Bone Marrow Mesenchymal Stem Cells Express Lubricin And Regenerate Superficial Layer Of Articular Cartilage In A Rat.

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Disclosures:

Introduction: Lubricin encoded by the PRG4 gene plays an important role in boundary lubrication of articular cartilage. In joint cavity, lubricin is mainly produced by synovial lining cells and chondrocytes in the superficial layer. According to previous reports, mesenchymal stem cells (MSCs) derived from synovium [1], adipose tissue [2] and muscle [3] produced lubricin in specific conditions, however, expression of lubricin by MSCs derived from bone marrow is hardly known. In this study, we investigated whether transplanted bone marrow MSCs (BM-MSCs) expressed lubricin and regenerated superficial zone in a rat osteochondral defect model.

Methods: 1. In vitro chondrogenesis
2.5 × 10^5 human BM-MSCs were pelleted, centrifuged, and cultured in 400μl chondrogenic medium supplemented with or without 10 ng/ml TGF-β3. The pellets at 21 days were embedded in paraffin, cut into 5 μm thick sections, and stained with safranin-O and immunostained with anti-lubricin antibody. Chondrogenesis was evaluated with Bern score, and expression of lubricin with intensity and extent score [2].

2. RT-PCR
RNA was extracted from monolayered cells and pellets at 7 days. Real-time PCR analysis was carried out using a LightCycler 480 instrument (Roche) to assess mRNA expression of PRG4, Sox-9, and aggrecan.

3. Western blot
Conditioned medium in pellet culture between 17 days and 21 days was collected for lubricin protein accumulation by western blot. 10μl medium was loaded on each lane and separated by 4-20% gel electrophoresis. Membranes were incubated with anti-lubricin antibody (H-140 Santa Cruz).

4. Preparation of aggregate of rat
2.5 × 10^5 BM-MSCs were prepared, and resuspended in 35μl of CCM, and plated on an inverted culture dish lid. Then, the lid was inverted, and placed on a culture dish. The cells were cultured for 3 days in hanging drops [4].

5. In vivo transplantation
Lewis female rats at 10-14 weeks were used in this experiment. Under anesthesia, full-thickness osteochondral defects (diameter 1.5 mm, 1.0 mm deep) were created in the trochlear groove of the femur. Four aggregates derived from GFP transgenic rat were transplanted to the defect. For the control group, the defect was left empty. Animals were sacrificed at 4 weeks and at 12 weeks after the operation (n = 7 at each time).

6. Histological examination
The dissected distal femurs were embedded in paraffin blocks. Sagittal sections at 5 μm thick were stained with safranin-O. The samples were immunostained with type II collagen. Histological sections of the repaired tissue were analyzed using a grading system which were modified from the repaired cartilage score described by Wakitani. Layer thickness and cell density of superficial layer in sections stained with safranin-O were also measured.

7. Fluorescent microscopic examination
For fluorescent microscopic examination, cryosections at 12 μm were prepared. Specimens were used to detect PRG4 expression stained with anti PRG4 antibody (H-140 Santa Cruz), and goat anti-mouse IgG labeled with Alexa fluor 555 (Invitrogen). Background nuclei were counterstained with Hoechst 33342 (Invitrogen).

8. Transmission electron microscopy
Ultrathin sections at 90 nm were collected on copper grids, double-stained with uranyl acetate and lead citrate, and then examined by transmission electron microscopy (H-7100, Hitachi, Hitachinaka, Japan).

9. Statistical analyses
Comparisons between two groups were analyzed using the Mann-Whitney U test in in vivo study and the paired t-test in in vitro study. Comparisons between multigroups were analyzed using the Kruskal-Wallis test and the Scheffe test. P-values less than
0.05 were considered to be statistically significant.

**Results:** Pellets cultured with TGF-β3 showed higher stainability of safranin-o and lubricin expression than without TGF-β3 (Fig.1AB). Cartilage pellet cultured with TGF-β3 at day 21 expressed lubricin entirely and especially higher in the superficial layer. The lubricin mRNA expression in pellets increased at day 7 (Fig.1D). Supplementation of TGF-β3 significantly increased mRNA expression of lubricin, SOX-9, and aggregan in comparison with that of day 0 (P<0.05, n=5) (Fig.1 D). Western blot analysis for the conditioned media showed that lubricin protein expression was higher when cultured with TGF-β3 than when cultured without TGF-β3 (Fig. 1E).

In vivo analysis, four aggregates derived from GFP transgenic rat was transplanted to full-thickness osteochondral defects in the trochlear groove of the femur (Fig.2). Macroscopically GFP positive cells were observed continuously through 12 weeks (Fig. 3A) Histologically at 4 weeks in MSCs group defect was filled with cartilage matrix stained safranin-O and type 2 collagen strongly (Fig. 3B). Contrarily, in the control group, cartilage matrix formation appeared poor. At 12 weeks in the MSCs group, the border between bone and articular cartilage moved upward, and thickness of the regenerated cartilage became to similar to the adjacent one. In the control group, the defect was still filled with fibrous tissue. The Wakitani score in the MSCs group was significantly better than that in the control group at both 4 and 12 weeks (Fig. 3C).

By fluorescent microscopy at 4 weeks, a lot of GFP positive cells were detected in the repaired tissue (Fig. 4). Lubricin expression was observed in the surface of the repaired tissue. A part of cells in the superficial layer were GFP positive and expressed lubricin. In the superficial layer of the repaired tissue, layer thickness and cell density in the MSCs group were closer to those in the normal cartilage than those in the control group (Fig 5A, B). By polarizing microscope observation, brightness of superficial layer in the MSCs group was higher than that in the control group (Fig. 5A).

By TEM at 12 weeks, in the control group, the surface of the articular cartilage was irregular and many fibroblasts were observed in the superficial layer indicated with arrow, while in the normal and MSCs groups, the surface was smooth and flat cells were observed (Fig. 6). The structure and cell morphology in the superficial layer in the MSCs group was closer to normal cartilage than in the control group.

**Discussion:** Transplantation of aggregates of BM-MSCs to osteochondral defect achieved articular cartilage regeneration. This result corresponded to aggregates of synovial MSCs in a rabbit model [5]. BM-MSCs were able to produce lubricin in vitro, and to differentiate superficial cells expressed lubricin in addition to chondrocytes of middle and deep layer in vivo. We demonstrated that MSCs transplantation promoted articular cartilage regeneration including superficial layer.

**Significance:** BM-MSCs were able to produce lubricin both in vivo and in vitro, showing usefulness of MSCs-based therapy for articular cartilage injury.

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