Bone 51 (2012) 704–713

Contents lists available at SciVerse ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Synthesis and inflammatory response of a novel silk fibroin scaffold containing BMP7 adenovirus for bone regeneration

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ARTICLE INFO

Article history:
Received 10 January 2012
Revised 15 June 2012
Accepted 18 June 2012
Available online 13 July 2012

Edited by: David Fyhrie

Keywords:
Biomedical applications
Biomimetics
Drug delivery
Medical applications
Bone healing

ABSTRACT

Gene therapy has garnished tremendous awareness for the repair of osseous defects. It exhibits high efficiency gene transfer and osteogenic differentiation potential making it well suitable for the sustained delivery of growth factors to local tissues. In the present study a simplified solution-based in situ biomimetic synthesis method is demonstrated for bone morphogenetic protein 7 (BMP7) adenovirus combined with silk fibroin scaffolds. This scaffold not only provides the three dimensional space for bone ingrowth, but also releases the BMP7 adenovirus which targets its secretion by host cells in vivo. Scaffolds were tested both in vitro for their osteogenic potential as well as in vivo in a critical-size calvarial defect in mice. Scaffolds loaded with bone morphogenetic protein 7 adenovirus (adBMP7) were able to sustain release of adBMP7 for up to 21 days and support cell proliferation and differentiation to bone forming osteoblasts. Calvarial defects treated with scaffolds containing adBMP7 significantly induced new bone formation in vivo. To demonstrate immuno-compatibility with host tissues, IL-2, IL-6 and TNF-α were measured up to 4 weeks post-implantation. Although these scaffolds demonstrated an initial pro-inflammatory response, levels of IL-2, IL-6 and TNF-α returned to baseline control values at either 2 or 4 weeks post-implantation demonstrating long term compatibility for growth factor delivery via gene therapy. The results from the present study indicate the promise of gene delivery scaffold systems for robust, low cost, and high quality bone tissue engineering applications.

1. Introduction

The delivery of osteogenic growth factors has garnished tremendous awareness for the repair of osseous defects. Several investigators have pursued the development of numerous ex vivo and in vivo strategies to improve bone regeneration [1–6]. Currently available recombinant growth factors approved for clinical use by the Food and Drug Administration (FDA) include human recombinant BMP-2 and BMP-7 [7]. Although findings from animal studies have been impressive, the effects may not necessarily translate directly to clinical trials [8–10]. The reasons remain unclear but may relate to growth factor delivery systems. In two clinical trials, large doses of BMP were required to induce adequate bone repair, suggesting that the mode of BMP delivery still requires further optimization [8,9].

Although addition of tissue-specific recombinant growth factors to scaffolds has generated positive outcomes [1–3], limitations in their biological activity remain problematic due to their poor in vivo stability [11,12]. Recombinant growth factors pose two major challenges: their half-life in vivo is transient (on the order of minutes to hours) and the protein chemistry of their active sites after implantation remains questionable. Thus, supplemental local growth factor production via gene transfer could be superior to bolus delivery methods [13,14]. Simply stated, gene therapy consists of the insertion of genes into an individual's cell either directly or indirectly with a matrix to promote a specific biological activity. Adenovirus vectors have several characteristics making them well suited for this type of gene therapy [15,16]. They exhibit extremely high transduction efficiencies and do not integrate into the host genome [17]. When adenovirus is delivered in vivo, host cells transfected will produce elevated protein expression, followed by the eventual loss of the adenovirus by cell attrition and cell turnover. Because these proteins are synthesized and released by the host cells, it creates an advantage over regular recombinant proteins resulting in improved protein activity and prolonged protein release until its eventual degradation [18–20].

Equally as important for bone tissue engineering is the fabrication of an osteoconductive scaffold. Simulated body fluid (SBF) suggested by Kokubo was a good simulation of the osteoproduction environment in osseous tissue and has proven an effective method to study the bioactivity of biomaterials and the mechanism of bone bonding [21]. SBF as an apatite source causes the spontaneous and in situ precipitation of calcium phosphates. In the present study, the fabrication

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doi:10.1016/j.bone.2012.06.029

of a simplified solution-based in situ biomimetic syntheses method was used carrying adenovirus BMP7 (adBMP7) combined with silk fibroin scaffolds. This scaffold not only provides the three dimensional space for cell and bone ingrowth, but also releases the BMP7 adenovirus which targets its secretion by host cells in vivo.

The aims and objectives of the present study were to test the efficiency of BMP-7 encoding adenoviruses impregnated within the silk scaffolds during the mineralization process in vitro. Furthermore, the ability for silk scaffolds to release adBMP7 over time and its subsequent release of BMP7 protein by bone marrow-derived stem cells (BMSCs) and participate towards their differentiation to osteoblasts was assessed in vitro. The osteo-inductivity and new bone formation of adBMP7 silk scaffolds were analyzed in a critical-sized skull defect mouse model, and the concentrations of IL-2, IL-6 and TNF-α were quantified simultaneously to demonstrate the immuno-compatibility of adBMP7 scaffolds in vivo. We hypothesized that by optimizing the delivery system for the release of adBMP7, the ability for a sustained release of local growth factor BMP7 would improve bone healing in vivo.

2. Materials and methods

2.1. Materials

All materials were purchased from Sigma Aldrich (Shanghai, China) unless otherwise stated. All other reagents were analytical grade. Silk fibroin protein was extracted from mulberry silk cocoons following a standard extraction procedure [22]. Recombinant adenoviruses were created using the Ad-Easy system (Stratagene, La Jolla, USA). Production of the recombinant viruses (Ad-BMP7) were performed as previously described [23], and viral titers were estimated by the tissue-culture infective dose (TCID50), which obtained 1.8×10^{11} PFU/ml. All cell culture media were supplied by Invitrogen (Grand Island, NY, USA).

