Bone regeneration by implantation of adipose-derived stromal cells expressing BMP-2

Huiwu Li a,b, Kerong Dai a,b,*, Tingting Tang a,b, Xiaoling Zhang b, Mengning Yan a, Jueren Lou c

a Department of Orthopaedic Surgery, Ninth People’s Hospital, Shanghai Jiaotong University School of Medicine, 639 Zhizaoju Road, Shanghai 200011, PR China
b Health and Science Center, SIBS CAS and SSMU, 225 South Chongqing Road, Shanghai 200025, PR China
c Shanghai Institute of Biological Products, 1262 West Road Yanan, Shanghai 200052, PR China

Received 25 February 2007
Available online 15 March 2007

Abstract

In this study, we reported that the adipose-derived stromal cells (ADSCs) genetically modified by bone morphogenetic protein 2 (BMP-2) healed critical-sized canine ulnar bone defects. First, the osteogenic and adipogenic differentiation potential of the ADSCs derived from canine adipose tissue were demonstrated. And then the cells were modified by the BMP-2 gene and the expression and bone-induction ability of BMP-2 were identified. Finally, the cells modified by BMP-2 gene were applied to a β-tricalcium phosphate (TCP) carrier and implanted into ulnar bone defects in the canine model. After 16 weeks, radiographic, histological, and histomorphometry analysis showed that ADSCs modified by BMP-2 gene produced a significant increase of newly formed bone area and healed or partly healed all of the bone defects. We conclude that ADSCs modified by the BMP-2 gene can enhance the repair of critical-sized bone defects in large animals.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Mesenchymal stem cells; Adipose tissue; Bone morphogenetic proteins; Gene therapy; Bone repair; Cell differentiation; Dogs; Ulna; Animal experimentation; Biocompatible materials

In combination with appropriate biomaterials and cytokine, autologous or syngenic mesenchymal stem cells have been shown to significantly enhance bone repair in animal fracture models [1–3]. Here, we report that canine adipose tissue contains mesenchymal stromal cells that have the potential to differentiate into bone and adipose tissue and can heal critically sized bone defects while transduced with adenovirus mediated BMP-2 gene.

Several cell populations used as gene delivery vehicles have been studied, such as bone marrow stromal cells (BMSCs), muscle-derived stem cells, and skin fibroblasts [4–6]. Compared with these cells, adipose-derived stromal cells (ADSCs) are easier to obtain, carry relatively lower donor site morbidity, and are available in large numbers. On the other hand, as mesenchymal stem cells, the ADSCs modified by BMP gene have the capacity to perform both paracrine and autocrine responses and not only secrete target protein but also participate in new-bone formation by differentiating into osteogenic cells [7,8].

Although the osteogenic potential of ADSCs has been demonstrated, in vivo experiments with human ADSCs have occurred exclusively through xenotransplantation into immunodeficient recipient animals and with small animal ADSCs have occurred through allotransplantation [5,9]. There is no report of autologous ADSCs for improving bone formation in large animals. The purpose of this study was to examine the osteogenic potential of canine ADSCs in an ulnar bone defect model.
large animal model such as the canine model and use of autologous ADSCs to treat bone defects have the advantage of more closely approximating human bone physiology and its clinical application in future.

Materials and methods

Isolation and culture of canine ADSCs. All animal experimental protocols were approved by the Animal Experiment Committee of Shanghai Jiaotong University School of Medicine. Adult beagle dogs were used for this study. They were anesthetized and the fat (4 × 3 × 1 cm³) was harvested from the back and extensively washed with phosphate-buffered saline (PBS). The fat was then excised, finely minced, and digested with 0.075% collagenase in a 37 °C water bath shaker at 150 rpm for 45 min. The collagenase was then neutralized with an equal volume of Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). Then, the cell suspension was centrifuged at 250g for 10 min. The supernatant was discarded, and the cell fraction containing ADSCs was resuspended in DMEM and filtered through a 150-μm nylon mesh to remove cellular debris and incubated for 48 h at 37 °C in 5% CO₂ in ordinary medium (DMEM, 10% FBS, and 1% antibiotic/antimycotic solution). Following incubation, the plates were washed extensively with PBS to remove residual nonadherent red blood cells. The collected cells were maintained at 37 °C/5% CO₂.

