Increase in excitatory amino acid concentration and transporters expression in osteoarthritic knees of anterior cruciate ligament transected rabbits

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Summary

Objective: The present study aimed to determine the role of excitatory amino acids (EAAs) and EAA transporters (EAATs) in an osteoarthritis (OA) model of rabbit knees.

Methods: OA was induced in New Zealand white male rabbits by anterior cruciate ligament transection (ACLT) in one knee of one hind limb; the other knee left unoperated. Rabbits that received ACLT of knee were assigned to the ACLT group (n = 6), while a sham-operated group (n = 6) underwent arthrotomy with no ACLT. Six naive rabbits that received no surgery were used as normal control. The width of the knee joint was measured to determine the severity of joint inflammation. Before operation and at 10, 20, and 30 weeks after operation, knee joint dialysates were collected by microdialysis and assayed for EAAs by high-performance liquid chromatography. Gross morphology and histopathology and EAATs glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) expression in the articular cartilage of the knees were evaluated by immunohistochemistry and western blot analysis.

Results: In the ACLT knees, a significant increase in the joint width was observed (5.3 ± 0.9 mm, P < 0.05) at 30 weeks after operation, while the sham-operated and naive knees showed no difference as compared with the basal values. The concentrations (μM) of aspartate and glutamate in knee dialysates at 30 weeks after ACLT in naive, sham, and ACLT were 0.36 ± 0.07 and 4.5 ± 1.10; 0.38 ± 0.09 and 4.61 ± 1.11; 0.67 ± 0.18 and 9.71 ± 2.89, respectively. Levels of glutamate and aspartate in the dialysates obtained from the ACLT knees increased by 213.3 ± 29.6% and 187.5 ± 33.8% (P < 0.05) when compared to those in the sham-operated knees. Both naive and ACLT chondrocytes were positively stained by antibodies against GLAST and GLT-1. GLAST and GLT-1 protein expressions were significantly increased in the ACLT knees (P < 0.05).

Conclusion: Our findings indicate an involvement of EAAs and EAATs in the pathogenesis of OA in ACLT rabbits.

Key words: Osteoarthritis, Anterior cruciate ligament, Microdialysis, Glutamate, Excitatory amino acid transporters, Glutamate transporters.

Introduction

Osteoarthritis (OA) is the most prevalent form of arthritis in the United States and is often associated with significant disability and impaired quality of life. OA is a synovial joint disorder that is characterized by the destruction of the articular cartilage accompanied by inflammation. Patients with rupture of the anterior cruciate ligament (ACL) develop post-traumatic OA of the knee. Restoration of knee stability provides symptomatic relief but does not reduce the...
degenerative changes in the ACL-injured knee. This suggests that the development of post-traumatic OA not only has a biomechanical origin but may also involve biochemical changes.

Glutamate is the best-known excitatory neurotransmitter in the mammalian central nervous system (CNS), being responsible for up to one-third of the excitatory synaptic functions. Growing evidence indicates that glutamate also plays a role in cell signaling in peripheral tissues such as the bone. Glutamate increases in the innervated axons of inflamed knee joints and the synovial fluid of arthritic patients. The injection of a kaolin/carrageenan mixture into the knee joint induces an immediate increase in the glutamate and aspartate levels in the joint; this increase persists for hours. We previously reported that glutamate and aspartate levels are significantly increased in the dialysates of ACLT rat knees and suggested that excitatory amino acids (EAAs) play a role in early OA development. The physiological extracellular concentration of glutamate is regulated by the action of high-affinity, sodium-dependent EAA transporters (EAATs). EAATs are the key mechanisms for the clearance and maintenance of extracellular glutamate concentrations at excitatory synapses and thus for the termination of glutamate signaling. At least five EAATs have been identified. Glutamate/aspartate transporter (GLAST, EAAT1), glutamate transporter1-1 (GLT-1, EAAT2), EAA carrier 1 (EAAC1, EAAT3), EAAT 4 (EAAT4), and EAAT5. Mason et al. first demonstrated glutamate signaling and downregulation of GLAST mRNA by mechanical loading in rat bone. Mechanical regulation of neural EAATs expression has recently been described in bones, suggesting that EAAs play a role in paracrine intercellular communication. Over the last few years, a small but growing number of researchers have provided increased evidence to support the role of glutamate as an important mediator of bone cell functions. Thus far, however, little attention has been paid to EAAT expression in the knee joint and its role in OA development. The present study, using the ACLT rabbit model, examined the concentration change in EAAs during OA development and the concomitant expression of GLAST, GLT-1, and EAAC1 in the articular cartilage of the knee joints.

