Published in final edited form as:

Exp Cell Res. 2011 June 10; 317(10): 1454–1464. doi:10.1016/j.yexcr.2011.03.014.

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

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Abstract

Tetracycline antibiotics, including Doxycycline (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.

Keywords

Doxycycline; Osteoclast; RANK ligand; MMP-9; MAPKs; NFATc1

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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 infectious diseases, such as Lyme disease and ehrlichiosis [3]. In addition to their antibiotic activity, TCs and their chemically modified analogues (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- κ B 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 -Tubulin (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 (C₂₇H₃₃N₃O₅S) 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 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/cm² 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 μ g/ml). This highest concentration of Dox (2 μ g/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/cm², 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 discs (diameter = 5 mm; ALPCO Diagnostics, Windham, NH) [18]. Briefly, RAW264.7 cells at 5×10^3 cells/cm² were cultured on dentin discs in 96-well culture plates for 6 days in medium C with or without Dox (2 μ g/mL). After incubation, the cells were removed by washing with 10% sodium hypochlorite, and the dentin discs 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 % Glycerol, and 1% (v/v) Triton X-100. The cell lysates were subjected to ultrasonic treatment for 8 sec on ice. The proteins in the cell

lysate were separated using SDS/PAGE (12 % gel) electrophoresis and electrophoretically transferred onto a nitrocellulose (NC) membrane (Bio-Rad Laboratories, Hercules, CA). The NC membranes were blocked with 5 % skim milk for 1 hr, followed by the reaction with rabbit anti-mouse phosphorylated ERK antibody (1:1000), rabbit anti-mouse total ERK antibody (1:1000), rabbit anti-mouse phosphorylated p38 antibody (1:1000), rabbit anti-mouse total p38 antibody (1:500), rabbit anti-mouse phosphorylated JNK antibody (1:500), rabbit anti-mouse total JNK antibody (1:1000), goat anti-mouse MMP-9 antibody (1:1000) and mouse anti-mouse α -tubulin antibody (1:1000) at 4 °C overnight. After the NC membrane was washed, it was incubated with peroxidase-conjugated donkey anti-rabbit IgG antibody (1:5000), peroxidase-conjugated rabbit anti-goat IgG antibody (1:5000), or peroxidase-conjugated goat anti-mouse IgG antibody (1:5000) for 1 hr at room temperature. After further washing, the localization of antibody specific to the molecule of interest on the NC membrane was developed by using Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA).

Gelatin Zymography

The amount of total protein in each lysate corrected as described above was measured using Quant-iTTM protein assay kit (Invitrogen, Carlsbad, CA, USA), and an equal amount of protein was separated by electrophoresis in 10% Tris-Glycine zymogram gelatin gel (Invitrogen) under non-reducing conditions. The proteins separated in the gel, the zymogram gel was developed in Developing Buffer supplied by the manufacturer (Invitrogen). Subsequently, the developed gelatin gel was stained with colloidal blue stain (Invitrogen), allowing the proteases to be visualized as clear bands on the gelatin gel.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cells by using RNA-Bee (TEL-TEST, Friendswood, TX). Complementary DNA was synthesized from the freshly isolated total RNA (1 mg) by using SuperScriptTM-II transcriptase (Invitrogen) following the manufacturer's instructions. The synthesized cDNA was reacted and amplified with specific PCR primers in the presence of Taq polymerase (Hot Start Taq, Qiagen, Valencia, CA). The primer sequences are as follows: NFATc1 forward 5'-GATGCTGAACCTGAGACGCC-3'; NFATc1 reverse 5'-GCCACCAGCCAGTCTGGTGT-3'; TRAP forward 5'-ACACAGTGATGCTGTGTGGCAACTC-3'; TRAP reverse 5'-CCAGAGGCTTCCACATATATGATGG-3'; cathepsin K forward 5'-CTGAAGATGCTTTCCCATATGTGGG-3'; cathepsin K reverse 5'-GCAGGCGTTGTTCTTATTCCGAGC-3'; β -actin forward 5'-GACGGGGTCACCCACACTGT-3'; β -actin reverse 5'-AGGAGCAATGATCTTGATCTTC-3'. The amplified PCR products were separated by electrophoresis in 1.5% agarose gels. The PCR products in the gels were visualized by SYBR safeTM (Invitrogen) and scanned using AlphaImager® (San Leandro, CA).

