Glutamate Suppresses Osteoclastogenesis through the Cystine/Glutamate Antiporter

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Previous studies have demonstrated functional expression of different glutamate receptor subtypes (GluRs) in both osteoblasts and osteoclasts. In the present study, we investigated the possible functional expression by osteoclasts of different glutamatergic signaling machineries including GluRs. In disagreement with the aforementioned prevailing view, no mRNA expression was found for all GluRs examined in primary cultured mouse osteoclasts differentiated from bone marrow precursors. Constitutive expression of mRNA was seen with glutamate transporters, such as excitatory amino acid transporters and cystine/glutamate antiporter, in primary osteoclasts. Glutamate significantly inhibited osteoclastogenesis at a concentration over 500 $\mu\text{mol/L}$ in both primary osteoclasts and preosteoclastic RAW264.7 cells without affecting the cell viability in a manner sensitive to the antiporter inhibitor. In RAW264.7 cells stably overexpressing the cystine/glutamate antiporter, the inhibition by glutamate was more conspicuous than in cells transfected with empty vector alone. The systemic administration of glutamate significantly prevented the decreased bone mineral density in both femur and tibia in addition to increased osteoclastic indices in ovariectomized mice *in vivo*. These results suggest that glutamate may play a pivotal role in mechanisms associated with osteoclastogenesis through the cystine/glutamate antiporter functionally expressed by osteoclasts devoid of any GluRs cloned to date. (*Am J Pathol* 2007, 170:1277–1290; DOI: 10.2353/ajpath.2007.061039)

L-Glutamate (Glu) is currently believed to play a role as an excitatory amino acid neurotransmitter in the mammalian central nervous system. In the central nervous system, Glu is supposed to mediate excitatory neurotransmission through particular Glu receptors (GluRs)

categorized into two major groups.^{1,2} One is ionotropic Glu-gated ion channels (iGluRs) that are further classified into $\text{DL-}\alpha\text{-amino-3-hydroxy-5-methylisoxazole-4-propionate}$ (AMPA), kainate (KA), and *N*-methyl-D-aspartate (NMDA) subtypes,^{3,4} whereas the other is G-protein-coupled metabotropic receptors (mGluRs) classified into three functional groups, group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8) subtypes.^{5,6} Excitatory amino acid transporters (EAATs) are required for the termination of signal transduction mediated by Glu as well as for the prevention of neurotoxicity mediated by this endogenous excitotoxin in the central nervous system. These transporters are classified into five different subtypes, including Glu aspartate transporter (GLAST) (EAAT1), Glu transporter-1 (GLT-1) (EAAT2), excitatory amino acid carrier (EAAC1) (EAAT3), EAAT4, and EAAT5 to date.^{7,8} In addition to the Glu transport systems mentioned above, a sodium-independent, chloride-dependent high-affinity Glu uptake system termed the cystine/Glu antiporter has been identified in many tissues.^{9,10} This antiporter is a heterodimeric complex between the CD98 heavy chain, also referred to as 4F2hc, ubiquitously present in various tissues and the xCT light chain responsible for determination of the substrate specificity. In addition, the third Glu transport system, which is sodium-dependent with cystine, Glu, and aspartate as substrates, has also been found in rat alveolar type 2 cells and in astrocytes.^{11,12} Moreover, vesicular Glu transporters (VGLUTs) are essential for signal output through the condensation of Glu into vesicular constituents for subsequent exocytotic release. Within the central nervous system, both

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VGLUT1¹³ and VGLUT2¹⁴ isoforms are supposed to suffice for the definition of an excitatory neuronal phenotype, whereas VGLUT3 is expressed in a number of cells shown to release Glu through exocytosis including dopaminergic, GABAergic, and serotonergic neurons as well as astrocytes.¹⁵

On the other hand, two distinct cell types are known to regulate in a sophisticated fashion bone formation and maintenance in bone tissues.^{16,17} These are bone-forming osteoblasts and bone-resorbing osteoclasts. The osteoblast lineage is derived from primitive multipotent mesenchymal stem cells with potential to differentiate into bone marrow stromal cells, chondrocytes, muscles, and adipocytes,¹⁸ whereas osteoclasts are multinucleated cells (MNCs) derived from hematopoietic stem cells shared with macrophage and dendritic cell lineages.^{19,20} Osteoclastogenesis is a multistep process dependent on the intimate cellular interaction of myeloid preosteoclastic precursors with either osteoblasts or stromal cells under the influence by a wide range of local autocrine and/or paracrine factors such as macrophage colony-stimulating factor (M-CSF)^{21,22} and receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL).^{23,24} In addition to these factors, recent studies have raised the possibility that Glu may be one of the endogenous factors used for intercellular communications in bone through activation of NMDA receptors expressed by bone-resorbing osteoclasts^{25–27} as seen in bone-forming osteoblasts.^{28–31} For example, the addition of an NMDA receptor antagonist inhibits cell differentiation and bone-resorbing activities in cultured osteoclasts expressing both NR1 and NR2 subunits required for the heteromeric assembly to functional channels.^{25–27} In these previous studies using bone marrow stromal cells, however, the possibility that functional expression of glutamatergic signaling molecules may be at least in part derived from osteoblasts contaminated in cultured preparations is not ruled out.

In the present study, therefore, we have attempted to evaluate the possible expression and functionality of a variety of different glutamatergic signaling machineries in primary cultured mouse osteoclasts devoid of contamination with both osteoblasts and stromal cells through the usage of recombinant mouse RANKL and M-CSF, which are both key extracellular regulators produced and released by osteoblasts and osteogenic stromal cells, for differentiation of hematopoietic bone marrow precursors prepared after Ficoll gradient centrifugation.