METHODS

ANIMAL MODEL

The use of animals conformed to the Guiding Principles in the Care and Use of Animals of the American Physiology Society and was approved by the Animal Care and Use Committee of the National Sun Yat-Sen University, Taiwan. A total of 25 healthy New Zealand white adult male rabbits (body weight: 1530–1620 g) were used in this study. OA was induced in New Zealand white male rabbits via ACL transection (ACLT) in one knee of one hind limb; the other knee was left unoperated. Rabbits that received ACLT were sacrificed, and the rabbit knees were dissected immediately after death. For the separation of the synovial membrane from the medial and lateral compartments of the knee was dissected from the underlying tissues for histological examination, as previously described.

MEASUREMENT OF EAAs

High-performance liquid chromatography (HPLC) with a fluorescence detector (Lumapump 126, Beckman Instruments Inc., Fullerton, CA, USA) was used. The protocol for EAA detection was according to our previously described methods. External standards containing 0, 10⁻⁸, 10⁻⁷, 10⁻⁶, or 10⁻⁵ M standard amino acids were run before and after each sample group. The detection sensitivity was 10⁻⁹ M. All standards and samples were analyzed in duplicate.

INFLAMMATION, GROSS MORPHOLOGY, AND HISTOPATHOLOGICAL EXAMINATIONS OF KNEE JOINTS

The severity of knee joint inflammation was reflected by an increase in the hind limb knee joint width. The width of the bilateral hind limb knee joints was measured from the medial to the lateral aspect of the joint line by using calipers before (baseline) and at 5, 10, 15, 20, 25, and 30 weeks after operation. At week 30 after surgery and microdialysis sample collection, the rabbits were sacrificed, and the rabbit knees were dissected immediately after death. The knee was cut in the mid-saggital plane, washed in running tap water, and paraffin-embedded in an automatic processor (Autotechnicon mono 2, Technion Co., Chauncy, NY, USA). Serial articular cartilage sections (2 μm) were cut using a microtome (Microm HM940E, Walldorf, Germany) from the central weight-bearing surface of the medial femoral condyles of the ACLT, sham-operated, and naive knees. Cartilage was stained with hematoxylin/eosin (H&E) and safranin-O/fast green stains to assess general morphology and matrix proteoglycans. Microscopic examination of the articular cartilage in the medial femoral condyles was graded according to the Mankin’s grading system. A representative specimen of the synovial membrane from the medial and lateral compartments of the knee was dissected from the underlying tissues for histological examination, as previously described.

IMMUNOHISTOCHEMISTRY FOR EAATs

Cartilage specimens were processed for immunohistochemistry, as described previously. Sections (2 μm) of paraffin-embedded specimens were placed on slides, deparaffinized with xylene, dehydrated in graded series of ethanol, and the endogenous peroxidase activity was quenched by 30 min of incubation in 0.3% H₂O₂ in methanol at room temperature. The antigenic retrieval was achieved by enzymatic digestion with proteinase K (Sigma; 20 μg/mL) in phosphate buffered saline (PBS) for 20 min at room temperature in a humid chamber. The slides were incubated with the primary antibody against either GLT-1 (1:100 dilution; Chemicon) or GLT-1 (1:50 dilution; BD Biosciences Pharmingen) in 0.3% Triton X-100 in PBS overnight at 4 °C in a humidified chamber. Thereafter, sections were treated with avidin–biotin complex (ABC) technique using the ABC kit (Vectastain ABC kit; Vector, Burlingame, CA, USA). Sections from the negative control samples were incubated with PBS without antibody. The images were viewed using a Leica DM-1000 microscope (Leica, Heidelberg, Germany) and captured using a SPOT CCD RT-slider integrating camera (Diagnostic Instruments Inc., USA).