Effect of Dox on systemic homeostatic bone metabolism in mice

BALB/c mice (6-week-old males, 5 mice/group) were treated with either Dox (30mg/kg/day in water solution) or with control water for 30 days (p.o. every single day). Afterwards, the animals were sacrificed, and the measurement of BMD (g/cm²) of whole body and femur was performed using a PIXImusTM bone mineral densitometer (Lunar Corp., Madison, WI). Further, the femur of each animal was scanned with a high-resolution micro-CT (b-cube AG, Schlieren-Zürich, Switzerland).

sRANKL/LPS-induced bone resorption in calvaria

sRANKL/LPS-induced bone resorption mouse model was employed as described previously [19]. Briefly, BALB/c mice (6-week-old males) received a supra-calvaria injection (50 μ l/site/day) of a combination of sRANKL (5 μ g/ml) and LPS (500 μ g/ml), or PBS as a control group, for three consecutive days. The animals were treated with daily administration of Dox (30 mg/kg), or PBS by oral inoculation. All mice were sacrificed 10 days after the first sRANKL and LPS injection. The calvariae were fixed and decalcified in 10% EDTA solution for 21 days. These calvariae were embedded in Tissue-Tek (Sakura, Torrance, CA), and coronal sections (8 μ m in thickness) were obtained using a cryostat. The sections were stained for TRAP, and the nuclei were counter-stained with methyl green. A half portion of calvaria was homogenized in lysis buffer (0.1% SDS, 1% NP-40, 1% Triton X-100, 25 mM Tris and 150 mM NaCl). After adjusting the protein concentration by using the Quant-iTTM protein assay kit, samples of equal amount were subjected to gelatin gel zymography and Western blot analyses for MMP-9 as described above.

TRAP activity assay

The homogenized calvaria tissue proteins obtained as described above were reacted with p-nitro-phenol-phosphate in 150 mM tartrate buffer (pH 5.5) supplemented with 1 mM MgCl₂. After 2 h incubation at 37°C, the reaction was stopped by 2N NaOH. The absorbance at 405 nm was measured by a spectrophotometer as TRAP activity.

Statistical analysis

The results were expressed as mean $\pm$ standard deviations (SD). Statistical differences between the mean values of control and experimental groups were analyzed by using ANOVA or Student's *t* test at a significance level of 5%.

Results

Dox suppressed RANKL-mediated OCgenesis in vitro

To examine whether Dox inhibits RANKL-mediated OCgenesis, RAW264.7 cells were stimulated with sRANKL in the presence or absence of Dox. RANKL stimulation induced OCgenesis as demonstrated by the emergence of TRAP⁺ multinuclear osteoclastic cells in RAW264.7 cells; however, the addition of Dox to the RAW264.7 cell culture suppressed the RANKL-mediated OCgenesis (Fig. 1A upper panel). Moreover, Dox suppressed the RANKL-induced OCgenesis in a dose-dependent manner (Fig. 1B). Dox treatment also inhibited the formation of resorption pits induced by the RAW264.7 cells stimulated with sRANKL (Fig. 1 A bottom panel). To confirm this inhibitory effect of Dox, OCgenesis assay was performed using the BMM cells. Consistent with the results of RAW264.7 cells, Dox treatment also suppressed the emergence of TRAP⁺ multinuclear cells induced by the stimulation of BMM cells with sRANKL in a dose-dependent manner (Fig. 1C and 1D). Based on such parallel and comparable results between RAW264.7 and BMM cells in response to the effects of Dox on RANKL-mediated OCgenesis, RAW264.7 cells were utilized for the OCgenesis assays in the following experiments.