Materials and Methods

Materials

[3,4-³H]-Glu (1587.3 GBq/mmol) was purchased from New England Nuclear/DuPont (Boston, MA). [¹⁴C]-cysteine (9.25 GBq/mmol) was from Perkin-Elmer (Boston, MA). Taq polymerase was obtained from Takara (Tokyo, Japan). Bio-Rad protein assay kit was provided by Bio-Rad Laboratories (Hercules, CA). A rabbit polyclonal an-

tibody against xCT subunit was from TransGenic (Kumamoto, Japan). A rabbit polyclonal antibody against GLT-1 isoform was purchased from Chemicon International (Temecula, CA). A rabbit polyclonal antibody against EAAT4 isoform was obtained from Alpha Diagnostic International (San Antonio, TX). An anti-rabbit IgG antibody was supplied by DAKO A/S (Glostrup, Denmark). Recombinant mouse M-CSF and recombinant mouse RANKL were purchased from R&D Systems International (Minneapolis, MN). A Ficoll-Paque Plus and cycle sequencing kit was supplied by Amersham Pharmacia Biotech (Buckinghamshire, UK). Naphtol AS-MX phosphate and fast red violet Lurina-Bertani salt were obtained from Sigma (St. Louis, MO). A pGL3-xCT promoter was a generous gift from Dr. H. Sato (Yamagata University, Japan). A pNF- κ B-Luc was provided by Stratagene (La Jolla, CA). TA cloning vector, pGL3 basic vector, and Dual luciferase assay system were purchased from Promega (Madison, WI). Lipofectamine reagent and Plus reagent were supplied by Invitrogen (San Diego, CA). ISOGEN was obtained from WAKO (Osaka, Japan). AMPA, KA, NMDA, 3,5-dihydroxyphenylglycine (DHPG), (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV), L-(1)-2-amino-4-phosphonobutyrate (L-AP4), dihydrokainate (DHK), L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC), and L-threo- β -hydroxyaspartate (THA) were provided by Tocris Cookson (Bristol, UK). Other chemicals used were all of the highest purity commercially available.

Culture of Primary Osteoclasts and Tartrate-Resistant Acid Phosphatase (TRAP) Staining

The protocol used here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to use alternatives to *in vivo* techniques. Osteoclasts were prepared from bone marrows according to the procedures previously described³² with minor modifications. In brief, bone marrows were prepared from tibia and femur of Std-ddY male mice at 4 weeks of age and cultured for 24 hours with M-CSF at 10 ng/ml in α -minimal essential medium (α MEM) containing 10% fetal bovine serum. After culturing for 24 hours in the presence of M-CSF alone, supernatants were collected by gentle aspiration, followed by the lamination of nonadherent cells in supernatants on Ficoll gradient and subsequent centrifugation at 500 $\times$ g for 15 minutes. Cells fractionated in the monocyte fraction were defined as preosteoclasts throughout this study. These preosteoclasts were collected and suspended in MEM containing 10% fetal bovine serum, M-CSF at 20 ng/ml, and RANKL at 20 ng/ml. Cells were then plated at a density of 1 $\times$ 10⁵ cells/cm², followed by culturing in MEM containing 10% fetal bovine serum, 20 ng/ml M-CSF, and 20 ng/ml RANKL at 37°C under 5% CO₂ for 5 consecutive days unless indicated otherwise. For TRAP staining, cultured cells were fixed with 10% formalin in phosphate-buffered saline for 10

minutes, and subsequently with ethanol-acetone (50:50; v/v) for 1 minute at room temperature. Cells were then incubated in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate as a substrate and fast red violet LB salt as a dye in the presence of 50 mmol/L sodium tartrate. TRAP-positive cells with more than five nuclei were scored as TRAP-positive MNCs. For double staining with the osteoblastic marker protein alkaline phosphatase, cells were first stained with TRAP and further incubated in 100 mmol/L Tris-HCl buffer (pH 9.5) containing 100 mmol/L NaCl, 50 mmol/L MgCl₂, 375 μ g/ml nitroblue tetrazolium chloride, and 188 μ g/ml 5-bromo-4-chloro-3-indolyl phosphate for different periods to obtain the most appropriate pictures.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

cDNA was synthesized with the oligo-dT primer and reverse transcriptase from extracted total RNA. PCR amplification was performed using specific primers, and PCR products were subcloned into a TA cloning vector for determination of DNA sequences by ABI Prism 310 Genetic Analyzer (Perkin-Elmer) using a cycle sequencing kit. Quantitative analysis was done with primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. PCR products were quantified by using a densitograph, followed by the calculation of ratios of expression of mRNA for each gene over that for glyceraldehyde-3-phosphate dehydrogenase.

Immunoblotting Analysis

Cultured osteoclasts were homogenized in 20 mmol/L Tris-HCl buffer (pH 7.5) containing protease inhibitors, followed by centrifugation at 4°C for 30 minutes at 100,000 $\times g$ as described elsewhere. Pellets thus obtained were dissolved in 10 mmol/L Tris-HCl buffer containing 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol, followed by boiling for 10 minutes and subsequent loading of an aliquot for electrophoresis on a 7.5% SDS-polyacrylamide gel toward blotting to a polyvinylidene fluoride membrane. After blocking by 5% skim milk dissolved in 20 mmol/L Tris-HCl buffer (pH 7.5) containing 137 mmol/L NaCl and 0.05% Tween 20 (TBST), the membrane was incubated with one of antibodies against GLT-1, EAAT4, and xCT adequately diluted with TBST containing 1% skim milk and then with the secondary antibody conjugated with horseradish peroxidase. Finally, the membrane was incubated with enhanced chemiluminescence detection reagent to detect immunoreactive proteins, followed by exposure to X-ray films for different periods to obtain films appropriate for subsequent quantitative densitometry.

Determination of [³H]Glu and [¹⁴C]Cystine Accumulation

Primary osteoclasts were cultured, followed by washing with HEPES Krebs-Ringer (HKR) (125 mmol/L NaCl, 3.5

mmol/L KCl, 1.5 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 1.25 mmol/L KH₂PO₄, 25 mmol/L NaHCO₃, 10 mmol/L HEPES, and 10 mmol/L D-glucose, pH 7.4) buffer twice and subsequent incubation in HKR buffer at 37°C for 1 hour in a 5% CO₂ incubator. For determination of the accumulation in preosteoclasts, preosteoclasts were briefly cultured in the presence of M-CSF alone for 6 hours for the cell adhesion to dishes before the washing with HKR buffer as described above. Cells were then incubated with 1 μ mol/L [³H]Glu or [¹⁴C]cystine at 2°C or 37°C for 1 to 60 minutes, unless indicated otherwise.³³ Reaction was terminated by the aspiration of buffer, followed by superficial rinsing with ice-cold HKR buffer containing 1 mmol/L unlabeled Glu or cystine at 2°C three times to remove extracellular [³H]Glu or [¹⁴C]cystine and subsequent solubilization with 0.1 mol/L NaOH for liquid scintillation spectrometry using 3 ml of scintillation cocktail (clear sol I). Protein concentration was determined with a Bio-Rad Protein Assay Kit.

