Decoy receptor 3 suppresses RANKL-induced osteoclastogenesis via down-regulating NFATc1 and enhancing cell apoptosis

Chia-Pi Cheng¹, Ming-Jen Sheu², Huey-Kang Sytwu³ and Deh-Ming Chang⁴

Abstract

Objective. Decoy receptor 3 (DCR3) has been known to modulate immune functions of monocyte or macrophage. In the present study, we investigated the mechanism and the effect of DCR3 on RANK ligand (RANKL)-induced osteoclastogenesis.

Methods. We treated cells with DCR3 in RANKL-induced osteoclastogenesis to monitor osteoclast formation by tartrate-resistant acid phosphatase (TRAP) staining. Osteoclast activity was assessed by pit formation assay. The mechanism of inhibition was studied by biochemical analysis such as RT–PCR and immunoblotting. In addition, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell apoptosis and apoptosis signalling were evaluated by immunoblotting and using flow cytometry.

Results. DCR3 inhibited RANKL-induced TRAP⁺ multinucleated cells and inhibited RANKL-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation and nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1) nuclear translocation in RAW264.7 cells. Also, DCR3 significantly inhibited the bone-resorbing activity of mature osteoclasts. Moreover, DCR3 enhanced RANKL-induced cell apoptosis and enhanced RANKL-induced Fas ligand expression. The mechanisms were mediated via the intrinsic cytochrome c and activated caspase 9 apoptosis pathway.

Conclusion. We postulated that the inhibitory activity of DCR3 on osteoclastogenesis occurs via down-regulation of RANKL-induced NFATc1 expression and induction of cell apoptosis. Our results postulated DCR3 as a possible new remedy against inflammatory bone destruction.

Key words: osteoclast, decoy receptor 3, NFATc1, FASLG.

Introduction

RA is a chronic inflammatory autoimmune disease characterized by the presence of synovitis and bone erosion concomitant with pannus formation and the destruction of joint cartilage [1–3]. An increasing body of evidence has demonstrated that osteoclasts play a pivotal role in bone resorption in inflammatory joint diseases. Multinucleated giant cells with the phenotypic features of osteoclasts predominate at erosion sites in RA and CIA animal models. Furthermore, it has been reported that mice lacking osteoclasts were resistant to arthritis-induced bone erosion [4]. These findings indicate that the application of anti-osteoclastic medications that inhibit osteoclastogenesis at inflammatory sites might be promising for the treatment of RA.

Decoy receptor 3 (DCR3) is a soluble protein that belongs to the TNF receptor superfamily [5]. DCR3 interacts with its natural ligands, including Fas ligand (FASLG), lymphotoxinlike, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT) and TNF-like protein 1A (TL1A) [6–8]. Previous studies has shown that DCR3 plays a multiplicity of roles in the immune system. DCR3 prevents...
heart allograft rejection [9], promotes cancer cell growth by escaping immune surveillance [10, 11] and ameliorates many animal models of autoimmune disease [12–15]. Investigations also found that DCR3 can modulate macrophage and dendritic cell differentiation and maturation [16, 17]. Furthermore, increasing evidence shows that natural ligands of DCR3, LIGHT and TL1A would promote osteoclastogenesis and bone resorption in inflammatory arthritides [18–20]. Recently, DCR3 was reported to be highly expressed in serum of patients with RA [21]. However, we have described found that global expression of DCR3 attenuated disease severity in CIA mice [22]. Also, the regulatory effects of DCR3 in the inflammatory disease RA or CIA under osteoclastogenesis were not well understood.

In the present study, we assessed the effects of DCR3 on RANKL-induced osteoclastogenesis in human peripheral blood mononuclear cells (PBMCs), RAW264.7 cells and murine bone marrow-derived macrophage (BMM) cells. Moreover, we evaluated the possible mechanism of DCR3 in RA inflammation under RANKL-induced osteoclastogenesis.