Only the Tetracycline family, but not other antibiotics, demonstrated inhibition of RANKL-mediated OCgenesis

In the past, only Dox or CMTs were tested for their inhibitory effect on RANKL-mediated OCgenesis [8]. Therefore, it is unknown if other antibiotics might also be able to suppress RANKL-mediated OCgenesis. Accordingly, the effects of a battery of antibiotics on RAW264.7 cells stimulated by sRANKL were examined. Similar to Dox, 0.2 μ g/ml and 2 μ g/ml of Min or 2 μ g/ml of Tet suppressed the RANKL-induced TRAP⁺ multinuclear cells

in RAW 264.7 (Fig. 2A). On the other hand, the other antibiotics tested, including Amo, Van, Bac, Spc, and Gen, showed no effect on OCgenesis stimulated by sRANKL (Fig. 2B). These findings suggested that only the Tetracycline family compounds, including Dox, possess inhibitory effect on RANKL-induced *in vitro* OCgenesis.

Dox suppressed Cathepsin K and TRAP mRNA expression, but neither MAPKs phosphorylation nor NFAT-c1 mRNA expression in RAW264.7 cells stimulated with sRANKL

To elucidate the mechanism underlying the inhibition of RANKL-induced OCgenesis by Dox, we used RAW264.7 cells to test the effect of Dox on MAPKs phosphorylation and mRNA expression of the down-stream transcriptional factor of MAPKs, NFAT-c1, which, taken together, compose the main streams in RANKL signaling cascades. In contrast to the induction of NFAT-c1 mRNA expression which occurs during the early stage of RANKL-induced osteoclastogenesis (within 24 hours from sRANKL-stimulation), expression of genes that indicate osteoclast maturation, including Cathepsin K or TRAP (more than 48 hours from sRANKL-stimulation), were also observed in RAW264.7. sRANKL stimulation elevated the phosphorylation levels of all three major MAPKs, including p38, ERK, and JNK, in a time-dependent manner (Fig. 3A). However, Dox did not affect the increase of MAPKs phosphorylation in the RANKL-stimulated RAW264.7 cells (Fig. 3A). Dox also had no effect on the up-regulation of NFAT-c1 mRNA expression induced by stimulation of RAW264.7 cells with sRANKL for 24 hr (Fig. 3B). Contrary to the lack of effect of Dox on these early-stage RANKL-induced signaling events, i.e., MAPKs phosphorylation and NFAT-c1 activation, Dox did significantly suppress RANKL-induced osteoclast maturation genes, Cathepsin K and TRAP mRNA (Fig. 3C). These results indicated that Dox-mediated inhibition of osteoclastogenesis resulted from the suppression of genes that are involved in the late stage of osteoclastogenesis in RAW264.7, while Dox did not affect the MAPK-NFAT-c1 cascade in RANKL signaling, which occurs in the early stage of OCgenesis.

Inhibition of MMP-9 activity by Dox may contribute to suppression of RANKL-induced OCgenesis

We hypothesized that inhibition of MMP-9 enzyme activity by Dox may be associated with its suppression of RANKL-induced OCgenesis in RAW264.7 cells based on the following lines of evidence: 1) the non-antibiotic biological property of Dox is derived from its inhibition of MMP activities [5, 6]; 2) MMP-9 expression is induced during the middle-stage of osteoclast differentiation following NFAT-c1 induction [14]; and 3) Dox can suppress the expression of late-stage osteoclastogenesis genes, i.e., TRAP and Cathepsin K (Fig. 3C). To examine this hypothesis, we first tested the effect of Dox on RANKL-induced MMP-9 expression and its activity. While Western blotting showed that RANKL stimulation induced MMP-9 expression in RAW264.7, Dox had no effect on the up-regulated expression of MMP-9 protein (Fig. 4A). RANKL-induced MMP-9 activity, on the other hand, was significantly suppressed by Dox treatment in the gelatin zymography assay (Fig. 4B), suggesting that Dox inhibits enzyme activity of MMP-9 without affecting the production of MMP-9 protein. To further investigate if inhibition of MMP-9 enzyme activity contributes to the suppression of RANKL-induced OCgenesis, effects of a chemical inhibitor for MMP-9 enzyme activity, MMP-9 inhibitor I, on the sRANKL-induced osteoclastogenesis in RAW264.7 cells was examined by TRAP-staining and RT-PCR. The induction of TRAP⁺ multinuclear cells in RANKL stimulated RAW264.7 cells was remarkably suppressed by treatment with MMP-9 chemical inhibitor in a dose-dependent manner (Fig. 4C and 4D). In addition, MMP-9 inhibitor I also significantly blocked the RANKL-induced Cathepsin K and TRAP mRNA expression in RAW 264.7. On the other hand, the exogenously applied an excess MMP-9 enzyme (1 µg/ml) attenuated the Dox-mediated inhibitory effects on the sRANKL-induced OCgenesis (Fig. 4F and 4G). It is

