J Orthop Sci (2013) 18:627–635
DOI 10.1007/s00776-013-0379-0

ORIGINAL ARTICLE

Treatment of cartilage defects by subchondral drilling combined with covering with atelocollagen membrane induces osteogenesis in a rat model

Michio Hamanishi · Tomoyuki Nakasa · Naosuke Kamei · Hiromi Kazusa · Goki Kamei · Mitsuo Ochi

Received: 31 July 2012 / Accepted: 1 March 2013 / Published online: 6 April 2013

Abstract
Background The coverage of the atelocollagen membrane at the chondral defect after subchondral drilling might improve the beneficial effects for cartilage repair because of the prevention of scattering and accumulation of cells and growth factors from bone marrow within the chondral defect. On the other hand, it might block cells and factors derived from the synovium or cause high pressure in the chondral defect, resulting in prevention of cells and growth factors gushing out from the bone marrow, which leads to disadvantages for cartilage repair.
Method We tested this hypothesis in a 2-mm-diameter chondral defect created in the articular cartilage of the patellar groove in a rat models. Defects were left untreated, or were drilled or drilled and covered with an atelocollagen membrane; healing was evaluated by histology and gene expression analysis using real-time polymerase chain reaction and immunohistochemistry.
Results Membrane coverage induced bone tissue ingrowth into the punched chondral defect. At 1 week, expression of TGFβ, Sox9, Runx2, osteocalcin, Col1a1, and Col2a1 in the drilling group was significantly higher than in the covering group. At 4 weeks, expressions of TGFβ, Runx2, and Col1a1 were all significantly higher in the drilling group, while Sox9, osteocalcin, and Col2a1 were significantly higher in the covering group. Immunohistochemistry demonstrated Sox9, osteocalcin, and type II collagen on the bony reparative tissue in the covering group.
Conclusions These results suggest that the atelocollagen membrane coverage resulted in inhibition of cartilage repair.

M. Hamanishi (✉) · T. Nakasa · N. Kamei · H. Kazusa · G. Kamei · M. Ochi
Department of Orthopaedic Surgery, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
e-mail: michiosprinter@yahoo.co.jp

N. Kamei
Division of Regeneration and Medicine, Hiroshima University Hospital, Hiroshima, Japan

Introduction

Articular cartilage has very limited healing potential, because it lacks a blood supply and is isolated from systemic regulation [1]. The most widely practiced methods of repairing defects are bone marrow stimulation techniques such as subchondral drilling, abrasion, and microfracture, procedures that aim to recruit bone marrow elements to repair cartilage defects [2–7]. Such procedures are thought to promote chondrogenesis, inducing formation of fibrous tissue, fibrocartilage, and/or hyaline cartilage by inducing migration of bone marrow mesenchymal stromal cells (MSCs) from the subchondral bone by bleeding. However, in experimental studies these techniques have resulted in the formation of fibrocartilaginous tissues [3, 7, 8]. There are several possible reasons why bone marrow-stimulating procedures do not always induce satisfactory results. One explanation is that the number of bone marrow MSCs may not be adequate to repair the lesion. Nishimori et al. [9] demonstrated that the addition of cultured bone marrow MSCs to a defect in combination with a bone marrow-stimulating procedure accelerated regeneration of articular cartilage in the defects better than the bone marrow-stimulating procedure alone.

One suggested reason why the number of bone marrow MSCs may be inadequate to repair the lesion could be that

they diffuse into the joint fluid and do not remain in the chondral defect. Therefore, we estimated that the use of an atelocollagen membrane to cover a chondral defect after subchondral drilling exerts beneficial effects on cartilage repair by preventing scattering of cells and growth factors from the bone marrow and causing them to accumulate within the chondral defect. The purpose of this study was to examine the effect and the mechanism of atelocollagen membrane coverage combined with subchondral drilling on cartilage regeneration.

