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Tetracyclines Convert the Osteoclastic-Differentiation Pathway of Progenitor Cells To Produce Dendritic Cell-like Cells

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Tetracyclines, such as doxycycline and minocycline, are used to suppress the growth of bacteria in patients with inflammatory diseases. Tetracyclines have been shown to prevent bone loss, but the mechanism involved is unknown. Osteoclasts and dendritic cells (DCs) are derived from common progenitors, such as bone marrow-derived macrophages (BMMs). In this article, we show that tetracyclines convert the differentiation pathway, resulting in DC-like cells not osteoclasts. Doxycycline and minocycline inhibited the receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis of BMMs, but they had no effects on cell growth and phagocytic activity. They influenced neither the proliferation nor the differentiation of bone-forming osteoblasts. Surprisingly, doxycycline and minocycline induced the expression of DC markers, CD11c and CD86, in BMMs in the presence of RANKL. STAT5 is involved in DC differentiation induced by GM-CSF. Midostaurin, a STAT5-signaling inhibitor, and an anti–GM-CSF–neutralizing Ab suppressed the differentiation induced by GM-CSF but not by tetracyclines. In vivo, the injection of tetracyclines into RANKL-injected mice and RANKL-transgenic mice suppressed RANKL-induced osteoclastogenesis and promoted the concomitant appearance of CD11c+ cells. These results suggested that tetracyclines prevent bone loss induced by local inflammation, including rheumatoid arthritis and periodontitis, through osteoclast–DC-like cell conversion. The Journal of Immunology, 2012, 188: 1772–1781.

Osteoclasts are bone-resorbing multinucleated cells derived from hematopoietic progenitors of the monocyte–macrophage lineage (1, 2). The differentiation of osteoclasts is tightly regulated by bone-forming osteoblasts (3). Osteoblasts express two cytokines essential for osteoclastic differentiation: M-CSF (4) and receptor activator of NF-κB ligand (RANKL) (5, 6). RANKL is inducibly expressed by osteoblasts in response to osteotropic hormones and factors, including 1α,25-dihydroxyvitamin D3, [1α,25(OH)2]D3, and PGE2. In contrast, M-CSF is constitutively expressed. Osteoblasts also produce osteoprotegerin (OPG), a soluble decoy receptor for RANKL, which inhibits osteoclastogenesis by blocking RANKL–receptor activator of NF-κB (RANK) interaction (8, 9). Osteoclast precursors, such as bone marrow-derived macrophages (BMMs), express c-Fms (M-CSF receptors) and RANK (RANKL receptors) and differentiate into osteoclasts in response to M-CSF and RANKL expressed by osteoblasts (6, 7).

The RANKL–RANK interaction leads to the activation of MAPKs, including JNK and p38, in osteoclast precursors (10, 11). MAPK signals play central roles in the regulation of osteoclastic differentiation (10). RANK signals also activate the transcription factor complex AP-1, through one of its components, c-Fos, and induce NFATc1, the master transcription factor of osteoclastic differentiation (12). Importantly, the RANKL-induced expression of NFATc1 is dependent on both MAPK and c-Fos pathways.

Dendritic cells (DCs) are APCs (13, 14); therefore, DCs are the preferred targets for immunotherapy in patients with autoimmune diseases and those with cancer (15). DCs and osteoclasts are derived from common progenitors, such as BMMs (10, 16). GM-CSF induces BMMs to differentiate into DCs through the activation of STAT5 (17, 18). BMMs obtained from Stat5a-deficient mice fail to differentiate into CD11c and CD86 double-positive DCs in response to GM-CSF (17). In contrast, GM-CSF strongly inhibits the osteoclastic differentiation of BMMs through suppression of c-Fos (16). These results suggested that the fate of common progenitors to become osteoclasts or DCs is tightly regulated by the up- and downregulation of the same signaling molecules, such as c-Fos.

