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Granulocyte macrophage – colony stimulating factor (GM-CSF) significantly enhances articular cartilage repair potential by microfracture

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SUMMARY

Objective: To investigate whether granulocyte macrophage-colony stimulating factor (GM-CSF) can be used to increase the number of mesenchymal stem cells (MSCs) in blood clots formed by microfracture arthroplasty (MFX) and whether it can improve the therapeutic outcome for cartilage repair.
Methods: Thirty-six New Zealand white rabbits were divided into four groups: (1) control, (2) GM-CSF, (3) MFX, and (4) GM-CSF + MFX. GM-CSF was administrated intravenously (IV) at 10 μg/kg body weight 20 min before the MFX surgery. The repaired tissues were retrieved and examined by histological observation, quantitative assessment, and biochemical assays at 4, 8, and 12 weeks after treatment. The number of MSCs was measured in the blood clots by the colony forming unit-fibroblast (CFU-F) assay. The kinetic profile and distribution of GM-CSF *in vivo* was also evaluated by near-Infrared (NIR) fluorescence imaging and enzyme-linked immune sorbent assay.
Results: In the histological observations and chemical assays examined at 4, 8, and 12 weeks, the MFX after GM-CSF administration showed better cartilage repair than the one without GM-CSF. The CFU-F assay showed a significantly larger amount of MSCs present in the blood clots of the GM-CSF + MFX group than in the blood clots of the other groups. The blood concentration of GM-CSF peaked at 10 min and decreased back to almost the initial level after a couple of hours. GM-CSF was distributed in many organs including the bone marrow but was not observed clearly in the joint cavity.
Conclusion: Intravenous administration of GM-CSF together with MFX could be a promising therapeutic protocol to enhance the repair of cartilage defects.

Introduction

Microfracture arthroplasty (MFX) is currently the most popular technique among bone marrow stimulation technique (BSTs) that transports endogenous cells from the bone marrow to cartilage defects[1,2], and its mechanism of cartilage regeneration has been examined intensively[3,4]. Shapiro *et al.*[5] showed the importance of bone marrow mesenchymal stem cells (MSCs) in cartilage repair, which depends significantly on the proliferation and differentiation of MSCs. Anraku *et al.*[6] also reported that undifferentiated MSCs migrate into cartilage defects and fill cavities within 4 days of their creation. A possible limitation of MFX is that the repaired cartilage lacks hyaline characteristics, which is possibly caused by insufficient amounts of MSCs retained in blood clots[7,8]. Therefore, efforts to preserve MSCs drained from the bone marrow are very important.

In a previous study, we found that amount of MSCs, which flows out from the subchondral bone, varied by area and amount of holes made by MFX[9]. Many reports have suggested that using biomaterials with MFX can help preserve MSCs in cartilage defects and promote cartilage repair[10–12]. We have also previously developed a thin biomaterial membrane to cover cartilage defects after MFX, which showed a superior result to the original protocol which left

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the defect naked to the joint cavity[13]. Preservation of more MSCs has the potential of increasing the efficiency of cartilage repair. Thus, increasing the outflow of MSCs from bone marrow could be another way to increase MFX's efficacy for cartilage repair. Similarly, studies have added more MSCs to cartilage defects from the outside[12–16]. Other studies have adopted different protocols for the co-treatment of growth factors like platelet-rich plasma, fibroblast growth factor, and chondrogenic inducers such as transforming growth factor-β (TGF-β) with MFX[1,17,18]. However, they still suffer from not having enough therapeutic benefits and/or being a burdensome procedure in terms of time and cost.

Granulocyte macrophage colony-stimulating factor (GM-CSF) has been investigated rigorously as a hematopoietic cytokine mobilizing hematopoietic stem cells (HSCs) for therapeutic transplantation[19–21]. GM-CSF is also known to induce the expression of monocyte chemoattractant protein-1 in monocyte cells[22], mediate the maturation of dendritic cells[23], and activate macrophages[24]. The effect of GM-CSF on the mobilization and proliferation of MSCs is expected but has not been investigated well. Xu *et al.*[25] showed that GM-CSF increases the number of colonies forming unit-fibroblast (CFU-F) from the bone marrow of acute leukemia patients. We have also found that GM-CSF increases the number of CFU-F in rats, both in bone marrow and peripheral blood, when injected intraperitoneally for 5 consecutive days[26]. Similar to the result of Xu *et al.*, our result showed that GM-CSF has a better activity to increase CFU-F than granulocyte colony stimulating factor (G-CSF). We hypothesized that treatment with GM-CSF can increase the number of MSCs and consequently, drain out numerous MSCs through MFX channels. It was expected that the increased MSCs by GM-CSF would eventually improve cartilage repair by MFX. We injected GM-CSF intravenously in a rabbit cartilage defect model, and MFX was performed 20 min thereafter. The number of MSCs in blood clots was measured by CFU-F assay. The effect of GM-CSF on cartilage repair after MFX was evaluated by histologic study.

