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The repair of osteochondral defects using baculovirus-mediated gene transfer with de-differentiated chondrocytes in bioreactor culture

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ABSTRACT

Baculovirus has emerged as a promising gene delivery vector. Hereby de-differentiated rabbit chondrocytes were transduced ex vivo with a recombinant baculovirus expressing BMP-2 (Bac-CB), seeded to scaffolds and cultured statically for 1 day (Bac-w0 group) or in a rotating-shaft bioreactor (RSB) for 1 week (Bac-w1 group) or 3 weeks (Bac-w3 group). Mock-transduced constructs were cultured statically for 1 day to serve as the control (Mock-w0 group). We unraveled that Bac-CB transduction and increasing culture time in the RSB yielded more mature cartilaginous constructs in vitro. Eight weeks after implanting into the rabbit osteochondral defects, Mock-w0 constructs failed to repair the lesion while Bac-w0 constructs resulted in augmented, yet incomplete, repair. Bac-w1 constructs yielded neocartilage layers rich in glycosaminoglycans and collagen II, but the integration between the graft and host cartilages was not complete. In contrast, Bac-w3 constructs led to the regeneration of hyaline cartilages as characterized by cartilage-like appearance, improved integration, chondrocytes clustered in lacunae, smooth and homogeneous matrix rich in collagen II and glycosaminoglycans but deficient in collagen I. In conclusion, combining baculovirus-modified de-differentiated chondrocytes and RSB culture creates constructs that repair osteochondral defects, and in vitro culture time dictates the construct maturation and subsequent in vivo repair.

1. Introduction

Weight-bearing articular cartilage comprises chondrocytes residing in the extracellular matrix (ECM) that primarily consists of collagen II (COL II) and glycosaminoglycans (GAGs) such as aggrecan. The structural integrity of articular cartilage may be disrupted owing to trauma or joint diseases, which ultimately leads to cartilage degeneration and debilitating pain. However, the avascular nature of cartilage restricts its ability to self-repair [1–3]. Although surgical procedures to treat cartilage defects (e.g. abrasion arthroplasty or microfracture) are adopted, they result in unwanted fibrocartilages [4,5]. Meanwhile, tissue transplantation is limited by the availability of donor tissues. To address the demand for donor tissues, tissue engineering approaches are being aggressively sought to produce engineered cartilages in vitro, which after implantation may facilitate the cartilage regeneration in vivo. One common strategy involves the immobilization of appropriate cells in porous scaffolds and cultivation of the cell/scaffold constructs in a bioreactor for tissue growth [6–8]. In this regard, we have developed a rotating-shaft bioreactor for two-phase cultivation of primary chondrocyte/scaffold constructs, which after 4-week culture develop into cartilaginous tissues with uniform cell distribution and abundant ECM deposition [9].

In addition, an alternative strategy emerging in recent years combines the concepts of cell therapy and gene therapy, by which the cells are genetically engineered ex vivo by a gene delivery vector, loaded to appropriate scaffolds and implanted into animals, hoping that the expressed factor (e.g. anabolic growth factor or regulatory transcription factor) can modulate cellular differentiation and accelerate tissue regeneration *in situ* [10–12]. Despite varying degrees of success, the commonly employed viral (e.g. retrovirus, lentivirus, adenovirus, adeno-associated virus and herpes simplex virus) and nonviral vectors (e.g. plasmid) possess their respective limitations (for review see [1,13]).

Aside from these vectors, baculovirus is an insect virus but is capable of efficiently transducing a broad range of mammalian cells, which prompts the development of baculovirus as a novel vector for in vitro and in vivo gene delivery. In recent years, the

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applications of baculovirus have expanded to cell-based assay development, eucaryotic protein display, study of gene function, viral vector production, delivery of vaccine immunogens, etc. (for review see [14–17]). Additionally, baculovirus can transduce rat articular chondrocytes with efficiencies up to 88% [18] without impeding cell proliferation and formation of cartilage-like constructs in vitro when the transduced cells are embedded in porous scaffolds and cultured in the RSB [19]. Furthermore, transduction of partially de-differentiated passage 3 (P3) rabbit chondrocytes with a recombinant baculovirus expressing BMP-2 (Bac-CB) remarkably promotes the secretion of cartilage-specific ECM [20]. Culture of the Bac-CB-transduced P3 cells in the RSB for 3 weeks synergistically enhances the formation of cartilaginous tissues with hyaline appearance, uniform cell distribution, cartilage-specific gene expression and ECM deposition [21].

