0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(6):2708–2715

doi: 10.1210/en.2007-0215

Overexpression of γ-Glutamyltransferase in Transgenic Mice Accelerates Bone Resorption and Causes Osteoporosis

Kiyoshi Hiramatsu, Yutaro Asaba, Sunao Takeshita, Yuji Nimura, Sawako Tatsumi, Nobuyoshi Katagiri, Shumpei Niida, Toshihiro Nakajima, Sakae Tanaka, Masako Ito, Gerard Karsenty, and Kyoji Ikeda

Department of Bone and Joint Disease (K.H., Y.A., S.Tak., S.Tat., N.K., S.N., K.I.) Research Institute, National Center for Geriatrics and Gerontology (NCGG), Obu, Aichi 474-8522, Japan; Department of Surgery (K.H., Y.A., Y.N.), Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan; National Institute of Biomedical Innovation (S.Tat.), Osaka 567-0085, Japan; Department of Genome Science (T.N.), Institute of Medical Science, St. Marianna University of Medicine, Kanagawa 216-8512, Japan; Department of Orthopedic Surgery (S.Tan.), University of Tokyo School of Medicine, Tokyo 113-8655, Japan; Department of Radiology (M.I.), Nagasaki University School of Medicine, Nagasaki 852-8501, Japan; and Department of Genetics and Development (G.K.), Columbia University, New York, New York 10032

We previously identified γ-glutamyltransferase (GGT) by expression cloning as a factor inducing osteoclast formation *in vitro*. To examine its pathogenic role *in vivo*, we generated transgenic mice that overexpressed GGT in a tissue-specific manner utilizing the Cre-loxP recombination system. Systemic as well as local production of GGT accelerated osteoclast development and bone resorption *in vivo* by increasing the sensitivity of bone marrow macrophages to receptor activator of nuclear factor-κB ligand, an essential cytokine for osteoclastogenesis. Mutated GGT devoid of enzyme activity was as potent as the wild-type molecule in inducing osteoclast formation, suggesting that GGT acts not as an enzyme but as a cytokine. Recombinant GGT protein increased receptor activator of nuclear factor-κB ligand expression in marrow stromal cells and also stimulated osteoclastogenesis from bone marrow macrophages at lower concentrations. Thus, GGT is implicated as being involved in diseases characterized by accelerated osteoclast development and bone destruction and provides a new target for therapeutic intervention. (*Endocrinology* 148: 2708–2715, 2007)

AGE-RELATED DISORDERS of the skeletal system are a major health problem worldwide (1, 2), and osteoclastic bone resorption plays a central role in the pathogenesis of bone and joint diseases, such as osteoporosis and rheumatoid arthritis (3). Osteoclasts are multinucleate, giant cells derived from hematopoietic progenitor cells of monocyte-macrophage lineage and are the only cell type that resorbs bone to maintain skeletal integrity and calcium homeostasis (4). The dysregulated generation of osteoclasts with resultant elevation of bone resorption that takes place after menopause and with aging or as an immune/inflammatory response leads to osteoporotic fractures and the joint destruction associated with rheumatoid arthritis (5). Genetic studies on human patients as well as mutant mice have disclosed critical molecules involved in osteoclast development, including cytokines, intracellular signaling molecules, and nuclear transcription factors (3, 4). Among these, the osteoclastogenic cytokines have received special attention, because they represent novel targets for the development of both diagnostic tools and antiresorptive drugs (6). For example, receptor activator of nuclear factor-κB ligand (RANKL), belonging to the TNF family (6), was identified as an essential cytokine for osteoclastogenesis, and mice deficient in RANKL were found to lack osteoclasts completely and to exhibit severe osteopetrosis (7).

In an attempt to identify new cytokines that stimulate osteoclast differentiation, we employed an expression cloning strategy, where cRNA from T lymphoma cells known to possess high bone resorption activity was injected into *Xenopus* oocytes (8). By assaying the conditioned medium for osteoclastogenic activity in murine marrow cultures, we identified γ-glutamyltransferase (GGT) as such a stimulator (9). GGT is an ectopeptidase that catalyzes the transfer of a γ-glutamyl moiety to an acceptor and plays a critical role in glutathione degradation and cysteine metabolism (10, 11). Mice deficient in GGT exhibit growth retardation, cataracts, and severe osteoporosis and die at 10–18 wk of age (12, 13). We have found that purified GGT from rat kidney is capable of inducing osteoclast formation in bone marrow cultures *in vitro* (9), which raises the possibility that its excess may also be involved in the bone and joint pathology characterized by enhanced osteoclastic bone resorption. The transgenic mice obtained by the Cre-loxP recombination system in the present study produce GGT systemically as well as locally, demonstrating that GGT indeed contributes to bone destruction, independently of its enzymatic activity, by acting as a local cytokine.

First Published Online March 15, 2007

Abbreviations: CT, Computed tomography; DPD, deoxypyridinoline; eGFP, enhanced green fluorescent protein; KO, knockout; M-CSF, macrophage colony-stimulating factor; pmtGGT, point-mutated GGT; RANKL, receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Materials and Methods

Reagents

Mouse macrophage colony-stimulating factor (M-CSF) and soluble RANKL were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal anti-GGT antibody was a generous gift from Dr. N. Taniguchi (Osaka University, Osaka, Japan).

Generation of transgenic mice and animal experiments

A transgenic cassette (floxed eGFP-GGT) carrying a CAG promoter and an enhanced green fluorescent protein (eGFP) flanked by loxP sites fused to mouse GGT cDNA with a polyadenylation signal was constructed. Mouse GGT cDNA was synthesized from RNA extracted from the kidney of C57BL/6 mouse, and Kozac sequences and *Mfe*I restriction sites were added by using a primer set (5′-CCGCAATTGCCACCATGAAGAATCGGTTTCTGGTGC-3′ and 5′-CCGCAATTGTCAGTAGCCAGCAGGTTCCCCGCC-3′). This GGT cDNA fragment was inserted into *Eco*RI-digested pCXP/loxGFP, which contained CAG promoter and eGFP (14). The transgenic construct was excised with *Sal*I and *Hin*dIII endonucleases and purified by using standard techniques.

