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Administration of Cyclooxygenase-2 Inhibitor Reduces Joint Inflammation but Exacerbates Osteopenia in IL-1α Transgenic Mice Due to GM-CSF Overproduction

Yasuo Niki,1* Hironari Takaishi,* Jiro Takito,* Takeshi Miyamoto,* Naoto Kosaki,* Hideo Matsumoto,* Yoshiaki Toyama,* and Norihiro Tada†

IL-1α transgenic (Tg) mice exhibit chronic inflammatory arthritis and subsequent osteopenia, with IL-1-induced GM-CSF playing an important role in the pathogenesis. This study analyzed the mechanisms underlying osteopenia in Tg mice, and the therapeutic effects of the cyclooxygenase-2 inhibitor celecoxib on such osteopenia. Inhibited osteoclast formation was observed in RANKL-treated bone marrow cell (BMC) cultures from Tg mice and coculture of Tg-derived BMCs and wild-type-derived primary osteoblasts (POBs). FACS analysis indicated that this inhibition was attributable to a decreased number of osteoclast precursors within Tg-derived BMCs. Moreover, in coculture of Tg-derived POBs and either Tg- or wild-type-derived BMCs, osteoclast formation was markedly inhibited because Tg-derived POBs produced abundant GM-CSF, known as a potent inhibitor of osteoclast differentiation. Histomorphometric analysis of Tg mice revealed that both bone formation and resorption were decreased, with bone formation decreased more prominently. Interestingly, administration of celecoxib resulted in further deterioration of osteopenia where bone formation was markedly suppressed, whereas bone resorption remained unchanged. These results were explained by our in vitro observation that celecoxib dose-dependently and dramatically decreased osteogenesis by Tg mouse-derived POBs in culture, whereas mRNA expressions of GM-CSF and M-CSF remained unchanged. Consequently, blockade of PGE2 may exert positive effects on excessively enhanced bone resorption observed in inflammatory bone disease, whereas negative effects may occur mainly through reduced bone formation, when bone resorption is constitutively down-regulated as seen in Tg mice. The Journal of Immunology, 2007, 179: 639–646.

Interleukin-1 is an immunomodulatory and proinflammatory cytokine that possesses a wide spectrum of biological activities, including stimulation of lymphocytes, pyrogenicity, and bone resorption (1–5). IL-1 is known as a potent activator of osteoclastic bone resorption observed in inflammatory and metabolic bone diseases such as rheumatoid arthritis, periodontitis, and postmenopausal osteoporosis (4, 5). Under these pathological situations, stimulatory actions of IL-1 in osteoclasts are mediated through both direct and indirect mechanisms. Differentiation of osteoclasts is indirectly induced by IL-1 through the induction of receptor activator of NF-κB ligand (RANKL)2 in osteoblasts (6), whereas extended survival and pit-forming activity of osteoclasts are promoted via direct action of IL-1 (7, 8). In addition, such bone-resorbing effects of IL-1 have been documented by in vivo experiments, showing that s.c. injection of IL-1 in mice increases osteoclast number and bone-resorbing activity (9, 10).

1 Abbreviations used in this paper: RANKL, receptor activator of NF-κB ligand; Tg, transgenic; POB, primary osteoblast; BMC, bone marrow cell; TRAP, tartrate-resistant acid phosphatase; COX, cyclooxygenase; 1,25(OH)2D3, 1α,25-dihydroxyvitamin D3; MNC, marrow monocyte cell; WT, wild type.

The prevention of PGE2 synthesis by pharmacological or genetic ablation of COX-2 inhibits PGE2-induced RANKL expression in osteoblasts and subsequent osteoclast formation (20, 21), resulting in recovery of osteopenia. Conversely, contrasting results have been reported with selective depletion of PGE2 action using a selective receptor antagonist directly abrogating the osteogenic potential of bone marrow and biasing bone metabolism toward

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osteoarthritis (22). The net balance of bone formation vs resorption following anti-PGE2 treatment may thus be changeable according to whether the dominant action of treatment is inhibition of the anabolic or catabolic actions of PGE2.