Inspired by these encouraging results, the primary goal of this study was to explore whether the constructs derived from the Bac-CB-transduced chondrocytes cultured in the RSB can repair cartilage defects in vivo. Besides, the 3-week culture required for the cartilaginous tissue formation increases the risks of contamination and operation cost, hence reduction of culture time may be desired. Therefore the second objective was to investigate how critically the in vitro culture time governs the construct properties and subsequent cartilage repair. Towards these ends, P3 rabbit chondrocytes were transduced ex vivo with Bac-CB, seeded to poly (L-lactide-*co*-glycolide) (PLGA) scaffolds and cultured in the dish for 1 day (Bac-w0 group). Alternatively, the transduced constructs were cultured in the RSB for 1 week (Bac-w1 group) or 3 weeks (Bac-w3 group). To serve as the control, P3 chondrocytes were mock transduced and cultured statically for 1 day (Mock-w0 group). The construct properties for each group were evaluated biochemically, biomechanically and genetically. These constructs were implanted into the osteochondral defects at the patellar grooves of New Zealand white (NZW) rabbits and the cartilage repair was assessed by macroscopic appearance, histological staining and immunohistochemical staining at 4 and 8 weeks post-implantation. The correlations between in vitro culture time, resultant construct properties and in vivo cartilage repair were elucidated and discussed.

2. Materials and methods

2.1. Cell, virus and transduction

All media, reagents and chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Primary chondrocytes were isolated from the articular cartilages of 2-day-old NZW rabbits as described [20], plated onto T-150 flasks and designated P0. The cells were cultured using Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin/streptomycin/amphotericin B (Biological Industries), and were serially passaged upon confluence. The recombinant baculovirus expressing BMP-2 under the control of cytomegalovirus immediate-early promoter was constructed previously and designated Bac-CB [20]. The virus was amplified, harvested, titrated and stored as described [20].

For virus transduction, P3 chondrocytes cultured overnight in the T-150 flasks (1.2×10^7 cells/flask) were washed with Dulbecco's phosphate-buffered saline (PBS, pH 7.4). For each flask, a certain volume of virus was diluted to 2 ml with TNM-FH medium to adjust the multiplicity of infection (MOI) to 75, followed by mixing with 8 ml PBS. Transduction was initiated by directly adding the virus-PBS solution to the cells and continued by gentle shaking on a rocking plate at room temperature for 8 h. After the incubation period, the cells were washed, replenished with 12 ml DMEM medium and incubated at 37 °C.

2.2. Scaffold, rotating-shaft bioreactor (RSB) and 3-dimensional culture

Porous PLGA scaffolds (porosity ≈ 85%) were fabricated to disk shape (≈5.2 mm in diameter, ≈3 mm in thickness) using a solvent casting/particulate leaching technique [22], sterilized with 70% alcohol and pre-wetted with fresh medium overnight prior to use.

P3 chondrocytes were mock-transduced or transduced as described above, and seeded into PLGA scaffolds (3.4×10^6 cells/scaffold) at 1 day post-transduction. One day later, the transduced (Bac-w0) or mock-transduced (Mock-w0) cell/scaffold constructs were subject to construct analyses or implantation. Additional transduced constructs were cultured in the RSB for 1 (Bac-w1) or 3 (Bac-w3) weeks. The RSB consisted of a water-jacketed glass cylinder, a sealing cap assembly and a stainless shaft on which 22-gauge needles were soldered [9]. The constructs were positioned on the individual needles (16 constructs per reactor) and half of the reactor space was filled with 100 ml medium containing 10% FBS, 50 μg/ml ascorbic acid and 1% penicillin/streptomycin/amphotericin B. The reactor was operated in a perfusion mode (medium flow rate = 0.2 ml/min, gas flow rate = 20 ml/min) with the shaft rotating at 10 rpm. The horizontal rotation of the shaft alternately exposed the constructs to gas and liquid phases, thereby leading to efficient oxygen and nutrient transfer, and periodically changing, mild shear stress exerting on the construct surfaces [9]. The direction of rotation (clockwise or counterclockwise) was switched daily and half of the spent medium was replaced by fresh medium every 3–4 days.