Materials and methods

Cell line and reagents

Murine RAW264.7 cells were obtained from the Food Industry Research and Development Institute (FIRDI) in Taiwan. Recombinant human DCR3 was purchased from R&D. Human and mouse recombinant RANKL and M-CSF were purchased from Peprotech (London, UK). Anti-NF-κBp65, anti-p38, anti-extracellular-signal-regulated kinase (ERK), anti-phospho-p38, anti-phospho-ERK and anti-caspase antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) came from Epitomics. Anti-TATA-box-binding protein (anti-TBP) came from Millipore. Anti-cytochrome c (H19) came from BioWorld Technology (Minneapolis, MN, USA). Anti-NFATc1 nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were purchased from Sigma Chemical Co (St Louis, MO, USA) or Wako Pure Chemical Industries Ltd (Osaka, Japan).

Cell culture, cell isolation and osteoclast differentiation

RAW264.7 cells were cultured with DMEM (Gibco BRL, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were grown in a humidified atmosphere containing 5% CO2 at 37°C. To induce osteoclast differentiation, RAW264.7 cells were suspended in α-minimal essential medium (MEM) containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin; seeded at 5 x 10^3 cells/well in a 96-well plate; and cultured with 50ng/ml soluble RANKL plus DCR3 or IgG control for 5 days. Human peripheral blood was obtained from healthy adult volunteers. PBMCs were isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare, Bucks, U.K.) and were re-suspended in α-MEM (Gibco BRL) supplemented with 10% heat-inactivated FBS, 30ng/ml M-CSF, penicillin (100 U/ml) and streptomycin (100 μg/ml). PBMCs were plated in 96-well plates (1.5 x 10^5 cells/well) and incubated overnight at 37°C. Non-adherent cells were then removed and the adherent cells were grown in the presence of 30ng/ml M-CSF and 50ng/ml RANKL as well as DCR3 or IgG for 5 days to generate osteoclasts. Murine osteoclasts were generated as previously described with minor modifications [23]. Briefly, BMM cells from normal DBA/1J mice were cultured overnight, and adherent BMM cells were harvested and cultured in the presence of 30ng/ml M-CSF and 50ng/ml RANKL as well as DCR3 or IgG for 5 days to generate osteoclasts. All differentiated media were replaced on day 3.

DCR3 plasmid transfection

The pCMV-hDCR3 construct was obtained as previously described [22]. RAW264.7 cells were transfected with DCR3 plasmid or empty vector using Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Briefly, plasmid was formulated with reagent and diluted in α-MEM then incubated with RAW264.7 cells. After 6 h incubation, the mixture was replaced by osteoclast differentiation medium.

Tartrate-resistant acid phosphatase staining

Cells were washed with PBS and fixed with 3.7% formaldehyde for 30 min. After washing with PBS, cells were incubated at 37°C in a humid and light-protected incubator for 1 h in the reaction mixture of the Leukocyte Acid Phosphatase Assay kit (Cat.387; Sigma), as directed by the manufacturer. Cells were washed three times with distilled water and tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells containing five or more nuclei were counted under a light microscope and photographed.

Pit formation assay

RAW264.7 cells were seeded onto 20 mm² dentine slices (Cat.3988; Corning, corning, NY, USA) in 24-well plates at a density of 1 x 10^5 cells per well to test osteoclast resorption activity. Then, the dentine slice was treated with 1 N NH₄OH with sonication for 5 min. Resorption pits on the dentine slices were visualized by staining with Mayer’s haematoxylin solution (Sigma). The ratio of the resorbed area to the total area was measured in four optical fields on a slice using NIH Image software (NIH, Bethesda, MD, USA) at 100-fold magnification.

RT-PCR analysis

Total RNA was isolated from cultured cells with TRizol reagent (Invitrogen Inc., USA) and reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). PCR was performed with mouse-specific primers,
shown in supplementary Table S1, available as supplementary data at Rheumatology Online. Thermal cycling parameters were 95°C for 5 min, followed by 25–30 cycles for 30 s at 95°C, 30 s at 61°C, 1 min at 72°C and 10 min at 72°C for the final elongation. The number of cycles for each gene was determined to be in the range of linear amplification through an optimization experiment. PCR products were separated on 1.2% agarose gels, visualized by ethidium bromide staining and analysed densitometrically using a Phosphoimager and Quantity One software. Optical densities for each gene were normalized to the corresponding values for GAPDH.

