Epigallocatechin-3-gallate diminishes cytokine-stimulated Cyr61 expression in human osteoblastic cells: a therapeutic potential for arthritis

Ping-Han Wu1, Sze-Kwan Lin2,3, Bor-Shiunn Lee3,4, Sang-Heng Kok2,3, Jyh-Horng Wang5, Kuo-Liang Hou4, Hsiang Yang3, Eddie Hsiang-Hua Lai2,3, Juo-Song Wang2,3 and Chi-Yuan Hong2,6,7

Abstract

Objective. To assess the effects of epigallocatechin-3-gallate (EGCG) on cytokine-induced Cyr61 synthesis in human osteoblastic cells and the associated signalling pathways. The therapeutic effect of EGCG on CIA in rats was also studied.

Methods. The expression of Cyr61 and NF-κB pathway molecules was examined by western blotting. CCL2 expression was assessed by northern blotting and ELISA. Interaction between NF-κB and Cyr61 promoter was evaluated by electrophoretic mobility shift assay. In rat CIA, osteoblastic expression of Cyr61 was examined by immunohistochemistry and disease progression was assessed by clinical, radiographic and histological examinations.

Results. EGCG inhibited Cyr61 expression stimulated by cytokines in primary human osteoblasts and human osteoblastic cell line U2OS. In U2OS, oncostatin M (OSM) induced IκB-α degradation through the mTOR/rictor/Akt pathway, and EGCG attenuated the action. Electrophoretic mobility shift assay revealed that the OSM-enhanced NF-κB/DNA binding was reduced by EGCG, possibly through abrogating nucleus localization of p65 and p50. Cyr61 enhanced OSM-induced expression of CCL2. Moreover, EGCG diminished OSM-stimulated CCL2 expression at least partially via suppressing Cyr61 induction. Co-distribution of CD68+ macrophages and Cyr61+ osteoblasts in osteolytic areas was obvious in the CIA model. Clinical, radiographic and immunohistochemical analyses revealed that administration of EGCG markedly diminished the severity of CIA, macrophage infiltration, and the number of Cyr61-synthesizing osteoblasts.

Conclusion. By modulating the mTOR/rictor/Akt/NF-κB pathway, EGCG attenuated Cyr61 production in osteoblastic cells and in turn diminished macrophage chemotaxis. Our data support the therapeutic potential of EGCG on arthritis.

Key words: CCN proteins, epigallocatechin-3-gallate, collagen-induced arthritis, bone resorption, NF-κB.

Introduction

Proinflammatory cytokines play an essential role in the development of RA [1]. In addition to TNF-α and IL-1 family cytokines, IL-6 family members and the signalling pathways downstream of the common gp130 receptor subunit are important in the pathogenesis of both murine and human inflammatory arthritis [2]. Oncostatin M (OSM) is a member of the IL-6 family, and many studies have demonstrated the stimulatory role of OSM in the progression of RA [3, 4]. Langdon et al. [5] detected an...
increased level of OSM in RA SF and concluded that OSM potentially modulated metalloproteinase activity. Our previous study demonstrated that OSM induced strong expression of CCL2, a potent chemoattractant for monocytes/macrophages, in human osteoblastic cells at both the mRNA and protein levels [4], supporting the significance of OSM in the pathogenesis of RA.

Cysteine-rich 61 (Cyr61; also known as CCN1) belongs to the CCN protein family, which includes six members, CCN1 to CCN6 [6]. Once synthesized, CCN proteins are secreted, associated with the cell surface or extracellular matrix through binding to integrins or heparan sulphate proteoglycans and serve as matricellular signalling molecules [7]. Functionally Cyr61 has been shown to promote angiogenesis, cell proliferation, adhesion, migration and differentiation. Cyr61 is implicated in many disease processes in which vascular proliferation may become dysregulated, such as neovascularization and pannus formation in RA [8]. A recent cDNA micro-array analysis of B cells from monozygotic twins revealed significantly higher expression of Cyr61 in the RA twin compared with the healthy co-twin, supporting the aetiological role of Cyr61 in RA development [9]. Recently Zhang et al. [10] found that Cyr61 played a critical role in IL-17-mediated proliferation of fibroblast-like synoviocytes in RA. In a rat model of bacteria-induced apical periodontitis, we found that Cyr61 played a critical role in IL-17-mediated proliferation of fibroblast-like synoviocytes in RA. In a rat model of bacteria-induced apical periodontitis, we found that osteoblastic expression of Cyr61 correlated with the severity of inflammation-associated bone resorption [11]. Nevertheless, the connection between Cyr61 and bone remodelling in RA pathogenesis remains unclear. Furthermore, signalling cascades mediating osteoblastic expression of Cyr61 in inflammation deserve further investigation.