2.3. Analyses of constructs

Mechanical strength of the constructs was measured through compression testing using a material testing machine at a cross-head speed of 1 mm/min. The Young's modulus was defined as the stress to strain ratio. After Young's modulus analysis, each construct was frozen, lyophilized and digested by papain. The GAG contents were quantified by mixing the papain-digested samples with 1,9-dimethylmethylene blue and reading the absorbance at 525 nm, with bovine trachea chondroitin sulfate as the standard [23]. Total collagen contents were determined spectrophotometrically from hydroxyproline concentration after acid hydrolysis (6 N HCl for 18 h at 115 °C) and reaction with chloramines-T and *p*-dimethylaminobenzaldehyde, using a hydroxyproline to collagen ratio of 1:9 [24].

For quantitative real-time RT-PCR (qRT-PCR) analyses, chondrocytes were released by digesting the constructs in 1% collagenase at 37 °C for 15 min. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and ≈1 μg of total RNA was used for cDNA synthesis with the Omniscript RT Kit (Qiagen). The primer sequences specific for the target gene and the internal control gene *gapdh* used for qRT-PCR are listed in Table 1. The real-time PCR was performed in an ABI 7300 real-time PCR system (Applied Biosystems) with SYBR Green PCR master mix (Applied Biosystems) under the condition of 15 s at 95 °C, 60 s at 60 °C, and the fluorescence intensity was recorded for 40 cycles.

2.4. Implantation of engineered constructs

Animal care and use were performed in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the National Science Council, Taiwan. Thirty NZW rabbits aged 12 weeks (2–2.5 kg, National Laboratory Animal Center, Taiwan) were anesthetized with Zoletil 50 (1 ml/kg body weight, Virbac) by intramuscular injection. Full-thickness cartilage defects (5 mm in diameter and 3 mm in depth) were created surgically at the patellar grooves of both knee joints and implanted with constructs by press-fit. The control constructs (Mock-w0, $n = 30$ in all) were implanted into the defects at the left or right knees randomly whereas experiment constructs (Bac-w0, Bac-w1 or Bac-w3, $n = 10$ for each group) were implanted into the contralateral knees. At 4 and 8 weeks post-implantation, animals were sacrificed and the knee joints were removed ($n = 15$ for the control constructs and $n = 5$ for each experiment construct at each time point) to assess the cartilage repair.

2.5. Histological and immunohistochemical staining

The graft sites were photographed for macroscopic observation. Samples were fixed in neutral-buffered formalin, embedded in paraffin and sectioned (8 μm thick). Sections were stained with Safranin-O for sulfated GAGs and hematoxylin and eosin (H&E) for histology and cell morphology. The sections were also de-paraffined and rehydrated for immunohistochemical staining specific for COL I and COL II. The sections were treated with Target Retrieval solution (DAKO) at 90 °C for 10 min, blocked with 2% bovine serum albumin for 1 h and then reacted with the primary antibody (mouse anti-collagen I or mouse anti-collagen II, 1:100 dilution,

Table 1
Primer sequences for qRT-PCR.

Marker gene		Sequence (5′ to 3′)
Aggrecan	Forward	AGCCTGCGCTCCAATGACT
	Reverse	TGGAACACGATGCCTTTCAC
COL II	Forward	GTCCAGGCAGAGGCAGGAA
	Reverse	GACACGGAGTAGCACCATCG
COL I	Forward	CCAGAGTGGAGCAGTGGTTAC
	Reverse	GCAGGTTTCGCCAGTAGAGAA
GAPDH	Forward	CTCTCTCAAGATTGTCAGCAAC
	Reverse	CACAATGCCGAAGTGGTCGT