As IL-1 is reportedly a strong inducer of COX-2 and subsequent PGE2 production (6, 23, 24), IL-1α Tg mice should offer a useful model for the analysis of COX-2 implications in the development of inflammatory arthritis and associated osteopenia. The present study analyzed the detailed mechanisms underlying osteopenia caused by genetically overproduced IL-1 and the effects of the COX-2 inhibitor celecoxib on inflammatory arthritis and osteopenia using IL-1α Tg mice. The results indicate that GM-CSF induced by genetically overproduced IL-1α constitutively inhibits osteoclastogenesis in Tg mice bone marrow, both in vivo and in vitro. Under such situations, systemic administration of celecoxib ameliorated joint inflammation, but osteopenia is rather exacerbated despite effective down-regulation of PGE2, essentially contradicting past lines of evidence supporting the osteoprotective effects of COX-2 inhibitors.

Materials and Methods

Generation of Tg mice

The generation of human IL-1α Tg mice has been previously described (11). Briefly, human IL-1α cDNA was ligated into a plasmid containing CAG promoter constructed by the first intron of the chicken β-globin gene and a portion of the rabbit β-globin gene. The resulting construct was used for microinjection, and two mouse lines, Tg1705 and Tg1706, were established. The Tg1706 line was backcrossed with C57/H129 mice for six to eight generations and used in all experiments. All procedures for animal care were approved by the animal management committee of Keio University (Tokyo, Japan).

Reagents

Collagenase, 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3), and anti-MEM were purchased from Wako Biochemicals. FBS was obtained from Life Technologies. Recombinant mouse M-CSF was from R&D Systems ( Minneapolis, MN), and recombinant mouse soluble RANKL was from PeproTech. PGE2 was purchased from Sigma-Aldrich. Celecoxib (4-[5- (4-methylphenyl)-3-(trifluoromethyl)-pyrazol-1-yl] benzenesulfonamide), which is known as a specific inhibitor of COX-2 (25), was obtained (Minneapolis, MN), and recombinant mouse soluble RANKL was from PeproTech. PGE2 was purchased from Sigma-Aldrich. Celecoxib (4-[5- (4-methylphenyl)-3-(trifluoromethyl)-pyrazol-1-yl] benzenesulfonamide), which is known as a specific inhibitor of COX-2 (25), was obtained from Pfizer.

Culture of bone marrow macrophages

Tibiae and femora were aseptically dissected from 8- to 10-wk-old male human IL-1α Tg mice and C57 wild-type (WT) mice. Bone ends were removed and the bone marrow was forced out into anti-MEM containing 10% FBS. After filtration through a Cell Strainer (BD Falcon) to remove bone particles, obtained bone marrow cells (BMCs) were cultured with M-CSF (50 ng/ml) and various concentrations of RANKL (0, 10, or 50 ng/ml) in 24-well plates (Corning) at 3.5 M-CSF (50 ng/ml) and various concentrations of RANKL (0, 10, or 50 ng/ml) in 24-well plates (Corning) at 3.5×10^6 cells/well. After 6 days, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). The number of osteoclasts was counted as TRAP-positive multinuclear cells (MNCs) containing ≥3 nuclei per cell.

Coculture of primary osteoblasts and BMCs

Calvariae were dissected from newborn Tg and WT mice. Primary osteoblasts (POBs) were obtained from calvariae by conventional enzymatic digestion using collagenase (26). POBs (3 × 10^5) and BMCs (3 × 10^5) were cocultured in anti-MEM containing 10% FBS in 24-well tissue culture plates (Corning). Cocultures were incubated with 10^−8 M 1,25(OH)2D3 and 10^−8 M PGE2 for 8 days. Cocultures were then fixed with 10% formalin (Wako Pure Chemicals) and stained with TRAP. The number of TRAP-positive MNCs containing ≥3 nuclei per cell was counted as osteoclasts.

Flow cytometry

The cell-staining procedure for flow cytometry was performed as previously described (23). The mAbs used in immunofluorescence staining were anti-F4/80 Ab (BM8; eBioscience), anti-c-Kit Ab (2B8; eBioscience), anti-Mac-1 Ab (M1/70; BD Pharmingen), and anti-c-Fms Ab (AFS98; eBioscience) and were labeled with PE, allophycocyanin, FITC, and PE, respectively. After 30-min incubation with fluorescein-labeled mAb, cells were washed twice with 5% FBS/PBS (washing buffer) and applied to flow cytometric analysis using FACSCalibur (BD Immuno- cytometry Systems).

