Nano-hydroxyapatite/collagen composite co-cultured with bone marrow-derived mesenchymal stem cells for repair of bone defects

Zhao Ji-dong, Qian Han-guang, Miao Zong-ning, Zhu Jian-zhong, Peng Wei

Abstract

BACKGROUND: There have been no effective means for clinical treatment of large regions of bone defects. Nano-hydroxyapatite/collagen (nHAC) composite would provide a new pathway for repair of bone defects owing to its similar structure to natural skeleton and better biocompatibility.

OBJECTIVE: To investigate the role of nHAC composite co-cultured with bone marrow-derived mesenchymal stem cells (BMSCs in repair of bone defects.

METHODS: Following isolation and culture, human BMSCs were co-cultured with nHAC composite. Gross observation, histological analysis, and electron microscope observation were performed to analyze osteogenesis for repair of bone defects in the clinic.

RESULTS AND CONCLUSION: Human nHAC could greatly proliferate *in vitro*. X-ray photography revealed that bone defects well healed after implantation of nHAC/BMSCs composite. These findings indicate that BMSCs exhibit osteogenic potential and nHAC is a satisfactory scaffold material for construction of tissue-engineered bone.

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INTRODUCTION

Bone grafting is a common mean for treatment of bone defects. Autologous bone grafting has become a gold standard of treatment of bone defects owing to its good immunological compatibility, but it is confined by some problems such as secondary surgery and donor numbers. There have been some problems hardly solved in the clinical allogenic and xenogeneic bone transplantation, such as transmission of pathogen, immunogenicity, and high infection rate[1-2]. Nano-hydroxyapatite/collagen (nHAC) composite resembles natural skeleton in structure and shows some features including good biocompatibility, rapid and good absorption, and osteoinduction[3-4]. Bone marrow-derived mesenchymal stem cells (BMSCs) are a kind of stem cells that have strong osteoinductive potential, are easily *in vitro* isolated and cultured, and rapidly proliferate[5-8].

The present study co-cultured nHAC composite and BMSCs *in vitro*, implanted them into the region of bone defects and observed osteogenesis by X-ray photography.

SUBJECTS AND METHODS

Design

A retrospective case analysis.

Time and setting

This study was performed at Department of Reparative and Reconstructive Surgery and Cell Laboratory, Wuxi Third People's Hospital, China between February 2006 and June 2009.

Subjects

A total of ten patients who suffered from bone defects or bone graft fusion and received bone grafting by tissue engineering in the Wuxi Third People's Hospital between February 2006 and June 2009 were included in this study.

Inclusion criteria: patients with bone defects or bone graft fusion; receiving treatment of tissue-engineered bone; without other related diseases; providing written informed consent and permitting follow-ups.

Ten patients consisted of eight males and two females and averaged 27.7 (range, 18–56) years of age. Among these patients, osteocystic lesion was found in two patients, obsolete nonunion fractures complicated by bone defects in six patients, fresh comminuted fracture of metacarpal bone in one patient and of phalange in one patient. Bone grafting regions included humerus (two patients), femur (two patients), tibia (three patients), radius (one patient), metacarpal bone (one patient), and phalange (one patient). All operations were approved by the Medical Ethics Committee, Wuxi Third People's Hospital and written informed consent of surgical methods was obtained from each patient.

Materials and equipments

Reagent/instrument	Source
DMEM-LG medium, fetal bovine serum, trypsin	Gibco, New York, NY, USA
Fluorescence-labeled mouse anti-human CD29-PE, CD34-FITC, CD44-PE, CD45-PE, HLA-dr-PE antibody	Ancell, Bayport, MN, USA
Percoll separation media	Amersham Pharmacia, Cambridge, England
EPICS XL-3 flow cytometer	BD Becton Dickinson, San Jose, CA, USA
XL220 scanning electron microscope	Philips, Amsterdam, the Netherlands