Methods

Experimental design

The experimental protocol was reviewed and approved by Laboratory Animal Research Center of Ajou University Medical Center's Ethics Committee for Animal Research (Institutional Review Board, IACUC No.2013-0046). Twenty -week-old male New Zealand white rabbits (KOATECH, Pyeongtaek, Korea) were used in this study. Thirty-six rabbits received a full-thickness chondral defect on the trochlear of both the left and right femurs and were divided into four experimental groups ($n = 9$ for each group): (1) control group with no additional treatment, (2) GM-CSF group treated with intravenous (IV) injection of 10 μg/kg GM-CSF (Cha Biotech, Seoul, Korea), (3) MFX group, and (4) GM-CSF and MFX (GM-CSF + MFX) group. Rabbits were sacrificed at 4, 8, and 12 weeks ($n = 3$/week/group), and osteochondral specimens at repair sites were obtained for histomorphochemical analysis. Statistical power of the sample size was approximately 94.6% when calculated by a *post hoc f* test (G-Power software) with an effect size $f = 1$ and error of $\alpha = 0.05$[27].

Surgical procedures

A full-thickness chondral defect was created on the trochlea of both femurs with 5 mm biopsy punch (Miltex, York, USA). A 1.5 mm curette was used to remove focal cartilage tissue and calcified layer in the defect area. MFX was performed with a mini-MFX awl to create three holes on the subchondral bone with a 3 mm depth and 2.0–2.5 mm apart from each other[9,28].

Histological and immunohistochemical analysis

After 4% formalin fixation followed by 5% nitric acid decalcification, femoral cartilage was dehydrated through a series of decreasing alcohol concentrations before being cleaned in xylene ($n = 3$/group/time point). Tissue sections 4 μm in thickness were prepared for histological analyses. Sections were stained with Safranin-O for glycosaminoglycan (GAG), hematoxylin and eosin (H&E) for morphologic analysis, and picrosirius red for total collagen. Sections were also analyzed for type I collagen, type X collagen (Abcam, Cambridge, UK), and type II collagen (Calbiochem, Temecula, CA) by immunohistochemistry. Safranin-O images were histologically evaluated with the 24-point modified O'Driscoll scoring system[29,30].

Biochemical analysis

To measure GAG content, femoral cartilage was freeze-dried and digested with papain-digestion solution. Total GAG content was spectrophotometrically measured with 1,9-dimethylmethylene blue colorimetric assay. Shark chondroitin sulfate was used as a standard[31]. To measure total collagen contents, repaired tissues were freeze-dried, and acid hydrolysis was completed with 4N HCl for 20 min at 110°C. Total collagen content of the samples was measured by chloramine-T hydroxyproline assay[32].

CFU-F assay

Number of MSCs drained into the cartilage defect after MFX was measured by CFU assay. Briefly, 100 μl of bone marrow were harvested from the cartilage defect after MFX. One million mononuclear cells (MNCs) were cultured in a six-well plate and maintained in alpha minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Gibco BRL, NY, USA). Colonies formed after 12 days were stained with 5% crystal violet solution (Sigma–Aldrich, MO, USA) in 100% methanol for 10 min.

Chondrogenic differentiation of human young chondrocytes, MSCs, and osteoarthritis (OA) chondrocytes in pellet culture

All experimental protocols were approved by Ajou University School of Medicine's Ethical Committee (AJIRB-BMR-SMP-14-145). Human chondrocytes (passage 2) were obtained from polydactyly excision tissues and bone marrow MSCs (passage 4) from trabecular bones. OA chondrocytes (passage 2) were obtained at the time of total knee replacement surgery (see Supplementary protocol for details on cell isolation and culture). For the pellet culture, 3×10^5 cells were centrifuged at 500*g* for 10 min in a 15 ml polypropylene tube, and cell pellets were basically cultured in chondrogenic defined medium containing high glucose Dulbecco's modified Eagle's medium (DMEM) with insulin-transferrin-selenium mixture, 50 mg/ml ascorbate 2-phosphate, 100 nM dexamethasone, 40 mg/ml proline, and 1.25 mg/ml BSA at 37°C under 5% CO_2 for 3 weeks. Samples were untreated or treated with 10 ng/ml TGF-β3 (R&D systems, NM, USA) or 0.01, 0.1, and 1 μg/ml GM-CSF (0.01 μg/ml for *in vivo* experiments). All pellets were sampled 3 weeks after culture and evaluated by Safranin-O stain.

Measurement of GM-CSF

After IV-injection of 10 μg/kg GM-CSF, samples were collected from the synovial fluid, bone marrow, and peripheral blood of rabbits after 20 min. For a time course experiment, peripheral blood was collected at 0, 10, and 20 min and 1, 6, 12 and 24 h after