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**Expression of Vascular Endothelial Growth Factor and Angiogenesis in Patellar  
Tendon Grafts in the Early Phase after Anterior Cruciate Ligament  
Reconstruction**

T. Yoshikawa, M.D.(1,2), H. Tohyama, M.D., Ph.D.(1), H. Enomoto, M.D., Ph.D.(2),  
H. Matsumoto, M.D., Ph.D.(2), Y. Toyama, M.D., Ph.D.(2), K. Yasuda, M.D., Ph.D.(1)

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- 1) Department of Sports Medicine and Joint Reconstruction Surgery, Hokkaido  
University School of Medicine, Sapporo, Japan
- 2) Department of Orthopaedic Surgery, Keio University School of Medicine,  
Tokyo, Japan

**Address correspondence to:** Harukazu Tohyama, MD, Department of Sports  
Medicine and Joint Reconstruction Surgery, Hokkaido University School of  
Medicine, Kita-15 Nishi-7 Kita-ku, Sapporo 060-8638, Japan.

Tel:+81(Japan)-11-706-7211

Fax:+81(Japan)-11-706-7822

E-mail:tohyama@med.hokudai.ac.jp

**ABSTRACT** The aim of this study was to clarify vascular endothelial growth factor (VEGF) expression and angiogenesis in the patellar tendon autograft in the early phase after ACL reconstruction using a rabbit model. The right knees of 30 Japanese white rabbits underwent ACL reconstruction using the medial third of the patellar tendon complex. We evaluated the grafted tendon at 1, 2, 3, 4, and 8 weeks after ACL reconstruction by immunohistology for proliferating cell nuclear antigen (PCNA), VEGF, and CD31, which is a marker for vascular endothelial cells. At 1 week, few cells were observed at the midsubstance of the grafted tendon. A number of proliferating cells were observed at the surface area of the patellar tendon graft 2 weeks after graft transplantation, while no vessel formation was observed in the graft at the same time. VEGF was highly expressed 2-to-3 weeks postoperatively. Vessel formation in the patellar tendon graft increased with time from 3 week to 8 weeks after ACL reconstruction. The rates of proliferating cells and VEGF-expressing cells decreased with time from 3 week to 8 weeks. This study has suggested that VEGF is involved in the graft remodeling process particularly at the early phase after ACL reconstruction.

**Key Words:** Anterior cruciate ligament reconstruction – Fibroblasts - Patellar tendon - Tendon graft - Vascular endothelial growth factor (VEGF)

## **INTRODUCTION**

In anterior cruciate ligament (ACL) reconstruction, intrinsic fibroblasts in the tendon grafted across the knee joint necrotize immediately after transplantation, and cellular repopulation from an extrinsic origin and revascularization occur sequentially [1,3,7,12].

A recent clinical case report suggests that the remodeling of the tendon graft takes longer than we expected [8]. Therefore, a new strategy needs to be developed to accelerate the remodeling of the tendon graft. To develop a new strategy for acceleration of the graft remodeling, we must understand the healing process of the grafted tendon after ACL reconstruction.

The formation of new blood vessels from pre-existing vessels is mediated by several angiogenic factors [21]. The most potent known angiogenic factor is vascular endothelial growth factor (VEGF). VEGF was detected in the synovial tissue of patients with rheumatoid arthritis [17], in osteoarthritic cartilage [19], and in degenerative tendon tissue [15]. In addition, VEGF has been detected during canine flexor tendon healing [5]. After harvesting for ACL reconstruction, autologous tendon grafts are separated from the circulation, and the tissue becomes necrotic. Hypoxia is a known potent stimulator for VEGF expression [2,13,15,16] and thus it seems likely that

decreased oxygen tension at the transition to the necrotic part may stimulate VEGF expression also in tendon grafts after ACL reconstruction.

Petersen et al. [18] reported that VEGF was expressed in the graft 6 to 24 weeks after ACL reconstruction. No studies, however, have been conducted to clarify the relationships between angiogenesis and VEGF expression in the early phase after ACL reconstruction. The purpose of this study was to clarify the temporal changes in VEGF expression and angiogenesis in the patellar tendon autograft in the early phase after ACL reconstruction using a rabbit model.