Nuclear factor-κB (NF-κB) is composed of various dimeric complexes of the Rel protein family members, including Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50) and NF-κB2 (p52) [12]. Of the NF-κB complexes, p65/p50 heterodimer is the most abundant and dominates in the modulation of target genes involved in inflammation and immune responses. In its latent form, NF-κB is usually bound to the IκB inhibitory proteins and located in the cytoplasm. On stimulation, the IκB kinase complexes (IKKs) induce phosphorylation and degradation of IκB with subsequent nucleus translocation of NF-κB [12]. In the nucleus, NF-κB binds to DNA consensus sequences and initiates gene transcription. Sequence analysis of the Cyr61 gene promoter region demonstrated the presence of a consensus binding site for NF-κB [13].

Akt has been reported to activate NF-κB either by inducing IκB degradation or mediating IKKζ kinase phosphorylation [14]. A recent study proposed that mTOR may activate p65, possibly through direct interactions between these two molecules [15]. Interactions between Akt and mTOR are rather complex. Akt could activate the mTOR complex 1 (mTORC1), which consists of mTOR, raptor and mLST8 [16], whereas other investigations showed that the mTOR complex 2 (mTORC2), containing mTOR, rictor and mLST8, catalysed the full activation of Akt by phosphorylating it at S473 [17].

Epigallocatechin-3-gallate (EGCG), the major component of brewed green tea from the leaves of Camellia sinensis, has been shown to exhibit protective effects against the occurrence of cancer, neuron degeneration and coronary artery diseases [18, 19]. Previous studies have demonstrated that EGCG suppresses the production of MMPs by synovial fibroblasts [20] and chondrocytes [21] stimulated with proinflammatory cytokines. Morinobu et al. showed that EGCG inhibited osteoclast differentiation and ameliorated experimental arthritis in mice [22]. In an earlier study, we also demonstrated that EGCG suppresses CCL2 production in osteoblasts and suppresses the development of CIA in rats [23]. However, information regarding the influences of EGCG on Cyr61 synthesis is relatively rare.

In this study, the modulating effects of EGCG on osteoblastic expression of Cyr61 induced by proinflammatory cytokines were examined. The involvement of mTOR/Akt/NF-κB signalling pathway in cytokine-stimulated Cyr61 expression and the inhibitory effect of EGCG were examined, using OSM as an example. We also assessed the effect of EGCG on Cyr61-induced synthesis of CCL2. In a rat model of CIA, the relationship of the therapeutic effect of EGCG to the osteoblastic expression of Cyr61 was investigated.

Materials and methods

Materials

Recombinant human TNF-α, IL-1β, IL-6 and OSM was from PeproTech (Rocky Hill, NJ, USA). Recombinant human Cyr61 was bought from Abnova (Taipei, Taiwan). The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was obtained from Sigma. LY294002, SH-5 and rapamycin were purchased from Calbiochem (Darmstadt, Germany). Antibodies and lentivirus shRNAs for raptor (plasmid 1857), rictor (plasmid 1854) and mTOR (plasmid 1856) were from Addgene (Boston, MA, USA). Scramble control shRNA (plasmid 1864) was also from Addgene. Cyr61 shRNA (V2LHS-236035) was obtained from OpenBiosystems (Huntsville, AL, USA). Antibodies for human and rat Cyr61, Akt, NF-κB p65 and p50 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-human phospho-Akt (Ser473), IκBα and phospho-IκBα (Ser32/36) antibodies were from Cell Signaling Technology (Beverly, MA, USA). The human CCL2 ELISA kit was purchased from Bender MedSystems (Vienna, Austria). The rat CCL2 ELISA kit was from Endogen (Woburn, MA, USA). Inactivated Mycobacterium tuberculosis and Freund’s incomplete adjuvant (IFA) were from Difco (Detroit, MI, USA).

Cell culture

Primary cultures of human bone marrow-derived osteoblasts were established as described previously [24]. Briefly, alveolar bone explants were harvested from five healthy patients (aged 20–40 years) receiving odontectomy of the third molar due to orthodontic considerations. U2OS, a human osteosarcoma cell line applied in several
studies as human osteoblastic cells [25, 26] was also used. Except for ELISA, U2OS was made quiescent in serum-free media for 24 h before treatment. All experiments were performed at least three times and representative results were shown. Informed consent was obtained from all patients and the study was approved by the Research Ethics Committee, National Taiwan University Hospital.