noteworthy that MMP-9 treatment alone did not increase the sRANKL-induced OCgenesis (Fig. 4G). These findings suggested that MMP-9 enzyme activity may play a crucial role in the late stage of RANKL-mediated OCgenesis that induces Cathepsin K and TRAP mRNA and that Dox can suppress RANKL-mediated OCgenesis by its inhibitory effect on MMP-9 enzyme activity.

Dox administration suppressed OCgenesis and MMP activities in vivo

To confirm the *in vivo* efficacy of Dox to inhibit RANKL-induced OCgenesis, we tested the effect of Dox on osteoclastogenesis induced in mouse calvaria. Since previous study reported that Min is able to decrease bone loss and increase bone formation [20], bone mineral density of whole body or femur in BALB/c mice treated with or without Dox was measured. Dox treatment did not affect the bone mineral density of either whole body or femur during the treatment period of 30 days (Fig. 5A). Furthermore, the evaluation of bone architecture using a Micro-CT showed that Dox treatment did not affect the trabecular bone surface density and cortical thickness (Fig 5B). These findings indicated that Dox does not affect systemic homeostatic bone metabolism. Then, to examine the effect of Dox on OCgenesis *in vivo*, osteoclastogenesis was induced by supra-calvaria injections with a mixture of sRANKL and LPS with or without administration of Dox. Ten days after injection with sRANKL/LPS, a remarkably increased number of TRAP-positive osteoclast-like cells were induced in the calvaria bone lacunae compared to the PBS injected groups (Fig. 5C). Most importantly, systemic administration of Dox reduced the sRANKL/LPS-induced TRAP-positive cells (Fig. 5C). Furthermore, similar to this result, TRAP enzyme activity assay showed that sRANKL/LPS injection enhanced the TRAP activity, but that its up-regulation was blocked by Dox administration (Fig. 5D). Finally, to examine whether Dox can decrease the RANKL-induced MMP-9 expression or its activity, Western blotting and gelatin zymography assays were performed. Consistent with the *in vitro* assays (Fig. 4), sRANKL/LPS stimulation enhanced MMP-9 protein expression in mice calvaria, but Dox did not affect the MMP-9 protein expression level (Fig. 5E). However, RANKL-induced MMP activity was remarkably abrogated by Dox treatment, as shown in the gelatin zymography assay (Fig. 5F). These findings suggested that Dox attenuates RANKL-induced OCgenesis *in vivo* without affecting the induction of MMP-9 protein while suppressing MMP-9's enzyme activity.

Discussion

The present study demonstrated that Dox suppresses RANKL-induced OCgenesis by its inhibitory effect of MMP-9 enzyme activity independent of the MAPK-NFATc1 signaling cascade. More specifically, such Dox-mediated suppression of MMP-9 enzyme activity resulted in halting RANKL-induced osteoclast maturation, as demonstrated by suppression of late-stage osteoclastogenesis signature genes for TRAP and cathepsin K mRNAs. It is noteworthy that MAPK-NFATc1 constitutes the main stream of RANKL signaling cascades during early-stage osteoclastogenesis (within 24 hours from RANKL-stimulation), while expression of molecules required for bone resorption function, such as TRAP and cathepsin K, is induced during the late stage of osteoclastogenesis (more than 48 hours from RANKL-stimulation). Furthermore, upon RANKL stimulation, Dox inhibited MMP-9 enzyme activity without affecting its protein expression level. Importantly, MMP-9 inhibitor I, the inhibitor of MMP-9 enzyme activity, also blocked the RANKL-induced OCgenesis and gene expression of osteoclast maturation markers, including TRAP and cathepsin K. On the other hand, exogenously applied excess MMP-9 abrogated the Dox-mediated inhibition of OCgenesis. The parallel results obtained by both Dox and MMP-9 inhibitor imply that the elevation of MMP-9 activity subsequent to RANK-MAPK-NFATc1 signaling activation may be essential to promote osteoclast maturation, which induces functional osteoclast gene

