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The synergistic effects of microfracture, perforated decalcified cortical bone matrix and adenovirus-bone morphogenetic protein-4 in cartilage defect repair

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ABSTRACT

We reported a technique for articular cartilage repair, consisting of microfracture, a biomaterial scaffold of perforated decalcified cortical bone matrix (DCBM) and adenovirus-bone morphogenetic protein-4 (Ad-BMP4) gene therapy. In the present study, we evaluated its effects on the quality and quantity for induction of articular cartilage regeneration. Full-thickness defects were created in the articular cartilage of the trochlear groove of rabbits. Four groups were assigned: Ad-BMP4/perforated DCBM composite (group I); perforated DCBM alone without Ad-BMP4 (group II); DCBM without perforated (group III) and microfracture alone (group IV). Animals were sacrificed 6, 12 and 24 weeks postoperation. The harvested tissues were analyzed by magnetic resonance image, scanning electron microscope, histological examination and immunohistochemistry. Group I showed vigorous and rapid repair leading to regeneration of hyaline articular cartilage at 6 weeks and to complete repair of articular cartilage and subchondral bone at 12 weeks. Groups II and III completely repaired the defect with hyaline cartilage at 24 weeks, but group II was more rapid than group III in the regeneration of repair tissue. In group IV the defects were concave and filled with fibrous tissue at 24 weeks. These findings demonstrated that this composite biotechnology can rapidly repair large areas of cartilage defect with regeneration of native hyaline articular cartilage.

1. Introduction

Damage to articular cartilage frequently cannot be repaired owing to its avascular nature which results in low intrinsic healing capacity. The destruction of articular cartilage leads eventually to secondary degenerative disease of the involved joint. Recently researchers have used culture-expanded autologous chondrocytes bonded with a scaffold to repair cartilage defects [1,2]. However, the results were either unsatisfactory or, even if satisfactory, the operation was too complicated for clinical application. Multiple surgical procedures have been developed to treat articular cartilage injuries, including abrasion arthroplasty [3,4], microfracturing [5] and osteochondral drilling [6], which achieved partially successful repair that led to fibrocartilage formation. Those surgical interventions are based on the theory of utilizing autogeneic marrow (stem cell niche) from the subchondral medullary cavity to repair the cartilage defect. However, those surgical interventions often cause insufficiency or overgrowth of the regenerated fibrocartilage. Furthermore, fibrocartilage is biochemically and biomechanically different from normal hyaline cartilage and unable to withstand repetitive load-bearing conditions at the articular surface [7]. The cause of the failure lies in not only the lack of a biomaterial scaffold which can regulate mesenchymal stem cells (MSCs) three-dimensionally, but also the shortage of directional induction.

To regenerate hyaline cartilage in situ, scaffolds have been used to carry endogenous MSCs into cartilage defects and regulate them. The scaffold needs mechanical stability and durability to withstand touch pressure from the articular surface. Furthermore, it should be cavernous to accommodate cell infiltration, absorbable and able to regenerate cartilage or bone. Decalcified cortical bone matrix (DCBM) is a three-dimensional collagenous scaffold with a similar structure to subchondral bone. Its structure is multiporous with many interconnecting channels which permit easy marrow penetration into the matrix. Considering that the inside and outer surface of DCBM is waterproof, we drilled holes in it as described in Fig. 7B to enable marrow cells to penetrate and diffuse inside.

Local administration of growth factors that can enhance cartilage healing is a potentially powerful alternative approach to cartilage

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repair. Several growth factors, including transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPs) and basic fibroblast growth factor (bFGF) can promote chondrocyte proliferation and extracellular matrix (ECM) synthesis in vitro and in vivo [8–11]. Studies observed remarkably high expression of chondrogenic markers in BMP4-transfected mesenchymal progenitor cells [12]. Local delivery of BMP4 enhanced muscle-derived stem cell chondrogenesis and improved articular cartilage repair [13].

In the present study, we attempted to combine three distinct approaches: three-dimensional regulation of repair by drilled DCBM, strongly stimulated regeneration of articular cartilage and subchondral bone by recruiting bone marrow MSCs to the osteochondral defect and local gene therapy with BMP4 to induce chondrocytic and osteogenic differentiation and stimulate cartilage and bone matrix production.

2. Materials and methods

2.1. Isolation of rabbit marrow MSCs and differentiation assay in vitro

MSCs were derived from bone marrow aspirates of 4-week rabbit taken from the distal femur. Each aspirate was combined with 25 ml of MSCs growth medium consisting of 89% DMEM–low glucose (Gibco BRL/Life Technologies Inc., Gathersburg, MD), 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and 1% antibiotic and 1% antimycotic (Gibco). Growth medium was changed twice each week. To characterize the pluripotential of MSCs, we induced MSCs towards adipocytes, chondrocytes and osteoblasts formation. Using the protocol described previously [14], adipogenic differentiation of MSCs was detected by oil red staining for lipid vacuoles, osteogenic differentiation by staining for alkaline phosphatase and chondrogenic differentiation by toluidine blue staining.