Western analysis, northern analysis, ELISA and MTT assay
Experiments were performed as previously described [24]. The results of western blot and northern blot were digitalized, and densitometric analysis was done by normalizing the data to the levels of reference molecules.

Immunoprecipitation assay
Experiments were performed as previously described [24].

Electrophoretic mobility shift assay
Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA) were performed as previously described [4]. Nuclear proteins were incubated with oligonucleotide probes containing binding sequences for NF-κB: 5′-GAACCTAAGTGGAACCTTCTAAAAC-3′ [13]. For supershift, antibodies against p65 and p50 were added to the incubation mixture for 30 min on ice then for 30 min at room temperature before electrophoresis.

Lentivirus production and transduction of U2OS
Gene silencing was performed by using lentiviral shRNAs. Recombinant lentiviruses were produced by co-transfecting 293FT cells with shRNA transferring plasmids and packaging plasmids using the calcium phosphate method. Cell culture supernatants were harvested at 48 h after transfection and filtered through a 0.45 μm filter. Culture supernatants containing lentivirus were added to U2OS in the presence of polybrene. Twenty-four hours later, the efficiency of transduction was assessed by detecting green fluorescent protein expression under flow cytometry. Stably transduced cells were selected by puromycin.

Animal model of CIA
Bovine type II collagen (BCII, Chondrex, Redmond, WA, USA) in acetic acid (4 mg/ml) was emulsified in an equal volume of Freund’s complete adjuvant (CFA), containing 2 mg/ml inactivated M. tuberculosis in IFA. CIA was induced in 20 male Sprague–Dawley rats (7–8 weeks old, weighing 150–180 g) by injection with 500 μg bovine type II collagen in CFA intradermally at the back and the base of the tail in volumes of 300 and 200 μl, respectively, at day 1. For booster, 300 μg BCII in IFA was given on day 7. One day before arthritis induction, rats (10 per each group) were given intraperitoneal injections of either EGCG (20 mg/kg) [23] or normal saline (NS, as control) on a daily basis until the rats were killed (day 35).

Measurement of serum CCL2 level in CIA rats
CIA induction and EGCG or NS administration were performed in another 10 animals as described earlier in the text. Blood samples were obtained from the rats (five animals for each of the NS and EGCG groups) by retro-orbital puncture on day 35. The serum concentration of CCL2 was determined by a rat CCL2 ELISA kit.

Clinical assessment of arthritis
Assessment was performed as previously described [24]. The rats were examined every other day by an investigator who was blinded regarding the treatment protocols. The severity of arthritis in each paw was graded on a scale of 0–4 as follows: 0 = normal joint; 1 = mild/moderate erythema and/or swelling in 1 joint; 2 = erythema and/or swelling in 1 joint; 3 = erythema and/or swelling in the entire paw and 4 = deformity and/or ankylosis. The sum of the scores for all four paws of each rat was used as the arthritis index. The mean arthritis index (the sum of the arthritis index for all animals divided by the number of animals) was used to denote the severity of CIA in each group.

Radiographic assessment of arthritis
Right ankle joints were placed in position on Kodak X-OMAT TL high-resolution specimen-imaging film (Eastman Kodak, Rochester, NY, USA) and radiographed with a Faxitron X-ray system (Model 43855A; Faxitron X-ray, Buffalo Grove, IL, USA). Images were shot at 26 kV for 10 s. Erosive changes were analysed using a semiquantitative scale as follows: grade 0 = no erosion, grade 1 = small, localized bone erosion in the small tarsal bones, grade 2 = several bone erosions in the small tarsal bones, grade 3 = erosions involving all small tarsal bones and grade 4 = erosions in the tibia, calcaneus, and talus [27].

