# Revascularization in the Tendon Graft Following Anterior Cruciate Ligament Reconstruction of the Knee: Its Mechanisms and Regulation

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In anterior cruciate ligament (ACL) reconstruction, slow graft maturation may result in graft failure or elongation during the postoperative rehabilitation period. Vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis. The findings of recent studies suggest that VEGF application is a potential strategy to accelerate angiogenesis in the graft after ACL reconstruction. However, the biomechanical results indicate that exogenous VEGF application decreases the stiffness of the grafted tendon at least temporarily. Therefore, we should take into account this adverse effect of exogenous VEGF application on the mechanical characteristics of the grafted tendon. (*Chang Gung Med J 2009;32:133-9*)



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Anterior cruciate ligament (ACL) reconstruction using a tendon autograft has been greatly improved over the last two decades.<sup>(1)</sup> In ACL reconstruction, however, the strength of the grafted tendon is reduced in the early phase after surgery and then gradually increases.<sup>(2:4)</sup> Recently, Delay et al. reported a human case of ACL reconstruction in which the core portion of the patellar tendon graft remained necrotic 18 months after surgery.<sup>(5)</sup> With damage to any tissue, cell infiltration from the blood system is thought to be required for tissue healing. The lack of vascularity within the ACL graft may induce degeneration or micro-ruptures of the grafted tendon dur-

ing the postoperative period. Therefore, slow graft maturation may result in graft failure or elongation during the postoperative rehabilitation period due to unknown causes. To improve these problems after ACL reconstruction, we aim to develop a new strategy to accelerate the remodeling of the tendon graft. This may enable more aggressive rehabilitation and an earlier return to rigorous sports for patients with ACL reconstruction.

Vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis, which involves activation, migration, and proliferation of endothelial cells in various pathological conditions.<sup>(6)</sup> In adults,

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apart from its association with tumors, VEGF has been detected in the synovial tissue of patients with rheumatoid arthritis,<sup>(7)</sup> in osteoarthritic cartilage,<sup>(8)</sup> and in degenerative tendon tissue.<sup>(9)</sup> VEGF has been detected during canine flexor tendon healing.<sup>(10)</sup> In addition, Corral et al. reported that an application of VEGF significantly enhanced not only angiogenesis but also wound healing in rabbit skin.<sup>(11)</sup> Therefore, there is a high possibility that an application of VEGF to a necrotized tendon graft enhances angiogenesis in the graft. In this article, the authors will review recent experimental studies of methods using VEGF application to enhance angiogenesis and healing of graft remodeling in the tendon graft after ACL reconstruction.

## Temporal changes in fibroblast proliferation, VEGF expression, and angiogenesis in the grafted tendon after ACL reconstruction

After their harvest for ACL reconstruction,

autologous tendon grafts are separated from the circulation and the tissue becomes necrotic, followed by ingrowth of hypercellular and hypervascular reparative tissue.<sup>(12,13)</sup> Hypoxia is a known potent stimulator of VEGF expression in solid tumors,<sup>(14)</sup> and thus it seems likely that decreased oxygen tension at the transition to necrosis may stimulate VEGF expression in tendon grafts after ACL reconstruction. We investigated temporal changes in the relationships between VEGF expression, fibroblast proliferation, and angiogenesis in the patellar tendon graft in the early phase after ACL reconstruction in a rabbit model. We showed that VEGF was highly expressed in proliferating extrinsic fibroblasts at 2 and 3 weeks (Figs. 1A and B).<sup>(15)</sup> From 4 weeks, although the ratio of VEGF-positive cells was reduced in the graft, angiogenesis still continued to be enhanced (Figs. 1A and C). In this period, vascular endothelial cells mainly produced VEGF, which might contribute to the promotion of localized angio-



**Fig. 1** Temporal changes in the relationships between vascular endothelial growth factor (VEGF) expression, fibroblast proliferation, and angiogenesis in a patellar tendon (PT) graft in the early phase after ACL reconstruction in a rabbit model. A: Expression of VEGF in the PT graft at 2 and 8 weeks (x 25) and the positive cell ratio. B: Expression of proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation in the PT graft at 2 and 8 weeks (x 25) and quantitative evaluation of cellular proliferation. C: Expression of CD31 as a marker of vascular endothelial cells in the PT graft at 3 and 8 weeks (x 25) and quantitative evaluation of angiogenesis with Chalkley counts. Adapted from Figure 1, Tohyama H, et, al.<sup>(26)</sup>

genesis. Peterson et al. studied the long-term expression of VEGF for 6 to 104 weeks after ACL reconstruction in sheep.<sup>(16)</sup> They showed strong immunostaining for VEGF in the synovial and subsynovial tissues in the periphery of the graft and within the invading reparative tissue at 6 weeks. At 24 weeks, however, the intensity of VEGF immunostaining decreased. At 52 and 104 weeks, the grafts were largely VEGF-negative. These findings suggested that VEGF mediates angiogenesis in an intra-articular tendon graft in the early remodeling phase after ACL reconstruction. Apparently, VEGF produced by fibroblasts induces revascularization in the graft, implying that VEGF application is a potential strategy for accelerating angiogenesis in the graft after ACL reconstruction.