Founder floxed eGFP-GGT mice were generated by microinjection of the DNA into fertilized eggs of C57BL/6 mice. Integration of the transgene was identified by green fluorescence and confirmed by Southern and PCR analyses of genomic DNA extracted from the tail, by using the following primers: 5′-GGTGTTCTGCCGCCAAGGGAAGG-3′ and 5′-GAGACACATCGACAAACTTTGGG-3′ for GGT and 5′-TGAACCGCATCGAGCTGAAGGG-3′ and 5′-TCCAGCAGGACCATGTGATCGC-3′ for eGFP. Floxed eGFP-GGT mice were crossed with CAG-Cre (15), RANKL knockout (KO) (7), and Col I-Cre (16) mice, to generate GGT-Tg, GGT-Tg/RANKL KO, and Col I-GGT mice, respectively.

Mice were raised under standard laboratory conditions at 24 ± 2 C and 50–60% humidity and allowed free access to tap water and commercial standard rodent chow (CE-2) containing 1.20% calcium, 1.08% phosphate, and 240 IU/100 g vitamin D_3 (Clea Japan Inc., Tokyo, Japan). Both male and female mice were analyzed at the age of 9 wk. Urine was collected during the final 24 h, and blood samples were centrifuged to obtain the serum.

All experiments were performed in accordance with NCGG's ethical guidelines for animal care, and the experimental protocols were approved by the animal care committee.

Bone and histological analyses

For bone analysis, right tibiae were dissected and stored in 70% ethanol for microcomputed tomography (micro-CT) scanning. Left tibiae were fixed in 4% paraformaldehyde, and bone histomorphometry was performed on un-decalcified sections, with tetracycline and calcein double labeling, as described previously (17). Histomorphometric parameters were measured at Niigata Bone Science Institute (Niigata, Japan).

Micro-CT scanning was performed on proximal tibiae by using a μCT-40 (SCANCO Medical AZ, Bassersdorf, Switzerland) with a resolution of 12 μm, and microstructure parameters were calculated three-dimensionally as described previously (18).

Biochemistry

Serum and urinary calcium, phosphate, creatinine, and GGT concentrations were determined by an autoanalyzer (Hitachi 7170, Tokyo, Japan). Urinary deoxypyridinoline (DPD) was measured with a Pyrilinks-D assay kit (Metra Biosystems Inc., Santa Clara, CA). Tissue GGT activity was determined using a γ-GTP C-TestWako kit (Wako Pure Chemical Industries Ltd., Osaka, Japan) with L-γ-glutamyl-*p*-*N*-ethyl-*N*-hydroxyethylaminoanilide as the substrate and glycylglycine as the acceptor, as described earlier (19).

Cell cultures and osteoclastogenesis assay in vitro

HEK293 cells were transfected with the transgenic cassette (floxed eGFP-GGT) described above, with or without Cre recombinase, to confirm that the DNA construct functioned as designed.

Bone marrow cells were isolated from the tibiae and femurs of 6- to 9-wk-old male C57BL/6J mice (SLC, Shizuoka, Japan), and osteoclastogenesis assays were performed as described previously (20). In brief, bone marrow cells were plated in culture dishes containing α-MEM, 10% heat-inactivated fetal bovine serum, antibiotics, and 1/10 volume of CMG14–12 culture supernatant (as a source of M-CSF). After 3 d culture, adherent cells were used as osteoclast precursor cells. Cells were then cultured with M-CSF (50 ng/ml) and soluble RANKL (50 ng/ml) for 4 d, fixed in 4% paraformaldehyde, and stained for tartrate-resistant acid phosphotase (TRAP). In some experiments, recombinant GGT was added at various concentrations to the culture. The number of multinucleate (at least three nuclei), TRAP-positive cells was counted as osteoclasts. Pit assay was performed on dentin slices as previously described (20), and analysis of pit area was performed on a Macintosh computer using the public domain NIH Image program (developed at the United States National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

Expression and purification of recombinant GGT

Recombinant GGT protein was generated using the baculovirus/insect cell system as described previously (21). In brief, human GGT cDNA in a pKBLgp64 vector (Oriental Yeast Co., Ltd, Tokyo, Japan) was cotranfected with DHBac10 (Invitrogen, San Diego, CA) using Cellfectin (Invitrogen) into Sf9 cells, which were then infected by the recombinant viruses. Cells were harvested, and the protein was extracted with 10 mM sodium phosphate buffer (pH 6.5)/1% Triton X-100 and further solubilized by protease treatment. The solubilized protein was purified through a hydroxylapatite column and isoelectric chromatography using PBE 94 (Pharmacia, Piscataway, NJ) and Polybuffer 74. The eluate was passed through a Sephacryl S-200 HR (Pharmacia) gel filtration column to remove the Polybuffer.

Site-directed mutagenesis of GGT and retroviral expression

Serine residues at 451 and 452 of mouse GGT were replaced by alanine by site-directed mutagenesis using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. In brief, oligonucleotide primers containing mutations (5′-GGTAAGCAGCCTCTTGCAGCCATGTGTCCCTCAATC-3′ and 5′-GATTGAGGGACACATGGCTGCAAGAGGCTGCTTACC-3′) were annealed to template DNA, and the mutant strand was synthesized and amplified by using *PfuTurbo* DNA polymerase (at 95 C for 30 sec, 55 C for 1 min, and 68 C for 5 min for 18 cycles). After the reaction products were treated with *Dpn*I endonuclease, the mutated DNA was transformed into XL 10-Gold ultracompetent cells. Plasmid DNA was prepared from the transformants, and the mutated sequences were confirmed.