Constructs and Luciferase Assay

Preosteoclastic cell line RAW264.7 cells were seeded at 5×10^4 cells/cm² and maintained at 37°C in a humidified 5% CO₂ incubator. The 3'-flanking region of mouse xCT was isolated from mouse genomic DNA by PCR using the following primers: 5'-AGAATTATGAACTTAATGCA-3' and 5'-CCCAGTAGGTAAAGCTATGTT-3'. The PCR-amplified DNA products were cloned into the pGL3-xCT. Reporter vectors were co-transfected with the TK-*Renilla* luciferase construct into RAW264.7 cells by the Lipofectamine method with Lipofectamine/Plus reagent for 1 hour in Opti-MEM after cell seeding. Medium was replaced with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum after transfection, followed by the exposure to a test drug for 48 hours. Firefly and *Renilla* luciferase values were determined using Dual Luciferase Assay system. Approximately 5% of cells expressed green fluorescent protein in RAW264.7 cells transfected with the EGFP-C2 plasmid under the transfection method used.

Establishment of Stable Transfectants

Preosteoclastic RAW264.7 cells were plated at a density of 1.5×10^5 cells/cm². After 24 hours, cells were stably transfected with pcDNA3.1 containing the full-length coding regions of both xCT and 4F2hc subunits (RAW264.7-xCT + 4F2hc) or with the empty vector (RAW264.7-EV) using 2 μ g of DNA and Lipofectamine and Plus reagent in 10 ml of medium. After 24 hours, and every 48 hours thereafter for 2 weeks, media were replaced with fresh media containing 600 μ g/ml G418. Pools of 15 clones of RAW264.7-xCT + 4F2hc were isolated for further studies. Pools of clones between passages 2 and 5 were used for this experiment.

Ovariectomy and Analysis of Skeletal Morphology

Eight-week-old female ddY mice were subjected to ovariectomy or sham operation. Mice were sacrificed by decapitation 30 days after ovariectomy, followed by the dissection of femora and tibiae and subsequent removal of adhering muscles around the bone for fixation with 70% ethanol. Bone mineral density was measured by single energy X-ray absorptiometry using a bone mineral analyzer (DCS-600R; Aloka Co., Tokyo, Japan). Histomorphometric analysis was performed using femur excised from mice 30 days after sham or ovariectomy operation. For toluidine blue O staining, femur was fixed in 70% ethanol, embedded in glycolmethacrylate, and sectioned in 3- μ m sections. The specimens were subjected to histomorphometric analyses under a light microscope with micrometer, using a semiautomatic image analyzing system (Osteoplan II; Carl Zeiss). Ovariectomized mice were also given with daily intraperitoneal administration of Glu at a dose of 1 mg/kg to 1 g/kg for 28 consecutive days from the next day and sacrificed 1 day after the last injection.

Determination of Glu Contents in Bone Marrow

Bone marrows were isolated from tibia of sham-operated or ovariectomized mice 24 hours after the last administration of Glu, followed by centrifugation at $20,000 \times g$ for 5 minutes and subsequent collection of the supernatant for the homogenization in 1 mol/L perchloric acid at a volume ratio of 4:1. Following centrifugation at $20,000 \times g$ for 5 minutes, the supernatant was collected for subsequent neutralization with sodium hydroxide and storage at -80°C . Glutamate was determined by the fluorometric method using β -NADP⁺ and glutamate dehydrogenase. Samples were incubated with 100 mmol/L β -NADP⁺ to make a final concentration of 2 mmol/L, and then with 80 U/ml glutamate dehydrogenase at a volume ratio of 1:1 for 5 minutes at 37°C , for subsequent measurement of Glu concentrations using a fluorescence microplate reader (MPT-100F; Corona Electric Co., Naka-Hitachi, Japan) with excitation at 340 nm and emission at 460 nm, respectively. In each experiment, known concentrations of Glu were determined in parallel as standards.

Data Analysis

Results are all expressed as the mean $\pm$ SE and the statistical significance was determined by the two-tailed and unpaired Student's *t*-test or the one-way analysis of variance with Bonferroni/Dunnett post hoc test.

Results

Expression Profiles of Glu Signaling Machineries

We first examined the expression profile of a variety of osteoclastic differentiation markers in mouse osteo-

clasts cultured as described above. Semiquantitative RT-PCR analysis revealed that mRNA expression was drastically increased for all osteoclastic marker genes examined in proportion to culture periods from 1 to 6 days. These included RANK, carbonic anhydrase II, matrix metalloproteinase-9, c-fms, cathepsin K, calcitonin receptor, and c-src, in addition to TRAP (data not shown). To evaluate the possible contamination with osteoblasts and/or stromal cells in these mouse cultured osteoclasts differentiated from bone marrow precursors in the presence of both M-CSF and RANKL, mRNA was extracted from these primary cultured osteoclasts for subsequent RT-PCR using specific primers for type I collagen and osteocalcin, which are both known as an osteoblastic marker. In preosteoclasts not exposed to RANKL and mature osteoclasts cultured for 5 days in the presence of both M-CSF and RANKL, mRNA expression was markedly found for glyceraldehyde-3-phosphate dehydrogenase, but not for either type I collagen or osteocalcin (data not shown). Therefore, subsequent experiments were done using cells isolated, prepared and cultured under adequate conditions described above as mouse primary cultured osteoclasts before and after the differentiation from bone marrow hematopoietic precursors.

To analyze the expression of Glu signaling machineries including GluRs and EAATs, mRNA was extracted from cultured primary osteoclasts for subsequent RT-PCR using specific primers for each molecule. Mouse whole brain exhibited marked expression of mRNA for all Glu signaling machineries examined. These included NR1, NR2A, NR2B, NR2C, and NR2D subunits of NMDA receptors, GluR1, GluR2, GluR3, and GluR4 subunits of AMPA receptors, GluR5, GluR6, GluR7, KA1, and KA2 subunits of KA receptors, mGluR1, mGluR2, mGluR3, mGluR4, mGluR5, mGluR6, mGluR7, and mGluR8 isoforms of mGluRs (Figure 1A), and GLAST, GLT-1, EAAC1, EAAT4, and EAAT5 isoforms of EAATs (Figure 1B), VGLUT1, and VGLUT2 isoforms of VGLUT (Figure 1C). In preosteoclasts cultured for 1 day in the presence of M-CSF alone after the isolation and mature osteoclasts cultured for 5 days in the presence of both M-CSF and RANKL, however, no mRNA expression was found for all GluRs and VGLUTs examined. In contrast to GluRs and VGLUTs, expression was seen with mRNA for GLT-1 and EAAT4, but not for GLAST, EAAC1, and EAAT5, isoforms of EAATs in primary osteoclasts before and after the differentiation from bone marrow precursors. Sequencing analysis on these amplified PCR products clearly confirmed the expression of mRNA for the corresponding Glu signaling machineries. In matured osteoclasts cultured for 5 days, moreover, high immunoreactivity was detected for EAAT4 (Figure 1D, a) but not for GLT-1 (Figure 1D, b) isoform on Western blotting. In membranes not treated with each primary antibody, no marked immunoblots were detected. Therefore, subsequent experiments focused on EAATs required for Glu transmembrane trafficking rather than GluRs essential for Glu signal input.

