CHEMOKINES OF HUMAN MESENCHYMAL STEM CELLS FOR INJURED-ARTICULAR CARTILAGE REPAIR

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Introduction
Articular cartilage has a limited capacity for self-regeneration after injury. Joint injury is characterized by increased activity of the pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α). Bone marrow-derived mesenchymal stem cells (BMSC) are good sources of repair since they can migrate directly to the injury site and differentiate to articular chondrocytes. The healing process is more effective if the cartilage defect is connected to the bone marrow. However, chondral defects do not heal as effectively due to the lack of sufficient reparative chondrocytes at the defect site and migration of surrounding chondrocytes and BMSCs to the injury site. Therefore, rapid induction of migration of a large number of BMSCs to the injury site can be an effective strategy for cartilage regeneration. Chemokines induce cells to migrate as quickly as in a few hours. The expression of chemokines is also induced by pro-inflammatory cytokines, such as IL-1β and TNF-α. Therefore, discovering the effective chemokines for the recruitment of BMSCs to the cartilage defects is required.

The purpose of this study was to investigate the chemokines for chemotaxis of human BMSCs stimulated by IL-1β and TNF-α as an in vitro injury condition.

Materials and Methods

Cell Culture
Human BMSCs were cultured in DMEM-low glucose medium containing 10% FBS and 1% of antibiotics and the medium was replaced every 3 days.

Treatment of Cytokines
After serum starvation of human BMSCs for 24 hours, human IL-1β or human 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.

RT-PCR
Total RNA was isolated from BMSCs using an RNeasy kit (Qiagen, USA). RT-PCR was performed to evaluate expression of 19 chemokine receptors and their ligands.

Reverse Dot Blot Hybridization
To evaluate changes in expression of 19 chemokine receptors and their ligands, sample DNA of chemokine receptors and chemokines were amplified using RT-PCR and spotted onto a nylon membrane in a Bio-Dot apparatus (Bio-Rad, Italy). Membranes were hybridized in 2×P-dCTP labeled cDNA of IL-1β or TNF-α-treated BMSCs. After washing of membranes, radioisotope-labeled sample signal was detected by fluorescent image analyzer (FLA-7000, Fujifilm). Obtained images of hybridized sample were quantitated with a densitometer.

Cell Proliferation Assay
After serum-starvation of BMSCs, IL-8, SDF-1, MCP-1, MIP-3α were treated to BMSCs from 1 to 4 days at 50–500 ng/mL. Culture medium was removed. Then, new DMEM-LG containing 10% FBS and MTT solution were added and incubated at 37°C for 4 hours. The intracellular formazan was solubilized by DMSO (Sigma). The absorbance of the produced formazan was measured at 570 nm and each measurement was performed in triplicate.

Wound Healing Assay and Live Cell Tracking
BMSCs were grown to confluency in silicon culture-inserts for wound healing assay (Ibidi, Munich, Germany) attached on tissue culture plates in DMEM-LG containing 10% FBS. After serum-starvation of BMSCs, culture-inserts were removed. IL-8, SDF-1, MCP-1, MIP-3α, were treated to BMSCs at 500 ng/mL. Migrated cells into wounded area were counted at 0, 1, 3, 5, 7, 9, 12, 24 and 48 hours. Cell counting was performed in triplicate by three observers. To calculate the velocity of cell migration, we tracked the migrating cells using microscopy (CK-41, Olympus, Tokyo, Japan), CCD camera (KP-D200L, HITACHI, Tokyo, Japan), capture software (TOMORO ver. FG 2.9.19, Olympus) and calculated using image J (Ibidi, Munich, Germany).

In vitro Chemotaxis Assay
To analyze migration of MSCs, a transwell assay with 24-well culture insert system containing 8 µm pores (Falcon, Dickinson and Company, Heidelberg, Germany) was used. Each insert used 10⁶ cells. After incubation for 6 hours, the cells were fixed using cold acetone-MtOH (1:1). After crystal violet staining, Wipe the unemigrated cells on the membrane with cotton brush and stained cells were counted. Cell counting was performed in triplicate by three observers.

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 3 donors. CCR2, CCR6, CCR7, CCR8, CCR10, CXCR1, CXCR5, CXCR7 were expressed in unstimulated MSCs obtained from 3 donors. To examine the changes in the expression of chemokine receptors by pro-inflammatory cytokines, we performed reverse dot-blotting with radioisotopelabeled 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 (Figure 1).

Collectively, in both RT-PCR and reverse dot-blotting, three receptors (CCR2, CCR6, CXCR2) and four ligands (MCP-1, MIP-3α, IL-8, and SDF-1) were expressed in unstimulated BMSCs and were increased in stimulated BMSCs by inflammatory cytokines.

In vitro chemotaxis using selected chemokines in BMSCs
We examined in vitro chemotactic capacity using MCP-1, MIP-3α, IL-8 and SDF-1 in human BMSCs. To investigate the migratory effect of chemokines, we performed cell proliferation assay, wound healing assay and live cell tracking using four chemokines such as IL-8, MCP-1, SDF-1 and MIP-3α. Cell proliferation was not affected by these chemokines. Cell migration was significantly increased by IL-8 and MIP-3α. We also performed in vitro chemotaxis using transwell inserts. In transwell assay, chemotaxis of BMSCs was significantly increased by IL-8 (3.3 fold) and MIP-3α (3.4 fold) compared to control.

Discussion
We have made an in vitro cartilage-injury condition using IL-1β or TNF-α, and investigated on the increased expression of chemokine receptors and their ligands in human BMSCs. Conclusively, we suggest that IL-8 and MIP-3α are useful inducing-factors for injured-articular cartilage regeneration.

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Figure 1 Chemokine expression in human hMSCs

Figure 2 In vitro chemotaxis of BMSCs

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