Retroviral vectors encoding wild-type and mutated GGT were used to transfect the retrovirus packaging cells, Plat-E (a gift from Dr. T. Kitamura, University of Tokyo) or GP2-293 (Clontech Laboratories, Inc., Palo Alto, CA). Osteoclast precursor cells were exposed to the retrovirus in the presence of polybrene (4 μg/ml) and M-CSF for 1 d and were subsequently treated with RANKL and M-CSF for 4 d in the presence of puromycin (1.6 μg/ml). Osteoclast differentiation was evaluated as described above. The retrovirus was also infected to primary osteoblasts isolated from newborn mouse calvaria, and alkaline phosphatase activity was assessed.

Northern and RT-PCR analyses

Total RNA was isolated with TRIzol Reagent (Invitrogen), and equal amounts (10 μg/lane) were fractionated on a 1.5% agarose gel. The specific mRNAs were detected by hybridization of Hybond N+ nylon membranes (Amersham Biosciences, Inc., Piscataway, NJ) with ^{32}P-labeled cDNA probes. For quantitative RT-PCR, total RNA (1 μg) was reverse transcribed by using Superscript III (Invitrogen); and samples were analyzed using a Light Cycler system (Roche Diagnostics, Basel, Switzerland). The primers included 5′-CCCGTGCTGATTCTCATCTT-3′ and 5′-GCATAGGCAAACCGAAAGG-3′ for GGT, 5′-TGGAAGGCTCATGGTTGGAT-3′ and 5′-CATTGATGGTGAGGTGTGCAA-3′ for RANKL, 5′-GCTGGCTACCACTGGAACTC-3′ and 5′-TGTGCACACCGTATCCTTGT-3′ for RANK, 5′-ACCTGTTCGTGAAACACACCA-3′ and 5′-CTCAGACTGTCCTTCAAGGC-3′ for c-*fos*, 5′-

GCAAAGATGGAAACGACCTTCTAC-3′,5′-GGCCAGGTTCAAGGTCA TGCTCT-3′ for c-*jun*, 5′-TGCAAGCGCTCACCGTCTAC-3′, 5′-ACCGACA-GATACTGCTCGCAAA-3′ for NFATc1, and 5′-TGCTGCCATTGTT-GATATGG-3′ and 5′-TCCACAGCTTTGATGACACC-3′ for EF-1α. The amount of target mRNA was corrected by that of EF-1α mRNA.

Western blot analysis

Cells were lysed in the buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VP_4, 1 mM NaF, and 1× protease inhibitor mixture. Cell lysates were boiled in the presence of SDS sample buffer [0.5 M Tris-HCl (pH 6.8), 10% glycerol, and 0.05% (wt/vol) bromophenol blue) for 5 min and subjected to electrophoresis on 10% SDS-PAGE. Proteins were transferred to Hybond-C extra nitrocellulose membranes from Amersham using a semidry blotter and incubated in blocking solution (5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20) for 1 h to reduce nonspecific binding. Membranes were then exposed to anti-GGT antibody overnight at 4 C, washed three times, and incubated with goat antirabbit IgG horseradish peroxidase-conjugated antibody for 1 h. Membranes were washed extensively, and enhanced chemiluminescence detection assay was performed according to the manufacturer's instructions.

Statistical analysis

Data are expressed as the mean ± SD. Statistical analysis was performed by Student's *t* test. Values were considered statistically significant at $P < 0.05$.

Results

Generation of GGT transgenic mouse

To test whether GGT could induce osteoclast formation and stimulate bone resorption *in vivo*, we used the Cre-loxP recombination system to generate transgenic mice that produced GGT systemically or locally. As schematically illustrated in Fig. 1A, we constructed a transgenic cassette that carries floxed eGFP fused to mouse GGT cDNA with a polyadenylation signal, under the control of the chicken β-actin (CAG) promoter driving ubiquitous gene expression. When HEK293 cells were transfected with this DNA construct, green fluorescence was observed (Fig. 1B) with little GGT activity in conditioned medium or cellular extracts (Fig. 1C). Upon the introduction of Cre recombinase, GGT activity as well as its mRNA was markedly induced (Fig. 1C), whereas the green fluorescence gradually weakened (Fig. 1B), indicating that the DNA cassette functioned as designed.

Nine transgenic founders were produced using the standard pronuclear injection protocol. Floxed eGFP-GGT mice were identified by their green color (Fig. 1D), and their genotype was confirmed by the presence of the GGT transgene in genomic DNA extracted from their tails (Fig. 1E) and by Southern hybridization (data not shown). Floxed eGFP-GGT male mice were then mated to female CAG-Cre transgenic mice to generate transgenic lines that produced GGT systemically. Of the four independent transgenic founders (GGT-Tg lines) obtained, two (A and B) were found to have lost the Cre gene and therefore were propagated for further investigation by mating to C57BL/6 wild-type females, because their progeny allowed avoidance of deleterious effects due to the presence of the Cre gene. Pups of both lines were born at the expected ratio, appeared normal, grew indistinguishably from wild-type mice, and were fertile. The two lines (A and B) displayed a very similar phenotype, and the results presented are from GGT-Tg line A.