Materials and methods

Animal preparation

All procedures were performed in accordance with the Guide for Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University (no. A10-97).

Male Sprague–Dawley rats (12 weeks old) were used in this study. A total of 50 knees of 26 rats were used. Before surgery, the animals were anesthetized with an intraperitoneal injection of 1 ml/kg pentobarbital sodium. The patella was everted through a medial approach. A chondral defect of 2 mm diameter was created in the articular cartilage of the patellar groove of the distal femur using a biopsy punch. The control group represented the natural course of healing of the chondral defect. In the drilling group, five holes were drilled into the cartilage using a 0.2-mm-diameter drill. In the covering group, we applied instant glue approved for clinical application (Aron Alpha A "Sankyo," Daiichi Sankyo, Tokyo, Japan), containing α-cyanoacrylate monomer, around the drilled defect and covered it with a recombinant peptide membrane (Fujifilm, Tokyo, Japan), which has low ecotoxicology and high uniformity, created using a yeast culture technique. The membrane was a rectangle of 4 × 5 mm and 5 μm thickness. No bleeding was observed in the untreated chondral defects, while bleeding derived from the bone marrow could be observed in chondral defects treated by drilling.

In the drilling and covering group, blood clots or reparative tissue in the defect were extracted and analyzed for expression of several factors as markers of chondrogenesis and osteogenesis, and extracellular matrix was analyzed by real-time polymerase chain reaction (PCR) at 1 and 4 weeks after creation of the defects. At 4 weeks after creation of the defects, the rats were euthanized by intraperitoneal injection of a lethal dose of pentobarbital sodium. The distal femora were resected en bloc and fixed in 4 % paraformaldehyde for 24 h at 4 °C. They were then decalcified in 0.5 M EDTA, then embedded in paraffin and cut into 5-μm sections serially along the sagittal plane that included the center of the defect, and histological evaluation was performed.

Quantitative reverse transcription polymerase chain reaction (real-time PCR)

To examine the expression of chondrogenic and osteogenic marker genes such as Col2a1, Sox9, TGFβ, Col1a1, Runx2, and osteocalcin, real-time PCR was performed using SYBR Green (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated from blood clots or reparative tissue that had been homogenized on ice with Trizol reagent (Invitrogen). One μg of total RNA was reverse-transcribed using the QuantiTect® Reverse Transcription Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol. Real-time PCR was performed using a Real-time PCR System (Applied Biosystems, Carlsbad, CA) in a 20-μl PCR mixture containing 1.0-μl template cDNA, 10 μl SYBR Green mix, 1.5 μM primer, and water to adjust the final volume to 20 μl. Primer sequences are listed in Table 1.

All reactions were performed in triplicate in a 96-well plate and incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The GAPDH gene was used as a control to normalize differences in total RNA levels between samples. A threshold cycle (C_T) was observed in the exponential phase of amplification, and quantification of relative expression levels was performed using standard curves for target genes and the endogenous control. Geometric means were used to calculate the $\Delta\Delta C_T$

Table 1 Primer sequences used for real time polymerase chain reaction (PCR)

Target genes	Primer sequence forward	Primer sequence reverse
TGF-β	5′-TATAGCAACAATTCGTGGCG-3′	5′-CAGAAGTTGGCATGGTAGCC-3′
Sox9	5-CGTCAACGGCTCCAGCA-3′	5′-TGCGCCCACACCATGA-3′
Runx2	5′-CACCCTCAAGAGCCTGAGTC-3′	5′-CAGACGGCTGAGTAGGGAAC-3′
Osteocalcin	5-GCATTCTGCCTCTCTGACCT-3′	5′-CTAAACGGTGGTGCCATAGA-3′
Col1a1	5′-TGCCGTGACCTCAAGATGT-3′	5-TGGGGI 1 IGGGCTGATGTA-3′
Col2a1	5′-CCCAGAACATCACCTACCAC-3′	5′-GGTACTCGATGATGGTCTTG-3′