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Functional expression of particular isoforms of excitatory amino acid transporters by rodent cartilage

Eiichi Hinoi [1], Liyang Wang [1], Akihiro Takemori, Yukio Yoneda *

Laboratory of Molecular Pharmacology, Division of Pharmaceutical Sciences, Kanazawa University Graduate School of Natural Science and Technology, Kanazawa, Ishikawa 920-1192, Japan

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Abstract

In the present study, we have attempted to demonstrate functional expression by the rodent cartilage of particular isoforms of excitatory amino acid transporters (EAATs) essentially required for central glutamatergic signal termination. Constitutive expression of mRNA was shown for the first time with the neuronal EAAT subtype excitatory amino acid carrier-1 (EAAC1), in addition to glial subtypes such as glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1), in rat costal chondrocytes cultured for 7–21 days on reverse transcription polymerase chain reaction (RT-PCR). Western blotting analysis confirmed the expression of corresponding proteins for both GLAST and GLT-1 in cultured chondrocytes. The accumulation of [^{3}H]glutamate (Glu) occurred in a temperature- and sodium-dependent manner with biochemical and pharmacological profiles similar to those seen for brain EAATs in chondrocytes cultured for 7 days, while [^{3}H]Glu accumulation consisted of a single component with a K_m of 39.1 ± 2.3 μM and a V_{max} of 1320 ± 120 pmol/mg protein/min, respectively. In organotypic cultured metatarsals isolated before vascularization from embryonic mice, where cells underwent maturational development from resting to proliferating, prehypertrophic, hypertrophic and calcified chondrocytes in a progressive order of cellular differentiation, moreover, mRNA expression was seen for GLAST, GLT-1 and EAAT4 but not for EAAC1 subtypes. Immunohistochemical analysis revealed distribution profiles different from each other with GLAST, GLT-1 and EAAT4 isoforms in sections of cultured metatarsals and isolated tibiae. These results suggest that extracellular Glu could be cleared up into intracellular locations through particular glial and/or neuronal EAAT isoforms functionally expressed by the rodent cartilage.

Keywords: Glutamate; EAATs; Chondrocytes; [^{3}H]Glutamate accumulation; Metatarsals; ATDC5

1. Introduction

In line with our previous findings in rat adrenal [1] and pituitary [2] glands, recent molecular biological studies have raised the possibility that the central excitatory amino acid neurotransmitter L-glutamate (Glu) may be one of the endogenous factors used for intercellular communications as a paracrine and/or autocrine substance in bone [3]. For example, reverse transcription polymerase chain reaction (RT-PCR) analysis reveals constitutive expression of mRNA for certain subunits of *N*-methyl-D-aspartate (NMDA) [4–6], DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionate [7] and kainate [7] receptor subtypes of ionotropic Glu receptors, as well as for metabotropic Glu receptor subtypes [6], in primary cultures of rat osteoblasts. In patch-clamped cultured rabbit osteoclasts, an antagonist at the NMDA receptor channel inhibits whole-cell membrane currents induced by Glu, with concomitant prevention of cellular differentiation of osteoclasts cultured together with bone marrow and osteoblasts [8]. The NMDA receptor antagonist dizocilpine markedly diminishes increases in both the activities of alkaline

Abbreviations: ALP, alkaline phosphatase; CCG-III, (2*S*,3*S*,4*R*)-2-(carboxycyclopropyl)glycine; CNS, central nervous system; DHK, dihydrokainate; DMEM, Dulbecco's modified Eagle's medium; EAAT, excitatory amino acid transporter; EAAC1, excitatory amino acid carrier-1; EAAT, excitatory amino acid transporter; FBS, fetal bovine serum; GLAST, glutamate aspartate transporter; GLT1, glutamate transporter-1; Glu, glutamate; HKR, HEPES Krebs-Ringer; L-THA, L-*threo*-β-hydroxyaspartate; NMDA, *N*-methyl-D-aspartate; PB, phosphate buffer; PBS, phosphate-buffered saline; PTH1R, parathyroid hormone type 1 receptors; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecylsulfate; T3MG, (±)-*threo*-3-methylglutamate; VGLUT, vesicular glutamate transporter

* Corresponding author. Tel.: +81 76 234 4471; fax: +81 76 234 4471.
E-mail address: yyoneda@anet.ne.jp (Y. Yoneda).

[1] These two authors equally contributed to this work.

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phosphatase (ALP) and Ca^{2+} accumulation, as well as expression of osteocalcin, during cellular maturation through inhibition of expression of core binding factor α-1 in cultured rat calvarial osteoblasts [9].

In central glutamatergic synapses, moreover, excitatory amino acid transporters (EAATs) need to be constitutively and functionally expressed for the termination of signaling mediated by this neuroactive amino acid, in addition to Glu receptors for the transformation of extracellular signals. EAATs are classified into five different subtypes including glutamate aspartate transporter (GLAST; EAAT1), glutamate transporter-1 (GLT-1; EAAT2), excitatory amino acid carrier-1 (EAAC1) (EAAT3), EAAT4 and EAAT5 so far [10]. Of these EAAT subtypes cloned to date, both the glial GLAST and GLT-1 are shown to reside in bone for mRNA and corresponding proteins [11,12], with the neuronal EAAC1 being absent [11]. However, we have shown constitutive expression of mRNA for EAAC1, in addition to both GLAST and GLT-1, by cultured rat calvarial osteoblasts that indeed accumulate [^{3}H]Glu in a temperature- and sodium-dependent manner [13]. A previous immunohistochemical analysis reveals the constitutive expression of GLAST in both osteoblasts and osteocytes, and of GLT-1 in mononuclear bone marrow cells, respectively [11]. Nevertheless, little attention has been paid to the possibility for cartilage to really express machineries required for central glutamatergic signaling compared to bone so far.

In the present article, therefore, we have focused on the evaluation of possible functional expression of particular isoforms of EAATs by cultured chondrocytes prepared from adult rat costicartilage, in addition to metatarsals isolated before vascularization from embryonic mice, where cells undergo maturational development from resting to proliferating, prehypertrophic, hypertrophic and calcified chondrocytes in an order of progressing differentiation without invasion by osteoblasts, osteoclasts and capillaries [14,15].

2. Materials and methods

2.1. Materials

[3,4-^{3}H]L-Glu (1587.3 GBq/mmol) was purchased from New England Nuclear/DuPont (Boston, MA, USA). QuickPrep Micro mRNA Purification Kit and Ready-To-Go You-Prime First-Strand Beads were supplied by Amersham Pharmacia Biotech (Buckinghamshire, UK) Taq polymerase was obtained from Takara (Tokyo, Japan). Quantum Prep Freeze N Squeeze DNA Gel Extraction Spin Columns and Bio-Rad Protein Assay Kit were provided by Bio-Rad Laboratories (Hercules, CA, USA). A guinea-pig polyclonal antibody against GLAST, a rabbit polyclonal antibody against GLT-1, and a rabbit polyclonal antibody against EAAT4 were purchased from Chemicon International (Temecula, CA, USA). An anti-guinea pig IgG antibody was provided by Vector Laboratories (Burlingame, CA). A rabbit polyclonal antibody against type II collagen was purchased from Rockland (Girbertsville, PA) and an anti-rabbit IgG antibody was supplied by DAKO A/S (Glostrup Denmark). (2*S*,3*S*,4*R*)-2-(Carboxycyclopropyl)-glycine (CCG-III), (±)-*threo*-3-methylglutamate (T3MG), L-*threo*-β-hydroxyaspartate (L-THA) and dihydrokainate (DHK) were provided by Tocris Cookson (Bristol, UK). Cell Counting Kit-8 was obtained from Dojindo (Osaka, Japan). Other chemicals used were all of the highest purity commercially available.