# Enhancement of angiogenesis in tendon grafts with VEGF

#### The rabbit in situ frozen-thawed ACL model

The *in situ* frozen-thawed ACL, which is anatomical but acellular, has been established as an ideal ACL graft model.<sup>(17,18)</sup> We histologically and

mechanically examined whether an application of VEGF would significantly enhance angiogenesis and significantly affect the mechanical properties of an in situ frozen-thawed rabbit ACL.<sup>(19)</sup> VEGF significantly enhanced vascular endothelial cell infiltration and revascularization in the ACL at 3 and 12 weeks, respectively (Fig. 2). However, the application provided no significant effects on the mechanical properties of the ACL at 12 weeks, although they were significantly weaker than those of the normal ACL at 12 weeks. We demonstrated that intra-articular administration of VEGF significantly accelerates angiogenesis in the ACL devitalized by in situ freeze-thaw treatment. Thus, VEGF has potential as a treatment to enhance revascularization of an autograft after ACL reconstruction surgery. However, there may be biological differences between a frozen-thawed ACL and an intra-articularly grafted tendon after ACL reconstruction.

#### The sheep ACL reconstruction model

We histologically and biomechanically examined the effects of an application of VEGF to a ham-



**Fig. 2** Immunohistochemistry for CD31 to identify vascular endothelial cells in the ACL after *in situ* freeze-thaw treatment without VEGF application (A: 3 weeks; B: 6 weeks; C: 12 weeks) and with VEGF application (D: 3 weeks; E: 6 weeks; F: 12 weeks). A: After *in situ* freeze-thaw treatment, few vascular endothelial cells are found at 3 weeks. B: At 6 weeks, vascular endothelial cells have formed vessels in the superficial portion of the ACL. C: The number of vessels with endothelial cells has decreased between 6 weeks and 12 weeks. D: Several vessels formed by endothelial cells are observed in the superficial portion of the ACL with VEGF application 3 weeks after *in situ* freeze-thaw treatment. E and F: Vessels with endothelial cells are more abundant in the ACL with VEGF application than that without VEGF application up to 12 weeks. Adapted from Figure 1, Figure 2, and Figure 3, Ju YJ, et, al.<sup>(19)</sup>

Chang Gung Med J Vol. 32 No. 2 March-April 2009 string graft in a sheep ACL reconstruction model.<sup>(20)</sup> Eighteen mature female sheep were divided into two groups. In Group I, we soaked the semitendinosus tendon in VEGF solution for 15 minutes and performed ACL reconstruction using this tendon. In Group II, we performed ACL reconstruction using the semitendinosus tendon soaked in phosphate-buffered saline (PBS) for 15 minutes. All animals were killed 12 weeks after ACL surgery to evaluate the graft histology and biomechanics.

At 12 weeks after ACL reconstruction, we found the VEGF-treated graft had a remarkable increase in synovial tissue with hypervascularity around the graft (Fig. 3). Histologically, the numbers of cells and blood vessels in the grafted tendon were greater in Group I than in Group II (Fig. 4). The anterior-posterior translation of the tibia relative to the femur was significantly larger in Group I than in Group II (Fig. 5). The stiffness of the femur-grafttibia complex was significantly lower in Group I than in Group II (Fig. 6A). The average value for the ultimate failure load of the femur-graft-tibia complex in Group I was lower than that of Group II, although we did not find a significant difference (Fig. 6B).

In this sheep study, we found that VEGF treatment promoted a remarkable increase in synovial tissue with hypervascularity around the graft 12 weeks



**Fig. 3** Gross appearances of the grafted tendons 12 weeks after ACL reconstruction in Groups I (A: with VEGF application) and II (B: without VEGF application). Grafted tendons were enveloped by synovium-like tissues (arrows) in the intercondylar notch in both groups. The synovium-like tissues were thicker in Group I than in Group II. Adapted from Figure 1, Yoshikawa T, et, al.<sup>(20)</sup>

after ACL reconstruction, and stimulated angiogenesis and cellular infiltration in the tendon. Thus, VEGF stimulates angiogenesis in the graft after the ACL reconstruction. However, our biomechanical evaluation showed that the linear stiffness of the VEGF-treated femur-graft-tibia complex was significantly lower than that of the PBS-treated graft at 12 weeks, although we could not show a statistical difference in the ultimate failure load between the grafts. In addition, the anterior-posterior translation



**Fig. 4** Histological findings in the grafted tendons 12 weeks after ACL reconstruction shown by hematoxylin-eosin stain (**x** 100) (A: Group I; B: Group II) and actin immunostain (**x** 75) (C: Group I, D: Group II) to detect the tunica intermedia of blood vessels. Adapted from Figure 2, Yoshikawa T, et, al.<sup>(20)</sup>



