Tensile properties of rat anterior cruciate ligament in collagen induced arthritis

K Nawata, M Enokida, D Yamasaki, T Minamizaki, H Hagino, Y Morio, R Teshima

Abstract
Objectives—To investigate the effects of collagen induced arthritis (CIA) on the tensile properties of rat anterior cruciate ligament (ACL).
Methods—The tensile strength, bone mineral density (BMD), and histology of ACL units from rats with CIA were investigated.
Results—The tensile strength of the ACL unit was significantly lower in the rats with CIA at 10 weeks after immunisation (ultimate failure load, 74.9% of the control; stiffness, 62.0% of the control). The major mode of failure was femoral avulsion, and the BMD was significantly lower in the rats with CIA. A histological examination of the ligament insertion in rats with CIA showed resorption of the cortical bone beneath the ACL insertion and an enlarged mineralised fibrocartilage zone.
Conclusions—These findings indicate that the decrease in tensile strength of ACL units correlated with histological changes in the ligament-bone attachment, such as bone resorption beneath the ligament insertion site and an enlargement of the mineralised fibrocartilage zone.

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Rheumatoid arthritis (RA) is a chronic systemic disease. The knee joints of patients with RA are commonly affected by ligamentous laxity.^{1–5} Although the instability is partly due to destruction of cartilage and bone in severely affected knees, knee stability is mainly provided by the ligaments, which are important components of the mechanical functioning of the knee. It is also known that laxity or instability may contribute to the development and progression of degeneration and attrition of the articular cartilage.^{6–8} However, little is known about changes in the tensile properties of ligaments in RA.

Collagen induced arthritis (CIA) is a well known disease model for RA. CIA resembles RA in a number of pathological, histological, and immunological aspects.^{9–11} Features of CIA include chronic synovitis, including inflammatory cell infiltration, pannus formation, destruction of cartilage, and bone erosion.

In this study we aimed at clarifying the changes in tensile properties and histology occurring in the anterior cruciate ligament (ACL) of rats with CIA.

Materials and methods

ANIMALS

Female Sprague-Dawley rats, 4 weeks of age, were obtained from Shimizu Laboratory Supply Co (Kyoto, Japan) and acclimatised for

four weeks under standard laboratory conditions. All rats were 8 weeks old at the beginning of the study. During the experimental period, tap water and commercially available food (CE-2, CLEA Japan Inc, Tokyo; calcium content 1.18 g/100 g; phosphorus content 1.09 g/100 g; vitamin D₃ content 250 10 U/100 g) were given freely. The lighting duration in the breeding room was 12 hours (7 00 am to 7 00 pm). The room temperature was 24°C. The experiment was approved by the Committee of Laboratory Animals, Faculty of Medicine, Tottori University.

EXPERIMENTAL DESIGN

Twenty rats were categorised by body weight into two groups of 10 animals each. In the first group (CIA group), weighing 141.0 (SD 9.95) g, arthritis was induced. The other group (control group), weighing 137.8 (2.76) g, served as the age matched controls. There was no significant difference in body weight between the groups (p=0.533). The clinical signs of arthritis and bone mineral density were evaluated by the same person for each rat. Rats were killed 10 weeks after the start of the experiment. The femur-ACL-tibia complex from the right knee of each rat was used for mechanical testing. The ACL from the left knee was examined histologically.

INDUCTION OF CIA

CIA was induced by established methods as previously described.⁹ Briefly, rats were injected intradermally around the root of the tail with 0.5 mg bovine type II collagen (Cosmo Bio Co, Tokyo, Japan) dissolved in 0.5 ml incomplete Freund's adjuvant (Difco). Seven days later, the rats were given a booster by the same method. A clinical evaluation of arthritis was performed by measuring the thickness of the hind foot using calipers every two weeks after immunisation.

BONE DENSITOMETRY

An in vivo bone mineral measurement was performed at eight weeks after immunisation by peripheral quantitative computed tomography (pQCT; XCT-960, Norland-Stratec). The rats were anaesthetised with 50 mg/kg body weight of ketamine hydrochloride and 10 mg/kg body weight of xylazine intraperitoneally. Measurements were made 2 mm apart (metaphysis) from the epiphysial line. A 0.30 mm voxel was used. A threshold for cortical bone of 0.93 and for trabecular bone of 0.63 was selected to establish a tomographic limit between the cortical and trabecular bone regions. The coefficient of variation for the measurement of bone density by pQCT was 1%.

