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Paracrine effect of inflammatory cytokine-activated bone marrow mesenchymal stem cells and its role in osteoblast function

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Mesenchymal stem cells (MSCs) have a crucial function in bone regeneration. Inflammation is a well-documented component of the osteogenic microenvironment. In the present study, we investigated whether stimulation of MSCs with inflammatory cytokines promotes osteogenesis through a paracrine mediator. MSCs were pre-stimulated with the inflammatory factors IFN-γ and TNF-α. After pre-stimulation, the MSC secretion levels of IL-6, HGF, VEGF, and TGF-β were significantly elevated ($p < 0.01$); however, the production of IL-2, IL-4, and IL-10 was not changed ($p > 0.05$). MG63, an osteoblast-like cell line, was cultured in different MSC-conditioned media. After treatment with conditioned media collected from MSCs pre-treated with cytokines, the proliferation and migration of MG63 cells were significantly improved, and the expression levels of the osteoblast differentiation markers ALP, COLI, OCN and OPN were significantly increased as revealed by a quantitative PCR analysis ($p < 0.05$). Furthermore, an immunofluorescence staining assay showed that more MG63 cells were OPN-positive, while an Alizarin red staining indicated the increased formation of calcium nodules in the IFN-γ and TNF-α combined pretreatment group. The results indicated that conditioned medium from inflammatory cytokine-activated MSCs can significantly promote osteoblast proliferation, migration, differentiation, and mineralization and ultimately enhance osteogenesis through paracrine mechanisms. These findings present a new direction for the clinical application of MSCs in the repair of bone defects.

[**Key words:** Bone marrow mesenchymal stem cells; Inflammatory cytokine; Osteogenesis; Paracrine; Bone regeneration]

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that can be potentially differentiated into a variety of cell types, including osteoblasts, adipocytes, chondrocytes, tenocytes and skeletal myocytes (1–3). *In vitro* investigations demonstrate that MSCs grow into adherent fibroblast-like cells that typically express CD44, CD73 and CD90 on their surface, with the absence of CD34, one type of hematopoietic marker (4). MSCs can be easily isolated from bone marrow, adipose tissue and umbilical cords and can be expanded *in vitro* while retaining their differentiation ability.

The capacity to secrete protective biologically active factors places MSCs among the most suitable tools for tissue regeneration (5). Other advantages that make MSCs suitable for therapy include their convenient isolation, trophic activity, lack of immunogenicity or ethical controversy (6), and potential to differentiate into specific cell types (7). Thus, therapies using MSCs have been widely used in the fields of regenerative medicine, cell therapy and immune modulation.

Interestingly, studies using cell tracking in tissue repair indicate limited new tissue formation by the differentiation of MSCs; instead, the newly formed cells are mostly retrieved from other precursor cells (8,9). A growing body of evidence supports the hypothesis that paracrine mechanisms mediated by cytokines released by stems cells function significantly in the reparative process (10–12). MSCs have been reported to exhibit immunosuppressive ability; however, this ability is not manifested under normal environments but rather requires the activation of inflammatory factors such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α (13–15). MSCs mediate immune responses through immunosuppressive factors, which also influence the surrounding precursor cells. Thus, the pre-treatment of MSCs with inflammatory cytokines may mimic their original paracrine effects.

In a healthy body, trauma is usually followed by inflammation, and inflammatory cells and factors are commonly present in injury sites. There are many inflammatory cytokines released at sites of injury, including TNF-α, IFN-γ, interleukin (IL)-1, IL-4 and IL-6 (16,17). Among them, IFN-γ has been reported as a key cytokine in the immunosuppressive function of MSCs. TNF-α plays an important role in bone healing by affecting MSC behavior in a dose–dependent manner (18,19); low dose (20 ng/mL) was shown to upregulate osteogenesis (19). In the present study, we investigated the possible role of TNF-α and IFN-γ in the paracrine effects of MSCs *in vitro*.

To date, many studies in the bone regeneration field focus on the paracrine effects of MSCs or the effect of inflammatory cytokines on MSC behavior, so in the present study, we chose IFN-γ and TNF-α to explore the effects of immunomodulatory-activated MSCs on osteoblasts. The results indicate that the paracrine effects of immunomodulatory-activated MSCs significantly enhance the ossification capability of osteoblasts.