Figure 1. Expression of Glu signaling machineries in primary osteoclasts. **A:** mRNA was isolated from mouse primary preosteoclasts not exposed to RANKL and mature osteoclasts cultured for 5 days for subsequent RT-PCR using primers specific for AMPA (**a**), KA (**b**), and NMDA (**c**) receptor subtypes of iGluRs, and group I mGluR (**d**), group II mGluR (**e**), and group III mGluR (**f**) subtypes of mGluRs. **B:** Preosteoclasts and mature osteoclasts were subjected to isolation of mRNA for subsequent RT-PCR using primers specific for different EAATs. **C:** Preosteoclasts and mature osteoclasts were subjected to isolation of mRNA for subsequent RT-PCR using primers specific for different VGLUTs. **D:** Osteoclasts were cultured for 5 days, followed by homogenization and subsequent centrifugation at $100,000 \times g$ for immunoblotting analysis using antibodies against EAAT4 (**a**) and GLT-1 (**b**) isoforms. Typical pictures are shown in the figure, whereas similar results were invariably obtained in at least three independent determinations. Br, whole brain; Re, retina.

[³H]Glu Accumulation in Cultured Osteoclasts

To evaluate the functionality of EAATs expressed, an attempt was made to determine whether the substrate Glu is indeed incorporated into these cultured osteoclasts. Cultured osteoclasts were incubated with $1 \mu\text{mol/L}$ [³H]Glu in HKR buffer at 2°C or 37°C for different periods up to 20 minutes, followed by rapid aspiration of buffer and subsequent rinsing with ice-cold buffer containing unlabeled Glu at 1 mmol/L to remove extracellularly located [³H]Glu. Under these experimental conditions, [³H]Glu incorporated was not markedly affected by the later addition of 1 mmol/L unlabeled Glu for rinsing in cultured osteoclasts (data not shown). The accumulation of [³H]Glu was almost 10 times higher in preosteoclasts cultured for an additional 6 hours in the presence of M-CSF alone than in mature osteoclasts cultured for 5 days in the presence of both M-CSF and RANKL throughout the incubation at 37°C (Figure 2). Indeed, [³H]Glu accumulation was linearly increased with the incubation time up to 5 minutes and reached a plateau within 10 minutes at 37°C in preosteoclasts (Figure 2A-a), whereas [³H]Glu accumulation was increased with incubation time up to 10 minutes and reached a plateau within 20 minutes at 37°C in mature osteoclasts (Figure 2A, b). No marked accumulation of [³H]Glu was seen even 10 minutes

after the initiation of incubation at 2°C in preosteoclasts and mature osteoclasts. Replacement of sodium chloride with choline chloride led to marked inhibition of the accumulation of [³H]Glu in mature osteoclasts (Figure 2B, b) without significantly affecting that in preosteoclasts cultured for an additional 6 hours in the presence of M-CSF alone (Figure 2B-a). By contrast, replacement with sodium gluconate induced a significant reduction of the accumulation of [³H]Glu in preosteoclasts cultured for an additional 6 hours in the presence of M-CSF alone with no marked decrease in mature osteoclasts.

Preosteoclasts cultured for an additional 6 hours in the presence of M-CSF alone were then incubated with $1 \mu\text{mol/L}$ [³H]Glu in HKR buffer containing a variety of amino acids at $100 \mu\text{mol/L}$ for 5 minutes at 37°C . Of the amino acids tested, both L-Glu and cystine were most effective in inhibiting [³H]Glu accumulation in these preosteoclasts with progressively less efficient inhibition by D-Glu and L-cysteine, whereas [³H]Glu accumulation was not significantly affected by L-aspartate, D-aspartate, L-alanine, and L-serine at $100 \mu\text{mol/L}$ (Figure 2C). Cells were also incubated with several Glu transporter inhibitors at $100 \mu\text{mol/L}$ for 5 minutes at 37°C . Of the five different inhibitors tested, homocysteic acid, a cystine/Glu antiporter inhibitor, was most

Figure 2. $[^3\text{H}]$ Glu accumulation in cultured osteoclasts. **A:** Preosteoclasts cultured for an additional 6 hours in the presence of M-CSF alone (**a**) and mature osteoclasts cultured for 5 days in the presence of both M-CSF and RANKL (**b**) were individually incubated with 1 $\mu\text{mol/L}$ $[^3\text{H}]$ Glu at 2°C or 37°C for different periods of up to 20 minutes in HKR buffer, followed by the gentle aspiration of buffer and subsequent rinsing with ice-cold buffer containing unlabeled Glu at 1 mmol/L. Values are the mean $\pm$ SE from 6 to 12 different experiments. **B:** Both preosteoclasts (**a**) and mature osteoclasts (**b**) similarly cultured were incubated with 1 $\mu\text{mol/L}$ $[^3\text{H}]$ Glu at 37°C for 10 minutes in HKR buffer where sodium chloride was replaced with equimolar choline chloride or sodium gluconate as needed. Values are the mean $\pm$ SE from 10 different experiments. **C:** The incubation was done with preosteoclasts cultured for an additional 6 hours in the presence of M-CSF alone in either the presence or absence of various amino acids at 100 $\mu\text{mol/L}$. **D:** The incubation was done with preosteoclasts cultured for an additional 6 hours in the presence of M-CSF alone in HKR buffer containing one of the inhibitors of different EAATs and cystine/Glu antiporter at 100 $\mu\text{mol/L}$. Values are the mean $\pm$ SE of three to seven independent experiments. * $P < 0.05$, ** $P < 0.01$, significantly different from each control value obtained with normal HKR buffer.

potent in inhibiting $[^3\text{H}]$ Glu accumulation than the EAAT inhibitors DHK, PDC, THA, and AβH in preosteoclasts cultured for an additional 6 hours in the presence of M-CSF alone (Figure 2D).