expression, such as TRAP and cathepsin K. In sum, the mechanism underlying the Dox-mediated inhibitory effect on osteoclastogenesis appeared to depend on its inhibition of MMP-9 enzyme activity independent of the MAPK-NFATc1 cascade.

It is true that RANKL-induced MAPK-NFATc1 signaling up-regulates MMP-9 expression during OCgenesis [14]. Nonetheless, although Dox inhibited RANKL-induced OCgenesis, Dox did not inhibit the expression of MMP-9 protein in response to RANKL stimulation. Such results appear contradictory. However, the finding that Dox targets MMP-9 enzyme activity, which is involved in the gene expression of osteoclast maturation makers, including TRAP and cathepsin K, indicated that Dox does affect the maturation stage of osteoclastogenesis, rather than its early stage, which involves the MAPK-NFATc1 signaling cascade. In the past, most attention was given to the development of anti-OCgenesis drugs that interrupt the ligation between RANK and RANKL or RANK-RANKL signaling [21, 22]. However, we have shown that Dox affects the late stage of cellular events, rather than the early signaling event, in osteoclasts precursors elicited by RANK-RANKL-ligation.

To the best of our knowledge, no *in vivo* study has yet been conducted to report the effect of Dox on RANKL-induced OCgenesis in the context of inflammation. Therefore, in the present study, we employed a mouse calvaria model injected with a mixture of sRANKL and LPS. Consistent with our *in vitro* study, Dox inhibited sRANKL/LPS-induced OCgenesis in calvaria along with inhibition of MMP-9 activity, while MMP-9 protein expression remained unaffected by Dox treatment. In this mouse model to induce TRAP+ cells in calvaria within 10 days, addition of LPS was requisite for the sRANKL-mediated OCgenesis, probably because inflammatory cytokines, such as TNF- α or IL-1 β , stimulated with LPS contributed to the promotion of RANKL-induced OCgenesis [23]. LPS by itself is not considered to cause OCgenesis because a previous study reported that *in vivo* LPS injection increases the generation of osteoclast precursors in bone marrow, but the maturation from these LPS-treated cells into TRAP+ cells requires RANKL [24]. Nonetheless, it was conceivable that Dox might exert its inhibitory effect on the OCgenesis induced in calvaria via suppression of proinflammatory cytokines induced by LPS. To address this possibility, we examined the effect of Dox on TNF- α production from RAW264.7 stimulated with 1 μ g/ml of LPS. Interestingly, Dox had no effect on LPS-induced TNF- α production (data not shown). This finding supported the idea that Dox might be solely and directly responsible for affecting RANKL stimulation in our mouse model.

We showed that Dox, which can suppress the enzyme activity of MMP-9 [25], as well as MMP-9 inhibitor (MMP-9 inhibitor I), down-regulated the expression of RANKL-induced osteoclast maturation genes in conjunction with the suppression of RANKL-induced OCgenesis. These findings indicated that MMP-9 induced by RANKL plays a role as an upstream effector of osteoclast gene expression and, as such, it may also be a regulator of OCgenesis. Previous studies reported that MMP inhibitor (RP59794) [26] or MMP-9 gene knockout [27] reduced osteoclast migration, which results in reduction of the resorption process in the growth plate, and, as a consequence, attenuated development of bone marrow cavity. However, since the latter studies treated the aspect of osteoclast migration, but not differentiation, ours is the first study to report the involvement of MMP-9 activity in RANKL-induced OCgenesis.