Administration of celecoxib

Four-week-old Tg and WT mice were divided into five groups of four mice each, and 10 or 100 mg/kg of celecoxib diluted in 0.5% methylcellulose was orally administered to each mouse every 2 days for 2 mo. All mice were killed at 8 wk after commencement of oral administration. At the end of the experiment, peripheral blood was collected by cardiac puncture for determination of PGE2 levels in serum.

Bone marrow density measurement

Femora were extracted from Tg mice and WT controls, and were fixed with 70% ethanol after the removal of soft tissues. Microcomputed tomography (CT) analysis of the femoral metaphysis and diaphysis was performed with a commercial x-ray analyzing system (NS-ELEX). Femoral bone marrow density was measured by dual-energy x-ray absorptiometry using a DCS-600R analyzer (Alola).

Histomorphometric measurement

Tibiae were removed from Tg mice and WT controls, fixed in 70% ethanol and embedded in glycol methacrylate without decalcification. Serial sections (3-μm thick) were cut using a microtome (model 2050; Reichert Jung). Alkaline phosphatase and TRAP staining was performed to identify the cellular components. For double-fluorescence labeling studies, all mice were s.c. injected with calcine (16 mg/kg body weight) at 11 and 4 days before sacrifice. Histomorphometric analysis of trabecular bone was performed in an area 1.8 mm long from 0.1 mm below the growth plate at the proximal tibial metaphysis using a semiautomated system (Osteoplan II; Carl Zeiss) with measurements made at a magnification of ×400. Nomenclature, symbols and units are those recommended by the American Society for Bone Mineral Research Histomorphometry Nomenclature Committee.

Clinical and histological assessment of arthritis

Clinical symptoms of arthritis in all four limbs were macroscopically evaluated according to a visual scoring system as reported previously (12). Arthritic joints were graded on a scale of 0–4. Scores for each mouse comprised the sum of scores for all four limbs, with a maximum score of 16. Clinical severity was also determined by quantifying changes in paw volume using plethysmometry (model 7140; Ugo Basile). In histological evaluations, paws and knees were dissected and fixed in formalin. Sagittal sections (6 μm) were prepared and stained using H&E. As previously described (12), synovial infiltration and cartilage destruction were scored on four semiserial sections of each specimen at 10-section intervals. Neutrophil infiltration and cartilage destruction were graded on a scale of 0–3 each. Scores for each mouse comprised the sum of scores for knee and ankle joints of a hind limb, with a maximum score of 12.

Quantification of cytokines and PGE2 production

Levels of mouse GM-CSF, human IL-1α, and mouse soluble RANKL secreted into culture medium were assayed by quantitative sandwich ELISA (R&D Systems) according to the instructions of the manufacturer. Levels of PGE2 in serum were also determined using a PGE2 assay kit (R&D Systems). Values were determined in triplicate. Sensitivity of the assay (minimum detectable dose) is <5.0 pg/ml for GM-CSF, 4.8 pg/ml for RANKL, <1.0 pg/ml for human IL-1α, and 10.1 pg/ml for PGE2.

Analysis of in vitro osteogenesis

For induction of osteogenesis, POBs were inoculated at a density of 5 × 10^5 cells/well in a 24-well plate, and cultured in medium supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, 100 nM dexamethasone (Sigma-Aldrich), and 200 ng/ml recombinant human bone morphogenetic protein BMP2 (R&D Systems). Alkaline phosphatase, Alizarin red, and von Kossa stainings were performed 7 days after confluence. For alkaline phosphatase staining, cells were fixed in 70% ethanol and stained for 10 min with a solution containing 0.1% naphthol AS-MX phosphate disodium salt (Sigma-Aldrich), 1% N,N-dimethylformamide (Wako Pure Chemicals), and 0.06% fast blue BB (Sigma-Aldrich). For Alizarin red, and von Kossa stainings were performed 7 days after confluence. For alkaline phosphatase staining, cells were fixed in 70% ethanol and stained for 10 min with a solution containing 0.1% naphthol AS-MX phosphate disodium salt (Sigma-Aldrich), 1% N,N-dimethylformamide (Wako Pure Chemicals), and 0.06% fast blue BB (Sigma-Aldrich). For Alizarin red staining, cells were fixed in 10% formalin/PBS and stained for 10 min with 2% Alizarin red (pH 4.0) (Sigma-Aldrich) solution. For von Kossa staining, cells were fixed with 100% ethanol and stained for 10 min with 5% silver nitrate solution (Wako Pure Chemicals) under UV light, then
incubated for 5 min with 5% sodium thiosulfate solution (Wako Pure Chemicals).

