Increased concentrations of neuro-excitatory amino acids in rat anterior cruciate ligament-transected knee joint dialysates: a microdialysis study

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Abstract

Changes in excitatory amino acid (EAA) levels were examined in the knee joint dialysates of rats with early osteoarthritis (OA). Early OA was induced by anterior cruciate ligament (ACL) transection in one knee and the contralateral knee was used as the sham-operated control, the side for ACL transection being assigned randomly. Twenty weeks after operation, knee joint dialysates were collected by microdialysis and assayed for EAAs by high performance liquid chromatography. The rats were then sacrificed for histopathological examination. Hematoxylin/eosin and Safranin-O staining showed cartilage fibrillation, clustering of chondrocytes, and a reduction in matrix proteoglycans at week 20 in the ACL-transsected knee, but not in the sham-operated knee. Levels of glutamate and aspartate in dialysates from the ACL-transsected knee were significantly increased by 92 ± 20.3% or 57 ± 17.5%, respectively, compared to those in the contralateral sham-operated knee. This increase may contribute to the pathogenesis of early OA.

Keywords: Osteoarthritis; Excitatory amino acid; Anterior cruciate ligament; Glutamate; Microdialysis

Introduction

Osteoarthritis (OA), a degenerative joint disease, has been considered to be caused by long-term mechanical stimulation and the aging process [12]. However, recent studies have revealed a role of the inflammatory process in the pathogenesis of OA [22,26,30], and potential mediators have been described in OA and rheumatoid arthritis (RA) [35]. Patients with rupture of the anterior cruciate ligament (ACL) develop post-traumatic OA of the knee [20,31]. Restoring knee stability through reconstruction provides symptomatic relief, but does not decrease the incidence of degenerative changes of the
ACL-transected knee [8,14]. This suggests that post-traumatic OA may not have a purely biomechanical origin and that chemical alterations may also be involved.

Excitatory amino acids (EAAs) are produced by the sensory neurons which mediate excitatory neurotransmission in the mammalian spinal cord [10,13]. A study on an inflammatory joint animal model showed an increase in spinal EAA levels [9]. The involvement of EAAs in peripheral nociceptive transduction has also been postulated in animal models of acute arthritis [2,4,5]. Injection of 3% kaolin/carrageenan into knee joints causes both chemical and mechanical irritation, and both peripheral and central mechanisms are involved in the articular inflammation and pain formation [2,29].

Glutamate transporters have been identified in bone, raising the possibility of the involvement of EAAs in paracrine signaling between bone cells [6,9,19]. The present study used an experimental model to examine the role of EAAs in early OA development. The use of the microdialysis technique allowed biochemical changes in joint dialysates to be continuously examined and the sham-operated knee was used as the control, thus eliminating individual variation. Our results showed an increase in EAA levels in the ACL-transected knee, but not in the contralateral sham-operated knee. We suggest that, in addition to mechanical stimulation, biochemical changes may be responsible, at least in part, for the development of early OA.

Materials and methods

Animal model

The use of rats conformed to the Guiding Principles in the Care and Use of Animals of our Institute and was approved by the Animal Care and Use Committee of our Institute. Three-month-old male Wistar rats (body weight 250–270 g) were housed individually on a 12-h light/dark cycle with food and water freely available. Rats with pre-existing anatomical or gut abnormalities were excluded. The side of the knee to undergo ACL transection or sham-operation was assigned randomly. For surgery, rats were anesthetized with 3% isoflurane in an oxygen/air mixture (1:1) at a flow rate of 3 l/min, anesthesia being considered adequate when no flexor withdrawal was seen when the foot was pinched. Body temperature was monitored and maintained at 37 °C using a temperature controller (CMA 150, Bioanalytical Systems Inc., USA) and a homeothermic blanket system. The knees were shaved and disinfected with betadine solution, then the medial parapatellar incision was made in the skin, and median arthroscopy performed. The patella was dislocated laterally and the knee placed in full flexion. The ACL was exposed and identified under direct vision and cut through the mid-substance. Adequacy of section was confirmed by a positive anterior draw sign. The joint was irrigated with normal saline, the capsule sutured with 4-0 vicryl, and the skin closed with 4-0 nylon mattress sutures. For the sham operation, the ACL of the contralateral knee was exposed, but not transected. This procedure was modified from the protocol described by Stoop et al. [32]. Cephazolin (20 mg/kg) was given intraperitoneally preoperatively and every 12 h for 3 days after operation for prophylactic infection control. Rats were allowed daily unrestricted cage activity after surgery. Wound healing, infection, and any other complications were monitored continuously during the 20 week observation period.