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MATERIALS AND METHODS

Cell culture The human osteogenesis cell line MG63 was obtained from the Institute of Orthopaedics, the First Affiliated Hospital of Soochow University, and cultured in Dulbecco's modified essential medium (DMEM) with low glucose (Hyclone, Logan, CT, USA) supplemented with 10% fetal bovine serum (Hyclone), 1% glutamine and 1% penicillin–streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Isolation of primary human mesenchymal stem cells Human bone marrow was collected from unrelated donors with informed consent according to the Declaration of Helsinki. The donors were all healthy and not receiving any medication. Primary human MSCs were obtained from bone marrow mononuclear cells by density gradient centrifugation using Histopaque-1077 solution (Sigma–Aldrich). Mononuclear cells were cultured in 100-mm tissue-culture dishes at a density of 2×10^6 cells per dish in DMEM-low glucose medium for 48 h. After incubation, non-adherent cells were removed by medium exchange. At sub-confluency, cells were continuously passaged.

Flow cytometry and differentiation capacity of MSCs Cells within passages 3 to 7 were used in the experiments. Flow cytometry was performed to analyze cell surface markers. Cells were detached with EDTA, incubated with the monoclonal antibodies FITC-CD34, PE-CD44, PE-CD90 and PE-CD73 (BD Biosciences), and analyzed by flow cytometry (FACSCalibur, BD Biosciences). Differentiation assays *in vitro* were performed according to the induction system of the osteogenic and the adipogenic lineages. Osteogenic differentiation was induced in an osteogenic medium (OM), consisting of low-glucose DMEM supplemented with 10% FBS, 50 mg/mL ascorbic acid, 10 mM β-glycerophosphate and 0.1 nM dexamethasone (Sigma). Adipogenic differentiation was induced in an adipogenic medium (low-glucose DMEM supplemented with 10% FBS, 100 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 200 μM indomethacin, and 10 μg/ml insulin). Each differentiation medium was changed every 2–3 days. Confirmation of the differentiation of the cells to adipocytes and osteocytes involved staining with oil-red O and alizarin red, respectively.

Preparation of conditioned medium The MSCs were divided into four groups: (i) control group: without inflammatory cytokines; (ii) IFN-γ group: pre-treated with IFN-γ (20 ng/mL); (iii) TNF-α group: pre-treated with TNF-α (20 ng/mL); and (iv) I+T group: pre-treated with IFN-γ and TNF-α (20 ng/mL each). After incubation for 24 h with or without inflammatory cytokines, the medium was removed, and fresh culture medium was added. Concentrations of cytokines were chosen after optimization studies (data not shown). After culturing for another 24 h, the MSC-conditioned medium was collected and filtered by 0.22-μm membranes.

Analyses of MSC-conditioned media The expression levels of transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) in MSC-conditioned media were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D System). IL-2, IL-4, IL-6, and IL-10 were determined by cytometric bead array (CBA, BD Biosciences). Assays were performed in duplicate, and readings were compared with standard curves obtained with standard proteins provided with the kits.

Cell proliferation assay A colorimetric assay (CCK-8, Beyotime) was used to determine cell proliferation. MG63 cells were seeded in a 96-well plate and incubated with 50% MSC-conditioned medium and 50% fresh DMEM (10% FBS). The conditioned media from non-inflammatory cytokine pre-treated MSCs were used as the control. After culturing at 37°C for 1, 3, or 5 d, cells were treated with CCK-8 reagent (Beyotime), and the optical density was monitored with a spectrometer at wavelength of 450 nm. Each experiment was performed in triplicate.

Wound healing assays A scratch assay was used to determine the wound healing potential of MG63 cells. MG63 cells were seeded onto a 6-well plate. At sub-confluence, a cell-free gap approximately 500 μm in width was created on the osteoblast monolayer. The cells were then incubated with different MSC-conditioned media or fresh DMEM (10% FBS). The conditioned media from non-inflammatory cytokines pre-treated MSCs were used as a control. After 48 h, cells were fixed in 4% paraformaldehyde and stained with acridine orange (Sigma). To quantify the number of migratory cells, fluorescence photographs were captured with a digital camera coupled to an inverted microscope (Olympus FV500). The number of migratory cells was counted using Image-Pro plus Software. The results represent the means of three independent assays.

