Adipogenesis of the mesenchymal stromal cells and bone oedema in rheumatoid arthritis

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Abstract

Objectives

Bone oedema is a pathological change in rheumatoid arthritis (RA) that is detectable by magnetic resonance imaging (MRI). Recent histological analyses revealed that a prominent feature of bone oedema is the replacement of adipose tissue with inflammatory cells. Here, we demonstrate the possible roles of mesenchymal stromal cells (MSCs) in bone oedema formation and the pathogenic potential of the cells in RA.

Methods

Adipogenesis of bone marrow-derived human MSCs was induced by a standard adipogenic induction medium in the presence or absence of cytokines. The cytokine productions from MSCs were screened by an antibody array system and confirmed by ELISA. The migration assay was performed to determine the locomotive abilities of undifferentiated MSCs or MSCs after adipogenesis. The expression of α smooth muscle actin (SMA) and F-actin was examined by immunostaining and phalloidin staining, respectively.

Results

TNF-α, interleukin (IL)-1β, IL-6, and TGF-β clearly inhibited the adipogenesis of MSCs. Production of IL-6 was markedly reduced, and IL-8 secretion was augmented in MSCs after adipogenesis. The mobility of MSCs after adipogenesis was clearly reduced in migration assays compared to that of undifferentiated MSCs. Consistent with these findings, SMA and F-actin expressions were clearly suppressed in MSCs committed to adipogenesis.

Conclusion

Our data suggest that the inflammatory milieu promotes bone oedema by blocking adipogenesis of MSCs. In bone oedema, the enhanced IL-6 production and the increased mobility of MSCs may contribute to the progression of RA. Therefore, bone oedema may be an important target lesion in the treatment of RA.

Key words

rheumatoid arthritis, adipogenesis, mesenchymal stem cells, PPAR-γ, lipoprotein lipase, fatty acid-binding proteins 4, F-actin, α-smooth muscle actin

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Introduction

Bone oedema is a pathological change that can be observed by magnetic resonance imaging (MRI) in early and advanced stages of rheumatoid arthritis (RA) (1, 2). Histological examinations of bone oedema revealed that the adipose tissue, which normally occupies the bone cavity, is replaced by inflammatory cells such as monocytes, fibroblasts, and osteoclasts (3). The extent of bone oedema is related to the disease progression of RA (4, 5); however, it is not clear how the lesion emerges in the bone marrow and to what extent it contributes to RA progression.

Mesenchymal stromal cells (MSCs) are specialised cells that can differentiate into mesenchymal lineage cells such as osteoblasts, adipocytes, and chondrocytes (6) and can expand rapidly without losing their multipotency (7). In addition, these cells possess immunosuppressive properties against T cells, natural killer cells, and dendritic cells (8), and it is suggested that MSCs have potential as a therapeutic tool for RA (9). On the other hand, the pathogenic roles of the cells were also implicated in RA (10). Thus, the role of MSCs in RA remains undetermined.

MSCs share many characteristics with fibroblast-like synovial cells (FLSs) in that they are spindle-shaped adherent cells expressing similar cell surface markers and differentiate into mesenchymal multilineage cells (6, 11). Using a synthetic peroxisome proliferator-activated receptor (PPAR)-γ ligand to induce FLSs to form adipocyte-like cells (12), we found that production of cytokines and proteases from FLSs was dramatically reduced (12). These data indicate the relationship between inflammation and adipogenesis of MSCs and these prompted us to investigate the functional change in MSCs through adipogenesis because this may be useful for understanding the pathophysiology of bone oedema in RA.

In the present study, we hypothesised that MSCs are a component of bone oedema, and that MSCs after adipogenesis are regarded as the adipose tissue in the normal bone marrow. On the basis of this hypothesis, we attempted to recapitulate bone oedema *in vitro* by using the adipogenesis induction system in MSCs and questioned whether the inflammatory milieu promotes bone oedema associated with RA progression.