Tetracyclines are widely used to treat infectious diseases (19). Minocycline and doxycycline were shown to prevent bone loss (20–23). Tetracyclines are now proposed to be therapeutic agents for diseases with bone loss, such as malignancy, arthritis, and periodontitis (20, 24, 25). Tetracyclines were shown to inhibit bone resorption, but the mechanisms of their inhibitory action remain largely unknown (26–29). In the current study, we examined the effects of doxycycline and minocycline on the formation of osteo-

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Abbreviations used in this article: ALP, alkaline phosphatase; BMM, bone marrow-derived macrophage; DC, dendritic cell; IκBα, NF-κB inhibitor; iNOS, inducible NO synthase; JNK, c-Jun N-terminal kinase; M-CSF, macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase; NFATc1, nuclear factor of activated T cells 1; NF-κB, nuclear factor κB; OPG, osteoprotegerin; RANK, receptor activator of NF-κB ligand; RANKL, receptor activator of NF-κB ligand; TG, transgenic; TRAP, tartrate-resistant acid phosphatase.
clasts in vitro and in vivo. Doxycycline and minocycline inhibited RANKL-induced osteoclastogenesis. To our knowledge, our study showed for the first time that tetracyclines convert the differentiation pathway, resulting in DC-like cells rather than osteoclasts, in the presence of RANKL in vitro and in vivo.

Materials and Methods

Mice and reagents

Seven-week-old male mice and newborn mice of the ddY strain were obtained from Japan SLC (Shizuoka, Japan) for experiments in vivo. C57BL/6 mice and C57BL/6 mice from Japan SLC were used for experiments in vivo. Rack1-transgenic (Tg) mice (genetic background of C57BL/6), which express a soluble form of mouse Rack1 using the human serum amyloid P component promoter, were generated in one of the authors’ laboratories (30). Rack1-Tg mice exhibit constitutively increased osteoclastic bone resorption. All procedures for animal care were approved by the Animal Management Committee of Matsumoto Dental University and were performed accordingly. Doxycycline, minocycline, and minocycline hydrate were purchased from Sigma (St. Louis, MO). Recombinant human RANKL, a fusion protein comprising GST and the extracellular domain of human RANKL (amino acid residues 140–317; GST-RANKL), was from Oriental Yeast (Tokyo, Japan). Recombinant human M-CSF (Leukoprol) was obtained from Kyowa Hakko (Tokyo). 1α,25(OH)2D3 and PGE2 were from Wako Pure Chemical Industries (Osaka, Japan). Recombinant mouse GM-CSF and an anti-mouse GM-CSF–neutralizing Ab were from R&D Systems (Minneapolis, MN). Fluorescent latex beads (Fluoresbrite) were purchased from Polysciences (Warrington, PA). Other chemicals and reagents were of analytical grade.

Experiments in vivo using RANKL–injected mice

Fifty microliters of minocycline (10 mg/kg body weight) or vehicle suspended in saline was injected daily into the s.c. tissue overlying calvariae of 7-wk-old C57BL/6 mice for 5 d, beginning on day 0. Twenty microliters of GST-RANKL (1 mg/kg body weight) suspended in type I collagen gel (Nitta Gelatin, Osaka, Japan) was injected for the last 2 d at the same site (31, 32). Mice were sacrificed on day 5. Calvariae were collected, fixed in 4% paraformaldehyde, decalcified with 10% EDTA, and embedded in paraffin. Histological sections were prepared and stained for tartrate-resistant acid phosphatase (TRAP; a marker enzyme of osteoclasts) or alkaline phosphatase (ALP; a marker enzyme of osteoblasts). The osteoclast number and surface/bone surface, respectively (33). The osteoblast number was determined by a histomorphometric analysis at the suture of calvariae and expressed as osteoclast number/bone perimeter and osteoclast surface/bone surface, respectively (33). The osteoblast number was determined by a histomorphometric analysis at the suture of calvariae (30).