WESTERN BLOT ANALYSIS FOR EAATs

Rabbits were anesthetized with isoflurane, and full-thickness pieces of the articular cartilages of the tibial plateau and femoral condyle were removed aseptically from the subchondral bone with a scalpel, washed with iced PBS, and cut into small pieces with scissors. The cartilage was homogenized in ice-cold lysis buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 2% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, and 1 μg/mL aprotinin) by using a rotor/stator homogenizer (2000 rpm, three times for 10 s each). The samples were then sonicated three times using a Microson ultrasonic cell disruptor (Heat Systems, Farmingdale, NY, USA) for 15 s each at 20% output power. The homogenates were centrifuged at 50,000 g (TX-100 Beckman, USA) for 30 min at 4 °C. For EAAT analysis, a 50 μg cartilage homogenate sample was obtained. In brief, an equal volume of sample buffer (2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue, 2% 2-mercaptoethanol, and 50 mM Tris–HCl at pH 7.5) was added to the sample, which was then loaded onto a 10% SDS–polyacrylamide gel, and...
electrophoresis was performed at 150 V for 60 min. The proteins were transferred to a polyvinylidene difluoride membrane (0.45 M pore size, Immobilon-P; Millipore) at 125 mA overnight at 4°C in a transfer buffer (50 mM Tris–HCl, 380 mM glycine, 1% SDS, and 20% methanol); thereafter, the membrane was blocked for 50 min at room temperature with 5% non-fat dry milk in 0.1% Tween-20 in 20 mM Tris–HCl and 137 mM NaCl at pH 7.4. Tissue extracts were incubated for 180 min at room temperature with antibodies against GLAST (EAAT1, 1:1000 dilution; Chemicon, Temecula, CA, USA), GLT-1 (EAAT2, 1:1000 dilution; BD Biosciences Pharmingen), and EAAC1 (EAAT3, 1:1000 dilution; Chemicon, Temecula, CA, USA). Immunoblotting was then performed using appropriate antibodies and then with horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Immuno Research Laboratories Inc., USA). The immunoreactive bands were visualized by enhanced chemiluminescence (ECL kit; Millipore, Bedford, MA, USA), and then the images were visualized using the UVP Biochem Imaging System. Relative densitometric quantification was then performed using LabWorks 4.0 software (UVP, Upland, CA, USA). The relative variations between the bands of the various treatment samples and the control group (right side of the naive knees) were calculated using the same image.

DATA AND STATISTICAL ANALYSIS

All data are presented as the mean ± standard deviation (SD) and were analyzed by one-way analysis of variance (ANOVA) according to the Student–Newman–Keuls post hoc test for multiple comparisons. For the immunoreactivity data, the intensity of each test band was expressed as the integrated optical density. A significant difference was defined as a P value of <0.05.

Results

KNEE JOINT WIDTH AND GROSS MORPHOLOGICAL CHANGES

The severity of knee inflammation, reflected by an increase in the width of the hind limb knee joint, was significant at 5, 10, 15, 20, 25, and 30 weeks after the surgery in the ACLT knee group. In the ACLT knees, the joint width was 5.3 ± 0.9 mm greater than the contralateral knee at 30 weeks after the operation, while the sham-operated knees and the naive knees exhibited no significant change in this regard (Fig. 1, P < 0.05). In the articular cartilage of the ACLT knees, an edematous change was observed, accompanied by mild focal fibrosis and pitting and thinning of the medial femoral condyle. Osteophytes were visible in all femoral condyles and tibial plateaus in the ACLT knee joints at 30 weeks after surgery. The macroscopic scores were 2.68 ± 0.78 in the ACLT group (Table I). In the sham-operated and naive knees of the rabbits, the cartilage

Data are expressed as mean ± SD. ACLT, rabbits that underwent ACLT of the knee; sham, rabbits that only underwent arthroscopy of the knee; naive, normal control rabbits that did not undergo any surgery. *P < 0.05 when compared with the sham-operated and naive groups.