Construction and placement of the microdialysis probe

Twenty weeks after ACL transection or sham operation, a microdialysis probe was implanted in each knee joint under isoflurane anesthesia, induced as described above. A 27-gauge needle attached to a tuberculin syringe was passed through the joint capsule lateral to the patellar ligament, then the microdialysis probe was inserted through the needle into the knee joint, following the protocol described by Lawand et al. [16], and its position checked by X-ray examination. The microdialysis probe was constructed using two 5 cm polyethylene tubes (0.008 in. inner diameter, 0.014 in. outer diameter) and a 1.5 cm cuprophan hollow fiber (300 μm outer diameter, 200 μm inner diameter, 50 kDa molecular weight cut-off, Filtral, AN 69-HF, Eicomp Co., Kyoto, Japan). A Nichrome-Formvar wire (0.0026 in. diameter, 12 cm) was passed through two polyacryl tubes (194 μm outer diameter, 102 μm inner diameter; 1 cm) and the cuprophan hollow fiber. The ends of the external silastic tubes were connected to a syringe pump (CMA-100, CMA/Microdialysis Inc., Solna, Sweden) for sample collection. The diagrammatic representation of the microdialysis in our rat model was shown in Fig. 1. The dialysis probe was perfused, at a flow rate of 5 μl/min, with modified Ringer’s solution of NaCl 8.60 mg/ml, 0.30 mg/ml KCl, and 0.33 mg/ml CaCl2 bubbled with 5% CO2 in 95% O2 to give a final pH of 7.4. After a 30 min equilibration, the dialysate was collected over the next 3 h. During the in vitro measurements, the recovery rate of the dialysis probe was 45% at an infusion rate of 5 μl/min. All samples were collected in polypropylene tubes, kept on ice, then frozen at −80 °C until assayed.

Measurement of excitatory amino acids

High performance liquid chromatography with a fluorescence detector (pump 126, Beckman Instruments Inc., Fullerton, CA, USA) was used as described in our previous report [34]. In brief, a reverse-phase C-18 column with o-phthaldialdehyde (27 mg, 1 ml of methanol, and 10 μl of 2-mercaptopentanol) pre-column derivatization and a fluorescence detector was used. Samples were injected onto the column, which was eluted at a flow rate of 0.45 ml/min with a linear gradient from 100% mobile phase A (20 mM sodium acetate, pH 7.2, 0.18% triethylamine, and 0.3% tetrahydrofuran) to 75% phase A, 25% phase B (100 mM sodium acetate:acetonitrile:methanol = 1:2:2) in 30 min, then for 5 min with 100% phase B. Using this protocol, glutamate, aspartate, serine, taurine and glutamine were successfully separated. 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.

Histopathological examination of joints

The width of the knee joint was measured bilaterally using calipers (AA847R, Aesculap, AG&CO, KG, German) before (baseline), and 4, 8, 12, 16, and 20 weeks after ACL transection. At week 20 after ACL transection, the rats were sacrificed by deep anesthesia with sodium pentobarbital (50 mg/kg), then perfused intracardially with heparinized saline (400 ml) followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4. The knees were disarticulated aseptically, then the joints were cut 0.5 cm above and below the joint line, stripped of muscle, fixed in 10% neutral buffered formalin for 2 days, then decalcified for one day in 10% formic acid, which was changed daily. After X-ray confirmation of decalcification, the joints were cut in the mid-sagittal plane, washed in running tap water, and paraffin-embedded in an automatic processor (Autotechnicon mon 2, Technicon Co, Chauncey, NY, USA). Serial articular cartilage sections (5 μm) were cut (Leica 2065 rotary microtome, Leica Instruments, Wetzlar, Germany) from the central weight-bearing surface of the medial femoral condyles of the ACL-transected and sham-operated knees. Synovial membrane specimens were carefully dissected from the suprapatellar notch and the medial tibiofemoral compartments. Ten slides were prepared from each knee. Cartilage was stained with hematoxylin/eosin (H/E) and Safranin-O stain to assess general morphology and the loss of proteoglycan in cartilage ground substance. The sections for Safranin-O stain were deparaffinized in xylene and hy-
drated in a graded series of solutions of ethanol in distilled water. The
staining solution, 0.5% (w/v) Safranin-O (Lot 713466; Fisher Scientific,
Fairlawn, NJ), was prepared in 0.1 mol/l of sodium acetate buffer at
pH 4.6. Staining was carried out for 10 min, and the sections were
dehydrated in ethanol solutions and cleared in xylene [15]. The syno-
vium was stained with H/E stain. All samples from both knees were
stained, and examined independently by two observers. The articular
cartilage in the medial femoral condyle was graded according to Man-
kin's histologic grading [18; this score assesses structure (0–6 points),
cellularity (0–3 points), matrix staining (0–4 points), and tidemark
integrity (0–1 points), and has a maximum of 14 points. The Mankin's
scores were divided into three stages: 0–6 points (stage I; mild degen-
erative change), 7–9 points (stage II; moderate degenerative change),
and 10 or more points (stage III; severe degenerative change, i.e., car-
tilage disorganization or complete cartilage loss with extensive expo-
sure of subchondral bone). Severity of synovitis was also evaluated
histologically [36].