2.2. Preparation of cultured chondrocytes

Cartilage was isolated from adult female Wistar rat ribs, followed by incubation at 37 °C for 10 min in calcium- and magnesium-free phosphate-buffered saline (PBS) containing 0.1% EDTA and subsequent digestion with collagenase in Dulbecco's modified Eagle's medium (DMEM) at 37 °C for 2.5 h. Cells were collected in DMEM containing 10% fetal bovine serum (FBS) and antibiotics and then centrifuged at 500 × *g* for 5 min. The pellets were suspended in DMEM containing 10% FBS. Cells were plated at a density of $4 \times 10^4/cm^2$, followed by culturing at 37 °C under 5% CO_2 for additional 6 days. Culture medium was exchanged to DMEM supplemented with 10% FBS and 50 μg/mL ascorbic acid for subsequent culturing for different periods of time up to 28 days. Medium was changed every 2–3 days.

Mouse chondral progenitor cell line ATDC5 cells were purchased from the RIKEN cell bank (Ibaraki, Japan) and maintained as described previously [16]. In brief, cells were plated at a density of $4 \times 10^4/cm^2$ in a maintenance medium of DMEM/Ham's F12 supplemented with 5% FBS, 10 μg/mL insulin, 10 μg/mL transferrin and 3×10^{-8} M sodium selenite at 37 °C under 5% CO_2. Medium was changed every second day.

2.3. Determination of alkaline phosphatase activity

Chondrocytes were plated at a density of 4×10^4 cells/cm^2 in 24-well dishes and cultured for different periods of time up to 28 days. Cells were washed twice with cold PBS, and then sonicated in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.1% Triton X-100. Assay buffer composed of 0.05 M 2-amino-2-methylpropanol, 2 mM $MgCl_2$ and 10 mM *p*-nitrophenylphosphoric acid was added at a volume of 200 μL into 10 μL of cell suspensions, followed by a reaction for 30 min at 37 °C and subsequent immediate determination of absorbance of *p*-nitrophenol at 405 nm.

2.4. Alcian blue staining

Chondrocytes were placed in 24-well plates and cultured for different periods of time up to 21 days. Cells were

rinsed with PBS twice and then stained for 30 min with 1% Alcian blue 8GS dissolved in 3% acetic acid. Cells were washed with 3% acetic acid for 30 s three times, and then photographed with an Olympus IMT-2-21 dissecting microscope. Cells stained were dissolved in 0.1% sodium dodecylsulfate (SDS) for subsequent quantification of the absorbance at 650 nm.

2.5. Isolation of type II collagen

Purification of collagen was performed from chondrocytes cultured for different periods of time up to 28 days. After being washed with PBS, cell layers were scraped and homogenized in 0.5 M acetic acid containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM *N*-ethylmaleimide and 2.5 mM EDTA). Cell homogenates were digested with pepsin (1 mg/mL) for 24 h at 4 °C and then centrifuged at 5000 × *g* for 30 min to obtain supernatants as a sample. The pH of the solution was then raised to 8.0 with NaOH to inactivate pepsin, followed by determination of protein contents according to the Lowry method using bovine serum albumin as standard.

2.6. Immunoblotting analysis

Cell homogenates prepared as described above were dissolved in 10 mM Tris–HCl buffer containing 10% glycerol, 2% SDS, 0.01% bromophenol blue and 5% 2-mercaptoethanol at a volume ratio of 1:4, followed by boiling for 10 min and subsequent loading of an aliquot for electrophoresis on a 4.5–7.5% gradient SDS–polyacrylamide gel at 15 mA/plate for 2 h at room temperature toward blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking by 5% skim milk dissolved in 20 mM Tris–HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20 (TBST), the membrane was incubated with one of antibodies against type II collagen, GLAST and GLT-1 adequately diluted with TBST containing 1% skim milk, and then with the anti-rabbit IgG antibody or anti-guinea pig IgG antibody conjugated with horseradish peroxidase. Finally, the membrane was incubated with ECL™ detection reagent to detect immunoreactive proteins, followed by exposure to X-ray film for different periods to obtain films appropriate for subsequent quantitative densitometry.

2.7. RT-PCR

Cultured chondrocytes were washed with PBS twice, followed by extraction of mRNA using mRNA purification kit and subsequent synthesis of complementary DNA (cDNA) with 12.5 μM random hexamer primers and first-strand beads. The reverse transcriptase reaction was run at 37 °C for 60 min, and an aliquot of synthesized cDNA was directly used for PCR performed in buffer containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of deoxy nucleotide triphosphate (dNTP), 20 pmol of each primer for the corresponding EAAT isoforms (Table 1) and 2 U of Taq DNA polymerase as described previously [17]. Cycling conditions were as follows: GLAST, GLT-1, EAAC1, EAAT4 and EAAT5, denaturation for 45 s at 95 °C, annealing for 60 s at 64 °C, extension for 40 s at 72 °C. PCR cycling number was 40 for both GLAST and EAAC1, and 32 for both GLT-1 and EAAT4, respectively. Electrophoresis was run for an aliquot of PCR amplification products on a 2% agarose gel, followed by detection of DNA with ethidium bromide. Appropriate PCR DNA products were extracted from agarose gel using DNA extraction spin columns, followed by sequencing by ABI Prism 310 Genetic Analyzer (Perkin-Elmer) using cycle sequencing kit.

2.8. Determination of [^{3}H]Glu accumulation

Chondrocytes were cultured for 7 days, followed by washing with HEPES Krebs-Ringer (HKR) (125 mM NaCl, 3.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES and 10 mM D-glucose, pH 7.4) buffer twice and subsequent incubation in HKR buffer at 37 °C for 1 h in 5% CO_2 incubator. Cells were then incubated with 1 μM [^{3}H]Glu at 2 °C or 37 °C for 1–20 min, in either the presence or absence of different

Table 1
Primers for EAATs

Subunits	Upstream (5′–3′)	Downstream (5′–3′)	Estimated base pair
Rat			
EAAT1 (GLAST)	GGGTTTTCATTGGAGGGTTGC	CCACGGGTTTCTCTGGTTCAT	572
EAAT2 (GLT-1)	GGGTCATCCTGGATGGAGGT	CGTGTCGTCATAAACGGACTG	328
EAAT3 (EAAC1)	GACTGGGAAATATTCCGCAAGT	CGCACAGCGGAATGTAACTGG	209
EAAT4	AGTCAACAACTATGAGCTGAACTTT	CTTATAGGGTTTCCCCAGGC	311
EAAT5	CCATGGTCATTGTGCTCACC	TCTAGGCTGGCAGTGGCCAG	355
Mouse			
EAAT1 (GLAST)	ATCCATTGGCCTCAGTGTTC	GTTGGACTGGGAGATGAGGA	594
EAAT2 (GLT-1)	ATCCACCCTGATGTGGTCAT	TTCATCCCGTCCTTGAACTC	572
EAAT3 (EAAC1)	GCTGTGCGGAAGAAAAGAAC	GTCTGGGTGAACGAGATGGT	538
EAAT4	CAGTCAGCGCTGTGATCATT	TTTTCCAGGATGCTGGTTTC	532

inhibitors, unless indicated otherwise [13]. Reaction was terminated by the aspiration of buffer, followed by superficial rinsing with ice-cold HKR buffer containing 1 mM unlabeled Glu at 2 °C three times and subsequent solubilization with 0.1 M NaOH for liquid scintillation spectrometry using 3 mL scintillation cocktail (clear sol I). Protein concentration was determined with a Bio-Rad Protein Assay Kit.

2.9. Embryonic metatarsal rudiment organ cultures

The three central metatarsal rudiments were isolated from ddY mouse embryos at 15.5 days post-gestation. Each of middle three metatarsals was placed in a well of a 24-well plate containing 1 mL of organ culture medium: MEM supplemented with 0.05 mg/mL ascorbic acid, 1 mM β-glycerophosphate and 0.25% FBS. These explants were grown at 37 °C in a humidified 5% CO_2 incubator for a period up to 5 days. On the day of the experiments, the total length of each bone rudiment and the length of each middle mineralized part determined by Alizarin Red staining were individually measured for subsequent calculation of mineralization ratio based on the length under an Olympus IMT-2-21 dissecting microscope. Similar results were invariably obtained with the calculation of mineralization ratios based on the areas in place of the lengths. Cultured metatarsals were washed with PBS twice, followed by extraction of mRNA using mRNA purification kit and subsequent RT-PCR analyses for EAATs as mentioned above.

