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

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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-mutated 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: 000–000.

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)2D3] and PGE2 (7). 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 precurors, 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 in 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; 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3; OPG, osteoprotegerin; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; Tg, transgenic; TRAP, tartrate-resistant acid phosphatase.

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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 vitro. C57BL/6 mice and BALB/c mice were used for experiments in vivo. RANKL-transgenic (Tg) mice (genetic background of C57BL/6), which express a soluble form of mouse RANKl using the human serum amyloid P component promoter, were generated in one of the authors’ laboratories (30). RANKl-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 midostaurin 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). IgG(OH)2D2 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 overlaying calvaria of 7-wk-old C57BL/6 mice for 5 d, beginning on day 0. Twenty microliters of GST-RANKL (1 mg/ml 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 were measured 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 overlaying calvaria 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 collected and 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 (RELATech, Wolfenbuttel, Germany), and an HRP-conjugated secondary anti-hamster Ab (Santa Cruz Biotechnology). The HRP-conjugated Ab was visualized with a 3,3′-diaminobenzidine kit (DAKO, Carpinteria, CA). The HRP-conjugated secondary Ab was visualized as 3,3′-diaminobenzidine (DAB). DAB was used as a chromagenic substrate, and DAB staining was performed to observe the localization of DCs and the effect of tetracyclines on DCs. For immunofluorescence staining, cells were incubated with the anti-CD11c Ab (HL3) and an FITC-conjugated anti-CD11b Ab (M1/70) (BD Biosciences, San Jose, CA). Stained cells were analyzed by flow cytometry (10). For immunofluorescence staining, cells were fixed, treated with 0.3% H2O2, and incubated with the FITC-conjugated anti-CD11c Ab and an HRP-conjugated secondary anti-FITC Ab. Cells were also stained with an anti-CD86 Ab (GL-1; BD Biosciences), followed by 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) in each cell. Phagocytic activity was expressed as the percentage of bead-positive cells.

**Formation of DCs in culture**

BMMs were prepared in cultures of bone marrow cells (2×10^5 cells/well) or BMMs (1×10^6 cells/well) were cultured in the presence of M-CSF (25 ng/ml) in RPMI 1640 medium (Sigma) supplemented with 5% FBS (10, 16). BMMs were further maintained in serum-free MEM for 2 d. In 12-wk-old C57BL/6 mice, 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). Osteoblast number/bone perimeter was determined by a histomorphometric analysis at the suture of calvariae (30).

**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 (IBT Pharmatech, Tokyo, Japan) using the following specific primers: mouse Opg (forward) and 5′-CATCAGACCTGAGATGTGTCG-3′ (reverse); mouse Rankl (forward) and 5′-CGCTGCTTTCTGCCTGTCG-3′ (reverse); mouse Opg, 5′-CCAGACAGTATGATACAGATATAC-AAGGCAGG-3′ (forward) and 5′-ATGAAGTGTCCTACGAAAGAC-CC-3′ (reverse); and mouse Gapdh, 5′-ACCACTGTCCTGACCAAC-3′ (forward) and 5′-TCCACACCGTGTCGACTGTA-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, Opg, and Gapdh were 516, 587, 630, and 452 bp, respectively.

**Western blot analysis**

Mouse BMMs (2×10^6 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–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 REGULATE OSTEOCLASTOGENESIS

FIGURE 2. Tetracyclines inhibit osteoclast formation in mouse cocultures. A, Formation of osteoclasts in mouse cocultures. Primary osteoblasts and bone marrow cells were cocultured for 7 d in the presence of 1α,25(OH)2D3 (10−8 M) and PGE2 (10−6 M) (1,25D + PGE2) with increasing concentrations of doxycycline and minocycline. Cells were fixed and stained for TRAP. TRAP+ multinucleated cells containing more than three nuclei were counted as osteoclasts. Results are expressed as the mean ± SD for five cultures. Scale bar, 200 µm. B and C, mRNA expression of M-CSF, Rankl, and Opg in osteoblasts. In B, osteoblasts were preincubated or not with minocycline (10 µg/ml) for 15 min and cultured for the periods indicated with or without minocycline in the presence of 1α,25(OH)2D3 (1,25D, 10−8 M). In C, osteoblasts were preincubated or not with doxycycline or minocycline (10 µg/ml) for 15 min and cultured for 24 h with or without tetracyclines in the presence or absence of 1α,25(OH)2D3 (1,25D, 10−8 M). Total RNA extracted from cells was subjected to RT-PCR analyses. D, Proliferation of osteoblasts. Osteoblasts were cultured for 48 h with or without doxycycline or minocycline (20 µg/ml). Cell proliferation was evaluated by the Alamar blue assay. Results are expressed as the mean ± SD for four cultures. E, Osteoblastic differentiation. Osteoblasts were cultured for 7 d in the presence of ascorbic acid (100 µg/ml) and β-glycerophosphate (5 mM), with or without doxycycline or minocycline (10 µg/ml), and stained for ALP. Scale bar, 50 µm. All experiments were performed three times with similar results. **p < 0.01, versus culture treated with 1α,25(OH)2D3 and PGE2.
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 BMM cultures, 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-
dependent of STAT5 signaling. We further examined whether an anti–GM-CSF–neutralizing Ab inhibited the effect of minocycline on DC-like cell differentiation (Fig. 5C). These cells were preincubated for 1 h with or without minocycline (3 μM) or GM-CSF (10 ng/ml) in the presence of RANKL (50 ng/ml) and M-CSF (25 ng/ml). Cells were then treated with anti–CD11c Ab (brown) and hematoxylin (purple). CD11c+ cells were counted. Results are expressed as the mean ± SD for six cultures. Arrows indicate multinucleated cells. Scale bar, 100 μm. **p < 0.01, versus culture treated with midostaurin.

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 exhibited 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 of 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). 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 osteo-

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

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<th>Treatment</th>
<th>Signals</th>
<th>Osteoclast Differentiation</th>
<th>DC-like Cell Differentiation</th>
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<tr>
<td></td>
<td>c-Fos</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.
DC differentiation was inhibited when c-Fos was expressed in the progenitors at an early stage of differentiation (16). Our preliminary experiments also showed that enforced expression of c-Fos in BMMs suppressed the DC-like cell differentiation induced by tetracyclines (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 inflammation, including rheumatoid arthritis and periodontitis. The development of a drug-delivery system for tetracyclines will facilitate their use in the suppression of bone loss induced by local inflammation, including rheumatoid arthritis and periodontitis.

Disclosures
The authors have no financial conflicts of interest.

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