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Mesenchymal stem cells markedly suppress inflammatory bone destruction in rats with adjuvant-induced arthritis

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Mesenchymal stem cells (MSCs) have potential to differentiate into multiple cell lineages. Recently, it was shown that MSCs also have anti-inflammatory and immunomodulatory functions. In this report, we investigated the regulatory function of MSCs in the development of inflammatory bone destruction in rats with adjuvant-induced arthritis (AA rats). MSCs were isolated from rat bone marrow tissues, expanded in the presence of basic FGF, and intraperitoneally injected into AA rats. MSC administration significantly suppressed inflammatory parameters: swelling score, swelling width, and thickness of hind paw. Radiographic evaluation indicated that MSC significantly suppressed bone destruction. Histological analysis showed that administration of MSCs markedly suppressed osteoclastogenesis in AA rats. To further delineate their effects on osteoclastogenesis, MSCs were added to *in vitro* bone marrow cultures undergoing osteoclastogenesis. MSCs significantly suppressed osteoclastogenesis in this system. Chemokine receptor expression in MSCs was assessed by RT-PCR, and a chemotactic assay was performed using a transwell culture system. MSCs showed significant chemotaxis to MIP-1α (CCL3) and SDF-1α (CXCL12), chemokines preferentially expressed in the area of inflammatory bone destruction. Furthermore, MSCs expressed IL-10 and osteoprotegerin, cytokines that suppress osteoclastogenesis. These data suggest that recruitment of MSC to the area of bone destruction in AA rats could suppress inflammatory bone destruction and raise the possibility that MSCs may have potential for the treatment of inflammatory bone destruction in arthritis.

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Mesenchymal stem cells (MSCs) are multipotent progenitor cells that can differentiate into cells of the mesenchymal lineage: osteoblasts, adipocytes, and chondrocytes.[1,2] MSCs also have potential to differentiate into neurons and lung cells.[3] Involvement of MSCs has been identified in various tissues including the bone marrow,[4] adipose tissue,[5] and umbilical cord.[6] In addition to their regenerative capacity, it was recently shown that MSCs have potent ability to modulate immune responses.[7] Because of their ability to suppress immune responses, MSCs have been applied in therapeutic approaches to various autoimmune diseases including graft-versus-host disease,[8] rheumatoid arthritis (RA),[9] systemic lupus erythematosus,[10] periodontitis,[11] and diabetes.[12]

RA is a chronic inflammatory disease, characterized by the severe destruction of articular bones and cartilage, as well as by marked pannus formation and infiltration of significant numbers of inflammatory cells into the synovial membrane.[13] Bone destruction is mediated by osteoclasts, multinucleated giant cells of hematopoietic origin.[14,15] Rats with adjuvant-induced arthritis (AA rats) have been utilized as an experimental model of human RA. A single intradermal injection of complete Freund's adjuvant (CFA) induces arthritis in rats in a highly predictable manner. In particular, severe

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bone destruction is reproducibly observed in the distal tibia and talus, constructing ankle joints.[16]

Chemokines have important roles in a diverse range of biological processes involving chemotaxis, cell activation, differentiation, and survival.[17–19] Macrophage inflammatory protein (MIP-1α/CCL3) has chemotactic activity for monocytes, lymphocytes, dendritic cells, eosinophils, and basophils and is known to activate human granulocytes. MIP-1α is produced by many cells, particularly by macrophages, dendritic cells, and lymphocytes.[20] This chemokine also acts as a chemotactic factor for mature osteoclasts[21] and for osteoclast precursors.[22] We have previously shown that MIP-1α is expressed in human bone tissues and stimulates osteoclastogenesis,[23,24] and we have shown that MIP-1α directly stimulates RANKL-dependent osteoclastogenesis.[25] MIP-1α is highly expressed in the areas of severe bone destruction of the distal tibia of AA-rats.[26] MIP-1α is also involved in pathological osteoclastogenesis associated with multiple myeloma.[27,28] Another chemokine, stromal-derived factor 1 α (SDF-1α/CXCL12), a CXC chemokine having potential ability to support hematopoietic stem cells and progenitors,[29] has the ability to promote migration of inflammatory cells *in vitro*.[30,31] During embryogenesis, it directs the migration of hematopoietic cells from fetal liver to bone marrow and the formation of blood vessels.[32] In adulthood, SDF-1α has an important role in angiogenesis by recruiting endothelial progenitor cells from bone marrow through a CXCR4-dependent mechanism.[33] In bone marrow, SDF-1α is mainly produced by bone marrow stromal cells. SDF-1α is also known to have a role in tumor metastasis; cancer cells expressing CXCR4 are attracted to metastatic target tissues that release SDF-1α.[34]

Here, we investigated the potential regulatory function of MSCs in inflammatory bone destruction in AA rats and examined the possibility that chemokines including MIP-1α and SDF-1α could induce the migration of MSCs to sites of inflammation.

MATERIALS AND METHODS

Preparation of MSCs

Lewis rats were obtained from Kyudo Co. Ltd (Tosu, Japan). Cells were flushed from the femur and tibia of 4-week-old female Lewis rat with α-MEM (GIBCO, Grand Island, NY), and were seeded into laminin-coated dishes (4×10^6 cells/60 mm dish) in α-MEM containing 10% (v/v) FBS (GIBCO, Grand Island, NY) and cultured for 1 week. After changing the medium, cells were cultured in the presence of 3 ng/ml basic FGF (Peprotech, London, UK). When the culture reached 80% confluency, cells were replated into a 100 mm dish. The cells were expanded in the presence of 3 ng/ml basic FGF, and cells of passage number 4 were used for experiments.

Confirmation of Expected Phenotypes of MSCs

Characterization of MSCs was assessed by confirming their potential for osteogenic, adipogenic, and chondrogenic differentiation. Cell surface markers were examined by flow cytometry.

Osteogenic Differentiation

Cells were seeded in 24-well plates (2×10^4 cells per well) and maintained for 28 days in α-MEM supplemented with 10 mM β-glycerophosphate (Sigma, St Louis, MO), 172 μM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma, St Louis, MO), and 10^{-8} M dexamethasone (Sigma, St Louis, MO), followed by staining with alizarin red S (Wako pure chemicals, Osaka, Japan) to detect calcium deposits.