2.10. In situ hybridization analysis

Metatarsals cultured for 5 days and tibiae isolated from embryonic mice at 15.5 days after gestation were individually fixed with 10% formalin neutral buffer solution, followed by decalcification with 20% EDTA and subsequent immersion in 30% sucrose overnight at 4 °C, respectively. Metatarsals and tibiae were then dissected for frozen sections with a thickness of 5 μm in a cryostat. Sections mounted as described above were fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 10 min at room temperature, followed by washing three times with 0.1 M PB, treating with 0.2 M HCl for 10 min, washing 3 times with 0.1 M PB, treating with 10 μg/mL proteinase K for 5 min, and washing three times with 0.1 M PB. Sections were then subjected to acetylation in 0.1 M triethanolamine/0.25% acetic anhydride for 10 min, followed by washing with 0.1 M PB and subsequent stepwise dehydration in 70, 80, 90, 95 and 100% ethanol for 5 min each. After being dried, sections were covered with the hybridization buffer [10% dextran sulfate, 5XSSC, 20 mM Tris–HCl (pH 8.0), 300 mM NaCl, 50% formamide, 1× Denhard's, and 500 μg/mL yeast tRNA] containing 25 μg/mL salmon sperm DNA at 65 °C for 1 h, and then incubated with hybridization buffer containing the digoxigenin (DIG)-labeled cRNA probe for parathyroid hormone type 1 receptors (PTH1R) at 65 °C for 16 h as described elsewhere [18]. Post-hybridization washes were done stepwise with 4XSSC at 65 °C for 20 min, 50% formamide in 2XSSC at 65 °C for 30 min, 10 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA and 500 mM NaCl (TNE buffer) at 37 °C for 10 min three times, 4 μg/mL RNase A in TNE buffer at 37 °C for 30 min, TNE buffer at 37 °C for 10 min, 2XSSC at 65 °C for 30 min, 0.2XSSC at 65 °C for 30 min, Buffer1 [100 mM Tris–HCl (pH 7.5), 150 mM NaCl] at room temperature for 10 min, and then 1.5% blocking buffer (1.5% blocking reagent in Buffer 1) at room temperature for 1 h. Subsequently, sections were washed with Buffer 1 at room temperature, and then incubated with 0.75 U/mL anti-DIG-AP Fab fragments in 0.5% blocking buffer containing 0.2% Tween at 4 °C for 16 h. After being washed four times with Buffer 1 containing 0.2% Tween at room temperature for 15 min, sections were treated with Buffer 2 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), and then with Buffer 2 containing 375 μg/mL nitro blue tetrazolium chloride and 188 μg/mL 5-bromo-4-chloro-3-indolyl phosphate for different periods to obtain pictures most appropriate for subsequent development. After washing with Buffer 2 at room temperature for 5 min, the development was stopped by incubation in 1× TE [10 mM Tris–HCl (pH 7.5), 1 mM EDTA (pH 8.0)].

2.11. Immunohistochemistry

Immunohistochemical detection of each EAAT isoform was done as described previously [18]. Metatarsals cultured for 5 days and tibiae isolated from embryonic mice at 15.5 days after gestation were individually fixed with 10% formalin neutral buffer solution, followed by decalcification with 20% EDTA and subsequent immersion in 30% sucrose overnight at 4 °C, respectively. Metatarsals and tibiae were then dissected for frozen sections with a thickness of 5 μm in a cryostat at −20 °C. Sections were fixed with 4% paraformaldehyde in PBS for 20 min, followed by washing with PBS, treating with 0.3% H_2O_2 in methanol for 30 min and washing with 70% ethanol for 5 min. After being washed with PBS, sections were subjected to blocking with PBS containing normal goat serum or BSA and 0.1% Triton X-100 at room temperature for 1 h. Sections were then reacted with antibodies against GLAST (1:40 dilution), GLT-1 (1:40 dilution) and EAAT4 (1:40 dilution) diluted with the same blocking buffer at room temperature overnight, followed by reaction with biotinylated anti-guinea pig IgG antibody at room temperature for 30 min and subsequent incubation with VECTASTAIN Elite ABC Reagent at room temperature for 1 h. Finally, immunostaining was done using 0.05% diaminobenzidine and 0.03% hydrogen peroxide using hematoxylin as counterstaining. Simultaneous experiments were invariably done in the absence of each primary antibody to confirm expression of the respective immunoreactive proteins.

2.12. Data analysis

Results are all expressed as the mean ± S.E. and the statistical significance was determined by the two-tailed and unpaired Students' *t*-test or the one-way analysis of variance ANOVA with Bonferroni/Dunnett post hoc test.

3. Results

3.1. Rat costal chondrocytes in culture

Costicartilage was isolated from adult female Wistar rat ribs, followed by digestion with collagenase and collection of cells toward subsequent plating for culture. Cultured cells underwent proliferation toward a confluent state for a period up to 14 days, followed by differentiation to form nodules on 14 days and subsequent calcification on 28 days (Fig. 1a). Expression of the chondral marker protein type II collagen was not detected in cells cultured for 3 days on Western blotting analysis, with gradually increased expression in costal chondrocytes cultured for 9–28 days with a peak at 15 days (Fig. 1b). In proportion to increasing culture periods up to 18 days, a linear increase was found in Alcian Blue staining used for detection of acidic mucopolysaccharide in cultured rat costal chondrocytes when quantified after extraction of the dye on spectrometry (Fig. 1c). A marked increase was also seen in the activity of ALP in chondrocytes cultured for a period of 4–18 days with a plateau thereafter up to 28 days (Fig. 1d).

3.2. Expression of EAAT mRNA in rat chondrocytes

In order to analyze expression of mRNA for EAATs, mRNA was extracted from samples for subsequent RT-PCR using specific primers for each EAAT. Rat whole brain exhibited marked expression of mRNA for GLT-1 and EAAC1, with expression of both GLAST and EAAT4 in rat cerebellum and EAAT5 mRNA in rat retina, respectively (Fig. 2a). In chondrocytes cultured for 7–21 days, mRNA was invariably expressed for GLAST, GLT-1 and EAAC1, but not for either EAAT4 or EAAT5 irrespective of the maturity of cultured chondrocytes. Sequencing analysis on amplified PCR products clearly confirmed the expression of mRNA for corresponding EAAT isoforms in immature and mature cultured chondrocytes. In chondrocytes cultured for 7–21 days, moreover, considerably weaker immunoblots were detected for both GLAST and GLT-1 at the corresponding molecular weight positions than those from rat whole brain on the gel when