2.2. Preparation of adenovirus combined scaffolds

Porous 3D silk fibroin scaffolds [24] were prepared by pouring regenerated viscous silk solutions (1–5 wt.%) into plastic Petri dishes, which were first cooled to 4 °C for 30 min and then immediately transferred to a freezer at −35 °C overnight to solidify the solvent and induce solid–liquid phase separation. The solidified mixture was maintained at −80 °C for 2 h and then transferred into a freeze drying vessel (OHRIST Beta 1–15, Germany) for 48 h until dry, after which the dried samples were treated with 70 vol.% ethanol for 12 h to induce crystallization and sterilization.

Following crystallization and sterilization, scaffolds were cut to 5×5×3 mm pieces. To each tube containing 1 mg scaffolds, 10 ml 3× SBF solution [25] and 2 ml adenovirus solution were added and placed into plastic dishes at 4 °C for 1 week. Silk fibroin scaffolds without adenovirus were prepared as control samples by placing the scaffold alone in 10 ml 3× SBF solution for 1 week. Control scaffolds were prepared in the same manner, and control/AdBMP7-containing scaffolds were standardized by the weight and volume.

2.3. Characterization of adenovirus combined scaffolds

The porous scaffold structures containing adenovirus were studied by scanning electron microscopy (SEM, QUANTA 200 Environmental SEM). Three independent samples containing human bone marrow stem cells (BMSCs) were cultured for 7 days on the scaffolds, observed by laser scanning confocal microscopy, then fixed with 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.3) for 3 h at room temperature, washed with PBS, and dehydrated in graded ethanol series. Samples were then critically point dried, coated with gold, and observed under SEM.

2.4. Release of viruses from scaffolds

Scaffolds (1 mg) were placed in 1.5 ml cryogenic vials containing 1 ml of sterile PBS (pH 7.4), and incubated at 37 °C in a shaking incubator for 21 days. At different time points (2, 4, 7, 14, 21 days), all media was removed and replaced by fresh PBS media. The number of released viruses was quantified by a plaque assay. Quantification of infectivity of the media removed from the different time point was performed by the end-point dilution method (TCID50) using 96-well plates and 293 cells.

2.5. Cell cultures

Human BMSCs were isolated and cultured as previously described [26]. One-milligram scaffolds containing adenovirus were incubated at 37 °C in complete growth media (DMEM plus serum) for 21 days. Media was collected at different time point to evaluate the infection efficiency on BMSCs. Transduction efficiencies were evaluated 48 h after cell infection by viruses and fluorescence emission of cells infected with adenovirus construct containing green fluorescence protein (Ad-GFP) by fluorescence microscopy (Leica® DM IRB inverted microscope).

To evaluate the release of protein in vitro, the scaffold was placed into 24-well plastic culture plates. One hundred-microliter cell suspensions containing 5×10^5 BMSCs were seeded into each scaffold. The BMP7 secreted into culture medium was determined using a commercial BMP7 ELISA kit (R&D Systems Inc, Minneapolis) according to the manufacturer's instructions at days 2, 4, 7, 14 and 21. At days 7 and 14, the cells were stained with Alizarin Red as previously described [27].

To evaluate cell proliferation on scaffolds in vitro, the scaffold was placed into 96-well plastic culture plates. Fifty-microliter cell suspensions containing 1×10^5 BMSCs were seeded into each scaffold. The cells were incubated for 1, 7 and 14 days, and then the medium was replaced with 100 μl PBS and 20 μl of CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA). Then the cell viability was determined by measuring the absorbance at 490 nm using a 550 BioRad plate-reader (Bio-Rad, Hertfordshire, UK) by MTS method.

To evaluate cell differentiation on scaffolds in vitro, the scaffold was placed into 24-well plastic culture plates, and a total of 10^6 BMP7 expressing MSCs was placed onto each scaffold. The medium was changed after 24 h to osteogenic differentiation medium(high glucose DMEM containing 10% FBS, 50 μM ascorbic acid 2-phosphate, 10 mM β-glycerol phosphate and 100 nM dexamethasone) which was changed every 3 days. On days 7 and 14, the samples were removed and total RNA was isolated using Tri-Reagent® (Sigma Aldrich) according to the manufacturer's protocol. Complementary DNA was synthesized from 1 μg of total RNA using SuperScript III (Invitrogen) following the manufacturer's protocol. RT-qPCR was performed in 25 μl reactions containing 12.5 μl SYBR green Master Mix (Applied Biosystems), 2.5 μl (10 μM) of each forward and reverse primer for each gene of interest for a final concentration of 20 pmol, 2.5 μl of cDNA template and RNA free water. Reactions were performed in triplicates to determine the expression of genes using primers for ALP (forward 5′ TCAGAAGCT CAACACCAACG 3′, reverse 5′ TTGTACGTCTTGGAGAGGGC 3′) and OCN (forward 5′ GCAAAGGTGCAGCCTTTGTG 3′, reverse 5′GGCTCCCAGCC ATTGATACAG 3′). The house keeping gene, 18S rRNA (forward 5′ TTCGGAACTGAGGCCATGAT 3′, reverse 5′ CGAACCTCCGACTTCGTTC 3′), was used as a control. The reaction was carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems), and the PCR amplification followed 1 cycle of 10 min at 95 °C, 40 cycles of 15 s at 95 and 60 °C for 1 min. Melting curve analysis was performed to validate specific amplicon amplification without genomic DNA contamination. Relative expression levels for each gene were normalized by the Ct value of the house keeping gene 18S rRNA and determined by using the △Ct method. The relative expression of each gene was