Curcumin inhibits the migration of osteoclast precursors and osteoclastogenesis by repressing CCL3 production

Zhengeng Liang¹, Yan Xue¹, Tao Wang¹, Qi Xie¹, Jiafu Lin² and Yu Wang³,⁴*

Abstract

Background: Curcumin can inhibit the osteoclastogenesis and the migration of several cells including macrophages. Osteoclast precursors (OCPs) are known to exist as bone marrow-derived macrophages (BMMs). This study aims to explore whether curcumin can prevent the fusion and differentiation of OCPs to mature osteoclasts by inhibiting OCP migration.

Methods: In this study, we investigated the role of curcumin in regulating the production of several chemokines (CCL2, CCL3 and CX3CL1) and the migration of OCPs by ELISA, Western blotting and Transwell assays. Furthermore, we explored the role of curcumin in the chemokines-related osteoclastogenesis using pharmacological intervention and virus infection, and used ovariectomized (OVX) mice (osteoporosis model) to explore the effect of curcumin on the production of specific chemokine in vivo.

Results: The results showed that curcumin significantly reduced the production of CCL3 in OCPs. Moreover, curcumin-inhibited the migration of OCPs was not affected by CCR1 (Receptor of CCL3) overexpression. Remarkably, curcumin-reduced osteoclastogenesis was significantly reversed by CCL3 addition, while CCR1 overexpression did not increase the osteoclastogenesis in the presence of curcumin. Furthermore, in vivo assays also showed that curcumin significantly reduced the production of CCL3 in OCPs in the trabecular bone of OVX mice.

Conclusions: In conclusion, curcumin prevents the migration of OCPs by reducing CCL3 production, ultimately inhibiting the formation of mature osteoclasts. Therefore, our study provides the clues for improving the clinical strategies of osteoporosis, dental implantation or orthodontic treatment.

Keywords: Curcumin, Osteoclast, Osteoclast precursors, Migration, CCL3

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Background
Bone integrity depends on the dynamic balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation, which is of great value in the stomatology and orthopedics. The excessive bone resorption causes the loss of bone mass, subsequently contributing to osteoporosis, which leads to the fracture as well as the failure of dental implantation or orthodontic treatment [1–3].

Curcumin is a kind of low-molecular-weight polyphenol compound isolated from Curcuma longa. Curcumin has a wide range of pharmacological activities, such as anti-inflammatory, anti-oxidation, lipid regulation, anti-virus, anti-infection, anti-tumor, anti-coagulation, anti-liver fibrosis and anti-atherosclerosis [4]. In addition, curcumin has obvious effect in the treatment of osteoporosis, which is conductive to fracture healing, implant repair and orthodontic treatment [5–7]. Its therapeutic effect has been broadly reported in maintaining bone integrity [5, 8–14]. Curcumin can significantly improve the bone mineral density of lumbar vertebrae in ovariectomized (OVX) rats [5]. Similar results are reported in other researches regarding rats [8–11]. Moreover, Heo, et al. reported that curcumin can also prevent bone loss in OVX mice [12, 13]. Curcumin can inhibit the formation of mature osteoclasts, which is involved in its bone-protective effect [10–14].

OCPs are known to exist as macrophages (bone marrow-derived macrophages, BMMs). The migration of OCP plays an essential role in fusion and differentiation of osteoclasts [15, 16]. Several chemokines are responsible for the above processes [17–20]. Curcumin can inhibit the mobility of multiple cells. Previous study has shown that curcumin inhibits the migration and invasion of glioma cells [21]. Curcumin can also inhibit the migration and invasion of human A549 lung cancer cells [22]. Similar results were reported in other studies [23–25]. What is more, curcumin can block the migration of macrophages [26, 27]. Thus, it has important research significance whether curcumin can regulate the migration of OCPs in the form of macrophages. Overall, we hypothesized that curcumin inhibits OCP migration, thus leading to the failure in the fusion and differentiation of osteoclasts.