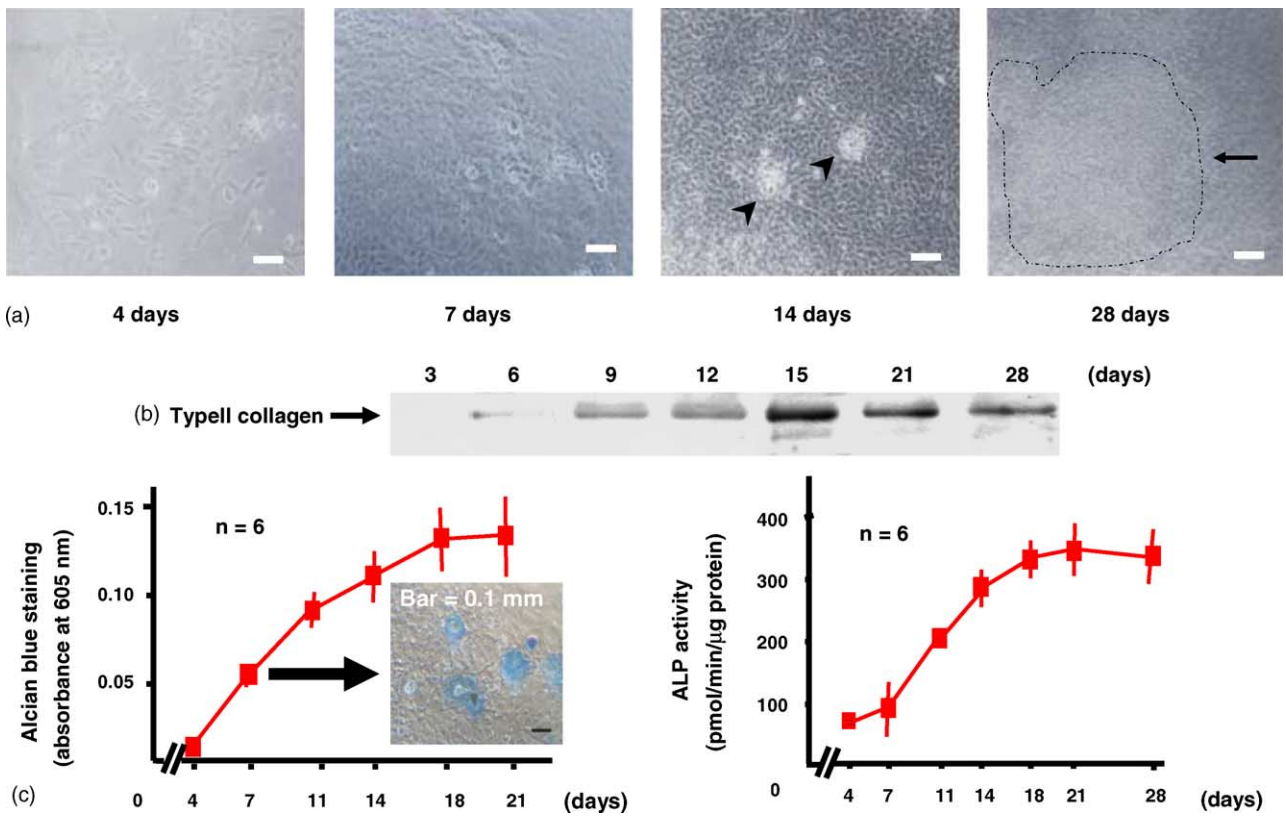

Fig. 1. Maturation of cultured chondrocytes. Costicartilages were isolated from adult rats, followed by digestion and subsequent cultivation of chondrocytes in DMEM for a period of 4–28 days. (a) Typical pictures are shown with phase contrast micrograph in the upper panels, where black arrowheads indicate nodules formed during culturing and a dotted line surrounds the area of calcification as shown by a black arrow. (b) Typical immunoblots are presented with type II collagen in the middle panels. (c) In the lower left panel, quantitative data are shown for Alcian Blue staining with a typical picture of chondrocytes cultured for 7 days in the inset. (d) In the lower right panel, the activity of ALP is shown. Values are the mean ± S.E. from six independent experiments.

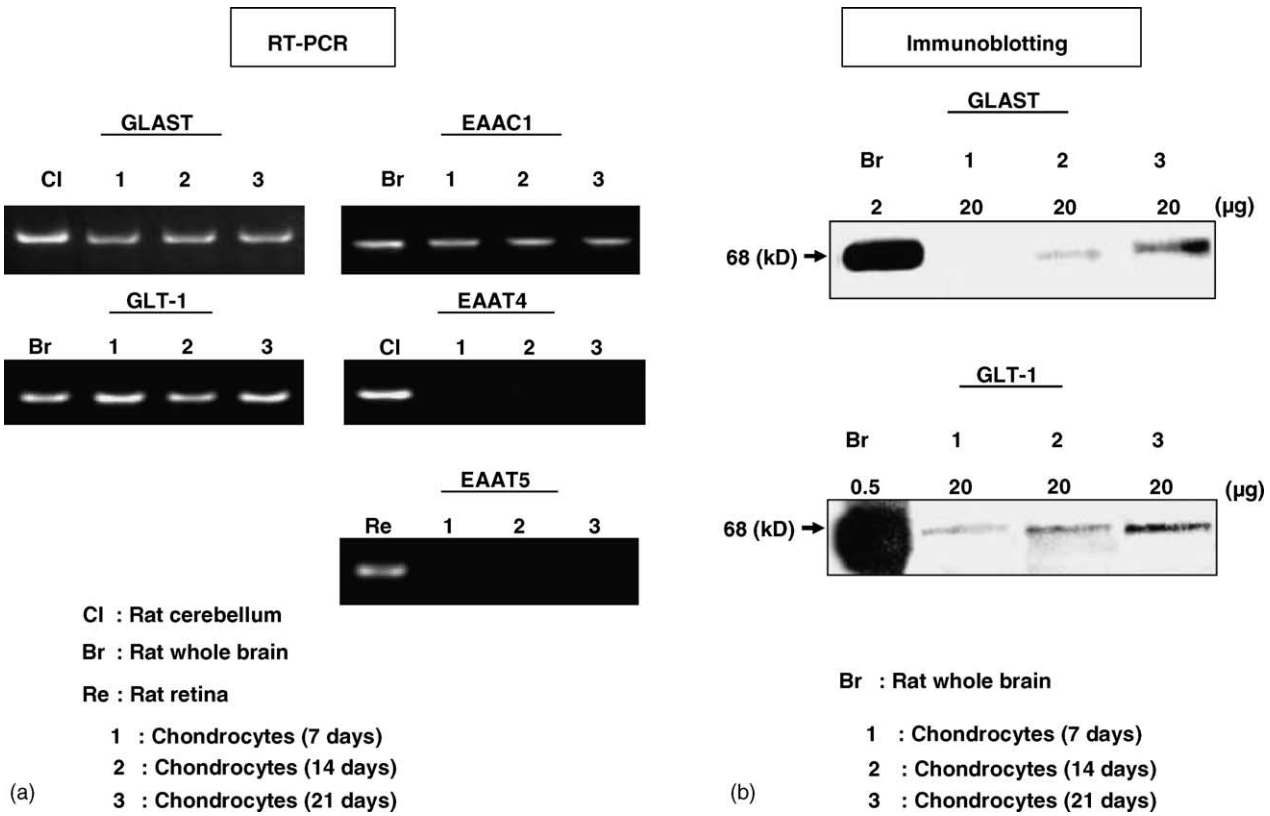

Fig. 2. Expression of EAATs in cultured chondrocytes. (a) Chondrocytes were cultured for 7–21 days, followed by isolation of mRNA and subsequent RT-PCR using primers specific for each rat EAAT. (b) Chondrocytes were cultured for 4–21 days, followed by homogenization and subsequent loading of an aliquot (20 μg protein) for immunoblotting assays using an antibody against GLAST or GLT-1. Rat whole brain, cerebellum and retina were also subjected to RT-PCR or immunoblotting simultaneously done under the same experimental conditions as positive controls. Typical pictures are shown in the figure with similar results in three separate determinations.

determined by Western blotting assays under the experimental conditions used (Fig. 2b).

3.3. [^{3}H]Glu uptake in rat chondrocytes

In order to evaluate the functionality of EAAT isoforms expressed by chondrocytes, an attempt was made to determine whether the substrate Glu is indeed incorporated through particular EAAT subtypes in these cultured cells. Chondrocytes cultured for 7–21 days were incubated with 1 μM [^{3}H]Glu in HKR buffer at 2 °C or 37 °C for different periods up to 100 min, followed by rapid aspiration of buffer and subsequent rinsing with ice-cold buffer containing unlabeled Glu at 1 mM three times. Under these experimental conditions [^{3}H]Glu accumulation was not markedly affected by the later addition of 1 mM unlabeled Glu in cultured chondrocytes (data not shown). The accumulation of [^{3}H]Glu almost linearly increased with incubation time up to 20 min and reached a plateau within 30 min at 37 °C in cells cultured for 7–21 days, while no marked accumulation of [^{3}H]Glu was seen even 30 min after the initiation of incubation at 2 °C in cells cultured for 7 days (Fig. 3a). In the chondrocyte cell line ATDC5 cells cultured for 7 days, a similarly linear increase was seen for [^{3}H]Glu accumulation for a period up to 20 min at 37 °C with a plateau thereafter up to 90 min, while no marked accumulation was detected for [^{3}H]Glu at 2 °C up to 30 min (Fig. 3b).

