**Correlation Between Graft Laxity and Myofibroblasts during Healing after Rabbit Anterior Cruciate Ligament Reconstruction**

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**ABSTRACT**

The relationship between graft laxity and histological changes during healing was evaluated in a rabbit anterior cruciate ligament (ACL) reconstruction model using an in situ freeze-thaw technique. Twenty-eight skeletally mature Japanese white male rabbits underwent a freeze-thaw procedure to the left ACL. The graft laxity was evaluated by measuring anterior-posterior translation applied with 10 N. Then, harvested ACLs were observed by immunohistochemistry and transmission electron microscopy for 24 weeks after surgery. The presence of fibroblasts and myofibroblasts was elucidated in fibroblast-like cells invading the healing graft. At 8 weeks, when cells invaded to the core of the femoral portion of the ligament, a transient graft laxity occurred. Then, the anterior-posterior knee laxity was reduced between 8 and 12 weeks. An increase of myofibroblasts was observed during the reduction in graft laxity. Myofibroblasts decreased in number after graft laxity disappeared. Our results suggest that myofibroblasts may relate to a reduction in ACL laxity.

**Introduction**

Myofibroblasts have phenotypical characteristics of both fibroblasts and smooth muscle cells [1], and can be identified by immunohistochemical staining with antibodies to α-smooth muscle actin [2]. Myofibroblasts have been found in wound contraction and contracted pathological tissues in diseases such as Dupuytren's contracture [3], breast carcinomas [4] and cirrhosis [5]. They play an important role in contraction of healing tissues [6].

After anterior cruciate ligament (ACL) reconstruction, an increase in graft laxity during healing is a significant problem. The increase in graft laxity is considered to be due to a decrease in graft strength [7] by fibroblast-like cells invasion during healing. However, while the graft strength is weak, a decrease in graft laxity with time has been reported in experimental studies [8]. There may be myofibroblasts in fibroblast-like cells invading a healing graft, and a decrease in graft laxity may also relate to myofibroblasts. Our aim was to investigate whether myofibroblasts appear or not during graft healing, and whether myofibroblasts relate to a change of graft laxity after rabbit ACL reconstruction.

**Materials and Methods**

**Animals**

Twenty-eight skeletally mature Japanese White male rabbits, weighing 3690±60g, were obtained from a licensed laboratory animal dealer. The ACL...
of the left knee was subjected to five freeze-thaw cycles [9]. The sham-operated contralateral knee served as the control for all evaluations. Four animals each were sacrificed at 2, 3, 4, 8, 12, 16, and 24 weeks postoperatively. After four paired specimens were evaluated for anterior-posterior translation, two ACLs were removed for light microscopy, and the remaining two for transmission electron microscopy.

This study was conducted in accordance with the standard of humane animal care as agreed upon the Osaka Medical College Experimental Animal Center.

**Operative Technique**

By sterile technique, the surgery was performed under intravenous pentobarbital anesthesia after an intramuscular injection of ketamine (20mg/kg). The left knee joint was approached through a medial parapatellar incision. The patella was displaced laterally, and the infrapatellar fat pad was mobilized and retracted to expose the ACL. A wooden blade was inserted between the posterior cruciate ligament (PCL) and the area of the femoral insertion of the ACL to protect the PCL from freezing. A specially designed probe with oozing liquid nitrogen was placed on the ACL for one minute. The probe was then removed, and the frozen ACL was thawed by irrigation with 15°C saline solution for two minutes. The freeze-thaw cycles were repeated five times [9]. The synovial tissue and joint capsule were closed as one layer, and closure of the subcutaneous tissue and skin completed the operation. The contralateral knee was similarly exposed without the freeze-thaw treatment. Postoperatively, the limbs were not immobilized and the animals were allowed to bear weight as tolerated.

**Gross Morphology**

At the end of the experimental period, the animals were sacrificed with an overdose of intravenous pentobarbital. The hindlimbs of the animals were removed. Then, the articular surfaces, menisci, and ACLs were grossly inspected.

**Anterior-Posterior Laxity**

The hindlimbs of the animals were dissected free of muscular tissue, leaving all periarticular connective tissues around the knee. Each specimen was mounted at 90 degrees of flexion in a special device that permitted 5 degrees of freedom: anterior-posterior, medial-lateral, and superior-inferior displacement, varus-valgus, and internal-external rotation. The knee specimen was subjected to a cyclic load of ±10 N applied at 90 degrees to the long axis of the tibia in an anterior-posterior direction for 10 cycles. Anterior-posterior laxity was defined as the displacement after the final load of ±10 N [10].