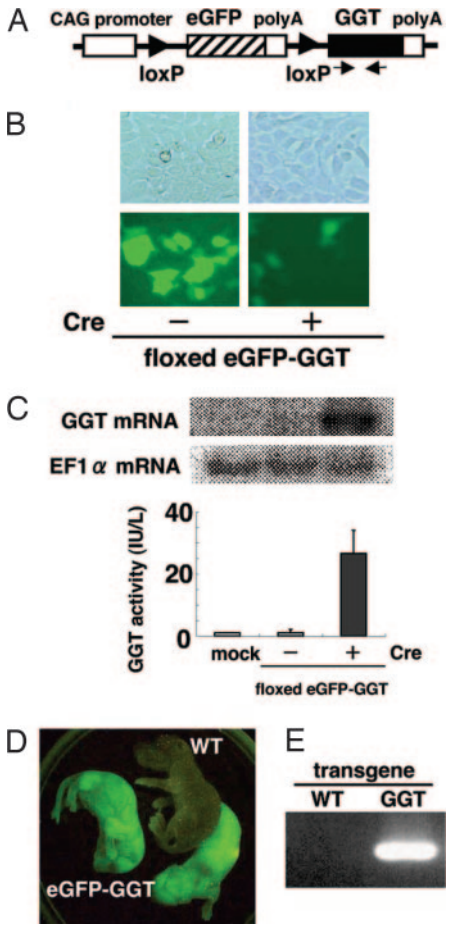

FIG. 1. Generation of floxed eGFP-GGT transgenic mice. A, Schematic representation of the transgenic construct (floxed eGFP-GGT). eGFP flanked by loxP sites fused to mouse GGT cDNA was placed under the control of a CAG promoter. *Arrows* indicate a set of primers used for confirming integration of the transgene. B and C, GFP expression in HEK293 cells transfected with the floxed eGFP-GGT construct without and with Cre recombinase transfection. *Green* fluorescence gradually decreased after introduction of the Cre gene (B), whereas the content of GGT mRNA and the activity of the enzyme in the culture supernatant were markedly induced after expression of Cre recombinase (C). D and E, Floxed GFP-GGT mice were identified by *green* color (D), and transgene expression was confirmed by PCR with the primer set shown in A (E). WT, Wild type.

Northern blot analysis of RNAs extracted from various tissues revealed that GGT mRNA was expressed predominantly in the kidney of the wild-type mouse, whereas it was found in a variety of tissues, including bone and bone marrow, in the GGT-Tg mouse (Fig. 2A). Production of actual GGT protein was also confirmed by measuring its enzymatic activity. In agreement with the mRNA tissue distribution, GGT enzymatic activity was highest in the kidney of wild-type mice, whereas the GGT-Tg mouse exhibited high enzyme activity in various tissues in addition to the kidney (Fig. 2B).

Systemic overexpression of GGT accelerates bone resorption and induces osteopenia with microstructural deterioration

GGT-Tg mice grew normally, and their body weight was indistinguishable from wild-type littermates (Fig. 2C). The data presented here are based on the analysis of 9-wk-old

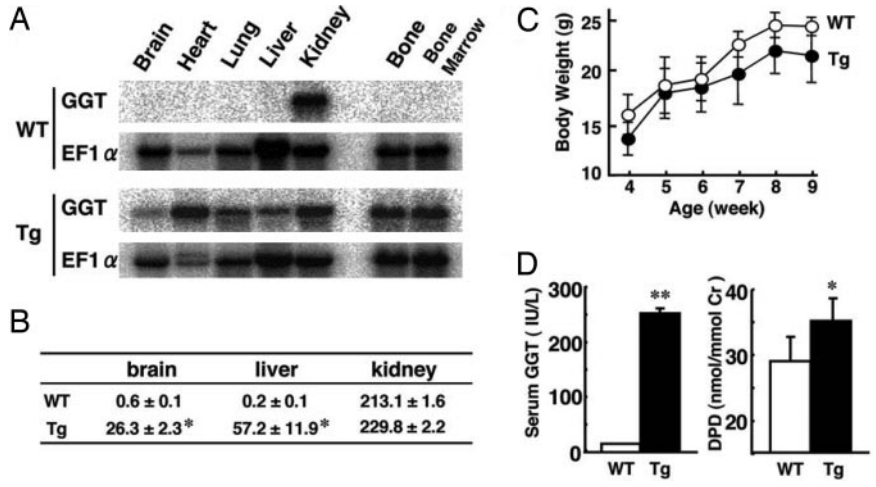

FIG. 2. Identification of GGT-Tg mice with increased GGT production. A, Tissue distribution of GGT mRNA by Northern blot analysis in various tissues from GGT-Tg mice and wild-type (WT) littermates. EF-1α mRNA was used as control for loading. B, GGT enzymatic activities (in IU/wet tissue weight) were determined in various tissues from GGT transgenic (Tg) mice and wild-type (WT) littermates. *, $P < 0.05$ (n = 3 in each group). C, Body weight of wild-type (WT) and GGT transgenic (Tg) mice. No difference was observed; n = 8 for each group. D, Serum GGT activity and urinary DPD excretion. Urinary DPD was corrected for creatinine (Cr). *, $P < 0.05$; **, $P < 0.01$ *vs.* WT (n = 8 for each group).

male mice, but a similar phenotype was observed in female mice. GGT-Tg mice exhibited a high serum concentration of GGT and significantly higher urinary excretion of DPD, a biochemical marker of bone resorption, compared with wild-type mice (Fig. 2D).

The effects of GGT overexpression on trabecular bone structure in GGT-Tg mice were analyzed by micro-CT scanning of the tibial metaphysis. As shown in Fig. 3, micro-CT images of the trabecular compartment of the proximal tibia reveal marked osteopenia with a substantial reduction in the three-dimensional-bone volume fraction (BV/TV). Microstructural analysis revealed the bone of GGT-Tg mice to be characterized by significant decreases in connectivity and trabecular thickness and number and by increases in trabecular separation and structure model index (Fig. 3B), indicating the trabeculae of GGT-Tg mice had become thinned, less connected, and converted from its normal plate-like structure to a more fragile, rod-like structure.

The effects of GGT excess on bone remodeling were further analyzed by histomorphometry at the proximal tibia. As shown in Fig. 4, as histological indices of bone resorption, the number of osteoclasts (N.Oc/BS), the bone surface covered by osteoclasts (Oc.S/BS), and the eroded surface (ES/BS), were all significantly elevated in GGT-Tg mice compared with the wild-

