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Excitatory amino acid transporters expressed by synovial fibroblasts in rats with collagen-induced arthritis

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Abstract

Although previous studies have demonstrated increased levels of the brain neurotransmitter glutamate (Glu) in the synovial fluid from patients with arthritis, not much attention has been paid to the possible role of Glu in joint synovial tissues to date. Constitutive expression of mRNA was for the first time shown with glutamate aspartate transporter, glutamate transporter-1 and excitatory amino acid carrier-1 (EAAC1), in addition to with particular ionotropic and metabotropic Glu receptors, in cultured synovial fibroblasts prepared from knee joints of male Lewis rats. Immunohistochemical analysis revealed high localization of immunoreactive EAAC1 at synovial tissues. The accumulation of [^{3}H]Glu occurred in a temperature- and sodium-dependent manner in cultured synovial fibroblasts, with a K_m of 23.1 ± 1.1 μM and a V_{max} of 237.1 ± 31.1 pmol/(mg protein min), respectively. In rats with arthritis induced by immunization to type-II collagen, marked increases were seen in hind paw volume, cytokine mRNA expression and Glu levels in synovial tissues, in addition to histological erosion. In cultured synovial fibroblasts prepared from these arthritic rats, [^{3}H]Glu accumulation was drastically increased with biochemical and pharmacological profiles similar to those seen in normal synovial fibroblasts. The exposure to Glu at 500 μM doubled the incorporation of 5-bromo-2′-deoxyuridine in cultured synovial fibroblasts of arthritic but not normal rats, without significantly affecting mRNA expression of different cytokines in both synovial fibroblasts. These results suggest that Glu may at least in part play a role in mechanisms associated with cellular proliferation through particular transporters functionally expressed by synovium in rheumatoid arthritis.

Keywords: EAATs; Glutamate; Rheumatoid arthritis; [^{3}H]glutamate accumulation; Cell proliferation; Synovial fibroblasts; GluRs

1. Introduction

Rheumatoid arthritis (RA) is a systemic disorder characterized by synovial inflammation and subsequent destruction and deformity of synovial joints. Although the pathogenesis and etiology of this disabling disease are not well understood to date, activated synovial fibroblasts are thought to be a key player in mechanisms underlying the joint destruction in RA [1,2]. Type-II collagen-induced arthritis (CIA) in Lewis rats is a widely used experimental animal model of inflammatory polyarthritis with clinical and pathological features similar to those seen with RA in humans, which are dependent on both humoral and cellular immunity to the immunizing antigen [3,4]. Previous studies have demonstrated a drastic

Abbreviations: A.B., Alcian Blue; AMPA, DL-α-amino-3-hydroxy-5-methylisoxasole-4-propionate; BrdU, 5-bromo-2′-deoxyuridine; CIA, collagen-induced arthritis; EAAC1, excitatory amino acid carrier-1; EAAT, excitatory amino acid transporter; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter-1; Glu, glutamate; H.E., Hematoxylin and Eosin; HKR, Hepes-buffered Krebs-Ringer; iGluR, ionotropic glutamate receptor; IL-1β, interleukin-1β; KA, kainate; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; RANKL, receptor activator nuclear factor-κB ligand; RT-PCR, reverse transcription polymerase chain reaction; RA, rheumatoid arthritis; TNF-α, tumor necrosis factor-α

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increase in endogenous levels of both glutamate (Glu) and aspartate in the synovial fluid obtained from patients with arthritis [5]. The elevation of Glu in synovial fluid is shown to be relevant to increased edema and sensitization to thermal hyperalgesia in experimental arthritis models [6,7].

On the other hand, Glu is one of the most abundant free amino acids with an excitatory neurotransmitter role in the vertebrate central nervous system (CNS), while recent trends are toward a role in neuronal differentiation, migration and survival in the developing brain [8,9]. The actions of extracellular Glu are mediated by membrane receptors, which can be divided into two major groups [9]. One is ionotropic Glu-gated ion channels (iGluRs) that are further classified into DL-α-amino-3-hydroxy-5-methylisoxasole-4-propionate (AMPA), kainate (KA) and *N*-methyl-D-aspartate (NMDA) subtypes according to sequential similarities as well as responsiveness to different agonists and antagonists [10,11], whereas the other is G-protein-coupled metabotropic receptors (mGluRs) that are a member of the class 3 G protein-coupled receptor family [12,13]. 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. These 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 [14,15]. Recently, evidence that glutamatergic signaling is also functional in non-neuronal tissues, such as bone, pancreas and skin, is accumulating in addition to an excitatory amino acid neurotransmitter role in the CNS in the literature [16,17]. In articulation, for example, we have shown the functional expression by cultured rat costal chondrocytes and mouse metatarsals isolated before vascularization of the glial EAAT isoforms GLAST and GLT-1 that are both required for signal termination at glutamatergic synapses in the brain [18]. It is now conceivable that Glu may act as a more widespread "cytokine" rather than a "neurotransmitter" to influence a variety of cellular activities in a range of different tissue types.

However, no much attention has been paid to the physiological and pathological significance of Glu in articular synovial tissues to date. In the present study, therefore, we have attempted to demonstrate the possible involvement of Glu in mechanisms underlying RA through evaluation of the functional expression of different glutamatergic signaling machineries using synovial fibroblasts as well as tissues prepared from CIA model rats.

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 guinea pig polyclonal antibody against GLT-1 and a goat polyclonal antibody against EAAC1 were purchased from Chemicon International (Temecula, CA, USA). An anti-guinea pig IgG antibody was provided by Vector Laboratories (Burlingame, CA, USA), and an anti-goat 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 (THA), L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) and dihydrokainate (DHK) were provided by Tocris Cookson (Bristol, UK). An adjuvant complete freund was from DIFCO laboratories (Detroit, MI, USA). Other chemicals used were all of the highest purity commercially available.

2.2. Culture of synovial fibroblasts

The protocol employed 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 utilize alternatives to in vivo techniques. Surface parts of synovial tissues were isolated from knee joints of male Lewis rats weighing 280–320 g, followed by washing with phosphate-buffered saline (PBS) and subsequent digestion with 0.2% collagenase in Dulbecco's modified Eagle's medium (DMEM) at 37 °C for 2 h. Synovial tissues were then treated with 0.2% collagenase and 0.25% trypsin at 37 °C for 2 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 1.8×10^3 cm^{-2}, followed by culturing at 37 °C under 5% CO_2 and subsequent usage for experiments after three to six passages as synovial fibroblasts.

2.3. Induction of collagen-induced arthritis

The experimental CIA model was generated by injecting to 8-week-old male Lewis rats with 500 μL emulsion containing 500 μg of type II collagen, intradermally into the base of the tail and back under anesthesia as described previously [3,19]. The basic emulsion was composed of 2 mg/mL bovine type II collagen dissolved in 0.05 M acetic acid and an equal volume of complete Freund's adjuvant. After 7 days and 14 days, a second and a third immunization booster (100 μg of type II collagen) was

administered. Four weeks after the first immunization, the limbs of rats were excised for subsequent biochemical and histological analyses.