**Immunohistochemical study**

The harvested ACLs were divided into two sides (femoral and tibial), and fixed with 10% neutral-buffered formalin for 3 days. Six-micrometer-thick sections were sliced from specimens embedded in paraffin. The slides were deparaffinized with xylene and then rehydrated with a graded series of ethanol. They were blocked with 3% hydrogen peroxide then washed with phosphate-buffered saline. Mouse anti-human α-smooth muscle actin monoclonal antibody (Sigma Chemical) was applied to the sample for 1 hour. Then, a secondary biotinylated horse anti-mouse IgG antibody was applied for 30 minutes, followed by an avidin-biotin peroxidase conjugate (ABC Elite; Vector Laboratories) for 30 minutes. Diaminobenzidine was used as a chromogen. Cell nuclei were counterstained with Harris's hematoxylin for 30 seconds, rinsed, and mounted. The stains used were hematoxylin and eosin. These were evaluated with light microscopy.

**Transmission Electron Microscopy**

The samples were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer for 5 hours and then transferred to 0.1 M cacodylate buffer (pH 7.4) until they were ready for processing. The samples were washed with fresh buffer and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 hours. They were then dehydrated in ethanol. The samples were immersed in propylene oxide for 30 minutes and then placed in a 1:1 dilution of Eponate 12 hard resin in propylene oxide overnight. Next, they were placed in pure resin for 3 hours and were put into molds containing fresh resin to polymerize overnight at 60°C. Ultrathin sections, approximately 80nm, were cut on an ultramicrotome. The sections were stained with uranyl acetate and lead acetate for 3 minutes. The grids were examined in a JEM-H7000 transmission electron microscope at ×1,000-30,000 magnification.
**Quantification of Myofibroblasts**

For quantification of cells containing \( \alpha \)-smooth muscle actin, the harvested ACLs were divided into two sides (femoral and tibial), and then stained by immunohistochemistry. In each specimen, the number of \( \alpha \)-smooth muscle actin positive fibroblast-like cells within the area at the center and the 4 corners of a microscopic field (\( \times 400 \) magnification) was counted.

**Statistical Analysis**

Mann-Whitney’s U-test was used to compare the differences of anterior-posterior translation between the experimental and control groups. Statistical significance was defined as \( P<0.05 \).

**Results**

**Gross Morphology**

Gross examination of the treated and sham-operated contralateral knees revealed no major degenerative changes in the articular cartilage and menisci. After 4 weeks postoperatively, the ACLs treated with the freeze-thaw procedure were more edematous than the controls. No intra-articular effusion or inflammation was observed.

**Anterior-Posterior Laxity**

There was a significant increase in anterior-posterior translation between the treated (1.93±0.08 mm) and the control knees (1.38±0.05 mm) at 8 weeks after surgery (\( p=0.019 \)). However, the difference was not statistically significant at other weeks postoperatively (Table 1). At 8 weeks, graft laxity increased transiently. Then, anterior-posterior knee laxity was reduced between 8 and 12 weeks.

**Histology**

The histological findings of controls and normal ACLs at each examined week were quite similar through 24 weeks postoperatively. At 2 weeks, no

<table>
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<tr>
<th>Table 1. Anterior-posterior translation measured at 90° of flexion (n=4 in each examined week)</th>
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<td><strong>Group</strong></td>
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op: group with the freeze-thaw treatment
sham: group without the freeze-thaw treatment
\(*\): significant difference compared with the sham operation group (\( p=0.019 \))
cell was observed in the frozen ACL (Fig. 1). At 3 weeks, fibroblast-like cells appeared in the superficial layer of the femoral side (Fig. 2). At 4 weeks, the cellular proliferation was observed throughout the matrix except in the core of the femoral portion, but no cell in the tibial site. At 8 weeks, fibroblast-like cells were seen throughout the femoral portion, and in the superficial layer of the tibial site. At 12 and 16 weeks, a further proliferation of fibroblast-like cells was observed in the tibial side, but acellular areas still remained in the core. At 24 weeks, cellular invasion into the grafts was seen throughout the ACLs. Throughout the 24 weeks after surgery, most invading cells had plump and oval nuclei.

An immunohistochemical study revealed

![Fig. 1](image1) No cell is seen in the frozen ACL at 2 weeks. (×200)

![Fig. 2](image2) Photomicrograph showing fibroblast-like cells in the superficial layer of the frozen ACL at 3 weeks. (×200)

![Fig. 3](image3) Alpha smooth muscle actin positive fibroblast-like cells at 12 weeks. (×800)

![Fig. 4](image4) Clusters of α-smooth muscle actin positive chondrocytes at 12 weeks. (×400)

Table 2. Number of fibroblast-like cells

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<tr>
<th>Postoperative weeks</th>
<th>α-SMA positive cells (mean ± S.E.)</th>
<th>Total number of cells (mean ± S.E.)</th>
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<tr>
<td>3</td>
<td>6 ± 2</td>
<td>237 ± 43</td>
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<td>4</td>
<td>104 ± 16</td>
<td>603 ± 77</td>
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<td>8</td>
<td>548 ± 60</td>
<td>1486 ± 202</td>
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<tr>
<td>12</td>
<td>2916 ± 227</td>
<td>3826 ± 164</td>
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<td>16</td>
<td>2259 ± 183</td>
<td>3557 ± 260</td>
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<tr>
<td>24</td>
<td>232 ± 30</td>
<td>3743 ± 283</td>
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myofibroblasts (Fig. 3) as \( \alpha \)-smooth muscle actin positive fibroblast-like cells. Myofibroblasts increased between 4 weeks (estimated to be approximately 104 ± 16) and 12 weeks (2916 ± 227) (Table 2). Then, myofibroblasts decreased, but remained at 24 weeks (232 ± 30).