Materials and methods

Adipogenesis of MSCs

Adipogenesis induction of human MSCs was carried out using the MSC differentiation adipogenesis BulletKit (Lonza, MD). Confluent MSCs were cultured in an adipogenic induction medium for 3 days, then cultured in an adipogenic maintenance medium for 2 weeks, with medium replacement every 3 days. In some experiments, MSCs were cultured in the presence of TNF-α (100 ng/ml), IL-1ß (10 ng/ml), IL-6 (100 ng/ml) with IL-6 receptor (100 ng/ml), or TGF-ß (10 ng/ml). The cytokines (purchased from R&D Systems, Inc., MN) were added whenever the medium was replaced (every 3 days). Control MSCs with only supplemented adipogenic maintenance medium were maintained on the same schedule. Lipid accumulation in the cells was visualised using the Lipid Assay kit (Wako Pure Chemical Industries, Ltd., Japan). For quantification of lipids, oil red O was extracted with the extraction buffer from washed and dried stained cells, and its absorbance was determined at 540 nm by Multiscan JX (Lab system).

Polymerase chain reaction

Total RNA was extracted from MSCs by using Trizol reagent (Invitrogen, CA), according to the manufacturer's instructions. For complementary DNA, total RNA (500 ng) was reverse-transcribed using ReverScript III (Wako Pure Chemical Industry, Japan). Specific messenger RNA (mRNA) was quantified by SYBR Green real-time PCR by using the Light Cycler (Roche Diagnostics, Germany). The following primer sequences were used: human *PPAR-γ* (Accession NM_015869.4) sense primer 5´- CTATTGACCCAGAAAGCGATT-3´ and anti-sense primer 5´-CATTACGGAGAGATCCACGGA-3´; human fatty acid binding protein 4 (*fabp4*) (Accession NM_001442.2) sense primer 5´-AACCTTAGATGGGGGTGTCCTG-3´ and

anti-sense primer 5´-TCGTGGAAGTGACGCCTTTC-3´; human lipoprotein lipase (*lpl*) (Accession NM_000237.2) sense primer 5´-ACAAGAGAGAACCAGACTCCAA-3´ and anti-sense primer 5´-AGGGTAGTTAAACTCCTCCTCC-3´; collagen-type I alpha 1 (*col1a1*) (Accession NM_000088) sense primer 5´-TAGGGTCTAGACATGTTCAGCTTTGT-3´ and anti-sense primer 5´-GTGATTGGTGGGATGTCTTCGTH-3´; human ribosomal RNA 18S (Accession NR_003286.2) sense primer 5´-AGGAATTCCCAGTAAGTGCG -3´ and anti-sense primer 5´- GCCTCACTAAACCATCCAA-3´. Ribosomal RNA 18S was used to verify equal loading. Specific amplification by the SYBR Green PCR was confirmed by performing a dissociation curve analysis for each primer pair. Differential gene expression was calculated with the threshold cycle (Ct), and relative quantification was calculated by the comparative Ct method. All experiments were performed in triplicate.

Cytokine production assay

After full induction of adipogenesis, treated cells were washed with PBS, and further cultured with adipogenesis maintenance medium for another 24 h. Supernatants of undifferentiated or adipogenesis-induced MSCs were analysed using the Human Inflammation Antibody Array 3 assay (RayBiotech, Inc., GA). The map of the array can be obtained at http://www.raybiotech.com/map/human_inflammation_G3_map.pdf. IL-6 was measured using a chemiluminescent enzyme immunoassay kit (Human IL-6 CLEIA Fujirebio, Fujirebio, Japan). IL-8 was measured using the IL-8 EASIA kit (BioSource Europe S.A., Belgium).