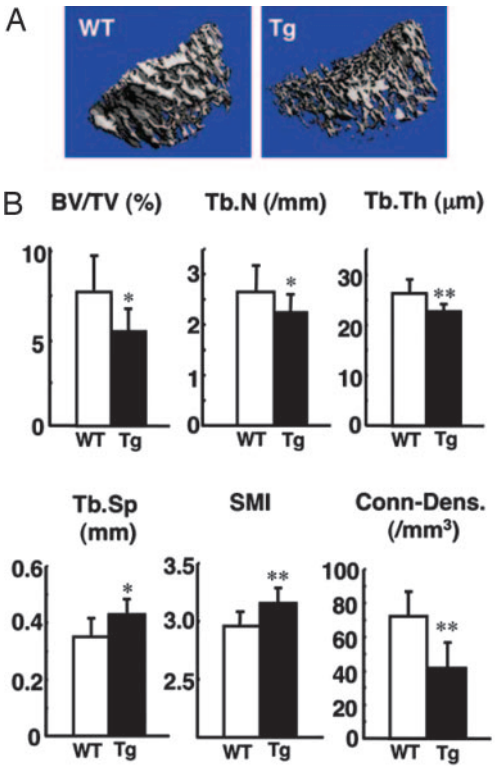

FIG. 3. GGT-Tg mice exhibit osteopenia with microstructural deterioration. A, Representative three-dimensional images obtained by micro-CT of trabecular bone at the tibiae of wild-type (WT) and of GGT transgenic (Tg) mice. B, Microstructural parameters are shown. BV/TV, Three-dimensional-bone volume fraction per tissue volume; Conn-Dens, connectivity density; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; SMI, structure model index. *, $P < 0.05$; **, $P < 0.01$ *vs.* WT (n = 7–9 for each group).

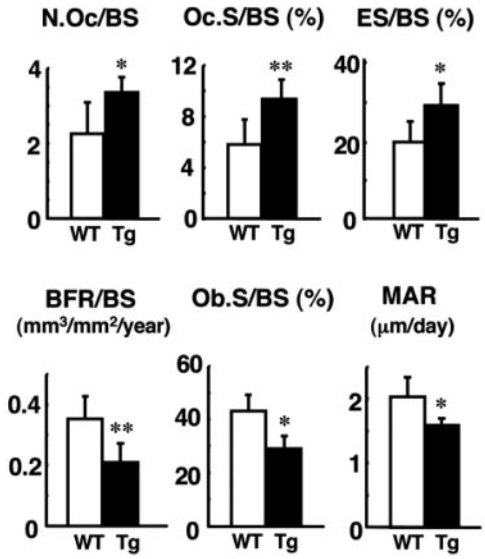

FIG. 4. GGT-Tg mice exhibit elevated bone resorption with reduced bone formation. Results of histomorphometry at the tibial metaphysis are shown. Note that GGT-Tg mice exhibit increases in indices of bone resorption (N.Oc, number of osteoclasts; Oc.S, osteoclast surface; ES, eroded surface) and decreases in parameters of bone formation (BFR, bone formation rate; Ob.S, osteoblast surface; MAR, mineral apposition rate). Data are normalized for bone surface (BS). *, $P < 0.05$; **, $P < 0.01$ *vs.* WT (n = 5 for each group).

type mice. Unexpectedly, the GGT-Tg mice revealed a lowered bone formation rate (BFR) with reduced osteoblast surface (Ob.S) and mineral apposition rate (MAR) (Fig. 4). Taken together with the elevated DPD excretion in GGT-Tg mice, these data suggest systemic overproduction of GGT accelerates bone resorption while suppressing bone formation, thus resulting in osteopenia.

Mechanism of accelerated bone resorption in GGT-Tg mouse

To gain further insight into the mechanism by which GGT stimulates osteoclast formation and bone resorption, we crossed GGT-Tg with mice lacking RANKL, an essential cytokine for osteoclastogenesis. Transgenic expression of GGT caused osteopenia on a RANKL$^{+/-}$ background (Fig. 5). Homozygous KO (RANKL$^{-/-}$) mice exhibited typical osteopetrosis with failure of tooth eruption and a complete absence of TRAP-positive osteoclasts, and GGT overexpression did not rescue these animals from the abnormal phenotypes in the absence of RANKL (Fig. 5). These results provide genetic evidence that RANKL is required for GGT to induce bone resorption.

Based on these findings, we investigated GGT modulation of the cellular response to RANKL in the presence of M-CSF. As shown in Fig. 6A, bone marrow macrophages derived from GGT-Tg mice gave rise to more and larger TRAP-positive multinucleate cells in response to RANKL, and quantitation of TRAP-positive cells with more than three nuclei indicated that the bone marrow macrophages from GGT-Tg mice generated significantly more osteoclasts than those from wild-type mice in response to RANKL. The TRAP-positive cells thus formed were functional, and those derived from GGT-Tg mice produced significantly more pits on dentin slices (Fig. 6B). These results suggest that GGT stimulates bone resorption by modulating the signaling of RANKL in osteoclast precursor cells of monocyte-macrophage lineage.

To further substantiate this concept, we extracted RNA from bone marrow cells and bone cells (cells attached to bone after flushing out the bone marrow) from both GGT-Tg and wild-type mice, and gene expression was examined by quantitative

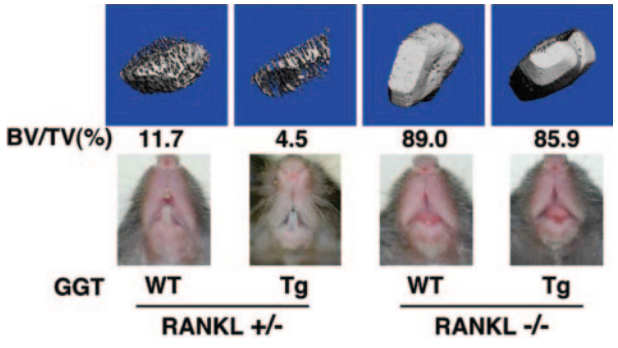

FIG. 5. RANKL is required for GGT to induce osteoclast formation. GGT-Tg mice were crossed with RANKL$^{+/-}$ mice to obtain the indicated genotypes. Representative micro-CT images of trabecular compartments of proximal tibiae are shown with the bone volume fraction (BV/TV) and the presence or absence of tooth eruption in the respective genotype. GGT-Tg induces osteopenia as a result of stimulated bone resorption on a RANKL$^{+/-}$ background, whereas GGT overexpression does not rescue RANKL$^{-/-}$ mice from their osteoclast-deficiency phenotypes, *i.e.* osteopetrosis and failure of tooth eruption.