2.4. Histological analysis

Synovial tissues were harvested and fixed with 10% formalin neutral buffer solution, followed by decalcification with 20% EDTA and subsequent immersion in 30% sucrose at 4 °C. Synovial tissues were then dissected for sections with a thickness of 5 μm in a cryostat. Sections were stained with Hematoxylin and Eosin (H.E.) and Alcian Blue (A.B.) under standard procedures, respectively. Sections were mounted in 30% glycerol and photographs of sections were taken using an Olympus microscope.

2.5. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Cultured synovial fibroblasts as well as synovial tissues were washed with PBS twice, followed by extraction of mRNA using mRNA purification kit and subsequent synthesis of cDNA with 12.5 μM random hexamer primers and first-strand beads. Reverse transcriptase reaction was run at 37 °C for 60 min and an aliquot of synthesized cDNA was directly used for PCR. PCR was performed in 10 mM Tris–HCl buffer (pH 8.3) containing 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dNTP (deoxy nucleotide triphosphate), 20 pmol of each primer for the corresponding GluRs, EAATs, cystine/Glu antiporter and cytokines (Table 1) and 2U Taq DNA polymerase as described previously [20]. The conditions of 32 PCR cycles for these primers were as follows: denaturation at 95 °C for 1 min; annealing at 55 °C for 1 min and extension at 72 °C for 1 min, respectively. An aliquot of PCR amplification products was run on 2% agarose gel, followed by detection of DNA with ethidium bromide. Rat whole brain was used as a positive control for the primers used in this study. The house keeping gene glycelaldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control with stable expression. To determine changes in mRNA expression, the density and area of each band of PCR products were analyzed with NIH image 1.6, followed by normalization into quantitative densitometric values over the corresponding PCR products of GAPDH. Appropriate PCR DNA products were extracted from agarose gel using DNA extraction spin columns, followed by sequencing by ABI Prism 310 Genetic Analyzer using cycle sequencing kit. Amplified PCR products were invariably subjected to sequential analysis for the confirmation of expression of the corresponding mRNA. As identical primers were used for amplification of GluR1 to GluR4 subunits of AMPA receptors, resultant PCR products were further digested with restriction enzymes specific for each subunit for determination of expression of mRNA for AMPA receptor subunits in cultured rat synovial fibroblasts.

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

Synovial fibroblasts were cultured, 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 1–60 min, unless indicated otherwise [21]. 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 to remove extracellular [^{3}H]Glu 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.

2.7. Immunohistochemistry

Immunohistochemical detection of each EAAT isoform was done as described previously [20]. Synovial tissues 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. Synovial tissues 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 (1:200 dilution), GLT-1 (1:200 dilution) and EAAC1 (1:200 dilution) diluted with the same blocking buffer at room temperature overnight, followed by reaction with biotinylated anti-guinea pig or goat 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. We have previously confirmed the adequacy of these primary antibodies against GLAST [22], GLT-1 [23] and EAAC1 [24] for reproducible immunohistochemical detection of the individual antigenic proteins on sections under the experimental conditions employed [20].

2.8. Determination of Glu contents

Surface parts of synovial tissues isolated from knee joints of male Lewis rats were washed three times with

Table 1

Genes	Upstream (5′-3′)	Downstream (5′-3′)	Region	Estimated base pair
iGluR				
NR1	ACGGAATGATGGGCGAGC	GGCATCCTTGTGTCGCTTGTAG	1761–2794	1033
NR2A	GGGGTTCTGCATCGACATCC	GACAGCAAAGAAGGCCCACAC	1593–2139	547
NR2B	GGGGTTCTGCATCGACATCC	GACAGCAAAGAAGGCCCACAC	1593–2139	547
NR2C	GGGGTTCTGCATCGACATCC	GACAGCAAAGAAGGCCCACAC	1593–2139	547
NR2D	CGATGGCGTCTGGAATGG	CTGGCAAGAAAGATGACCGC	1431–1977	465
GluR1	CCTTTGGCCTATGAGATCTGGATGTG	TCGTACCACCATTTGTTTTTCA	1386–1931	749
GluR2	CCTTTGGCCTATGAGATCTGGATGTG	TCGTACCACCATTTGTTTTTCA	1386–1931	749
GluR3	CCTTTGGCCTATGAGATCTGGATGTG	TCGTACCACCATTTGTTTTTCA	1386–1931	755
GluR4	CCTTTGGCCTATGAGATCTGGATGTG	TCGTACCACCATTTGTTTTTCA	1386–1931	749
GluR5	GGTTTTTCACCCTTATCATCAT	GCACTTCAGGGACATTCTCAG	2281–2745	692
GluR6	TATGTTCTGCTGGCTTGCTTG	GCACTTCAGGGACATTCTCAG	1761–2794	918
GluR7	GGTTTTTCACCCTTATCATCAT	TGCTCCCGTTCCGCTGTCTTGC	2062–2698	637
KA1	GGTGTAATCTCCTGGTCAAC	GATGCTTCTGAGTGTCTGAG	1824–2586	763
KA2	TCGCCCGTGTCCTCAACTCA	CACCGACACCTCCTCAGACT	2328–2725	398
mGluR				
mGluRI	CCAGTGATGTTCTCCATACC	CACTCTGGGTAGACTTGAGTG	5229–5589	361
mGluR2	TTTAGGTCAGAAGCCAGAGT	CAGTAACCATCCTCTCTATCC	2962–3211	250
mGluR3	TATTCTCAGTCCTCTGCAAG	TTGTAGCACATCACTACATACC	2698–2958	261
mGluR4	TCATTTTCTCTTCTGTTCCC	GACATGCTACACATCAGAGAC	4101–4439	340
mGluR5	CCCCAAACTCTCCAGTCT	ATTTTTCACCTCGGGTTC	3680–3889	210
mGluR6	CAAGTAGCAAGGTTGAGTGT	GGTTGTAGTGTTGGATCAAG	2774–3136	363
mGluR7	GAACTCTGTGAAAATGTAGACC	TTAGGGAGTCCAGAATTACAG	3068–3388	321
mGluR8	CGAGGGTTATAACTACCAGGT	TAGGTGCTGTGACAGATTTCT	1690–2128	440
EAAT				
GLAST	GGGTTTTCATTGGAGGGTTGC	CCACGGGTTTCTCTGGTTCAT	1240–1812	572
GLT-1	GGGTCATCCTGGATGGAGGT	CGTGTCGTCATAAACGGACTG	1345–1673	328
EAAC1	GACTGGGAAATATTCCGCAAGT	CGCACAGCGGAATGTAACTGG	940–1149	209
EAAT4	AGTCAACAACTATGAGCTGAACTTT	CTTATAGGGTTTCCCCAGGC	1464–1776	311
EAAT5	CCATGGTCATTGTGCTCACC	TCTAGGCTGGCAGTGGCCAG	1307–1661	355
Antiporter				
xCT	CCTGGCATTTGGACGCTACAT	TCAGAATTGCTGTGAGCTTGC	442–623	182
4F2hc	CTCCCAGGAAGATTTTAAAGACCTTCT	TTCATTTTGGTGGCTACAATGTCAG	730–876	141
Cytokine				
RANKL	GGTCGGGCAATTCTGAATT	GGGGAATTACAAAGTGCACCAG	957–1770	814
IL-1b	TGATGTTCCCATTAGACAGC	GAGGTGCTGATGTACCAGTT	421–798	378
TNF-α	CACGCTCTTCTGTCTACTGA	GGACTCCGTGATGTCTAAGT	135–675	541