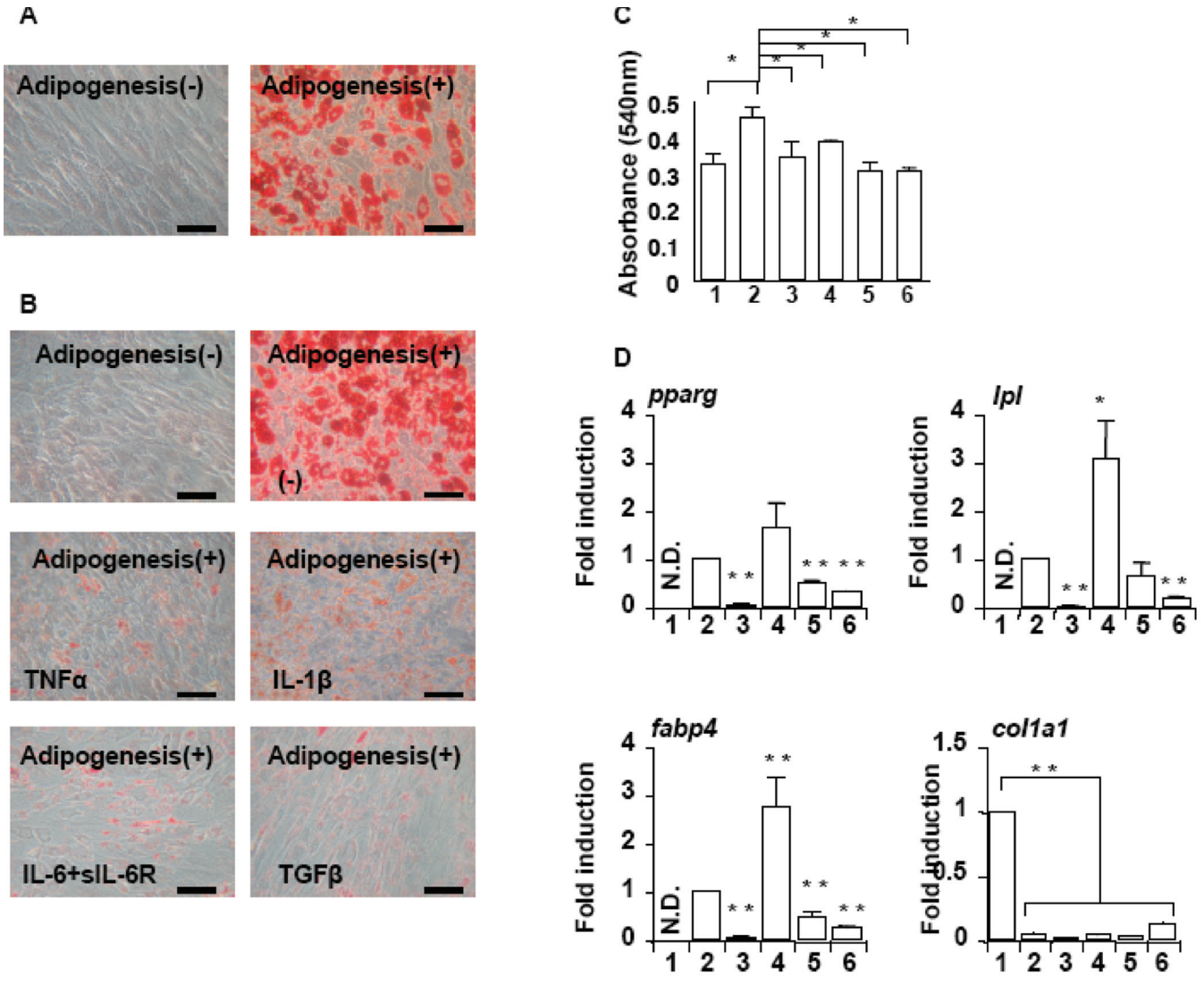

Fig. 1. Cytokines inhibit adipogenesis induction of MSCs. **A)** MSCs were cultured in the medium with (right panel) or without (left panel) adipogenesis induction supplements. Bar=60 μm. **B)** MSCs were cultured for adipogenesis in the absence or presence of TNF-α, IL-1β, IL-6 and soluble IL-6 receptor, or TGF-β. Lipid accumulation in MSCs was visualised by the lipid assay kit. Bar=60 μm. **C)** The quantity of lipids in the MSCs was determined by measuring the absorbance of the oil red O extracted from the cells. 1: non-adipogenesis. 2–6: adipogenesis. 2: No cytokine, 3: TNF-α, 4: IL-1β, 5: IL-6 and soluble IL-6 receptor, 6: TGF-β. *p<0.05. **D)** The expression of adipogenesis gene markers (pparg, lpl, fabp4) and col1a1. 1: non-adipogenesis. 2–6: adipogenesis. 2: No cytokine, 3: TNF-α, 4: IL-1β, 5: IL-6 and soluble IL-6 receptor, 6: TGF-β. *p<0.05, **p<0.01.

