Curcumin inhibits osteoclastogenic potential in PBMCs from rheumatoid arthritis patients via the suppression of MAPK/RANK/c-Fos/NFATc1 signaling pathways

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Abstract. The aim of the present study was to determine the effects of curcumin on the osteoclastogenic potential of peripheral blood mononuclear cells (PBMCs) obtained from patients with rheumatoid arthritis (RA), and to investigate the underlying molecular mechanisms. PBMCs from patients with RA (n=12) and healthy controls (n=10) were cultured to assess osteoclastogenic potential. The number of tartrate-resistant acid phosphatase-positive osteoclasts differentiated from PBMCs isolated from patients with RA was significantly increased compared with that of the healthy controls. In addition, the osteoclast number in patients with RA was correlated with the clinical indicators, Sharp score (r=0.810; P=0.001) and lumbar T-score (r=-0.685; P=0.014). Furthermore, the resorption area was increased in the RA group compared with the healthy controls. The mRNA and protein expression levels in PBMC-derived osteoclasts treated with curcumin were measured by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. Curcumin inhibited the osteoclastogenic potential of PBMCs, potentially by suppressing activation of extracellular signal-regulated kinases 1 and 2, p38 and c-Jun N-terminal kinase, and inhibiting receptor activator of nuclear factor κB (RANK), c-Fos and nuclear factor of activated T cells (NFATc1) expression. The results of the present study demonstrated that curcumin may inhibit the osteoclastogenic potential of PBMCs from patients with RA through the suppression of the mitogen-activated protein kinase/RANK/c-Fos/NFATc1 signaling pathways, and that curcumin may be a potential novel therapeutic agent for the treatment of bone deterioration in inflammatory diseases such as RA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by abnormal immune responses, chronic inflammation of peripheral joints and progressive bone destruction (1,2). Under the pathological conditions of RA, bone homeostasis is in persistent disequilibrium, resulting in uncoordinated bone formation and degradation, and is mediated by abnormal osteoclast formation (3). Osteoclasts are derived from osteoclast precursor cells (OPCs), which originate from the monocyte/macrophage lineage. Previous studies have demonstrated that the osteoblast-associated synthesis and secretion of receptor activator of nuclear factor κB ligand (RANKL) and osteoclast-associated RANK are essential in osteoclastogenesis, and that RANK on monocytes binds to RANKL, initiating osteoclast differentiation (4,5). Furthermore, macrophage-derived proinflammatory mediators in RA directly contribute to the degradation of articular cartilage and subchondral bone, and the activation of RA monocytes/macrophages occurs locally and in the peripheral circulation (6,7). Therefore, the present study investigated the osteoclastogenic potential of peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis patients.

Curcumin is the primary active ingredient of turmeric (Curcuma longa) and has been demonstrated to possess anti-inflammatory and anti-arthritis properties (8). Previous studies have revealed its underlying mechanisms of action as the induction of apoptosis in human fibroblast-like synoviocytes and protection against collagen-induced arthritis (9,10). A randomized pilot study in patients with RA indicated that curcumin treatment is safe and is not associated with any adverse events (11). Accumulating evidence suggests that signaling pathways malfunction in RA (12). Curcumin has been demonstrated to mediate the suppression of mitogen-activated protein kinases (MAPKs)/RANK and extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathways and thus promotes chondrogenic differentiation (13,14). Furthermore, curcumin inhibits osteoclast differentiation and function via the inhibition of the signalosome-associated kinase inhibitor of κB in a dose-dependent manner (15). Therefore, the present study aimed to investigate the effect of curcumin on osteoclastogenesis of PBMCs from patients with...
RA via the suppression of the MAPK/RANK/c-Fos/nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) signaling pathways.

**Materials and methods**

**Patients and PBMC culture.** A total of 12 patients and 10 healthy controls were recruited from the Department of Integrated Traditional Chinese and Western Medicine, Jinling Hospital (Nanjing, China), and written informed consent obtained. The study was approved by the Ethics Committee of the Department of Integrated Traditional Chinese and Western Medicine, Jinling Hospital. Human blood samples from the patients with RA and healthy controls were collected between January and December 2014.

PBMCs were separated from erythrocytes by density centrifugation at 650 × g, 18°C for 20 min, using Ficoll® PM 400 Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA) of blood samples, and were maintained in α-minimal essential medium (α-MEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified 5% CO2 incubator (Thermo Fisher Scientific, Inc.). The medium was replenished every second day.

