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Demineralized bone matrix gelatin as scaffold for osteochondral tissue engineering

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Abstract

To develop a single-unit osteochondral tissue with demineralized bone matrix gelatin (BMG), rabbit chondrocytes were cultured on demineralized bone matrix gelatin for 6 weeks. The engineered osteochondral tissue was characterized with histology, immunolocalization, TEM, SEM, biochemical assay, and gene expression analysis. About 1.3 mm viable neo-cartilage was produced on demineralized BMG. RT-PCR, immunohistochemistry, TEM, biochemical assay, and histology revealed hyaline-like cartilage with zonal layers, intense type II collagen expression, and abundant proteoglycan content formed upon BMG compared with normal cartilage. But hydroxyproline content and type I collagen gene and protein expressions were significantly lower. We consider engineering cartilage tissue with chondrocytes cultured on allogenic demineralized BMG is a good approach for osteochondral tissue engineering.

Keywords: Cartilage tissue engineering; Chondrocyte; Bone graft; Cell culture; Collagen

1. Introduction

Osteoarthritis of the knee and other weight-bearing joints is a major health problem in western societies. By the age of 40, nearly 90% of the population will harbor some degenerative changes in their weight-bearing joint surfaces and a minority will experience pain, stiffness and loss of function [1]. Unfortunately, the regenerative capacity of articular cartilage is limited, leaving only symptomatic treatment strategies such as nonsteroidal anti-inflammatory drugs or surgery for those with severe symptoms.

A variety of treatments have been attempted for those with focal, symptomatic osteochondral lesions. Current surgical approaches for this include various forms of abrasion chondroplasty, microfracture, transplantation of osteochondral plugs or the use of cultured autologous chondrocytes [2]. Although each of these treatment strategies has shown some temporary benefit in improving patient symptoms, no studies have yet documented the ability these forms of treatment to delay or halt the relentless progression of the joint towards end stage degeneration. Thus, most patients treated for early focal osteochondral lesions will ultimately require total joint replacement in the future as the disease process advances.

The focal treatment strategies for osteochondral defects are currently associated with a variety of risks and limitations including inadequate availability of donor tissue, donor site morbidity, poor attachment of the graft to the surrounding chondral surface, delamination of the chondral grafts or the formation of fibrocartilage with inferior mechanical properties at the treatment site [1,3,4]. In addition, the use of cultured autologous chondrocytes has been limited by the need for multiple arthroscopic procedures and the cost associated with the procedures [5]. Fortunately, in recent years, tissue-engineering approaches have been developed with the potential to improve the outcome of treatment for focal chondral defects [6–8].

The optimal-engineered osteochondral graft must address not only the damaged cartilage but also the underlying bone to allow for adequate subchondral repair and to support the overlying neo-cartilage. The tissue-engineered

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graft should produce tissue that is qualitatively similar to the native tissues at the site of the defect and incorporate quickly and fully into the host joint. Although many cell scaffolds have been described [8–10] for growing chondrocytes, most are not ideal for producing regeneration of both bone and cartilage at the site of the joint defect. Also, many cell scaffolds do not have an adequate mechanical integrity to support attachment of the graft to the host joint. Finally, host immune response to the cell scaffold severely limits the clinical usage of many of the bioresorbable scaffolds.

Autolyzed antigen-extracted allogenic (AAA) bone matrix gelatin (BMG) is a cell matrix produced by acid demineralization of whole bone [11,12]. In the process of producing this material, the soluble noncollagenous proteins are removed by sequential chemical extraction with dilute HCl, concentrated $CaCl_2$, and LiCl as a denaturant, while native insoluble bone morphogenetic proteins (BMPs) and noncollagenous proteins are kept. Some studies have been done to determine the sequence and mechanisms of bone and cartilage formation induced by BMG, which can induce differentiation of mesenchymal cells into chondrocytes in vitro [11–15]. After in vivo implantation, the BMG scaffold can be quickly resorbed, releasing bone morphogenetic proteins and other growth factors, which further stimulate a robust healing response. To our knowledge, the use of BMG as a scaffold for osteochondral tissue regeneration has not been reported.

In this study, we have produced and biologically and histologically characterized a potential osteochondral graft using autolyzed antigen-extracted allogenic BMG as scaffold. The aim is therefore to have neo-cartilage form above the BMG, which could serve a dual purpose both as an excellent scaffold for chondrogenesis and as a bone substitute because of its osteoinductive properties.