## **MATERIALS AND METHODS**

### **Experimental design**

A total of 33 skeletally mature Japanese White rabbits weighing  $3.3 \pm 0.2$  kg (Mean  $\pm$  SD) were used in this study. The right knee of each animal underwent ACL reconstruction using the medial third of the autologous patellar tendon (PT) complex, while the left knee underwent the sham operation. Six animals were euthanized for immunohistological evaluations for proliferating cell nuclear antigen (PCNA), VEGF, and CD31, which is a marker for vascular endothelial cells, at 1, 2, 4, and 8 weeks,

respectively. This study was approved by our institutional animal care and use committee.

### **Surgical procedure and postoperative management:**

Each animal was anesthetized with intravenous pentobarbital sodium and maintained on inhalation anesthesia with halothane. Using sterile technique, the right knee joint was approached through a medial parapatellar incision. The medial one-third of the PT complex, with its distal tibial insertion intact, was isolated and used as graft material [4]. This complex included a 2-cm portion of quadriceps fascia proximal to the patella and a thin flake of bone from the anterior surface. The ACL was transected at its tibial and femoral insertions and completely removed the native ACL including synovial tissue which coated the native ACL before ACL reconstruction. Using a drill with a drill guide, 2.7-mm tibial and femoral drill holes were made which allowed the PT graft to be placed intraarticularly to mimic the position of the normal ACL. A tensile force of 10 N measured with a spring scale was applied to the graft, and this tension was maintained. The graft was then secured to a 2.0-mm diameter cortical screw and washer. A fiberglass cast was used to immobilize the right knee for 1 week [1, 4].

Postoperatively, all animals resumed cage (52 cm in width, 35 cm in height, and 33 cm in depth) activity immediately.

### **Histological procedure:**

For histological observation, the femur–ACL–tibia complex was resected and fixed in a buffered 10% formalin solution, decalcified, and cast in paraffin blocks. Sections 4- $\mu$ m thick were sliced with a microtome along the longitudinal axis of the grafted tendon in the sagittal plane. First, one section from each block was stained with hematoxylin-eosin. For immunohistology, monoclonal anti-PCNA (proliferating cell nuclear antigen) antibody (Zymed Laboratories Inc.), mouse monoclonal anti-VEGF antibody (NeoMarkers), mouse monoclonal anti-CD31 antibody (DAKO) were used as primary antibodies. The sections were incubated with a universal immuno-peroxidase polymer solution (Histofine Simple Stain PO Multi, Nichirei, Tokyo, Japan). The reaction product was detected by the addition of a 0.02 % DAB (3,3'-diaminobenzidine) solution containing 0.02 % H<sub>2</sub>O<sub>2</sub> for 10 minutes. Finally, the sections were counterstained with hematoxylin.

### **Semiquantitative evaluation of immunohistology:**

All immunohistological sections were semiquantitatively evaluated at the midsubstance level of the graft by two independent observers who were not informed about the time duration between the surgery and the sacrifice for each specimen. We did not attempt to semiquantitatively evaluate the graft around the tibial or femoral attachment sites, since there were large location-dependent variations of immunohistological findings around the bone tunnels even in the same specimen. Each observer examined every slide three times, and the scores of each slide were averaged. To evaluate cell proliferation in the grafted tendon after ACL reconstruction, we semiquantitatively analyzed a "stained cell ratio" of PCNA. We defined any cell with a stained nucleus as "a positively stained cell" [22]. The ratio of the number of positively stained cells to the number of total cells found in a unit rectangular area (220  $\mu\text{m}$  x 330  $\mu\text{m}$ ) in a microscopic visual field was defined as "stained cell ratio". In the same manner as described above, we also analyzed a "stained cell ratio" of VEGF. To evaluate angiogenesis in the grafted tendon, we used the Chalkley counting method, which was established for the evaluation of tumor angiogenesis [9]. Briefly, the three most vascular areas ("hot-spots") with the highest number of microvessel profiles were chosen subjectively from each graft immunohistological section for CD31, which is a marker for vascular endothelial cells. A 25-point Chalkley eyepiece graticule (Leitz Orthoplan: Leica) was applied to each

hot-spot area at 200× magnification with a corresponding Chalkley grid area of 0.196 mm<sup>2</sup>. The graticule was oriented so that the maximum number of points was on or within areas of highlighted microvessel profiles at 200× magnification. The Chalkley count for an individual specimen was taken as the mean number of points on or within areas of highlighted microvessel profiles of 18 graticule counts.