Department of Orthopaedic Surgery, Faculty of Medicine, Tottori University, Yonago, Japan
K Nawata
M Enokida
D Yamasaki
T Minamizaki
H Hagino
Y Morio
R Teshima

Correspondence to:
Dr K Nawata, Department of Orthopaedic Surgery, Faculty of Medicine, Tottori University, Yonago, Tottori, 683–8504 Japan
nawata@grape.med.tottori-u.ac.jp

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TENSILE FAILURE TEST
All specimens were dissected just before testing. The femur-ACL-tibia preparation was carried out by adapting an established method.¹² The dissected specimens were mounted at approximately 80 degrees of knee flexion on a test fixture, which was designed so that the only relative motion that could occur between the tibia and femur was linear displacement nearly parallel to the axis of the ligament. A conventional tensile tester (Autograph AGS-H; Shimadzu, Kyoto, Japan) was used. The specimen was subjected to tensile testing to failure at an elongation rate of 10% of the initial ligament length per second.

HISTOLOGICAL EVALUATION
The left knees for histological examination were removed immediately after death, and the extra-articular soft tissues were dissected and fixed in 10% buffered formalin solution, decalcified, and cast in paraffin blocks. For each block, 10 µm sections were made through the femoral insertion, ACL, and tibial insertion and stained with haematoxylin and eosin for light microscopy.

STATISTICAL ANALYSIS
Calculations were performed with Stat-View 4.02J on a Macintosh. The onset time of arthritis was analysed with the repeated measure analysis of variance and Dunn tests. Student's *t* test was used to compare body weights, the bone mineral density, and the mechanical properties between groups. The level of significance for all analyses was *p*<0.05.

Results
CLINICAL COURSE OF ARTHRITIS
Arthritis occurred in the CIA group at two weeks after the primary immunisation, and hind foot swelling reached a plateau after four weeks of follow up (fig 1).

BONE DENSITOMETRY
Values of bone mineral density (BMD) in the CIA group were significantly lower than those in the control group at eight weeks after immunisation (*p*<0.01). Total BMD was 74.6%, trabecular BMD 60.7%, and cortical BMD 83.0% of the controls (table 1).

TENSILE PROPERTIES
The major mode of failure was femoral avulsion in the femur-ACL-tibia specimens of both groups (six of 10 specimens in the control

Figure 1 Changes in hind foot oedema. Each value is the mean and SEM. Open symbols represent control rats; closed symbols represent rats with collagen induced arthritis.

Table 1 Bone mineral density (BMD) at eight weeks after immunisation. Values are given as the mean (SD)

	Control	CIA†
Total BMD (mg/cm³)	583.3 (58.0)	434.9 (69.6)*
Trabecular BMD	402.1 (50.4)	244.2 (31.0)*
Cortical BMD	716.0 (46.4)	594.3 (102.8)*

*Significantly different from control (Student's *t* test, *p*<0.01).
†CIA = collagen induced arthritis.

Table 2 Tensile properties of the anterior cruciate ligament unit. Values are given as the mean (SD)

	Control	CIA†
Ultimate failure load (N)	26.7 (8.6)	20.0 (4.5)*
Stiffness (N/mm)	41.3 (19.3)	25.6 (10.1)*
Energy to failure (×10 ⁻² Nmm)	4.0 (3.8)	1.7 (0.7)

*Significantly different from control (Student's *t* test, *p*<0.05).
†CIA = collagen induced arthritis.

group and eight of 10 in the CIA group). The femur-ACL-tibia complex had a significantly lower ultimate failure load and stiffness in the CIA group than in the control group (*p*<0.05). Table 2 summarises the mechanical testing data.

HISTOLOGICAL FINDINGS
In the CIA group, subsynovial resorption of bone around the bone-pannus junction was seen at the peripheral margins of the ligament insertion site. Vascular foramina in the cortical bone beneath the ACL insertion were enlarged with inflammatory cell infiltration. The number of chondrocytes increased in the mineralised fibrocartilage zone, which was enlarged. No histological changes were seen in the ligament substance among rats with CIA compared with the control rats (fig 2).