2.2. In vitro transfection of MSCs with Ad-BMP4 and Ad-EGFP

Transfection of MSCs was performed when cells reached 80% confluence. The medium was removed and the cells were incubated with bone morphogenetic protein-4 recombinant adenovirus (Ad-BMP4) (provided by Dr. Lin Lin, Department of Sports Medicine, Peking University Third Hospital, China) or enhanced green fluorescent protein recombinant adenovirus (Ad-EGFP) at multiplicity of infection (MOI) = 300 in serum-free DMEM at 37 °C for 6 h, with agitation every 15 min. An equal volume of growth medium was then added. Cells were recovered with complete medium 24 h later and the medium was changed every 2 days. Cells were collected at different time points for in vitro assays. For transplantation in vivo, cells were harvested 24 h posttransduction.

2.3. Indirect immunofluorescence

Indirect immunofluorescence was performed as previously described [15]. MSCs were transfected with Ad-BMP4 and Ad-EGFP. The cells were incubated with a polyclonal antibody against rat BMP4 (Santa Cruz, CA, USA) for 1 h at a dilution of 1:50, followed by TRITC-conjugated rabbit anti-goat IgG (Zhongshan Biochemical, Beijing, China) at a dilution of 1:200 for a further 1 h. The cells were visualized with a fluorescence confocal microscope. Images of stained cells were captured using an Olympus IX70 microscope and SPOTRT image acquisition software.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The MSCs were transfected with Ad-BMP4. After 1 day, 3, 7 and 10 days, the concentration of BMP4, collagen I and collagen II expression by MSCs was measured using an ELISA kit (ADL, USA) according to the manufacturer's standard protocols. Secreted cytokines were standardized to the amount of DNA and expressed as pictogram cytokine per gram DNA. The untransduced MSCs were used as the control.

2.5. Preparation of implantation

Cortical bone specimens were obtained from the diaphyses of sheep femur. Specimens were demineralized by soaking in 0.5 M ethylene diamine tetraacetic acid (EDTA) at 4 °C, pH = 8.3, with fresh solution every day [16]. The EDTA was dissolved in de-ionized water and kept in plastic containers to avoid possible calcium contamination from glass. The replaced EDTA solution was analyzed by atomic absorption spectrophotometry to track the demineralization process, and radiographs of specimens allowed a visual check of demineralization. The decalcified cortical bones were incised into 1 × 1 cm pieces, stored at −80 °C and sterilized with cobalt-60 irradiation for 24 h on the day preceding implantation (Fig. 7A).

2.6. Animal experiments

Seventy-two New Zealand White rabbits weighing 2.5–3.0 kg (4–6 months) were used for the investigation. The rabbits were anesthetized by intravenous injection of 10 ml ethyl carbamate [0.2 g/ml]. After shaving, disinfection and draping, the right knee was opened by an anteromedial parapatellar incision and the patella was everted. Full-thickness articular osteochondral defects, 4 mm in diameter and 2 mm in depth, were created by corneal trephine in the trochlear groove of the right distal femur. And then microfracture was made into the medullary cavity. The sterilized DCBM was made into a cylinder 4 mm in diameter and 2 mm in height by a corneal trephine (Fig. 7A). Allowing marrow to penetrate the DCBM, holes were drilled through the cylinder of the DCBM with a drill bit 0.5 mm in diameter (Fig. 7A, B). Sufficiently soaked with adenovirus-BMP4, the cylinder of DCBM was implanted into the osteochondral defect. Bone marrow was infiltrated through the holes of the DCBM and mixed with Ad-BMP4. Rabbit knees were divided into four implant groups: group I implanted with Ad-BMP4/drilled DCBM composite ($n = 18$); group II implanted with drilled decalcified cortical bone matrix alone without Ad-BMP4 ($n = 18$); group III implanted with DCBM without drilled ($n = 18$) and group IV in which the osteochondral defects were left empty ($n = 18$). All the groups were treated with microfracture. In groups I, II and III, all implants were placed the same level with the surface of the adjacent cartilage. Wound closure was performed in layers. All the rabbits were kept in cages and had free access to food pellets and water. Six weeks, 12 weeks and 24-week after surgery, the rabbits were killed with an intravenous injection of 5 ml of pentobarbital (six rabbits in each group at each time point). All animals were purchased from Beijing Animal Administration Center. All animal experimental protocols were approved by the Animal Care and Use Committee of Peking University and are in compliance with the "Guide for the Care and Use of Laboratory Animals" (National Academy Press, NIH Publication No. 85-23, revised (1996)).

2.7. Histological scoring

In order to evaluate the histological repair of articular cartilage defects quantitively, we used a modified version of the grading scale which has been described by

Table 1
Histological scoring system[a]

Category	Points
Cell morphology	
Hyaline cartilage	4
Mostly hyaline cartilage	3
Mostly fibrocartilage	2
Mostly non-cartilage	1
Non-cartilage only	0
Matrix staining (metachromasia)	
Normal	3
Slightly reduced	2
Markedly reduced	1
No metachromatic stain	0
Structural integrity	
Normal	2
Slight disruption	1
Severe disintegration	0
Surface regularity[b]	
Smooth (>3/4)	3
Moderate (>1/2–3/4)	2
Irregular (1/4–1/2)	1
Severely irregular (<1/4)	0
Thickness of cartilage	
>2/3	2
1/3–2/3	1
<1/3	0
Regenerated subchondral bone	
Good	2
Moderate	1
Poor	0
Integration of donor with host adjacent cartilage	
Both edge integrated	2
One edge integrated	1
Neither edge integrated	0
Total maximum	18

[a] A modified version of the system described by Wakitani et al. [17].
[b] Total smooth area of repair cartilage compared with the entire area of the cartilaginous compartment of the defect.