ADAM-8 (a disintegrin and metalloproteinase-8), which is highly expressed at the late stage of OCgenesis [28], is required for the fusion between osteoclast precursors and their differentiation *in vitro* and *in vivo* [29]. ADAM (also known as the adamalysin family) is a family of peptidase proteins [30] and is classified as sheddase based on the ability to cut off or shed extracellular portions of transmembrane proteins [31]. Important to this study, MMP-9 also belongs to this peptidase family and, as such, possesses sheddase activity [31].

Since the enzyme activities that shed transmembrane proteins or other possible target proteins are required for OCgenesis, it appears that both ADAM-8 and MMP-9 are engaged in such shedding activities. Although the putative transmembrane protein that is shed by MMP-9 remains unclear, it is important to identify the target molecules of these sheddases required for OCgenesis because such molecule, as a result of shedding by MMP-9, is supposed to elicit the signal to activate the expression of osteoclast maturation genes. It is suspected that Dox-mediated suppression of OCgenesis results from the inhibition of shedding of such putative target molecule for MMP-9.

It is unclear why systemic Dox administration did not affect the *in vivo* homeostatic bone metabolism, while Dox inhibited *in vitro* RANKL-induced OCgenesis as well as OCgenesis induced in calvaria by RANKL/LPS. Zhou et al. demonstrated that Tet can inhibit RANKL-mediated *in vitro* OCgenesis, whereas systemically administered Tet does not affect the systemic bone turnover in rats [32]. Although the authors did not illustrate the mechanism underlying the Tet-mediated inhibition of *in vitro* OCgenesis [32], their results clearly support our findings using a mouse model. Although, in the present study, soluble recombinant RANKL was used for the *in vitro* OCgenesis as well as *in vivo* calvaria OCgenesis, membrane bound-RANKL expressed on osteoblasts or stromal cells is thought to be engaged in the homeostatic OCgenesis [10, 33]. Furthermore, osteoclast-associated receptor (OSCAR) and triggering receptor expressed by myeloid cells (TREM)-2 expressed on osteoblasts or stromal cells provide the co-stimulatory signals required for RANKL-mediated OCgenesis [34]. On the other hand, soluble RANKL released from activated T cells and B cells, in the absence of OSCAR/TREM-2, is thought to induce OCgenesis in the context of inflammatory bone resorption lesion [18]. Therefore, while future studies will need to elucidate, we presume that Dox's influence on OCgenesis in homeostatic bone metabolism differs from that on soluble RANKL-mediated OCgenesis, because the OCgenesis in homeostatic bone metabolism is induced by distinct cell signals elicited by membrane bound RANKL along with OSCAR/TREM-2 which appears to be absent in soluble RANKL-mediated OCgenesis.

In the present study, we tested the effect of a battery of antibiotics on RANKL-induced OCgenesis and showed that only TCs, such as Tet, Min, and Dox, inhibited OCgenesis. Periodontal disease is associated with a constellation of oral microorganisms that infect the gingival crevice [35]. These polymicrobial infections activate the immune system which results in the robust production of RANKL as a critical initiator of critical alveolar bone resorption [36]. Therefore, among a number of antibiotics, TCs may be the best potential remedy to treat periodontitis because of its bifunctional biological property, i.e., bactericidal effect and inhibition of RANKL-induced osteoclastogenesis. For example, the oral administration of submicrobial dose of Dox (20mg twice daily) as an adjunct therapy for conventional procedures for periodontal disease resulted in a significant improvement in tooth attachment as well as reduction of pocket depth and bleeding after probing [37]. Moreover, similar to Tet, local delivery of Dox also showed clinical potency on chronic periodontitis [38]. Dox has indeed been found to be a valuable adjunct to conventional mechanical therapies used for periodontitis [39, 40]. Accordingly, other TCs, Tet or Min, may also show a pharmaceutical advantage against periodontal disease.

Conclusion

In summary, Dox ameliorates RANKL-induced OCgenesis by its inhibitory effect on MMP-9 activity independent of MAPK-NFATc1 signaling cascade. Therefore, Dox may lead to the development of therapeutic approach for the bone resorptive disease caused by soluble RANKL based on its novel bioeffect which inhibits RANKL-mediated OCgenesis, in addition to its previously well known effect as a broad spectrum antibiotic.