Adipogenic Differentiation

Cells were seeded in 24-well plates (2×10^4 cells per well) and maintained for 21 days in α-MEM supplemented with 10% FBS, 10 μg/ml insulin (Sigma, St Louis, MO), 0.2 mM indomethacin (Sigma, St Louis, MO), 1 μM dexamethasone (Sigma, St Louis, MO), and 0.5 mM 3-isobutyl- 1methyl-xanthine (Sigma, St Louis, MO). After culture, the cells were stained with oil red O (Sigma, St Louis, MO).

Chondrogenic Differentiation

Cells (2×10^5 cells) were centrifuged in 15 ml plastic tubes at 2000 r.p.m., 5 min at 20 °C. Cell pellets were cultured for 4 days in 0.5 ml serum-free α-MEM supplemented with 6.25 ng/ml insulin (BD Biosciences, NJ), 6.25 ng/ml transferrin (BD Biosciences, NJ), 6.25 ng/ml selenite (BD Biosciences, NJ), 2 mM L-glutamine (GIBCO, Grand Island, NY), 10^{-7} M dexamethasone, 284 μM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 25 mM D-(+) glucose (Sigma, St Louis, MO), 100 μg/ml pyruvate (Sigma, St Louis, MO), and 10 ng/ml TGF-β1 (Peprotech, London, UK). Cultures were fed with 1 ml of the same supplemented medium every other day. The cells were collected at various time points up to 28 days and fixed with 4% (w/v) paraformaldehyde overnight. For histological analysis, the cell pellets were frozen in OCT compound and 5 μm sections were prepared. Sections were stained with 0.05% (w/v) toluidine blue (Waldeck GmbH & Co., Munster, Germany).

Flow Cytometric Analysis

After blocking with 3% (v/v) goat serum on ice, 30 min, cells were stained for 30 min on ice with phycoerythrin (PE)-conjugated monoclonal antibodies specific for CD29, CD90, CD45, and fluorescein-4-isothiocyanate (FITC)-conjugated antibody for CD8a (BioLegend, CA), or stained with PE-labeled or FITC-labeled isotype controls (BioLegend). After washing, cells were analyzed on a FACS Calibur (BD Biosciences, NJ) flow cytometer using Cell Quest software.

Culture for Osteoclastogenesis and Co-culture with MSCs

Osteoclasts were formed in a rat whole-bone marrow culture system as described previously.[35,36] In brief, bone marrow cells were obtained from the tibia and femur of 4-week-old

male SD rats. The cells were cultured in 24-multiwell culture plates (1×10^6 cells/ml per well) for forming osteoclast-like multinucleated cells in α-MEM containing 15% (v/v) FBS in the presence of 10^{-8} M 1α,25(OH)2D3 and 10% (v/v) heat-treated conditioned medium derived from the rat osteoblastic cell line ROS 17/2.8 (ht-ROSCM). After culture of bone marrow cells for 1 day, MSCs were added to each well with or without transwell membranes. At 4 days of culture, transwell membranes (upper wells) were removed, and the cells in the lower chambers were stained for tartrate-resistant acid phosphatase (TRAP) using an acid phosphatase staining kit (Sigma, St Louis, MO).

Induction of Adjuvant Arthritis in Lewis rats and Administration of MSCs

Adjuvant arthritis was induced as described previously.[26] Five-week-old female Lewis rats were anesthetized with diethyl ether and were intradermally injected at the base of the tail with complete Freund adjuvant (CFA) containing 25 mg/kg heat-killed *M. butyricum* (Difco Laboratories) suspended in mineral oil. In control experiments, rats were injected with mineral oil alone. MSCs (5×10^6 cells/rat) were injected intraperitoneally at the same time as the CFA injection.

Clinical Assessment of Arthritis

Evaluation of AA severity was performed every 3–4 days beginning on Day 7 after CFA injection. The arthritic severity in each paw was evaluated on a scale ranging from 0 to 4: 0, paws with no swelling and focal redness; 1, paws with swelling of finger joints; 2, paws with mild swelling of ankle or wrist joints; 3, paws with severe inflammation of the entire paw; and 4, paws with maximum swelling. The cumulative score for all four paws of each rat (maximum score = 16) was used as the polyarthritis index.

Histological Analysis

After fixation by perfusion with 4% (w/v) paraformaldehyde/phosphate buffer (pH 7.4), tissue blocks were taken from the hind paws (talus and tibia) of rats and immersed in the same fixative for overnight at 4 °C, followed by washing in PBS overnight at 4 °C. After decalcification in 10% (w/v) ethylene diamine tetra-acetate trisodium salts for 3 weeks at 4 °C and dehydration in an ethanol series, tissue blocks were embedded in paraffin. Sections were prepared for Hematoxylin/eosin (H/E) staining and TRAP staining.

Micro-Computed Tomographic Bone Analysis

Bone samples were analyzed by micro-computed tomography (micro CT) using a Skyscan 1076 scanner (Sky scan, Kontich, Belgium). A total of 222 slices (9 μm) were taken, which was equivalent to a 2 mm segment of spongy bone from the distal end of the tibia. Scanning was conducted at 48 kV and 200 μA to obtain the best contrast of bone and soft tissue. Data sets were reconstituted into three-dimensioned images using InstaRecon/NRecon software (Skyscan). Density values for soft tissue and bone tissue were calibrated according to the manufacturer's protocol. The bone volume/total tissue volume (BV/TV), bone surface/total volume (BS/TV), trabecular number (Tb.N), and trabecular separation (Tb.Sp) were calculated. The level of bone destruction was also analyzed using a soft X-ray system (SOFRON: SRO-M 50, Sofron Inc., Tokyo, Japan).