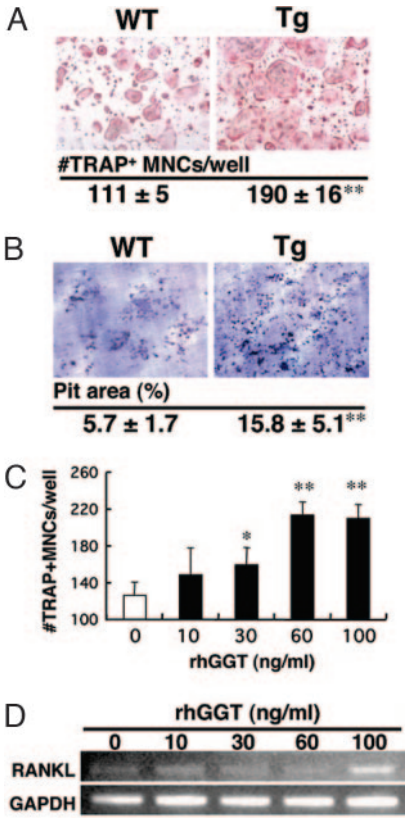

FIG. 6. GGT increases the sensitivity of bone marrow macrophages to RANKL. A, Bone marrow macrophages derived from GGT-Tg mice generated more and larger TRAP-positive osteoclasts than those from wild-type (WT) bone marrow in response to RANKL. *Ex vivo* culture was performed in the presence of M-CSF and RANKL, and the number of TRAP-positive cells with more than three nuclei was counted. B, Preosteoclasts were prepared from the bone marrow macrophages from GGT-Tg and WT mice in the presence of M-CSF and RANKL for 2 d, and then cells were incubated on dentin slices for 2 d. Resorption pits were stained with Coomassie brilliant blue. **, $P < 0.01$ *vs.* WT (n = 3 for each group). C, Recombinant human GGT (rhGGT) stimulated osteoclast formation in a dose-dependent manner. Bone marrow macrophages were cultured with M-CSF (50 ng/ml) and RANKL (25 ng/ml) in the absence or presence of the indicated concentrations of recombinant GGT protein. *, $P < 0.05$; **, $P < 0.01$ *vs.* control cultures without GGT (n = 3 for each group). D, Recombinant human GGT (rhGGT) stimulated RANKL expression. Marrow stromal cells were treated with increasing concentrations of recombinant GGT protein, and RANKL mRNA expression was analyzed by RT-PCR. GAPDH mRNA was used as an internal control.

RT-PCR. As shown in Fig. 7, expression levels of RANKL mRNA in the bone fraction and RANK mRNA in bone marrow cells did not differ significantly between the GGT-Tg and wild-type mice. In contrast, bone marrow cells from GGT-Tg mice exhibited significantly higher expression of c-*fos*, c-*jun*, and NFATc1 mRNA, which are transcription factors essential for osteoclastogenesis (22–24) (Fig. 7). The finding that critical positive regulators known to transmit the osteoclastogenic signal from the RANK receptor are up-regulated in bone marrow cells of GGT-Tg mice, although the expression of RANKL and RANK are unaltered, supports the concept that GGT acts on hematopoietic cells of monocyte-macrophage lineage to increase their sensitivity to RANKL, thereby stimulating osteoclast generation and bone resorption. Also consistent with this

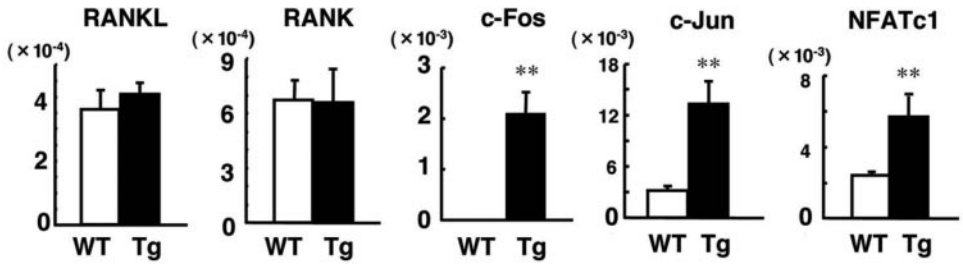

FIG. 7. Increased expression of c-Fos, c-Jun, and NFATc1 mRNA in GGT-Tg marrow cells. Expression of critical regulators of osteoclast differentiation (RANKL in bone fraction and other molecules in bone marrow fraction) in GGT-Tg *vs.* wild-type (WT) mice was examined. RNA was extracted from bone and bone marrow separately from three wild-type and three Tg mice, and mRNA levels were determined by quantitative RT-PCR. **, $P < 0.01$ *vs.* wild type.

notion are the results that recombinant GGT protein was capable of stimulating osteoclast formation from bone marrow macrophages in a dose-dependent manner, and a significant increase in osteoclastogenesis was observed at 30 ng/ml (Fig. 6C). As reported previously (9), recombinant GGT also increased the expression of RANKL mRNA in marrow stromal cells but only at the highest concentration of 100 ng/ml (Fig. 6D).

GGT-Tg mice produce GGT not only in skeletal tissue but also in various other tissues and exhibit elevated levels of circulating GGT (Fig. 2, A and D). To rule out the possibility that bone resorption was stimulated as a secondary effect of systemic overexpression of GGT and to obtain evidence that GGT acts locally in bone, we next generated transgenic lines that produce GGT only in bone tissue. Taking advantage of the original design of our floxed eGFP-GGT construction (Fig. 1A), which enables tissue-specific expression of GGT, we crossed floxed eGFP-GGT mice with Col I-Cre mice, which express Cre recombinase under the control of the 2.3-kb type I collagen promoter and have been shown to target transgenic expression specifically in osteoblasts (16), to generate Col I-GGT mice. The serum GGT concentration of Col I-GGT mice was not increased (<2 IU/liter), compared with the floxed eGFP-GGT mice used as a control, indicating that Col I-GGT mice are not characterized by systemic overproduction of GGT. TRAP staining of tibial sections revealed that significantly more TRAP-positive multinucleate osteoclasts were induced in the skeletal tissue of Col I-GGT mice than floxed eGFP-GGT mice (Fig. 8A). These results suggest that systemic elevation of GGT is not a prerequisite for its osteoclastogenic action and are consistent with the concept that GGT acts locally on bone in stimulating osteoclast formation.