Histological examination and immunohistochemistry
The histopathological score was evaluated using a scale of severity ranging from 1 to 4, where 1 = hyperplasia of the synovial membrane and presence of polymorphonuclear infiltrates; 2 = pannus and fibrous tissue formation and focal subchondral bone erosion; 3 = articular cartilage destruction and bone erosion and 4 = extensive articular cartilage destruction and bone erosion [24]. Immunohistochemical staining was performed as previously described [28]. Briefly, tissue sections were deparaffinized and rehydrated and treated with 3% H2O2 in methanol to remove endogenous peroxidase activity. After blocking nonspecific binding with 10% normal goat serum (Zymed Laboratories, San Francisco, CA, USA), sections were incubated overnight with polyclonal rabbit anti-human/rat Cyr61 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal goat anti-rat CD68 (rat macrophage marker, Serotec, Oxford, UK) antibody at 4°C. Bonded antibodies were detected by sequential incubation with biotinylated anti-rabbit or anti-goat IgG antibody (Promega, Madison, WI, USA), streptavidin-peroxidase conjugate and diaminobenzidine. The sections were then counterstained with haematoxylin.
mounted with Permount and examined by a light microscope. The field in each section exhibiting strongest inflammation was selected and examined under microscopic high power field (HPF) at ×400 magnification. The number of osteoblasts lining the bone surface and the number of Cyr61-positive osteoblasts were counted. The data were converted to a percentage of Cyr61-positive osteoblasts lining the bone surface.

Statistical analysis
Data were subjected to ANOVA analysis for multiple comparisons and then Fisher’s protected least significant difference test. \( P < 0.05 \) was considered statistically significant.

Results
EGCG inhibits cytokine-stimulated Cyr61 expression in human bone marrow-derived osteoblasts and U2OS cells
Western analysis showed that IL-1β, TNF-α, IL-6 and OSM stimulated Cyr61 expression in human bone marrow-derived osteoblasts (Fig. 1A) and U2OS cells (Fig. 1B). The stimulating effects were time dependent and usually peaked at 12 to 24h in both cell types. EGCG attenuated cytokine-enhanced Cyr61 expression in both types of osteoblastic cell (Fig. 1C and D). We assessed the level of Cyr61 in the culture medium of cells treated with OSM, alone or in combination with EGCG, and found that EGCG also diminished secretion of Cyr61 into the culture medium of U2OS (Fig. 1E). MTT assay revealed that exogenous Cyr61 enhanced, whereas EGCG did not exert any influence on the proliferation of both cell types (Fig. 1F and G). The two cell types showed a similar response to the cytokines tested, but U2OS appeared to be a better producer of Cyr61 and has more stable biologic behavior. Therefore we used U2OS cells to study the transcriptional regulation of Cyr61 expression.

mTOR/rictor/Akt/p65 signalling pathway mediated OSM-induced Cyr61 production
LY294002 (PI3K inhibitor) or SH-5 (Akt inhibitor) attenuated Cyr61 production, whereas the inhibitory effect of rapamycin (mTOR/raptor complex inhibitor) was minimal (Fig. 2A). Inhibition of the raptor gene by raptor shRNA did not significantly reduce Cyr61 expression. Note the pronounced alleviation of raptor shRNA on raptor synthesis. (Fig. 2B). Figure 2C confirmed the gene-silencing activities of rictor and mTOR shRNAs. Knockdown of rictor or mTOR attenuated Cyr61 production (Fig. 2C) and Akt activation (Fig. 2D). SH-5, mTOR and rictor shRNAs inhibited the degradation of IκBα (Fig. 2E) and nuclear translocation of p65 (Fig. 2F) induced by OSM. Short hairpin RNA targeting p65 effectively blocked p65 gene expression (Fig. 2G) and inhibited OSM-induced Cyr61 synthesis in U2OS (Fig. 2H). Note that vector or scramble shRNA did not change the levels of OSM-stimulated Cyr61 (supplementary Fig. 1A, available as supplementary data at Rheumatology Online) or nuclear p65 (supplementary

EGCG attenuates mTOR/rictor/Akt/NF-κB activation induced by OSM
To explore the effects of EGCG on the interactions among rictor, mTOR, and Akt, cell lysates immunoprecipitated with mTOR were immunoblotted with anti-rictor and anti-Akt antibodies. Formation of the mTOR/rictor/Akt complex was induced by OSM but was significantly inhibited by EGCG (Fig. 3A). In addition, EGCG attenuated OSM-induced phosphorylation of Akt (Fig. 3B). In U2OS cells, EGCG suppressed IκBα degradation induced by OSM (Fig. 3C). EGCG also inhibited nuclear translocation of NF-κB p65 (Fig. 3D) and p50 (Fig. 3E) induced by OSM. EMSA, using a probe containing NF-κB consensus sequence, showed that marked NF-κB/DNA interaction occurred at 30 min after OSM stimulation. Both p65 and p50 were identified in the DNA-protein complex and EGCG diminished the induced NF-κB/DNA interaction (Fig. 3F).