Osteogenic and adipogenic differentiation of ADSCs

Osteogenic differentiation. Osteogenic differentiation was induced by culturing ADSCs in osteogenic medium: DMEM containing 10% FBS, 10 μM dexamethasone, 10 μM 1, 25-(OH)₂-VitD₃, 50 μM ascorbate-2-phosphate, and 10 mM β-glycerophosphate. The media were changed every 3 days. Osteogenesis was assessed with alkaline phosphatase and von Kossa staining at 7, 14, 21, and 28 days after initial osteogenic induction. The protocols have been described by Zuk et al. [8].

Adipogenic differentiation. Adipogenic differentiation was induced by culturing ADSCs in adipogenic medium: DMEM containing 10% FBS, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin, and 0.5 mM isobutyl-methylxanthine. The differentiation was assessed by Oil Red O staining as an indicator of intracellular lipid accumulation at 7, 14, 21, 28 days after initial adipogenic induction [8].

BMP-2 gene transduction

BMP-2 expression in ADSCs. Adenovirus containing hBMP-2-cDNA (Ad-hBMP-2) and Lac-Z cDNA (Ad-Lac-Z) was constructed as described in previous reports [10]. The ADSCs were transduced by adenovirus overnight with a multiplicity of infection (MOI pfu/cell) of 150. The transduction efficiency of the ADSC cell population was evaluated using Lac-Z transduction and staining. On the fifth day after infection, immunoprecipitation/Western blot were performed to detect BMP-2 expression. The method has been described by Dai et al. [10].

Ectopic bone formation in nude mouse. To demonstrate biological activity of the BMP-2 secreted by ADSCs, the Ad-hBMP-2 transduced ADSCs were trypsinized, and 5 × 10⁴ cells were suspended in 100 μL phosphate-buffered saline. The cell suspensions were injected into the triceps surae muscle of nude mice using a gas-tight syringe (n = 5). Separate animals injected with the ADSCs transduced with Ad-Lac-Z or not transduced served as controls. After 4 weeks, the animals were sacrificed, radiographed, and the posterior hindlimb muscle dissection was dissected from the limb. The specimens were decalcified in HCl, processed, embedded in paraffin, and sectioned. Hematoxylin and eosin (H&E) staining was then performed.

Repair of canine ulnar bone defects with tissue-engineered ADSCs

Preparation of cell-TCP constructs. The pieces of porous β-tricalcium phosphate (TCP) ceramic granules (porosity of 60% and mean pore size of 400 μm, provided by Institut de Recherche sur les Biomateriaux et les Biotecnologies, Université du Littoral Côte d’Opale, Berck sur Mer, France) were 3 × 3 × 3 mm. They were sterilized by autoclaving which did not affect the TCP composition.

The ADSCs harvested from dogs’ adipose tissues were cultured in osteogenic medium and subcultured once they had become confluent. After 21 days, the cells were used to mix with TCP granules. Three days before mix, the ADSCs were transduced with Ad-hBMP-2. The ADSCs cultured in ordinary medium were used as a control. Before mix, the cells were detached and resuspended in the media at a cellular density of 2 × 10⁶/mL. The cell suspensions were slowly added to the TCP granules until the TCP granules were soaked in the suspensions completely. The cell-TCP constructs were maintained at 37 °C in 5% CO₂. After incubation for 1 h, 1 day, and 5 days, respectively, the cell-TCP constructs were consecutively rinsed two times with phosphate-buffered saline (PBS), fixed in 2% formalin for 2 h and broken into two halves. The broken surface was sprinkled with aurum powder and processed for scanning electron microscopy (SEM XL-30 Philips Holland) examination to evaluate cell adhesion and cell penetration. Cell-TCP constructs were co-cultured in vitro for a period of 7 days prior to surgery and were then implanted into the ulnar defects of the recipient dogs.