**Fig. 5** Anterior-posterior (A-P) translation of the tibia relative to the femur between  $\pm 10$  anterior-posterior load at 30° (A), 60° (B), and 90° (C) knee flexion. Adapted from Figure 3, Yoshikawa T, et, al.<sup>(20)</sup>



**Fig. 6** Structural properties of the femur-graft-tibia complex. A: Linear stiffness. B: Ultimate failure load. C: Absorbed energy. D: Elongation at failure. Adapted from Figure 4, Yoshikawa T, et, al.<sup>(20)</sup>

of the knee in Group I was significantly larger than that in Group II. Therefore, these findings showed that VEGF treatment increased knee laxity and decreased graft stiffness after ACL reconstruction, at least temporarily. We do not know exactly why VEGF application reduced the stiffness of the grafted tendon. Shrive et al. studied a medial collateral ligament injury model in the rabbit and found that the area of newly formed vessels, infiltrative cells, and disorderly arranged collagen fibers in the scar tissue was inversely correlated with the mechanical strength of the scar tissue and that newly formed vessels and infiltrative cells might act as "flaws" and enhance the deterioration of the mechanical properties of the grafted tendon.<sup>(21)</sup> Therefore, newly formed vessels and infiltrative cells induced by VEGF administration might cause deterioration in the mechanical properties of the ACL graft due to soft tissue flaws. In addition, it has been reported that VEGF promotes matrix metalloproteinase (MMP) production by some types of cells.<sup>(6,22-24)</sup> Therefore, VEGF-induced MMPs may directly digest the matrix of the graft.

Biomechanical findings in the sheep ACL reconstruction model were different from those of

our rabbit in situ frozen-thawed ACL model,<sup>(19)</sup> while both of these studies indicated that recombinant VEGF application stimulated angiogenesis in the ACL graft or the ACL after necrosis. That is, the adverse effects of VEGF on the mechanical characteristics were greater in the sheep study than in the rabbit in situ frozen-thawed ACL. There are several possible reasons for this difference. The first is that biological differences between the free tendon graft model and the *in situ* frozen-thawed ACL model induce different effects on the mechanical characteristics of the ACL graft. The in situ frozen-thawed ACL model did not create any bone tunnels. Therefore, the inflammation in the knee joint was considered to be extremely severe in the ACL reconstruction model with a free tendon graft compared with the in situ frozen-thawed ACL model. Inflammation might enhance the adverse biological effects of VEGF on the mechanical characteristics of the ACL graft. In addition, there might be some contribution from bone marrow-derived cells. The second possibility is the difference in the methods of recombinant VEGF application between these studies. In our rabbit study, recombinant VEGF was applied to the ACL via a single injection to the knee joint, while in the sheep study, the graft was soaked in VEFG solution for 15 minuets. Soaking a graft might be more effective than a single intra-articular injection. The third possibility is the adverse effect of VEGF on healing in the bone tunnel. Evaluation of graft healing in bone tunnels cannot be done with an in situ frozen-thawed model. In the sheep study, we biomechanically evaluated the femur-graft-tibia complex. Therefore, VEGF might mainly cause deterioration in the mechanical characteristics of the graft-bone interfaces rather than the graft substance. However, this third possibility is unlikely because all grafted tendons failed at graft substance of the ACL graft 12 weeks after the surgery during failure tensile testing. Although we do not know an exact reason for the difference in the adverse effects of VEGF on the ACL graft, we think that clinical ACL reconstruction cases are more similar to the sheep ACL reconstruction model than the rabbit in situ frozenthawed ACL model.

# Future direction of VEGF application in ACL reconstruction

VEGF is widely used for patients with extensive

tissue ischemia in whom primary vascular reconstruction procedures are not feasible or have previously failed.<sup>(25)</sup> Early clinical data provided evidence that VEGF application can achieve beneficial angiogenesis with minimal side-effects. Our experimental findings imply that an application of recombinant VEGF therapy can enhance revascularization in the graft as well as cellular infiltration after ACL reconstruction. However, the biomechanical results indicate that exogenous VEGF application decreases the stiffness of the grafted tendon at least temporarily after ACL reconstruction. Therefore, if we intend to apply exogenous VEGF the adverse effects on the mechanical characteristics of the grafted tendon need to be considered. In addition, recent advances in ACL graft biology may bring new strategies in therapeutic options to prevent this adverse effect.<sup>(26)</sup> On the other hand, the healing between tendon grafts and bone at the tendon-bone junction involves a complex biochemical, biomechanical and biological cascade. The effects of increased angiogenesis mediated by VEGF on bonding between graft and bone at the tendon-bone junction is beyond the scope of this review. Recently, many therapeutic methods using physical stimulation such as extracorporeal shockwaves were shown to be effective in enhancing neovascularization with VEGF expression.(27) In addition, Wang et al. showed that extracorporeal shockwaves improve the mechanical strength at the tendon-bone interface in a bone tunnel in rabbits after ACL reconstruction surgery.<sup>(28)</sup> Therefore, indirect enhancement of VEGF using physical stimulation is another possible strategy to accelerate graft remodeling without weakening the graft-bone contacts after ACL reconstruction.

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