EGCG suppresses OSM/Cyr61-stimulated CCL2 expressions
To elucidate the role of Cyr61 in macrophage recruitment, we examined its effect on the expression of CCL2 in U2OS cells. Northern blot showed that Cyr61 induced the transcription of CCL2 mRNA in a time-dependent manner (Fig. 4A). ELISA revealed increased CCL2 secretion into the culture medium after Cyr61 treatment (Fig. 4B). A gene silencing experiment showed that shRNA targeting Cyr61 successfully suppressed its expression (Fig. 4C). Knockdown of Cyr61 or addition of EGCG significantly attenuated OSM-enhanced CCL2 synthesis. EGCG further enhanced the repressive effect of Cyr61 shRNA on CCL2 induction (Fig. 4D and E). Additive effects on CCL2 gene expression (Fig. 4F) and protein synthesis (Fig. 4G) were noted when OSM and Cyr61 were added together. EGCG diminished CCL2 expression stimulated by OSM or Cyr61. Moreover, the alleviating effect of EGCG on CCL2 induction by OSM was partially restored by exogenous Cyr61 (Fig. 4F and G).

EGCG inhibits the progression of CIA
No fluctuation in animal body weight was noted during the experimental course. Clinical signs of arthritis exacerbated gradually, whereas administration of EGCG attenuated the severity of CIA. At the end of the experiment, the mean (±s.d.) arthritis index was 9.0 (2.1) in the control group and 3.9 (1.9) in the EGCG rats (Fig. 5A). Radiographic examination of the ankle joints of the control group revealed bone erosions usually involved all small tarsal bones. The articular surfaces of tarsal bones were very irregular, and the talocalcaneal joint space was frequently obliterated (Fig. 5C). In the EGCG group, bone erosions were occasionally found in the tarsal bones. Most of the articular surfaces of the tarsal bones were smooth, and the talocalcaneal joint space was preserved (Fig. 5D). Semiquantitative analysis of arthritis progression by
Fig. 1 EGCG attenuated Cyr61 synthesis in osteoblastic cells.

(A) Primary human osteoblasts (HOB) and (B) U2OS were incubated for various time points with 10 ng/ml IL-1β, TNF-α, IL-6 and OSM. (C) HOB and (D) U2OS cells were incubated for 24 h with each of the cytokines in combination with 10 μg/ml EGCG (3 h before the addition of cytokines). Cyr61 levels were assayed by western blot analysis. For each cytokine, upper panel: Cyr61, lower panel: β-actin. (E) Cyr61 secretion into the culture medium of U2OS cells was assessed by western blot. U2OS cells were treated with OSM for 24 h, alone or combined with 10 μg/ml EGCG (3 h before the addition of cytokines). Bar graph (A-E) data were quantified by densitometric analysis and expressed as fold change relative to untreated control. Values are mean (s.d.) of three independent experiments. *P < 0.05 vs control; **P < 0.05 vs cytokines alone. The effects of EGCG and exogenous Cyr61 (200 ng/ml) on the growth of (F) HOB and (G) U2OS were examined by MTT assay. Values are mean (s.d.) of three independent experiments. *P < 0.05 vs control.
Fig. 2 mTOR/rictor/Akt/p65 pathway-mediated Cyr61 production.

(A) U2OS cells were stimulated for 24 h with OSM, alone or in combination with 50 μM LY294002, 8 μM SH-5 or 50 ng/ml rapamycin (inhibitors were added 3 h before the addition of OSM). Cyr61 was determined by western blot. (B) U2OS cells were transduced with raptor shRNA, followed by incubation with OSM for 24 h. The levels of Cyr61 and raptor were assayed by western blot. (C) U2OS cells were transduced with rictor or mTOR shRNA, followed by incubation with OSM for 24 h. The levels of Cyr61 were assayed by western blot. (D) U2OS cells were transduced with rictor or mTOR shRNA. After treating with OSM for 30 min, the levels of phospho-S473-Akt were determined by western blot. U2OS cells were either pretreated with SH-5 for 3 h or transduced with rictor or mTOR shRNA and then incubated for 60 min with OSM. The levels of IκBα (E) and nuclear p65 (F) were determined by western blot. (G) U2OS was transduced with shRNA targeting NF-κB p65 and the gene silencing effect was confirmed by western blot. (H) U2OS cells transduced with p65 shRNA were stimulated with OSM for 24 h and the levels of Cyr61 were determined. Bar graph (A-H) data were quantified by densitometric analysis and expressed as fold change relative to untreated controls. Values are mean (±S.D.) of three independent experiments. *P < 0.05 vs control; **P < 0.05 vs OSM alone.
FIG. 3 EGCG inactivated the mTOR/riCTOR/Akt/NF-κB pathway.