**RT-PCR analysis**

POBs cultured in anti-MEM containing 10% FBS were treated with $10^{-8}$ M 1,25(OH)$_2$D$_3$ and $10^{-6}$ M PGE$_2$, when cells reached confluence. In some cultures, various concentrations of celecoxib were added. After 3 days of culture, total RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies). Reverse transcription of cDNA was performed using SuperScript III (Invitrogen Life Technologies). PCR was performed with a Titanium PCR kit (Clontech Laboratories), and β-actin was used as an internal control. The number of PCR cycles was selected in the linear amplification phase for each primer. For calculation of relative mRNA expression, the mRNA level for each cytokine was normalized with that of corresponding β-actin using NIH Image 1.6 software. Primers used for RT-PCR were as follows: human IL-1α (sense) 5'-TTAC AGCACACAGGCCACCTT-3', (antisense) 5'-CGTGACGTTGCAAGTCAAGTGGT-3'; mouse alkaline phosphatase (sense) 5'-CCAAGACTACAACACCAACGC-3', (antisense) 5'-AAATCTGATAGAGGTTT-3'; mouse RANKL (sense) 5'-TATGATGGCAGGCTCATGTGG-3', (antisense) 5'-TGTCCTGAACATTGTAAGACG-3'; mouse GM-CSF (sense) 5'-GGAAGCATGTAGAGGCCATCA-3', (antisense) 5'-GGAAGCATGTAGAGGCCATCA-3', (antisense) 5'-TGGCTTGGCTTGG-3'; mouse M-CSF (sense) 5'-CTCTGGC-3', (antisense) 5'-GCCGAGGAGGAGG-3'; mouse alkaline phosphatase (sense) 5'-TCCG-3', (antisense) 5'-ACGCACGATTTCCTCTCAGC-3'.

**Statistical analysis**

Results are basically expressed as mean ± SD. Statistical differences between groups were determined using one-way ANOVA followed by post hoc testing using Bonferroni’s method (StatView-J 5.0 statistical software; SAS Institute). Values of $p < 0.05$ were considered statistically significant.

**Results**

**Inhibition of osteoclastogenesis in Tg mice**

Previous histomorphometric analyses have revealed that in our Tg mice, human IL-1α overproduction results in inhibited bone formation and resorption compared with WT mice, but inhibition of bone formation exceeds that of bone resorption, leading to systemic osteopenia (15). We first examined in vitro osteoclast formation induced by RANKL in cultures of bone marrow macrophages derived from Tg mice. TRAP-positive MNCs were cocultured with POBs derived from calvariae of Tg or control mice. Under stimulation with 1,25(OH)$_2$D$_3$, formation of TRAP-positive MNCs was prominent in coculture of control POBs/BMCs, but such formation was reduced by 78%, 66%, and 35% in cocultures of Tg BMCs/POBs, control BMCs/POBs, and Tg BMCs/control POBs, respectively, compared with control POBs/BMCs (Fig. 2). Consequently, osteoclastogenesis may be inhibited when either POBs or BMCs were derived from Tg mice. In addition, POBs appeared to contribute to such inhibition of osteoclastogenes similarly closely than BMCs.

