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In vitro and *in vivo* induction of bone formation based on *ex vivo* gene therapy using rat adipose-derived adult stem cells expressing BMP-7

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Background

Adipose-derived adult stem (ADAS) cells are multipotent cells capable of differentiating into osteoblasts, adipocytes and chondrocytes. The aim of this study was to determine whether BMP-7-expressing ADAS cells would elicit bone formation in vitro *and* in vivo.

Methods

ADAS cells were harvested from Lewis rats and transduced with adenovirus carrying the recombinant human bone morphogenetic protein-7 (Ad-BMP-7) gene. Untransduced cells and cells transduced with adenovirus carrying the enhanced green fluorescence protein (Ad-EGFP) gene served as controls. BMP-7 expression was assessed by RT-PCR, immunofluorescence on day 1, and Western blot on days 4, 8 and 12. Alkaline phosphatase (ALP) activity was assayed on days 2, 4, 6, 8, 10 and 12. Osteocalcin production and bone nodule formation were detected by immunohistochemistry and von Kossa stain on day 12. A total of 1×10^6 cells mixed with type I collagen were implanted into the subcutaneous pocket in Lewis rat and subjected to histologic analysis 1, 2 and 4 weeks post-implantation.

Results

The Ad-BMP-7-transduced ADAS cells expressed BMP-7 at both mRNA and protein levels. ALP activity was detected in Ad-BMP-7-transduced cells from day 2 to day 12, peaking on day 8. Osteocalcin production and matrix mineralization further confirmed that these cells differentiated into osteoblasts and induced bone formation in vitro. *Histologic examination revealed that implantation of BMP-7-expressing ADAS cells could induce new bone formation* in vivo.

Discussion

ADAS cells would be a promising source of adult autologous stem cells for BMP gene therapy and tissue engineering.

Keywords

Adipose-derived adult stem cells, BMP-7, differentiation, gene therapy.

Introduction

The repair of massive segmental bone defects and non-healing fractures remains a challenging problem in orthopedic surgery. Although conventional autologous bone or allograft bone grafting is currently available for treatment of these orthopedic disorders, early expectations of these methods have yet to be realized. Recent advances in cell and molecular biology have led to the development of a hybrid approach that combines bone tissue engineering with gene therapy to accelerate fracture healing and bridge segmental bone defects. In this alternative strategy, the core technique is the transfer of osteo-inductive genes to pluripotent stem cells followed by re-introduction of these genetically engineered cells into the body to serve as a platform for long-term delivery of osteo-inductive growth factors [1]. Bone morphogenetic proteins (BMP), especially BMP-2, -4 and -7, are strong osteo-inductive molecules that can drive the differentiation of pluripotent stem cells toward the osteoblastic lineage and stimulate new bone formation *in vitro* and *in vivo* [2–5]. Adenovirus vector is an appropriate candidate gene transfer system for the induction of bone regeneration because of its high

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transduction efficiency, low toxicity, ease of use and extrachromosomal life cycle [6]. However, the optimal cell type for adenovirus-mediated BMP gene therapy still needs to be determined.

Currently, there are two general types of pluripotent stem cell potentially useful for gene therapy and tissue engineering: embryonic stem cells and adult autologous stem cells. The embryonic stem cells are of limited practical use because of ethical considerations. In contrast, autologous stem cells are a more promising option because they are immunocompatible and there are fewer ethical issues to consider. To date, BM has been the major source of autologous stem cells. However, obtaining these cells necessitates a potentially painful BM biopsy under anesthesia and usually results in the harvesting of only a small number of adult stem cells that require further expansion *in vitro* to obtain a clinically significant number of cells [7]. Recent studies have indicated that adipose-derived adult stem (ADAS) cells are capable of differentiating along multiple mesenchymal cell lineages (osteoblasts, adipocytes, chondrocytes and myoblasts) [7–11]. Under osteogenic culture conditions, these cells can differentiate into osteoblasts [10]. Large numbers of autologous ADAS cells can easily be obtained from fat [8]. Hence, these cells are a promising substrate for the clinical application of bone tissue engineering. However, whether ADAS cells could be effectively used in an *ex vivo* system (requiring harvesting, manipulation and reimplantation) for osteo-inductive regional gene therapy requires further investigation.

In this study, we aimed to:

- determine whether ADAS cells could be successfully transduced with an adenoviral vector carrying the BMP-7 gene and expressing the BMP-7 protein
- observe whether these BMP-7-expressing cells could display the differentiated osteoblast phenotype *in vitro*
- investigate whether implantation of collagen-wrapped BMP-7-expressing cells could induce new bone formation *in vivo*.

Methods

Animals

Thirty-three 6-week-old Lewis rats, weighing about 250 g, were purchased from Beijing Animal Administration Center (Beijing, China). All animal experimental protocols were approved by the Animal Care and Use Committee of Peking University (Beijing, China) and were in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Academy Press (NIH Publication No. 85–23, revised 1996 [12]).

Isolation and culture of ADAS cells

Epididymal fat was obtained from 6-six-week-old normal Lewis rats. Finely minced fat was digested with 0.1% collagenase at 37°C with shaking at 200 r.p.m. for 40 min. The collagenase was then neutralized with an equal volume of culture medium containing 10% FBS (HyClone, Logan, UT, USA). The digested tissue was serially filtered through 250-μm and 100-μm nylon mesh to remove undigested debris, and centrifuged at 200 r.p.m. for 5 min. The supernatant containing mature adipocytes was discarded and the remaining cell pellet was incubated with 160 mm NH_4Cl at room temperature for 10 min to lyse erythrocytes. The cell suspension was centrifuged at 800 r.p.m. for 5 min and the cell pellet resuspended in DMEM (HyClone) with 10% FBS and 1% penicillin/streptomycin. The isolated ADAS cells were plated at a density of 5×10^5/ 25-cm^2 flask and cultured at 37°C in humidified 5% CO_2 with media changes every 3 days. The primary cells were defined as 'passage 0' and passaged at a ratio of 1:3 when they reached confluence.

Construction of recombinant adenovirus harboring human BMP-7 gene

The first generation (E1-, E3-) recombinant adenovirus carrying the human BMP-7 gene was constructed using the AdEasy-1 system [13] (kindly provided by Dr TC He, Hopkins Oncology Center, USA). Briefly, the full-length human BMP-7 cDNA (1.3 kb, cloned by RT-PCR from HEK293 cells using the following primers: forward, 5′-GTG GTA CCG ATG CAC GTG CGC TCA CTG-3′; reverse, 5′-AGA AGA TCT CTC GGA GGA GCT AGT GGC AG-3′) was inserted into the *Kpn*I and *Not*I restriction sites of the shuttle vector pAdTrack-CMV. The resultant plasmid was linearized by digestion with *Pme*I, and subsequently cotransformed into *E. coli* BJ5183 with an adenoviral backbone plasmid (pAdEasy-1). Recombinants were selected for kanamycin resistance and recombination was confirmed by digestion with *Pac*I and *EcoR*I. Finally, the linearized recombinant plasmid was transfected into 293A cells using the lipofectamine (Invitrogen, Carlsbad, CA, USA) method for adenovirus packaging. The primary recombinant adenovirus with