Data and statistical analysis

All data presented as mean ± SEM. Data on the knee joint width
was using analyzed using one-way ANOVA with Fisher’s PLSD post
hoc tests for multiple comparisons. Data on the histopathological
changes and excitatory amino acids were analyzed using paired Stu-
dent’s t-test for statistical analysis, a p value < 0.05 being considered
significant.

Results

One rat died due to deep anesthesia, and another rat died 2 weeks after ACL transection due to wound infec-
tion were excluded from the study. All ACL transected
knees showed complete transection of the ACL at sacri-
cifice, with only a stump remaining.

Knee joint width and gross morphologic changes

In the ACL-transected joints, the joint width at 20
weeks after operation was 2.6 ± 0.3 mm greater than
the baseline value, while the sham-operated knee
showed no change, the difference between the two joints
being significant (Fig. 2, *p < 0.05). In the articular carti-
lage of the ACL-transected knees, an edematous change

![Fig. 1. Schematic diagram of the knee joint microdialysis setup and microdialysis probe construction. (A) The position of the dialysis probe in the knee joint. (B) The construction structure of the microdialysis probe.](image)

![Fig. 2. Time-course of joint width changes after operation. The widths of the bilateral knee joints were measured in each rat before, and at 4, 8, 12, 16, and 20 weeks after ACL transection. A significant increase in the width of the ACL-transected knee joint was seen compared to the sham-operated knee (*p < 0.05). Data (mean ± SEM) are expressed as the difference in knee width between the values at each time point and baseline (before surgery).](image)
was seen, together with mild focal fibrillation and pitting and thinning of the medial femoral condyle. The synovium from the ACL-transected knee exhibited congestion and an inflammatory reaction. Articular cartilage from the contralateral sham-operated knee appeared normal, with a glistening, smooth surface, and no cartilage defects or osteophytes were observed, while the synovium showed no hyperemia or evidence of synovitis.

**Microscopic findings**

On histopathological examination using H/E or Safranin-O staining, cartilage at the medial femoral condyle in most of the ACL-transected knees exhibited the characteristic pathological changes of early OA (Fig. 3). The mean Mankin score for cartilage from the ACL-transected knee was 5.93 ± 0.21, while that for cartilage from the sham-operated knee was 1.35 ± 0.14 ($p < 0.05$, Table 1). The ACL-transected knees showed surface fraying, chondrocyte clustering, and proteoglycan reduction (Fig. 3). In the sham-operated knees, a thin, glistening, smooth lamina filled with flattened chondrocytes was observed, and no loss of proteoglycan was seen in the matrix by Safranin-O stain (Fig. 3). Table 1 also shows the scores for the evaluation of the synovial tissues in the ACL-transected and sham-operated knees. Mild to moderate inflammation (8.5 ± 2.8) was noted in the ACL transected knees compared to the sham-operated knees (1.2 ± 0.3) ($p < 0.05$, Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Osteoarthritic score</th>
<th>Synovitis score</th>
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<tbody>
<tr>
<td>OA ($n = 11$)</td>
<td>5.93 ± 0.21*</td>
<td>8.5 ± 2.8*</td>
</tr>
<tr>
<td>Sham ($n = 14$)</td>
<td>1.35 ± 0.14</td>
<td>1.2 ± 0.3</td>
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* Denotes a significance difference ($p < 0.05$) in the paired $t$-test compared to the sham-operated knee. Data are expressed as mean ± SEM.

The synovium in the contralateral sham-operated knee showed a normal pattern (Fig. 4).

**Excitatory amino acid concentrations in knee joint dialysates**

Twenty weeks after ACL transection, levels of aspartate and glutamate in dialysates from the ACL-transected knee were significantly increased by 57 ± 17.5% ($n = 14$) and 92 ± 20.3% ($n = 15$), respectively, when compared to those in the contralateral sham-operated knee dialysates ($p < 0.05$, Fig. 5). The concentration of serine, taurine and glutamine in the dialysates showed no significant difference between ACL-transected knee and the contralateral sham-operated knee (data not shown).