MICROSCOPIC FINDINGS

Specimens obtained from the ACLT knees exhibited focal fibrillation extending through the superficial zone up to the superficial zone—mid zone region. Moreover, loss of safranin-O/fast green staining in the calcified cartilage layer and cleft, depletion of chondrocytes in the cartilage layer, hyperplasia of chondrocytes, and irregularity of the bony trabeculum at the junction between the calcified cartilage and subchondral layer were observed in the ACLT knees. In the sham-operated and naive knees, the histological appearance of the medial femoral condyles was normal; the cartilage surface was smooth with no superficial zone discontinuity. The matrix and associated chondrocytes were organized into three appropriately oriented, well-ordered zones. No enlargement, distortion, or proliferative changes were observed in the chondrocytes. Further, preservation of safranin-O/fast green staining, normal chondrocytes in the calcified cartilage layer, and smooth bony trabeculum in the subchondral layer were observed. Significant differences in the histological Mankin score for OA were observed between the ACLT knees (8.50 ± 0.93) and both the sham-operated (1.83 ± 0.62) and naive knees (1.20 ± 0.53) (P < 0.05) (Table II). The synovium of the ACLT knees was thick with focal villi and

<table>
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<th>Group</th>
<th>Osteoarthritic score</th>
<th>Synovitis score</th>
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<tbody>
<tr>
<td>ACLT (n = 6)</td>
<td>8.50 ± 0.38*</td>
<td>9.50 ± 0.60*</td>
</tr>
<tr>
<td>Sham (n = 6)</td>
<td>1.83 ± 0.42</td>
<td>2.01 ± 0.26</td>
</tr>
<tr>
<td>Naive (n = 6)</td>
<td>1.20 ± 0.22</td>
<td>1.83 ± 0.34</td>
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Data are expressed as mean ± SD. ACLT, rabbits that underwent ACLT of the knee; sham, rabbits that only underwent arthroscopy of the knee; naive, normal control rabbits that did not undergo any surgery. For details on the osteoarthritic score (Mankin score) and the synovitis score, refer to the Methods. *P < 0.05 when compared with the sham-operated and naive groups.

Fig. 1. Time-course of joint width changes after operation. In the ACLT knees, the joint width at 30 weeks after surgery was 5.3 ± 0.4 mm greater than its baseline value, while it did not change significantly in the sham-operated and naive knees (P < 0.05).
exhibited hyperplasia of the lining cells, with moderate mono-
nuclear cell infiltration. The histology of the synovium was
normal in the sham-operated and naive knees. The synovitis
scores are shown in Table II; the score was significantly
higher in the ACLT group (9.50 ± 1.47, \(P < 0.05\)) than in
both the sham-operated (2.01 ± 0.64) and naive groups
(1.83 ± 0.83) (Table II).

EAA LEVELS IN THE KNEE JOINT DIALYSATES

Table III shows the concentration of aspartate and
glutamate in the knee dialysates of baseline (before
surgery) and at 10, 20, and 30 weeks after surgery in the
knees of naive, sham, and ACLT groups. No significant
difference was observed in the baseline level of EAs among
the three groups. For statistics analysis consider
the aspartate and glutamate level in the contralateral knee
as the 100% control. As shown in Fig. 2, at week 30 after
the ACLT surgery, the levels of aspartate and glutamate
in the ACLT knee dialysates were 187.5 ± 33.8% and
213.3 ± 29.6%, respectively (considering the corresponding
levels in the contralateral knee as 100%). A significant
increase in the glutamate level was observed in the ACLT
knees (213.3 ± 29.6%) \((P < 0.05)\) when compared with
that in the sham-operated knees (Fig. 2[B]). In brief, from
week 10 to week 30 after surgery, both the aspartate and
 glutamate levels were significantly increased in the joint
dialysates of the ACLT knees when compared with those
in the sham-operated knees \((P < 0.05)\). No difference was
observed in the aspartate and glutamate concentrations
between the right and left naive knees, and between naive
and sham-operated joints (data not shown).

