Possible Involvement of Glutamatergic Signaling Machineries in Pathophysiology of Rheumatoid Arthritis

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Abstract. The prevailing view is that L-glutamate (Glu) functions as an excitatory amino acid neurotransmitter through a number of molecular machineries required for the neurocrine signaling at synapses in the brain. These include Glu receptors for signal input, Glu transporters for signal termination, and vesicular Glu transporters for signal output through exocytotic release. Although relatively little attention has been paid to the functional expression of these molecules required for glutamatergic signaling in peripheral tissues, recent molecular biological analyses including ours give rise to a novel function for Glu as an extracellular signal mediator in the autocrine and/or paracrine system in several peripheral and non-neuronal tissues, including bone and cartilage. In particular, a drastic increase is demonstrated in the endogenous levels of both Glu and aspartate in the synovial fluid with intimate relevance to increased edema and sensitization to thermal hyperalgesia in experimental arthritis models. However, to date, there is only limited information about the physiological and pathological significance of glutamatergic signaling machineries expressed by articular synovial tissues. In this review, we have outlined the role of Glu in synovial fibroblasts in addition to the possible involvement of glutamatergic signaling machineries in the pathogenesis of joint diseases such as rheumatoid arthritis.

Keywords: glutamate, glutamate receptor, glutamate transporter, rheumatoid arthritis, synovial fibroblast

1. Glutamatergic signaling machineries

1.1. Glutamate receptors

The excitatory amino acid L-glutamic acid (Glu) is believed to be a neurotransmitter in the mammalian central nervous system (CNS). Membrane receptors for Glu (GluRs) are endowed to transform extracellular first messenger signals carried by Glu into intracellular second messenger signals and nowadays categorized into the two major subclasses, ionotropic (iGluRs) and metabotropic (mGluRs) receptors, according to their differential intracellular signal transduction mechanisms and molecular homologies (Fig. 1) (1 – 3). These iGluRs and mGluRs are supposed to mediate the excitatory neurotransmission with a pivotal role in synaptic plasticity such as learning and memory in the CNS (4, 5). In addition, excessive overactivation of iGluRs is thought to participate in the chronic neurodegenerative disorders such as Alzheimer’s disease, Huntington’s disease, and amyotrophic lateral sclerosis, as well as the neurodegeneration following a wide range of neurological insults including ischemia, trauma, hypoglycemia, and epileptic seizures (6 – 8). The former iGluRs are classified into DL-α-amino-3-hydroxy-5-methylisoxasole-4-propionate (AMPA), kainate (KA), and N-methyl-D-aspartate (NMDA) receptors, which are all associated with ion channels permeable to particular cations, in line with the sequence homology and exogenous agonists (9, 10). For example, AMPA-receptor channels consist of different subunits including GluR1, GluR2, GluR3, and GluR4 subunits (also referred to as GluA subunits) with usually higher permeability for Na⁺ than Ca²⁺ ions, whereas KA-receptor channels are constructed through combination among GluR5, GluR6, and GluR7 subunits (also referred
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Fig. 1. Classification of glutamate receptors. GluRs are divided into two major classes, metabotropic (mGluRs) and ionotropic (iGluRs) receptors, according to their differential intracellular signal transduction mechanisms as well as sequence homologies. The former class is subdivided into three distinct subtypes, including groups I, II, and III, while the latter is subclassified into NMDA, AMPA, and KA receptors.

to as GluK subunits) with high permeability for Na+ rather than Ca2+ ions. Both KA1 and KA2 subunits are supposed to participate in the expression of functional KA-receptor channels with high affinity in association with other KA-receptor subunits described above (10). By contrast, NMDA-receptor channels are highly permeable to Ca2+, with sensitivity to blockade by Mg2+ in a voltage-dependent manner (11, 12). Functional NMDA-receptor channels are comprised of heteromeric assemblies between the essential NR1 subunit and one of four different NR2 (A – D) subunits (also referred to as GluN subunits), in addition to one of two dominant negative NR3 (A – B) subunits. On the basis of agonist preference and intracellular second messenger, the mGluR family comprises at least eight independent subunits (mGluR1 to mGluR8) so far with classification into the three distinct subtypes with seven transmembrane domains, including group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8) isoforms (13, 14). The group I subtype stimulates the formation of inositol-1,4,5-triphosphate and diacylglycerol, while both group II and III subtypes induce a reduction of intracellular cyclic AMP.