**Osteoclast differentiation.** Isolated PBMCs were seeded onto plates. Non-adherent cells were harvested after 24 h and were seeded (3.5 x 10^5 cells/well in 96-well plates) onto glass coverslips in the presence of 50 ng/ml recombinant human macrophage colony-stimulating factor (rM-CSF; R&D Systems China Co., Ltd., Shanghai, China) and 100 ng/ml rhRANKL (R&D Systems China Co., Ltd.) for 14 days. Osteoclasts with ≥3 nuclei/cell were identified as tartrate-resistant acid phosphatase (TRAP)-positive cells. Osteoclast differentiation was additionally confirmed by the expression of RANK using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To investigate the effect of curcumin treatment, 0-10 μM curcumin was added to wells for the 14 days of osteoclast differentiation.

**Cell viability detection by Cell Counting kit-8 (CCK-8).** PBMCs (1 x 10^5/well) were seeded in 96-well plates (three wells per group) and treated with with curcumin (0-40 μM) for 48 h. Subsequently, 10 μl CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well, and cell viability was measured at 490 nm using an enzyme-linked immunosorbent assay reader (BioTek Instruments, Inc., Winooski, VT, USA), according to the manufacturer's instructions.

**TRAP staining and bone resorption pit assay.** Cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100. Fixed cells were subjected to an assay for TRAP activity using an Acid Phosphatase, Leukocyte (TRAP) kit (Sigma-Aldrich) according to the manufacturer's instructions. Images were captured with a digital camera attached to the microscope (Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany). TRAP positive multinucleated cells (≥3 nuclei) were identified as osteoclasts, and the number of osteoclasts was counted in 10 fields per sample.

PBMCs were seeded onto 100-μm thick bovine bone slices and incubated with α-MEM containing 50 ng/ml M-CSF and 100 ng/ml RANKL. After 21 days, cells were removed by sonication and the bovine bone slices were stained with 0.25% toluidine blue (Sigma-Aldrich) to identify resorption pits. In addition, resorption lacunae were visualized using a Hitachi S-3400N scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

**RT-qPCR.** RNA was extracted from the PBMCs using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Synthesis of cDNA was performed on 2 μg total RNA using Moloney Murine Leukemia Virus reverse transcriptase (Promega Corporation, Madison, WI, USA) and oligo dT 15 primers (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR was performed using the Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Inc.). Reaction mixes (20 μl) were prepared using the TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Following initial denaturation at 95°C for 3 min, 40 cycles were performed of denaturation at 95°C for 15 sec, annealing at 56°C for 20 sec and extension at 72°C for 20 sec. The Cq (quantification cycle fluorescence value) was calculated using SDS software, version 2.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.), and the relative expression levels of RANK mRNA were calculated using the 2^ΔΔCq method (16) and normalized to the internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primer sequences were used: Forward, 5'-CCTCGGGGCTGGAATCGTCCG-3' and reverse, 5'-CTGACACACGATGATGACCTT-3' for RANK; and forward, 5'-CAAGGGAGGTGATAGCTT-3' and reverse, 5'-GACCAAACGCCTTCATACCTC-3' for GAPDH.

**Western blotting.** PBMCs were homogenized and lysed in NP-40 buffer (Beyotime Institute of Biotechnology, Haimen, China). Following 5-10 min boiling, cells were centrifuged at 10,000 x g, 4°C for 10 min to obtain the supernatant. Protein samples (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (EMD Millpore, Billerica, MA, USA). Membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline and 0.1% (w/v) Tween 20 (TBST), and incubated with the following primary antibodies: Rabbit anti-RANK (1:500; sc-9072), mouse anti-c-Fos (1:1,000; sc-27143), mouse anti-NFATc1 (1:1,000; sc-17834), mouse anti-phosphorylated (p)-ERK1/2 (1:1,000; sc-163521), mouse anti-ERK1/2 (1:1,000; sc-514302), rabbit anti-p-p38 (1:1,000; sc-17852-R), mouse anti-p38 (1:1,000; sc-81621), goat anti-p-c-Jun N-terminal kinase (JNK) (1:1,000; sc-12882) and mouse anti-JNK (1:1,000; sc-137019) all from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and rabbit anti-β-actin antibody (1:2,000; catalog no. AP0060; BioWorld Technology, Inc., St. Louis Park, MN, USA), at 4°C overnight. Following three washes with TBST, membranes were incubated with the following secondary antibodies conjugated to horseradish peroxidase: Donkey anti-goat IgG (1:10,000; sc-2020), donkey anti-mouse IgG (1:10,000; sc-2096) and goat anti-rabbit IgG (1:10,000; catalog no. sc-204) from Santa
Cruz Biotechnology, Inc. at a dilution of 1:10,000 -1:20,000. Following a 1-h incubation at 37˚C, membranes were washed three times with TBST. Blots were visualized using an enhanced chemiluminescence system (Amersham; GE Healthcare Life Sciences, Chalfont, UK). Signals were densitometrically assessed using Quantity One® software version 4.5 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to the β-actin signals to correct for unequal loading.