IMMUNOHISTOCHEMISTRY OF EAATs

The immunolocalization of GLAST and GLT-1 protein
expression in cartilage specimens from naive and ACLT
groups were examined. As shown in Fig. 3, GLAST and
GLT-1 were located in chondrocytes of the superficial and
transitional zones of naive and ACLT knees. Figure 3(A
and B) shows the staining of GLAST and GLT-1 expression
in naive knees, while Fig. 3(C and D) shows the staining of
GLAST and GLT-1 expression in the ACLT knees. From the
four individual observations, we found that no staining was
observed in the negative control (Fig. 3(E and F)). Both
naive and ACLT chondrocytes were positively stained with antibodies against GLAST and GLT-1.

EFFECTS OF ACLT ON THE GLAST AND GLT-1 PROTEIN
EXPRESSIONS IN ARTICULAR CARTILAGE OF RABBIT
KNEE JOINTS

As shown in Fig. 4(A), the expression of GLAST and
GLT-1 proteins in knee cartilage homogenates (50 µg)
was analyzed by western blot of naive (right and left knees),
sham and ACLT knees at week 30 after surgery. The
GLAST antibody exhibited a band at ~55 kDa (open arrow)
and the GLT-1 exhibited a band at ~54 kDa (closed arrow).
Figure 4(B and C) shows that the quantification of GLAST
and GLT-1 protein expression. The GLAST protein

<table>
<thead>
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<th>Time after surgery</th>
<th>Naive/L</th>
<th>Sham</th>
<th>ACLT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp Glu</td>
<td>Asp Glu</td>
<td>Asp Glu</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.35 ± 0.08</td>
<td>4.11 ± 1.24</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td>10th</td>
<td>0.38 ± 0.08</td>
<td>4.36 ± 1.13</td>
<td>0.38 ± 0.08</td>
</tr>
<tr>
<td>20th</td>
<td>0.37 ± 0.08</td>
<td>4.37 ± 1.02</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td>30th</td>
<td>0.36 ± 0.07</td>
<td>4.50 ± 1.10</td>
<td>0.38 ± 0.09</td>
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Data are expressed as mean ± SD (µM) of six rabbits. Naive/L: left knee of naive rats.

Fig. 2. The EAA levels in the knee joint dialysates were measured
at 0 (baseline), 5, 10, 20, and 30 weeks after the surgery. The
level in the contralateral knee was considered as 100%. A significant
increase in the glutamate level was observed in the ACLT knees
(213.3 ± 29.6%, \(P < 0.05\)) when compared with that in the sham-operated knees. The difference in the aspartate concentration
was also significant between the ACLT knees and the sham-operated
knees (187.5 ± 33.8%). Both the aspartate (A) and glutamate
(B) levels increased significantly from week 10 to week 30 after the
surgery in the knee joint dialysates of the ACLT knee \((P < 0.05)\)
when compared with those in the sham-operated knees. \(*P < 0.05\) when compared with those in the sham-operated knee; L, left side of
the naive knee.
expression significantly increased in the cartilage of the ACLT and the sham-operated knees when compared with that in the naïve (both right and left) knees ($P < 0.05$). Moreover, the GLAST protein expression was significantly higher in the ACLT knees than in the sham-operated knee [Fig. 4(B)]. As observed in Fig. 4(C), GLT-1 expression was only significantly upregulated in the cartilage of the ACLT knees when compared with that in the sham-operated and naïve (both right and left) knees. No difference in the GLAST and GLT-1 protein expressions was observed between the right and left knees of the naïve rabbits. In the present study, EAAC1 protein expression was not observed in the cartilage of any of the knees.

**Discussions**

The present study confirmed that OA developed 30 weeks following ACLT in rabbits, and a concomitant increase in the glutamate and aspartate concentrations released was observed in the ACLT knees but not in the sham-operated knees. Both naïve and ACLT chondrocytes were positively stained with antibodies against GLAST and
GLT-1. More importantly, we observed changes in the EAAT expression in the articular cartilage of the ACLT knees. As compared to the sham-operated and naïve knees, the GLAST and GLT-1 protein expression increased significantly in the articular cartilage of the ACLT knees. To our knowledge, this paper is the first study to directly demonstrate the functional expression of specific EAATs in articular cartilage in relation to the pathogenesis of OA following ACLT of the knees in an animal model.