(A) U2OS was incubated with OSM in combination with EGCG (10 μg/ml, 3 h before the addition of OSM) or not for 20 min. The protein extracts were immunoprecipitated with anti-mTOR antibody and subjected to western blot analysis by antibodies against rictor, mTOR and Akt. (B) U2OS was treated with OSM, alone or in combination with EGCG (10 μg/ml) for 30 min. The levels of phospho-S473-Akt were determined. Cells were treated with OSM (10 ng/ml) for 30 (C) or 60 min (D and E), alone or in combination with EGCG (10 μg/ml). Cytoplasmic levels of IκBα (C) and the levels of nuclear p65 (D) and p50 (E) were determined by western blot. (F) Nuclear extracts were subjected to EMSA with a supershift assay using antibodies against p65 and p50. Arrowhead indicates the NF-κB/protein complex. Arrows: supershift positions of p65 and p50. Bar graph (A-E) data were quantified by densitometric analysis and expressed as fold change relative to untreated controls. Values are mean (s.o.) of three independent experiments. *P < 0.05 vs control; **P < 0.05 vs OSM alone.
EGCG alleviated OSM/Cyr61-induced CCL2 expression.

(A) U2OS cells were incubated with Cyr61 (200 ng/ml) for 0, 4, 8, 12 and 24 h. CCL2 mRNA levels were assayed by northern blotting. **(B)** Cells were treated with various concentrations of Cyr61 for 24 h and the amounts of CCL2 released into the culture medium were quantified by ELISA. Values are mean (S.D.) of three independent experiments. *P < 0.05 vs control; **P < 0.05 vs 100 ng/ml Cyr61. U2OS cells were transduced with shRNA targeting Cyr61. The gene silencing effect was confirmed by western blotting (C). Cells transduced with Cyr61 shRNA were stimulated by OSM (10 ng/ml) and EGCG (10 μg/ml; 3 h before the addition of OSM), alone or in combination for 8 (D) or 24 h (E) and CCL2 expression was assessed by northern analysis (D) or ELISA (E). Bar graph (A, C and D) data were quantified by densitometric analysis and expressed as fold change relative to untreated control. Values are mean (S.D.) of three independent experiments. *P < 0.05 vs control; **P < 0.05 vs OSM alone; ***P < 0.05 vs OSM + Cyr61 shRNA transfection. (F and G) Values are mean (S.D.) of three independent experiments. *, X, +: P < 0.05 vs control; **, XX, ++: P < 0.05 vs the respective stimulant alone.
Radiography revealed elevated scores in the control group, whereas administration of EGCG reduced the severity of CIA (3.3 ± 0.5 vs 1.9 ± 0.8) (Fig. 5B). In another 10 CIA rats receiving EGCG or NS administration (five per each group), serum CCL2 levels elevated significantly in control rats at the final stage of the experiment (250 ± 82.3 pg/ml). EGCG reduced the amount of circulating CCL2 (136 ± 45.3 pg/ml) (Fig. 5E).

Histopathological examination revealed extensive pannus formation, resulting in pronounced destruction of the articular surface in the control animals (Fig. 6A). Under HPF, prominent inflammatory cell infiltration associated with zig-zag bone resorption was noted (Fig. 6C). Immunohistochemistry demonstrated marked Cyr61 expression in osteoblasts rimming the osteolytic areas (Fig. 6E). In addition, prominent CD68 signals were observed in the macrophages close to the resorption lacunae (Fig. 6G). In contrast, EGCG markedly alleviated joint destruction, as evidenced by the preservation of joint contour (Fig. 6B). HPF revealed decreased inflammatory cell infiltration, with much less erosion of cartilage and bone (Fig. 6D). EGCG also reduced the number of.