Throughout the 24 weeks, myofibroblasts were more plentiful in the sections from femoral portions than those from the tibial sites.

At 8, 12, and 16 weeks, the ligament showed focal areas of acellular collagen scaffold being invaded by clusters of \( \alpha \)-smooth muscle actin positive chondrocytes at the insertion of the tibial side (Fig. 4).

**Transmission Electron Microscopy**

Control specimens showed that fibroblasts throughout the extracellular matrix displayed typical fibroblast morphology: a slender fusiform and a smoothly contoured nucleus, a well developed Golgi area, and numerous, often dilated cisternae of rough endoplasmic reticulum, with scattered mitochondria and microfilaments.

Specimens of frozen ACLs demonstrated cells displaying myofibroblastic features: a nucleus with indentations (Fig. 5a), stress fiber, dense bodies (Fig. 5b), pinocytotic vesicles (Fig. 5c), and a gap junction (Fig. 5d). Myofibroblasts produced a predominance of small-diameter collagen fibrils (Fig. 5a).

**Discussion**

Myofibroblasts have been found in contracted pathological tissues in diseases such as Dupuytren's contracture [3] and frozen shoulder [11]. Faryniarz and co-workers [12] reported the appearance of myofibroblasts after a medial collateral ligament injury in a rabbit model. Faryniarz [12] and others [13; 14] demonstrated the presence of myofibroblasts in ligament scar tissues during the healing period when the ligament was known to contract.

Clinically, laxity of the reconstructed ACL was evaluated by the anterior instability of the knee.
However, the experimental study of graft laxity during healing was insufficient. Jackson and co-workers [8] documented a reduction in anterior-posterior displacement values in the 6-month autografts, compared with 6 weeks, after ACL reconstruction using a goat model. Ng and co-workers [15] also stated that the anteroposterior laxity of the grafts at less than 1 year was significantly higher than that of the 3-year grafts, after goat ACL reconstruction. Clinically, Blecher and Richmond [16] reported transient laxity of a reconstructed ACL around the end of pregnancy disappeared at 5 months postpartum. Ihara [17] and Kurosaka [18] reported spontaneous recovery of anterior laxity of the knee after ACL injury. Daniel [19] and Buss [20] also reported successful results in conservative treatments after acute ACL injuries, while histological changes of the ACL during healing were not observed in these reports. Only Jackson and co-workers [8] stated that a reduction in anterior laxity was associated with a prolific production of small-diameter collagen fibrils and an increased cross-sectional area of the reconstructed ACL in the goat model.

In the present study, we used an in situ freeze-thaw model [9]. The freeze-thaw technique represents the ideal operative placement of a biological graft. In Jackson's study [8] using a goat model, no instability of the knee joint occurred during graft healing. However, in our study with a rabbit model, a transient laxity of the frozen ACL occurred at 8 weeks when fibroblast-like cells invaded throughout the graft of a femoral portion, and improved at 12 weeks. The transient laxity of the ACL in our study may be explained by the differences that we observed at shorter intervals using a rabbit model whose knees usually flex.

Immunohistochemical findings and transmission electron microscopy showed that fibroblast-like cells invading grafts during healing included myofibroblasts. Myofibroblasts were increased between 4 and 12 weeks postoperatively, and then decreased in number to 24 weeks. Anterior-posterior translation was reduced between 8 and 12 weeks after surgery. These results demonstrated an increase in the number of myofibroblasts during a reduction of graft laxity. Myofibroblasts may relate to a reduction in graft laxity. Gabbiani and co-workers [21] stated myofibroblasts principally produced type-III collagen in the granulation tissue of healing wounds. In our observation as well as Gabbiani's [21], myofibroblasts produce a predominance of small-diameter collagen fibrils. Our results support the observations of Jackson and co-workers [8] that a reduction in anterior-posterior translation was associated with a prolific production of small-diameter collagen fibrils.

In the present study, although the ligament strength was not studied, a predominance of small-diameter collagen fibrils was observed during a reduction in graft laxity. It was assumed that an increase of type-III collagen during ligament healing decreased graft strength [22]. Clinically, a decrease of graft laxity does not correspond to an increase in graft strength. Recently, cytokines transforming fibroblasts to myofibroblasts have been studied [23; 24]. In the future, cytokines inducing myofibroblasts may improve the treatment of ACL injury.

References


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