Wound-healing assay

Cell migration was assessed by the wound-healing assay (13). Briefly, uninduced and adipogenesis-induced MSCs cultured on 6-well dishes were mechanically damaged using a pipette tip and allowed to migrate. Cells were photographed through an inverted microscope before and after lipid accumulation was visualised using the Lipid assay kit.

Cell staining

MSCs were seeded onto coverslips (Thermo Fisher Scientific, MA), and adipogenesis-induced cells were fixed in 4% paraformaldehyde for 15 min and permeabilised using 100% chilled methanol for 10 min. After rinsing with PBS, the cells were incubated for 1 h with a blocking buffer (5% normal horse serum in PBS containing 0.02% sodium azide). Cells were incubated for 1 h with an anti-smooth muscle actin antibody (DAKO), diluted (1:100) in blocking buffer, followed by 3 washes with PBS, followed by incubation for 1 h at room temperature with Cy2 labelled anti mouse antibodies (Jackson Immunoresearch, ML) diluted to 1:100 in a blocking buffer containing 0.5 μg/ml Hoechst 33258 dye. After washing with PBS, coverslips were mounted in a polyvinyl mounting medium. For F-actin visualisation, phalloidin, alexa flour 488conjugate (Lonza. MD) was used according to the manufacturer's protocol. Cells were viewed and photographed with a BZ-9000 HS BIOREVO (KYENCE, Japan). The images were merged and analysed using Adobe Photoshop (version 11).

Statistical analysis

Data were expressed as mean with standard deviations (SD). Statistical significance was determined using the Student's *t*-test, with a *p*-value of less than 0.05 denoting a statistically significant difference.

Results

Cytokines block MSC adipogenesis

Adipogenesis of MSCs was demonstrated by marked lipid deposition (Fig. 1A). The effect of cytokines on the adipogenesis of MSCs was evaluated by culturing the cells in an adipogenic induction protocol in the presence of TNF-α, IL-1β, IL-6, or TGF-β. As shown in Fig. 1B-C, each cytokine clearly inhibited the adipogenesis of MSCs. As shown in Figure 1D, the adipogenesis gene markers *pparg, lpl,* and *fabp4* became detectable in MSCs after adipogenesis, regardless of cytokine treatment. However, the expression levels of these genes were altered. TNF-α, IL-6, or TGF-β clearly suppressed the mRNA expression of the adipogenesis markers. Interestingly, IL-1β upregulated the *lpl* and *fabp4* mRNA expression levels significantly. The expressions of *col1a1* mRNA were significantly suppressed by adipogenesis induction regardless of cytokine treatment.