RT-PCR

Total RNA was prepared from cultured cells or frozen (in liquid nitrogen) tissue powder from ankle joints of hind paws using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. After synthesizing cDNA from the total RNA using of oligo dT primers, PCR reactions were performed using TPersonal (Biometra, Göttingen, Germany). The PCR primers used were as follows: rat CCR1 forward, 5′-ATG AGA GCC TGA AGA CGT GGA A-3′; rat CCR1 reverse, 5′-GGA TTG ACA CAA CAG TGG GTG TAG-3′; rat OPG forward, 5′-TCC GGA AAC AGA GAA GCA AC; rat OPG reverse, 5′-CAC TGC TTT CAC AGA GGT CA-3′; rat CCR2 forward, 5′-CTA CTC AGG AAT CCT CCA CA-3′; rat CCR2 reverse, 5′-GGA GAG ATA CCT TCG GAA CT-3′; rat CCR3 forward, 5′-CTG CCT CCA CTG TAT TCC CT; rat CCR3 reverse, 5′-AGC CAG GTA CCT GTC GAT TG-3′; rat CXCR4 forward, 5′-CAA GCA AGG ATG TGA GTT CGA G-3′; rat CXCR4 reverse, 5′-GCG TAA GTG TTA GCT GGA GTG A-3′; rat TGFβ1 forward, 5′-GCC CTG GAT ACC AAC TAC TG-3′; rat TGFβ1 reverse, 5′-GAC CCA CGT AGT AGA CGA TG-3′; rat IFNγ forward, 5′-TTC ATG AGC ATC GCC AAG TTC GAG-3′; rat IFNγ reverse, 5′-TGA CAG CTT TGT GCT GGA TCT GTG-3′; rat IL-10 forward, 5′-AGA CCA GCA AAG GCC ATT CC-3′; rat IL-10 reverse, 5′-CAA GGC TTG GCA ACC CAA GT-3′; rat MIP-1α forward, 5′-TCA GCA CCA TGA AGG TCT CCA-3′; rat MIP-1α reverse, 5′-GCT GCC TCT AAT CTC AGG CAT T-3′; rat SDF forward, 5′-CGC TCT GCA TCA GTG ACG GTA A-3′; rat SDF reverse, 5′-GGA AAG TCC TTT GGG CTG TTG TG-3′; rat GAPDH forward, 5′-CCC AAT GTA TCC GTT GTG GAT CTG-3′; rat GAPDH reverse, 5′-GTG GTC CAG GGT TTC TTA CTC CTT-3′. PCR products were amplified using the following parameters: 94 °C for 30 s, 60 °C for 30 s, 68 °C for 1 min. PCR products were subjected to electrophoresis on 2% (w/v) agarose gels and visualized by ethidium bromide staining with UV light illumination.

Transwell Migration Assay

Migration assays were carried out by using a transwell culture system involving inserts with membrane having 8 μm pores (BD Biosciences, NJ). MSCs (6×10^5 cells) were inoculated in 100 μl into the upper chamber. In the lower chamber, chemokines (SDF-1α, MIP-1α, or MCP1) were added as the chemoattractant. After culture for 10 h, the upper membrane surface was scraped gently with a swab. The membranes were fixed in 4% (w/v) paraformaldehyde for 15 min and stained with hematoxylin for 20 min. The number of migrated cells

per unit area was determined by counting five randomly selected fields of the membrane under a microscope at a magnification of $\times 100$.

Western Blotting

Western blot analysis was performed mainly as described previously.[37] Ten micrograms of proteins was subjected to 13% (w/v) SDS–PAGE followed by transfer to nitrocellulose sheets. After blocking in 5% (w/v) nonfat dry milk in TBS for 4 h at room temperature, these sheets were reacted with an anti-mouse OPG antibody for 4 h at room temperature. After rinsing in TBS-0.05% (v/v) Tween 20, protein bands were detected with anti-rabbit IgG conjugated with horse radish peroxidase followed by detection with ECL-plus system (GE Healthcare Life Sciences, Uppsala, Sweden). Fluorescence illuminating bands were detected by using X-ray autoradiography film (GE Healthcare).

Ethics Committee Approval

All animal experiments were performed according to the guidelines for 'Care and use of Animals of Kyushu University'.

Statistical Analysis

Data are presented as the mean ± s.e.m. Statistical analysis was performed using Student's *t*-test. Data shown are representative results from three or more independent experiments.

RESULTS

Characterization of basic FGF-Dependent Bone Marrow Stromal Cells

To determine whether MSC-like cells isolated from rat bone marrow in the presence basic FGF are real MSCs, we analyzed expression of cell surface markers and capacity for multidifferentiation. Flow cytometric analysis showed that MSC-like cells expressed nonhematopoietic markers CD90 (95.9%) and CD29 (79.8%) but not hematopoietic makers CD31 (0.3%) or CD45 (0.1%) (Figure 1a). In addition, fluorescence microscopic analysis showed that MSC-like cells were intensely stained for CD90 and moderately stained for CD29 but almost negative for CD31 and CD45 (Figure 1b). We next examined multidifferentiation potential of these cells. After culturing the MSC-like cells in osteogenic medium for