Twenty rats were immunized with BCII in CFA on day 1 and BCII in IFA on day 7. Animals (10 per each group) were injected with normal saline or EGCG on a daily basis until they were killed (day 35). The severity of arthritis was quantified by the clinical arthritis index (A) and semiquantitative radiographic score (B). Note the radiographic images of ankle joints from the normal saline (C) or EGCG (E) group. In another 10 rats, CIA induction and EGCG or normal saline administration (five per each group) were performed similarly. Blood samples were obtained by retro-orbital puncture on day 35. The serum concentration of CCL2 was determined by ELISA (E). *P < 0.05 vs control.
Fig. 6 EGCG abrogated osteoblastic expression of Cyr61 and macrophage infiltration in the ankle joints of CIA rats.

In the control group (A, C, E and G), extensive destruction of bone by pannus (A, arrows) with heavy infiltration of inflammatory cells is noted (A and C). Marked expression of Cyr61 in osteoblasts (arrowheads) overlying the osteolytic areas is found (E). CD68 is clearly visible in macrophages (arrowheads) adjacent to resorption lacunae (G). In the EGCG group (B, D, F and H), less bone erosion and inflammatory cell infiltration are observed (B and D). A reduced number of Cyr61+ osteoblasts (F, arrowheads) associated with decreased infiltration of CD68-positive macrophages (H, arrowheads) is noted. (A-D: H&E stain, E and F: immunohistochemical stain for Cyr61, G and H: immunohistochemical stain for CD68; original magnification: A and B: x40, C and D: x200, E and H: x400).
Cyr61+ osteoblasts (Fig. 6F) and recruitment of macrophages (Fig. 6H).

Discussion

In the study we showed that proinflammatory cytokines stimulated the expression of Cyr61 in human bone marrow-derived osteoblasts and U2OS cells. The inductive effects were suppressed by EGCG in both cell types. Exogenous Cyr61 enhanced the growth of both types of osteoblastic cell, whereas addition of EGCG did not exhibit any effect on cell proliferation. Our findings were consistent with those from previous studies that showed that Cyr61 enhanced the proliferation of osteoblasts [29] and EGCG had no effect [30]. Although varied in magnitude, all cytokines tested show stimulatory action on Cyr61 synthesis. Furthermore, EGCG had similar suppressive effects on Cyr61 production induced by various cytokines. OSM was used in the mechanistic study of Cyr61 expression in osteoblasts since our previous studies have demonstrated the significance of OSM/osteoblast interactions in inflammatory bone diseases [4, 24].

OSM-induced Cyr61 synthesis was diminished by LY294002 and SH-5, but not rapamycin. Rapamycin is a specific inhibitor of mTORC1 with high potency. Under the concentrations between 5 nM and 1 μM, rapamycin could disrupt the assembly of the mTOR/raptor complex within 30 min [31]. In our experiment, cells were treated with 50 ng/ml, approximately 55 nM rapamycin for 24 h, with only a minimal decrease in Cyr61 production. Furthermore, we inhibited raptor expression with raptor shRNA and similarly no significant reduction of Cyr61 synthesis was noted. These data implied that although PI3K/Akt is important for OSM action, mTORC1 is not the major downstream mediator. In contrast, Akt is the downstream target of rapamycin-insensitive mTOR/riCTOR complex (mTORC2) [17] and can activate NF-κB either by inducing IκB degradation or mediating IKKα kinase phosphorylation [14]. In U2OS, OSM induced a physical interaction between the mTOR/riCTOR complex and Akt, which is consistent with the proposal that mTOR/riCTOR is a direct Akt kinase [17]. Moreover, because no specific inhibitors of rictor and mTOR are available, we silenced these genes by shRNAs and found attenuation of OSM-induced Akt activation, degradation of IκBα and Cyr61 production. These results suggested a role of mTOR/riCTOR in OSM-induced Akt/NF-κB signalling.

NF-κB is crucial for the mediation of inflammatory responses and it is known that Cyr61 promoter contains NF-κB consensus sequence [13]. We examined the effect of OSM on NF-κB activity and found that OSM induced Akt-dependent IκB degradation. Moreover, p65 shRNA effectively blocked OSM-enhanced Cyr61 expression in U2OS. EMSA and supershift assay revealed increased DNA binding with nuclear p65 and p50 after OSM stimulation. The findings suggested the importance of p65/p50 heterodimer in OSM-induced Cyr61 synthesis. In the study we showed for the first time that cytokine-induced Cyr61 expression is mediated via NF-κB signalling. In addition, activation of NF-κB by OSM is mTOR/riCTOR/Akt-dependent. This may to a certain extent correlate with the findings of a recent study demonstrating that transcription of p65-mediated gene required activation of mTOR-related pathways [15].

