s)) of the materials were recorded in a Rigaku D/max-RB

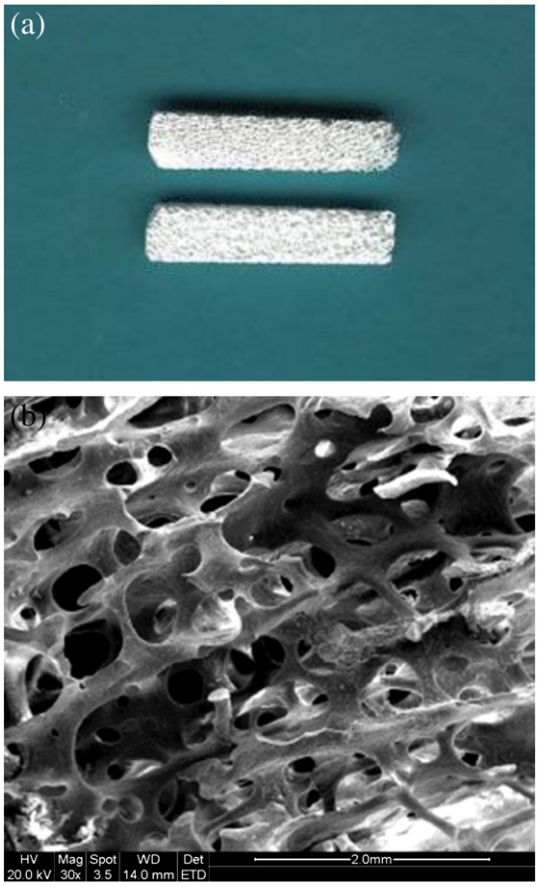

Fig. 2. (a) Macrostructure of TBC. (b) SEM image of TBC (magnification: 30×).