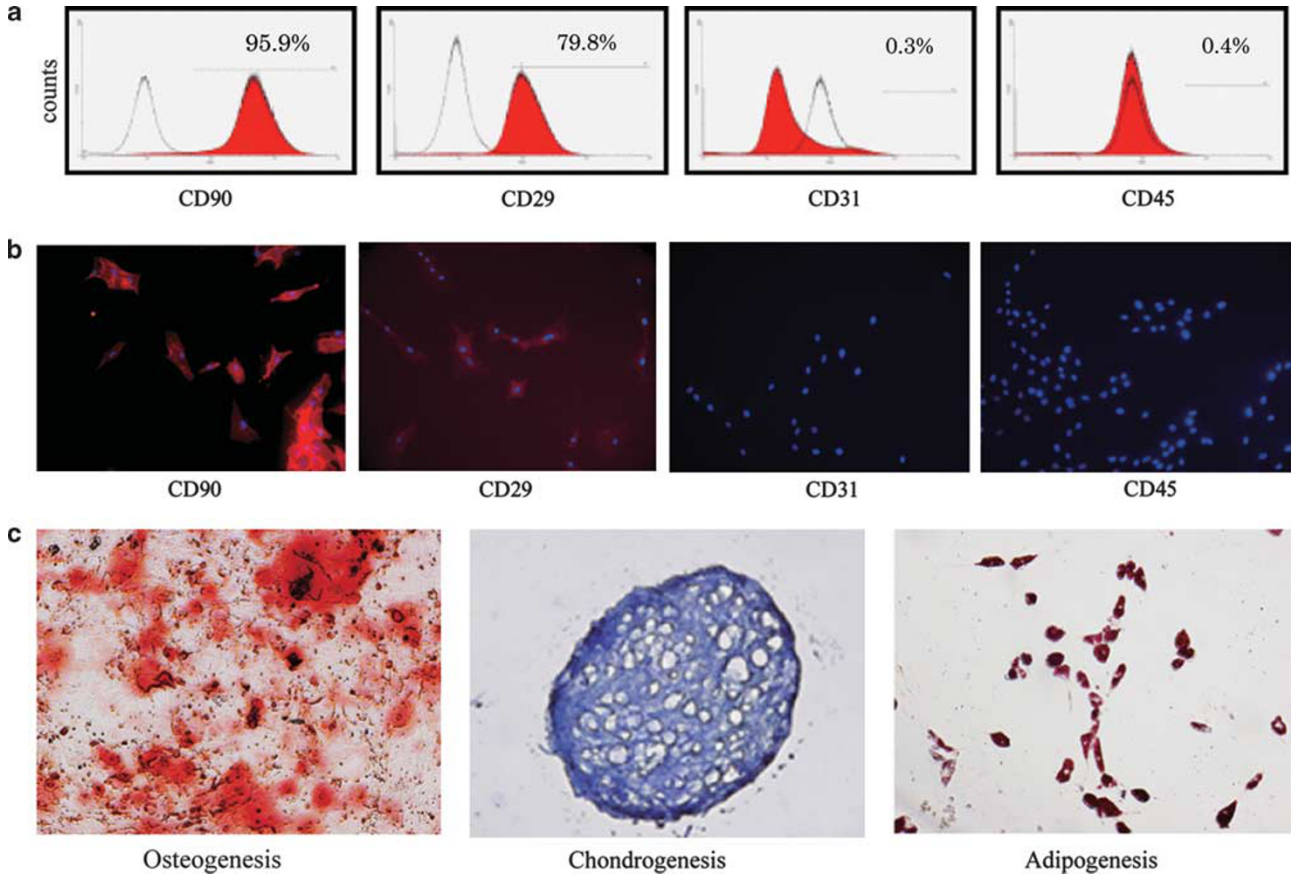

Figure 1 Characterization of rat MSCs. (**a**) Examination of cell surface markers. Trypsinized MSCs were stained with the PE- or FITC-conjugated antibody specific to CD90, CD29, CD31, or CD45, followed by flow cytometric analysis. Dotted line: isotype control. (**b**) Fluorescence microscopic observation of MSCs. Cultured cells were stained with an antibody specific to each antigen and observed by fluorescence microscopy (red fluorescence). Nuclei were stained with DAPI (blue fluorescence). (**c**) Differentiation of MSCs into multiple lineages. Culture conditions for differentiation into each lineage are described in Materials and Methods. Left panel: osteogenesis, center panel: chondrogenesis, right panel: adipogenesis.

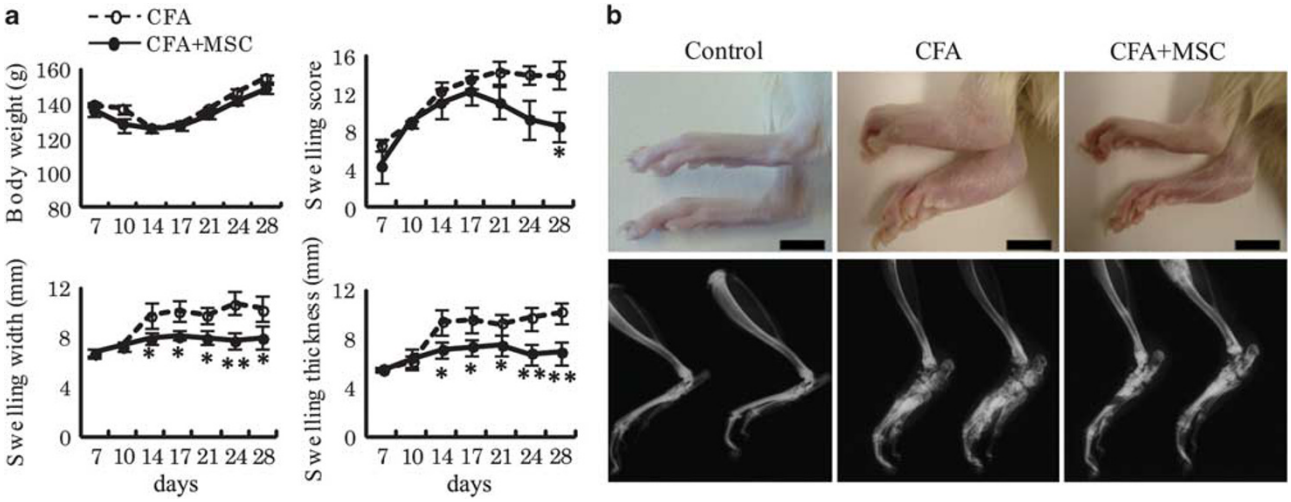

Figure 2 Suppressive effect of MSCs on AA rats. Adjuvant arthritis was induced in Lewis rats by injection of complete Freund's adjuvant (CFA). MSCs were administered to AA rats (CFA + MSC) as described in Materials and Methods. (**a**) Suppression of inflammation parameters by MSCs. Swelling score of the fore and hind paw (**a**, upper right), swelling width and thickness (**a**, lower left and right, respectively) and the body weight (**a**, upper left) were measured every 3 or 4 days from day 7 to day 28 after CFA injection (X-axis). Open circles: Rats injected with CFA only. Closed circle: Rats injected with CFA and MSCs; $n = 8$ from 4 rats, $^{*}P < 0.05$, $^{**}P < 0.01$. (**b**) Marked suppression of bone destruction by MSCs. Upper panels: Suppression of swelling of the ankle joints by MSCs. Bars 10 mm. Lower panels: Radiograms. At 28 days after CFA injection, animals were euthanized, and limbs were fixed and excised followed by X-ray analysis. Control: rats treated only with mineral oil, CFA: rats treated only with CFA. CFA + MSC: rats treated with CFA and MSCs.

4 weeks, numerous cell aggregates having calcium deposits were observed by Alizarin Red staining (Figure 1c left panel). In adipogenic medium, fat droplets stained with Oil red O were formed in MSCs after culture for 3 weeks (Figure 1c right panel). Pellets of MSC-like cells were cultured in chondrogenic medium for 4 weeks, followed by staining with toluidine blue. Apparent chondrogenesis was observed (Figure 1c center panel). Thus, the MSC-like cells isolated from rat bone marrow in the presence of basic FGF fulfilled the criteria for mesenchymal stem cells, and we utilized the term 'MSC' to indicate these basic-FGF-dependent cells obtained from bone marrow.

