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Application of Growth Factors for Enhancement of Mechanical Strength of Grafted Tendon Following Anterior Cruciate Ligament Reconstruction

Harukazu Tohyama and Kazunori Yasuda

Department of Sports Medicine, Hokkaido University School of Medicine, Sapporo, Japan

1. Introduction

Anterior cruciate ligament (ACL) injury is a relatively common knee injury during sports activities (Uhorchak, 2003). A torn ACL usually occurs through a twisting force being applied to the knee whilst the foot is firmly planted on the ground or upon landing (Boden, 2000). The traditional surgical treatment for ACL rupture is ACL reconstruction by an autogenous tendon graft. However, fibroblasts of the tendon graft are necrotized immediately after transplantation of an autogenous tendon graft, and, then, extrinsic fibroblasts infiltrate in the graft (Amiel, 1986; Arnoczky, 1982; Kleiner 1986). During this process, the grafted tendon weakens in the early phase after ACL reconstruction surgery, even if the grafted tendon is subjected in the mechanically physiological condition (Jackson 1991). In addition, a case report of histology of patellar tendon graft 18 months after ACL reconstruction suggested that the cell infiltration into a core portion of the graft occurs very slowly after ligament reconstruction (Delay, 2002). The slow graft maturation may result in graft failure during the postoperative rehabilitation period. It has been known that growth factors enhance proliferation, migration, and matrix synthesis of cells in vitro (Deie, 1997; DesRosiers, 1996; Kobayashi, 2000; Marui, 1997; Scherping, 1997; Schmidt, 1995). The authors conducted a series of animal experimental studies for the application of growth factors to ligament reconstruction. We will review our recent experimental studies that intended to enhance mechanical strength of grafted tendon after ligament reconstruction using growth factors.

2. Biological characteristics of infiltrative fibroblasts into the necrotized tendon

Previous studies have demonstrated that, in the grafted tendon for ligament reconstruction, fibroblast repopulation from an extrinsic origin occurs with revascularization after intrinsic fibroblasts in the tendon are necrotized (Kleiner 1986). The authors have reported that infiltration of the extrinsic fibroblasts results in mechanical deterioration of the extracellular matrix of the grafted tendon (Tohyama 2000, Tohyama 2006). Thus, the infiltrative

fibroblasts play an important role in remodeling of the autogenous tendon graft. Infiltrative fibroblasts repopulating in the skin wound are phenotypically distinct from normal dermal fibroblasts. Amiel et al. (1995) and Hannafin et al. (1999) reported that proliferation and migration characteristics of the ligament fibroblasts depended on their origin. Therefore, there is a high possibility that extrinsic fibroblasts infiltrating in the necrotized tendon have significantly different biological characteristics, compared with the intrinsic fibroblasts in the normal tendon. To understand the remodeling of the tendon autograft in ligament reconstruction, it is necessary to clarify differences in biological characteristics between the infiltrative and intrinsic fibroblasts.

We have compared the biological characteristics of infiltrative fibroblasts into the patellar tendon after fibroblast necrosis using an in situ freeze-thaw procedure to normal patellar tendon fibroblasts (Ikema, 2005; Tohyama, 2007). The in situ frozen-thawed patellar tendon simulates ligament reconstruction with the patellar tendon graft under ideal condition. To obtain the infiltrative fibroblasts, we performed an in situ freeze-thaw treatment on the patellar tendon to kill the intrinsic fibroblasts (Fig. 1). In this in situ freeze-thaw treatment, the patellar tendon was frozen with liquid nitrogen for 1 minute. The frozen patellar tendon was then thawed by physiological saline solution. We confirmed that this procedure killed 97% to 100% of intrinsic fibroblasts in the rabbit patellar tendon. After this treatment, only the extrinsic fibroblasts were available to repopulate in the patellar tendon (Tohyama, 2000). Six weeks later, the patellar tendons were harvested and placed in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. A confluent monolayer formed in 2 weeks. Thus, infiltrative cells, >95% fibroblast-like as confirmed by microscopic analysis, were obtained from the right patellar tendon. For comparison, the untreated patellar tendon was similarly incubated and normal fibroblasts were isolated in the same manner.