Experiments in vivo using Rankl-Tg mice

Fifty microliters of minocycline (10 mg/kg body weight), doxycycline (10 mg/kg body weight), or vehicle (saline) was injected daily into the s.c. tissue of calvariae of 12-wk-old Rankl-Tg mice or C57BL/6 mice for 5 d, beginning on day 0. Mice were sacrificed on day 5. Calvariae were then collected and processed for bone histomorphometry, as described above. Osteoclast number/bone perimeter was determined. Calvariae were also processed for the detection of DC-like cells. For Ag retrieval, calvarial sections were autoclaved in an Ag-retrieval buffer (Nichirei Bioscience, Tokyo, Japan) at 121°C for 10 min and treated with 0.3% H2O2. The sections were incubated with a hamster anti-CD11c Ab (REL/1Tech, Wolfenbuttel, Germany), and an HRP-conjugated secondary anti-hamster Ab (Santa Cruz Biotechnology, Santa Cruz, CA). The TRAP-conjugated Ab was visualized with a 3,3′-diaminobenzidine kit (Dako, Carpinteria, CA). Sections were counterstained with hematoxylin. CD11c+ cells were counted as DC-like cells. To detect minocycline accumulated in calcified tissues, minocycline (10 mg/kg body weight) or vehicle (saline) was injected daily into the s.c. tissue on 7-wk-old C57BL/6 mice. Calvariae were recovered on day 5. Calvarial sections were prepared, and minocycline was observed using fluorescence microscopy.

Formation of osteoclasts in cultures

Primary osteoclasts were prepared from newborn mouse calvariae, as described (34). Primary osteoclasts (1 × 104 cells/well) were cocultured with bone marrow cells (2 × 105 cells/well) in oMEM (Sigma) containing 10% FBS (JRH Biosciences, Lenexa, KS) in 48-well plates (0.5 ml/well). Cocultures were treated with increasing concentrations of doxycycline or minocycline in the presence of 1α,25(OH)2D3 (10-8 M) and PGE2 (10-6 M). After 7 d, cells were fixed and stained for TRAP. TRAP+ cells containing three or more nuclei were counted as osteoclasts.

Mouse BMMS were prepared as osteoclast precursors (35). BMMS were cultured in 96-well plates (1 × 104 cells/well) in the presence of RANKL (100 ng/ml) and M-CSF (50 ng/ml) with increasing concentrations of doxycycline or minocycline. After 3 d, cells were fixed and stained for TRAP. TRAP+ cells containing three or more nuclei were counted as osteoclasts.

Cell viability assay

Osteoclasts (1 × 104 cells/well) or BMMS (1 × 104 cells/well) were cultured for 1 d in oMEM containing 10% FBS in 96-well plates and further treated with doxycycline or minocycline (20 μg/ml) for specific periods. The viability of cells was measured using the Alamar blue assay (Biosource, Camarillo, CA).

Differentiation of osteoclasts

BMMS (1 × 105 cells/well) were cultured in 12-well plates for the presence of M-CSF (50 ng/ml), with or without minocycline (3 or 10 μM). BMMS were further maintained in serum-free oMEM for 4 h. Then, fluorescent latex beads (1:500) were added to the cultures for 40 min, as described (10). Cells were fixed and stained with rhodamine-conjugated phalloidin to visualize F-actin, as well as with DAPI to visualize nuclei. Cells incorporating beads (bead-positive cells) were divided into two groups according to the number of beads (>50 and <50) incorporated in each cell. Phagocytic activity was expressed as the percentage of bead-positive cells.

Fluorescence microscopy

BMMS (1 × 105 cells/well) were cultured on 18-mm coverslips in 12-well plates for the presence of M-CSF (50 ng/ml), with or without minocycline (10 and 50 μM). BMMS were further maintained in serum-free oMEM for 4 h. Then, fluorescent latex beads (1:500) were added to the cultures for 40 min, as described (10). Cells were fixed and stained with rhodamine-conjugated phalloidin to visualize F-actin, as well as with DAPI to visualize nuclei. Cells incorporating beads (bead-positive cells) were divided into two groups according to the number of beads (>50 and <50) incorporated in each cell. Phagocytic activity was expressed as the percentage of bead-positive cells.

Phagocytosis assay

BMMS (1 × 105 cells/well) were cultured in 96-well plates for 4 d, with or without GM-CSF (25 ng/ml) and M-CSF (25 ng/ml). After 7 d, cells were fixed and processed for ALP staining (36).

