J Orthop Sci
DOI 10.1007/s00776-014-0574-7

ORIGINAL ARTICLE

Comparison of repair between cartilage and osteocartilage defects in rabbits using similarly manipulated scaffold-free cartilage-like constructs

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Received: 14 November 2013 / Accepted: 14 April 2014

Abstract
Background Articular cartilage has a limited capacity for spontaneous repair, and its repair remains a clinical challenge. The purpose of this study was to prepare scaffold-free cartilage-like constructs and evaluate the feasibility of their use for the treatment of cartilage and osteocartilage defects in vivo.
Methods The scaffold-free constructs were prepared by chondrocytes isolated from the articular cartilage of rabbits using a high-density three-dimensional culture system. Two different defects, i.e., a chondral defect without oozing blood and an osteochondral defect with oozing blood, of 4-mm diameter, were created on the patellar groove of rabbits and forwarded to in vivo trials. In each defect, the constructs cut into 4-mm-diameter cylinders were grafted at the bottom of the defects. As a control, defects were only made on the contralateral knee joint in each rabbit. At 2, 4, 8 and 12 weeks after surgery, six rabbits in each group were evaluated macroscopically and histologically.
Results In vitro, histological examination revealed that the constructs have the character of hyaline cartilage with a potential adhesiveness to surrounding tissue. In vivo, in two control groups, incomplete spontaneous cartilage repair was observed in the osteochondral defects, whereas no repair was observed in the chondral defects. In the two treated groups, the surviving constructs in chondral defects showed significantly better repair compared to those in osteochondral defects.
Conclusions It is possible for a chondral defect to be repaired by scaffold-free constructs in certain conditions. Establishing the optimal environment suitable for cartilage repair is warranted.

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Introduction

It is well known that articular cartilage has a very limited capacity for repair, since it is avascular and aneural. Even if an osteochondral defect is repaired spontaneously, it is composed of fibrocartilage, not the original hyaline cartilage. Current treatment methods for cartilage repair include drilling, debridement, microfracture, mosaic plasty and autologous chondrocyte implantation (ACI). However, none of these methods provide ideal cartilage repair, which remains an important clinical challenge.

As another approach that has been developed to improve the quality of cartilage healing, the implantation of engineered chondral grafts has been reported. But when chondrocytes were cultured in the monolayer, they rapidly dedifferentiated during proliferation and lost their original cartilage-specific character. To prevent this unfavorable dedifferentiation, three-dimensional (3D) culture techniques using artificial matrices, such as embedding in agarose or alginate, have been reported. These methods can produce implantable constructs [1–3]; however, it is possible that the scaffold constructs can cause immunological problems such as acute rejection, foreign body reactions and/or fibroblastic overgrowth [4, 5]. Thus, the scaffold-free cartilage-like constructs (SFCs) could be an excellent alternative.

A high-density scaffold-free 3D environment enables cell-to-cell interactions and closely mimics the physiological conditions of precartilage in vivo [6]. Uenaka et al. [7]

Published online: 02 May 2014

reported a comprehensive scaffold-free culture system to develop cartilage-like constructs using rats. These SFCs maintain the character of chondrocytes in vitro; however, the feasibility of this technique for other animals was not examined. In addition, the use of these SFCs for the treatment of cartilage defects in vivo has not been evaluated yet. The aims of the present study are to confirm the availability of developing SFCs using rabbits in vitro and evaluate the feasibility of their use for the treatment of cartilage defects at different cartilage depths in vivo using a rabbit model.

Materials and methods

The experiments were conducted according to the guidelines for animal experiments of the Animal Research and Care Committee at Shiga University of Medical Science.