Immunoblotting analysis

Nuclear extracts (NEs) were prepared according to the method of Andrews and Faller [24]. In brief, RAW264.7 cells treated with DCR3 or IgG control in the absence or presence of 50 ng/ml RANKL were harvested, washed with PBS, suspended in 400 μl of buffer A and incubated on ice for 15 min. Nuclei were pelleted by centrifugation for 5 min at 18000g, and the supernatant was collected as the cytoplasmic fraction. The nuclei were resuspended in 40 μl of buffer C, incubated on ice for 20 min and centrifuged for 5 min at 18000g at 4°C. The supernatant was used as the NE. Equivalent amounts of protein were loaded for SDS-PAGE, and immunoblotting was performed using specific antibodies for p38, phospho-p38, ERK, phospho-ERK, c-Jun N-terminal kinase (JNK), phospho-JNK, NF-κBp65, NFATc1, Caspase (3, 6, 9, 12), cytochrome c, TBP, β-actin and GAPDH.

NFATc1 immunofluorescent staining

RAW264.7 cells were seeded onto glass coverslips and then incubated with 30 ng/ml M-CSF and 50 ng/ml RANKL in the presence of DCR3 or IgG control. The distribution of NFATc1 protein 48 h after stimulation was assessed according to previously published protocols [25]. Coverslips were removed, washed in PBS, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with 5% BSA and incubated with a specific anti-NFATc1 monoclonal antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. Cells were washed in PBS, incubated for 2 h with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After the immunostaining procedures, cells were nuclear-stained with DAPI (Sigma). Fluorescence was visualized using a Leica fluorescence microscope (Leica DMI4000B, Wetzlar, Germany). The percentage of cells displaying nuclear staining was then quantified and 100 cells per group were measured from three separate coverslips per group.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cells (1 × 10⁴/well) were seeded in a 96-well plate with medium supplemented with 10% FBS and treated with various concentrations of DCR3 for 24 and 48 h, and then washed three times with PBS and treated with medium containing 500 μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 30 min at 37°C. Cells were then washed with PBS and solubilized in 100 μl of dimethyl sulfoxide (DMSO). The intracellular purple formazan concentrations were determined at 550 nm in an ELISA plate reader.

Apoptosis assay

The Annexin V-phycocerythrin (PE) Apoptosis Detection Kit (eBioscience, San Diego, CA, USA) was used according to the manufacturer’s instructions. Briefly, cells were collected and stained with Annexin V-PE, a calcium-dependent phosphoserine-binding protein, for 15 min, and then with 7-amino-actinomycin D (7-AAD), a viability dye. Cells were analysed by flow cytometry (Beckman Coulter, Fullerton, CA, USA), where Annexin V-PE-positive and 7-AAD-negative cells were defined as apoptotic.

Flow cytometric analysis

RAW264.7 cells and PBMCs were cultured in osteoclast differentiation medium with DCR3 or IgG control for 48 h. Cells were trypsinized, washed twice with PBS, stained with PE-conjugated anti-CD178 (anti-Fas ligand) antibody (eBioscience) for 30 min at room temperature, washed twice with PBS and protected from light until data collection. All experiments were analysed by FACSCalibur (BD Biosciences, San Jose, California, USA) and CellQuest Pro software (BD Biosciences, San Jose, California, USA).

Statistical analysis

Data were shown as means (s.o.) and were analysed using one-way analysis of variance (ANOVA) with Newman–Keuls multiple comparisons on post tests. P < 0.05 was considered to be statistically significant.