PCR amplification of reverse-transcribed mRNA

For the semiquantitative RT-PCR analysis, total cellular RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from the total RNA with oligo (dT)12–18 primers and subjected to PCR amplification with EX Taq polymerase (Takara Biochemicals, Shiga, Japan) using the following specific primers: mouse M-csf, 5′-GAGAAGACTTAGTGGTAC-3′ (forward) and 5′-TAC-TTGCGAGGTTCACC-3′ (reverse); mouse Rankl, 5′-GCGTCTTGTT-CCTGTACCTGTGAGC-3′ (forward) and 5′-TCGTGCTCCCTCCTTT-TGTACAGG-3′ (reverse); mouse Opg, 5′-CAGAGAAGACTTAGTGGTAC-3′ (forward) and 5′-ATGAGAGCTGTCGTTGAGAAGAG-CC-3′ (reverse); and mouse Gadph, 5′-ACCACGTCATGTCATACGAC-3′ (forward) and 5′-TCCACACCCGGTTGGTCC-3′ (reverse). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. Sizes of the PCR products for mouse M-csf, Rankl, and Gadph were 516, 587, 630, and 452 bp, respectively.

Western blot analysis

Mouse BMMS (2 × 105 cells/well) were cultured in 60-mm dishes with test chemicals for specific periods. Cells were lysed in a lysis buffer (0.1% Nonidet P-40, 20 mM Tris [pH 7.5], 50 mM β-glycerophosphate, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM Na3VO4, and 1× protease...
inhibitors mixture; Sigma) (37). Whole-cell extract was electrophoresed on a 10% SDS-polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane (Clear blot P membrane, Atto, Tokyo), and incubated with primary Abs. The bound Abs were visualized using an electro-generated chemiluminescence system (Amersham, Piscataway, NJ), followed by exposure to x-ray film. The following were used as primary Abs: anti–c-Fos Ab (H-125) from Santa Cruz Biotechnology; anti–NFATc1 Ab (7A6) from Affinity Bio Reagents (Golden, CO); anti–p-ERK Ab, anti-ERK Ab, anti–p-p38 Ab, anti-p38 Ab, anti–p-STAT5 Ab, and anti-STAT5 Ab from Cell Signaling Technology (Beverly, MA); and anti–β-actin Ab (AC-74) from Sigma.

Statistical analysis

All experiments in vitro and in vivo were performed at least twice, with similar results. Results are expressed as the mean ± SD for three or more cultures. The significance of differences was determined using the Student t test. Differences were considered significant at *p < 0.05 and **p < 0.01.

Results

We first examined whether the administration of minocycline in vivo inhibits RANKL-induced bone resorption using RANKL-injected mice (31, 32). Minocycline or vehicle was injected daily into the s.c. tissue overlying calvariae of mice for 5 d. GST-RANKL was injected into the same area for the last 2 d (Fig. 1A). Calvarial sections of RANKL-injected mice exhibited enhanced osteoclastic bone resorption (Fig. 1B). Both osteoclast surface and osteoclast number were significantly increased in RANKL-injected mice compared with vehicle-injected mice (Fig. 1C). Daily injections of minocycline reduced osteoclast surface and osteoclast number in RANKL-injected mice to the control levels. Osteoclast surface in the control mice was slightly decreased by the minocycline injection, but the difference was not significant. Daily injections of minocycline affected neither ALP activities in osteoblasts nor osteoblast number in RANKL-injected mice, suggesting that injected minocycline may not affect bone formation in this model (Fig. 1D, 1E). Furthermore, the minocycline treatment did not seem to affect histological features in the calvariae.