ice-cold PBS, followed by homogenization in PBS and subsequent incubation with 1 M perchloric acid at a volume ratio of 4:1. Following centrifugation at 20,000 × *g* for 5 min, the supernatant was collected for subsequent neutralization with sodium hydroxide and storage at −80 °C. Endogenous Glu was determined by the fluorometric method using β-$NADP^+$ and glutamate dehydrogenase. Sample were incubated with 100 mM β-$NADP^+$ to make a final concentration of 2 mM, and then with 80 U/mL glutamate dehydrogenase at a volume ratio of 1:1 for 5 min at 37 °C, followed by measurement of Glu concentrations in a fluorescence microplate reader (MPT-100F; Cprpna Elec. 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.

2.9. Analysis of cell proliferation

The 5-bromo-2′-deoxyuridine (BrdU) method was conducted to analyze the proliferating cells present in cultured synovial fibroblasts. Synovial fibroblasts were cultured in the presence of Glu at concentrations of below 500 μM for 24 h at 37 °C in either the presence or absence of EAAT inhibitors, followed by the addition of 10 μM BrdU and subsequent determination of BrdU incorporation by Cell Proliferation ELISA BrdU Kit according to the manufacturer's instructions.

2.10. 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. Expression profiles of Glu signaling machineries

In order to analyze expression of mRNA for Glu signaling machineries including GluRs, EAATs and cystine/Glu antiporter, mRNA was extracted from cultured synovial fibroblasts prepared from normal rats for subsequent RT-PCR using specific primers for each molecule. Rat whole brain exhibited marked expression of mRNA for all Glu signaling machineries examined in this study (Fig. 1A). These included NR1 and NR2A–D subunits of NMDA receptors, GluR1–4 subunits of AMPA receptors, GluR5–7, KA1 and KA2 subunits of KA receptors, mGluR1-8 isoforms of mGluRs, GLAST, GLT-1, EAAC1, EAAT4 and EAAT5 isoforms of EAATs, and both xCT and 4F2hc subunits of the cystine/Glu antiporter. In cultured rat synovial fibroblasts, expression was also found with mRNA for NR2D subunit, but not for NR1 and NR2A–C subunits (Fig. 1A-i). Expression of mRNA was detected for KA2 subunit, but not for other subunits including GluR5, GluR6, GluR7 and KA1 subunits. Moreover, mRNA expression was seen for GluR3 subunit, but not for GluR1, GluR2 and GluR4 subunits.

RT-PCR analysis was further conducted using specific primers for each mGluR. Expression of mRNA was seen for only mGluR8 isoform of the group III subtype, while no expression of mRNA was found for group I (mGluR1 and mGluR5) and group II (mGluR2 and mGluR3) subtypes in cultured rat synovial fibroblasts (Fig. 1A-ii). In addition to GluRs, expression was seen with mRNA for GLAST, GLT-1 and EAAC1, but not for EAAT4 and EAAT5, isoforms of EAATs, whereas expression of both xCT and 4F2hc subunits was also found with cystine/Glu antiporter in cultured rat synovial fibroblasts (Fig. 1A-iii). Sequencing analysis on these amplified PCR products clearly confirmed the expression of mRNA for the corresponding Glu signaling machineries.

Synovial tissues were dissected from rat knees for frozen sections to evaluate distribution profiles of each EAAT isoform on immunohistochemical analysis. In these sections, highly immunoreactive cells were detected for EAAC1 isoform in synovial membranes as revealed by their morphology and location, but neither GLAST nor GLT-1 (Fig. 1B left three panels). In sections not treated with each primary antibody, no marked immunoreactivity