In vitro experiments

High-density culture for SFCs

SFCs using rabbit chondrocytes were prepared according to the previous report with slight modifications [7]. In brief, Japanese white rabbits weighing 2.0 kg (approximately 10 weeks old Kitayama Labs, Nagano, Japan) were killed by an intravenous injection of an overdose of sodium pentobarbital. Articular cartilage slices from the hip, knee and shoulder joints were obtained. Subsequently, these cartilage slices were minced and then digested in Dulbecco's modified Eagle's medium (DMEM, MP Biomedecals, Solon, OH, USA). Chondrocytes were isolated with 0.1 % trypsin/ethylenediaminetetraacetate (EDTA, Kanto Chemical, Tokyo, Japan) in phosphate-buffered saline (PBS, DS Pharma, Osaka, Japan) for 20 min and 0.25 % collagenase (Worthington Biochemical, Lakewood, NJ, USA) in DMEM supplemented with 10 % fetal bovine serum (FBS, MP Biomedicals) and antibiotics (penicillin 10,000 U/ml, MP Biomedicals; streptomycin 100 μg/ml, MP Biomedicals; amphotericin B 0.25 μg/ml, DS Pharma) (culture medium) for 4 h at 37 °C in a culture tube. Chondrocyte suspensions were strained through a 100-μm cell strainer (BD Falcon, Bedford, MA, USA). The collected chondrocytes were centrifuged, washed and re-suspended in culture medium. Chondrocytes were seeded on 500-cm^2 square dishes at 2×10^3 cells/cm^2 at 37 °C in 5 % CO_2 and 95 % humidity. Around 4 weeks later, the primary cultured cells reached a confluence and were detached using 0.05 % trypsin/EDTA for 3 min at 37 °C. The cells were centrifuged, washed three times in DMEM and re-suspended in culture medium. A synthetic culture membrane (Transwell, 6.5 mm; Corning, Lowell, MA, USA) with a pore size of 0.4 μm was placed into each well of a 6-well plate, and a prepared suspension with 6×10^6 cells/cm^2 chondrocytes was added on top of the membrane. The cells precipitated and accumulated on top of the membrane at 2 h, after which 12 ml of culture medium supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich, Steinheim, Germany) was added to each well to allow oxygen and nutrient diffusion. The medium of each well was changed every 2 days. After 1 week, we obtained gelatinous SFCs.

After 1 week of high-density culture, the produced constructs were stable enough to be handled by surgical forceps and were regular in form. Our pilot study revealed that the constructs in 3-day culture were too fragile to be implanted; in turn, those in 2-week culture were too thick to be implanted into our shallow defects (data not shown). Furthermore, long-term cultured tissue equivalents in vitro have possible problems in adequately integrating with the neighboring cartilage when implanted [8]. Taking all these findings into account, we employed 1 week as an optimal culture period for our study.

Histological evaluation

The constructs were fixed in 20 % formalin at room temperature for 2 days and then embedded in paraffin. Each specimen was cut axially into 3-μm-thick sections and deparaffinized, dehydrated and used for subsequent staining with toluidine blue as the immunohistochemical stain for type I and II collagens and fibronectin. We used a mouse monoclonal antibody against human type I and type II collagens that has been verified to crossreact with rabbit type I and type II collagens (Daiichi Fine Chemical, Toyama, Japan). For the immunohistochemical staining, endogenous peroxidase activity was blocked by 0.3 % hydrogen peroxide. The sections were incubated with the antibody against type I collagen (diluted 1:200 with Tris buffered saline) or with an antibody against type II collagen (diluted 1:5,000 with Tris buffered saline), at 4 °C overnight. Antibody binding was visualized by incubation with the Histofine Simple Stain_kit (Nichirei Bioscience, Tokyo, Japan) in combination with DAB solution. The sections were then counterstained with hematoxylin. In the immunohistochemical staining for fibronectin, we investigated using the same procedure as for the mouse monoclonal antibody against fibronectin (Abcam, Cambridge, UK) diluted 200 fold. We immunostained for CD68 at 4 weeks in the OI group using the mouse monoclonal antibody against CD68 (Dako, Tokyo, Japan).

In vivo experiments

Implantation procedure

Rabbits weighing 3.0 kg (approximately 18 weeks old; Kitayama Labs) were anaesthetized by intramuscular