3.4. Biochemical profiles of [^{3}H]Glu uptake in rat chondrocytes

In order to confirm the net incorporation of [^{3}H]Glu into intracellular locations, chondrocytes cultured for 7 days were incubated with 1 μM [^{3}H]Glu at 37 °C for 5 min in HKR buffer containing the membrane selective surfactant digitonin at different concentrations. As shown in Fig. 4a [^{3}H]Glu accumulation was significantly inhibited by the addition of digitonin in a concentration-dependent manner at a concentration of above 10 μg/mL. The accumulation was completely abolished by digitonin at 100 μg/mL.

[^{3}H]Glu accumulation was also significantly inhibited in a concentration-dependent manner by both the potassium ionophore valinomycin (Fig. 4b) and the sodium ionophore monensin (Fig. 4c) at 0.1–10 μM. Replacement of sodium chloride with choline chloride led to marked abolition of the accumulation of [^{3}H]Glu in a concentration-dependent manner, while replacement with sodium gluconate induced a slight but statistically significant decrease in the accumulation of [^{3}H]Glu in chondrocytes cultured for 7 days (Fig. 4d).

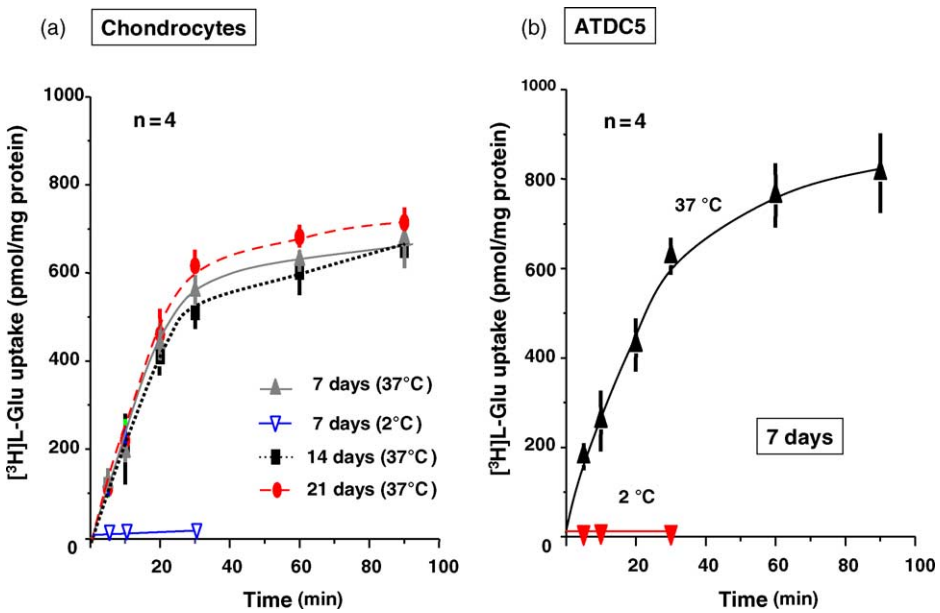

Fig. 3. [^{3}H]Glu accumulation in cultured chondrocytes. (a) Chondrocytes cultured for 7–21 days were incubated with 1 μM [^{3}H]Glu at 2 or 37 °C for different periods of up to 90 min in HKR buffer, followed by aspiration of buffer and subsequent rinsing with buffer containing unlabeled Glu at 1 mM. (b) The chondral progenitor cell line ATDC5 cells were cultured for 7 days and incubated with 1 μM [^{3}H]Glu at 2 or 37 °C for different periods up to 90 min. Values are the mean ± S.E. from four independent experiments.

3.5. Kinetics of [^{3}H]Glu uptake in rat chondrocytes

Chondrocytes cultured for 7 days were incubated with different concentrations of [^{3}H]Glu at a concentration range of 1– 250 μM at 37 °C for 5 min in HKR buffer for determination of saturation isotherms. The accumulation increased with increasing concentrations of [^{3}H]Glu followed by a saturable profile at a concentration above

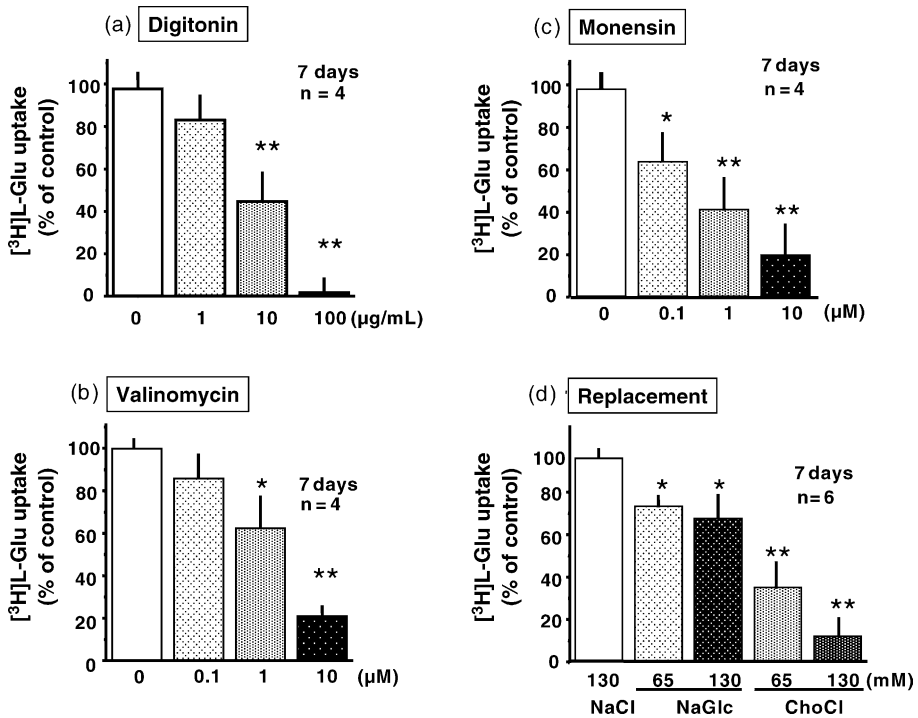

Fig. 4. Biochemical properties of [^{3}H]Glu accumulation in cultured chondrocytes. Chondrocytes cultured for 7 days were incubated with 1 μM [^{3}H]Glu at 37 °C for 5 min in HKR buffer containing (a) digitonin at concentrations of below 100 μg/mL, (b) valinomycin and (c) monensin at a concentration range of up to 10 μM. (d) Chondrocytes cultured for 7 days were also incubated with 1 μM [^{3}H]Glu at 37 °C for 5 min in HKR buffer where sodium chloride was replaced with equimolar choline chloride (ChoCl) or sodium gluconate (NaGlc) as needed. Values are the mean ± S.E. of four to eight independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, significantly different from each control value obtained with normal HKR buffer.