Methods

Construction of tissue-engineered bone

According to previous reports[9-11], under sterile condition, ilial bone marrow was taken from a 29-year-old healthy male adult volunteer. A total of 5 mL heparinized bone marrow was mixed with the same amount of phosphate buffered saline (PBS), triturated with a pipette, and centrifuged at 1 800 r/min at room temperature for 20 minutes. After supernatant removal and PBS addition, the resultant product was re-suspended. Following cell concentration adjustment at 10^{11}/L, 6 mL cell suspension was slowly added into a 15 mL centrifuge tube in which 4 mL Percoll separation medium (1.073 g/mL) was pre-added, followed by a 20-minute centrifugation at 1 800 r/min. The interface cells were collected, re-suspended using DMEM supplemented with 10% fetal bovine serum, and then inoculated into a culture flask at 10^5/cm^2. Subsequently, cells were incubated at 37 ℃ under 5%CO_2 and saturated humidity and passaged at 1: 3 using 0.25% trypsin at 80% confluency.

Identification of BMSCs

According to previous reports[12-14], passage 2 cells that survived well were washed three times using D-Hank's solution, digested with 2.5 g/L trypsin, and concentration adjusted to 5×10^9/L using PBS supplemented with 1% fetal bovine serum. A total of 50 μL cell suspension was incubated at 4 ℃ for 30 minutes after addition of mouse anti-human CD34-FITC, CD45-PE, CD29-PE, CD44-PE, CD11b-PE monoclonal antibodies, washed with PBS to discard non-binding antibodies, and fixed with 10 g/L paraformaldehyde. Finally, cell surface antigen expression was detected through the use of EPICS XL-3 flow cytometer.

Preparation of scaffold materials

Scaffold materials were nHAC composite[3] provided by Department of Materials, Tsinghua University, China. The nHAC showed the feature of microstructure of natural skeleton and was made of 57% hydroxyapatite, 30% collagen, and 13% polylactic acid. As required, the nHAC composite was chopped into small blocks with a size of 6 mm× 6 mm× 4 mm, sterilized under ^{60}Co, and infiltrated into DMEM before use.

Co-culture of nHAC and BMSCs

In accordance with previous reports[15-17], passages 2–3 cells were collected and adjusted to 10^9/L. nHAC composite was added to a 24-well plate in which 100 μL medium was pre-added. Then 100 μL cell suspension was added to the 24-well plate. Following a 2-hour incubation at 37 ℃ under 5% CO_2 and saturated humidity, the 24-well plate was overturned and additional 100 μL cell suspension was added, followed by a 2-hour incubation at 37 ℃ under 5% CO_2 and saturated humidity, serving as experimental group. In the blank control group, cell culture was performed in the common culture flask under the same culture condition as experimental group.

Preparation of scanning electron microscope samples

Following previous reports[18-19], co-cultured samples were fixed with 2.5% glutaraldehyde (prepared with 0.1 mol/L PBS, pH 7.4), post-fixed with 1% osmium tetroxide, dehydrated in a gradual series of ethanol, and critical point dried with CO_2 or treated with a gradual series of methyl cyanide. In the vacuum drying apparatus, methyl cyanide was abstracted through the use of water pump. Following gold spraying, samples were observed under scanning electron microscope.

Clinical research

Two patients with fresh comminuted fracture received surgery at 14 days after fracture, and eight patients with obsolete fracture received surgery at 6–18 months. For osteocystic lesion patients, tissue-engineered bone was implanted immediately after focus removal.

Surgical methods

For fresh comminuted fracture of metacarpal bone and phalange complicated by a part bone defects, tissue-engineered bone was implanted into bone defect region followed by steel plate fixation 2 weeks after admission. For obsolete nonunion fracture, three-dimensional CT reconstruction was performed prior to surgery to determine bone defect region and gross shape for preparation of pre-shaped tissue-engineered bone; during surgery, following clearing of fractured stumps, tunneling of medullary cavity, and steel plate fixation, pre-shaped tissue-engineered bone was inserted into the bone defect region and fixed with absorbable thread. For osteocystic lesion, focus was thoroughly erased and bone defects region was filled with tissue-engineered bone particles after repeated washes. Following bone grafting, wounds were washed with antibiotic-containing rinse solution and sutured layer by layer. Negative pressure drainage was performed for 24–48 hours.

