**In situ** recruitment of human bone marrow-derived mesenchymal stem cells for articular cartilage regeneration

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Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs) are good sources of repair since they can migrate directly to the injury site and differentiate to articular chondrocytes. Articular chondral defects do not completely heal due to lack of chondrocytes at the defect site and the insufficient migration of surrounding chondrocytes and BMSCs to the injury site. In this study, the chemotaxis of BMSCs toward selected chemokines which may give rise to a complete regeneration of articular cartilage was investigated.

Materials and Methods

-Treatment of Cytokines-
IL-1β or TNF-α was added to DMEM-low glucose medium without FBS. At 4, 24, and 48 hours of IL-1β or TNF-α treatment, cells were harvested.

-Reverse Dot Blot Hybridization:
Sample DNA of chemokine receptors were spotted onto a nylon membrane. Membranes were hybridized in 3P-dCTP labeled DNA of IL-1β or TNF-α-treated BMSCs. And then, the signals of radiolabeled samples were detected by fluorescent image analyzer. Images of detected signals were quantitated with a densitometer.

-Cell Proliferation and Differentiation Assay-
After serum-starvation of BMSCs, chemokines were treated to BMSCs. DMEM-LG containing 10% FBS and MTT solution were added and incubated for 4 hours. The absorbance of the produced formazan was measured at 570 nm and each measurement was performed in triplicate. To differentiate, the BMSCs were grown in osteogenic or chondrogenic differentiation media for 14 or 21 days.

-Wound Healing Assay and Live Cell Tracking-
BMSCs were grown to confluency in silicon culture-inserts for wound healing assay attached on tissue culture plates. After serum-starvation of BMSCs, culture-inserts were removed. IL-8, SDF-1, MCP-1, MIP-3α, were treated to BMSCs. Migrated cells into wounded area were counted. Cell counting was performed in triplicate by three observers. To calculate the velocity of cell migration, we tracked the migrating cells using microscopy, CCD camera, capture software and image J.

-Chemotaxis Assay-
To analyze migration of MSCs in vitro, a transwell assay with 24-well culture-insert system containing 5μm pores was used. After incubation for 6 hours, the cells were stained with crystalviolet dye. After the unmigrated cells on the membrane were wiped, cell counting was performed in triplicate by three observers. In vivo chemotaxis of human BMSCs was performed with eXplore Optix system. Briefly, After subcutaneous implantation of PLGA scaffolds (3(D)x4(T)mm disk) containing saline or chemokines into the back of nude mice, NIR-labeled human BMSCs (1x10⁶ cells) were tail-injected. Signals of NIR-labeled human BMSCs were detected with eXplore Optix system at every 3 days.

-Histology-
At 6 weeks after operation, inserted scaffolds were closely examined, photographed, and harvested. The scaffolds and adjacent tissues were evaluated for subdermal tissue formation. To confirm the recruitment of the injected human BMSCs and the autologous inflammatory cells in the scaffolds containing chemokines, all specimens were fixed in 10% formalin for 3 days and embedded in paraffin and cut into 5 μm-thick sections. H&E staining, confocal microscopy, and immunofluorescence using CD11b-specific antibody for macrophages and neutrophils, and CD45-specific antibody for B, T cells were performed.

Results

-Increased chemokine receptors and ligands by IL-1β or TNF-α in BMSCs-
To examine the expression of chemokines and cytokine receptors in unstimulated human BMSCs, we performed RT-PCR of 19 chemokine receptors with human BMSCs from three donors. CCR2, CCR6, CCR7, CCR8, CCR10, CXCR1, CXCR5, CXCR7 were expressed in unstimulated BMSCs obtained from three donors. To examine the changes in the expression of chemokine receptors by pro-inflammatory cytokines, we performed reverse dot-blotting with radiolabeled cDNA of human BMSCs stimulated by IL-1β or TNF-α for 4, 24, and 48 hours. In stimulated BMSCs, CCR2 and MCP-1; CCR6 and MIP-3α; CXCR1 and IL-8; SDF-1 were increased.

Collectively, in both RT-PCR and reverse dot-blotting, CCR2, CCR6, CXCR1, and CXCR2 were expressed in unstimulated BMSCs and were increased in stimulated BMSCs by inflammatory cytokines.

The effect of chemokines on cell proliferation and differentiation

To test the effect on the cell proliferation and differentiation, we performed MTT assay and osteo- or chondro-specific staining assay using four chemokines (IL-8, MCP-1, SDF-1 and MIP-3α). According to the results, cell proliferation and osteo- or chondrogenic differentiation of BMSCs were not affected by four chemokines.

Chemotaxis of BMSCs using selected chemokines in vitro and vivo

We examined in vitro chemotactic capacity using MCP-1, MIP-3α, IL-8 and SDF-1 in human BMSCs. In wound healing assay and live cell tracking using four chemokines such as IL-8, MCP-1, SDF-1 and MIP-3α. Cell migration was significantly increased by IL-8 and MIP-3α. In transwell assay, chemotaxis of BMSCs in vitro was significantly increased by IL-8 (3.3 fold) and MIP-3α (3.4 fold). In the animal test, tail-injected human BMSCs were significantly recruited toward IL-8 and MIP-3α in vivo (fig 1).

Figure 1 Chemotaxis of BMSCs in vitro (left) and in vivo (right).

Histological analysis
To investigate the inflammatory reaction, tissue formation and the recruited inflammatory cells and BMSCs, H&E staining and immunohistochemistry assay were performed. There was no inflammatory reaction in all groups, and the tissue formation was significantly increased by fibroblastic cells which are perhaps chemokine-recruited BMSCs (Fig 2). Macrophages and neutrophils were rarely found around blood vessels in all groups. CD45 positive B, T cells were also found around vessels in the scaffolds with MIP-3α a few more than scaffolds with PBS or IL-8. However, there was no meaningful difference among all groups.

Figure 2 H&E staining of harvested scaffolds.

Discussion

ILS and MIP3a were induced recruitment of human BMSCs without inflammation. Thus, both IL-8 and MIP-3α will help us to overcome difficulties of current approaches such as the fibrillation of regenerated cartilage, the loss-of-function of in vitro expanded cells and the limitation of donor sites. Conclusively, it is thought that both IL-8 and MIP-3α assist with the repair of damaged articular cartilage and the development of new treatment for cartilage regeneration.

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