1.2. Glutamate transporters

Glu transporters are thought to be essential for the prevention of neurotoxicity mediated by Glu as well as for the termination of signal transduction mediated by Glu in the CNS. To date, these Glu transporters are categorized into 5 different subtypes: glutamate aspartate transporter (GLAST) (EAAT1: excitatory amino acid transporter 1) (15, 16), glutamate transporter-1 (GLT-1) (EAAT2) (17), excitatory amino acid carrier (EAAC1) (EAAT3) (18), EAAT4 (19), and EAAT5 (20) (Table 1). These Glu transporters display heterologous regional and cellular expression profiles. Both GLAST and GLT-1 are localized to astrocytes, with GLAST predominating in the cerebellum and GLT-1 in the cortex and forebrain, respectively (21). EAAC1 is localized to neurons throughout the CNS (22), whereas EAAT4 localization is largely restricted to cerebellar Purkinje cells (19). EAAT5 has been shown to exclusively reside in the retina (20) and specifically on a photoreceptor and bipolar rod and cone cells (23). In addition, a sodium-independent, chloride-dependent high affinity Glu uptake system named cystine/Glu antiporter has been demonstrated in plasma membranes of different cells in several tissues. This antiporter is composed of a heterodimeric assembly between 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 specific-

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<td>Subtype</td>
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| GLAST   | brain, retina, testis, bone
<p>| GLT-1   | brain, retina, liver   |
| EAAC1   | Glu, Asp              | brain, intestine, kidney, retina, liver, heart |
| EAAT4   | brain, placenta       |
| EAAT5   | retina, liver         |</p>
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<th>Subunit</th>
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<td>xCT</td>
<td>Glu, Cystine</td>
<td>ubiquitous</td>
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<td>4F2hc</td>
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Glu transporters are grouped into 2 major subtypes including EAATs, such as GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5, and the cystine/Glu antiporter composed of 4F2hc and xCT subunits.
ity (Table 1) (24). In general, the rate of cystine uptake by the cystine/Glu antiporter is a determining factor in the regulation of intracellular reduced glutathione (GSH) levels. Extracellular Glu is taken up in exchange for intracellular cystine through the cystine/Glu antiporter under the condition of high extracellular Glu concentration, which leads to intracellular GSH depletion toward cell death mediated by oxidative stress in a non-excotoxic manner (25, 26).

Three different subtypes of vesicular glutamate transporters (VGLUTs) (VGLUT1, VGLUT2, and VGLUT3) have recently been characterized in the mechanism relevant to Glu transport for the condensation into synaptic vesicles at nerve terminals of glutamatergic neurons within the CNS. While the distribution of VGLUT1 and VGLUT2 in the brain is highly complementary to one another, both isoforms have been identified on synaptic vesicles at excitatory synapses (27). The expression of VGLUT1 and VGLUT2 would account for the exocytotic release of Glu in all known glutamatergic neurons, while VGLUT3 is expressed by a number of cells previously suggested to release Glu through exocytosis including dopaminergic, GABAergic, and serotonergic neurons as well as astroglia (28).