Methods
Animals
4 ~ 8-week-old C57BL/6 female mice (19 ~ 24g) were obtained from Gempharmatech co.ltd (Nanjing, China). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Chifeng Municipal Hospital (No.44002100017774). They were placed in a normal environment with room temperature of 20 ~ 30°C and humidity of 60–75%, and received a normal laboratory diet (Agway RMH 3000 animal chow; Arlington Heights, IL, USA).

Reagents
M-CSF (Macrophage-Colony stimulating Factor, No. 31502) and RANKL (Receptor Activator for Nuclear Factor-Kb, No. 31511) were purchased from Peprotech (Rocky Hill, NJ, USA). Curcumin (No. C1386) and TRAP staining kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit CCL3 (No. ab9781), CCR1 (No. ab19013), and β-actin antibodies were purchased from Abcam (Cambridge, England). After dissolving in 1% BSA, different working concentrations of curcumin (0, 5, 10, 15, 25 μM) were prepared by complete α-minimum Eagle’s medium (α-MEM).

Isolation and induction of OCPs
The mice were killed by cervical dislocation, and the tibiae of mice were washed with serum-free α-MEM. Bone marrow cells were incubated with α-MEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) for 24 h. Non-adherent cells were harvested, and induced to BMMs (adherent cells; used as OCPs) using M-CSF (20 ng/ml) as previously described [16, 28]. Cells were cultured in the humidified atmosphere at 37°C and 5% CO₂.

Osteoclast differentiation assay
OCPs (2 × 10⁴ cells/well) were incubated in 48-well plate in α-MEM containing M-CSF (20 ng/ml) plus RANKL (100 ng/ml) along with other specific reagents for 5 days to form mature osteoclasts. Osteoclasts differentiation was observed by Tartrate resistant acid phosphatase (TRAP) staining. TRAP⁺ cells with more than three nuclei were considered as mature osteoclasts. TRAP⁺ cells with more than five nuclei were considered as large osteoclasts. The ratio of large osteoclasts to total osteoclasts is used to express the size of osteoclasts.

Cell migration assay
Cell migration was observed by using the Transwell system (24-well insert, Millipore, Billerica, MA, USA). OCPs (1 × 10⁵/well), grown in α-MEM supplemented with 2% FBS, were placed in the upper chamber exposed to the supernatant of OCPs containing recombinant chemokine CCL2 (10 ng/mL) placed in the lower chamber. Cells were incubated and allowed to migrate overnight (16 h) and then cells migrating into lower surface of the inserts were fixed, stained with 1% crystal violet, and photographed. Migration was measured by counting the number of stained cells.
Lentivirus transduction
Full-length CCR1 cDNA was amplified by reverse transcription-PCR using the total RNA extracted from 293 cells. The designed primers were as following:

5′-ATGAGTCCTAGGAAAGCCTGATTGTC-3′ and 5′-CTTCTTGGCTGTGCATCATGA-3′.

The recombinant lentivirus encoding the CCR1 cDNA (LV-CCR1), and the control vectors (LV-Cont) were constructed using the lentivirus construction kit in 293 cells as previously described (GeneCopoeia; Washington, Maryland, USA) [29]. After 2 days, the supernatants were collected and cells were incubated in medium containing lentivirus and polybrene (7.5 μg/ml) at multiplicity of infection (MOI) 10. The transduced cells were selected by puromycin (7.5 μg/ml). The transduction efficiencies were detected by Western blotting or qRT-PCR analysis.

ELISA analysis
OCPs treated with different working concentrations of curcumin were centrifuged, and the supernatants were extracted. The concentration of CCL2, CCL3 and CX3CL1 in supernatants were detected by using ELISA kits (Cell Signaling Technology, MA, USA). The assays were carried out according to the manufacturer’s instructions. The detection of CCL3 concentration in serum from different mice was the same as above.

Western blotting (WB) analysis
The lysate proteins from cells with different interventions were prepared. The lysates were filled into 10% SDS-PAGE gels and transferred onto the polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After sealing with 5% skim milk for 2 h at room temperature, PVDF membranes were incubated with anti-CCL3 antibodies at 4 °C overnight, and the secondary antibodies at room temperature for 1 h (6 in each group). For immunofluorescence, the sections were incubated in citrate buffer at 60 °C overnight to expose antigens. Subsequently, the sections were incubated in the primary antibody for 1 h at room temperature. The signal was visualized using a Chemiluminescence system (Pierce, Rockford, IL, USA) or manual exposure.