### **Statistical Analysis:**

The one-way analysis of variance (ANOVA) was used to determine statistical differences in each parameter among the postoperative periods. If we found statistical differences between periods, we used Fisher's PLSD tests for post hoc multiple comparisons. The significance limit was set at p=0.05.

## **RESULTS**

At 1 week, few cells were observed at the midsubstance of the grafted tendon (Fig. 1-A). Therefore, we did not attempt any immunohistological examination of the patellar tendon grafts 1 week after the operation. At 2 weeks, a number of infiltrative cells were observed within the peripheral area of the patellar tendon graft (Fig. 1-B). Moving from the peripheral to the central areas of the graft, the number of fibroblast-like cell nuclei

decreased. The central portion of the graft was acellular. Cells exhibiting PCNA-positive nuclei were frequently found at the superficial portion of the tendon graft at 2 weeks (Fig. 2-A). VEGF-positive cells scattered at the similar area where PCNA-positive cells existed (Fig. 2-C). No vascular endothelial cells were observed at this period. At 3 weeks, a number of cells exhibiting PCNA-positive nuclei as well as VEGF-positive cells were still found at the superficial portion of the tendon graft. Vascular endothelial cells appeared at the relatively deep portion apart from the surface area of the graft tendon in spite of the lack of vessel formation at this time (Fig. 2-E). At 4 weeks, the number of PCNA-positive cells and of VEGF-positive cells was relatively decreased in the patellar tendon graft compared with the 2 to 3-week specimens. On the other hand, the number of vascular endothelial cells increased compared with the 2 to 3-week old grafts. At 8 weeks, fibroblasts were scattered in the core portion of the patellar tendon graft (Fig. 1-C). Among these cells, PCNA-positive cells or VEGF-positive cells were not frequently observed in the patellar tendon graft (Figs. 2-B and D). At 8 weeks, a number of vessel formations were frequently found in the tendon graft (Fig. 2-F). The sham operation did not affect histology of the ACL after 2 weeks (Fig. 1-D).

The postoperative period significantly affected the PCNA-positive cell ratio (Fig. 3-A)( $p < 0.0001$ ). The positive ratio at 2 weeks was significantly higher than those at 4 and 8 weeks (versus 4-weeks:  $p < 0.0001$ ; versus 8-weeks:  $p < 0.0001$ ), while there were no significant differences in the ratios between 2 and 3 weeks ( $p = 0.1261$ ). The PCNA-positive cell ratio at 3 weeks was significantly higher than those at 4 and 8 weeks (versus 4-weeks:  $p = 0.006$ , versus 8-weeks:  $p < 0.0001$ ). Concerning VEGF expression, an ANOVA showed a significant effect of the time-period on the positive cell ratio (Fig 3-B)( $p < 0.0001$ ). The positive ratio at 2 weeks was significantly higher than those at 4 and 8 weeks (versus 4-weeks:  $p < 0.0001$ , versus 8-weeks:  $p < 0.0001$ ), while there were no significant differences in the ratios between 2 and 3 weeks ( $p = 0.9295$ ). The VEGF-positive cell ratio at 3 weeks was significantly higher than those at 4 and 8 weeks (versus 4-weeks:  $p < 0.0001$ , versus 8-weeks:  $p < 0.0001$ ). Chalkley counts significantly increased with time (Fig. 3-C)( $p < 0.0001$ ). Chalkley counts were significantly higher at 4 and 8 weeks than at 2 weeks (versus 4-weeks:  $p < 0.0001$ , versus 8-weeks:  $p = 0.001$ ) and 3 weeks (versus 4-weeks:  $p < 0.0002$ , versus 8-weeks:  $p < 0.0001$ ). There were no significant differences in Chalkley counts between 2 and 3 weeks ( $p = 0.0084$ ) or between 4 and 8 weeks ( $p = 0.4431$ ).

