EXPERIMENTAL CELL RESEARCH 317 (2011) 1454–1464

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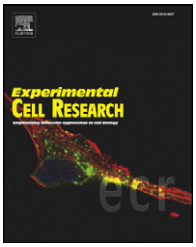

Research Article

Inhibition of matrix metalloproteinase-9 activity by doxycycline ameliorates RANK ligand-induced osteoclast differentiation *in vitro* and *in vivo*

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ARTICLE INFORMATION

Article Chronology:
Received 4 November 2010
Revised version received
13 March 2011
Accepted 15 March 2011
Available online 21 March 2011

Keywords:
Doxycycline
Osteoclast
RANK ligand
MMP-9
MAPKs
NFATc1

ABSTRACT

Tetracycline antibiotics, including doxycycli\e (DOX), have been used to treat bone resorptive diseases, partially because of their activity to suppress osteoclastogenesis induced by receptor activator of nuclear factor kappa B ligand (RANKL). However, their precise inhibitory mechanism remains unclear. Therefore, the present study examined the effect of Dox on osteoclastogenesis signaling induced by RANKL, both *in vitro* and *in vivo*. Although Dox inhibited RANKL-induced osteoclastogenesis and down-modulated the mRNA expression of functional osteoclast markers, including tartrate-resistant acid phosphatase (TRAP) and cathepsin K, Dox neither affected RANKL-induced MAPKs phosphorylation nor NFATc1 gene expression in RAW264.7 murine monocytic cells. Gelatin zymography and Western blot analyses showed that Dox down-regulated the enzyme activity of RANKL-induced MMP-9, but without affecting its protein expression. Furthermore, MMP-9 enzyme inhibitor also attenuated both RANKL-induced osteoclastogenesis and up-regulation of TRAP and cathepsin K mRNA expression, indicating that MMP-9 enzyme action is engaged in the promotion of RANKL-induced osteoclastogenesis. Finally, Dox treatment abrogated RANKL-induced osteoclastogenesis and TRAP activity in mouse calvaria along with the suppression of MMP9 enzyme activity, again without affecting the expression of MMP9 protein. These findings suggested that Dox inhibits RANKL-induced osteoclastogenesis by its inhibitory effect on MMP-9 enzyme activity independent of the MAPK-NFATc1 signaling cascade.

Introduction

Bone resorption is facilitated by osteoclasts, unique multinucleated giant cells, which are derived from monocyte–macrophage lineage cells originated in bone marrow. While osteoclasts play a critical role in the physiological bone remodeling process, they are also engaged in pathological bone resorption in bone lytic diseases, such as rheumatoid arthritis, osteomyelitis, osteoporosis and periodontal disease [1,2].

The tetracycline family compounds (TCs) are well known and widely used as antibiotics to treat common infections, as well as rare

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0014-4827/$ – see front matter
doi:10.1016/j.yexcr.2011.03.014

infectious diseases, such as Lyme disease and ehrlichiosis [3]. In addition to their antibiotic activity, TCs and their chemically modified analogs (CMTs) are known to suppress bone resorption, both *in vitro* and *in vivo* [4]. At first, the inhibition of bone loss was solely attributed to the ability of TCs to inhibit the enzymatic action of matrix metalloproteinases (MMPs), especially collagenases and gelatinases [5,6]. However, Bettany et al. demonstrated that TCs and CMTs can directly induce cell apoptosis in osteoclasts [7]. Furthermore, following the latter study, Holmes et al. revealed that doxycycline (Dox) and CMTs can down-regulate *in vitro* osteoclastogenesis from human peripheral blood mononuclear cells stimulated with receptor activator of NF-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) [8]. This accumulating evidence suggested that TCs or CMTs can suppress bone resorption by inhibiting not only the bone lytic enzyme activity of osteoclasts (i.e., inhibition of MMPs activity) but also their RANKL-induced differentiation. Thus far, however, the precise mechanism underlying the ability of TCs or CMTs to inhibit RANKL-induced OCgenesis has been unclear.

Differentiation of osteoclasts from osteoclast precursor cells, or osteoclastogenesis (OCgenesis), and the activation of bone resorption function by mature osteoclasts are events that require RANKL and its permissive factor M-CSF to induce the expression of RANK, a receptor for RANKL [9]. RANKL ligation to RANK then leads to recruitment of TNF receptor-associated factor 6 to the cytoplasmic domain of RANK, which, in turn, results in the activation of distinct signaling cascades mediated by mitogen-activated protein kinases (MAPKs), including c-jun N-terminal kinase (JNK), p38 MAPK (p38), and extracellular signal-regulated kinase (ERK) [10]. Once the MAPKs signaling cascade is activated, nuclear factor of activated T cells (NFAT)c1 is elicited as a master transcription factor for osteoclast differentiation [10,11]. It is also true that auto-amplified NFATc1 plays a key role in up-regulating expressions of genes required for osteoclast maturation, such as TRAP [12], cathepsin K [13], or MMP-9 [14], which are requisite for the bone resorption processes mediated by mature osteoclasts.