Quantitative real-time PCR (qRT-PCR) analysis
According to the manufacturer’s protocol, total RNA was extracted with Trizol reagent (Invitrogen). The designed primers were as following: CCR1, 5′-TTGGACGATTTGACAGAT-3′ (Forward) and 5′-TGATGCCAAAAGTAACAGTTCG-3′ (Reverse); Cyclophilin A (Housekeeping), 5′-CGAGCTCTGAGCACTGGA-3′ (Forward) and 5′-TGCCGTGTAAAGTCCACCACC-3′ (Reverse). qRT-PCR was carried out by using SYBR Premix Ex TaqTM kit (TakaRa; Tokyo, Japan) on ABI7500 PCR machine (Applied Biosystems; Waltham, MA, USA) according to manufacturer’s instructions.

Identification of the experimental doses of curcumin
The range of the in vivo doses for curcumin administration was selected based on the previous study [14]. The experimental dose of curcumin was determined by comparing the recovery of bone loss in OVX mice among each group (4 g of diet containing 150, 175, 200 mg/kg curcumin, for 4 weeks; n = 5, per group). The pre-experiments show that curcumin at 200 mg/kg had the most obvious function in recovering bone mass. Thus, 200 mg/kg curcumin was used as the in vivo dose.

Ovariectomized mice model
Under anaesthesia, together, 10-weeks-old mice were subjected to bilateral ovariectomies or sham surgery. Three days later, SHAM mice were fed 4 g of the control diet each day and OVX mice were fed 4 g of the control diet or the diet containing 200 mg/kg curcumin each day. After 4 weeks of administration, SHAM and OVX mice were killed by cervical dislocation. Blood samples were centrifuged for serum isolation and stored in −80 °C for ELISA analysis. The femurs were harvested, wrapped with gauze soaked in 0.9% salt water and stored at −20 °C.

Micro-computed tomography (micro-CT)
Three-dimensional (3D) reconstructions of the cancellous bones in distal femur metaphysis were performed by using Micro-CT (ICT-80, Scanco Medical AG, Bassersdorf, Switzerland). The voxel size of Micro-CT is 10 μm. The parameters contain bone mineral density (BMD), bone volume density (BV/TV), trabecular number (Tb.N) and trabecular spacing (Tb.Sp) (8 in each group).

The staining analyses in tissues
The femurs of mice were fixed in 4% PFA for 48 h, and decalcified in 10% EDTA (pH 7.3) for 2 weeks at 4 °C. Then all samples were dehydrated by graded ethanol and embedded in paraffin. The sections (5 μm thick) were stained by using the TRAP kit. The osteoclasts number of TRAP-stained sections was counted by using an eyepiece grid (8 in each group). For immunofluorescence analysis, the sections were incubated in citrate buffer at 60 °C overnight to expose antigens. Subsequently, the sections were incubated in the primary antibodies at 4 °C overnight, and the secondary antibodies at room temperature for 1 h (6 in each group).

Statistical analysis
Statistical analyses were carried out by using GraphPad Prism Software. All data are expressed as mean ± SEM. The statistical differences were analysed by one-way ANOVA or Student’s t-test. Bonferroni test was used for
Results
Curcumin inhibited CCL3 production in OCPs
Firstly, the role of curcumin in the production of several chemokines in OCPs needs to be investigated. ELISA results showed that without RANKL, curcumin reduced the secretion of supernatant CCL3 in a concentration-dependent manner from 5 μM to 25 μM (Fig. 1b). However, curcumin only reduced the secretion of CCL2 or CX3CL1 at 10 or 5 μM, respectively (Fig. 1a, c). Besides, all concentrations of curcumin also inhibited CCL3 secretion while exerting no effects on the secretion of CCL2 or CX3CL1 in the presence of RANKL (Fig. 1d-f). Western blotting assays showed that curcumin reduced the expression level of CCL3 in OCPs at 25 μM in the presence of RANKL and at 10, 15 and 25 μM without RANKL (Fig. 1g, h). It suggested that curcumin could inhibit the production of CCL3 in OCPs.