Postoperative management

Routine nursing was necessary. Antibiotics (cephazolin 2.0 + 500 mL physiological saline) were intravenously administered, twice a day, for a total of 3–5 days. Plaster external fixation or functional bracing was used. In addition, routine functional exercise was indispensable.

Main outcome measures

BMSCs surface antigen expression was detected by flow cytometer; cells growth on nHAC was observed under scanning electron microscope, and repair of bone defects was assessed through the use of X-ray photography.

Design, enforcement and evaluation

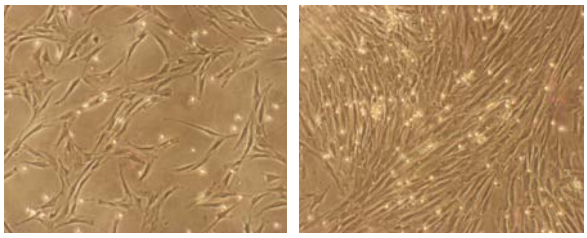
Zhao Ji-dong designed this study. Zhao Ji-dong, Qian Han-guang, Miao Zong-ning, and Peng Wei performed this study. Zhao Ji-dong and Zhu Jian-zhong evaluated experimental data. All authors received professional training. Blind method evaluation was utilized.

RESULTS

Isolation, culture, and identification of BMSCs

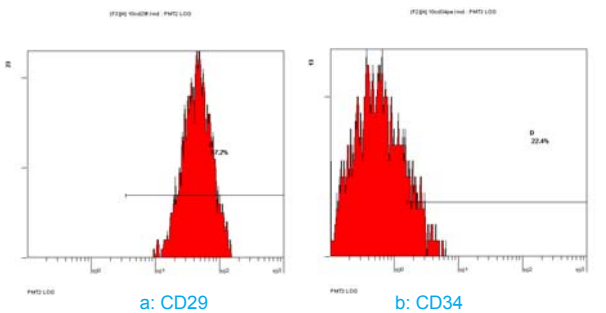
Following 1.037 g/mL Percoll centrifugation step, mononuclear and nucleated cells could be easily separated from bone marrow. After 4 hours of primary culture, adherent cells could be observed. Forty-eight hours later, medium was replaced to

discard dead cells and other suspending cells. Adherent cells exhibited clone-like growth pattern and presented a scale-shaped morphology with a large cell body as well as a nucleus located in the center. Generally it needed around 1 week for cells to cover the bottom of the culture flask and even only 3–5 days after passage (Figure 1). Flow cytometry results showed that the cells cultured were negative for CD34, CD45, HLA-DR and positive for CD29, CD4, CD105 (Figure 2).

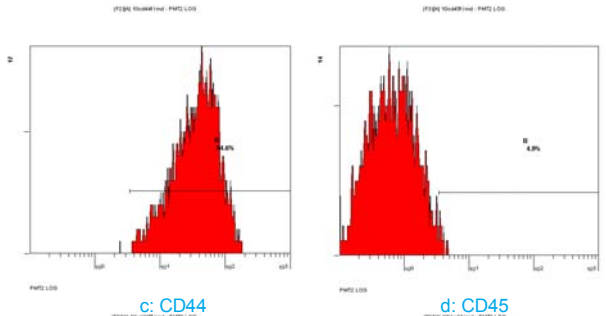


a: Primary culture for 7 d b: Subculture for 3 d

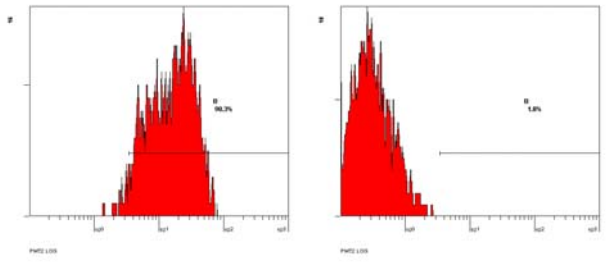
Figure 1 Primary culture and subculture of human bone marrow-derived mesenchymal stem cells (×40)



a: CD29 b: CD34



c: CD44 d: CD45



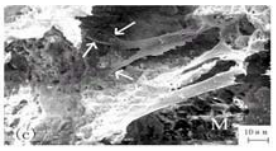
e: CD105 f: HLA-dr

Figure 2 Flow cytometry analysis of human bone marrow-derived mesenchymal stem cells