Acknowledgments

This research study was supported by NIDCR grants DE016276, DE-03420, DE-09018, DE-14551, DE-18499 and DE-19917 and FAPESP 05/54580.

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Fig. 1. Dox suppressed RANKL-mediated osteoclast differentiation in vitro

(A and B) RAW264.7 cells were stimulated with sRANKL (50 ng/ml) for 5 days in the presence or absence of indicated concentration of Dox on culture plate (A top and B) or dentin disk (A bottom). (A top) Cells were fixed and stained for TRAP. (B) TRAP+ multinuclear cells were counted. Data represent mean $\pm$ SD of three cultures. * $P < 0.05$; ** $P < 0.01$; significantly lower than sRANKL stimulation by Student's t test. (A bottom) Cells were washed out, and dentin discs were stained by Toluidine blue. The arrows indicate resorption pit. (C and D) BMM cells were cultured with sRANKL (50ng/ml) and M-CSF (10 ng/ml) in the presence or absence of indicated concentration of Dox. (C) Cells were fixed and stained for TRAP. (D) TRAP+ multinuclear cells were counted. Data represent mean $\pm$ SD of three cultures. ** $P < 0.01$; significantly lower than sRANKL stimulation by Student's t test.

Fig. 2. Only Tetracycline family, but not other antibiotics, showed the inhibition of RANKL-mediated osteoclast differentiation

(A and B) RAW264.7 cells were stimulated with sRANKL (50 ng/ml) for 5 days in the presence or absence of indicated concentration of a battery of antibiotics shown in the figure. The concentrations of control antibiotics to Dox were chosen to be equal to, or higher than, the sub-antibiotic concentration of Dox. TRAP+ multinuclear cells were counted. Data represent mean $\pm$ SD of three cultures. * $P < 0.05$; ** $P < 0.01$; significantly lower than sRANKL stimulation by Student's t test.

Fig. 3. Dox abrogated Cathepsin K and TRAP mRNA expression, but not MAPKs phosphorylation and NFAT-c1 transcription in RAW 264.7 stimulated with sRANKL

(A) RAW264.7 cells were exposed to sRANKL (50 ng/ml) for the indicated times in the presence or absence of 2 μg/ml of Dox. The phosphorylated or total ERK, p38, and JNK levels in the cell lysates were analyzed by immunoblotting. The relative density of phospho-p38, ERK, or JNK expression detected in the blot-membrane was calculated and expressed as a ratio to that of total-p38, ERK, or JNK, respectively. Data are expressed as the means $\pm$ SD of three cultures. # $P < 0.05$; significantly elevated compared to no-sRANKL/no-reagent control by Student's t test. N.D; no statistical difference was detected. (B and C) RAW264.7 cells were stimulated with sRANKL (50 ng/ml) for 24 hr (B) or 48 hr (C) in the presence or

absence of indicated concentration of Dox. Total mRNA was extracted and (B) NFATc1 and β -actin or (C) TRAP, cathepsin K and β -actin mRNA expression were analyzed by RT-PCR. Data are representative of three independent experiments. Relative density of amplified PCR-products for NFAT-c1 (B), Cathepsin K (C), or TRAP (C) was calculated and expressed as a ratio to that of encoding β -actin. Data are expressed as the means $\pm$ SD of three experiments. # $P < 0.05$; significantly elevated compared to no-sRANKL/no-reagent control by Student's t test. * $P < 0.05$, ** $P < 0.01$; significantly lower than sRANKL stimulation by Student's t test. N.D; no statistical difference was detected.

Fig. 4. Inhibition of MMP-9 activity by Dox may contribute to the suppression of RANKL-induced osteoclast differentiation