Discussion
Several clinical studies have focused on changes in knee laxity in patients with RA using quantitative measurements.^{2 3 5} Wada *et al* reported that anteroposterior knee laxity increased in RA knees, and that a high rate of morphological damage to the cruciate ligaments was seen.⁵ However, the mechanism of these laxity changes has not been clarified.
This is the first study, to our knowledge, to clarify the effects of CIA on the tensile and histological properties of rat ACL. Our findings show that the tensile strength of the ACL was significantly lower in rats with CIA (ultimate failure load, 74.9% of the control; stiffness, 62.0% of the control). The major mode of failure was femoral avulsion, and the BMD was significantly lower in the rats with CIA. A histological examination of the ligament insertion in rats with CIA showed resorption of the cortical bone beneath the ACL insertion and an enlarged mineralised fibrocartilage zone. These findings suggest that structural and mechanical changes occur in the ligament insertion of the ACL in CIA.
Periarticular osteoporosis is a common clinical feature of RA. Histomorphometric studies in the periarticular bone of patients with RA showed the presence of increased bone resorption through osteoclast activation.^{13 14} Several studies have also shown bone loss in arthritis

Figure 2 Light micrographs of femoral insertion sites of the anterior cruciate ligament (ACL) (haematoxylin and eosin). (A) Group with collagen induced arthritis (×100). Photomicrograph shows bone resorption around the bone-pannus at the peripheral margins of the ligament insertion site and the enlarged mineralised fibrocartilage zone. (B) Enlarged vascular foramina with inflammatory cell infiltration in cortical bone beneath the ACL insertion is shown (×200). (C) Control group (×100).

experimental models.¹⁵⁻¹⁹ In this study the cortical BMD was significantly lower in rats with CIA, and enlarged vascular foramina in the cortical bone beneath the ACL insertion and subsynovial resorption of bone at the peripheral margins of the ligament insertion site were seen.

Dolgo-Saburoff in 1929, using a cat patellar-tendon insertion into the tibia, described four zones: tendon, fibrocartilage, mineralised fibrocartilage, and bone.²⁰ Cooper and Misor studied dog ligament insertions. They proposed that the fibrocartilage zone and the mineralised fibrocartilage zone might afford a gradual transmission of the tensile force between ligament and bone, and act as a growth zone for ligament and underlying bone.²¹ In this study the mineralised fibrocartilage zone was enlarged with an increased number of chondrocytes in rats with CIA. It was considered that these histological changes resulted from a reduced functioning of the growth zone in CIA. Consequently, an enlargement of the mineralised fibrocartilage zone would affect the viscoelastic properties of the ligament insertion.

The decreased mechanical strength of the bone-ACL-bone complex from rats with CIA is considered to be due to these histological changes at the ligament-bone insertion site, in bone, the fibrocartilage zone, or both. The changes in the relation between load and ligament elongation in CIA were shown as a decrease in ligament stiffness. These results indicate that the projected *in vivo* functional capacity of the ligament unit would be altered by CIA.

This study had several limitations. Firstly, we used young rats and therefore the structures of the ligament insertions are different from those of skeletally mature humans. We selected young rats because they are usually used in experimental studies on arthritis, and an arthritis model of the skeletally mature rat has not been established. Secondly, the most common mode of failure was a femoral avulsion in this study. Noyes and Grood reported that the major mode of ACL failure in tensile failure tests was ligament disruption in young adult humans and avulsion of the bone beneath the ligament insertion site in older humans.²² Noyes *et al* also reported that in adult rhesus monkeys the major mode of ACL failure changed from a predominance of tibial avulsion at slow strain rate to ligament disruption at a fast rate.¹² On the other hand, Danto and Woo reported that in skeletally mature rabbits, strain rate had no effect on the mode of failure, and most ACL units failed at the insertion site.²³ This variability in the mode of failure may be due to multiple factors, such as animal species, age, and testing procedures used. Finally, there were no histological changes in the ACL substance of rats with CIA despite their decreased stiffness. A biochemical analysis of the collagen metabolism in ACL would be needed to clarify this observation.²⁴

In conclusion, our findings showed that the tensile strength of the ACL unit was decreased in CIA. The effect of CIA on the ACL unit

depended on the histological characteristics of the ligament-bone attachment, such as bone resorption beneath the ligament insertion site and an enlargement of the mineralised fibrocartilage zone.

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