Recently investigators began to recognize the influences of EGCG on mTOR activity. Most of the experiments reported the suppressive effects of EGCG on mTOR synthesis [32] or phosphorylation [33]. In contrast, our data showed that EGCG inhibited the OSM-induced binding between mTOR/riCTOR and Akt, resulting in diminished Akt activation, reduced degradation of IκBα and finally decreased nuclear translocation of p50 and p65. Taken together, our results proposed that EGCG could attenuate OSM-stimulated Cyr61 activation by suppressing the mTORC2/Akt/IκB/NF-κB signalling pathway. To the best of our knowledge, the inhibitory effect of EGCG on cross-talk between mTOR/riCTOR and Akt has never been shown before. More interestingly, Akt is frequently deregulated in tumor cells that have lost their normal phosphatase and tensin homologue (PTEN) activity [16, 34]. For those cancer cells, the mTOR/riCTOR complex has been proposed to be a valuable drug target [17]. In this regard, our results also highlighted the potential benefit of EGCG as an anti-cancer agent. We previously reported that EGCG could alleviate CCL2 expression in OSM-stimulated human osteoblastic cells via its action on Raf-1. In that model, EGCG attenuated Raf-1 phosphorylation at Ser338, resulting in reduced mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) activity, diminished interaction between AP-1 and CCL2 promoter and finally diminished CCL2 production [23]. Because Raf-1 can also activate NF-κB [35], the results of our previous study also underlined the importance of the NF-κB pathway in the anti-inflammatory action of EGCG on osteoblast.

We found that Cyr61 enhanced the OSM-induced expression of CCL2 in osteoblastic cells and an additive effect was noted when OSM and Cyr61 were added together. Although not completely, knockdown of Cyr61 prominently repressed CCL2 synthesis. CCL2 primarily modulates chemotaxis of macrophages/monocytes and its expression has been detected in pathological conditions associated with macrophage aggregation, including RA [24]. Our results implied that Cyr61 could also stimulate macrophage recruitment to promote RA pathogenesis, in addition to its effect on mediating the proliferation of fibroblast-like synoviocytes reported recently [10]. Moreover, EGCG diminished OSM-induced CCL2 expression to a certain extent via suppressing Cyr61 induction. However, other signalling pathways, such as AP-1 and CCL2, might also be involved, as EGCG exerted an additional effect after the knockdown of Cyr61 (Fig. 4D).

To assess the clinical relevance of EGCG action, a rat model of CIA was used. Radiographically, profound deformity of joint architecture was found in control animals. Histopathological examinations and immunohistochemical stains demonstrated typical microscopic features of arthritis, aggregation of proliferating vessels, and CD68+...
macrophages around Cyr61+ osteoblasts in areas of bone resorption. Our results substantiated the significance of Cyr61 in RA development. In contrast, image and microscopic analyses revealed that administration of EGCG diminished the extent of joint destruction, accompanied by a decreased number of Cyr61+ osteoblasts. These findings implied a therapeutic benefit of EGCG on RA.

In conclusion, excessive Cyr61 may be pathogenic in inflammatory conditions, especially in the presence of other proinflammatory cytokines. EGCG attenuates cytokine-stimulated Cyr61 expression in human osteoblastic cells and, at least for OSM, this action is mediated by suppression of the mTORC2/Akt/ltre/B/NF-NkB signalling pathway. EGCG also alleviates the progression of CIA, at least partially due to the reduction of Cyr61 synthesis in osteoblasts. Our results imply a therapeutic potential of EGCG on arthritis.

### Rheumatology key messages

- EGCG diminishes cytokine-stimulated Cyr61 expression in osteoblastic cells via suppression of the mTORC2/Akt/ltre/B/NF-NkB signalling pathway.
- EGCG alleviates CIA progression via abolition of Cyr61 synthesis in osteoblasts.

### References


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### Disclosure statement:
The authors have declared no conflicts of interest.

### Supplementary data

Supplementary data are available at Rheumatology Online.


14 Ozes, Mayo, Gustin et al. NF-kB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 1999;401:82-5.


24 Kok SH, Hong CY, Kuo MY et al. Oncostatin M-induced CCL2 transcription in osteoblastic cells is mediated by multiple levels of STAT-1 and STAT-3 signaling: an implication for the pathogenesis of arthritis. Arthritis Rheum 2009;60:1451-62.