## DISCUSSION

This is the first report to clarify the temporal changes in VEGF expression, fibroblast proliferation, and angiogenesis in the patellar tendon graft at the early phase after rabbit ACL reconstruction. ~~We found a number of proliferating cells at the surface area of the patellar tendon graft 2 weeks after the graft transplantation, while no vessel formation was observed in the graft at the same time. Around the proliferating cells, VEGF was highly expressed 2 to 3 weeks postoperatively. Vessel formation in the patellar tendon graft increased with time from 3 week to 8 weeks after ACL reconstruction. However, the rates of proliferating cells and the VEGF-expressing cells decreased with time from 3 weeks to 8 weeks. We believe that the findings of the present study provide useful information to clarify the healing process of the patellar tendon graft after ACL reconstruction as discussed below.~~ Kleiner et al. [11] showed that the cellular population of the autograft is derived entirely from a source other than the native patellar tendon fibroblasts by graft sequestration experiments. The results of their study are consistent with our findings that cellular necrosis is found in the autogenous patellar tendon graft 1 week after ACL reconstruction and that 2-week old autografts are populated by a peripheral rim of cells with high PCNA-positive rates. The present study also demonstrated that cellular repopulation in the patellar tendon graft is observed

before vascular formation in the graft. Kleiner et al [12] also reported that remarkable cellular proliferation occurred in the rabbit patellar tendon graft at 3 weeks after ACL reconstruction, while vascular injection revealed the absence of a blood supply to the graft. These findings of their study and our study indicate that cellular repopulation in the tendon graft after necrosis occurs without any vascular contribution within 3 weeks after transplantation.

We also found that VEGF was expressed in 70-80% of cells which had infiltrated into the patellar tendon graft 2 to 3 weeks after ACL reconstruction in this study. Previous studies showed that hypoxia induces VEGF expression in various kinds of cells [2, 10, 14, 15, 17]. Kleiner et al. [12] found increased collagen metabolic activities of the graft tissue 7 to 21 days after ACL reconstruction in the rabbit model. The present study showed that approximately half of the cells were PCNA-positive indicating high proliferation activity in the patellar tendon graft 2 weeks after transplantation. Based on these findings, we assumed that these high metabolic and proliferation activities of cells in the patellar tendon graft without a blood supply yield a hypoxia condition at the early period after ACL reconstruction and then hypoxia, in response to these high cellular activities, promotes VEGF production at 2 to 3 weeks after surgery.

We consider that synovial tissues of the knee joint and/or the bone marrow around the bone tunnels were possible origins of the VEGF producing cells in the tendon graft after ACL reconstruction. Nagashima et al [14] reported that VEGF polypeptide was slightly expressed in synovial lining cells and fibroblasts surrounding microvessels in the synovial tissue of osteoarthritis and normal joints. Therefore, the VEGF producing cells in the tendon graft might come from the synovial tissue of the knee joint. On the other hand, Uchida et al [23] found that VEGF was strongly detected in angioblasts, osteoprogenitor cells, and osteoblasts during the healing process of bone and bone marrow after drill-hole injury. Their findings suggested a possibility that the VEGF producing cells in the tendon graft after ACL reconstruction might originate from bone marrow around the bone tunnels. In addition, Ushiyama et al. [24] found that the expression of VEGF were localised in immature adipocytes, interstitial undifferentiated mesenchymal cells, and vascular endothelial cells in the infrapatellar fat pad of osteoarthritis and normal joints. The infrapatellar fat pad might be another source of VEGF producing cells in the tendon graft after ACL reconstruction, because previous microvascular studies showed that some new vessels in the ACL graft originate from the infrapatellar fat pad [3].

VEGF induces angiogenesis and endothelial cell proliferation and it plays an important role in regulating vasculogenesis. Most types of cells, but usually not endothelial cells themselves, secrete VEGF. VEGF can also stimulate cell migration [21]. In the present study, significant angiogenesis was found in the grafted tendon at 3 to 4 weeks after ACL reconstruction. Therefore, VEGF secreted by proliferating cells in the grafted tendon may contribute to angiogenesis in the ACL graft. In addition, the vascular formation in the graft may diminish the hypoxia condition in the graft and may then reduce the VEGF expression in the tendon graft after 4 weeks. Petersen et al [18] found that VEGF immunostaining decreased in the Achilles tendon graft for sheep ACL graft with time. These grafts were largely VEGF-negative at 52 and 104 weeks. In spite of different animal models, the findings of the present study are generally consistent with those of the study by Petersen et al. [18].