Results

Effects of DCR3 on RANKL-induced osteoclast differentiation

We evaluated the effect of DCR3 on osteoclastogenesis by TRAP, a specific marker of osteoclast differentiation, using osteoclast precursor cell line RAW264.7. DCR3 decreased the number of multinucleated osteoclasts in a dose-dependent manner (Fig. 1A). DCR3 alone had no effect on osteoclast formation (supplementary Fig. S1, available as supplementary data at Rheumatology Online). The expression pattern of DCR3 protein was elevated to a peak at 72 h after plasmid transfection (supplementary Fig. S2, available as supplementary data at Rheumatology Online). The expression pattern of DCR3 protein was elevated to a peak at 72 h after plasmid transfection (supplementary Fig. S2, available as supplementary data at Rheumatology Online). The expression pattern of DCR3 on osteoclastogenesis were further confirmed by using primary BMM cells and human PBMCs. BMM cells and PBMCs were cultured in the presence of M-CSF (30 ng/ml) and RANKL (50 ng/ml) together with DCR3 or IgG (10 μg/ml) for 5 days. DCR3 reduced the number of TRAP-positive multinucleated cells generated with
Fig. 1 Effects of DCR3 on RANKL-induced osteoclast differentiation of PBMCs, BMM cells and RAW264.7.

(A) RAW264.7 cells were treated with serial dilution of DCR3 (2.5, 5 and 10 μg/ml) or IgG control (10 μg/ml) in the presence of RANKL (50 ng/ml) for 5 days. (B) BMM cells were treated with serial dilution of DCR3 (2.5, 5 and 10 μg/ml) or IgG control (10 μg/ml) in the presence of RANKL (50 ng/ml) and M-CSF (30 ng/ml) for 5 days. (C) PBMCs were treated with DCR3 (10 μg/ml) or IgG control (10 μg/ml) in the presence of RANKL (50 ng/ml) and M-CSF (30 ng/ml) for 5 days. After incubation, the cells were fixed and stained for TRAP and TRAP⁺ multinucleated cells containing more than three nuclei were counted as multinucleated osteoclasts. Data represent the means (s.d.) of more than three cultures. *P < 0.05, ***P < 0.001.
78.46% ± 7.05% inhibition at 10 μg/ml concentrations on BMM cells, respectively (Fig. 1B). Interestingly, we found that TRAP-expressing cells in the DCR3-treated group seem to have defects and diminished cell numbers compared with the IgG control in PBMCs, BMM and RAW264.7 cells. Also, the cytoplasmic region of osteoclast was not fused well in DCR3-treated PBMCs as shown by the pointed arrows in Fig. 1C (white arrow pointed common fused osteoclast, black arrow pointed bad fusion of osteoclast).

**Effects of DCR3 on bone resorption in cell cultures**

To examine the effects of DCR3 on osteoclastic bone resorption, resorption pit formation was assessed. RAW264.7 cells were plated onto dentine slices and then differentiated into osteoclasts by RANKL treatment for 5 days in the presence of DCR3 or IgG control. After being cultured for 5 days, many resorption pits were formed on the bone slices. RANKL-evoked bone resorption was diminished by coincubation addition of DCR3 (Fig. 2). This result indicated that DCR3 prevented osteoclastic bone resorption by inhibiting osteoclastogenesis.

**Effects of DCR3 on NFATc1-regulated gene expression during RANKL-induced osteoclast development**

Osteoclast differentiation is associated with up-regulation of specific genes in response to RANKL and RANK binding. The c-Fos and NFATc1 genes have been reported to play a pivotal role in osteoclast differentiation [26]. Therefore, we examined the effects of DCR3 on the RANKL-induced regulation of c-Fos and NFATc1 expression and assessed whether there were any effects on TRAP, osteoclast-associated receptor (OSCAR), and cathepsin K (CTSK) expression using GAPDH as an internal control. RAW264.7 cells were treated with DCR3 or IgG control and stimulated with osteoclast differentiation medium at various time points. Results revealed that c-Fos and NFATc1 mRNA levels were increased in response to RANKL, but both c-Fos and NFATc1 expression was significantly inhibited by DCR3. DC-STAMP and CTSK mRNA expression was partially inhibited by DCR3 (Fig. 3). However, TRAP and OSCAR were not affected by DCR3 treatment. This raises the possibility that DCR3 may inhibit osteoclast differentiation through the inhibition of RANKL-induced c-Fos and NFATc1 expression.