To determine whether the enzymatic activity is required for the bone-resorbing activity, we introduced mutations in two consecutive serine residues at 451 and 452, which are known to be critical for the catalytic activity, by substituting them with alanine (S451A/S452A double mutant). When the point-mutated GGT (pmtGGT) was expressed in HEK293 cells at the same level as the wild-type molecule, the enzyme activity was barely detectable in the conditioned medium or in whole cell extract (Fig. 8B). We then introduced the wild-type or S451A/S452A GGT into bone marrow macrophages through retroviral infection and assessed their osteoclastogenic potential. The results indicate that mutant GGT with essentially no enzyme activity induced osteoclast formation as potently as the wild-type GGT (Fig. 8C). Thus, these results can be taken as evidence that GGT can induce osteoclast development independently of its enzyme activity and raise the possibility that GGT acts as a cytokine on hematopoietic cells in the bone marrow. When the wild-type or pmtGGT was introduced into primary osteoblasts by retroviral infection, both molecules reduced alkaline phosphatase activity (Fig. 8D), suggesting that GGT can act directly on osteoblasts to suppress their differentiation.

Discussion

GGT is recognized as a marker of alcohol consumption and liver disease, and increased serum GGT enzyme activity is a biochemical hallmark of patients with alcoholism, fatty liver disease, and biliary tract disorders such as primary biliary cirrhosis (25). Although it is known that these clinical conditions are risk factors for or complicated by skeletal abnormalities (26–28), the underlying molecular mechanisms are not fully understood. It is demonstrated in the present study that an excess of GGT causes osteopenia and microstructural deterioration characteristic of osteoporosis through an acceleration of osteoclast generation and bone resorption. The serum GGT concentrations in the transgenic model in this study are in the range of 200 IU/liter, a level often seen in individuals with moderately excessive alcohol consumption and in patients with liver and biliary tract diseases. Together with our *in vitro* data showing that purified GGT protein at 100 IU/liter is capable of inducing osteoclast formation in bone marrow cultures (9), it is likely that even moderately elevated circulating levels of GGT would contribute to the bone loss associated with these conditions.

Whereas serum GGT activity derives mainly from the liver, GGT is abundantly expressed in the kidney, pancreas, seminal vesicle, and small intestine, where this ectoenzyme is located on the apical membrane (11). GGT is anchored to the plasma membrane with most of the molecule exposed to extracellular space (11) and is thought to be released by shedding into the serum as well as urine (29), although the exact molecular form in the extracellular fluid as well as the mechanism of shedding remains to be determined. GGT catalyzes the transfer of a γ-glutamyl moiety to an acceptor and plays a critical role in glutathione degradation and cysteine metabolism (10, 11). Mice deficient in GGT exhibit accelerated aging, such as gray fur, cataracts, sexual dysfunction, and severe osteoporosis, and die at the early age of 10–18 wk due to the lack of GGT enzyme activity and the resultant cysteine deficiency (12, 13). Osteopenia in GGT-deficient mice is associated with decreased bone formation, which is treatable by supplementation with *N*-acetylcysteine, suggesting that GGT also plays an important physiological role in regulating bone formation through cysteine metabolism (13). Taken together with the current results, both a deficiency and an excess of GGT result in osteoporosis. However, the osteoporosis is exerted through distinct mechanisms;

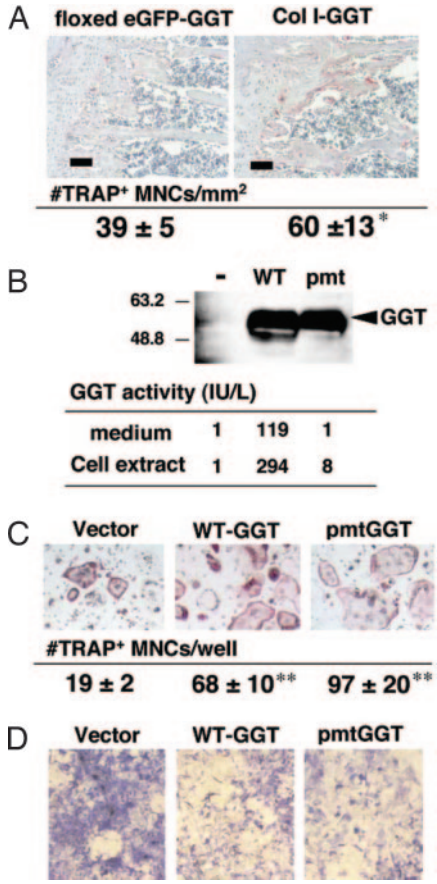

FIG. 8. GGT acts on osteoclast precursors and stimulates osteoclastogenesis independently of its enzyme activity. A, Floxed eGFP-GGT mice were crossed with Col I-Cre mice to generate Col I-GGT mice that expressed GGT specifically in their osteoblasts. The results of staining for TRAP in tibial sections from Col I-GGT mice and floxed eGFP-GGT mice as a control are shown. The number of TRAP-positive multinucleate cells (MNCs) was counted. *, $P < 0.05$ *vs.* wild-type (n = 3 for each group). B and C, pmtGGT with defective enzymatic activity is capable of inducing osteoclast differentiation. S451A/S452A double-mutated GGT (pmtGGT) was introduced into HEK293 cells at the same level as the wild-type (WT) protein, and GGT activity in the conditioned media and cell lysates was determined (B). Bone marrow macrophages were infected with retroviral vectors encoding WT-GGT or pmtGGT and cultured in the presence of M-CSF and RANKL, after which the cells were stained for TRAP activity (C). Note that pmtGGT as well as WT-GGT generated more and larger TRAP-positive osteoclasts. **, $P < 0.01$ *vs.* vector-infected cultures (n = 4 for each group). D, Primary osteoblasts isolated from the calvaria were infected with retroviral vectors encoding WT-GGT or pmtGGT and cultured under osteogenic conditions, after which the cells were stained for alkaline phosphatase activity.

i.e. the former is caused by suppression of bone formation due to systemic deficiency in enzymatic activity, whereas the latter is mainly due to an acceleration of bone resorption by an excess of GGT, which is independent of the enzyme activity and reflects a local effect. The latter nonenzymatic activity of GGT may have been masked by the gene knockout, and appreciating its physiological importance would require the identification of its mediator, possibly the putative receptor.