2. Glutamatergic machineries in bone and cartilage

2.1. Bone

Bone formation and maintenance are known to be sophisticatedly regulated by two distinct cell types: bone-forming osteoblast and bone-resorbing osteoclast. Imbalance between these cells leads to pathogenesis as well as etiology of certain metabolic bone diseases including osteoporosis, Paget’s disease, and osteopetrosis (29 – 31). The balancing mechanism at least in part involves not only the paracrine (autocrine) control by insulin-like growth factor and fibroblast growth factor, but also the endocrine control by estrogen and parathyroid hormone (29 – 31). Recent studies have raised the possibility that Glu may be one of the endogenous paracrine (autocrine) factors used for intercellular communications in bone cells. Osteoblasts constitutively express mRNA for non-NMDA receptors such as the GluR3 subunit of AMPA receptors as well as KA1 and KA2 subunits of KA receptors (32), while AMPA receptors modulate the exocytotic release of Glu from cultured osteoblasts (33, 34). Moreover, several independent lines of evidence have demonstrated that functional NMDA receptors are also expressed in osteoblasts and osteoclasts as revealed by both in vivo and in vitro studies (35 – 38). In cultured osteoclasts, the NMDA-receptor antagonist dizocilpine (MK-801) inhibits cell differentiation (35), while Glu induces elevation of intracellular free Ca\textsuperscript{2+} in a manner sensitive to antagonism by MK-801 in the human osteoblastic cell lines MG63 and SaOS-2 (36). In addition, we have shown that NMDA receptors could predominantly modulate cell differentiation through a mechanism associated with expression of runt-related transcription factor 2, which is an essential transcription factor for osteoblast differentiation, during cellular maturation in osteoblasts. Prior stimulation of group I/group II mGluR agonist leads to reduction of NMDA-induced whole cell current in cultured osteoblasts, whereas constitutive expression is shown for particular mGluRs (mGluR4 and/or mGluR8) that are negatively coupled to adenyl cyclase to inhibit the formation of cAMP stimulated by forskolin in cultured osteoblasts (39). No marked differences are seen in a variety of phenotypes including mandible and long bone size, morphology, trabeculation, regions of muscle attachment, resorption lacunae, and areas of formation versus resorption of bone, compared with wild-type siblings in mice devoid of GLAST (40), while mechanical loading is shown to down-regulate GLAST expressed in osteocytes when determined by immunohistochemistry (41). In addition, we have demonstrated the possible involvement of the cystine/Glu antiporter in the mechanisms underlying osteoclastogenesis (42) as well as osteoblastogenesis (43) during bone remodeling.

2.2. Cartilage

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 (44). Through this endochondral ossification, the cartilaginous rudiment, which is a tightly regulated area of both differentiation and maturation of chondrocytes, undergoes developmental growth. Within the cartilaginous rudiment, chondrocytes differentiate, progressing through the resting, proliferating, hypertrophic, and calcifying stages, which lead to mineralization of the cartilage matrix around the central region of the rudiment in the area of hypertrophic chondrocytes. 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 (45). Although less attention has been paid to the functional expression by chondrocytes of glutamatergic signaling machineries in cartilage than in bone to date, we have recently demonstrated the functional expression of particular Glu signaling machineries in chondrocytes (46 – 48). The group III mGluR agonist l-(1)-2-amino-4-phosphonobutyrate drastically inhibits chondral mineralization in a manner...
sensitive to an antagonist in cultured mouse embryonic metatarsals isolated before vascularization (46), while the addition of AMPA markedly evokes the release of endogenous Glu into incubation medium from cultured rat costal chondrocytes with further potentiation by the AMPA receptor desensitization–blocker cyclothiazide (47). Extracellular Glu is taken up into intracellular locations through particular EAAT isoforms functionally expressed by the rodent chondrocytes (48). In addition, Glu could cooperatively regulate cellular differentiation toward mineralization through a mechanism associated with apoptosis after depletion of intracellular GSH due to the possible retrograde operation of the cystine/Glu antiporter, in addition to the activation of group III mGluR, in chondrocytes (49).

3. Rheumatoid arthritis

3.1. Induction of collagen-induced arthritis (CIA) and culture of synovial fibroblasts

Rheumatoid arthritis (RA) is a systemic disorder characterized by synovial inflammation together with subsequent destruction and deformity of synovial joints. RA has a prevalence of approximately 1% in the world population. Although the pathogenesis and etiology of this disabling disease are not well understood to date, several research groups have demonstrated an increased oxidative enzyme activity, along with decreased antioxidant levels in the sera and synovial fluids in human subjects with RA. Patients with RA have been reported to have lower serum levels of anti-oxidants including vitamin E, vitamin C, and β-carotene in comparison with controls (50, 51). Oxidative stress–induced transformation of synovial fibroblasts into proliferating cells is thought to be a key event in mechanisms underlying the joint destruction in RA (52, 53). Type-II 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 is dependent on both humoral and cellular immunity to the immunizing antigen (54, 55).