As a next experiment, osteoclast formation was examined in the coculture of BMCs and POBs. Tg- or control-derived BMCs were cocultured with POBs derived from calvariae of Tg or control mice. Under stimulation with 1,25(OH)$_2$D$_3$, formation of TRAP-positive MNCs was prominent in coculture of control POBs/BMCs, but such formation was reduced by 78%, 66%, and 35% in cocultures of Tg BMCs/POBs, control BMCs/POBs, and Tg BMCs/control POBs, respectively, compared with control POBs/BMCs (Fig. 2). Consequently, osteoclastogenesis may be inhibited when either POBs or BMCs were derived from Tg mice. In addition, POBs appeared to contribute to such inhibition of osteoclastogenesis more closely than BMCs.
Accelerated PGE\(_2\), RANKL, and GM-CSF production in Tg mouse-derived POBs

Because inhibition of osteoclastogenesis was closely related to Tg mouse-derived POBs, production of bone metabolism-related molecules by POBs was analyzed at the mRNA and protein levels using RT-PCR and specific ELISA (Fig. 3). As expected, Tg mouse-derived POBs displayed high levels of RANKL and GM-CSF mRNA in addition to transgene-derived human IL-1\(\beta\). Furthermore, significantly high levels of PGE\(_2\) and GM-CSF secreted into culture medium were observed in Tg mice. Interestingly, GM-CSF was prominently induced at both mRNA and protein levels in Tg mice as compared with control mice, whereas levels of M-CSF mRNA were equivalent between Tg and control mice. Conversely, levels of alkaline phosphatase mRNA were diminished in Tg mice compared with control mice.

FACS analysis of monocyte/macrophage progenitor cells in Tg mice

To characterize monocyte/macrophage progenitor cells in bone marrow, cellular phenotypes of bone marrow mononuclear cells were analyzed using FACS. After gating on c-Kit\(^{+}\) cells, which involve a majority of hemopoietic progenitor cells in bone marrow, cells were subdivided according to Mac-1 (CD11b) and c-Fms (Fig. 4A). In Tg mice bone marrow, c-Fms\(^{+}\)Mac-1\(^{dull}\) (Fig. 4, R1) and c-Fms\(^{+}\)Mac-1\(^{high}\) (Fig. 4, R2) cells accounted for ~50% and 4% of c-Kit\(^{+}\) cells, respectively. These data indicate that bone marrow of Tg mice includes a significantly larger number of c-Fms\(^{+}\)Mac-1\(^{high}\) cells compared with WT control. In contrast, the number of c-Fms\(^{+}\)Mac-1\(^{dull}\) cells was lower in Tg mice than in WT controls. According to previous reports, c-Fms\(^{+}\)Mac-1\(^{dull}\) cells comprise a large number of osteoclast precursor cells, whereas c-Fms\(^{+}\)Mac-1\(^{high}\) cells mainly comprise cells committed to becoming mature macrophages or dendritic cells (27, 28), suggesting that Tg bone marrow progenitor cells tend to be committed to become macrophages or dendritic cells rather than osteoclasts. Furthermore, the number of F4/80\(^{+}\) cells, known as a highly differentiated phenotype of a macrophage lineage, was significantly higher in Tg mice than in controls (Fig. 4B), reflecting the predominance of c-Fms\(^{+}\)Mac-1\(^{dull}\) cells in c-Kit\(^{+}\) hemopoietic cells in Tg mice bone marrow.

Anti-inflammatory effects of COX-2 inhibitor in IL-1\(\beta\) Tg mice

Because IL-1 is widely accepted as a strong inducer of COX-2 (6, 23, 24) and COX-2-induced PGE\(_2\) has been implicated in several inflammatory arthritis models in mice (16–19), the anti-inflammatory effects of the COX-2 inhibitor celecoxib were examined in Tg mice. After administration of celecoxib for 2 mo, serum levels of PGE\(_2\) constitutively increased in Tg mice were efficiently decreased with increasing dosages of celecoxib. In particular, PGE\(_2\) levels in Tg mice declined to levels almost 642 EFFECTS OF COX-2 INHIBITOR ON ARTHRITIS IN IL-1\(\beta\) Tg MICE

FIGURE 3. Up-regulated production of RANKL, PGE\(_2\), and GM-CSF in Tg mouse-derived POBs. A, mRNA expression of transgene-derived human IL-1\(\beta\) (hIL-1\(\beta\)), RANKL, alkaline phosphatase (ALP), M-CSF, and GM-CSF in POBs prepared from Tg and control mouse calvariae. B, Levels of human IL-1\(\beta\), PGE\(_2\), soluble RANKL, and GM-CSF in conditioned medium of POBs were determined using specific ELISA. Results are expressed as mean ± SD of quadruplicate wells from three different mice. * p < 0.001 between Tg and control mice.