It is well-known that knee instability following a complete ACL tear often induces OA and is accompanied by degradation of the articular cartilage matrix. Consistent with our previous study, OA developed in the rabbits 30 weeks following ACLT in the present study. Synovial inflammation was observed to play an important role in OA progression. The mild-to-moderate inflammatory changes observed in the synovium of the ACLT knees in our study were compatible with those observed in previous studies. Previous studies have demonstrated a drastic increase in endogenous glutamate levels (6.25 vs 326 μM) in the synovial fluid obtained from the knees of rheumatoid arthritis (RA) patients, whereas the elevation of glutamate in the synovial fluid was shown to be relevant to the edema and the thermal hyperalgesia in experimental arthritis models. In our present study, the increase in the knee joint width indicated an increase in inflammation with time, and a corresponding increase in the amount of synovial fluid and the concentrations of glutamate and aspartate in the joint dialysates of ACLT knees (Fig. 2).

Glutamate has been detected in the network of nerve fibers transversing the bone marrow as well as in osteoblasts, where it may be released due to exocytosis. OA-associated pain may arise due to glutamate release from the axons innervating the inflamed region. Flood et al. demonstrated that MK-801 (a non-competitive antagonist of N-methyl-D-aspartate (NMDA) receptors) in RA fibroblast-like synoviocytes induced an increase in matrix metalloproteinase-2 release, whereas non-NMDA ionotropic glutamate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBOX) inhibited interleukin-6 production; this suggested that glutamate receptor antagonist may influence the RA pathology. Moreover, glutamate receptor antagonists inhibit the proliferation of synovial fibroblasts, suggesting that these antagonists exert a disease-modifying effect on synovial proliferation occurring in RA. In the present study, from week 10 to week 30 after ACLT, increased levels of EAA glutamate and aspartate were observed in the ACLT knee dialysates.

Fig. 4. Western blots of GLAST and GLT-1 (A) in various knee cartilage extracts. The knee cartilages were collected at 30 weeks after ACLT surgery. Lanes show samples from right side of the naïve knee (lane no. 1), left side of the naïve knee (lane no. 2), sham-operated knee (lane no. 3), and ACLT knee (lane no. 4). The GLAST antibody exhibited a band at ~55 kDa (closed arrow) and the GLT-1 exhibited a band at ~54 kDa (open arrow). The densitometric quantifications for GLAST (B) and GLT-1 (C) were normalized to that for total loading protein (50 μg) from knee cartilage homogenates, and each bar represents the mean ± SD of five independent experimental results. *P < 0.05 when compared to the right knee of the naïve rabbits; #P < 0.05 when compared to the sham-operated knees. R = right side of the naïve knee; L = left side of the naïve knee. Bands marked with * in (A) are non-specific binding of secondary antibody.
as compared to those in the sham-operated knee dialysates; this is consistent with our previous study, where early OA development occurred 20 weeks after the ACLT with a concomitant increase in the glutamate and aspartate concentrations in the ACLT knees but not in the sham-operated knees of Wistar rats. These biochemical changes with regard to the EAAs elevation in the dialysates of ACLT knees are attributed to the pathogenesis of OA.