Surgical procedure. A bilateral-ulnar segmental defect model was used to evaluate bone healing in fourteen 12- to 15-kg adult beagle dogs. Before surgery, radiographs were obtained to verify skeletal maturity and lack of bone pathology. The animals were anesthetized. A lateral incision approximately 5 cm in length was made and extraperiosteal exposure of the ulna was obtained. A 2.5-cm segmental osteoporoietic defect was created in the midulna. A 2.5-mm Kirschner wire was inserted into the intramedullary canal for stability. The 24 defect sites were randomly divided into four groups and filled with granular TCP alone, granular TCP plus ADSCs, granular TCP plus osteogenic-induced ADSCs, or TCP plus transduced ADSCs (in this group, the osteogenic-induced ADSCs were transduced with Ad-hBMP-2), respectively. Four defect sites were left untreated. One day after surgery, all animals were fully able to walk and they were active and bearing weight. The experimental model has been described by Grundel [11].

Radiographic examination. After surgery, the position of the implants relative to the bone defects was monitored and bone healing progress was evaluated with X-rays taken at the anteroposterior and mediolateral planes on the first day and post-operatively at 4, 8, 12 and 16 weeks.

Deposition rate of new bone. To evaluate the deposition rate of new bone, tetracycline fluorescence bone labels (25 mg/kg) were administered through a venous route at 6 and 8 weeks. At 16 weeks, the dogs were sacrificed; the specimens were fixed in 10% formalin solution and embedded in methylmethacrylate. All tissues were cut lengthwise with a circular water-cooled diamond saw. Sections closest to the longitudinal mid-sagittal plane were selected. These sections were ground down to a thickness of 100 μm, polished, and observed under a fluorescent microscope for the tetracycline labeling. The mean tetracycline double labeling interval was determined by a Zeiss Axioscope Imaging system, and the interval was divided by administration time interval (that was 2 weeks), and the mean value showed the rate of new-bone deposition per day.

Histological and histomorphometric analysis. The sections observed under a fluorescent microscope for the tetracycline labeling were then surface-stained with Steven blue and van Gieson picro-fuchsin. The stained sections from each group were observed under microscope and scanned using a 3-CCD video camera that was linked to an image processing system (Zeiss Axioscope Imaging system). Residual TCP surface area (RSA) and newly formed bone surface area (BSA) were determined and converted to a percentage of bone defect total surface area (TSA).

Statistical method. For the new-bone deposition rate and histomorphometric analysis, a one-way analysis of variance (ANOVA) was used to compare means across groups. A multiple comparison procedure (Student–Newman–Keuls test) was used to determine the significance of differences among means. For all analyses, P < 0.05 level was used to indicate statistical significance.
Results

Osteogenic differentiation of ADSCs

Canine ADSCs placed in osteogenic medium exhibited changes in cell morphology after 7 days in culture. The cells changed from an elongated fibroblastic shape to a rounder shape. The cells were stained positively for ALP activity after 14 days of culture in osteogenic medium (Fig. 1A). In contrast, the cells cultured in ordinary medium did not show evidence of ALP expression (Fig. 1B). The calcified ECM were confirmed by von Kossa staining after 28 days of culture in osteogenic medium (Fig. 1C), whereas a few scattered black spots were observed in the control group (Fig. 1D).

Adipogenic differentiation of ADSCs

Canine ADSCs placed in adipogenic medium differentiated towards adipogenic lineage after 21 days post-induction (Fig. 1E and F). The Oil Red O-containing ADSCs cells exhibited an expanded morphology with the majority of the intracellular volume occupied by lipid droplets, consistent with the phenotype of mature adipocytes.