**Discussion**

In the present study, early OA development was seen 20 weeks after ACL transection in rats and a concomi-

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**Fig. 3.** Histopathological examination of the medial femoral condyles. (A) Degenerative changes (fibrillation formation and chondrocyte clustering) in the knee joint at 20 weeks after ACL transection (H/E stain, ×200). (B) Normal knee articular cartilage in the sham-operated knee (H/E stain, ×200). (C) Reduction of matrix proteoglycan in the ACL-transected knee and fibrillation of articular surface is also shown (arrow) (Safranin-O stain, ×200). (D) Normal matrix proteoglycan in the sham-operated joint. Note the smooth articular surface (arrow), absence of fibrillation and persistence of pericellular staining (Safranin-O stain, ×200).
tant increase in glutamate and aspartate concentrations was observed in the ACL-transsected knee, but not the sham-operated knee. This is the first report of such findings. The diagnosis of early OA is based on early histopathologic changes in the cartilage, including fibrillation of the superficial zone of the cartilage, extending laterally from the superficial to the deep layer, a net loss of proteoglycans and type II collagen, and chondrocyte clustering [27]. In a longitudinal study in the ACL-transected dog model for post-traumatic OA, Brandt et al. [3] reported that the degenerative changes seen in the knee joint 54 months post-ACL transection are similar to those seen in late-stage OA in humans. The late-stage OA in patients is characterized by a progressive loss of cartilage, sclerosis changes in the subchondral bone, and marginal new bone formation [8]. In our study, in which ACL transection of the knee of Wistar rats, followed by normal daily activity for 20 weeks, was used to study the early phase of OA, the histologic changes observed in the OA cartilage from the ACL-transected knees were similar to those described previously in ACL-transsected knee dogs [25]. In OA, synovial inflammation plays an important role in the disease process [11,28]. The mild to moderate inflammatory changes seen in the synovial membrane in the OA knees in our study were compatible with those in previous reports [7,11]. These results support the use of this animal model for studying the effect of mechanical stress on biochemical changes in the injured knee.

In vitro studies have demonstrated that EAAs play an important role in nociceptive transmission in various animal models of pain [1]. Tracey et al. [33] reported that both glutamate and aspartate are present in the cell bodies and terminals of nociceptive primary afferents. In addition to the effect of EAAs in the CNS, glutamate was recently shown to play a role in sensory transduction in the periphery [2,23]. Intra-articular injection of EAAs into the knee joint results in thermal hyperalgesia and mechanical allodynia, which are attenuated by local injection of N-methyl-D-aspartate (NMDA) or non-NMDA receptor antagonists [17]. In the inflamed state in arthritic patients, an increase in the glutamate

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**Fig. 4.** Histopathology of synovium. (A) The ACL-transsected knee shows synovial lining cell proliferation with palisading and a marked mixed cellular infiltration, consisting mainly of monocytes in the underlying tissues (arrow) (H/E stain, original magnification, ×200). (B) Synovial membrane from a sham-operated knee at 20 weeks after surgery. There is no synovial lining cell hyperplasia and only very slight cellular infiltration is seen in the underlying tissue (H/E stain, original magnification, ×200).

**Fig. 5.** EAA concentrations in joint dialysates of ACL-transsected and sham-operated knees at 20 weeks after operation. In the ACL-transsected knee, aspartate and glutamate concentration were increased by 57 ± 17.5% (A, n = 14) and 92 ± 20.3% (B, n = 15) of those in the contralateral sham-operated knee (p < 0.05).
concentration is observed not only in axons in the inflamed region [4], but also in the synovial fluid [21]. Electrophysiological studies have also shown that blockade of glutamatergic NMDA receptors in the knee joint prevents the development of peripheral nociceptive sensitization of inflammatory joints [16]. The observation that NMDA receptors are expressed in human and rat osteoblasts and osteoclasts suggests a role of the glutamate signal pathway in bone cells [6,24]. Lawand et al. [16] reported that glutamate is released from neuronal endings in inflammatory joints in rats and that this is prevented by direct injection of lidocaine into the knee joint. A clinical study showed that the aspartate concentration is increased two-fold in the synovial fluid of arthritic patients compared to controls [21]. Consistent with this, our results showed that glutamate and aspartate levels were significantly increased in dialysates from ACL-transected knees, suggesting that EAAs play a role in early OA development. However, our present results do not exclude a contribution of other biological substances in the early OA knee joint.

In conclusion, our results show that early OA of the knee joint is seen in rats at 20 weeks after ACL transection. A parallel increase in EAA levels in dialysates from the injured joint is observed and may be involved in the process of OA development. Such biochemical changes in the dialysate from ACL-transected knees could be a marker for the development of OA and act as a predictor for the prognosis of ACL transection. This offers the potential for medical therapy aimed at inhibiting EAA release to prevent the development of OA in injured knees. Further studies are required to determine the importance of EAAs in early OA development and their possible contribution to progressive joint damage.

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