This study aimed to elucidate the molecular mechanism underlying Dox-mediated inhibition of RANKL-induced OCgenesis. Therefore, the MAPKs-NFATc1 signaling cascade and osteoclast maturation marker genes, including TRAP, cathepsin K, and MMP-9, were examined *in vitro* using RAW264.7 murine monocytic cells followed by *in vivo* OCgenesis assay using mouse calvaria. The biological effects of TCs or CMTs are thought to be derived from their inhibition of MMP-9's enzymatic actions [5,6], but it is unclear if inhibition of MMP-9 by these drugs is solely responsible for their suppression of RANKL-induced OCgenesis. Therefore, we investigated the effects of MMP-9 enzyme inhibition mediated by Dox, as well as a chemical inhibitor for MMP9 enzyme, on RANKL-induced OCgenesis and osteoclast maturation marker genes in RAW264.7. Finally, by using an *in vivo* mouse calvaria model, the efficacy of Dox and its inhibitory effects on MMP-9 enzyme action on RANKL-induced OCgenesis was confirmed. For the first time, we report here that Dox does suppress RANKL-induced OCgenesis by its inhibitory effect of MMP activity independent of the MAPK-NFATc1 signaling cascade.

Materials and methods

Reagents and antibodies

sRANKL and M-CSF were purchased from Peprotech (Rocky Hill, NJ). MMP-9 was obtained from R&D Systems (Minneapolis, MN). Antibodies for rabbit anti-mouse total ERK, rabbit anti-mouse total p38, rabbit anti-mouse total JNK, rabbit anti-mouse phosphorylated ERK, rabbit anti-mouse phosphorylated p38, and rabbit anti-mouse phosphorylated JNK were purchased from Cell Signaling Technology (Beverly, MA). Goat anti-mouse MMP-9 antibody and mouse anti-mouse α-Tublin (B-7) antibody were from Santa Cruz (Santa Cruz, CA). Peroxidase-conjugated donkey anti-rabbit IgG antibody was purchased from Jackson Immunoresearch (West Grove, PA), and peroxidase-conjugated rabbit anti-goat IgG antibody and peroxidase-conjugated goat anti-mouse IgG antibody were obtained from Sigma (Louis, MO). Doxycycline (Dox), amoxicillin (Amo), vancomycin (Van), bacitracin (Bac), spectinomycin (Spc), gentamicin (Gen), tetracycline (Tet) and minocycline (Min) were all purchased from Sigma. MMP-9 inhibitor I ($C_{27}H_{33}N_3O_5S$) was purchased from Calbiochem (San Diego, CA).

Mice

BALB/c mice (6 to 8-week-old males) were kept in a conventional room with a 12-h light–dark cycle at constant temperature. The experimental procedures employed in this study were approved by the Forsyth IACUC.

Osteoclast culture

Bone marrow-derived monocyte-macrophage cells (BMM) cells were generated as described previously [15]. In brief, nonadherent bone marrow-derived monocyte–macrophage cells derived from BALB/c mice were seeded and cultured in a-minimal essential medium (α-MEM) (Sigma) with 10% fetal bovine serum (FBS) (Thermo Scientific, Waltham, MA) containing 10 ng/ml M-CSF (medium A). After 2 days, adherent cells were used as BMM cells after washing out the nonadherent cells, including lymphocytes. For osteoclastogenesis, BMMs were seeded at 2×10^5 cells/cm^2 in α-MEM with 10% FBS and cultured in medium A containing with sRANKL (50 ng/ml) (medium B) with or without Dox (0.2 and 2.0 μg/ml). This highest concentration of Dox (2 μg/ml) used in the *in vitro* study was chosen from the minimum inhibitory concentration of several bacteria, including *Staphylococcus aureus*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis* [16,17]. RAW264.7 cells (ATCC, Manassas, VA) were plated at 5×10^3 cells/cm^2, and 12 h later the medium was changed into α-MEM with 10% FBS containing sRANKL (50 ng/ml) (medium C) with or without each antibiotic. After 6 days for BMMs or 5 days for RAW264.7 cells, cells were fixed by 4% paraformaldehyde, washed with phosphate buffered saline, and stained for TRAP. TRAP-positive multinucleated (>3 nuclei) cells were counted as osteoclast-like cells. To determine the bone resorption activity by osteoclast-like cells induced by sRANKL, a pit formation assay was performed by using dentin disks (diameter = 5 mm; ALPCO Diagnostics, Windham, NH) [18]. Briefly, RAW264.7 cells at 5×10^3 cells/cm^2 were cultured on dentin disks in 96-well culture plates for 6 days in medium C with or without Dox (2 μg/mL). After incubation, the cells were removed by washing with 10% sodium hypochlorite, and the dentin disks were then stained by a 0.5% Toluidine Blue solution.

Protein extraction and immunoblotting

Cells were lysed in buffer containing 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.1% SDS, 1% NP-40, 10%