BMP-2 gene transduction and expression in ADSCs

The transduction efficiency of ADSCs using ad-Lac-Z averaged 90%. Western blot analysis of supernatants confirmed ADSCs were successfully transfected with the BMP-2 gene. The cells secreted BMP-2 protein, as shown by a 16 KDa band that corresponds to the size of BMP-2. No or little BMP-2 expression was detected in ad-Lac-Z transduced and nontransduced ADSCs groups (data not shown).

Ectopic bone formation in nude mouse

All mice survived until the day of sacrifice. Radiographic evidence of bone formation was evident at 4 weeks in all five mice after injection with Ad-hBMP-2 transduced ADSCs (Fig. 2A). H&E staining shows the presence of
Fig. 2. (A) X-ray demonstrated that Ad-hBMP-2 transduced ADSCs produced ectopic bone formation in hindlimb muscle of nude mice at four weeks. (B) H&E staining showed ectopic bone tissues lay mainly in the periphery of the injected cellular droplets, and only a few bone tissues lay in the center.

Fig. 3. Radiographic follow-ups. (A–C) TCP alone: (A) immediately post-operatively, (B) 8 weeks, and (C) 16 weeks. (D–F) TCP plus ADSCs: (D) immediately post-operatively, (E) 8 weeks, and (F) 16 weeks. (G–I) TCP plus osteogenic-induced ADSCs: (G) immediately post-operatively, (H) 8 weeks, and (I) 16 weeks. (J–L) TCP plus transduced ADSCs: (J) immediately post-operatively, (K) 8 weeks, and (L) 16 weeks.
new-bone tissue surrounded by myofibers. The bone tissue mainly lay in the periphery of the injected cell droplets, only few bone tissues in the center (Fig. 2B). No radiographic or microscopic evidence of ectopic bone formation was found for animals injected with Ad-Lac-Z transduced ADSCs and nontransduced ADSCs.

SEM examination of cell-TCP constructs

SEM showed that in the center of the TCP granule, the cells were in abundance and adhered to the surface of the TCP by 1 h. By day 1, the cells spread flat and fibroblast-like on the surface within an abundant surrounding matrix. By day 5, variform ADSCs filled the TCP apertures that were interconnected with each other (data not shown).

In vivo evaluation of cell-TCP constructs

All dogs survived until the day of sacrifice. All incisions healed uneventfully without infection.

Radiographic examination

The defects without implants never healed spontaneously. Osteogenesis spread slowly through the granular TCP in defects filled with TCP alone, confirming its osteoconductivity. However, osteogenesis never led to bone defect union. At 16 weeks, most of the materials had been resorbed, and the bone defects were evident on both views (Fig. 3A–C). Defects filled with TCP plus ADSCs were also radiolucent in all animals at 16 weeks, indicating massive TCP resorption and little bone apposition (Fig. 3D–F). All of the dogs grafted with TCP plus osteogenic-induced ADSCs had good early bone formation from both ends of the intact bone into the granules by 4 weeks. At eight weeks, there appeared noticeable mineralized callus formation, but a defect area was evident in the middle portion of the defect in three of the six ulnae and the radiolucent area persisted until 16 weeks. Another three ulnae appeared partly healed at 16 weeks (Fig. 3G–I). In the group with TCP plus transduced ADSCs, the early bone formation was more evident than in the other three groups. By 8 weeks, large areas of mineralized callus have formed. All of the six defects had at least one side healed cortex at 16 weeks (Fig. 3J–L). The situation of bone healing was showed in Table 1.

Rate of new-bone deposition

The tetracycline double labeling interval showed the calcification deposition of the four groups after 6 weeks. With TCP alone or with TCP plus ADSCs, the rate of calcification deposition was 4.88 ± 3.04 and 4.42 ± 2.26 μm/day, respectively. Compared with TCP plus osteogenic-induced ADSCs and TCP plus transduced ADSCs, there was no difference among the four groups (Table 2).