FIGURE 4. FACS analysis of phenotypic characteristics of BMCs derived from Tg mice (Tg) and WT controls (Cont.). A, Expression of c-Fms and Mac-1 (CD11b) on BMCs was analyzed. After gating on c-Kit\(^{+}\) cells, the cells were further subdivided into the following two fractions based on expression of c-Fms and Mac-1: c-Fms\(^{+}\)Mac-1\(^{dull}\) (R1) and c-Fms\(^{+}\)Mac-1\(^{high}\) (R2). Representative data from four different mice are shown. Percentages of R1 and R2 fractions to c-Kit\(^{+}\) cells are shown (far right). B, Expression of F4/80 on BMCs was analyzed. Percentages of F4/80\(^{+}\) cells in Tg and control mice are shown (far right). Representative data from four different mice are shown. Results are expressed as mean ± SD. * p < 0.05 comparing Tg and control mice.
equivalent to levels in WT controls, when 100 mg/kg/day celecoxib was administered (Fig. 5A). Degree of paw swelling and macroscopic scores also decreased in a dose-dependent manner in parallel with reduced PGE\textsubscript{2} levels in celecoxib-treated mice. Histological severity of arthritis after celecoxib treatment tended to display a similar but nonsignificant decrease (Fig. 5B).

**Effects of COX-2 inhibitor on osteopenia in Tg mice**

IL-1\textalpha overproduction in Tg mice resulted in reduced bone marrow density in the whole tibia, compared with WT control. Loss of bone marrow density was more prominent in the proximal and distal portions than in the central portion (data not shown). Tg mice administered celecoxib at 100 mg/kg/day displayed significant reductions in bone marrow density compared with Tg mice treated using vehicle alone (Fig. 6A). Likewise, WT control treated with 100 mg/kg/day tended to display a nonsignificant reduction in bone marrow density compared with control treated using vehicle alone. Effects of celecoxib were further analyzed using histomorphometry. Osteopenia in Tg mice has been attributed to decreased bone formation rather than increased bone resorption (15), and identical results were obtained in the present study. Various parameters for trabecular bone structure indicated trabecular bone loss in vehicle-treated Tg mice as compared with vehicle-treated WT control (Fig. 6A). Parameters for bone formation, including mineral apposition rate and mineralizing surface/bone surface ratio, were inhibited in vehicle-treated Tg mice, whereas indices of bone resorption, including eroded surface/bone surface ratio and osteoclast number/bone perimeter ratio, did not differ between vehicle-treated Tg mice and WT controls (Fig. 6C). Interestingly, administration of celecoxib resulted in loss of trabecular bone, supporting the bone marrow density data (Fig. 6B). According to the results of histomorphometry, bone formation parameters such as mineral apposition rate and mineralizing surface/bone surface ratio were further suppressed after celecoxib administration.

**FIGURE 5.** Anti-inflammatory effects of the COX-2 inhibitor celecoxib on arthritis in Tg mice. Tg mice (Tg) and WT controls (Cont) were orally administered as follows: Tg mice, vehicle only (Tg0); 10 mg/kg celecoxib (Tg10); 100 mg/kg celecoxib (Tg100) and WT mice, vehicle only (Cont0); 100 mg/kg celecoxib (Cont100). A, After 2-mo administration, serum levels of PGE\textsubscript{2} were measured in celecoxib- and vehicle-treated Tg mice and vehicle-treated WT controls. B, Antiarthritic effects of celecoxib were evaluated both clinically and histologically using the scoring system. Simultaneously, volumes of hind paws were measured. Each value for paw volume represents the mean of both hind paws. *p < 0.001; **p < 0.05 between the two groups.