Administration of MSCs Ameliorates Development of Arthritis and Suppresses Bone Destruction in AA Rats

To observe the regulatory effect of MSCs on the development of arthritis, rats were injected with CFA to induce AA with or without administration of MSCs. Although administration of MSCs did not significantly affect body weight loss observed in CFA-injected rats, significant suppression was observed in inflammation parameters including swelling score, swelling width, and swelling thickness (Figures 2a and b). Radiographic analysis of the hind paws at 28 days after CFA injection showed a marked suppression of bone destruction in the distal tibia in the MSC-injected group, whereas a remarkable extent of bone destruction was observed among animals not receiving MSCs (Figure 2b). In histological analysis, CFA-injected animals exhibited a marked infiltration of inflammatory cells, deformity of ankle joints, an increase in the thickness of synovial membrane, and severe bone destruction (Figure 3a). These symptoms were significantly suppressed by administration of MSCs. To evaluate formation and localization of osteoclasts, sections were stained with TRAP. Although numerous osteoclasts were observed in large areas of the distal tibia in the CFA-injected group, only a limited number of osteoclasts were observed in the distal metaphyseal area of tibia in MSC-injected animals (Figure 3b); a similar outcome was observed in control rats injected with vehicle only.

Quantitative Demonstration of MSC-Mediated Suppression of Bone Destruction by Micro CT

We used micro CT to quantitatively examine bone structure and trabecular bone mass of the distal tibia. Significant bone destruction was apparent in bone comprising ankle joints of AA rats; however, in contrast, MSC-injected animals showed almost normal morphology (Figure 4a). Quantitative analysis indicated that administration of MSCs significantly increased trabecular bone mass (BV/TV) and reduced trabecular bone space Tb.Sp in comparison to these parameters in rats injected only with CFA (Figure 4b). These *in vivo* data suggest that administration of MSCs significantly suppressed osteoclast-mediated bone destruction.

MSCs Inhibit Osteoclast Differentiation *In Vitro*

In order to determine if the suppressive effect of MSCs on osteoclastogenesis observed *in vitro* is a consequence of a direct interaction of MSCs with osteoclast precursors, MSCs were cocultured with osteoclast precursors in osteoclastogenic culture conditions. Osteoclast formation was markedly suppressed by the addition of MSCs. Significant inhibition

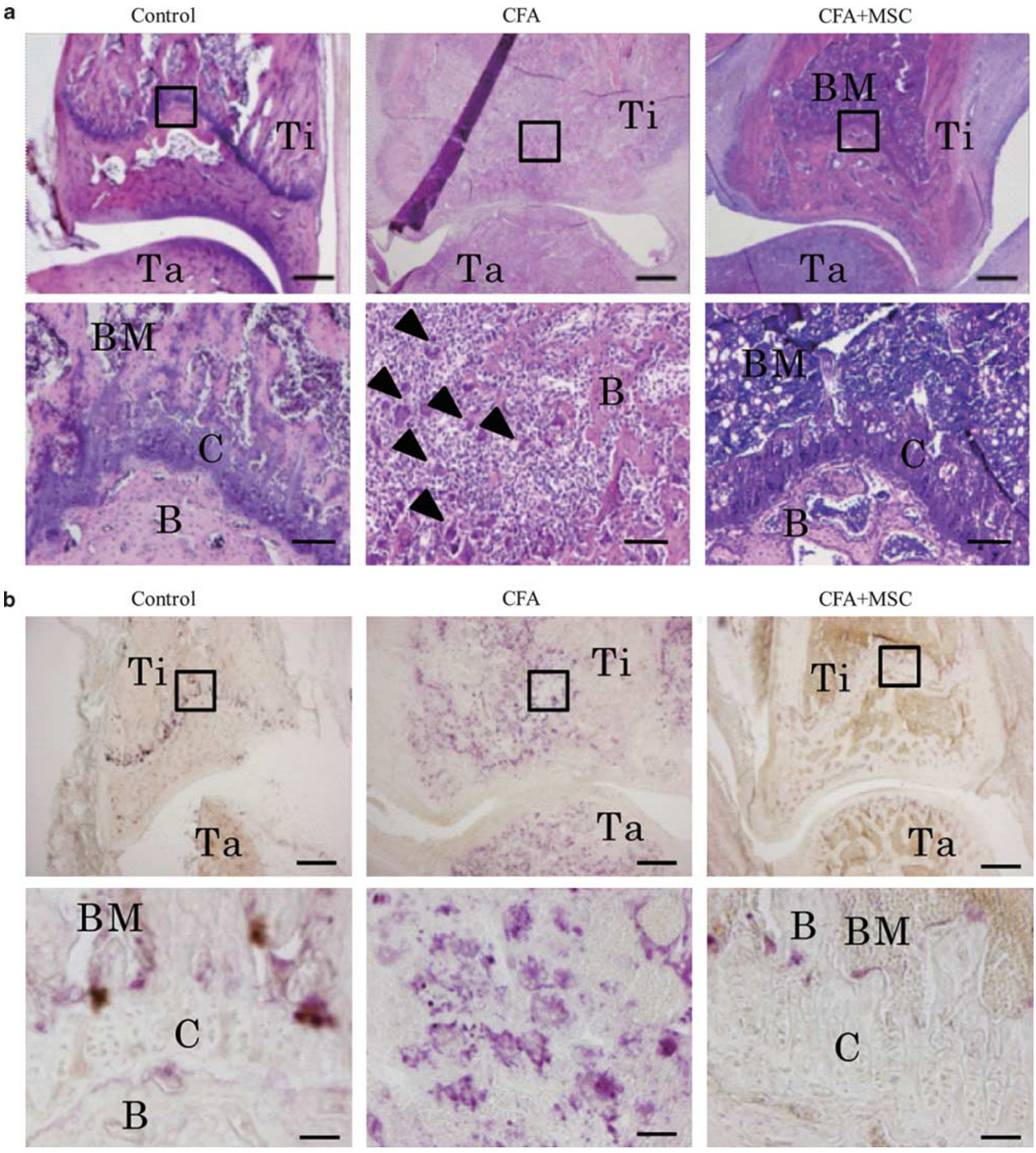

Figure 3 Marked suppression of bone destruction and osteoclastogenesis by MSCs in AA rats. Histological samples were obtained from ankle joints of rats utilized in Figure 2. (**a**) H/E staining of the ankle joints. Control: rats injected only with Mineral oil. CFA: rats injected with CFA. CFA + MSC: rats injected with CFA and MSCs. Arrow heads: osteoclasts. Ti: tibia, Ta: talus, B: bone, BM: bone marrow; Bars 300 μm. (**b**) TRAP staining of ankle joints. Control, CFA, and CFA + MSCs were as described above. B: bone, BM: bone marrow, C: cartilage. Bar 50 μm. Red stained cells are osteoclasts. Lower panels (in **a** and **b**) show a higher magnification of the squared areas in upper panels. Data represent the typical photographs among four rats.