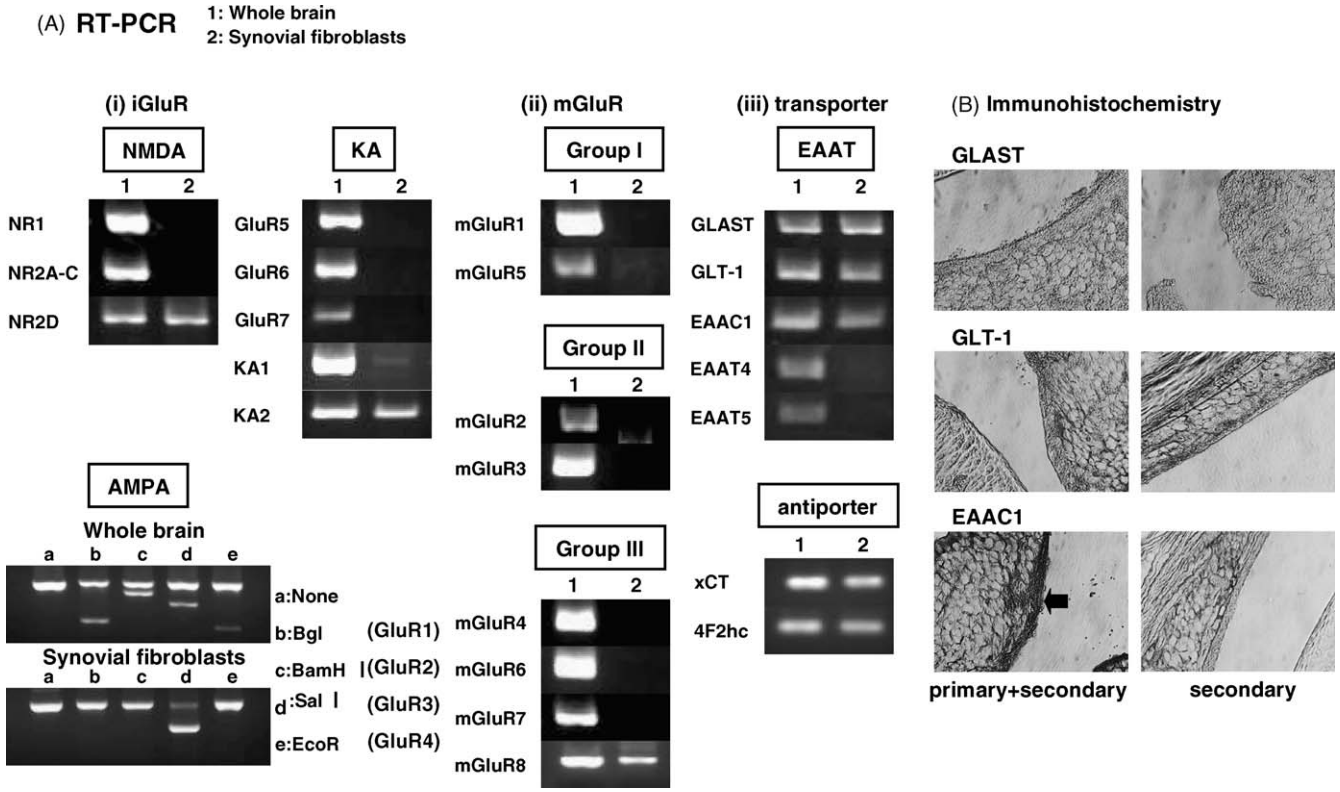

Fig. 1. Expression of Glu signaling machineries in rat synovium. (A) mRNA was isolated from cultured rat synovial fibroblasts for subsequent RT-PCR using primers specific for each iGluR (i), mGluR (ii) and Glu transporters (iii). Typical pictures are shown in the figure with similar results in three separate determinations. (B) Synovial tissues were isolated from Lewis rats, followed by fixation with formalin and subsequent dissection of frozen sections in a cryostat. Sections were then subjected to detection of the individual EAAT isoforms on immunohistochemical analysis using an antibody against GLAST, GLT-1 or EAAC1. Typical micrographic pictures are shown in this figure, while similar results were invariably obtained in at least three independent determinations. The black arrow indicates the location of high immunoreactivity.

was detected in any synovial tissues (Fig. 1B right three panels). Therefore, subsequent experiments focused on EAATs required for Glu membrane trafficking rather than GluRs essential for Glu signal input.

3.2. [^{3}H]Glu uptake in cultured synovial fibroblasts

In order to evaluate the functionality of different transporters expressed, an attempt was made to determine whether the substrate Glu is indeed incorporated into these cultured synovial fibroblasts. Cultured synovial fibroblasts were incubated with 1 μM [^{3}H]Glu in HKR buffer at 2 °C or 37 °C for different periods up to 60 min, followed by rapid aspiration of buffer and subsequent rinsing with ice-cold buffer containing unlabeled Glu at 1 mM to remove extracellularly located [^{3}H]Glu. Under these experimental conditions, [^{3}H]Glu incorporated was not markedly affected by the later addition of 1 mM unlabeled Glu for rinsing in cultured synovial fibroblasts (data not shown). The accumulation of [^{3}H]Glu was almost linearly increased with incubation time up to 30 min and reached a plateau within 60 min at 37 °C, while no marked accumulation of [^{3}H]Glu was seen even 60 min after the initiation of incubation at 2 °C (Fig. 2A).

Cultured cells were incubated with [^{3}H]Glu at different concentrations of 1–100 μM at 37 °C for 20 min in HKR buffer for determination of saturation isotherms. The accumulation was increased with increasing concentrations of [^{3}H]Glu, followed by a saturable profile at a concentration above 40 μM within a concentration range of up to 100 μM (Fig. 2B). Woolf–Hanes plot analysis revealed that [^{3}H]Glu accumulation consisted of a single component with a K_m value of 23.1 ± 1.1 μM and a V_{max} value of 237.1 ± 31.1 pmol/(mg protein min), respectively, at the substrate concentration range employed (Fig. 2B, inset).

3.3. Confirmation of validity of CIA model

To evaluate the validity of CIA model rats employed, several biochemical and histological analyses were performed. Apparent swelling was seen in hind paws of model rats obtained 28 days after the first immunization to type II collagen (Fig. 3A, left panel), with a significant increase in the hind paw volume of model rats (Fig. 3A, right panel). Histological analyses revealed that distinct morphological differences were observed in synovial tissues prepared from the knee joints of normal (Fig. 3B-i) and model (Fig. 3B-ii) rats on H.E. staining. In particular, H.E. staining showed severe synovitis consisting of hyperplastic synovium with fibroblast-like cells in knee joints of CIA model rats (Fig. 3B-ii). In addition, severe destruction of cartilage as well as bone was detected in model rats by staining with both H.E. (Fig. 3B-iii) and A.B. (Fig. 3B-iv) as marked by black arrowheads. Compared to normal synovial tissues, endogenous levels of different cytokines, including receptor activator of NF-κB ligand (RANKL), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), were significantly increased in synovial tissues isolated from model rats (Fig. 3C). Under these conditions, moreover, Glu levels were almost tripled in synovial tissues from model rats compared with that from normal animals (Fig. 3D). These data clearly showed that the experimental model for CIA was indeed completed in rats employed at 28 days after the first immunization to type II collagen with successive second and third immunization.

3.4. Comparison between synovium of normal and CIA model rats

In order to evaluate possible differential expression profiles in the aforementioned CIA model rats, semi-

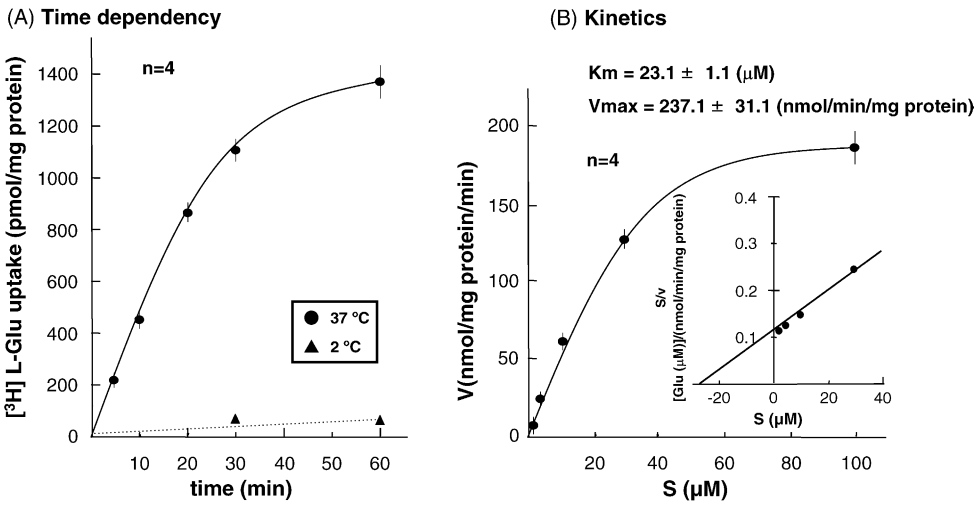

Fig. 2. [^{3}H]Glu accumulation in cultured synovial fibroblasts. (A) Cultured fibroblasts were incubated with 1 μM [^{3}H]Glu at 2 or 37 °C for different periods of up to 60 min in HKR buffer, followed by aspiration of buffer and subsequent rinsing with buffer containing unlabeled Glu at 1 mM. (B) Cultured fibroblasts were incubated with [^{3}H]Glu at different concentrations from 1 to 100 μM for 5 min at 37 °C for determination of [^{3}H]Glu accumulation. *Inset*: Woolf–Hanes plot analysis was done with the data obtained from saturation isotherms for [^{3}H]Glu accumulation. Values are the mean ± S.E. from four independent experiments.