We examined effects of doxycycline and minocycline on the formation of osteoclasts in vitro. Mouse primary osteoblasts and bone marrow cells were cocultured in the presence of 1α,25(OH)2D3 and PGE2, with or without increasing concentrations of
tetracyclines (Fig. 2A). TRAP⁺ osteoclasts were formed within 7 d in response to 1α,25(OH)₂D₃ and PGE₂. Tetracyclines inhibited the osteoclastogenesis induced by 1α,25(OH)₂D₃ and PGE₂ (Fig. 2A) in a dose-dependent manner. 1α,25(OH)₂D₃ stimulates the formation of osteoclasts in cocultures through the induction of RANKL expression and suppression of OPG expression in osteoblasts (7). Treatment of osteoblasts with 1α,25(OH)₂D₃ enhanced expression of Rankl mRNA and suppressed that of Opg mRNA within 8 h (Fig. 2B). Minocycline affected neither Rankl mRNA expression nor Opg mRNA expression in osteoblasts treated with 1α,25(OH)₂D₃ at any time point examined. Doxycycline, as well as minocycline, showed no effect on the expression of Rankl and Opg mRNAs in osteoblasts treated or not with 1α,25(OH)₂D₃ (Fig. 2C). Expression of M-csf mRNA in osteoblasts was not affected by minocycline, doxycycline, or 1α,25(OH)₂D₃. Osteoblasts were cultured with a high concentration (20 μg/ml) of doxycycline and minocycline, and cell proliferation was monitored (Fig. 2D). The proliferation of osteoblasts was not affected by doxycycline or minocycline at any time point examined. Osteoblasts were cultured with doxycycline and minocycline at 10 μg/ml for 7 d, and osteoblastic differentiation was evaluated by ALP staining (Fig. 2E).

We next examined the effects of doxycycline and minocycline on the formation of osteoclasts in BMM cultures treated with RANKL and M-CSF (Fig. 3A). RANKL stimulated osteoclasts to form in the presence of M-CSF. Doxycycline and minocycline inhibited RANKL-induced osteoclastogenesis in a dose-dependent manner. RANKL–RANK signals activate the transcription factor complex AP-1 through the induction of one of its components, c-Fos (38). These signals in osteoclast precursors enhance the expression of NFATc1 (12). Treatment of BMMs with RANKL increased levels of c-Fos and NFATc1 protein within 12 and 24 h, respectively. The expression of these transcription factors in BMMs was strongly inhibited by simultaneous treatment with doxycycline or minocycline (Fig. 3B, 3C). MAPK signals play central roles in the regulation of osteoclastic differentiation (10). Treatment of BMMs with RANKL induced phosphorylation of ERK and p38 within 5 and 10 min, respectively. The phosphorylation of these MAPKs was also inhibited by prior and simultaneous treatment with tetracyclines (Fig. 3D, 3E).

We next examined the effects of doxycycline and minocycline on the proliferation and phagocytic activity of BMMs (Fig. 3F, 3G). M-CSF stimulated the proliferation of BMMs. Neither doxycycline nor minocycline affected the M-CSF–supported proliferation of BMMs. When cultured with latex beads, BMMs phagocytosed great quantities of the beads (Fig. 3G). Minocycline showed no effects on the phagocytic activity of BMMs. Doxycycline also failed to affect the phagocytosis of BMMs (data not shown).
Tetracyclines were shown to inhibit the function of osteoclasts (26). We confirmed that minocycline inhibited osteoclastic function by decreasing the survival of osteoclasts (Supplemental Fig. 1). These results suggested that tetracyclines specifically suppressed bone resorption-related events, such as osteoclastic differentiation and function.
DCs and osteoclasts are derived from common progenitors (10, 13, 14, 16). During experiments in vitro, we noticed that cells with an appearance similar to DCs emerged in BMM cultures treated with tetracyclines. BMMs were then cultured for 4 d with minocycline or GM-CSF, a well-known inducer of DCs, in the presence of RANKL and M-CSF (Fig. 4A). RANKL stimulated multinucleated cells (osteoclasts) to form in the presence of M-CSF. Minocycline inhibited the formation of osteoclasts but induced the appearance of DC-like cells with elongated processes in BMM cultures treated with RANKL. As expected, GM-CSF stimulated the differentiation of BMMs into DCs. This process was examined further by flow cytometry using Abs against CD11c (a marker of DCs) and CD11b (a marker of macrophages) (Fig. 4B). GM-CSF strongly induced the differentiation of BMMs into CD11c+ cells, even in the presence of RANKL and M-CSF. Minocycline did not induce the differentiation of BMMs into CD11c+ cells in the presence of M-CSF but the absence of RANKL; however, it did so in the presence of RANKL. More than 30% of BMMs differentiated into CD11c+ cells in response to minocycline (3 μg/ml) and RANKL. Treatment of BMMs with a high concentration of minocycline (10 μg/ml) resulted in rather small effects on the differentiation of CD11c+ cells (data not shown). This suggested that the suppression of osteoclast differentiation and induction of DC differentiation by tetracyclines may be regulated by different signals. CD86 is a marker of mature DCs. GM-CSF treatment increased the number of CD86+ cells, as well as CD11c+ cells, in BMM cultures (Fig. 4C). Similarly, the numbers of CD86+ cells and CD11c+ cells increased in response to minocycline and RANKL (Fig. 4C). Doxycycline also induced the differentiation of BMMs into DC-like cells in the presence of RANKL (data not shown).

