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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 naïve 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 naïve 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 naïve, 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 $213.3 \pm 29.6\%$ and $187.5 \pm 33.8\%$ ($P < 0.05$) when compared to those in the sham-operated knees. Both naïve 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[1]. OA is a synovial joint disorder that is characterized by the destruction of the articular cartilage accompanied by inflammation[2,3]. Patients with rupture of the anterior cruciate ligament (ACL) develop post-traumatic OA of the knee[4]. Restoration of knee stability provides symptomatic relief but does not reduce the

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degenerative changes in the ACL-injured knee[5]. This suggests that the development of post-traumatic OA not only has a biomechanical origin but may also involve biochemical changes.

Glutamate is a well-known excitatory neurotransmitter in the mammalian central nervous system (CNS), being responsible for up to one-third of the excitatory synaptic functions[6]. Growing evidence indicates that glutamate also plays a role in cell signaling in peripheral tissues such as the bone[7–9]. Glutamate increases in the innervated axons of inflamed knee joints[10] and the synovial fluid of arthritic patients[11]. 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[12]. 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[13]. The physiological extracellular concentration of glutamate is regulated by the action of high-affinity, sodium-dependent EAA transporters (EAATs)[6]. EAATs are the key mechanism 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[6,14]. Glutamate/aspartate transporter (GLAST, EAAT1), glutamate transporter-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[15]. Mechanical regulation of neural EAATs expression has recently been described in bones, suggesting that EAAs play a role in paracrine intercellular communication[15]. Over the last few years, a small but growing number of researchers have provided increasing evidence to support the role of glutamate as an important mediator of bone cell functions[7,8]. 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 assigned to the ACLT group ($n=6$), while the sham-operated group ($n=6$) underwent arthrotomy with no ACLT. Six naïve rabbits that received no surgery were used as normal control. For the sham operation, the ACL of the knee was exposed but not transected. This procedure was modified from the protocol described by Stoop *et al.*[16]. Rabbits were allowed daily unrestricted cage activity after surgery. Wound healing and infection or any other complications were monitored continuously during the 30-week observation period.

CONSTRUCTION AND PLACEMENT OF THE MICRODIALYSIS PROBE

A microdialysis probe was implanted in each rabbit knee joint under isoflurane anesthesia for joint dialysate collection before (baseline) and at 5, 10, 20, and 30 weeks after the ACLT or sham operation. The construction and placement of the microdialysis probe were done according to our previous reports[13,17]. For the *in vitro* measurement, the average recovery rate of the dialysis probe was $19.5 \pm 5.2\%$ at an infusion rate of 1.5 μL/min of eight different microdialysis probes. All samples were collected in polypropylene tubes, kept on ice, and then frozen at −80°C until assayed.

MEASUREMENT OF EAAs

High-performance liquid chromatography (HPLC) with a fluorescence detector (pump 126, Beckman Instruments Inc., Fullerton, CA, USA) was used. The protocol for EAA detection was according to our previously described methods[18]. 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^{-8} 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 disarticulated immediately after death. Gross morphologic changes in the cartilage of the medial femoral condyle were examined according to previously described methods[19,20]. The joints were cut 0.5 cm above and below the joint line, fixed in 10% neutral buffered formalin for 3 days, and then decalcified for 2 weeks in buffered 12.5% ethylene diamine tetraacetic acid (EDTA)/formalin solution. The joints were cut in the mid-saggital plane, washed in running tap water, and paraffin-embedded in an automatic processor (Autotechnicon mono 2, Technion Co., Chauncey, NY, USA). Serial articular cartilage sections (2 μm) were cut using a microtome (Microm HM340E, Walldorf, Germany) from the central weight-bearing surface of the medial femoral condyles of the ACLT, sham-operated, and naïve 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[21]. 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[22].

IMMUNOHISTOCHEMISTRY FOR EAATs

Cartilage specimens were processed for immunohistochemistry, as described previous[23]. 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% H_2O_2 in methanol at room temperature. The antigen was retrieved by enzymatic digestion with proteinase K (Sigma; 20 mM) 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 GLAST (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 g/mL phenylmethylsulfonyl fluoride, and 1 μg/mL aprotinin) by using a rotor/stator homogenizer (2000 rpm, three times for 10 s each). All 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* (TXL-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 7.2] was added to the sample, which was then loaded onto a 10% SDS–polyacrylamide gel, and