**Effects of DCR3 on RANKL-induced MAPK activation in osteoclasts**

Several reports have indicated that RANKL induces the activation of three well-known mitogen-activated protein kinases (MAPKs) (ERK, JNK and p38) in osteoclast precursors [27]. These kinases (especially ERK and JNK) also participate in c-Fos and c-Jun activation in osteoclast precursors [28, 29]. Based on the findings that DCR3 suppressed RANKL-induced c-Fos mRNA expression, we next investigated whether or not MAPKs were involved in the inhibition of osteoclastogenesis by DCR3 in RAW264.7. RANKL (50 ng/ml) markedly activated p38 and ERK phosphorylation when measured at the early time point of 8 and 15 min. Results indicated that RANKL-induced phosphorylation was inhibited by DCR3 (Fig. 4).

**Effects of DCR3 on RANKL-induced nuclear translocation and expression of NF-κB and NFATc1**

Activation of NF-κB is important in the activation and survival of mature osteoclasts, as well as osteoclastogenesis [30]. NFATc1 is one of the key transcription factors involved in osteoclast differentiation and autoamplification by RANKL [26]. In the inactive state, NFATc1 is retained in the cytoplasm. When being activated, NFATc1 is translocated from the cytoplasm into the nucleus. Therefore, we performed western blot to analyse NF-κB-p65 and NFATc1 in NEs and cytoplasmic extracts (CEs). The data showed that NF-κB-p65 and NFATc1 in NEs dramatically increased after 48 h treatment by RANKL. However, treatment of DCR3 inhibited NF-κB-p65 and NFATc1 nuclear translocation (Fig. 5A), suggesting that DCR3 suppressed RANKL-induced NF-κB-p65 and NFATc1 nuclear translocation and activation during osteoclastogenesis in RAW264.7 cells. To further confirm whether DCR3 suppressed NFATc1 nuclear translocation at the protein level, we examined the effect of DCR3 on the nuclear translocation of NFATc1 in RANKL-activated RAW264.7 by immunofluorescent staining. As shown in Fig. 5B, the majority of NFATc1 was located primarily in the cytoplasm. Upon RANKL stimulation, nuclear accumulation of NFATc1 was markedly increased by RANKL stimulation for 48 h compared with the levels in cells without RANKL treatment. These elevated levels of nuclear NFATc1 were significantly reduced by DCR3.

**Effects of DCR3 on viability and apoptosis of RANKL-induced osteoclasts**

A previous study has shown that both NF-κB and NFATc1 are required for maintaining RANKL-induced osteoclast survival [30]. Also, Fas ligand was involving in RANKL-induced osteoclast formation and apoptosis [31]. Based on the findings that DCR3 suppressed RANKL-induced NF-κB and NFATc1 translocation, we examined the effect of DCR3 on cell viability and apoptosis in RANKL-induced osteoclastogenesis. We treated RAW264.7 cells with various concentrations of DCR3 for 24 and 48 h and then measured cell viability by an MTT assay. DCR3 alone did not affect cell viability (Fig. 6A). However, DCR3 diminished cell viability ~30% in RANKL-induced RAW264.7 cells at 48 h (Fig. 6B). A ligand of DCR3, Fas ligand, also increased when treated with DCR3 under RANKL stimulation in both RAW264.7 and PBMCs (Fig. 6C). Moreover, apoptosis assay confirmed that DCR3 treatment increased in both apoptotic cells 2-fold under RANKL stimulation (Fig. 6D). In addition, it has been reported that caspase 3 was essential to promote osteoclast differentiation [32]. We found that cleaved caspase 3 was inhibited at early stage, 24 h after DCR3.
Fig. 2 Effects of DCR3 on resorption pit formation.