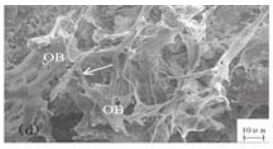
At 2 days after surgery, cells attached to porous nHAC material, and at 5 days, they completely spread over this material (Figure 3b). Thereafter, long pseudopodia of cells in the nHAC pores clenched the surface of materials, and cells reached multilayer status at 1 week, and fiber bundles interlaced between cells (Figure 3d). While at this time, there was still a monolayer of cells in the blank control group (Figure 3a).

a: Blank control group, 1 wk after surgery (bar: 60 μm)

b: Experimental group, 5 d after surgery (bar: 10 μm)



c: Experimental group, 6 d after surgery (bar: 10 μm)



d: Experimental group, 1 wk after surgery (bar: 10 μm)

Figure 3 Microstructure of nano-hydroxyapatite/collagen/bone marrow-derived mesenchymal stem cell composite

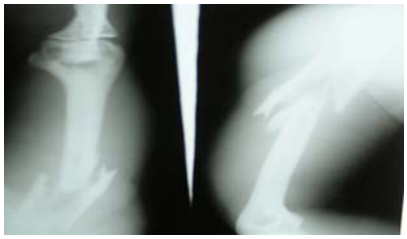
Clinical effects

The amount of postoperative drainage was 10–230 mL. All wounds healed by first intention. For fresh fracture and osteocystic lesion patients, bone union occurred at 3 months. The healing time for bone nonunion complicated by bone defects was 2.5–6 months. Joint function of affected limbs well recovered in fresh fracture and osteocystic lesion patients[20]. Of three patients followed up for over 12 months, one had removed internal fixation and resumed normal life.

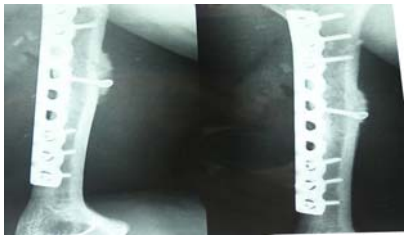
Illustrative case

A 27-year-old male patient with trauma-caused open, comminuted fracture of middle segment of the right humerus (Figure 4a) was admitted on November 23rd, 2003. After admission, 20 mL ilial bone marrow was taken from the patient for preparation of tissue-engineered bone. Open reduction and internal fixation of fracture was performed when open wound healed, *i.e.*, at 16 days after surgery. During surgery, 10-pore LC-DCP bracing was used. Bone defect region was filled with prefabricated 1/4 tube-shaped tissue-engineered bone, and binding fixation of steel wire was necessary. After surgery, under the protection of bracing, functional exercise of shoulder

and elbow joints should be performed and X-ray films were taken periodically for observation of fracture healing (Figure 4b). After surgery, shoulder and elbow functions recovered to normal.



a: Prior to surgery



b: 6 wks after surgery

Figure 4 Repair effects of nano-hydroxyapatite/collagen/bone marrow-derived mesenchymal stem cell composite on bone defects

DISCUSSION

To date, there have been no effective means to treat large regions of bone defects. Bone tissue engineering brings new hope to solve this problem. Bone tissue engineering is an approach that uses proper materials as the scaffold for cell growing, *in vitro* co-cultures materials and cells for a period of time to prepare tissue-engineered bone, and finally implants the tissue-engineered bone into the body for repair of defects[21-22]. nHAC/PLA composite prepared based on the concept of bionics mimics the component and structure of natural skeleton and can be used as scaffold material for bone tissue engineering. Seed cells are the source of osteoblasts and BMSCs are the best[23]. Among adult stem cells, BMSCs are a kind of multipotent stem cells that can be isolated from bone marrow and can be *in vivo* and *in vitro* induce-differentiated into various mesenchymal tissue cells, involving bone, cartilage, tendon, fat and bone marrow stroma[24].