Expression of Cystine/Glu Antiporter and Regulation by RANKL

Therefore, subsequent experiments focused on the cystine/Glu antiporter required for bidirectional Glu membrane transport rather than EAATs. To analyze expression of mRNA for the cystine/Glu antiporter, RT-PCR was conducted for both xCT and 4F2hc subunits essential for the functional heteromeric assembly in primary osteoclasts. Although xCT mRNA was seen in preosteoclasts with a dramatic decrease in matured cells cultured for 5 days, constitutive expression of mRNA was found for 4F2hc subunit in both preosteoclasts and matured osteoclasts (Figure 3A). In addition to temperature-dependent $[^3\text{H}]$ Glu incorporation, indeed, $[^{14}\text{C}]$ cystine was also incorporated into preosteoclasts in a temperature-dependent manner (Figure 3B). To further examine the expression of xCT subunit, preosteoclasts were cultured in ei-

ther the presence or absence of RANKL for an additional 24 hours, followed by the determination of xCT expression at both mRNA and protein levels by RT-PCR and immunoblotting techniques. Exposure to RANKL for 24 hours markedly decreased xCT expression at both mRNA (Figure 3C) and protein (Figure 3D) levels, without affecting 4F2hc mRNA expression, as seen in matured osteoclasts. To investigate whether xCT mRNA expression is regulated by RANKL at a transcriptional or post-transcriptional level, the reporter assay was done in the preosteoclastic RAW264.7 cells using xCT promoter (Figure 3E-a) and xCT promoter containing 3'UTR of xCT mRNA (Figure 3E, b). Exposure to RANKL did not significantly affect the xCT promoter activity in RAW264.7 cells (Figure 3E, a), whereas the activity of xCT promoter containing xCT 3'UTR was significantly decreased by 50% in cells exposed to RANKL for 24 hours (Figure 3E).

Glu Inhibits RANKL-Induced Differentiation in Primary Osteoclasts

To evaluate the possible effect of Glu on osteoclast differentiation, preosteoclasts were cultured in MEM con-

Figure 3. Expression of cystine/Glu antiporter and regulation by RANKL in osteoclasts. **A:** mRNA was isolated from preosteoclasts and mature osteoclasts for subsequent RT-PCR using primers specific for xCT or 4F2hc subunit. Typical pictures are shown in the figure with similar results in three separate determinations. Br, whole brain. **B:** Preosteoclasts cultured for an additional 6 hours in the presence of M-CSF alone were incubated with 1 μ mol/L [14 C]cystine at 2°C or 37°C for 10 minutes. **C:** Preosteoclasts were cultured in either the presence or absence of 20 ng/ml RANKL for 24 hours, followed by the isolation of mRNA and subsequent RT-PCR using primers specific for xCT or 4F2hc subunit. **D:** Preosteoclasts were cultured in either the presence or absence of 20 ng/ml RANKL for 24 hours, followed by the homogenization and subsequent centrifugation at 100,000 $\times$ g for immunoblotting analysis using an antibody against xCT subunit. Typical pictures are shown in the figure, whereas similar results were invariably obtained in at least three independent determinations. **E:** Preosteoclastic RAW264.7 cells were transiently transfected with xCT promoter (a) or xCT promoter containing 3' UTR (b) of xCT mRNA, followed by cultivation in either the presence or absence of 20 ng/ml RANKL for 48 hours and subsequent cell harvest for determination of the luciferase activity. Values are the mean $\pm$ SE from four different experiments. ** $P < 0.01$, significantly different from control value obtained in the absence of RANKL.

taining both M-CSF and RANKL in either the presence or absence of Glu at concentrations of below 1 mmol/L for 5 consecutive days. Sustained exposure to Glu at 500 μ mol/L markedly inhibited the formation of TRAP-positive MNCs with a pavement shape (Figure 4A, top panel), whereas quantitative calculation revealed that Glu significantly decreased the number of TRAP-positive MNCs in a concentration-dependent manner at concentrations of 1 μ mol/L to 1 mmol/L (Figure 4A, bottom panel). By contrast, no significant changes were seen in the number of TRAP-positive MNCs in cells cultured for 5 days with different iGluR agonists such as AMPA, KA, and NMDA, or mGluR agonists including DHPG, DCG-IV, and L-AP4, at 100 μ mol/L (Figure 4B). Preosteoclasts were exposed to 500 μ mol/L Glu at different days from 0 to 5 days, followed by TRAP staining at 5 days. The number of TRAP-positive MNCs was significantly decreased following the sustained exposure to Glu for 3 to 5 consecutive days, whereas Glu did not significantly affect the number of TRAP-positive MNCs at 500 μ mol/L when exposed after 3 days until the day of TRAP staining (Figure 4C). However, no significant alternation was found in the cel-

lular viability in preosteoclasts cultured in MEM containing both M-CSF and RANKL in the presence of Glu at concentrations of over 500 μ mol/L for 5 consecutive days when determined by both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (Figure 4D, a) and lactate dehydrogenase release (Figure 4D, b). In cells exposed to Glu at 0.5 to 1 mmol/L for 5 days, moreover, a significant decrease was seen in intracellular glutathione (GSH) levels (Figure 4D, c).

Glu Inhibits RANKL-Induced Differentiation in RAW264.7 Cells

Preosteoclastic RAW264.7 cells were cultured in either the presence or absence of Glu at concentrations of below 1 mmol/L for 4 consecutive days, followed by TRAP staining. Sustained exposure to Glu at a concentration of over 500 μ mol/L significantly inhibited the formation of TRAP-positive MNCs (Figure 5A, left panel), whereas quantification clearly showed a significant decrease by Glu in the number of TRAP-positive MNCs in a

Figure 4. Effect of Glu on cell differentiation of primary osteoclasts. **A:** Preosteoclasts were cultured for 5 days with 20 ng/ml M-CSF and 20 ng/ml RANKL in either the presence or absence of Glu at a concentration range from 1 $\mu\text{mol/L}$ to 1 mmol/L. Cultured cells were then fixed with 10% formalin, followed by staining for TRAP and subsequent counting of the number of MNCs positive to TRAP staining. Typical pictures are shown in the top panel, whereas quantitative data are shown in the bottom panel. **B:** Preosteoclasts were cultured with M-CSF and RANKL in either the presence or absence of different GluR agonists, followed by determination of the number of TRAP-positive MNCs. **C:** Preosteoclasts were cultured with M-CSF and RANKL, followed by the addition of Glu at 500 $\mu\text{mol/L}$ on different days from 0 to 5 days and subsequent additional culture for up to 5 days. **D:** Preosteoclasts were cultured with M-CSF and RANKL in either the presence or absence of Glu at 0.5 or 1 mmol/L for 5 days, followed by the determination of the cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (a), lactate dehydrogenase activity (b), and GSH contents (c). Values are the mean $\pm$ SE of four independent experiments. ** $P < 0.01$, significantly different from each control value obtained in the absence of Glu.

concentration-dependent manner (Figure 5A, right panel). An attempt was next made to elucidate underlying mechanisms for the inhibition by Glu of osteoclastic differentiation in RAW264.7 cells. The decrease by Glu was not significantly affected by the simultaneous addition of EAAT inhibitors such as DHK and THA but was prevented by the addition of homocysteic acid, another substrate for the cystine/Glu antiporter, in addition to GSH, at 0.5 mmol/L (Figure 5B).