Clinical assessments. The Sharp score of patients with RA was calculated using Multix Select DR X-ray (Siemens AG, Munich, Germany), as described previously (17), from the measurements of arthritis (scale, 0-5) and joint space abnormality (scale, 0-4).

The T-score of Patients with RA was calculated as described previously (18), from the measurement of the bone mineral density of lumbar vertebra L1-L4 by dual-energy X-ray absorptiometry.

Statistical analysis. Data are expressed as the mean ± standard deviation. All statistical analyses were performed using GraphPad Prism software, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Correlations were assessed by Spearman's coefficient rho (ρ) with a 95% confidence interval. Groups were compared using one-way analysis of variance, followed by Tukey's multiple comparison test as a post hoc test to compare the mean values of each group. P<0.05 was considered to indicate a statistically significant difference.

Results

Enhanced osteoclastogenic potential in patients with RA. The osteoclastogenic potential of PBMCs from patients with RA was induced in the presence of 50 ng/ml M-CSF and 100 ng/ml RANKL. A total of three days later, PBMCs were adhesive and narrowly elongated, following six days fibroblast-like cells were significantly increased, and PBMCs were in cell condensation following nine days of culture. Polynuclear giant cells were observed at day 14. (B) Observation of osteoclast morphology in PBMCs from RA patients using TRAP staining following 14 days in culture. Cells were fixed in 4% formalin, permeabilized with 0.1% Triton X-100 and stained with TRAP solution. (C) PBMCs isolated from RA patients and healthy controls were cultured with 50 ng/ml M-CSF and 100 ng/ml RANKL for 14 days and TRAP staining performed. The number of osteoclasts (TRAP-positive cells containing ≥3 nuclei/cell counted in 10 fields of each sample) differentiated from PBMCs from patients with RA was significantly increased compared with healthy controls. Data are expressed as the mean ± standard deviation, n=10 per group. PBMCs, peripheral blood mononuclear cells; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor κB ligand; TRAP, tartrate-resistant acid phosphatase; RA, rheumatoid arthritis; OC, osteoclasts.
patients with RA was significantly increased compared with healthy controls (P=0.002; Fig. 1C). The resorption area was measured as a separate indicator of osteoclast formation. The bone resorption pits on bone slices were stained with toluidine blue, and resorption areas were analyzed by light and electron microscopy. The results demonstrated that the lacunar number and area of bone resorption were increased in the RA group compared with the healthy control group (Fig. 2A and B). These results confirmed the increased osteoclastogenic potential of PBMCs isolated from patients with RA.

**Correlation between osteoclastogenic potential and clinical indicators.** To determine whether there was a correlation between the number of osteoclasts and a clinical indicator of disease, the Sharp score was calculated by assessing articulation in the two hands of patients with RA. The Sharp score was significantly positively correlated with the number of osteoclasts in patients with RA. (B) Linear correlation plot of the osteoclast number and lumbar T-score in patients with RA. A significant negative correlation was detected between the osteoclast number and the lumbar T-score in patients with RA. RA, rheumatoid arthritis; TRAP, tartrate-resistant acid phosphatase; PBMCs, peripheral blood mononuclear cells; OC, osteoclasts.