Curcumin-inhibited OCP migration was not affected by CCR1 overexpression
We clarified that curcumin inhibited CCL3 production in OCPs. The relationship between OCP migration and curcumin administration also requires exploration. To achieve this goal, we used chemokine CCL2 and the supernatant of OCPs treated by curcumin to induce the migration of OCPs, and observed the effect of CCR1 overexpression on OCP migration using lentivirus transduction. For subsequent experiments, the most effective concentration (25 μM) of curcumin was applied. The expression of viral genes was detected at protein and mRNA level (Fig. 2a). As shown in Fig. 2b, c, the migration of OCPs driven by CCL2 plus the supernatant of OCPs was reduced by curcumin. However, CCR1 overexpression increased the migration of OCPs while having no effect on the role of curcumin in OCP migration (Fig. 2b, c). These results suggested that curcumin could inhibit OCP migration induced by CCL3.

CCL3 significantly reversed curcumin-reduced osteoclastogenesis
The role of specific chemokines in the osteoclast differentiation inhibited by curcumin is also worth to clarify. As shown in Fig. 3a-d, all three chemokines did not affect the number of total osteoclasts and large osteoclasts as well as the size of osteoclasts (defined as the ratio of large osteoclasts/total osteoclasts) under the induction of RANKL plus M-CSF. However, the osteoclast parameters above were reduced by curcumin, which was reversed by the addition of all three chemokines (Fig. 3a-d). Nevertheless, among three chemokines, CCL3 had the strongest ability to reverse the reduced osteoclastogenesis (Fig. 3a-d), proving CCL3 plays an indispensable role in curcumin-inhibited osteoclast differentiation.

![Fig. 1 Curcumin inhibited CCL3 production in OCPs. (a-c) After treatment with different concentrations of curcumin for 12 h, the levels of CCL2, CCL3 or CX3CL1 secreted from OCPs are assessed by ELISA assays. (d-f) After treatment with different concentrations of curcumin for 12 h in the presence of RANKL, the levels of CCL2, CCL3 or CX3CL1 secreted from OCPs are assessed by ELISA assays. (a-f) Compared among each group, letters p < 0.05 by one-way ANOVA. (g, h) After treatment with different concentrations of curcumin for 8 h in the presence or absence of RANKL, the protein levels of CCL3 in the OCPs are detected by Western Blotting. Compared among each group, letters p < 0.05 by one-way ANOVA. Data are expressed as mean ± SEM from three independent experiments. CUR, curcumin.](RETRACTED_IMAGE)
CCR1 overexpression did not promote the osteoclastogenesis in the presence of curcumin

In order to further investigate the relation between CCL3 and curcumin-regulated osteoclastogenesis, we observed the effect of CCR1 overexpression on the osteoclast differentiation treated by curcumin through lentivirus transduction. The results showed that although CCR1 overexpression increased osteoclast number, large osteoclast number and osteoclast size, there were no statistical differences between the CCR1-overexpressed group and the control group in the presence of curcumin (Fig. 4a–d), supporting that CCR1 overexpression is invalid in enhancing the osteoclastogenesis as curcumin blocks CCL3 production.

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Fig. 2 Curcumin-inhibited OCP migration was not affected by CCR1 overexpression. (a) Protein and mRNA levels of CCR1 in viruses (LV-Cont or LV-CCR1)-transduced OCPs. (b) OCPs was treated with 25 μM curcumin or PBS for 24 h, and CCL3 (10 ng/ml) was added into the supernatant of treated OCPs. The migration of OCPs exposed to the above supernatant placed in the lower chamber is observed by Transwell assays. Scale bar, 100 μm. (c) The histogram shows the number of migrated OCPs in B, *P < 0.05 by Student’s t-test; **P < 0.05 by one-way ANOVA. Data are expressed as mean ± SEM from three independent experiments. CUR, curcumin; OCP, osteoclast precursor; SUP, supernatant; SUP/OCP-PBS, supernatant of OCPs treated with PBS; SUP/OCP-CUR, supernatant of OCPs treated with curcumin