RAW264.7 cells were transiently transfected with the luciferase reporter plasmid containing five NF- κ B-binding sequences, followed by exposure to RANKL in either the presence or absence of Glu for 48 hours and subsequent cell harvest for determination of the luciferase activity. The addition of RANKL at 10 ng/ml tripled the luciferase activity in RAW264.7 cells transfected with NF- κ B reporter plasmid, whereas the further addition of Glu at concentrations over 0.5 mmol/L significantly prevented the increase by RANKL to the control level (Figure 5C). Moreover, both homocysteic acid and GSH were effective in significantly preventing the inhibition by Glu of RANKL-dependent NF- κ B reporter activity (Figure 5D).

Effect of Stable Overexpression of Both xCT and 4F2hc Subunits on Differentiation in RAW264.7 Cells

To assess the role of the cystine/Glu antiporter in osteoclast differentiation, preosteoclastic RAW264.7 cells were stably transfected with pcDNA3.1 containing the full-length coding regions of xCT and 4F2hc subunits (RAW264.7-xCT + 4F2hc) or with empty vector (RAW264.7-EV). Expression levels of both xCT and 4F2hc subunits were examined by semiquantitative RT-PCR, immunoblotting, and uptake analyses. Several clones of cells transfected with xCT and 4F2hc subunits showed markedly elevated expression of xCT and 4F2hc subunits compared with cells transfected with empty vector alone. Figure 6 shows representative results for mRNA (Figure 6A) and corresponding protein (Figure 6B) among different clones tested with RAW264.7 cells. In addition to the up-regulation of xCT and 4F2hc expression, stable overexpression of xCT and 4F2hc subunits more than tripled the activity to incorporate [^3H]Glu (Figure 6C) and [^{14}C]cystine (Figure 6D) in RAW264.7 cells.

Figure 5. Effect of Glu on cell differentiation of preosteoclastic RAW264.7 cells. **A:** RAW264.7 cells were cultured for 4 days with 20 ng/ml RANKL in either the presence or absence of Glu at a concentration range from 0.1 to 1 mmol/L, followed by determination of the number of TRAP-positive MNCs. Typical pictures are shown in the left panel, whereas quantitative data are shown in the right panel. **B:** RAW264.7 cells were cultured with RANKL in either the presence or absence of different test drugs, followed by determination of the number of TRAP-positive MNCs. **C:** RAW264.7 cells were transiently transfected with the reporter plasmid of NF-κB, followed by further cultivation with RANKL at 1 to 50 ng/ml in either the presence or absence of Glu at 0.1 to 1 mmol/L for 48 hours and subsequent cell harvest for determination of the luciferase activity. **D:** Cells were transiently transfected with the reporter plasmid of NF-κB, followed by further cultivation with 10 ng/ml RANKL and 500 μmol/L Glu in either the presence or absence of homocysteic acid or GSH at 500 μmol/L for 48 hours and subsequent cell harvest for determination of the luciferase activity. Values are the mean ± SE from four independent experiments.

To investigate whether xCT and 4F2hc indeed affect osteoclast differentiation, both RAW264.7-EV and RAW264.7-xCT + 4F2hc cells were cultured for 4 days with 10 ng/ml RANKL in either the presence or absence of Glu. The number of TRAP-positive MNCs was similarly increased in proportion to the culture duration from 1 to 4 days in both RAW264.7-EV and RAW264.7-xCT + 4F2hc cells (Figure 6E). In addition, sustained exposure to Glu at a concentration of over 500 μmol/L significantly decreased the number of TRAP-positive MNCs in a concentration-dependent manner in both RAW264.7-EV and RAW264.7-xCT + 4F2hc cells (Figure 6F). Cultured cells were exposed to 500 μmol/L Glu at different days of culture from 0 to 4 days, followed by TRAP staining at 4 days. In RAW264.7-xCT + 4F2hc cells, Glu was more efficient in decreasing the number of TRAP-positive MNCs than in RAW264.7-EV when exposed for a period longer than 1 day up to 3 days (Figure 6G). However, no significant difference was seen in the inhibition between both cells exposed to Glu for 4 days. Therefore, stable overexpression of both xCT and 4F2hc subunits would

facilitate the inhibition by Glu of osteoclastogenesis in RAW264.7 cells *in vitro*.

Effect of Glu on Ovariectomy-Induced Bone Loss

To examine the possible functional significance of the *in vitro* inhibition by Glu of osteoclastogenesis, we next conducted daily intraperitoneal administration of Glu at different doses in ovariectomized mice for subsequent determination of the bone mineral density in addition to different histomorphometric parameters 1 day after the last injection. The administration of Glu at a dose of 1 g/kg did not significantly affect the drastic decrease by ovariectomy in uterine weight determined 30 days after operation (Figure 7A), whereas the daily administration of 1 g/kg Glu for 28 days significantly increased Glu concentrations in tibial bone marrows irrespective of ovariectomy when determined 24 hours after the last administration (Figure 7B). Ovariectomy induced a significant reduction

Figure 6. Effect of Glu on cell differentiation in xCT/4F2hc stable transfectants. RAW264.7 cells were stably transfected with xCT/4F2hc expression vectors or empty vector (EV), followed by determination of expression levels of xCT and 4F2hc subunits by semiquantitative RT-PCR (**A**), immunoblotting (**B**), and [3 H]Glu (**C**) and [14 C]cystine (**D**) incorporation assays. Values are the mean $\pm$ SE obtained in four independent experiments. ** $P < 0.01$, significantly different from each control value obtained in cells transfected with EV. **E:** RAW264.7-xCT/4F2hc and RAW264.7-EV cells were cultured for 4 days with 20 ng/ml RANKL, followed by determination of the number of TRAP-positive MNCs. **F:** RAW264.7-xCT/4F2hc and RAW264.7-EV cells were cultured for 4 days with 20 ng/ml RANKL in either the presence or absence of Glu at a concentration range from 1 μ mol/L to 1 mmol/L, followed by determination of the number of TRAP-positive MNCs. **G:** Both RAW264.7-xCT/4F2hc and RAW264.7-EV cells were cultured with 20 ng/ml RANKL, in either the presence or absence of 500 μ mol/L Glu added on different days from 0 to 3 days, for up to 4 days. Values are all of the mean $\pm$ SE of eight independent experiments. * $P < 0.05$, ** $P < 0.01$, significantly different from each control value obtained in cells with EV. # $P < 0.05$, ## $P < 0.01$, significantly different from the value obtained in cells transfected with EV alone.