GM-CSF-induced DC differentiation requires an increase in p-STAT5 (17, 18). We then examined the effects of midostaurin hydrate (midostaurin), an inhibitor of the phosphorylation of STAT5 (39), on the differentiation induced by minocycline in comparison with that induced by GM-CSF (Fig. 5A). Midostaurin inhibited the differentiation induced by GM-CSF but not minocycline. Similar results were obtained using doxycycline (data not shown). Midostaurin had no effect on the formation of osteoclasts in BMM cultures treated with RANKL and M-CSF (Fig. 5A, arrows in left panels). We next examined the effect of minocycline on the phosphorylation of STAT5 (Fig. 5B). GM-CSF induced the phosphorylation of STAT5 in BMMs, but minocycline did not. Midostaurin (3 μM) inhibited the phosphorylation of STAT5 in BMM cultures treated with GM-CSF. These results suggested that the DC-like cell differentiation induced by tetracyclines is inde-
pendent of STAT5 signaling. We further examined whether an anti–GM-CSF–neutralizing Ab inhibited the effect of minocycline on DC-like cell differentiation in comparison with that of GM-CSF (Fig. 5C). The anti–GM-CSF Ab inhibited GM-CSF–induced, but not minocycline-induced, differentiation of BMMs into CD11c+ cells (Fig. 5C). These results strongly suggested that the stimulatory effects of minocycline on the differentiation of DC-like cells are not mediated by GM-CSF.

Finally, we examined the effect of tetracycline on the formation of DC-like cells and osteoclasts in vivo using Rankl-Tg mice (30) (Fig. 6). Rankl-Tg mice exhibit increased osteoclastic bone resorption because of constitutively elevated levels of RANKL expression. Minocycline (10 mg/kg body weight) and doxycycline (10 mg/kg body weight) were injected daily for 5 d into the s.c. tissue on calvariae of Rankl-Tg mice. Calvariae were recovered on day 5. A few CD11c+ cells were observed in calvarial sections of Rankl-Tg mice, as well as control C57BL/6 mice (Fig. 6A). Daily injections of minocycline and doxycycline for 5 d significantly increased the number of CD11c+ cells in calvariae. Osteoclast number was significantly higher in Rankl-Tg mice than in C57BL/6 mice (Fig. 6B). Administration of minocycline and doxycycline to Rankl-Tg mice returned the number to the control level. Fluorescence microscopy revealed that minocycline accumulated in the calvarial calcified tissue (Fig. 6C). Neither the CD11c+ cell number nor the osteoclast number in the control C57BL/6 mice was significantly affected by treatment with tetracyclines (data not shown). These results suggested that tetracyclines not only inhibit osteoclastic differentiation but also induce DC-like cell differentiation in the presence of RANKL in vivo (Fig. 6D, Table I).