Bone marrow stromal cells have been found to be a kind of mesenchymal stem cells that exhibit not only the feature of stem cells, *i.e.*, self-renewal and multi-lineage differentiation potentials, but also plasticity, *i.e.*, under certain condition, specified cells can differentiate and dedifferentiate towards various directions[25]. Tissue-engineered bone requires a considerable number of functional cells inoculated onto scaffold material, generally 10^6–10^7 order of magnitude[26]. BMSCs easily survive and proliferate within a short period of time, which meet the requirement of tissue-engineered bone. A previous study demonstrated that integrin family members CD29, adhesion molecule CD44 and CD105 are the important markers of BMSCs[27]. BMSCs do not express hematopoietic cell surface antigens, such as hematopoietic progenitor cell antigen CD34, leukocyte common antigen CD45, and leukocyte activation antigen CD11b. Results from this study were in accordance with above findings, indicating that cells cultured are mesenchymal stem cells. Results from this study suggest that BMSCs are a kind of satisfactory functional cells for repair of bone defects owing to its great amplification, easy harvesting, and no immunological rejection in autologous application.

Substrate materials are the basic framework for cell attachment and the place for cell metabolism, and their morphology and function directly influence the morphology and function of constitutive tissue. The topological structure of polymer scaffold surface yields important influences on cell morphology, adhesion, spreading, directed-growth, and biological activity[28-30]. Ripamonti *et al*[31] reported that an ideal substrate material for bone tissue engineering should correspond to the following requirements: (1) enable rapid invasion of vessels and mesenchymal stem cells and help to contact with inducing factors adhering on material; (2) can be gradually degraded and absorbed and finally disappear from implantation region; (3) without immunological rejection after implantation *in vivo*; (4) easy to trim its outline to match defects of different shapes; (5) able to provide mechanical support if necessary; (6) able to produce best osteogenesis at the lowest dose of inducing factors.

nHAC material used in this study shows similar structure to natural skeleton and benefits cell adhesion, growth, collagen secretion, and calcification. The nano-sized crystal on nHAC surface is easily phagocytized and degraded by cells, which meet the requirement that new bone formation matches to material reduction in speed and enables normal reconstruction of natural bone. Scanning electron microscope results demonstrated that nHAC possesses large pore diameter and high porosity which favor cell proliferation. Immonological rejection has not been found in the clinical application. nHAC material used in this study can be made into proper three-dimensional shapes according to defects shape provided by preoperative three-dimensional reconstruction, which shortens intraoperative re-shaping time and operation time, and reduces hemorrhage volume. The mechanical strength of nHAC meets the requirement of bone grafting. So nHAC is an ideal bone substitute material.

In the process of *in vitro* construction of tissue-engineered bone, functional cells and substrate materials are always the research hotspots. Nano-sized bioactive material used in this study is a developing direction. BMSCs as functional cells show a wide range of application prospects. Clinical application of tissue-engineered bone is in its infancy and cannot completely meet various clinical requirements. Clinical patients have great individual difference, show complicated disease conditions, and suffer from various degrees of bone defects. Through the cooperation of multidisciplinary specialists, bone tissue engineering will acquire more achievements and better serve human being.

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纳米晶羟基磷灰石胶原复合骨髓间充质干细胞修复骨缺损

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摘要

背景：临床上对大范围骨缺损还没有很有效的治疗手段，而纳米晶羟基磷灰石胶原复合材料与天然骨骼的结构类似，具有较好的生物相容性，可能为修复骨缺损提供新的途径。

目的：观察纳米晶羟基磷灰石胶原材料复合骨髓间充质干细胞在修复骨缺损中的作用。

方法：分离培养人骨髓间充质干细胞，与纳米晶羟基磷灰石胶原材料于体外联合培养；通过大体观察、组织学分析及电镜观察了解成骨情况，进一步临床应用于修复骨缺损。

结果与结论：人骨髓间充质干细胞在体外可以大量扩增，复合细胞的材料植入骨缺损处后，X 射线摄片动态观察可见骨缺损处连接良好。说明骨髓间充质干细胞具有成骨细胞作用，纳米晶羟基磷灰石胶原材料是一种很好的构建组织工程骨的支架材料。

关键词：骨髓间充质干细胞；羟基磷灰石；纳米材料；组织工程；骨

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