of bone mineral density in both total tibia and total femur when determined by single-energy X-ray absorptiometry at 30 days later (Figure 7C). The daily administration of Glu for 28 consecutive days significantly prevented the reduction of bone mineral density in total tibia at a dose over 10 mg/kg (Figure 7C, a) and in total femur at a dose over 100 mg/kg (Figure 7C, b), respectively. No significant alternation of bone mineral density was observed in total femur of sham-operated mice with the daily intraperitoneal administration of Glu at a dose of 1000 mg/kg for 28 consecutive days.

Micro-CT analysis clearly showed marked bone loss in the cancellous bone, but not in the cortical bone, in ovariectomized mice on 30 days after operation (Figure 8A), whereas the daily intraperitoneal administration of 1 g/kg for 28 days markedly prevented bone loss in the cancellous bone without affecting the cortical bone density on micro-CT analysis. In ovariectomized mice, moreover, a significant decrease was seen in bone volume/tissue volume (BV/TV) ratio (Figure 8B, a) with significant increases in the extent of eroded surface (ES/BS; Figure 8B, c), the number of osteoclasts on bone surfaces (Oc no; Figure 8B, d) and the extent of bone surface covered by osteoclasts (Oc surface; Figure 8B, e) by histomor-

phometric analysis 30 days after operation. However, ovariectomy did not significantly affect the extent of bone surface covered by osteoblasts (Ob surface; Figure 8B, b). The daily administration of 1 g/kg Glu for 28 consecutive days was invariably effective in significantly preventing alterations of different bone parameters in ovariectomized mice when determined 30 days after operation.

Discussion

The essential importance of the present findings is that Glu markedly inhibited the cellular differentiation toward maturation in association with the decreased endogenous level of intracellular GSH as a consequence of the retrograde operation of the bidirectional cystine/Glu antiporter functionally expressed in mouse osteoclasts differentiated from bone marrow hematopoietic precursors *in vitro*. In addition, the systemic daily administration of Glu significantly prevented the ovariectomy-induced decrease in bone mineral density in addition to increases in different osteoclastic indices *in vivo*. To our knowledge, this paper deals with the first direct demonstration of

Figure 7. Effect of Glu administration on bone mineral density. Eight-week-old female ddY mice were subjected to ovariectomy (OVX) or sham operation, followed by the daily intraperitoneal administration of Glu at 1 to 1000 mg/kg for 28 consecutive days from the next day. Mice were sacrificed 1 day after the last injection of Glu for determination of uterine weight (**A**), Glu contents of tibial bone marrow (**B**), and bone mineral density (**C**) of both tibia (**a**) and femur (**b**) by single-energy X-ray absorptiometry. Values are the mean $\pm$ SE from different numbers of independent experiments shown in the figure. ** $P < 0.01$, significantly different from each control value obtained in sham-operated mice. * $P < 0.05$, ** $P < 0.01$, significantly different from the value obtained in OVX mice.

functional expression of the cystine/Glu antiporter required for the cellular differentiation by preosteoclastic RAW264.7 cells and by primary osteoclasts differentiated from bone marrow precursors. Although several previous studies including ours have already demonstrated the functional expression of different EAATs, such as GLAST and GLT-1 isoforms, in bone and cartilage, no direct evidence is available for a pivotal role of the cystine/Glu antiporter in osteoclastogenesis in the literature to date.

Recent studies have raised the possibility that Glu may be one of the endogenous paracrine (autocrine) factors used for intercellular communications in bone.^{25,34} In mammalian osteoblasts constitutive expression is shown with mRNA for non-NMDA receptors such as GluR3 subunit of AMPA receptors as well as KA1 and KA2 subunits of KA receptors,³⁵ whereas activation of AMPA receptors modulates the exocytotic release of Glu from cultured osteoblasts.^{36,37} Moreover, previous studies reveal the functional expression of NMDA receptor channels in osteoblasts. For example, Glu induces an elevation of intracellular-free Ca^{2+} in a manner sensitive to antagonism by the NMDA receptor antagonist dizocilpine (MK-801) in the human osteoblastic cell line.³⁸ We have also demonstrated marked suppression of the cellular differentiation by sustained exposure to different NMDA receptor antagonists in a manner dependent on Runx2, which is the master regulator of osteoblast differentiation, in cultured rat calvarial osteoblasts.³⁹ Functional expression is also shown with mGluR1 isoform in rat femoral osteoblasts⁴⁰

and with both mGluR4 and mGluR8 isoforms in rat calvarial osteoblasts,⁴¹ respectively.

In addition to the aforementioned analyses on different molecules required for glutamatergic signal input in osteoblasts, on the other hand, the possible functional expression of GluRs has also been evaluated in osteoclasts in the literature. Beside the expression of NR1 subunit essential for a heteromeric assembly of functional NMDA receptor channels, for instance, particular NR2 subunits are expressed in cultured osteoclasts where the addition of an NMDA receptor antagonist inhibits cellular differentiation and bone resorption activity.^{25,27,33} Secretion of Glu accumulated in transcytotic vesicles by VGLUT1 is also shown in cultured osteoclasts.⁴² In the present study, by contrast, mRNA expression was exclusively seen for GLT-1 and EAAT4 isoforms, but neither for other EAAT isoforms nor for any iGluR and mGluR subtypes including NMDA receptors, in primary cultured mouse osteoclasts differentiated from monocyte/macrophage progenitor cells in the presence of M-CSF and RANKL. The paradox could be accounted for by taking into consideration the differences in both species and protocols for the preparation of osteoclasts between the previous and present studies. In the present study, we used primary cultured mouse osteoclasts devoid of the contamination with both osteoblasts and stromal cells through the usage of recombinant mouse RANKL and M-CSF, which are both produced by osteoblasts and osteogenic stromal cells. In contrast to the previous studies using the

Figure 8. Micro-CT and histomorphometric analyses. Eight-week-old female ddY mice were subjected to ovariectomy (OVX) or sham operation, followed by the daily intraperitoneal administration of 1 g/kg Glu for 28 consecutive days from the next day and subsequent analysis on micro-CT (**A**) and histomorphometric bone (**B**) parameters. Values are the mean $\pm$ SE from three or four different experiments. * $P < 0.05$, ** $P < 0.01$, significantly different from each control value obtained in sham-operated mice. # $P < 0.05$, significantly different from the value obtained in OVX mice.

co-culture system containing at least 10% osteogenic stromal cells,⁴³ the high predominance of osteoclasts is validated in the present culture system where marked increases were seen in the expression of different osteoclastic marker genes during cell maturation along with the absence of either osteoblastic or stromal cells responsible for the expression of alkaline phosphatase, type I collagen, and osteocalcin.