Curcumin inhibits osteoclastogenic potential of PBMCs from patients with RA. To investigate the cytotoxicity of curcumin, PBMCs isolated from healthy controls were incubated with various concentrations of curcumin for 48 h. The CCK-8 assay revealed that PBMCs had similar viability following treatment with 0-10 µM curcumin for 48 h. However, viability was significantly reduced at concentrations of ≥20 µM (20 µM, P=0.031; 40 µM, P=0.006; Fig. 4A). To evaluate the inhibitory effects of curcumin on the osteoclastogenesis of PBMCs isolated from RA patients, M-CSF and RANKL-treated cells were exposed to concentrations of curcumin <20 µM. The results demonstrated that curcumin treatment inhibited the number of osteoclasts generated in a dose-dependent manner (2.5 µM, P=0.012; 5 µM, P=0.003; 10 µM, P<0.001; Fig. 4B). This reduction...
in osteoclast differentiation following curcumin treatment was confirmed by the dose-dependent reduction in RANK mRNA (2.5 µM, P=0.018; 5 µM, P=0.008; 10 µM, P<0.001; Fig. 4C) and protein (2.5 µM, P=0.035; 5 µM, P=0.009; 10 µM, P<0.001; Fig. 4D) expression levels. c-Fos and NFATc1 are crucial for osteoclast differentiation (5,19). Therefore, it was examined whether curcumin inhibited the osteoclastogenic potential of PBMCs from patients with RA through regulation of the expression of c-Fos and NFATc1 in response to M-CSF and RANKL stimulation. The western
blotting results demonstrated that the protein expression levels of c-Fos and NFATc1 were significantly suppressed in PBMCs from RA patients by curcumin in a dose-dependent manner (P<0.001 at all curcumin concentrations; Fig. 4E). To determine the involvement of signaling pathways and the molecular mechanisms underlying the effects of curcumin on M-CSF and RANKL-stimulated osteoclast differentiation of PBMCs from patients with RA, the activation of MAPKs in PBMCs from RA patients was evaluated. The results indicated that curcumin inhibited the protein expression levels of p-ERK1/2, p-p38 and p-JNK in PBMCs from RA patients in a dose-dependent manner (P<0.001 at all curcumin concentrations; Fig. 4F). These results suggest that curcumin inhibited M-CSF and RANKL-stimulated osteoclast differentiation via intracellular MAPK signaling pathways.

**Discussion**

Curcumin has been demonstrated to possess anti-inflammatory activities in interleukin (IL)-1β-stimulated human chondrocytes (14) and murine macrophages (3). In RA, pro-inflammatory cytokines, including IL-1, -6, -8 and -11 and tumor necrosis factor α, have been reported to be osteoclastogenic (3). However, the role of curcumin in RA and its contribution to the inhibition of the osteoclastogenic potential of PBMCs remains unclear. In the present study, the anti-osteoclastogenic effect of curcumin and its underlying mechanisms were investigated.

Monocytes migrate out of the peripheral blood and into inflammatory tissue where they differentiate into resident macrophages and dendritic cells, which secrete a variety of inflammatory cytokines involved in the pathogenesis of RA (20,21). In the present study, PBMCs from patients with RA were isolated to investigate the systemic enhancement of osteoclastogenesis in RA. The results confirmed the increased osteoclastogenic potential of PBMCs isolated from patients with RA compared with healthy controls. Furthermore, increased numbers of mature osteoclasts were observed in PBMC cultures from patients with RA compared with healthy controls. These results suggest that PBMCs may contribute to osteoclast formation in the presence of osteoclastogenic cytokines or RANKL in RA patients. Therefore, RA may have direct effects on bone metabolism. Ikić et al (7) demonstrated that PBMC-derived OPGs transdifferentiate into osteoclasts in the presence of M-CSF and RANKL in vitro, suggesting that inflammatory factors may directly contribute to osteoclastogenesis. The results of the present study confirm a number of previous studies (22,23), simultaneously, the Sharp score was positively correlated with osteoclast number, and lumbar T-score was negatively correlated with osteoclast number.

Previous studies indicate that inflammatory cytokines enhance osteoclastogenesis via a RANKL–RANK dependent mechanism, upregulating the expression levels of RANK on osteoclast precursors and increasing their sensitivity to RANKL, which may result in bone erosion in RA (3,24,25). In the present study, curcumin inhibited the number of osteoclasts generated from PBMCs in a dose-dependent manner, and reduced the RANK mRNA and protein expression levels in PBMCs from patients with RA. It has been previously demonstrated that RANKL activates multiple signaling pathways in osteoclast precursors via RANK and stimulates critical transcription factors for osteoclast differentiation (26,27). NFATc1 is a crucial transcription factor that is expressed in osteoclast precursors through Ca²⁺ oscillation, MAPKs and c-Fos or RANK in response to RANKL (28). In the present study, curcumin inhibited the osteoclastogenic potential of PBMCs from patients with RA, potentially via the suppression of ERK1/2, p38 and JNK activation, and the inhibition of c-Fos and NFATc1 expression. In conclusion, the results of the present study suggest that curcumin inhibits osteoclast formation via preventing the phosphorylation of components of the MAPK signaling pathways, and that it may be a potential novel therapeutic agent for managing osteoporosis or bone deterioration in inflammatory diseases including RA. However, future studies are required to demonstrate the anti-osteoclastogenic potential of curcumin in animal models of osteoporosis and RA.

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