The first weakness of the present study is that we used a rabbit ACL reconstruction model. there is a huge difference between rabbit ACL reconstruction and the sports field, in particular, in the mechanical properties of the ACL grafts. For example, previous biomechanical studies suggested that a significant joint instability might persist after ACL reconstruction in rabbits [4]. Therefore, mechanical factors induced by joint instability might have influenced the results of the present study.

However, it is considered that believe that biological process in the tendon graft after ACL reconstruction, i.e. ischemic necrosis, cellar repopulation, revascularization, and matrix remodeling, in the rabbit model is essentially similar to that in clinical ACL reconstruction. Therefore, we believe that the changes in VEGF expression in the tendon graft in clinical cases are similar to those in the rabbit experiment, although actual time-courses may be slightly different between the present study and clinical cases. The second weakness of the present study is that we evaluated cellular proliferation activities, VEGF expression, and vascular density of the graft by a semiquantitative method, and that an unknown intraobserver or interobserver difference also might have influenced the results.

In conclusion, our data strongly suggest that VEGF is involved in the subsequent angiogenesis in the graft after ACL reconstruction. Regarding clinical relevance, recent evidence suggests that recombinant VEGF therapy may provide the added stimulus to the healing of certain types of injuries. For example, Corral [6] reported that an application of VEGF improved granulation tissue formation in ischemic dermal ulcers in the rabbit ear. Zhang et al. [25] reported that tensile strength was statistically higher in the incisional wound group and in the ischemic flap wounds with VEGF treatment compared to the ischemic flaps with no treatment. They also reported

that ischemic wound repair with VEGF treatment had a significantly higher level of microvessel density than the ischemic wounds without treatment. In the present study, we confirmed the lack of VEGF or vascularities in the patellar tendon graft at the early period after ACL reconstruction. Therefore, exogenous VEGF application at the early period after the surgery may promote neovascularization and increase mechanical strength of the tendon graft for ACL reconstruction. Further research has to determine if VEGF might be a tool to accelerate the process of ACL graft remodeling.