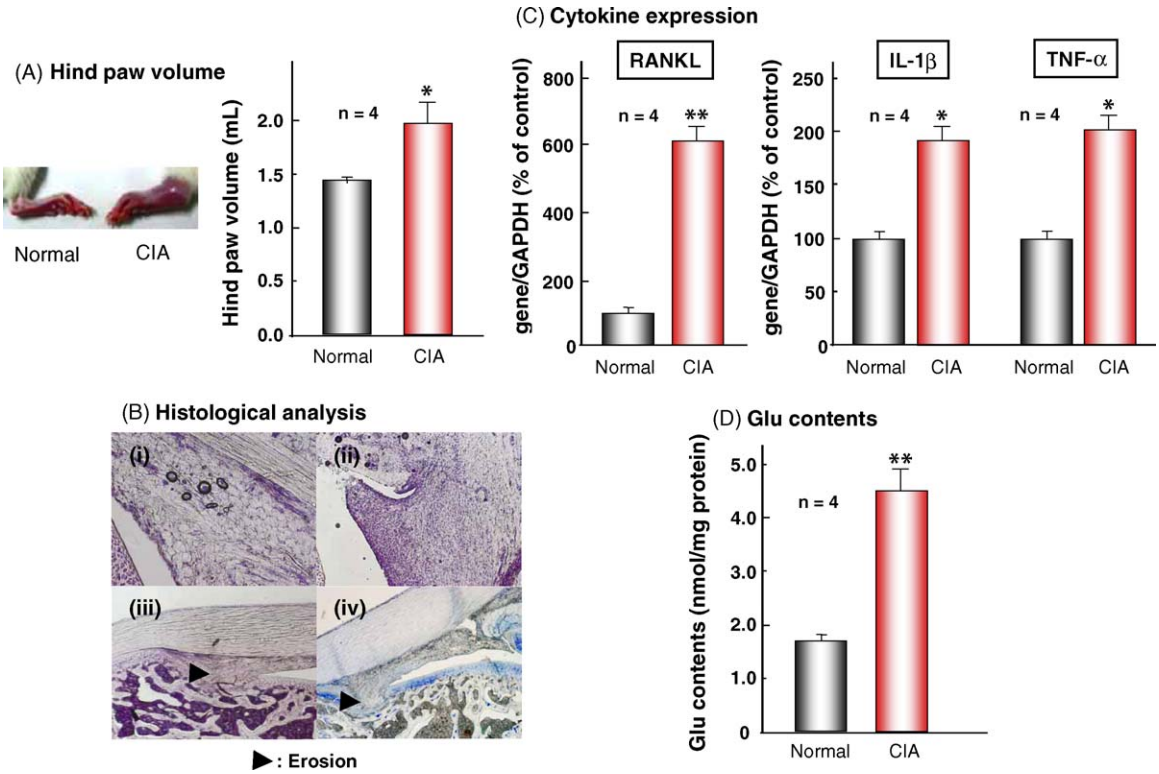

Fig. 3. Biochemical and histological evaluation of CIA model rats. (A) Hind paw volume was determined with normal and CIA model rats on 28 days after the first immunization. (B) Histological analyses were conducted in synovial sections of normal and CIA model rats by staining with H.E. (i, normal rat; ii, model rat; iii, model rat) and A.B. (iv, model rat). The black arrowhead indicates the location of severe erosion. (C) Quantification of the expression of different cytokines, including RANKL, IL-1β and TNF-α, was done on the basis of expression of the housekeeping gene GAPDH. (D) Synovial tissues were isolated from normal and CIA model rats, followed by determination of Glu contents by the fluorometric method. Values are the mean ± S.E. of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from each control value obtained with normal rats.

quantitative RT-PCR was conducted with several Glu signaling machineries expressed in synovial tissues. As shown in Fig. 4A-i, however, no significant alterations were found in expression profiles of mRNA for NR2D, GluR3 and KA2 subunits of iGluRs and mGluR8 isoform of mGluRs in synovial tissues isolated from both normal and CIA model animals at 28 days after the first immunization. Similarly, no significant change was seen in expression of mRNA for GLAST, GLT-1 and EAAC1 isoforms of EAATs (Fig. 4A-ii), and for both xCT and 4F2hc subunits of cystine/Glu antiporter (Fig. 4A-iii) in synovial tissues isolated from both normal and CIA model animals when analyzed on 7 and 28 days after the first immunization.

We therefore next tried to compare the activity of Glu incorporation in cultured synovial fibroblasts prepared at different days after the first immunization to type-II collagen in CIA model rats. [^{3}H]Glu accumulation was invariably increased linearly up to 30 min and reached a plateau within 60 min at 37 °C in cultured synovial fibroblasts irrespective of the immunization period (Fig. 4B). No marked accumulation of [^{3}H]Glu was seen even 60 min after the initiation of incubation at 2 °C in all situations (data not shown). However, [^{3}H]Glu accumulation was highest throughout the incubation at 37 °C in synovial fibroblasts prepared from 7 days after the first immunization with gradually declined activities in proportion to days after the first immunization up to 28 days in spite of the second and third immunization.

Replacement of sodium chloride with choline chloride led to marked abolition of the accumulation of [^{3}H]Glu in cultured synovial fibroblasts prepared from normal and CIA rats at 28 days after the first immunization (Fig. 5A). For evaluation of pharmacological profiles, cultured synovial fibroblasts were incubated with 1 μM [^{3}H]Glu in HKR buffer containing a variety of amino acids at 1 mM for 20 min at 37 °C. Of the amino acids tested, L-Glu and L- and D-aspartate were effective in significantly inhibiting [^{3}H]Glu accumulation in cultured synovial fibroblasts prepared from normal and CIA rats at 28 days after the first immunization with D-Glu and cystine being ineffective at 1 mM (Fig. 5B). Of the five different EAAT inhibitors tested at a concentration of 100 μM, the non-selective inhibitors THA, CCG-III and PDC significantly inhibited [^{3}H]Glu accumulation, while GLT-1 inhibitors such as T3MG and DHK did not significantly affect [^{3}H]Glu accumulation in cultured synovial fibroblasts prepared from normal and CIA rats at 28 days after the first immunization (Fig. 5C). In addition, L-homocysteate, a cystine/Glu antiporter inhibitor, was also ineffective in significantly inhibiting [^{3}H]Glu accumulation irrespective of fibroblasts examined.