Histological and histomorphometric analysis

Histological examination showed defects left untreated were invaded by a dense, almost avascular, fibrous tissue after 16 weeks, indicating that scarring had occurred, rather than bone regeneration. Defects filled with TCP alone and TCP plus ADSCs displayed some small areas of woven bone at the proximal and distal ends of the cortices but showed entirely fibrous tissue in the middle. Defects filled with TCP plus osteogenic-induced ADSCs produced much larger areas of bone than those seen with TCP alone or TCP plus ADSCs. New-woven bone could be seen in intimate contact with the cut ends of the cortices but cortical continuity was never restored in three out of six ulnae. Defects filled with TCP plus transduced ADSCs showed substantial new-bone formation has occurred, leading to a continuous span of bone across the defect. The TCP had almost disappeared after 16 weeks in all groups. Only a few scattered fragments remained embedded in bone or fibrous tissue (Fig. 4A–D).

The bone regeneration ability of all implants was assessed by measuring newly formed bone surface areas by histomorphometry analysis. Adding ADSCs to TCP did not significantly increase new-bone area as compared with TCP alone. In contrast, TCP plus osteogenic-induced ADSCs and TCP plus transduced ADSCs produced a significant increase of newly formed bone area as compared with TCP alone or TCP plus ADSCs, and there was more new-bone formation in the TCP plus transduced ADSCs group than in the TCP plus osteogenic-induced ADSCs group. The difference was significant. The percentages of RSA to TSA in the four groups were less than 5%. All of

### Table 1

<table>
<thead>
<tr>
<th>Radiographic review</th>
<th>TCP alone</th>
<th>TCP plus ADSCs</th>
<th>TCP plus osteogenic-induced ADSCs</th>
<th>TCP plus transduced ADSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not healed</td>
<td>6/6</td>
<td>6/6</td>
<td>3/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Partly healed</td>
<td>0/6</td>
<td>0/6</td>
<td>3/6</td>
<td>4/6</td>
</tr>
<tr>
<td>One side cortex healed</td>
<td>0/6</td>
<td>0/6</td>
<td>2/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Two side cortices healed</td>
<td>0/6</td>
<td>0/6</td>
<td>1/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Three side cortices healed</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>2/6</td>
</tr>
<tr>
<td>Totally healed</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>2/6</td>
</tr>
</tbody>
</table>

### Table 2

The calcification deposition rate analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Deposition rate (μm/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP alone</td>
<td>4.88 ± 3.04</td>
</tr>
<tr>
<td>TCP plus ADSCs</td>
<td>4.42 ± 2.26</td>
</tr>
<tr>
<td>TCP plus osteogenic-induced ADSCs</td>
<td>6.47 ± 2.71</td>
</tr>
<tr>
<td>TCP plus transduced ADSCs</td>
<td>5.45 ± 2.98</td>
</tr>
</tbody>
</table>

* Values are means ± SD.
the data confirmed the radiological and histological results (Table 3).

Discussion

This is the first study of adipose-derived stromal cells harvested from canine. The study demonstrates that adipose tissue from the canine back contains a cell population which can differentiate into both osteoblasts and adipocytes in appropriate medium and is permissive to adenovirus infection. The autologous ADSCs modified by the BMP-2 gene in a TCP carrier can repair critical-sized ulnar bone defects in the canine.

Granular rather than block TCP has been used in the experiment because: (1) in vitro, when the TCP were soaked into the cell supernatant, the cells only penetrated 2–3 mm deep (unpublished data). Therefore, the cells are able to penetrate into the center of the granular TCP (confirmed by SEM observation) much easier than into block TCP; (2) in vivo, the bone penetration into the granules was generally better than that into the block form. In a block, bone invasion is limited by void size and geometry, and new capillaries must grow down channels to initiate bone formation. Far less space relative to the amount of material is available in the block form for neovascularization. In the granular form, capillaries can grow easily throughout the open spaces between the granules and maintain the viability of BMP-2 producing ADSCs; and (3) the ulna is not the major weight-bearing bone, making it more amenable to use of granular TCP.