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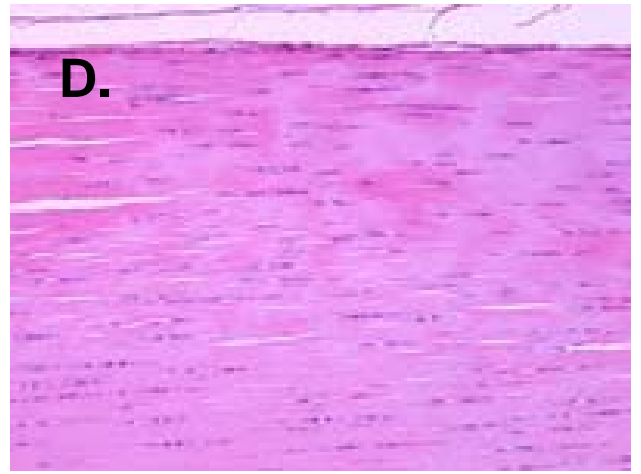
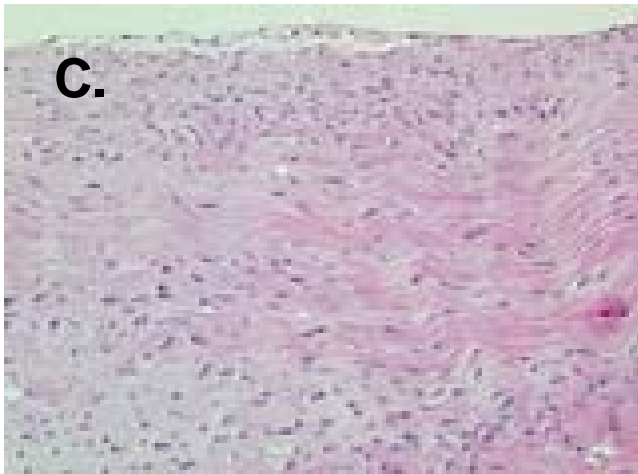
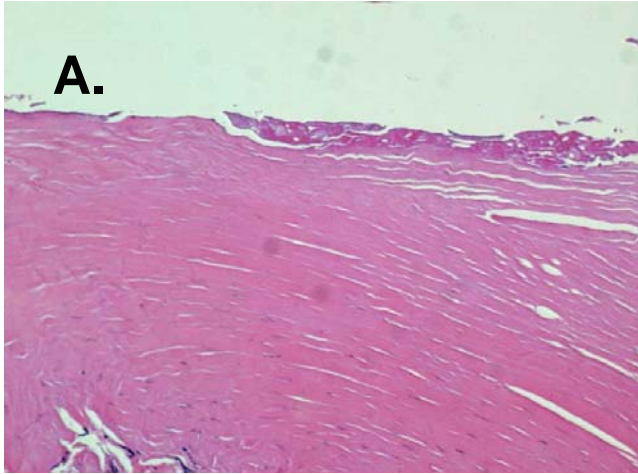
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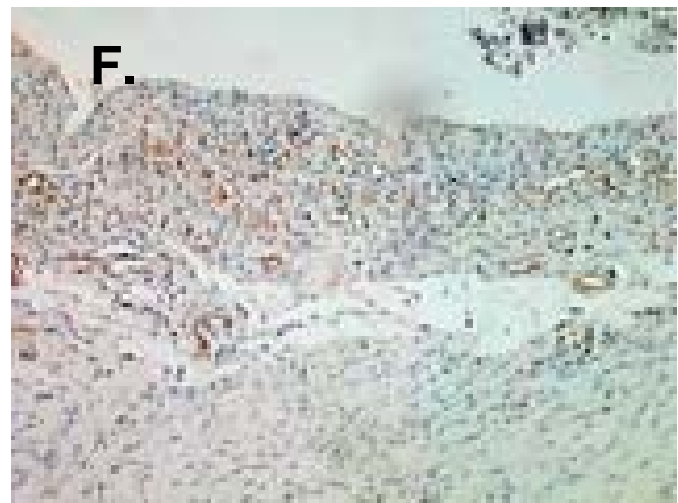
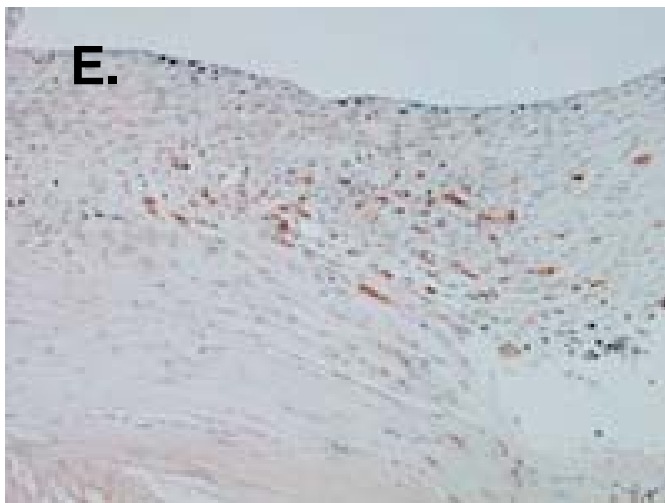
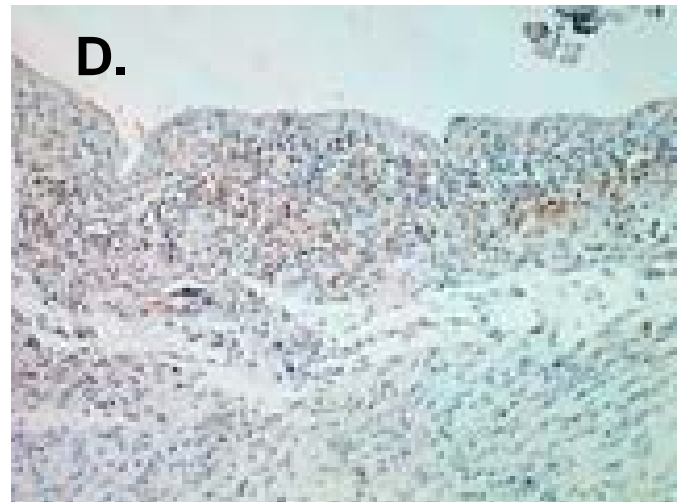
## Figure Legends

**Figure 1** Histological findings of the patellar tendon graft after ACL reconstruction (x 50, HE stain). **A:** At 1 week, few cells were observed at the midsubstance of the grafted tendon. **B:** At 2 weeks, granulation tissue was seen within the peripheral area of the patellar tendon graft. The central portion of the graft was still acellular. **C:** At 8 weeks, fibroblasts were scattered in the core portion of the patellar tendon graft at 8 weeks. **D:** At 8 weeks, histological findings of anterior cruciate ligament after the sham operation were similar to those of normal anterior cruciate ligament.