**FIGURE 6.** Effects of celecoxib on bone marrow density and histomorphometry. A, Bone marrow density (BMD) of whole femora was measured in celecoxib- and vehicle-treated Tg mice and WT controls using dual-energy x-ray absorptiometry. B, Histomorphometric analysis of trabecular bone in tibiae obtained from celecoxib-treated Tg mice and WT controls. Some Tg and WT mice were treated with vehicle alone. Trabecular bone samples were measured in an area 1.8 mm long from 0.1 mm below the growth plate at the proximal tibial metaphysis. Several parameters for trabecular bone (Tb) structure, including bone volume fraction (BV/TV, bone volume/total tissue volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; and Tb.Sp., trabecular separation) are shown. Results are expressed as a ratio to WT controls treated with vehicle alone and are the mean ± SD for four different mice. C, Parameters for bone formation ratios, including mineral apposition rate (MAR) and mineralizing surface/bone surface (MS/BS), and for resorption, such as eroded surface/bone surface (ES/BS) and osteoclast number/bone perimeter (NOc/BPm) are shown. Values are expressed as the ratio to WT controls treated with vehicle alone, and represent mean ± SD for four different mice. *p < 0.05; **p < 0.001 between the two groups.
whereas bone resorption parameters such as eroded surface/bone surface and osteoclast number/bone perimeter ratios remained largely unchanged (Fig. 6C). Consequently, COX-2 inhibitor exerts negative effects on bone marrow density and trabecular bone quality mainly through reduced bone formation, indicating that blockade of PGE$_2$ exacerbates osteopenia in Tg mice.

**Effects of COX-2 inhibitor on mRNA expression by POBs**

Because osteoblasts appeared to play a key role in osteopenia of Tg mice, the effects of celecoxib on mRNA expression of bone metabolism-related agents in POBs were analyzed using RT-PCR. Accelerated RANKL mRNA expression promoted by transgene-derived IL-1$\alpha$ was markedly reduced in response to 0.1 mM celecoxib in Tg mouse POBs, and similar inhibitory effects were observed in control POBs (Fig. 7A). Conversely, constitutively decreased expression of alkaline phosphatase mRNA in Tg mice POBs was further reduced in response to celecoxib treatment. In contrast, neither M-CSF nor GM-CSF mRNA expression displayed any response to celecoxib treatment and, importantly, GM-CSF expression remained rather high even after celecoxib treatment. According to analysis of the dose-dependent effects of celecoxib on each mRNA expression, RANKL mRNA levels were clearly decreased with increasing doses of celecoxib in Tg mouse-derived POBs, whereas GM-CSF and M-CSF remained unchanged irrespective of celecoxib dose (Fig. 7B). Interestingly, when in vitro osteogenesis by Tg mouse-derived POBs was assessed using alkaline phosphatase, von Kossa, and Alizarin red staining, the bone-forming ability of POBs dramatically decreased in response to celecoxib in a dose-dependent manner (Fig. 7, C and D).

**Discussion**

As previously reported, IL-1$\alpha$-induced GM-CSF may play a pathogenic role in not only synovial tissue, but also bone marrow in IL-1$\alpha$ Tg mice (11). Indeed, GM-CSF production was dramatically up-regulated at both mRNA and protein levels in synovocytes and POBs derived from Tg mice. GM-CSF has been reported as a strong inhibitor of osteoclast differentiation acting at the early stage (13). The inhibitory effects of GM-CSF on osteoclasts were well reflected in our FACS results of Tg mouse-derived BMCS, showing that the proportion of c-Fms$^+$Mac-1$^{high}$ cells, which reportedly include abundant osteoclast precursor cells, were decreased, whereas c-Fms$^+$Mac-1$^{low}$ cells comprising mature macrophages, granulocytes, and cells committed to becoming dendritic cells were increased (27, 28). These differences in phenotypic characteristics of BMCS between Tg and control mice were also identified by the observation of the increased number of F4/80$^+$ cells and CD11c cells and the decreased number of RANK$^+$ cells, which represent macrophage, dendritic cell, and osteoclast precursors, respectively (or unpublished observation).