EAATs are known to be responsible for maintaining the homeostasis of the extracellular glutamate concentration; they protect neurons against harmful overstimulation of glutamatergic receptors. Glutamate cytotoxicity is only evident at a high concentration of 10 mM. Genever et al. have previously demonstrated that osteoblasts express GLAST and rapidly accumulate [3H]-glutamate; this provides a possible molecular mechanism for intracellular glutamate accumulation. In our present study, by immunohistochemical analysis, we found an expression of EAAT isoforms (GLAST and GLT-1) in the chondrocytes of naive and ACLT cartilage (Fig. 3). Cultural rat costal chondrocytes were demonstrated to express the glial EAAT GLT-1. To our knowledge, this is the first report of the EAAT protein expression in chondrocytes of naive and ACLT-induced OA knee cartilage. Our results confirm EAAT expression in chondrocytes. The molecular weights of GLAST and GLT-1 proteins in the CNS were different from those reported in the previous studies; GLAST and GLT-1 immunoreactive bands were detected at 65-70 kDa and 65-70 kDa, respectively, in the neuronal tissue. In the present study, we found that GLAST protein expression significantly increased in the cartilage of the ACLT and sham-operated knees when compared with that in the naive and ACLT-induced cartilage (Fig. 4B); the immunoreactive bands of GLAST and GLT-1 were found at ~55 kDa and ~54 kDa, respectively. Several studies have demonstrated that the molecular weights of GLAST and GLT-1 range from 65 to 70 kDa and 54 to 55 kDa, which correspond to their glycosylated and non-glycosylated forms, respectively. Danbolt et al. and Raunser et al. also found a 73 kDa band corresponding to glycosylated GLT-1 and a 55 kDa band corresponding to non-glycosylated GLT-1. Moreover, Conradt et al. also revealed a 65 kDa band corresponding to the glycosylated GLAST and a 56 kDa band for the non-glycosylated GLAST. Both the glycosylation and non-glycosylation of GLAST and GLT-1 did not affect the transport activities. Another possibility of the different molecular weights of EAATs (GLAST and GLT-1) is splice variant. A splice variant of GLAST, namely, GLAST-1a, lacking of exon-3 is expressed in the brain and brain, and its non-glycosylated form has a molecular weights of 54 kDa. Moreover, the exon-9 skipping form of GLAST-1b expresses predominantly in the plasma membrane of apparently abnormal neurons. It suggested that the equivalent exon-9 skipping form of GLAST (GLAST-1b) is a negative effector of function. The exon-9 skipping form of GLT-1 was cloned from human tissues and was initially described selectively present in the brain of patients with motor neuron disease of amyotrophic lateral sclerosis, and this splice variant was a causal factor for the pathogenesis of this disease.

In our present study, from the result of western blot analysis, it was suggested that both GLAST and GLT-1 proteins expressed in cartilage were not glycosylated. It is interesting that an increase in GLAST expression was observed in sham-operated knees as compared to naive knees in our present study; the reason for this feature is yet unclear and requires further investigation. Among the EAAT subtypes, both GLAST and GLT-1 mRNA and proteins have been demonstrated in bone, while the EAAC1 immunoreactivity is absent. However, by the immunohistochemical analysis, EAAC1 protein expression was found in the synovial tissue. In contrast, our present study failed to demonstrate the protein expression of EAAC1 in the knees of all examined rabbits. Collectively, our findings suggest the possibility of a compensatory mechanism of glutamatergic signaling pathways for this increase in EAAT isoform expression and EA receptor in the articular cartilage of OA knees. However, the roles of GLAST and GLT-1 in this regard remain unclear. Our present results do not exclude the contribution of other biological substances to the development of OA in ACLT knees.

In conclusion, our results show that OA of the knee is observed in rabbits at 30 weeks after ALC. An associated increase observed in the glutamate and aspartate levels in the dialysates of the ACLT knees may be involved in the process of OA development. Furthermore, an increase in GLAST and GLT-1 expressions in the ACLT knee cartilage was also demonstrated in immunohistochemistry and western blot data. Understanding the role of glutamate and the regulation of its uptake in animal models of ACLT-induced OA will be helpful for elucidating the mechanisms underlying the pathogenesis of OA and for developing new therapeutic strategies.

Conflict of interest
None.

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Author contributions
Dr. Wong had full access to all of the study data and assumes responsibility for its integrity and for the accuracy of the data analysis.

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Data acquisition: Yen-Hsuan Jean, Zhi-Hong Wen, Yi-Chen Chang, Shih-Peng Hsieh, An-Kuo Chou, and Jin-Ding Lin.

Data analysis and interpretation: Jin-Ding Lin, Chi-Chieh Tang, Wu-Fu Chen, An-Kuo Chou, Zhi-Hong Wen, and Yi-Chen Chang.

Manuscript preparation: Yen-Hsuan Jean, Zhi-Hong Wen, Yi-Chen Chang, and Jin-Ding Lin.

Statistical analysis: Zhi-Hong Wen, Yi-Chen Chang, Chi-Chieh Tang, and Jin-Ding Lin.

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