(A and B) RAW264.7 cells were exposed to sRANKL (50 ng/ml) for 24 hr in the presence or absence of 2 µg/ml of Dox. The amount of protein isolated from harvested cells was adjusted and subjected to (A) immunoblotting for MMP-9 and α -tubulin expression or (B) gelatin zymography to analyze MMP activity. Data are representative of three independent experiments. (A) The relative density of MMP-9 expression was calculated and expressed as a ratio to that of the α -tubulin. (B) The band density was quantified as MMP activity. Data are expressed as the means $\pm$ SD of three experiments. # $P < 0.05$; significantly elevated compared to no-sRANKL/no-Dox control by Student's t test. * $P < 0.05$, ** $P < 0.01$; significantly lower than sRANKL stimulation by Student's t test. N.D; no statistical difference was detected. (C and D) RAW264.7 cells were stimulated with sRANKL (50 ng/ml) for 5 days in the presence or absence of indicated concentrations of MMP-9 inhibitor, MMP-9 inhibitor I. (Based on the manufacturer's report, IC_{50} for MMP-9 by MMP-9 inhibitor I, i.e. 5 nM, is at least 10 fold lower than that for its secondary target; MMP-1 [$IC_{50} = 1.05 \mu M$] and MMP-13 [$IC_{50} = 113 \text{ nM}$]). (C) Cells were fixed and stained for TRAP. (D) TRAP+ multinuclear cells were counted. Data represent mean $\pm$ SD of three cultures. ** $P < 0.01$; significantly lower than sRANKL stimulation by Student's t test. (E) RAW264.7 cells were stimulated with sRANKL (50 ng/ml) for 48 hr in the presence or absence of indicated 10 nM of MMP-9 inhibitor I. Total mRNA was extracted, and TRAP, cathepsin K and β -actin mRNA expressions were analyzed by RT-PCR. Data are representative of three independent experiments. Relative density of PCR-products for Cathepsin K and TRAP were calculated and expressed as a ratio to that for β -actin. Data are expressed as the means $\pm$ SD of three experiments. # $P < 0.05$; significantly elevated compared to no-sRANKL/no-inhibitor control by Student's t test. * $P < 0.05$, ** $P < 0.01$; significantly lower than sRANKL stimulation by Student's t test. (F and G) RAW264.7 cells were stimulated with sRANKL (50 ng/ml) for 5 days in the presence or absence of Dox (2 µg/ml) and indicated concentration of MMP-9. (F) Cells were fixed and stained for TRAP. (G) TRAP+ multinuclear cells were counted. Data represent mean $\pm$ SD of three cultures. # $P < 0.05$; significantly elevated compared to no-sRANKL/no-reagent control by Student's t test. * $P < 0.05$, ** $P < 0.01$; significantly lower than sRANKL stimulation by Student's t test. N.D; no statistical difference was detected.

Fig. 5. Dox administration suppressed osteoclast differentiation and MMP activities in vivo (A and B) BALB/c mice (8-week-old males, n=5 mice/group) were treated with either regular water or 30 mg/kg of body weight of Dox solution water for 30 days (p.o. every single day), after which the bone mineral density of whole body or femur was measured by a bone mineral densitometer (A) or the distal metaphysis of femur was scanned with micro-CT (B). Data are the mean $\pm$ SD of four mice per group. (C-F) Mice (6-week-old males, n=6 mice/group) received a combined injection of sRANKL (5 μ g/ml) and LPS (500 μ g/ml) or PBS for 10 days (i.p. every 3 days). The animals were treated with daily administration of Dox (30 mg/kg), or PBS by oral inoculation. All mice were sacrificed 10 days after the first sRANKL and LPS injection. (C) The calvaria were fixed and decalcified. These calvariae were embedded, and coronal sections were obtained. The sections were stained for TRAP, and the nuclei were counter-stained with methyl green. (D-F) The calvariae were homogenized in lysis buffer as described in the Materials and Methods section. After adjusting the protein concentration, TRAP enzyme activity assay was performed (D). The measurement of mean $\pm$ SD of TRAP activity in each group is depicted. * $P < 0.05$; significantly higher than PBS injection group by Student's t test. (E and F) The equal amount of protein was subjected to (E) immunoblotting for MMP-9 expression and (F) gelatin gel zymography for MMP-9 activity. Data are representative of three independent experiments. The band density was quantified as (E) MMP-9 expression and (F) MMP activity, respectively. Data are expressed as the means $\pm$ SD of three experiments. # $P < 0.05$; significantly higher than control (Lane 1) by Student's t test. ** $P < 0.01$; significantly lower than sRANKL stimulation by Student's t test.