The experimental CIA model is usually achieved by intradermally injecting 500 μl emulsion containing 500 μg of type II collagen into the base of the tail and back under anesthesia in 8-week-old male Lewis rats (54, 56). The basic emulsion is 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, followed by administration of second and third immunization boosters (100 μg of type II collagen) 7 and 14 days later, respectively. Four weeks after the first immunization, the knee joints of rats are excised for subsequent biochemical and histological analyses, in addition to isolation of synovial fibroblasts for culture (Fig. 2) (57). Apparent swelling is seen in hind paws of model rats obtained 28 days after the first immunization to type II collagen (Fig. 3A). Distinct morphological differences are seen in synovial tissues prepared from the knee joints between normal and model rats on both Hematoxylin and Eosin (H.E.) staining and Alcian Blue (A.B.) staining. In particular, H.E. staining would clearly show severe synovitis consisting of hyperplastic synovium with fibroblast-like cells in knee joints of CIA model rats. In addition, severe destructions of cartilage as well as bone are detected in model rats by staining with both H.E. and A.B. as marked by black arrowheads in the figure (Fig. 3B).

For the in vitro investigation using cultured synovial fibroblasts, by contrast, surface parts of synovial tissues are isolated from knee joints of rats, followed by washing with PBS and subsequent digestion with 0.2% collagenase in Dulbecco’s modified eagle 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 20% FBS. Cells were plated at a density of $1.8 \times 10^3$/cm$^2$, followed by culturing at 37°C under 5% CO$_2$ and subsequent usage for experiments after 3 to 6 passages as synovial fibroblasts (Fig. 4) (57) to minimize the contamination with cells other than fibroblasts. However, cultured cells after 3 to 6 passages are highly enriched of morphologically uniform fibroblastic cells free of macrophages (58, 59).

Indeed, synovial tissues are known to consist of at least 3 different cell types, including type A, type B, and type D, according to the morphology and expression profiles of surface antigens (60, 61). Type A cells are round macrophage-like cells expressing monocyte lineage antigens, with phagocytic properties and lysosomal enzymes. Type B cells are elongated fibroblast-like cells expressing fibroblast-associated antigens, which are usually called 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 contain all the cell types described above (type A, type B, and type D) when judged from their morphology.

### 3.2. Functional expression of glutamatergic signaling machineries in synovial fibroblasts

To evaluate possible expression profiles of mRNA for glutamatergic signaling machineries including GluRs, EAATs, and cystine/Glu antipporter in synovial tissues, mRNA is extracted from cultured synovial fibroblasts prepared from normal and CIA model rats at 28 days after the first immunization for subsequent RT-PCR using specific primers for each molecule. Rat whole brain exhibits marked expression of mRNA for all glutamatergic signaling machineries examined, while mRNA expression is found for particular glutamatergic signaling machineries in synovial fibroblasts isolated from both normal and CIA model rats. As shown in Table 2, however, no significant alterations are found in expression profiles of mRNA for NR2D, GluR3, and KA2 subunits of iGluRs; mGluR8 isoform of mGluRs; GLAST, GLT-1
and EAAC1 isoforms of EAATs; and xCT and 4F2hc subunits of the cystine/Glu antiporter in synovial tissues isolated from both normal and CIA model animals at 28 days after the first immunization. In addition, highly immunoreactive cells are detected for the EAAC1 isoform in synovial membranes as revealed by their morphology and location, but neither GLAST nor GLT-1 by immunohistochemical analysis.