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Fig. 3 CCL3 significantly reversed curcumin-reduced osteoclastogenesis. (a) The representative TRAP staining images of differentiated osteoclasts derived from OCPs treated with 25 μM curcumin or PBS in the presence of M-CSF, RANKL and vehicle (DMSO) or three chemokines (10 ng/mL CCL3; 5 ng/mL CCL3; 10 nM CX3CL1) for 5 days. Scale bar, 200 μm. (b–d) The histogram shows the quantitative results regarding mature osteoclasts (more than three nuclei), large osteoclasts (more than five nuclei) or osteoclast size (the ratio of large osteoclasts/total osteoclasts) in A. Data are expressed as mean ± SEM from three independent experiments. *P < 0.05 by one-way ANOVA. CUR, curcumin; M, M-CSF; R, RANKL
**Fig. 4** CCR1 overexpression did not promote the osteoclastogenesis in the presence of curcumin. (a) The representative TRAP staining images of osteoclasts derived from viruses-transduced OCPs under the combined intervention of M-CSF, RANKL and 25 μM curcumin or PBS for 5 days. Scale bar, 200 μm. (b-d) The histogram shows the quantitative results regarding mature osteoclasts (more than three nuclei), large osteoclasts (more than five nuclei) or osteoclast size (the ratio of large osteoclasts/total osteoclasts) in A. Data are expressed as mean ± SEM from three independent experiments, *P < 0.05 by Student’s t-test; ns, no significance. CUR, curcumin; M, M-CSF; R, RANKL.

**Fig. 5** Curcumin inhibited CCL3 production of OCPs in the trabecular bone of OVX mice. OVX or sham-operated 10-weeks-old WT mice were fed 4 g of the control diet or the diet containing 200 mg/kg curcumin each day (for 4 weeks; n = 6 ~ 10, per group). (a) The typical femoral 3D MicroCT reconstructed images in each group. Scale bar, 100 μm. (b) The typical TRAP-stained femoral sections in each group (red arrowheads). Scale bar, 5 μm. (c) Femoral sections were stained with green and red fluorochromes for RANK and CCL3, respectively, and observed under fluorescent microscopy. The overlaps of RANK and CCL3 are indicated by white arrows (yellow fluorescence). Scale bar, 2.5 μm. Data are representative images among 6 independent samples with unanimous results. (d-g) The trabecular bone parameters (BMD, BV/TV, Tb.N and Tb.Sp) were analysed by using Micro-CT. (h) The calculation regarding osteoclasts number per millimeter of trabecular bone surface. (i) Serum levels of CCL3 were measured using ELISA kits. Data are expressed as the mean ± SEM. *P < 0.05. CUR, curcumin.
Curcumin inhibited CCL3 production by OCPs in the trabecular bone of OVX mice

We documented that curcumin could inhibit CCL3 production in OCPs from in vitro experiments. Next, we evaluated the in vivo role of curcumin in the OCPs of the trabecular bone using OVX mice. Oral curcumin (200 mg/kg/d for 4 weeks) was employed for the in vivo study. Micro-CT results showed that trabecular bone loss in OVX mice was ameliorated by curcumin administration (Fig. 5a). Bone parameter analysis of femurs showed that curcumin efficiently increased BMD, BV/TV and Tb. N and decreased Tb.Sp in OVX mice (Fig. 5d-g). Likewise, TRAP staining showed that the trabecular areas of OVX mice had more osteoclasts, which were partially reversed by curcumin administration (Fig. 5b, h). These data prove the effectiveness of curcumin dosage in our experimental system.

More importantly, we found that curcumin significantly inhibited CCL3 serum level in OVX mice (Fig. 5i). In order to make clear the relationship between curcumin-treated CCL3 production and trabecular OCPs, we performed the double immunofluorescence staining of RANK (specific marker of OCPs attached to bone matrix) and CCL3 on mice bone sections. The results showed that compared with SHAM mice, the CCL3 expression of the RANK+ cells in the trabecular bone of OVX mice (defined as yellow overlapping fluorescence of CCL3 and RANK) significantly increased (Fig. 5c). However, curcumin effectively reversed the increased overlap of CCL3 and RANK (Fig. 5c). These data indicated that OVX promotes CCL3 production in trabecular OCPs, which is reversed by curcumin treatment.