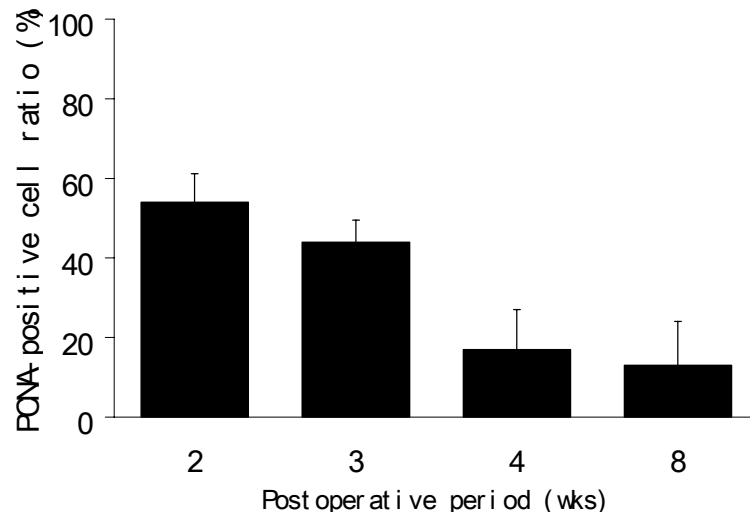
**Figure 2** Immunohistologies for PCNA (A: 2 weeks, B: 8 weeks), VEGF (C: 2 weeks, D: 8 weeks), and CD31 (E: 3 weeks, F: 8 weeks) of the patellar tendon graft after ACL reconstruction. **A:** Cells exhibiting PCNA-positive nucleus were frequently found at the superficial portion of the tendon graft at 2 weeks. **B:** At 8 weeks, few PCNA-positive cells were observed in the patellar tendon graft. **C:** At 2 weeks, VEGF-positive cells scattered at the similar area where PCNA-positive cells existed. **D:** At 8 weeks, VEGF-positive cells were seldom observed in the patellar tendon graft. **E:** At 3 weeks, vascular endothelial cells appeared at the midsubstance portion apart from the surface area of the graft tendon in spite of lack of vessel formation at this time. **F:** At 8 weeks, a number of vessel formations were observed in the tendon graft.

**Figure 3** Semiquantitative analysis of immunohistological examinations (A: PCNA-positive cell ratio, B: VEGF-positive cell ratio, and C: Chalkley counts).

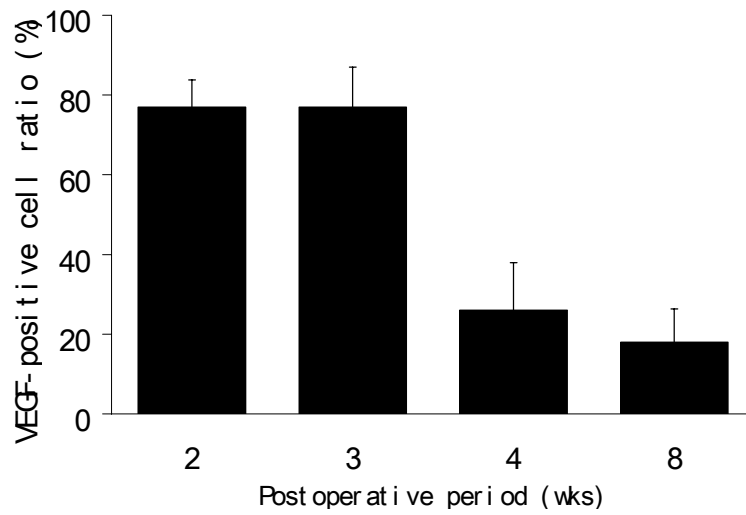




**A.**



**B.**



**C.**