was observed following addition of as few as 100 MSCs (Figure 5a) when MSCs were directly cocultured with bone marrow cells. In indirect cocultures using transwells, osteoclastogenesis was suppressed by the addition of 1×10^3 MSCs (Figure 5b). Expression profiling of cytokines in MSCs (Figure 5c) revealed mRNA for TGF-β, OPG, and IL-10 but

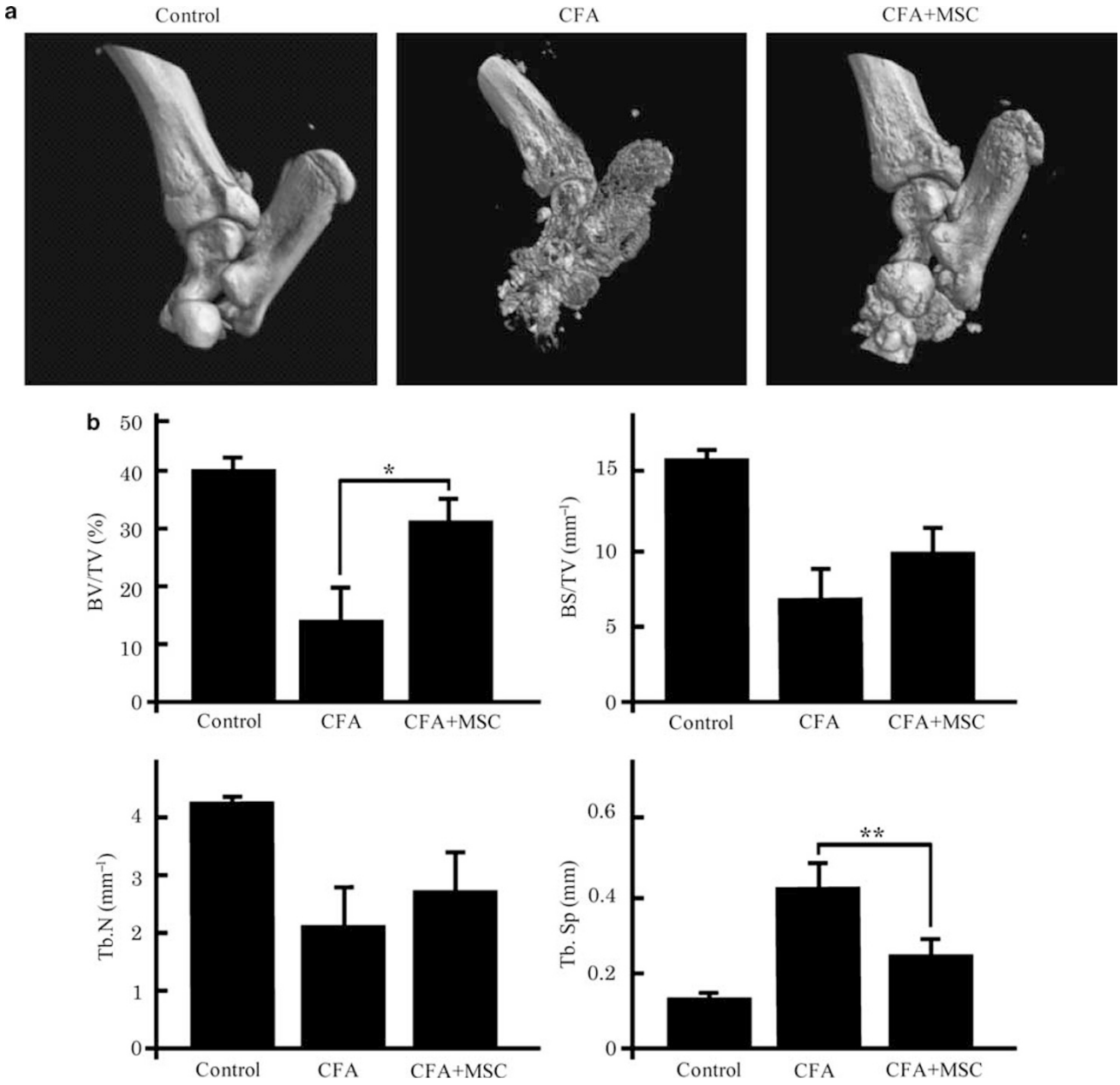

Figure 4 Marked suppression of bone destruction by MSCs revealed by micro CT analysis of ankle joints of AA rats. Control: rats injected only with mineral oil. CFA: rats injected with CFA. CFA + MSC: rats injected with CFA + MSC. (**a**) Three-dimensional view of ankle joints reconstituted from micro CT data. (**b**) Quantitative analysis of trabecular bone mass in the distal tibia by micro CT; $n = 6$ (joints) (from 3 rats), $^{*}P < 0.05$, $^{**}P < 0.01$.

not IFN-γ, which have been reported to regulate osteoclastogenesis. Expression of OPG protein in MSCs was confirmed by use of western blotting (Figure 5d). These data suggest that the suppressive activity of MSCs on osteoclastogenesis is likely mediated by these cytokines secreted from MSCs.

Assessment for Migration Potential of MSCs

MSCs are thought to have potential to migrate to inflammatory areas, and we have previously demonstrated high expression of MIP-1α in sites of bone destruction in AA rats.[26] Before assessing chemotaxis by MSCs, we confirmed expression of chemokine receptors by RT-PCR. MSCs expressed several chemokine receptors including CCR1, CCR3, and CXCR4, but not CCR2 (Figure 6a). To assess the possibility that MSCs are able to migrate to the site of bone destruction in AA rats, ankle joint tissues were excised and RNA was extracted. Semi-quantitative RT-PCR analysis of AA rat ankle joints showed expression of mRNA for MIP-1α (CCL3; CCR3 ligand) and SDF-1α (CXCL12; CXCR4 ligand) (Figure 6b). Administration of MSCs did not affect expression levels of