Discussion

Curcumin has a blocking function on osteoclastogenesis [10–14], but the explicit mechanism still remains vague, which leaves an interesting scientific issue for the research. Curcumin has been shown to inhibit cell migration in other cells, especially macrophages [26, 27]. In the current study, we reveal the first evidence regarding the effect of curcumin on repressing OCP migration, which causes the reduction in the osteoclastogenesis.

Multiple chemokines are involved in cell migration. CCL2, CCL3 and CX3CL1 are responsible for the mobility of OCPs and the fusion and differentiation of osteoclasts.

Fig. 6 The current working model regarding curcumin-inhibited OCP migration during the osteoclastogenesis. CCL3 promote the recruitment of OCPs by binding to CCR1 on OCPs, which leads to the fusion and differentiation of osteoclasts. Curcumin inhibits the migration of OCPs and osteoclastogenesis by repressing the production of CCL3 in OCPs. OC, osteoclast; OCP, osteoclast precursor.
Our experimental data suggested that curcumin inhibited the production of CCL3 but not CCL2 and CX3CL1 in OCPs. What’s more, although curcumin inhibited OCP migration, CCR1 overexpression by lentivirus transduction did not affect curcumin-regulated OCP migration. The above results were attributed to that CCL3 production is blocked under the intervention of curcumin, which causes that CCR1 up-regulation can not enhance the migratory ability of OCPs. The molecular mechanism underlying curcumin-inhibited CCL3 production requires further studies. Moreover, it was observed that RANKL enhanced CCL3 production, which is in line with the previous reports [30]. Our results showed that curcumin could reduce CCL3 production in OCPs with or without RANKL, which laid a theoretical foundation for further research.

Besides, although all the three chemokines could reversed curcumin-inhibited osteoclastogenesis, CCL3 was more effective than the other two chemokines in reversing the reduced osteoclastogenesis. Furthermore, under curcumin intervention, CCL3 made osteoclasts largest, proving the essential role of CCL3 on inhibiting OCP migration upon the above conditions. Our experimental results confirmed the role of curcumin-inhibited CCL3 production in preventing OCP recruitment and fusion. Taken together, curcumin could inhibit the osteoclastogenesis by repressing CCL3 production. Moreover, under curcumin administration, CCR1 overexpression could not promote the osteoclastogenesis. Given the blocking effect of curcumin on CCL3, CCR1 overexpression lost the ability to promote the osteoclastogenesis, which further verified the above theory. Overall, there is a close relationship among curcumin-inhibited CCL3 production, the migration of OCPs and the osteoclastogenesis. The osteoprotective effect of curcumin on OVX mice has been confirmed [12, 13]. Our in vivo experiments proved that curcumin not only prevented bone loss and osteoclastogenesis but also inhibited the CCL3 production of the trabecular bone in OVX mice, which is consistent with the results in vitro. The detailed molecular mechanism of curcumin-regulated CCL3 production in OCPs requires further research. Our current working model regarding curcumin-regulated OCP migration during the osteoclastogenesis is described in Fig. 6.

Conclusions
Curcumin is considered as the inhibitor of osteoclastogenesis and cell migration (especially macrophages). Nevertheless, the effect of OCP migration on curcumin-inhibited osteoclastogenesis still remains unknown.

From the perspective of cell migration, this study is the first to reveal a novel mechanism underlying curcumin-inhibited osteoclastogenesis. Our experimental data clarified the role of curcumin in inhibiting the migration of OCPs due to its blocking function on the production of CCL3, which is involved in curcumin-regulated osteoclastogenesis. Our study not only explores the novel mechanism underlying curcumin-regulated osteoclastogenesis, but also provides the clues for improving the therapeutic strategies of curcumin in preventing bone loss.
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