In vitro osteoclast formation was substantially inhibited in Tg mice, and both BMCs and POBs appear to play a key role in this inhibition (Figs. 1 and 2). Inhibited formation of TRAP-positive MNCs in bone marrow culture of Tg mice (Fig. 1) and in coculture of Tg mice BMCs and control POBs (Fig. 2) was attributable to an increased number of osteoclast precursors within the bone marrow of Tg mice as defined by FACS analysis. When POBs were Tg mouse-derived during coculture of BMCs and POBs, formation of TRAP-positive MNCs was markedly inhibited, potentially because POBs were potent producers of GM-CSF in Tg mice bone marrow, and such POB-derived GM-CSF may directly inhibit spontaneous differentiation of BMCs toward osteoclasts.

In contrast, osteogenic capacity should be considered to explain osteopenia in Tg mice. IL-1$\alpha$ is widely known as a catabolic agent in bone metabolism, although anabolic actions of PGE$_2$ induced by IL-1 are also widely accepted and may affect osteoblastic bone formation in Tg mice (29). Several studies have shown that PGE$_2$ stimulates new bone trabeculae and increased osteoblast numbers and activity (30, 31). In addition, systemic administration of PGE$_2$ dramatically increases bone formation and bone mass (32, 33). However, despite increased levels of PGE$_2$, histomorphometric analysis of Tg mice revealed that new bone formation was actually abrogated, and RT-PCR analysis of Tg mouse-derived POBs indicated a decreased level of alkaline phosphatase mRNA (Fig. 3), both suggesting that the catabolic effects of IL-1 exceed anabolic effects in our Tg mice.

Regarding the therapeutic effects of COX-2 inhibitors, oral administration of celecoxib in Tg mice resulted in further deterioration of osteopenia, contrary to the expectations from recent knowledge. Inhibition of COX-2 theoretically reduces levels of PGE$_2$ and subsequent RANKL expression, leading to reduced osteoclast formation and bone resorption. However, because GM-CSF is spontaneously produced by overproduced IL-1 in Tg mice, osteoclastic bone resorption is markedly and constitutively inhibited by GM-CSF, suggesting that the inhibitory effects of celecoxib on bone resorption through reduced production of RANKL by osteoblasts may become minimized and can be neglected in Tg mice. Consequently, during celecoxib treatment, inhibition of PGE$_2$-induced bone formation by osteoblasts may far outweigh inhibition of PGE$_2$-induced bone resorption, resulting in further exacerbation of osteopenia. Actually, in our study, in vitro osteogenesis by Tg mouse-derived POBs was markedly inhibited in response to celecoxib (Fig. 7, C and D). Interesting results have recently suggested

![FIGURE 7. Effects of celecoxib on mRNA expression of bone metabolism-related agents in POBs. Tg or WT control (Cont.) mouse-derived calvarial POBs were cultured with or without indicated concentrations of celecoxib for 3 days. A, RT-PCR analysis of mRNA expression levels in POBs. Ethidium bromide-stained RT-PCR products are shown. B, Dose-dependent effects of celecoxib on each mRNA expression. C, After 7-day culture, the effects of celecoxib on osteogenesis in 24-well plates were assessed using alkaline phosphatase (ALP), Alizarin red, and von Kossa stainings. D, Relative intensity of Alizarin red staining was expressed as a ratio to control POBs not cultured without celecoxib (Cxb0). Results are mean ± SD of triplicate wells from three different mice. *+ p < 0.001.](http://www.jimmunol.org/Downloadedfrom)
that genetic ablation of COX-2 or administration of COX-2 inhibitors under normal physiological conditions reduces bone formation more than bone resorption (34, 35). In contrast, other studies dealing with collagen-induced arthritis in COX-2 knockout mice and administration of COX-2 inhibitors in several animal arthritis models have concluded that blockade of COX-2 ameliorates not only joint inflammation, but also osteopenia (19, 36). Combined with these contrasting lines of evidences, COX-2 and subsequent PGE2 production may play an important role in certain pathological conditions with enhanced bone resorption, but may predominantly contribute to bone formation under normal physiological condition.

Recently, a close relationship between the immune and skeletal systems has attracted a great deal of attention due to the observation that inflammatory osteopenia is a common sequela associated with several medical conditions with enhanced bone resorption, but may predominantly contribute to bone formation under normal physiological condition.

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Disclosures

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