Discussion

Minocycline and doxycycline inhibited RANKL-induced osteoclastic differentiation by suppressing MAPKs and c-Fos in BMMs, as well as induced DC-like cells to form from BMMs in a STAT5-independent manner. We examined the effects of tetracycline administration in vivo on bone resorption in two mouse models: one model exhibits rapid bone resorption after RANKL injection, and the other constitutively enhances bone resorption by the Rankl transgene. Using these models, we showed that the administration of tetracyclines in vivo inhibited RANKL-induced osteoclastogenesis and enhanced DC-like cell differentiation. Tetracyclines also suppressed the pit-forming activity of osteoclasts. These
results suggested that tetracyclines can be used as potential anti-bone resorption agents with DC-like cell-inducing properties.

MAPK and c-Fos signals play essential roles in osteoclastic differentiation (10, 38, 40). Tetracyclines inhibited both the phosphorylation of MAPKs and the expression of c-Fos in BMMs. These results suggested that the inhibition of MAPK and c-Fos signals is essential for tetracyclines-suppressed osteoclastic differentiation (Table I). Miyamoto et al. (16) reported that GM-CSF inhibited RANKL-induced osteoclast formation along with the suppression of c-Fos, and enforced expression of c-Fos in osteoclast progenitors.

Table I. Effects of GM-CSF, RANKL, and tetracyclines on signal transduction in BMMs and on their differentiation into osteoclasts and DC-like cells

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>c-Fos</th>
<th>p38</th>
<th>ERK</th>
<th>STAT5</th>
<th>Osteoclast Differentiation</th>
<th>DC-like Cell Differentiation</th>
</tr>
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<tr>
<td>GM-CSF (+ RANKL)</td>
<td>↓*</td>
<td>N.D.</td>
<td>N.D.</td>
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<td>RANKL</td>
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Arrows indicate suppression and acceleration of signals in BMMs, as well as their differentiation into osteoclasts and DC-like cells.

*BMMs were treated with GM-CSF (+ RANKL), RANKL, or RANKL + tetracyclines.

*Signals listed were examined in this study.

*Reported by Miyamoto et al. (16).

N.D., not done.
eralized nodules (42). Consistent with this, minocycline at 10 μg/ml delayed the formation of minocycline-suppressed osteoclast formation (data not shown). These results suggested that the suppression of c-Fos signals is important for DC differentiation.

Tetracyclines inhibited MAPKs in addition to c-Fos. Li et al. (10) reported that inhibition of p38 in BMMs by SB203580, a p38 MAPK-signaling inhibitor, suppressed RANKL-induced osteoclastic differentiation but not GM-CSF-induced DC differentiation. These results suggested that MAPK signals are involved in osteoclastic differentiation but not in DC differentiation.

To our surprise, tetracyclines induced DC-like cell differentiation without causing the phosphorylation of STAT5 in BMMs. Midostaurin suppressed GM-CSF-induced DC differentiation but not tetracycline-induced DC-like cell differentiation. We also examined the effect of minocycline on Gm-csf mRNA expression in osteoblasts cultured with 1α,25(OH)2D3, as well as in BMMs cultured with RANKL. Minocycline failed to affect Gm-csf mRNA expression in osteoblasts and in BMMs in culture conditions similar to osteoclast-formation assays (data not shown). These results suggested that the effects of tetracyclines on DC-like cell differentiation are not mediated by GM-CSF–STAT5 signaling. In fact, it is reported that DCs exist in vivo. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345: 442–444.


Supplementary Fig. 1. Minocycline inhibits osteoclastic function in cultures.