It is notable that the ADSCs without induction or modification cannot heal critical-sized bone defects in the canine. The result is similar to that reported by Peterson [9]. In their study, the ADSCs alone could not heal the critical-sized femoral bone defect in nude rat. The histomorphometry analysis in our study showed the amount of new-bone formation caused by ADSCs plus TCP is as

<table>
<thead>
<tr>
<th>Group</th>
<th>BSA/TSA (%)</th>
<th>RSA/TSA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP alone</td>
<td>10.95 ± 3.10</td>
<td>2.28 ± 0.68</td>
</tr>
<tr>
<td>TCP plus ADSCs</td>
<td>12.86 ± 6.0</td>
<td>3.83 ± 2.23</td>
</tr>
<tr>
<td>TCP plus osteogenic-induced ADSCs</td>
<td>25.38 ± 8.24*</td>
<td>2.79 ± 1.06</td>
</tr>
<tr>
<td>TCP plus transduced ADSCs</td>
<td>39.95 ± 8.55**</td>
<td>0.99 ± 0.46</td>
</tr>
</tbody>
</table>

BSA: newly formed bone surface area.
RSA: residual TCP surface area.
TSA: total surface area of bone defect.
* P < 0.05 compared with TCP alone and TCP plus ADSCs.
** P < 0.01 compared with TCP alone and TCP plus ADSCs.

---

Fig. 4. Steven blue and van Gieson staining. (A) With TCP alone, defects displayed some small areas of woven bone at the proximal and distal ends but entirely fibrous tissue in the middle. (B) With TCP plus ADSCs, the bone formation situation was similar to that with TCP alone. (C) With TCP plus osteogenic-induced ADSCs, defects produced large areas of bone at the cut end of cortices. The residual TCP appeared black and new-bone tissue presented within the pores or encompassed the TCP fragments. (D) In the TCP plus transduced ADSCs group, defects showed substantial new lamellar and woven bone formation. Cortical continuity was achieved between the edges of the defects.
much as that caused by TCP alone. The results indicate that the ADSC itself does not have the osteogenic potential. In contrast, Petite et al. reported that the BMSCs alone can enhance new-bone formation in repair of sheep critical-sized metatarsal bone defect [12]. The osteogenic difference between ADSCs and BMSCs may be due to several factors: (1) the BMSCs may have osteogenic capacity in vivo not possessed by the ADSCs; or (2) the BMSCs taken from bone marrow in their study may have been heterogeneous, blended with osteoblast or osteoprogenitor cells, and the cell blends may then have enhanced the bone formation. Comparative studies of monoclonal ADSCs and BMSCs will be helpful.

It is also important to note that although the osteogenic-induced ADSCs caused new-bone formation, the amount of new bone is apparently less than that caused by BMP-2 gene modified ADSCs. The bone defects still remained in three out of six cases. The results may be due to: (1) the osteogenic capacity of osteogenic-induced ADSCs being limited and not enough to heal a critical-sized bone defect; (2) the degeneration rate of TCP possibly being faster than that of new-bone formation caused by osteogenic-induced ADSCs. The fast degeneration of TCP may have impeded bone defect healing; or (3) as the construction of tissue-engineered bone is complicated; the conditions of construction in our study may not be the most favorable for new-bone formation.

Our findings demonstrate that ADSCs transduced with BMP-2 gene in a TCP carrier can elicit true bone regeneration with the approximate disappearance of the biomaterial and formation of cortical bone in a bone defect. Thus, this study may provide insight into the clinical repair of long bone defect with gene modified ADSCs.

Acknowledgments

The research was supported by the National Natural Science Foundation of China (30571879), Ph.D. Programs Foundation of Ministry of Education of China, and Shanghai Science and Technology Committee Major Program.

References