3.3. Possible involvement of glutamatergic signaling machineries in pathophysiology of RA

Previous studies have demonstrated a drastic increase in endogenous levels of both Glu and aspartate in the synovial fluid obtained from patients with arthritis (62). The elevation of Glu in synovial fluid is shown to be relevant to increased edema and sensitization to thermal hyperalgesia in experimental arthritis models (63, 64). However, not much attention has been paid to the physiological and pathological significance of glutamatergic signaling machineries in articular synovial tissues to date. We have therefore attempted to demonstrate the possible involvement of glutamatergic signaling machineries in mechanisms underlying RA through evaluation of the functional expression of Glu transporters using synovial fibroblasts prepared from normal and CIA model rats.

To evaluate the possible differential functionality of Glu transporters expressed in synovial fibroblasts, [3H]Glu incorporation activity is determined in cultured synovial fibroblasts prepared at different days after the first immunization of type II collagen in CIA model rats. [3H]Glu incorporation is invariably increased linearly up to 30 min with a plateau within 60 min at 37°C in cultured synovial fibroblasts irrespective of the immunization period, while [3H]Glu incorporation is highest throughout the incubation in synovial fibroblasts prepared from 7 days after the first immunization with gradually decreasing activities in proportion to days after the first immunization up to 28 days in spite of the second and third immunization. The non-selective EAAT inhibitors 1-threo-β-hydroxyaspartate (THA), (2S,3S,4R)-2-(carboxycyclopropyl)glycine (CCG-III), and 1-trans-pyrrolidine-2,4-dicarboxylic acid significantly inhibit [3H]Glu incorporation in cultured synovial fibroblasts prepared from normal and CIA rats at 28 days after the first immunization, while neither GLT-1 inhibitors, such as (±)-threo-3-methylglutamate and dihydrokainate, nor the cystine/Glu antiporter inhibitor homocysteic acid significantly affect [3H]Glu incorporation in cultured synovial fibroblasts. The underlying mechanism for facilitated [3H]Glu incorporation in synovial fibroblasts of CIA model rats, accordingly, remains to be elucidated in future studies.

To determine whether Glu indeed modulates the functionality of synovial fibroblasts, cellular proliferative activity is determined in synovial fibroblasts prepared from normal and arthritic animals. Although the exposure to Glu does not significantly affect 5-bromo-2′-deoxyuridine (BrdU) incorporation in cultured synovial fibroblasts, a significant increase is seen in BrdU incorporation in arthritic rat fibroblasts cultured in the presence of Glu. In addition, simultaneous treatment of Glu with the non-selective EAAT inhibitor THA or CCG-III significantly inhibits the Glu-induced promotion of BrdU incorporation in arthritic rat fibroblasts. On
the contrary, no marked change is found in expression of cytokines such as receptor activator nuclear factor-κB ligand, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) irrespective of the presence of Glu in both normal and arthritic rat fibroblasts in culture.

4. Conclusion

The clinical characteristics of RA include chronic inflammation of systemic joints associated with overgrowth of synovial fibroblasts, which eventually causes cartilage and bone destruction in the joint. Several cytokines such as TNF-α, IL-1β, and IL-6 produced by activated macrophages, in turn, stimulate the overgrowth of synovial fibroblasts to form a mass of synovial tissue, called the pannus, which invades the bone and cartilage through osteoclast activation along with protease production during the course of inflammation (65 – 67). It is widely and generally accepted that primary proliferation of synovial fibroblasts would play a critical role in the pathogenesis of RA with regards to bone and cartilage destructions. We have demonstrated that Glu stimulates the proliferation rate in synovial fibroblasts from CIA model rats in a manner sensitive to EAAT inhibitors, but not in those from normal rats. In addition, [3H]Glu incorporation was increased in synovial fibroblasts of arthritic rats at the initial stage after first immunization. Taken together, it is conceivable that RA would promote the incorporation of extracellular Glu to stimulate the cellular proliferation in synovial fibroblasts at an initial proliferation stage in RA, which subsequently leads to the pathological destructions of both cartilage and bone in the joint in association with several cytokines released from activated macrophages (Fig. 5).

Evaluation of synovial EAATs could therefore 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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