In this study, we have for the first time demonstrated the possible alternating expression of particular EAAT isoforms and cystine/Glu antiporter during osteoclastogenesis. The luciferase analysis on xCT promoter suggests that the cystine/Glu antiporter expression may be down-regulated during osteoclastogenesis induced by RANKL through a mechanism related to the stability of corresponding mRNA in osteoclasts. Preosteoclasts would highly express the cystine/Glu antiporter responsible for sodium-independent and chloride-dependent [³H]Glu accumulation in addition to EAAT subtypes such as GLT-1 and EAAT4 isoforms, whereas mature osteoclasts could preferentially express particular EAAT subtypes involved in sodium-dependent and chloride-independent [³H]Glu accumulation. Biochemical and pharmacological profiling is suggestive of the predominant expression of the cystine/Glu antiporter in preosteoclasts. Several independent lines of evidence indicate the functional expression by osteoblasts of particular Glu transporters required for the signal termination at glutamatergic synapses in the brain. Of EAAT subtypes cloned to date, both GLAST and GLT-1 isoforms are shown to reside in bone with respect to mRNA and cor-

responding proteins.³⁴ Mechanical loading is shown to down-regulate GLAST isoform expressed in osteocytes when determined by immunohistochemistry.³⁴ Moreover, we have recently shown temperature- and sodium-dependent [³H]Glu accumulation in cultured rat calvarial osteoblasts in addition to expression of particular EAAT mRNA.³³ The present investigation gives rise to an idea that the cystine/Glu antiporter would play a pivotal role in osteoclastogenesis at an early differentiation stage through regulation of intracellular GSH levels for subsequent modulation of NF- κ B promoter activity. The functional significance of EAAT subtypes expressed in preosteoclasts and mature osteoclasts, however, is not clarified in this study. One possible speculation is that EAAT subtypes may regulate the extracellular level of Glu responsible for the retrograde operation of the cystine/Glu antiporter during early osteoclastogenesis.

In fact, the rate of cystine incorporation is a crucial determinant for the regulation of GSH concentrations in the cytoplasm. Under the condition of high extracellular Glu levels, Glu is taken up into cells in exchange for intracellular cystine through the retrograde operation of the bidirectional cystine/Glu antiporter. This retrograde exchange would result in depletion of intracellular GSH attributable to the decreased cystine level in the cytoplasm, which often leads to cell death in several different cells.^{44,45} The fact that Glu significantly suppressed cell differentiation without cell death is unfavorable for the possible involvement of the cytotoxicity after GSH depletion or the excitotoxicity mediated by particular GluRs in mechanisms underlying the inhibition by extracellular Glu

of osteoclastogenesis. Both cellular differentiation and function could be stimulated by reactive oxygen species through intracellular signaling molecules essential for osteoclastogenesis including NF- κ B and c-Jun amino terminal kinase in osteoclasts, whereas antioxidants would inhibit osteoclastic differentiation along with NF- κ B activation.^{46–48} In this study, however, Glu markedly inhibited cellular differentiation in association with the decreased intracellular GSH for subsequent modulation of RANKL-induced NF- κ B activity in osteoclasts. The exact mechanism underlying the inhibition by Glu, thus, remains to be elucidated in future studies. Glutamate could thus play a dual role in mechanisms underlying osteogenesis: 1) facilitation of osteoblastogenesis through activation of NMDA receptors expressed by osteoblasts for subsequent induction of runx2; and 2) inhibition of osteoclastogenesis through retrograde driving of the cystine/Glu antiporter expressed by osteoclasts for subsequent depletion of intracellular GSH. The alternating expression profiles of glutamatergic signaling machineries would thus be a determinant critical for the vector of osteogenesis toward bone remodeling.

It is noteworthy that the daily administration of Glu prevented ovariectomy-induced bone loss in mice *in vivo*. Our previous findings on the inhibition of osteoblastogenesis by different NMDA receptor antagonists *in vitro*³⁹ argue in favor of an idea that the prevention by Glu would be brought about by suppression of osteoclastogenesis through retrograde operation of the cystine/Glu antiporter expressed in osteoclasts as well as by stimulation of osteoblastogenesis through activation of NMDA receptors expressed in osteoblasts *in vivo*. However, histomorphometric analysis revealed that Glu prevented the ovariectomy-induced alternations of different osteoclastic indices, including the number of osteoclasts on bone surface, the extent of bone surface covered by osteoclasts, and the extent of eroded surface, without significantly affecting the number of osteoblasts on bone surface. Taken together, it is conceivable that the prevention by Glu administration would be mainly mediated by the inhibition of osteoclastogenesis through the depletion of intracellular GSH after the oppositely directed driving of the cystine/Glu antiporter expressed by osteoclasts rather than the stimulation of osteoblastogenesis through activation of NMDA receptors expressed by osteoblasts against bone loss by ovariectomy in mice.

It thus seems that Glu may be protective against bone loss induced by ovariectomy through retrograde driving of the cystine/Glu antiporter expressed by osteoclasts rather than activation of NMDA receptors expressed by osteoblasts. The activity of the cystine/Glu antiporter would be a determinant of the progress of osteoclastogenesis through modulation of the endogenous level of GSH that plays a pivotal role as a predominant antioxidant in osteoclasts. Therefore, the cystine/Glu antiporter would be a novel target of a great benefit for the discovery and development of strategies useful for the therapy and treatment of a variety of bone diseases such as postmenopausal osteoporosis in humans. This study could thus deal with the prime development of an innovative and novel interdisciplinary field on “neuro-osteol-

ogy” as a scientific bridge between bone and brain biology.

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