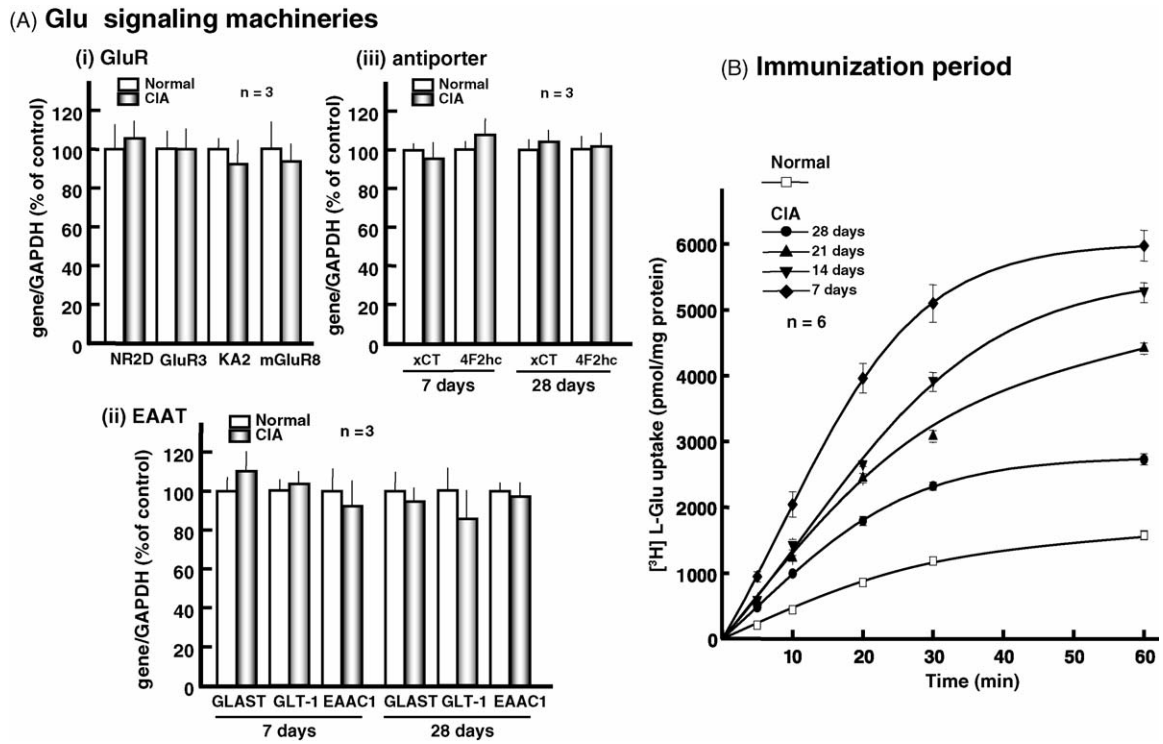

Fig. 4. Comparison between normal and CIA model rats. (A, i–iii) mRNA was isolated from synovial tissues from both normal and CIA model rats on 7 days and 28 days after the first immunization, followed by RT-PCR using primers for different Glu signaling machineries expressed. Values are the mean ± S.E. of three separate animals. (B) Cultured synovial fibroblasts prepared on different days after the first immunization were incubated with 1 μM [^{3}H]Glu at 37 °C for different periods of up to 60 min in HKR buffer, followed by aspiration of buffer and subsequent rinsing with buffer. Values are the mean ± S.E. of six independent experiments.

3.5. *Effect of Glu on proliferation and cytokine expression in control and arthritic fibroblasts*

An attempt was next made to determine whether Glu indeed modulates the functionality of synovial fibroblasts. For this purpose, we at first investigated the effect of culture with Glu on the cellular proliferative activity by using the BrdU incorporation method. Synovial fibroblasts prepared from normal and arthritic animals were further cultured in the presence of Glu at concentrations of 100–

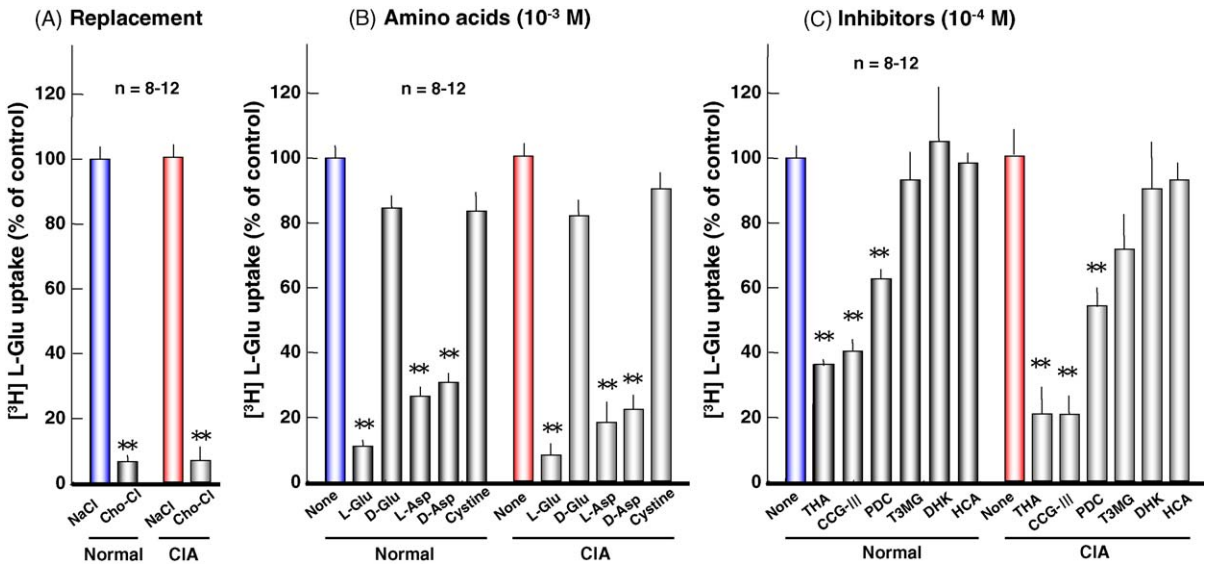

Fig. 5. Pharmacological properties of [^{3}H]Glu accumulation in cultured synovial fibroblasts. (A) Cultured fibroblasts prepared from normal and CIA model rats on 28 days after the first immunization were incubated with 1 μM [^{3}H]Glu at 37 °C for 20 min in HKR buffer where sodium chloride was replaced with equimolar choline chloride (Cho-Cl) as needed. (B) The incubation was also done in either the presence or absence of various amino acids at 1 mM. (C) The incubation was done in HKR buffer containing one of the inhibitors of different EAATs and cystine/Glu antiporter at a concentration of 100 μM. Values are the mean ± S.E. of 8–12 independent experiments. $^{**}P < 0.01$, significantly different from each control value obtained with normal HKR buffer.