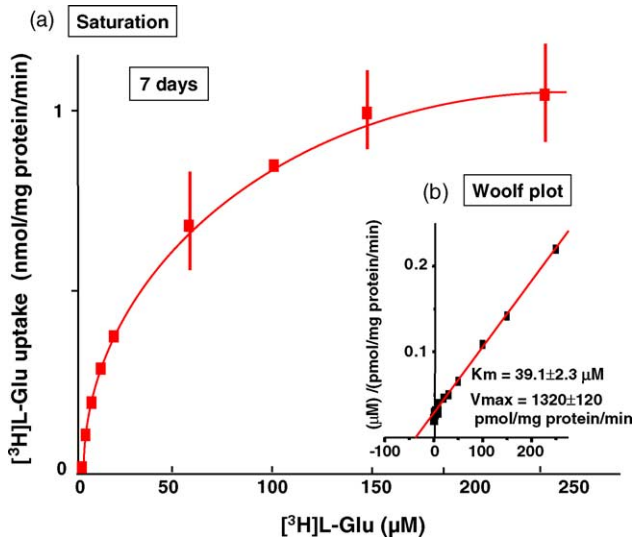

Fig. 5. Kinetic analysis on [^{3}H]Glu accumulation in cultured chondrocytes. Chondrocytes were cultured for 7 days, followed by incubation with [^{3}H]Glu at different concentrations from 1 to 250 μM for 5 min at 37 °C for determination of [^{3}H]Glu accumulation. Values are the mean ± S.E. of three separate determinations (a). Inset: Woolf-Hanes plot analysis was done with the data obtained for [^{3}H]Glu accumulation in chondrocytes cultured for 7 days (b).

150 μM within a concentration range of up to 250 μM in chondrocytes cultured for 7 days (Fig. 5a). Woolf-Hanes plot analysis revealed that [^{3}H]Glu accumulation consisted of a single component within a substrate concentration range of 1–250 μM in chondrocytes cultured for 7 days (Fig. 5b). In chondrocytes cultured for 7 days [^{3}H]Glu accumulation was composed of one single component with a K_m value of 39.1 ± 2.3 μM and a V_{max} value of 1320 ± 120 pmol/min/mg protein, respectively, at the substrate concentration range employed.

3.6. Pharmacological profiles of [^{3}H]Glu uptake in rat chondrocytes

Chondrocytes cultured for 7 days were incubated with 1 μM [^{3}H]Glu in HKR buffer containing a variety of amino acids at 1 mM for 5 min at 37 °C. Of the amino acids tested, L-Glu was most effective in inhibiting [^{3}H]Glu accumulation in chondrocytes cultured for 7 days with progressively less efficient inhibition by L- and D-aspartate, cystine and L-cysteine, while [^{3}H]Glu accumulation was not significantly affected by D-Glu, L-alanine and L-serine at 1 mM (Fig. 6a). Cells were also incubated with several EAAT inhibitors at a concentration range of 0.1 μM to 1 mM for 5 min at 37 °C. Of the 4 different inhibitors tested, GLT-1 inhibitors such as T3MG (IC_{50} = 3.7 ± 0.22 μM) and DHK (IC_{50} = 1.8 ± 0.13 μM) were more potent in inhibiting [^{3}H]Glu accumulation than the non-selective inhibitors L-CCG-III (IC_{50} = 89.3 ± 10.2 μM) and THA (IC_{50} = 22.1 ± 2.4 μM) (Fig. 6b).

3.7. Expression of EAAT mRNA in mouse metatarsals

Metatarsals before vascularization were isolated from embryonic mice at 15.5 days after gestation, followed by organotypic culture for different periods up to 5 days. In proportion to increasing culture days, marked increases were seen in both the total length of the cartilage rudiment

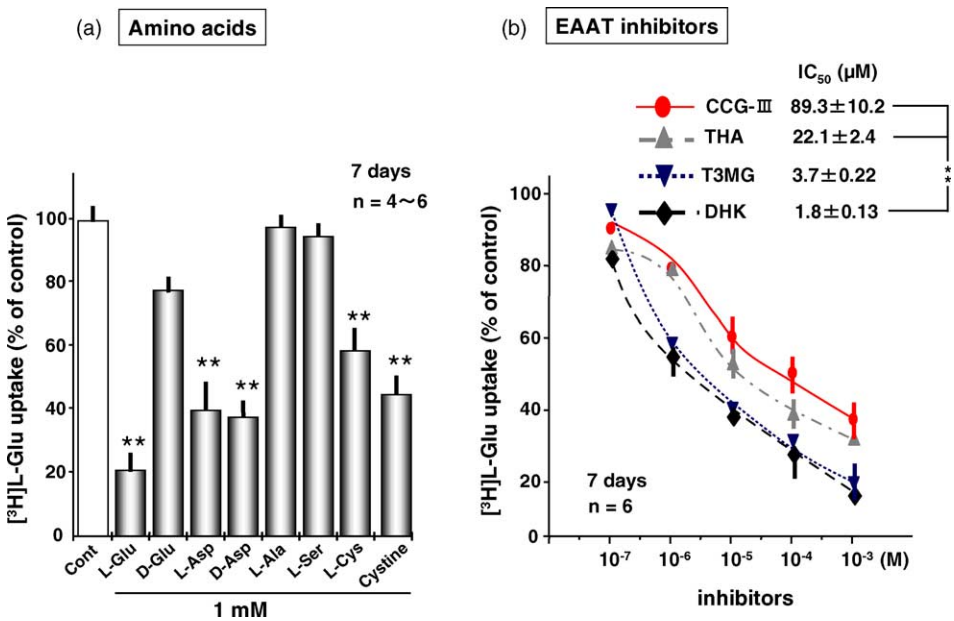

Fig. 6. Pharmacological properties of [^{3}H]Glu accumulation in cultured chondrocytes. (a) Chondrocytes cultured for 7 days were incubated with 1 μM [^{3}H]Glu at 37 °C for 5 min in either the presence or absence of various amino acids at 1 mM. Values are the mean of 4–6 separate determinations. $^{**}P < 0.01$, significantly different from each control value obtained in the absence of any test compounds added. (b) Chondrocytes cultured for 7 days were incubated with 1 μM [^{3}H]Glu at 37 °C for 5 min in HKR buffer containing the four different EAAT inhibitors at a concentration range of 0.1 μM to 1 mM. Values are the mean ± S.E. from six independent experiments. $^{**}P < 0.01$, significantly different from the value obtained in the presence of DHK.