2. Experimental materials and methods

2.1. Preparation of bone matrix gelatin (BMG) grafts

Demineralized bone matrix gelatin was prepared from segments of distal femur that had been harvested from freshly euthanized adult New Zealand white rabbits. All soft tissues were removed and the bones were washed in sterile deionized water. The cleaned bones were extracted for 1.5 h in a 1:1 mixture of chloroform and methanol (30 ml/g of bone), and then subjected to the following steps: (1) Demineralization at 2 °C with 0.6 M hydrochloric acid (60 mg/g) overnight; (2) Washed with sterile deionized water to pH 7.4; (3) Washed with 2 M $CaCl_2$ for 1 h at 2 °C; (4) Washed with 0.5 M EDTA for 1 h; (5) Washed with 8 M LiCl for 1 h; (6) Washed with deionized water at 55 °C. Between each step the bone material was washed with sterile deionized water. The bone matrix gelatin was then incubated with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 units/ml penicillin, and 100 mg/ml streptomycin for 1 h at 37 °C. BMG was cut into 5 mm × 5 mm in size and stored at −70 °C until used.

2.2. Isolation of chondrocytes and culture in vitro

According to our published chondrocytes isolation protocol [16], 1 g of articular cartilage was harvested aseptically from the femora and tibiae of a male New Zealand white rabbit that were weighted 3.5–4.5 kg. The cartilage was diced, digested in 0.1% (weight/volume) collagenase type II (Worthington, Freehold, HJ) for 3–6 h at 37 °C, and the harvested chondrocytes were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 gm/l glucose, 10% fetal bovine serum, 50 mg/l sodium ascobate, 10 mM HEPES, 100 units/ml penicillin, and 100 mg/ml streptomycin. Primary chondrocytes were plated in 2–3 T-75 cm^2 flasks and cultured without passage in a humidified, 5% CO_2 incubator for 5–7 days.

Passage 1 (P1) chondrocytes at 90% confluence were then seeded onto the BMG. Briefly, the BMG scaffolds were placed at the bottom of 50 ml Falcon centrifuge tubes. Then, 2×10^6 cells were plated on each BMG scaffold and cultured in 10 ml of Dulbecco's modified Eagle's medium containing 4.5 gm/l glucose, 20% fetal bovine serum, 50 mg/l ascorbic acid, 10 mM HEPES, 100 units/ml penicillin, and 100 mg/ml streptomycin for 6 weeks. The medium was changed every 3 days.

2.3. Morphology and histology of engineered cartilage

After 6 weeks of culture in vitro, a cartilaginous tissue cap was formed around the BMG scaffold; the cartilaginous tissue was particularly prominent on the top of BMG. Histological characterization and organization of the engineered cartilage were evaluated microscopically on sections that were stained with hematoxylin and eosin (H&E) and Safranin-O.

2.4. Confocal laser scanning microscopy (CLSM) cell viability (live/dead assay)

The viability of each construct was assessed employing cell viability fluorescence markers (Live/dead kit, Molecular Probes, Eugene, USA) [17]. Live tissue from the regenerated cartilaginous cap was sliced and incubated in live/dead buffer consisting of calcein-AM (1 μM) and ethidium homodimer (8 μM) in PBS for 1 h. In this assay, Ethidium homodimer-1 enters into the cells with compromised cell membranes and subsequently intercalates into their nucleic acids. Thus, a cell with compromised cell membranes has a red fluorescence and is classified as dead. Calcein-AM penetrates the membranes of living cells, where ubiquitous cytoplasmic esterases cleave the molecule to produce a green fluorescence. The sections were then visualized using a laser scanning confocal microscope (LSM410, Zeiss, Germany) [18].

2.5. Collagen immunolocalization

Type II collagen was immunolocalized in engineered cartilage. Freshly harvested tissue was preserved in Optimal Cutting Temperature (OCT) Compound (Sakura, Torrance, CA) and 6 μm sections were prepared using a Leica CM3050 cryomicrotome (Leica, Bannockburn, IL) at −30 °C. Sections were incubated with monoclonal mice anti-type II collagen (Sigma, St. Louis, MO), followed by fluorescein isothiocyanate (FITC) labeled goat anti-mice IgG (American Qualex, San Clemente, CA). Fluorescence was visualized with a Nikon Eclipse E600 microscope (Nikon, Melville, NY) equipped with a digital camera and Adobe Photoshop 7.0 software (Adobe system Incorporated, San Jose, CA).

2.6. Western-blot

Engineered cartilage and normal cartilage samples were harvested and preserved in tissue protein extraction reagent (Pierce, Rockford, IL). The tissue samples were homogenized with a rotor–stator homogenizer (IKA Labortechnik, Germany), 20 μl supernatant solution from either engineered cartilage or normal cartilage and 5 μl 5 × loading buffer were mixed and heated at 100 °C for 10 min. Protein samples were separated by electrophoresis in 10% polyacrylamide gels on a mini-gel apparatus (Bio-Rad, Hercules, CA, USA) at 200 V for 40 min. Western blot transfer of the gel components to nitrocellulose was carried out under semidry conditions