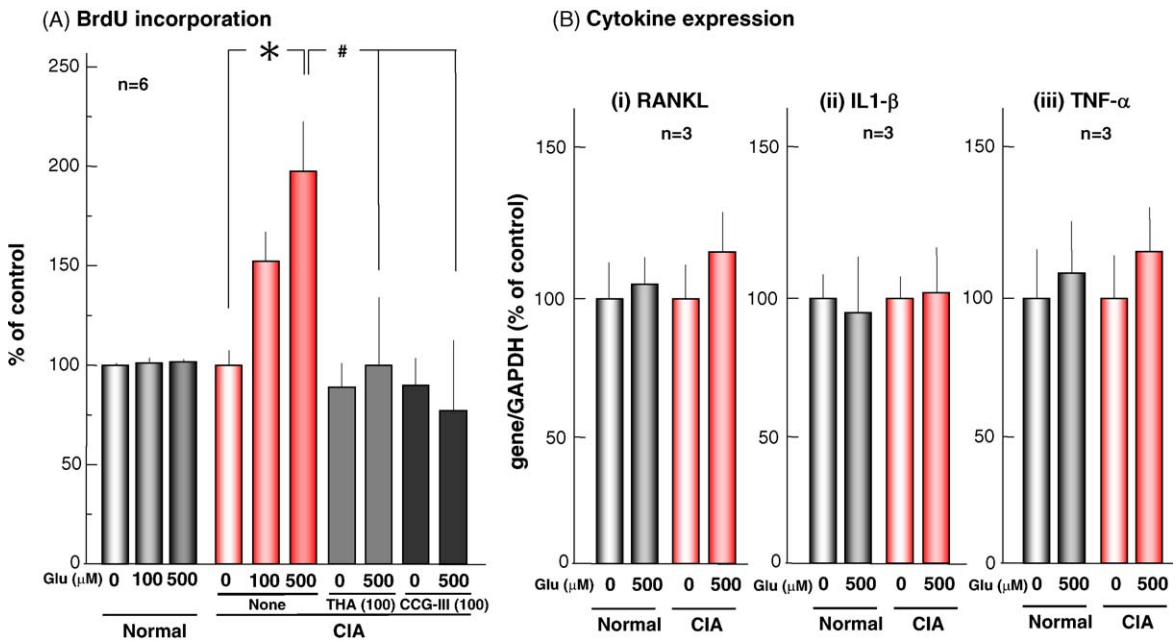

Fig. 6. Effect of Glu on cell proliferation and cytokine expression in cultured synovial fibroblasts. (A) Synovial fibroblasts were prepared from normal and arthritic animals on 28 days after the first immunization. Cells were further cultured in the presence of Glu at a concentration range of up to 500 μM in either the presence or absence of EAAT inhibitors at 100 μM for 24 h, followed by incubation with BrdU for 2 h and subsequent determination of BrdU incorporation by enzyme immunoassays. Values are the mean ± S.E. of six separate determinations. $^{*}P < 0.05$, significantly different from each control value obtained in the absence of any test compounds added. $^{\#}P < 0.05$, significantly different from the value obtained in the presence of Glu alone. (B, i–iii) Cultured fibroblasts were incubated in either the presence or absence of Glu at 500 μM for 24 h, followed by cell harvest and subsequent determination of mRNA for different cytokines on semi-quantitative RT-PCR. Values are the mean ± S.E. obtained in three independent determinations.

500 μM for 24 h, followed by the addition of BrdU and subsequent determination of BrdU incorporation. Although the exposure to Glu did not significantly affect BrdU incorporation in cultured normal synovial fibroblasts at the concentrations used, a significant increase was seen in BrdU incorporation in arthritic rat fibroblasts cultured in the presence of Glu at 500 μM (Fig. 6A). However, the non-selective EAAT inhibitors THA and CCG-III at 100 μM significantly inhibited the increase by Glu at 500 μM of BrdU incorporation in arthritic rat fibroblasts, without significantly affecting that in normal rat fibroblasts, when cultured together with Glu.

Synovial fibroblasts were also cultured in either the presence or absence of 500 μM Glu for additional 24 h, followed by extraction of mRNA and subsequent semi-quantitative RT-PCR using specific primers for RANKL, IL-1β and TNF-α. Although expression of mRNA was seen for all cytokines examined, semi-quantitative RT-PCR revealed that no marked change was found in expression of any cytokines tested irrespective of the presence of Glu in both normal and arthritic rat fibroblasts in culture (Fig. 6B).

4. Discussion

The essential importance of the present findings is that the rat synovium functionally expressed particular EAAT isoforms required for bi-directional Glu membrane trafficking rather than GluRs essential for Glu signal input from extracellular to intracellular locations. The fact that [^{3}H]Glu accumulation was drastically increased in cultured synovial fibroblasts prepared from CIA model rats at the initial stage is favorable for a possible key role of Glu transport across cell membranes in the pathogenesis of arthritis. The reason why the increase in [^{3}H]Glu accumulation was gradually declined in proportion to the completion of immunization is not clear so far. One possible speculation is that drastically increased Glu transport would prime the stimulation of cellular proliferation, as seen in RA, in synovial tissues immunized to type-II collagen at an initial stage. The present finding that the culture with Glu exclusively doubled the incorporation of BrdU in a manner sensitive to EAAT inhibitors in synovial fibroblasts of arthritic rats only, without affecting cytokine mRNA expression, gives support to this idea. The exact mechanism as well as functional significance of the stimulation by Glu, however, remains to be elucidated. To our knowledge, at any rate, this paper deals with the first direct demonstration of the functional expression of particular isoforms of EAATs by synovium in relation to the pathogenesis of arthritis. In patients with arthritis such as rheumatoid arthritis, in fact, a drastic increase is seen in the endogenous level of Glu in synovial fluids with markedly activated proliferation of synovial fibroblasts [1,2,5].

A splice variant of GLAST lacking exon 3 is expressed in both bone and brain [25], while several N-terminal or C-terminal splicing variants of GLT-1 are also found in different rat, mouse and human tissues [26]. Although PCR primers used are for all known splicing variants of GLAST and GLT-1, in this study, the antibody used, which is directed against the C-terminal peptide of wild type GLT-1, could not recognize C-terminal splicing variants of GLT-1 isoform. The possibility that the inconsistent results between RT-PCR and immunohistochemistry analyses may be caused by the expression of C-terminal splicing variants of GLT-1 isoform in synovium, therefore, is not ruled out. In contrast to cultured rat costal chondrocytes expressing the glial EAAT isoform GLT-1 [18], however, the non-selective EAAT inhibitors PDC, CCG-III and THA, but not the GLT-1 selective inhibitors T3MG and DHK, significantly inhibited temperature- and sodium-dependent [^{3}H]Glu accumulation in cultured rat synovial fibroblasts in the present study. This means that the glial EAAT isoform GLT-1 would be less responsible for mediating Glu transport across cell membranes in synovial tissues than either the other glial isoform GLAST or the neuronal isoform EAAC1, amongst different isoforms expressed by cultured rat synovial fibroblasts with respect to mRNA on RT-PCR analysis. In addition to these pharmacological profiles, the present immunohistochemical analysis could support predominant expression of EAAC1 isoform in synovial tissues. There is accumulating evidence that the putative neuronal EAAC1 isoform is expressed outside the CNS in kidney, heart, muscle, lung, placenta and liver [27,28], indeed, whereas expression of mRNA is shown for the putative glial EAAT isoforms GLAST and GLT-1 in particular restricted structures of rat testis [20]. 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.