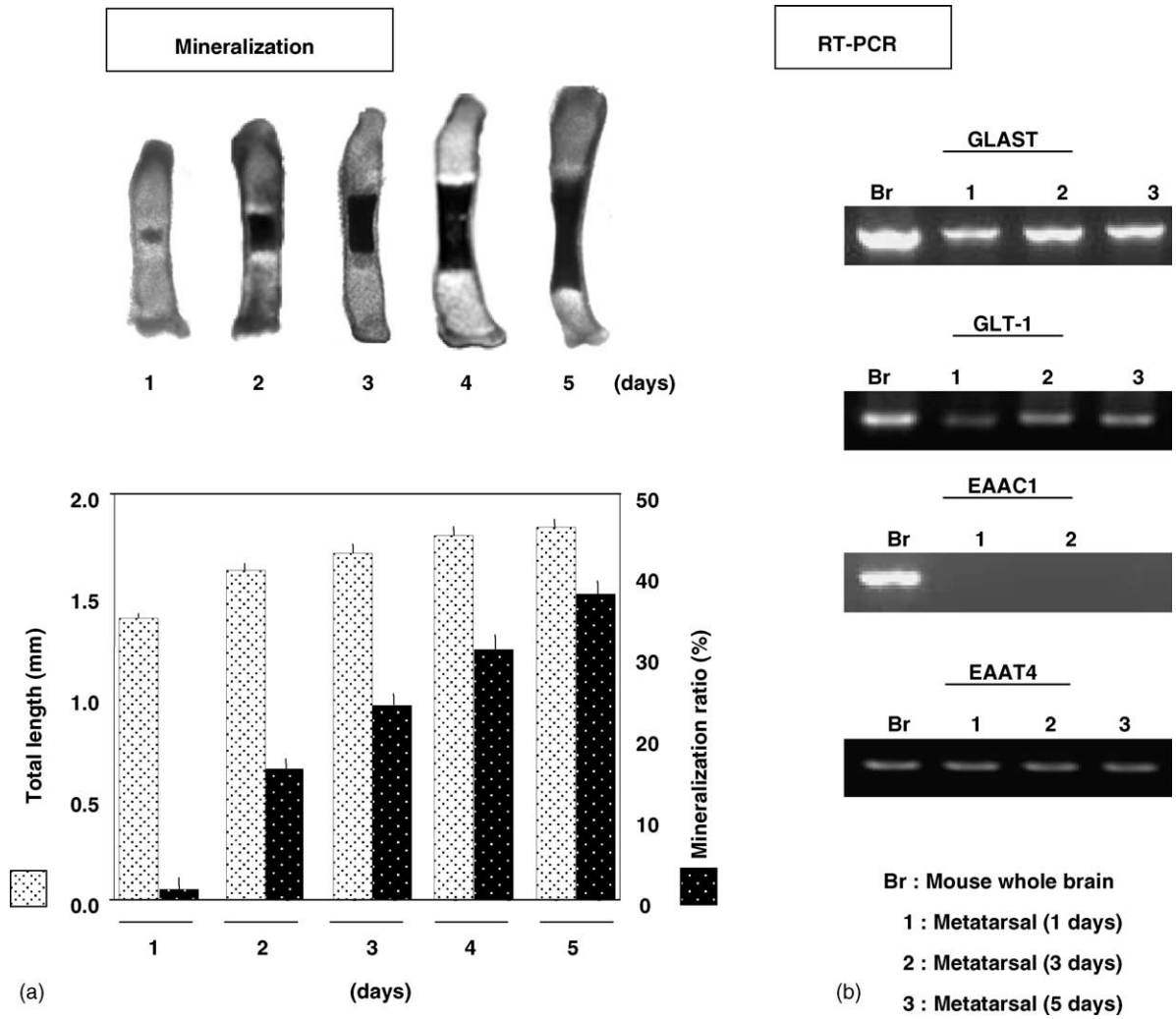

Fig. 7. Expression of EAATs in cultured metatarsals. (a) Metatarsal rudiments before vascularization were isolated from embryonic mice at 15.5 days after gestation, followed by organ culture in MEM for a period up to 5 days and subsequent determination of the total length and mineralization ratio as described in the text. Typical pictures are shown in the left panels, while quantitative data are shown in the right panels as the mean ± S.E. in four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from each control value obtained in metatarsals cultured for 1 day. (b) Metatarsals before vascularization were cultured in MEM for different days up to 5 days, followed by extraction of mRNA and subsequent RT-PCR using primers specific for each mouse EAAT. Mouse whole brain was used as a positive control. These experiments were repeated at least four times with similar expression profiles.

and the length of the middle mineralized part in cultured metatarsals (Fig. 7a). Similar mineralization ratios were invariably obtained by the calculation based on the areas of the mineralized part assessed by Alizarin Red staining in cultured metatarsals (data not shown). In these cultured metatarsals, constitutive expression was seen with mRNA for GLAST, GLT-1 and EAAT4, but not for EAAC1, irrespective of the culture period (Fig. 7b).

3.8. Expression profiles of EAATs in mouse metatarsals and tibiae

Metatarsals cultured for 5 days were dissected for frozen sections to evaluate distribution profiles of each EAAT in particular chondral cell types. In these sections, expression of mRNA for PPR was highly restricted to prehypertrophic chondrocytes, but not in other chondral cells at particular differentiation stages, on in situ hybridization analysis (Fig. 8a, left panel). These included resting, proliferating, hypertrophic and calcified chondrocytes. Immunoreactive cells were invariably detected for GLAST, GLT-1 and EAAT4 in hypertrophic chondrocytes in these cultured metatarsals, while a high immunoreactivity was only seen for GLT-1, but neither GLAST nor EAAT4, in resting to proliferating chondrocytes (Fig. 8a, right three panels). In prehypertrophic chondrocytes, moreover, immunoreactive cells were detected for both GLT-1 and EAAT4, but not for GLAST. In sections not treated with each primary antibody, no marked immunoreactivity was detected in any chondral cells seen in cultured metatarsals (data not shown).

In order to confirm the expression of particular isoforms of EAATs in chondrocytes, tibiae were isolated from embryonic mice at 15.5 days after gestation, followed

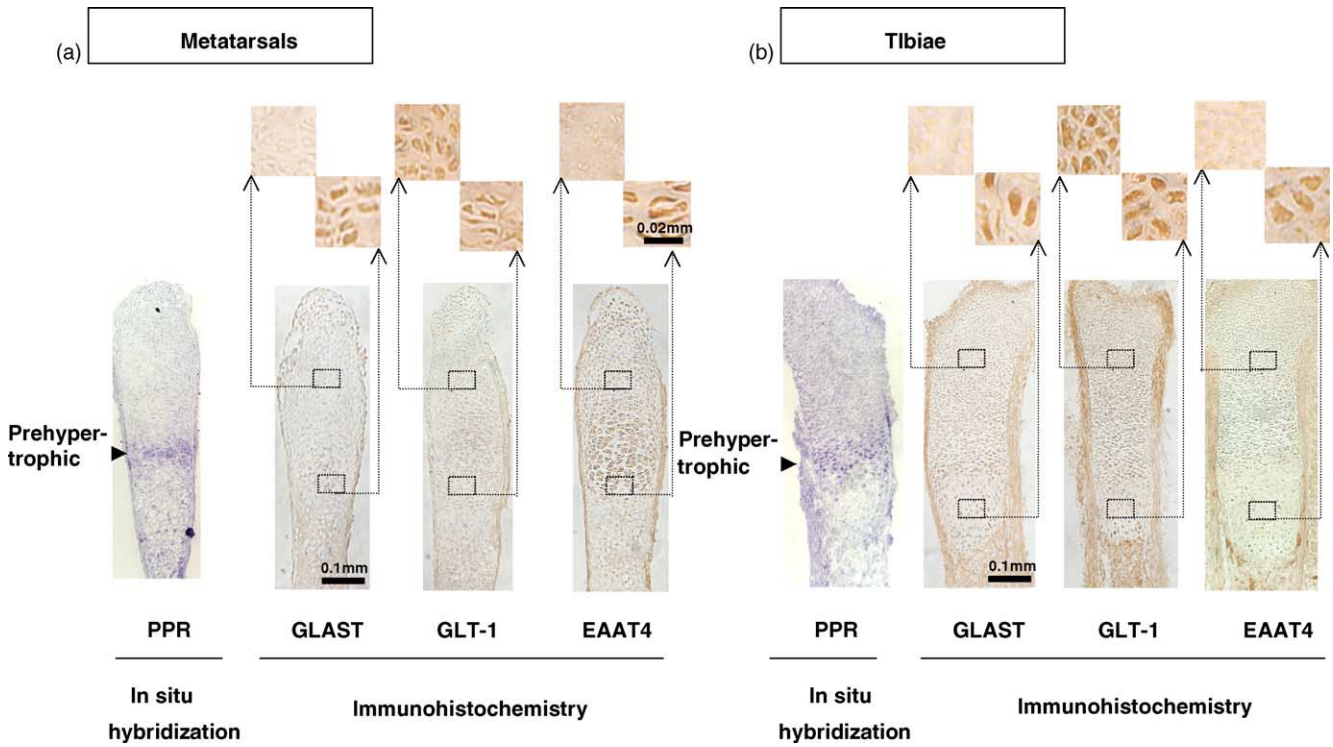

Fig. 8. Distribution profiles of EAATs in cultured metatarsals and isolated tibiae. (a) Metatarsals before vascularization were cultured in MEM for 5 days, followed by fixation with formalin and subsequent dissection of frozen sections in a cryostat for in situ hybridization analysis using a specific probe for PTH1R. Sections were also subjected to detection of the individual EAAT isoforms on immunohistochemical analysis using an antibody against GLAST, GLT-1 or EAAT4. Typical micrographic pictures are shown in this figure, while similar results were invariably obtained in at least three independent determinations. (b) Tibiae were isolated from embryonic mice at 15.5 days after gestation, followed by fixation with formalin and subsequent dissection of frozen sections in a cryostat for in situ hybridization analysis using a specific probe for PPR. Sections were also subjected to detection of the individual EAAT isoforms on immunohistochemical analysis using an antibody against GLAST, GLT-1 or EAAT4. Typical micrographic pictures are shown in this figure, while similar results were invariably obtained in at least three independent determinations.