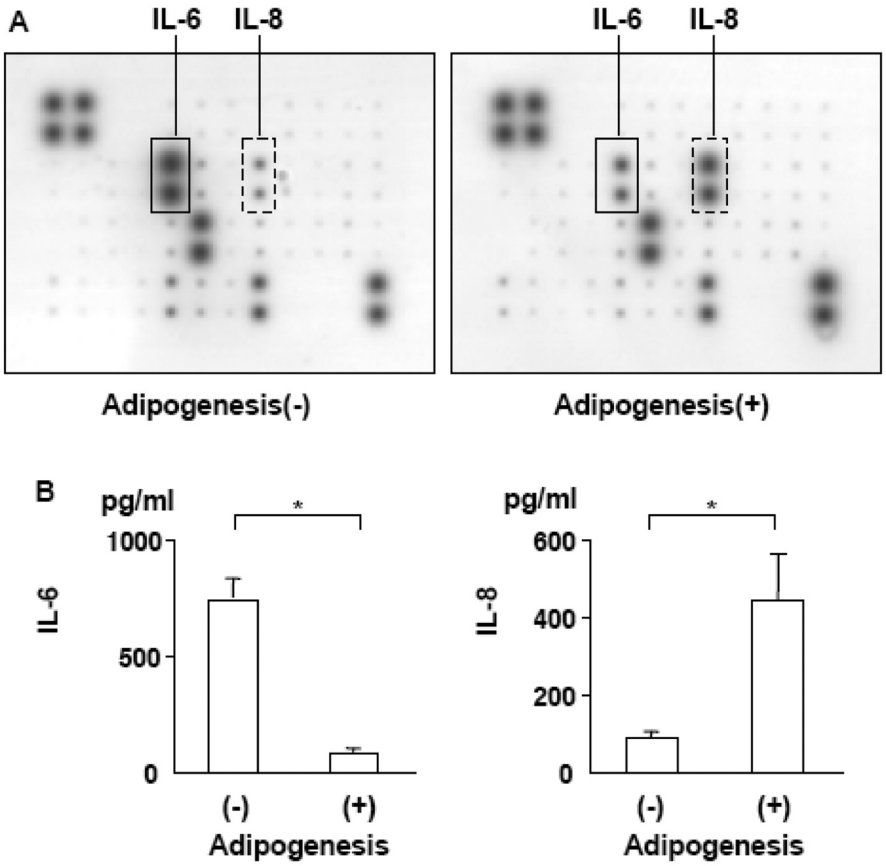

Fig. 2. Comparison of cytokine production in MSCs with or without adipogenesis induction. **A)** MSCs were cultured with (+) or without (-) adipogenesis induction. Supernatants were analysed by the Human Inflammation Antibody Array assay. **B)** The productions of IL-6 or IL-8 from MSCs cultured with (+) or without (-) adipogenesis induction were determined by ELISA. Each value is expressed as mean (SD) of 3 independent experiments. *$p<0.01$.

IL-6 production is inhibited in MSCs after adipogenesis

By comparing the comprehensive cytokine changes in the supernatants from undifferentiated and adipogenesis-induced MSCs using the Human Inflammation Antibody Array assay, we found a marked reduction of IL-6 in MSCs after adipogenesis (Fig. 2A). This finding is consistent with our previous study that showed decreased IL-6 production by FLSs during PPAR-γ ligand-mediated adipocyte-like differentiation (12). Conversely, IL-8 secretion was augmented after adipogenesis in MSCs. IL-6 and IL-8 changes were further confirmed by ELISA (Fig. 2B left and right, respectively).

Adipogenesis and migration ability of MSCs

Fibroblast-like cell invasion in bone oedema is an important pathological feature of RA. Using a wound-healing assay to examine the migration potential of undifferentiated or adipogenesis-induced MSCs, we found that MSCs with lipid accumulation did not migrate