Measurement of calcium accumulation MG63 cells were loaded on a 6-well culture plate containing different MSC-conditioned media or fresh DMEM at a density of 1×10^5 cells per well. The conditioned media from the non-inflammatory cytokines pre-treated MSCs were used as a control. After 24 h, the media were removed, and the cells were fixed with 4% glutaraldehyde. Then, the cells were stained with Alizarin red S (Sigma, pH 8.3) and photographed using an optical microscope. The integrated optical density (IOD) was determined to assess the extent of mineralization in the cells.

Quantitative PCR MG63 cells were seeded in 6-well plates at an initial density of 1×10^5 cells/well and cultured with various MSC-conditioned media or fresh DMEM (10% FBS) for 7 days. The conditioned media from non-inflammatory cytokines pre-treated MSCs were used as a control. Total RNA was extracted using Trizol reagent (Invitrogen). Total RNA (1500 ng) was reverse-transcribed to cDNA using a Prime Script First Strand cDNA Synthesis Kit (Thermo) according to the manufacturer's instructions. The sequence of primers is listed in Table 1. Thermocycler conditions included an initial hold at 50°C for 2 min and then 95°C for 10 min. A two-step PCR program with the following conditions followed: 95°C for 15 s and 60°C for 60 s, repeated for 40 cycles while data were continuously collected and quantitatively analyzed. SYBR quantitative real-time PCR was performed using an ABI 7500 sequence detector according to the manufacturer's instructions; data were normalized against the Ct (threshold cycle) of the GAPDH control. The expression level of mRNA is presented as fold-changes relative to the control.

Immunofluorescence staining MG63 cells were seeded on a 12-well plate with coverslips and cultured with different MSC-conditioned media. After treatment, the MG63 cells on coverslips were fixed with 4% glutaraldehyde and permeabilized with 0.2% Triton X-100. Then, fixed cells were incubated with an anti-osteopontin antibody (OPN, Boster) and a FITC-conjugated secondary antibody. Cell nuclei were stained with 4,6-diamino-2-phenylindole (Invitrogen). The images of the stained cells were examined by a confocal laser scanning microscope (Olympus FV500).

Statistical analysis Data were analyzed using GraphPad Prism 4 software and are presented as the mean ± S.E. Student's *t*-test was used to compare the mean values of two groups. Data from three or more groups were compared using one-way analysis of variance followed by Dunnett's post hoc test. A p value of <0.05 was considered statistically significant.

RESULTS

Characterization of human MSCs Primary human MSCs were isolated from the bone marrow of healthy donors. In culture, these cells formed small colonies and had a spindle-shaped appearance (Fig. 1A). In flow cytometry, the MSCs expressed high levels of CD44 (99.8%), CD73 (91.6%) and CD90 (96.5%) but were negative for CD34 (Fig. 1B). When cultured in a defined differentiation medium, the primary MSCs had the potential for osteogenesis and adipogenesis (Fig. 1C, D). The results indicated that the primary cells isolated from bone marrow were characterized as MSCs with differentiate potentials.

Modulation of cytokine and growth factor expression in MSCs by pre-stimulation with the inflammatory cytokines IFN-γ and TNF-α Using ELISA and flow cytometry, it was demonstrated that MSCs secreted various cytokines and growth factors and that the secretion levels were increased when MSCs were pre-treated with IFN-γ and TNF-α. As shown in Fig. 2, the levels of VEGF and IL-6 were significantly elevated in MSCs pre-treated with IFN-γ and TNF-α, either alone ($p < 0.05$) or in combination ($p < 0.01$), over those in MSCs without inflammatory cytokine pre-treatment; the I+T group was significantly higher than other groups ($p < 0.01$). A similar change was found in the HGF level ($p < 0.05$). However, the levels of TGF-β were only increased in the culture medium of MSCs pre-treated with both IFN-γ and TNF-α ($p < 0.01$). Notably, the levels of IL-2, IL-4, and IL-10 did not change among the non-inflammatory cytokines control and the MSCs pre-treated with a single or combined inflammatory cytokines ($p > 0.05$).

TABLE 1. The sequence of primer used in real-time PCR.

Gene name	Sense	Antisense	Product size (bp)
ALP	CCTCGTTGACACCTGGAAGA	CTGTTCAGCTCGTACTGCATGT	150
OCN (BGLAP)	GAGGGCAGCGAGGTAGTGA	ATGTGGTCAGCCAACTCGTC	140
COLI	GTGGAAACCCGAGCCCTGCC	CAGACGGGACAGCACTCGCC	129