(A) RAW264.7 cells were seeded on bone slices with 10 μg/ml of DCR3 or IgG control in the presence of RANKL (50 ng/ml). After incubation for 5 days, the dentine slices were recovered from the culture and were subjected to Mayer's haematoxylin staining to visualize resorption pits. Arrows indicate pit area. (B) Percentage of resorbed area was determined using the NIH Image software. Data represent the mean (s.d.) of more than four slices. **P < 0.01.
RAW264.7 cells were treated for 12 and 24 h with 10 μg/ml DCR3 or IgG control in the presence of RANKL (50 ng/ml). Total RNA was isolated with TRIzol, and 1 μg of total RNA was used to transcribe cDNA. cDNA was used as a template for PCR with mouse-specific primers. A representative result of at least three independent experiments is shown. Statistical analysis represents the mean (s.d.). *P < 0.05, **P < 0.01. R + G: RANKL + IgG; R + D: RANKL + DCR3.
treatment under RANKL stimulation. Apoptotic mechanisms were involved in intrinsic cytochrome c release, cleaved caspase 9 and delayed activated caspase 3 at 48 h after RANKL plus DCR3 treatment (Fig. 6E). Taken together, this suggested that DCR3 inhibited cell survival on RANKL-induced osteoclastogenesis by enhancing cell apoptosis.

Discussion

DCR3 is a soluble protein known for anti-inflammatory effects in many autoimmune animal models [12, 13, 15]. Also, DCR3/Fc can modulate macrophage differentiation by suppressing several macrophage makers (CD14, CD16, CD68 and CD206), M-CSF receptor and phagocytic ability [16]. Yang et al. [33] found that DCR3 itself could induce osteoclast formation, bypass the NF-κB signalling pathway and induce TNF-α expression. Also, Tang et al. [34] found that phosphoglycerate kinase (PGK) promoter-driven DCR3 transgenic mice had attenuated in bone mass. However, Lam et al. [35] found that TNF-α-induced osteoclastogenesis was RANKL dependent. Also, Tai et al. [36] found that macrophage-specific CD68 promoter-driven DCR3 transgenic mice could induce tumour-associated macrophages, IL-1ra and IL-10, reduce pro-inflammatory cytokines (TNF-α and IL-6) and not be affected in MMP2, MMP9 or development. Although DCR3 may play a controversial role in monocyte and macrophage differentiation, most of the data showed that phosphorylated kinases (ERK, JNK and p38) and CD51/61 marker, presented by Yang et al. [33] showed lower expression in DCR3 treated alone than in RANKL-stimulated osteoclast precursor cells. Also, Edwards et al. [20] found that DCR3 dose-dependently inhibited LIGHT-induced osteoclast formation and DCR3 alone had no effect on osteoclast formation and resorptive activity of osteoclasts. Accumulating evidence suggests that DCR3-treated monocyte and macrophage lineage cells were dominant M2 macrophage phenotype [36]. We postulated that the PGK promoter-driven DCR3 transgenic mouse model induced a low level osteolytic phenotype, which may result from loss of function of Fas or Fas ligand, as in gld or lpr mice [37]. In our previous study, we showed that DCR3 can attenuate CIA when mice were treated before the onset of the disease [22], and this study further demonstrated that DCR3 is able to suppress inflammation-induced osteoclast differentiation in RANKL stimulation.

Fig. 4 Effects of DCR3 on the activation of MAPKs in RAW264.7 cells.

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RAW264.7 cells were serum-starved for 16 h and treated with 10 μg/ml DCR3 or IgG in the presence of RANKL (50 ng/ml) stimulation for 8, 15 or 30 min. Cell extracts were analysed by western blot using antibodies specifically directed against the phosphorylated forms of MAPKs, compared with data obtained with antibodies directed against the unphosphorylated states of the kinases. Equal amounts of protein were loaded in each lane as demonstrated by the level of β-actin. A representative result of at least three independent experiments is shown. N: RAW264.7 cells; R: RANKL; R + G: RANKL + IgG; R + D: RANKL + DCR3.
FIG. 5 Effects of DCR3 on RANKL-induced nuclear translocation of NF-κB-p65 and NFATc1.

(A) Western blot analysis. RAW264.7 cells were serum-starved overnight and then incubated with RANKL (50 ng/ml) for 48 h in the absence or presence of DCR3. CE and NE were analysed by western blot using antibody specifically directed against NF-κB-p65 and NFATc1 protein. Equal amounts of protein were loaded in each lane as demonstrated by the level of TBP (NE) and GAPDH (CE).