In addition to the aforementioned EAAT isoforms, sodium-independent, chloride-dependent high affinity Glu uptake system has been demonstrated in several tissues. Glutathione, a major ubiquitous antioxidant, is a tripeptide (γ-glutamylcysteinylglycine) synthesized from cysteine, Glu and glycine. Since the availability of cysteine in body fluids is extremely low, the cysteine for GSH synthesis is provided by the intracellular reduction of cystine, which is taken up through cystine/Glu antiporter [29]. This antiporter is a heterodimeric complex composed of the CD98 heavy chain also referred to as 4F2hc, which is ubiquitously present in various tissues, and the xCT light chain, which determines the substrate specificity [30]. In general, the rate of cystine incorporation by the cystine/Glu antiporter is a crucial determinant for the regulation of endogenous levels of GSH. Under the condition of high concentration of extracellular Glu, moreover, extracellular Glu is taken up in exchange for intracellular cystine through the cystine/Glu antiporter, leading to intracellular GSH depletion and subsequent possible cell death in several cells [31,32]. Thus, intracellular GSH depletion by competitive inhibition of cystine uptake is a non-excitotoxic mechanism for Glu-induced cell death. In addition, a third Glu transport system has also been described in rat alveolar type 2 cells [33,34] and in astrocytes [35]. This transport system is sodium-dependent, and its substrates are cystine, Glu and aspartate. Accordingly, Glu and cystine would compete for the entry into cells as seen in the cystine/Glu antiporter but not in EAATs. In this study, however, [^{3}H]Glu accumulation was not significantly inhibited by the addition of the substrates cystine and homocysteate, in spite of the expression of mRNA for the antiporter system by synovial fibroblasts prepared from normal and arthritic rats. In our preliminary experiments, no significant difference was detected in total GSH contents in synovial tissues isolated from normal and CIA model animals (unpublished data). It is, thus, conceivable that [^{3}H]Glu accumulation observed in this study is mediated by particular isoforms of EAATs, but not by either cystine/Glu antiporter or the third Glu transport system, expressed by synovial fibroblasts irrespective of arthritis.

It should be noted that immunization to type-II collagen led to a drastic increase in [^{3}H]Glu accumulation activity without affecting profiles of mRNA expression for particular glutamatergic machineries required for Glu transport across cell membranes. In recent studies, several novel proteins are isolated and characterized with respect to the selective interaction with the neuronal EAAT subtypes EAAC1 and EAAT4 through the respective intracellular carboxyl terminal domains in rat brains [36,37]. The individual interactions are shown to lead to a marked decrease in the activity of [^{3}H]Glu accumulation by EAAC1, but a profound increase in that by EAAT4. These previous findings could account for the aforementioned paradox between expression of mRNA and corresponding protein for the EAAC1 isoform in synovial fibroblasts prepared from CIA model rats. The arthritis would induce decreased expression of the interacting protein without affecting expression of the EAAC1 subtype itself by synovial fibroblasts. However, the possible participation of posttranscriptional modifications in the conflicting data is not ruled out. Nevertheless, the facilitated [^{3}H]Glu accumulation could account for the marked increase in the endogenous level of Glu in synovial tissues of CIA model rats.

A possible discouraging criticism is that the functional expression may be derived from other cells contaminated in cultured rat synovial fibroblasts. Synovial tissues are known to indeed consist of at least three different cell

types, including type A, type B and type D, according to the morphology and expression of surface antigens [38,39]. Type A cells are round macrophage-like cells expressing monocyte lineage antigens, and shown to have phagocytic properties and to contain lysosomal enzymes. Type B cells are elongated fibroblast-like cells expressing fibroblast-associated antigens, which are usually called as synovial fibroblasts. Type D cells are of dendritic appearance and thought to be specific for RA. Primary cultured synovial fibroblasts isolated from articular synovial tissues are containing all cell types described above (type A, type B and type D) when judged from their morphology. In the present study, however, we have used cells after three to six passages for experiments to minimize the contamination with cells other than fibroblasts. Cultured cells after three to six passages are highly enriched of morphologically uniform fibroblastic cells free of macrophages [40,41]. The mechanism for consolidation of the increased activity of [^{3}H]Glu accumulation in synovial fibroblasts after three to six passages, however, should await future evaluation.

One of the interesting finding obtained in this study is that synovial fibroblasts expressed mRNA for particular subtypes of GluRs, in addition to EAAT isoforms. Functional NMDA receptor channels are comprised of heteromeric assemblies between the essential NR1 subunit and one of four different NR2 (A–D) subunits [9]. Expression of the NR2 subunit alone does not lead to composition of functional ion channels in any expression systems, while coexpression of each NR2 subunit with NR1 subunit results in expression of functional channels permeable to Ca^{2+} in mammalian cells. Similarly, KA receptor channels are constructed through the combination among GluR5, GluR6 and GluR7 subunits. Both KA1 and KA2 subunits are also supposed to participate in functional expression of KA receptor channels together with GluR5-7 subunits, while expression of KA1 or KA2 subunit alone does not lead to composition of functional KA receptor channels [10,11]. On the contrary, AMPA receptor subunits could constitute functional receptor channels through homomeric assemblies with each subunit. The possibility that the rat synovium may express functional AMPA receptor channels as well as group III mGluR subtype for glutamatergic signal input is not ruled out so far. If functional GluRs required for signal input are really absent from synovial fibroblasts, by contrast, the transport across cell membranes is the only one process to modulate cell proliferation seen after the exposure to Glu in cultured cells. This means that extracellular Glu signals could be transformed into intracellular signals through EAATs, but not GluRs, across cell membranes in synovial fibroblasts.

As mentioned above, the clinical characters of RA include the chronic inflammation of systemic joints in association with the overgrowth of synovial fibroblasts, which eventually causes cartilage and bone destruction in the joint [1,2]. Several cytokines such as TNF-α, IL-1 and IL-6 produced by activated macrophages, in turn, stimulate the overgrowth of synovial fibroblasts to form a mass of synovial tissue, called pannus, which invades both bone and cartilage through osteoclast activation and protease production during the course of inflammation [42–44]. It is widely and generally accepted that primary proliferation of synovial fibroblasts would play a critical role in pathogenesis of RA including bone and cartilage destruction. In this study, Glu stimulated the proliferation rate in synovial fibroblasts from CIA model rats in a manner sensitive to EAAT inhibitors, but not from normal rats. In addition, [^{3}H]Glu accumulation was increased in synovial fibroblasts of arthritic rats at the initial stage after the first immunization. Taken together, it is thus conceivable that intracellular Glu incorporated through increased Glu transport activity would prime the cellular proliferation in synovial fibroblasts at an initial stage in arthritic rats, which results in the pathogenesis of arthritis including cartilage and bone destruction in the joint. There is accumulating evidence that Glu plays a key role in the proliferation in tumor cells and in neuronal precursor cells through activation of particular GluRs including NMDA receptor. To data, nevertheless, no much attention has been paid to the correlation between functional expression of EAATs and cellar proliferation.

It thus appears that extracellular Glu may be incorporated into intracellular locations through particular isoforms of EAATs for possible modulation of cell proliferation in synovial fibroblasts. Evaluation of synovial EAATs could be of a great benefit for the future elucidation of molecular mechanisms underlying the crisis of a variety of diseases relevant to facilitated cell proliferation in synovial tissues including RA and osteoarthritis.

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