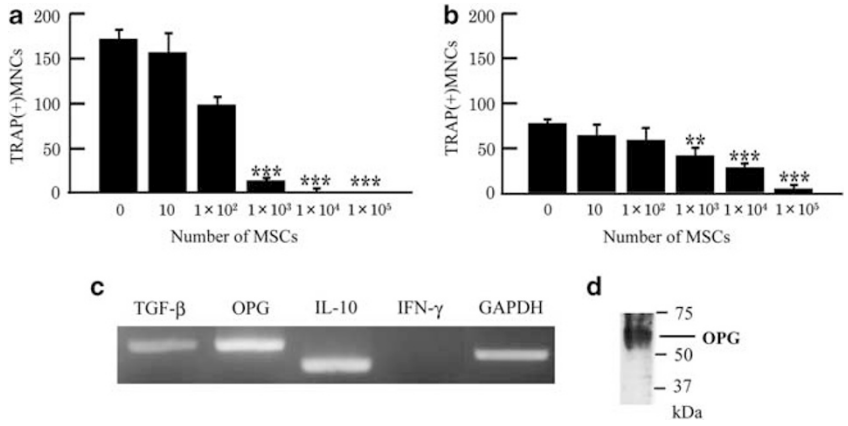

Figure 5 Inhibition of osteoclastogenesis by MSCs evaluated in coculture system. (**a**) Bone marrow cells were cultured for forming osteoclasts in the presence of various numbers of MSCs. (**b**) MSCs and bone marrow cells were cultured in a transwell culture system. MSCs were plated in the upper chamber; bone marrow cells were plated in the lower chamber. After 4 days of culture for osteoclastogenesis, osteoclasts were stained for TRAP and compared with controls cultured in the absence of MSCs. Data are a representative example of three independent experiments; $^{**}P<0.01$, $^{***}P<0.001$. (**c**) Expression of inhibitory cytokines in MSCs. Total RNA was extracted from cultured MSCs followed by analysis by RT-PCR. (**d**) Expression of OPG protein in MSCs. Cell lysates were obtained from rat MSCs followed by western blot analysis.

these chemotactic factors in AA rat ankle joints. We performed *in vitro* migration assays and found that MSCs exhibited significant chemotaxis against MIP-1α (CCR3 ligand) and SDF-1α (CXCR4 ligand), but not MCP1 (CCR2 ligand) (Figures 6c and d). These results correspond to the chemokine receptor expression profile in MSCs.

DISCUSSION

Multiple mechanisms have been reported to be involved in the immunosuppressive properties of MSCs. Both direct cell–cell contact and/or soluble factors have been reported including prostaglandin E2 (PGE2),[38] indoleamine 2, 3-dioxigenase (IDO),[39] inducible nitric oxide synthase (iNOS),[40] transforming growth factor-β (TGF-β),[41] and programmed cell death ligand1 (PDL1).[42] We focused on TGF-β, IL-10, and OPG and confirmed the expression of these cytokines in rat MSCs in the current study. Although TGF-β induces formation of osteoclasts by recruitment of precursors and monocytes from murine bone marrow,[43] TGF-β inhibits osteoclastogenesis in human bone marrow culture systems.[44] Although the role of TGF-β in osteoclastogenesis is controversial, it clearly affects osteoclast differentiation. We have previously shown that IL-10 inhibits osteoclastogenesis in a rat bone marrow culture system.[45] Hong *et al*[46] also reported that IL-10 functions as an inhibitor of osteoclast formation by suppressing IL-6 production in osteoclast precursors. IL-10 released by a new inflammation-regulated lentiviral system efficiently attenuates zymosan-induced arthritis, in which IL-10 released by MSCs acts as a suppressive factor on arthritis.[47] OPG is a well-known decoy receptor for RANKL and has the ability to inhibit osteoclastogenesis.[48] Recently, Oshita *et al*[49] demonstrated that human MSCs express OPG and inhibit osteoclast formation *in vitro*. It may be that combinations of the inhibitory factors IL-10 and OPG secreted from MSCs efficiently suppress osteoclastogenesis. In the current study, osteoclastogenesis was inhibited by coculture with MSCs, even in a transwell culture system. Inhibitory factor OPG and IL-10 are thought to inhibit osteoclastogenesis. In the current work, efficiency of inhibition mediated by MSCs was 10-fold higher in the direct co-culture system in comparison with the transwell culture system. As few as 100 MSCs significantly inhibited osteoclastogenesis in the direct coculture system, suggesting the presence of some putative potent inhibitory molecules on the surface of MSCs. Further studies are required to elucidate such regulators.

MSCs have been shown to have potential to penetrate into inflammatory sites.[50] In the current study, we have demonstrated suppressive activity of MSCs on inflammatory bone destruction associated with adjuvant arthritis *in vivo*. With respect to the destiny of MSCs injected into AA rats, it was hard to trace the trafficking of injected MSC in AA rats as the number of injected MSCs was limited to only 5×10^6 cells per rat.

Although we could not directly detect injected MSCs in joint tissues, we obtained lines of evidence indicating a potent ability of MSCs to be recruited to the sites of inflammation. We demonstrated that MSCs expressed chemokine receptors CCR1, CCR3, and CXCR4, but not CCR2. In accordance with these data, MSCs exhibited significant chemotaxis against MIP-1α (CCL3), the ligand for CCR1, and SDF-1α (CXCL12), the ligand for CXCR4, but not against MCP1 (CCL2), the ligand for CCR2. Furthermore, we demonstrated expression of mRNA for MIP-1α and SDF-1α in the ankle joints of AA rats. As the expression levels of these chemokines were not significantly altered when MSCs were injected into