(B) Immunofluorescent analysis. RAW264.7 cells were treated with 10 μg/ml DCR3 or IgG control in the presence of RANKL (50 ng/ml) stimulation for 24 and 48 h. After the time indicated, the cells were stained with anti-NFATc1 antibody (green) and DAPI (nuclear staining, red) (upper panels). The nuclear localization of NFATc1 was confirmed in merged images of RAW264.7 cells treated with RANKL (yellow). NFATc1-positive nuclei were counted in RAW264.7 cells (lower panel). Data represent the mean (±S.D.) of more than three cultures. ***P < 0.001. N: RAW264.7 cells; R: RANKL; R + G: RANKL + IgG; R + D: RANKL + DCR3.
Fig. 6 Effect of DCR3 on viability of RANKL-stimulated RAW264.7 cells.

(A) RAW264.7 cells were seeded onto 96-well plate. Then the cells were treated for 48 h with the indicated concentrations of DCR3 or IgG control. After 48 h, MTT solution (500 μg/ml) was added to each well. The optical density was read at 550 nm in an ELISA plate reader after 30 min of incubation. Cell viability was determined relative to the control.

(B) RAW264.7 cells were treated for 24 and 48 h with the indicated concentrations of DCR3 or IgG control in the presence of RANKL (50 ng/ml). Then the cells were also analysed by MTT assay.

(C) RAW264.7 cells or PBMCs were treated for 48 h with the indicated concentrations of DCR3 or IgG control in the presence of RANKL (50 ng/ml). After 48 h, anti-Fas ligand was stained and analysed by flow cytometry.

(D) RAW264.7 cells were treated for 48 h with 10 μg/ml of DCR3 or IgG control in the presence of RANKL (50 ng/ml). After 48 h, cells were harvested for Annexin V apoptosis assay kit.

(E) RAW264.7 cells were treated for 24 h (left panel) and 48 h (right panel) with 10 μg/ml of DCR3 or IgG control in the presence of RANKL (50 ng/ml). After 24 h and 48 h, cells were harvested for Caspase immunoblotting (ratio C/P means cleaved/procaspase; cytochrome c ratio was normalized to GAPDH). A representative result of at least three independent experiments is shown. Data represent the mean (s.d.) of more than three cultures. *P < 0.05, **P < 0.01, ***P < 0.001.
The RANKL–RANK axis is essential for osteoclastogenesis [38, 39]. Binding of RANKL to RANK strongly activates NF-κB to translocate into the nucleus where it enhances transcription of target genes [40]. Also, RANKL activates a series of intracellular signalling pathways including JNK, ERK, p38 MAPK and transcription factors such as AP-1 (c-Fos) and NFATc1. NFATc1 is a key mediator of osteoclastogenesis that autoamplifies and conducts the expression of osteoclast-specific genes including TRAP, calcitonin receptor, OSCAR and CTSK [41, 42]. In the present study, we found that DCR3 decreased the phosphorylation of ERK, JNK and p38 in response to RANKL. Furthermore, DCR3 also significantly suppressed not only RANKL-induced c-Fos expression but also NFATc1 nuclear transport. As a result of down-regulation of NFATc1 expression, NFATc1-mediated osteoclastogenic genes such as DC-STAMP and CTSK were also concomitantly inhibited by DCR3. However, TRAP and OSCAR, two osteoclast related genes, were not down-regulated. TRAP has been known to be highly expressed by osteoclasts and activated macrophages [43]. OSCAR has been proved to costimulate osteoclastogenesis via immunoreceptor tyrosine-based activation motifs, such as the common Fc receptor γ [44]. This means that DCR3 may not be involved in complete pathways of suppression of macrophage activation or the whole osteoclastogenesis process like FcRγ. On the other hand, macrophage fusion is one of the critical steps in the generation of osteoclasts or giant cells and to affect resorption activity [45, 46]. A previous study proved that cell adhesion molecules such as monocyte chemoattractant protein 1 (MCP1) also stimulated osteoclast fusion by regulating NFATc1 downstream gene, DC-STAMP [47]. Also, DCR3 has proved efficient in down-regulating MCP1 expression [13]. Here, we found that DCR3 inhibits the bone resorptive activity of osteoclast. Also, DCR3 suppressed DC-STAMP mRNA expression. Taken together, we postulate that DCR3 inhibited osteoclastogenesis via down-regulation of RANKL-induced NFATc1 expression. DCR3 inhibited RANKL-induced osteoclast formation by down-regulating DC-STAMP expression, suppressing osteoclast fusion and decreasing phagocytic activity.