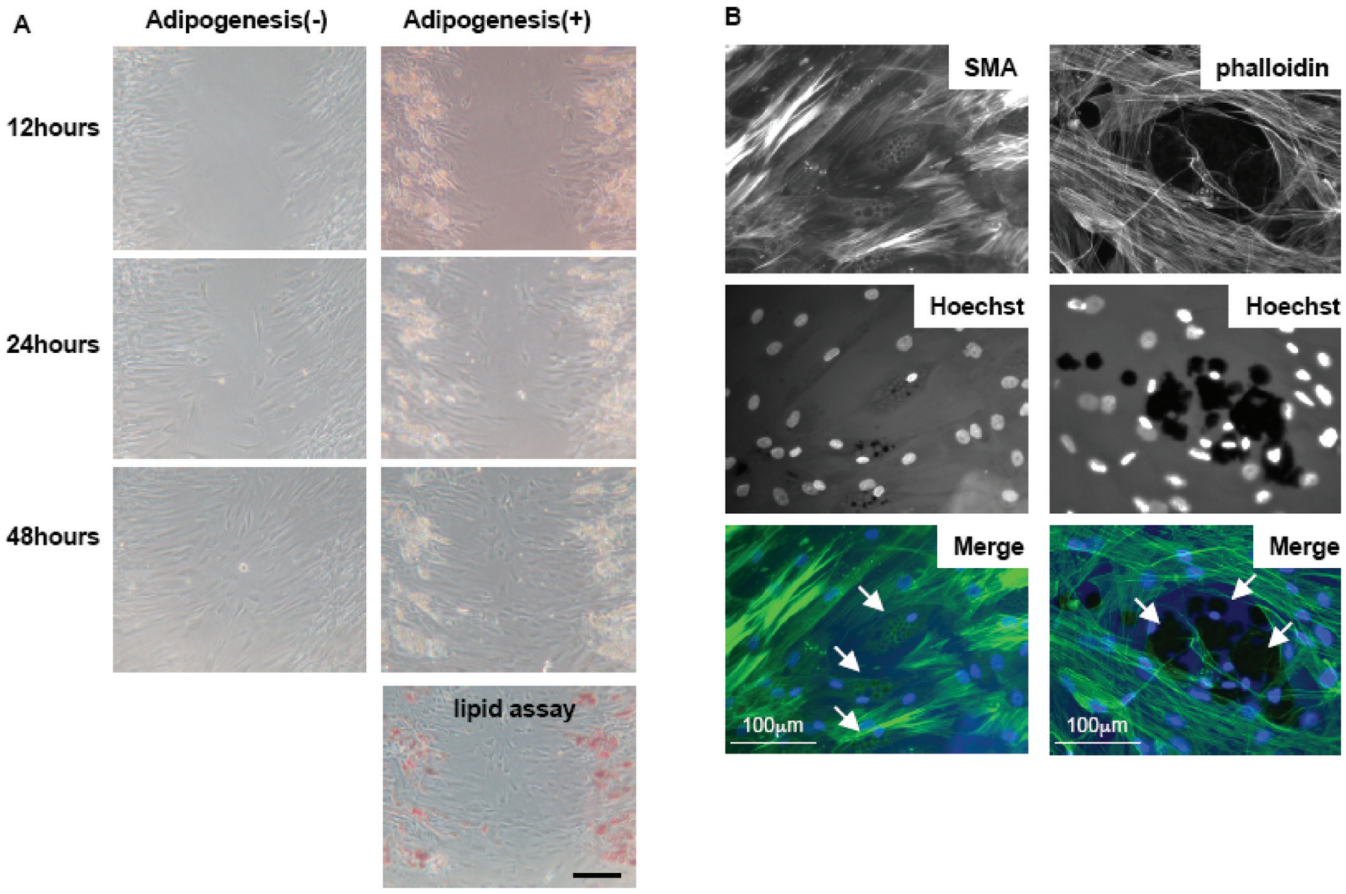

Fig. 3. The migration ability of MSCs after adipogenesis. **A)** Wound-healing assays were carried out as described in *Materials and Methods* by using MSCs cultured in the adipogenesis induction medium. Lipid accumulation was visualised using the lipid assay kit (bottom panel). Bar=200 μm. **B)** Immunostaining of SMA (left) and F-actin labelled using phalloidin (right) in MSCs after adipogenesis (green in merge). Hoechst staining was used to visualise nuclei (blue in merge). The arrows indicate MSCs with lipid accumulation.

into the mechanically damaged space (Fig. 3A). Immunostained α smooth muscle actin (SMA) and phalloidin-labelled F-actin (14) visualised in MSCs after adipogenesis (arrows in Fig. 3B) revealed a pattern that was markedly different from the so-called stress fiber pattern associated with mobility observed in undifferentiated MSCs.

Discussion

This study demonstrates two findings. First, the inflammatory cytokines block the adipogenesis of MSCs (Fig. 1). Second, adipogenesis attenuates the potent pathogenic functions of MSCs such as IL-6 production and cell migration (Fig. 2-3).

The inhibition of adipocyte differentiation by cytokines has been well described (15). Thus, it is plausible that inflammation also blocks adipogenesis of MSCs in the bone marrow to bring about bone oedema as an early lesion of RA. The adipogenesis inhibition by cytokines does not depend on a single pathway because the expression levels of adipogenesis markers in IL-1β treated MSCs were different from the cells treated with other cytokines (Fig. 1C).

IL-6 is a crucial cytokine for the development of RA. Here, we have demonstrated that IL-6 prevents MSCs from differentiating into adipocytes and that undifferentiated MSCs persist to secrete IL-6, suggesting that future *in vivo* experiments exploring the role of IL-6 in the development of bone oedema and RA are warranted.

This study demonstrated that undifferentiated MSCs have a high migration capacity compared to MSCs after adipogenesis. Since inflammatory cells and proliferating synovial cells in inflamed joints are thought to break cortical bone to generate bone oedema (1), we propose that MSCs may be involved in this cortical break since they can vigorously migrate and secrete IL-6. Further basic and clinical research is necessary to examine whether MSCs may be a potential therapeutic target or serve as an indicator for an affirmative treatment in RA.

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