The results of experiments exploiting targeted overexpression of GGT in osteoblasts driven by a type I collagen promoter suggest not only systemic elevation of GGT but also local production of GGT is sufficient to stimulate osteoclast development. The findings that retroviral transduction of GGT as well as the addition of recombinant GGT protein in bone marrow macrophages stimulates osteoclastogenesis and that bone marrow macrophages from GGT-Tg mice stimulate increased osteoclasts in *ex vivo* cultures further support the concept that hematopoietic cells, especially those of monocyte/macrophage lineage, are the target of GGT in bone. Two cytokines, M-CSF and RANKL, are essential for these progenitor cells to undergo terminal differentiation to become mature, bone-resorbing osteoclasts. Genetic evidence has been obtained by crossing GGT-Tg with RANKL-deficient mice, which shows RANKL is required for GGT action, because GGT is not able to induce osteoclastogenesis in the absence of RANKL. Furthermore, our data show osteoclast progenitor cells from GGT-Tg mice exhibit an enhanced responsiveness to RANKL, resulting in increased generation of osteoclasts at the cellular level and increased expression of critical regulators of osteoclastogenesis, *i.e.* c-Fos, c-Jun, and nuclear factor of activated T cells (NFATc1), at the molecular level. The finding that neither RANKL expression in stromal cells nor RANK expression in bone marrow cells is altered in the presence of an excess of GGT further supports the concept that GGT modulates the signal transduction pathway after RANKL has bound RANK on the surface of osteoclast progenitors. We previously reported the addition of purified GGT protein to marrow stromal cells increased RANKL mRNA and protein at 3 d post addition, which prompted us to hypothesize that GGT stimulates osteoclast formation indirectly through increased RANKL expression (9). The stimulatory effect of GGT on RANKL expression in marrow stromal cells has been confirmed in the current study using recombinant GGT protein but needed a greater concentration (100 ng/ml) than the minimal concentration that exerted the direct effect on bone marrow macrophages (30 ng/ml). Thus, although the exact reason for the difference between these findings and the unaltered RANKL expression in the stromal cells of GGT-Tg mice is not clear, it is likely that hematopoietic cells are more sensitive to the GGT effect than are stromal cells. Also, differences in the molecular form of GGT and/or its duration of action (*i.e.* days in the *in vitro* cultures *vs.* the chronic overexpression for a period of months in the transgenic mice) may be involved. GGT is similar to TGF-β in that both cytokines enhance osteoclast differentiation from hematopoietic cells in response to RANKL (30). However, GGT is dissimilar to TGF-β in that GGT stimulates RANKL expression, whereas TGF-β decreases RANKL and increases OPG expression in stromal cells (31), resulting in complex effects on osteoclastic bone resorption.

Finally and most importantly, the osteoclastogenic function of GGT does not require its enzymatic activity, because the introduction of point mutations in the amino acids critical for enzyme activity had no affect on the osteoclast-inducing activity. These results suggest that a functional domain of GGT required for stimulating osteoclast differentiation is dissociable from its enzymatic activity, and raise the intriguing potential of GGT acting as a local osteoclastogenic cytokine through interaction with a receptor molecule. Alternatively, GGT may modulate the availability of RANKL to RANK through protein-protein interaction. Additional studies are required to identify the putative GGT receptor on osteoclast progenitor cells and to determine whether GGT converges on signaling pathways downstream of the RANK receptor in a cell-autonomous fash-

ion or whether GGT action is mediated indirectly through yet another extracellular signaling molecule.

In conclusion, this study has uncovered a novel function for GGT in the regulation of osteoclast development. This does not require the enzyme activity and may represent a novel mode of action as a cytokine. Given the involvement of GGT in bone and joint diseases characterized by accelerated osteoclast development, GGT may be a novel target for the development of diagnostics and therapeutics.

Acknowledgments

We thank Drs. Yoshitaka Ikeda (Saga University), Naoyuki Taniguchi (Osaka University), and Yasuyuki Ishizuka (Applied Cell Biotechnologies, Inc.) for valuable suggestions and anti-GGT antibody; Dr. Tatsuo Suda (Research Center for Genomic Medicine, Saitama Medical School) for critical reading of the manuscript; Dr. Jun-ichi Miyazaki (Osaka University) for providing the CAG-Cre mice; Dr. Josef Penninger for the RANKL KO mice; Dr. Noboru Motoyama (NCGG) for advice on transgenic construction; Ms. Kumi Tsutsumi and Mrs. Mie Suzuki for technical assistance; members of our Department (NCGG) for stimulating discussion; and Drs. Akihiro Yasui and Shinji Fukada (National Hospital for Geriatric Medicine, NCGG) and Dr. Toshiyuki Arai (Department of Surgery, Nagoya University School of Medicine) for encouragement and support throughout the study. Pacific Edit reviewed the manuscript before submission.

Received February 14, 2007. Accepted March 5, 2007.

Address all correspondence and requests for reprints to: Kyoji Ikeda, M.D., at Department of Bone and Joint Disease, Research Institute, National Center for Geriatrics and Gerontology (NCGG), 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan. E-mail: kikeda@nils.go.jp.

This work was supported by a grant-in-aid for Longevity Science from the Ministry of Health and Welfare of Japan (to K.I.) and a grant for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan (MF-14 and 06-31 to K.I.).

The authors have nothing to disclose.

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