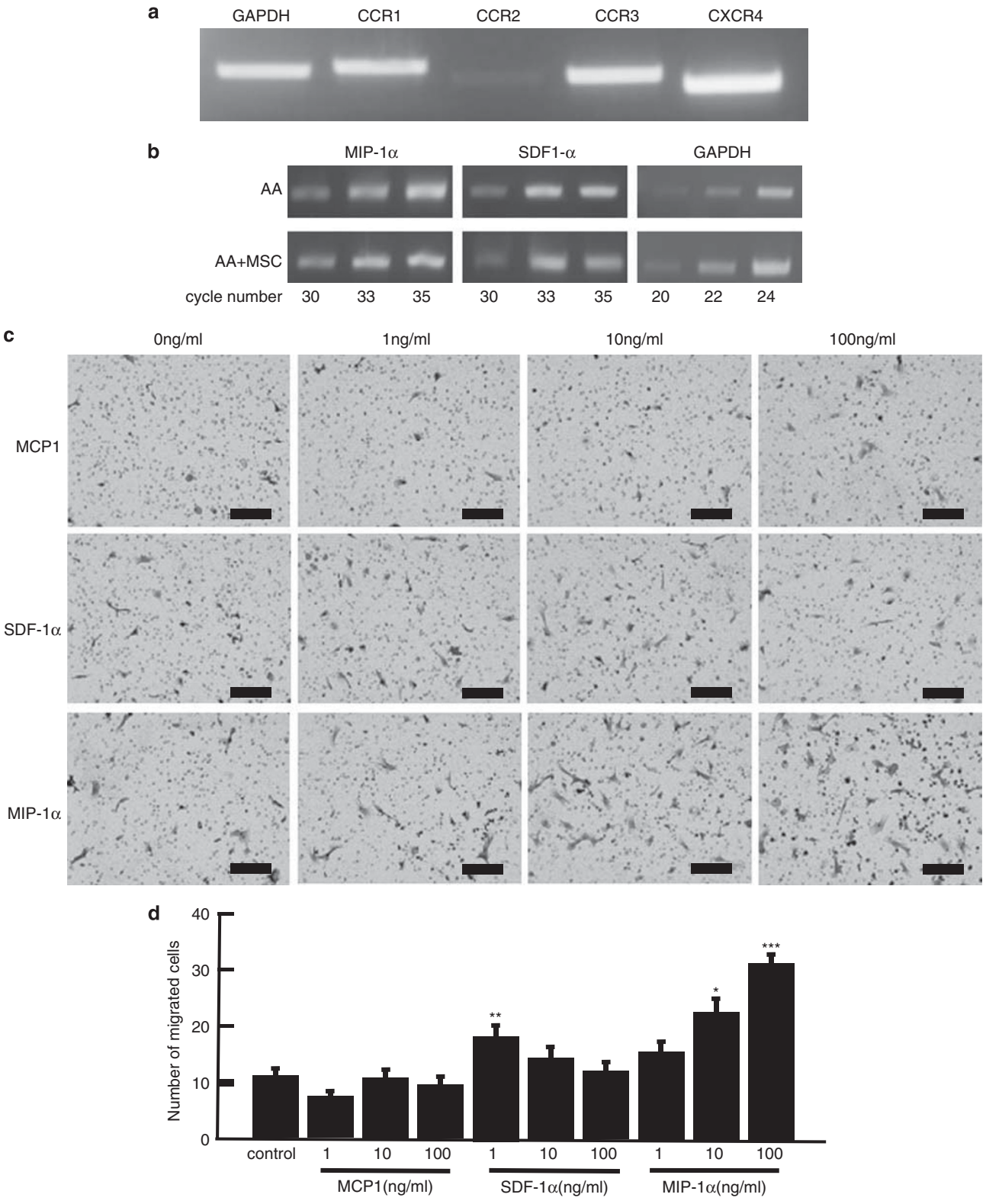

Figure 6 Expression of chemokine receptors in MSCs, and chemotaxis against chemokines. (**a**) Expression of chemokine receptors in MSCs evaluated by RT-PCR analysis. Total RNA was extracted from cultured MSCs followed by RT-PCR. (**b**) Expression of mRNA for MIP-1α and SDF-1α in sites of bone destruction in AA rats. Total RNA was extracted from the frozen ankle joint tissues from three rats per treatment conditions. Similar data were obtained in the duplicate experiments. Semi-quantitative RT-PCR analysis of ankle joints of AA rats with or without MSCs. (**c**, **d**) Chemotactic activity of MSCs against chemokines. MSCs inoculated in the upper chambers of transwell culture plates were cultured in the presence of the indicated chemokine in the lower chambers. After 10 h, migrated MSCs were observed microscopically (**c**) and were counted (**d**); $^{**}P<0.01$, $^{***}P<0.001$; Bar, 200 μm.

AA rats, the continuous expression of MIP-1α and SDF-1α in the inflammatory sites in the MSC-injected rats would contribute to homing of the injected MSCs into the area of inflammation for an extended period of time. Such continuous homing of MSCs might result in efficient inhibition of osteoclastogenesis. In our previous works, we demonstrated that MIP-1α, which is abundantly expressed in the distal tibia, contributes to the efficient recruitment of osteoclast progenitors to the sites of bone destruction in AA rats.[26]

MIP-1α-expressing ED-1-positive macrophages are frequently recruited to the inflammatory area before initiating bone destruction.[26] The chemotactic activity mediated by MIP-1α/CCR1 could partly explain why MSCs are efficiently recruited to the site of inflammation.[51] The biological effect of MIP-1α on MSCs has not been reported. It was assumed that MSCs respond to MIP-1α and SDF-1α through specific receptors and migrate to the inflammatory area of the ankle joints. Park *et al*[52] reported successful amelioration of type-II-collagen-induced arthritis by the administration of TGF-β-transduced MSCs. In their experiments, injected MSCs expressing enhanced green fluorescent protein (GFP) were recruited to the inflamed joints, which was efficiently detected by the anti-GFP antibody. The authors demonstrated the expression of CCR1, CCR4, CCR7, and CXCR4 in MCSs. In our current study, we have detected CCR1, CCR3, and CXCR4 in rat MSCs, suggesting an involvement of chemokines for the specific recruitment of MSCs to the site of inflammation also in our experimental system of AA rats.

SDF-1α and its receptor CXCR4 are also considered to be essential for recruiting hematopoietic stem cells or progenitors from the stem cell niche to the affected site.[29] SDF1-α is associated with cell survival, proliferation, and adhesion. CXCR4 and SDF-1α knockout mice undergo prenatal death due to severe anemia resulting from the failure in bone marrow development.[53] In these models, defects in the movement of hematopoietic stem cells from fetal liver to the bone results in hypoplasia of the bone marrow niche. The ability of SDF-1α to act as a chemotactic factor for MSCs could be important for creating a specialized niche in inflammatory sites.

In conclusion, MSCs suppressed not only inflammation but also bone destruction in AA rats and inhibited osteoclastogenesis, possibly through secreting OPG and IL-10. Chemokine receptors CCR1 and CXCR4 were expressed in MSCs, and their respective ligands, MIP-1α and SDF-1α, were expressed in the hind paws of AA rats, suggesting the potential recruitment of MSCs to the inflammatory sites. The current study thus demonstrates a potential use of MSCs for the treatment of inflammatory bone destruction.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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