(A) Pit formation assay. Mouse osteoblasts and bone marrow cells were cocultured for 6 days in the presence of $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) and PGE$_2$ ($10^{-6}$ M) in 10 cm dishes pre-coated with type I collagen gel. All the cells including osteoclasts were recovered from the coculture by using
0.2% collagenase. Aliquots of the crude osteoclast preparations were cultured on dentine slices in the presence or absence of minocycline (10 μg/ml). After 24 hours, dentine slices were recovered. Cells were removed from the dentine slices, and the slices were stained with Mayer’s hematoxylin to identify resorption pits. The number of resorption pits was counted. Some dentine slices were processed for TRAP and F-actin staining. Scale bar, 100 μm. (B) Survival assay. The purity of osteoclasts in the crude osteoclast preparations was about 5%. To purify osteoclasts, the crude preparation was plated in 48-well culture dishes. After incubation for 6 hours, osteoblasts were removed by treatment with trypsin-EDTA. Purified osteoclasts were cultured with or without minocycline (3 μg/ml, 10 μg/ml). After 18 hours, cells were fixed and stained for TRAP. TRAP-positive cells containing more than three nuclei were counted as osteoclasts. All experiments were performed at least three times with similar results. Results are expressed as the mean ± SD for eight cultures. Significantly different from the control culture, **p<0.01. Scale bar, 200 μm.
Supplementary Figure 2. Enforced expression of c-Fos in BMMs cannot rescue the minocycline-suppressed osteoclast formation.
pMX-c-Fos was constructed by inserting cDNA of c-Fos into a pMX vector. pMX-empty was used as a control. Retroviral packaging was performed by transfection of plasmids into Plate-E using FuGene-6 transfection reagen. (A) Expression of c-Fos. BMMs were infected with pMX-empty or pMX-c-Fos retroviruses, and cultured with M-CSF (50 ng/ml) in 6-well plates (1 x 10^6 cells/well) for 1 day. The cells were further cultured in the presence of M-CSF (50 ng/ml) for 3 days. Expressions of c-Fos and β-actin were detected by immunoblotting. (B) Formation of osteoclasts in BMM cultures treated with minocycline. BMMs were infected with pMX-empty or pMX-c-Fos retroviruses, and cultured with M-CSF (50 ng/ml) in 96-well plates (1 x 10^4 cells/well) for 1 day. The cells were further cultured in the presence of RANKL (100 ng/ml) and M-CSF (50 ng/ml) with or without minocycline (10 μg/ml) for 3 days. The cells were fixed and stained for TRAP. TRAP-positive cells containing more than three nuclei were counted as osteoclasts. (C) Formation of osteoclasts in BMM cultures treated with GM-CSF. BMMs were infected with pMX-empty or pMX-c-Fos retroviruses, and cultured with M-CSF (50 ng/ml) in 96-well plates (1 x 10^4 cells/well) for 1 day. The cells were further cultured in the presence of RANKL (100 ng/ml) and M-CSF (50 ng/ml) with or without GM-CSF (10 ng/ml) for 3 days. The cells were fixed and stained for TRAP. TRAP-positive cells containing more than three nuclei were counted as osteoclasts. All experiments were performed three times with similar results. Results are expressed as the mean ± SD for five cultures. Significantly different between the two treatment groups, **p<0.01. Scale bar, 200 μm.
Supplementary Figure 3. The kinetics of both minocycline-induced DC-like cell differentiation and inhibitory effects of minocycline on osteoclast formation.

(A) Effects of minocycline on DC-like cell differentiation. BMMs were cultured for 3 days in the presence of RANKL (50 ng/ml) and M-CSF (25 ng/ml). These cultures were treated with minocycline according to four experimental designs illustrated in the left upper panel.
Minocycline (3 μg/ml) was added to the cultures for 3 days (Day 0~3), during the first day (Day 0~1), and during the last day (Day 2~3). BMMs were pre-cultured with minocycline for 1 day before the addition of RANKL and M-CSF (Day -1~0). The cells were stained with an anti-CD11c antibody (brown) and hematoxylin (purple). CD11c-positive cells were counted. Results are expressed as the mean ± SD for five cultures. Significantly different from the culture treated with RANKL, **p<0.01. Scale bar, 50 μm. (B) Effects of minocycline on osteoclast formation. BMMs were cultured according to the same experimental design in (A) with RANKL (100 ng/ml) and M-CSF (50 ng/ml). The cells were fixed and stained for TRAP. TRAP-positive cells containing more than three nuclei were counted as osteoclasts. Results are expressed as the mean ± SD for five cultures. Significantly different from the culture treated with RANKL, *p<0.05 and **p<0.01. Scale bar, 200 μm.