by immediate dissection of frozen sections for subsequent in situ hybridization and immunohistochemistry analyses as done with metatarsals cultured for 5 days. Similarly high localization was seen with expression of mRNA for PTH1R to prehypertrophic chondrocytes in tibiae on in situ hybridization evaluation (Fig. 8b, left panel). As seen in cultured metatarsals isolated before vascularization, immunohistochemical analysis revealed a wide distribution profile of immunoreactive GLT-1 in chondrocytes at different stages of differentiation, with immunoreactive GLAST being localized in prehypertrophic to hypertrophic chondrocytes (Fig. 8b, right three panels). Immunoreactive EAAT4 was also distributed from prehypertrophic to hypertrophic chondrocytes, but not in resting to proliferating cells, in these tibial sections.

4. Discussion

The essential importance of the present findings is that radiolabeled Glu was indeed accumulated in a temperature- and sodium-dependent manner in primary cultured rat costal chondrocytes that expressed mRNA and corresponding proteins for particular isoforms of EAATs. The accumulation of [^{3}H]Glu also occurred in chondrocytes with pharmacological profiles basically similar to those seen with astrocytes and/or neurons in the brain. To our knowledge, this paper deals with the first direct demonstration of the functional expression of particular isoforms of EAATs absolutely required for the central glutamatergic signaling system by cultured rat costal chondrocytes. Several independent lines of evidence indicate the constitutive expression of both mRNA and corresponding proteins for the glial EAAT subtypes GLAST and GLT-1 in rat bone [11,12], which is also confirmed with mRNA expression and [^{3}H]Glu accumulation using cultured rat calvarial osteoblasts in our previous study [13]. The fact that chondrocytes were superficially rinsed three times with buffer containing unlabeled Glu at a molar ratio of 1000 over radiolabeled Glu in the present experimentation, gives rise to an idea that [^{3}H]Glu accumulation shown here is really derived from the net incorporation of the radiolabeled substrate into intracellular spaces in cultured chondrocytes but not due to the simple adsorption and/or binding of the radioactive substrate to cellular surfaces. The almost complete abolition by digitonin indeed gives strong support to this idea.

By contrast, one possible discouraging speculation is that the chondral functional expression may be derived from osteoblastic cells contaminated in cultured chondrocytes isolated from rat costicartilage. In the present study,

however, immunohistochemical analysis clearly revealed the localized expression of immunoreactive EAAT isoforms in particular chondrocytes resided in tibiae and metatarsals, where we have confirmed the absence of the osteoblastic marker protein type-1 collagen on in situ hybridization (unpublished data). Moreover, metatarsals isolated on E15.5 from embryonic mice have been considered as pure cartilage rods devoid of osteoblasts, osteoclasts and capillaries even when cultured for 5 days after the isolation [14,15]. During embryogenesis the mesenchymal precursor cells differentiate into skeletal elements by forming a cartilaginous model, which then induces bone formation known as endochondral ossification in the vertebral column and long bones [19]. Shortly after the mineralization process takes place, most hypertrophic chondrocytes undergo the sustained apoptotic process. Upon death of chondrocytes after mineralization, osteoblasts, osteoclasts and capillaries begin to invade the cartilage matrix to produce new bone, leading to growth of endochondral bones [20]. Although it thus seems clear that EAAT isoforms are indeed expressed by chondrocytes in addition to osteoblasts, their exact functionality in mechanisms associated with endochondral ossification remains to be elucidated in future studies.

The more potent inhibition by the GLT-1 selective inhibitors T3MG and DHK than the non-selective EAAT inhibitors CCG-III and THA argues in favor of the view that the glial EAAT isoform GLT-1 would play a role more crucial for mediating the temperature- and sodium-dependent accumulation of [^{3}H]Glu compared to either the glial isoform GLAST or the neuronal isoform EAAC1, which are all expressed by cultured rat costal chondrocytes. Indeed, DHK specifically inhibits GLT-1-mediated Glu uptake with a K_i value of 23 μM, but has no significant effect on the activities of both GLAST and EAAC1 at concentrations below 1 mM when determined in transfected COS7 cells [21]. In addition to DHK, T3MG is also a selective inhibitor of GLT-1 with an IC_{50} value of 90 μM, while IC_{50} values are over 1 mM for GLAST and EAAC1 in transfected oocytes [22]. The absence of EAAC1 mRNA from cultured mouse metatarsals and/or of EAAT4 mRNA from cultured rat chondrocytes could be due to a difference between species used. Indeed, sequential homologies vary from an isoform to another isoform of EAATs between rats and mice, with EAAT5 being not cloned in mice to date [10,23]. Alternative expression between the neuronal isoforms EAAC1 and EAAT4 is thus conceivable in a particular situation. In a recent paper, furthermore, β-lactam antibiotics are shown to stimulate the expression of GLT-1 through increased transcription without affecting that of GLAST, EAAC1 and EAAT4 isoforms in cultured rat organotypic spinal cord slices [24]. Although penicillin was also included in culture medium for rat chondrocytes and mouse metatarsals in the present study, the possible artifactitious induction of GLT-1 during cultivation is ruled out by taking into consideration similar expression profiles between sections prepared from cultured metatarsals and isolated tibiae for different EAAT isoforms including GLT-1.

A splice variant of GLAST lacking exon 3 is expressed in both bone and brain [12], while several N-terminal or C-terminal splicing variants of GLT-1 are also found in different rat, mouse and human tissues [25]. Although PCR primers used are for all known splicing variants of GLAST and GLT-1, in this study, whereas the antibody used, which is directed against the C-terminal peptide AANGKSADCSVEEEPWKREK of wild-type GLT-1, could not recognize the C-terminal splicing variant of GLT-1. From the aforementioned point of view, conventional determination of [^{3}H]Glu accumulation is still crucial for elucidation of the functional significance as well as exact mechanism for regulation of cellular homeostasis by different EAAT isoforms expressed by particular neuronal and non-neuronal tissues, in addition to molecular biological strategies including RT-PCR, Western and Northern blotting, in situ hybridization and immunohistochemistry.

Glutamatergic innervations would be distributed not only in the CNS, but also in peripheral non-neuronal tissues including bone and cartilage. Glu could be classified into autacoids as done with histamine and serotonin, whereas a concept for the paracrine and/or autocrine signal mediator should be re-considered for establishment of the autocrine hypothesis for Glu. The lack of phenotypic alterations of the bone growth in mice knocked out of GLAST [26] would be brought about through the activity of Glu transport compensated by other EAAT subtypes co-expressed in osteoblasts even in the absence of GLAST. On the other hand, EAATs are not only required for the termination of glutamatergic signaling, but also needed for the disinhibition of the Glu/cystine antiporter xc^- [27] through the reduction of extracellular Glu concentrations. The antiporter is essential for the biosynthesis of glutathione that is also effective in preventing cell death by a variety of reactive oxygen species. The accumulation of extracellular Glu would result in the depletion of intracellular glutathione through inhibition of the activity of the antiporter required for the entry of extracellular cystine to provide a substrate for this protective tripeptide.

It thus appears that extracellular Glu may be incorporated into intracellular locations through particular isoforms of EAATs for possible subsequent events such as signal termination and/or glutathione synthesis in cartilage as well as in bone. Evaluation of chondral EAATs could be of a great benefit for the future elucidation of molecular mechanisms underlying the crisis of a variety of chondral diseases associated with the degenerative malfunction of chondrocytes including rheumatoid arthritis and osteoarthritis.

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