FASLG, LIGHT and TL1A, the natural ligands of DCR3, were highly expressed in RA patients and exacerbated arthritis severity in CIA mice treated with these ligands [19, 20, 48]. Also, FASLG and LIGHT could increase
We proved that DCR3 acted directly on modulation of clast differentiation and pit formation on dentine slices. RANKL-stimulated osteoclasts in monocyte macrophage lineage cells that Fas ligand was elevated by the synergistic effect of RANKL and DCR3 in monocyte macrophage lineage cells under RANKL stimulation. Our results regard, we tested whether or not DCR3 was involved in treatment induced dendritic cell apoptosis [51]. In this regard, we tested whether or not DCR3 was involved in osteoclast apoptosis after RANKL stimulation. Our results showed that DCR3 induced RAW264.7 cell apoptosis by enhancing Fas ligand expression, inducing intrinsic cytochrome c and activating caspase 9 under RANKL stimulation. However, the mechanisms may be further clarified that Fas ligand was elevated by the synergistic effect of RANKL and DCR3 in monocyte macrophage lineage cells in RA.

In conclusion, we provide the first evidence that DCR3 inhibited exibitory effects on RANKL-stimulated osteoclast differentiation and pit formation on dentine slices. We proved that DCR3 acted directly on modulation of macrophage differentiation by suppressing NF-κB and NFATc1 translocation and suppressing downstream gene expression. We found that DCR3 inhibited RANKL-stimulated cell fusion of RAW264.7, mouse BMM cells and human PBMCs and decreased the number of large osteoclasts. We also found that DCR3 delayed caspase 3 activation and promoted cytokrome c, caspase 9 and Fas ligand expression for inducing cell apoptosis. Taken together, our data might decipher the possible molecular mechanisms by which DCR3 mitigates CIA-evoked bone destruction and might make DCR3 a potential novel therapeutic medicine for bone disorders such as RA and osteoporosis by fine-tuning RANKL-induced osteoclast differentiation as well as functions.

**Rheumatology key messages**

- The present study revealed reduced osteoclastogenic activity in DCR3-treated monocyte/macrophage cells under RANKL stimulation.
- Modulation of DCR3 may represent a new approach against inflammatory induced osteolytic diseases such as RA.

**Funding:** This work was supported by the National Science Council, Taiwan, Republic of China (NSC-97-2628-B-016-001-MY3 and NSC-99-2628-B-016-001-MY3 to D.-M.C.), and by Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan (TSGH-C99-007-S01 and TSGH-C100-005-007-8-S01).

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

**Supplementary data**

Supplementary data are available at Rheumatology Online.

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Clinical vignette

Temporal ultrasonography findings in temporal arteritis: early disappearance of halo sign after only 2 days of steroid treatment

Temporal arteritis (TA) is the most common chronic systemic vasculitis, frequently involving superficial temporal arteries, hence the name. Diagnosis of TA is based on clinical, laboratory and histological findings, as recommended by the ACR criteria. Nevertheless, over the past few years colour Doppler ultrasonography (CDU) of temporal arteries has emerged as a valid non-invasive diagnostic tool. The main specific CDU finding is the halo sign, a dark hypoechoic circumferential wall thickening around the artery lumen predominantly due to an acute inflammatory process of the arterial wall that tends to disappear a mean of about 2 weeks after the start of steroid treatment [1].

We observed the case of a 79-year-old woman with TA in which the halo sign disappeared after only 2 days of steroid treatment (Fig. 1). This case underlines that temporal CDU aspects have to be considered when performing CDU of temporal arteries, making it desirable to perform this examination before starting steroid therapy. Considering that current guidelines are very clear about the importance of starting high-dose steroids immediately on suspicion of TA, CDU must be performed immediately rather than delaying steroids while this examination is being arranged.

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

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