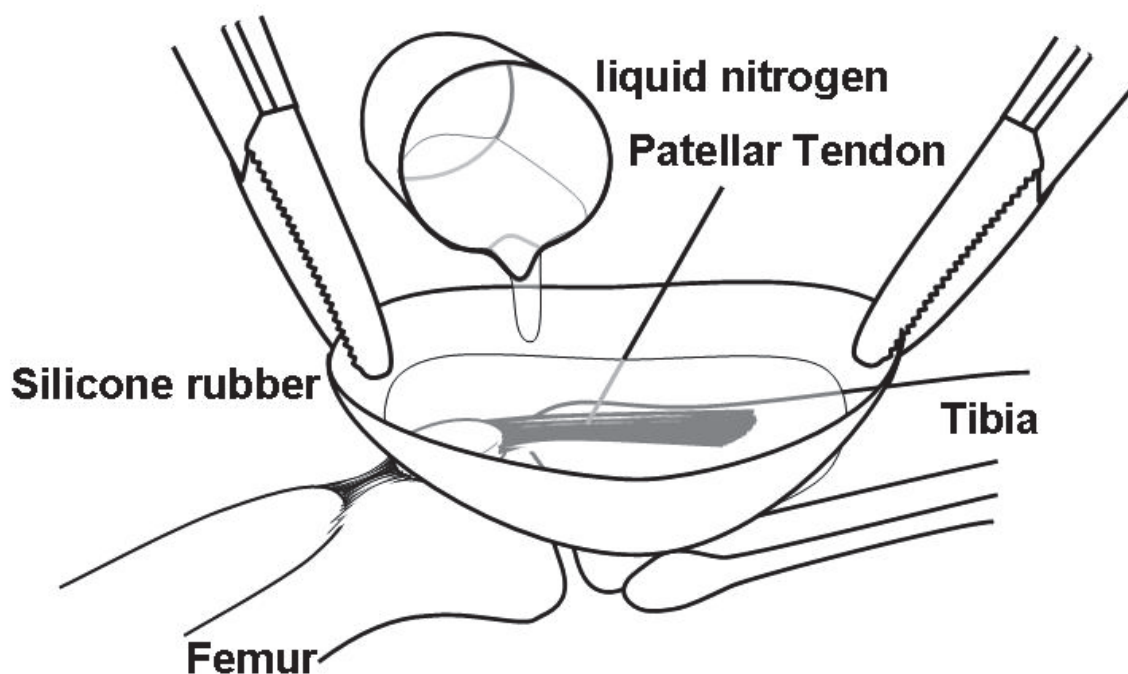
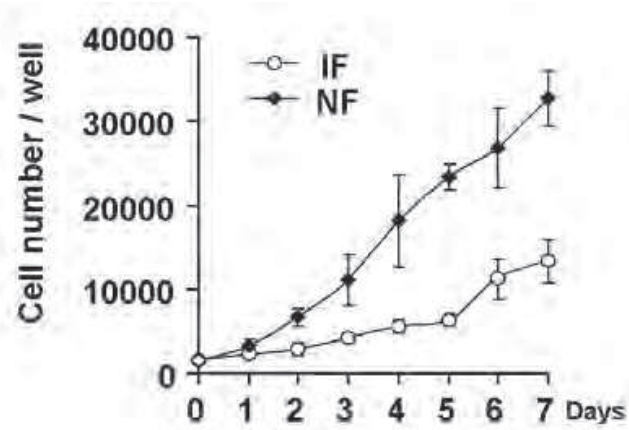
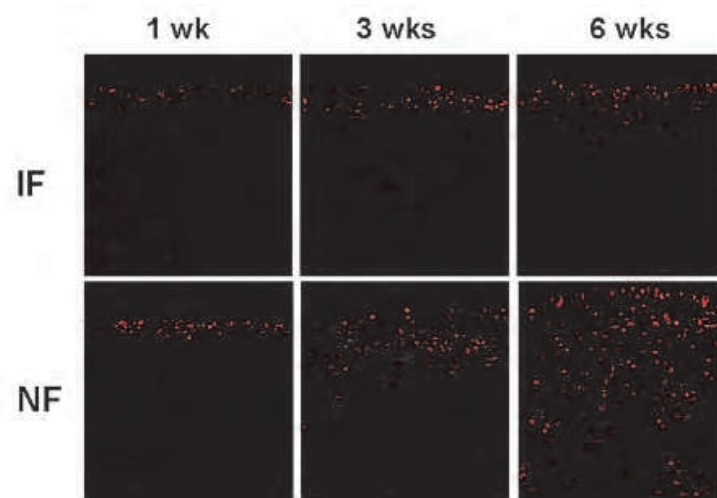


Fig. 1. The in situ freeze-thaw treatment for necrotizing intrinsic fibroblasts in the patellar tendon (From ref. Ikema (2005))

a.



b.



c.

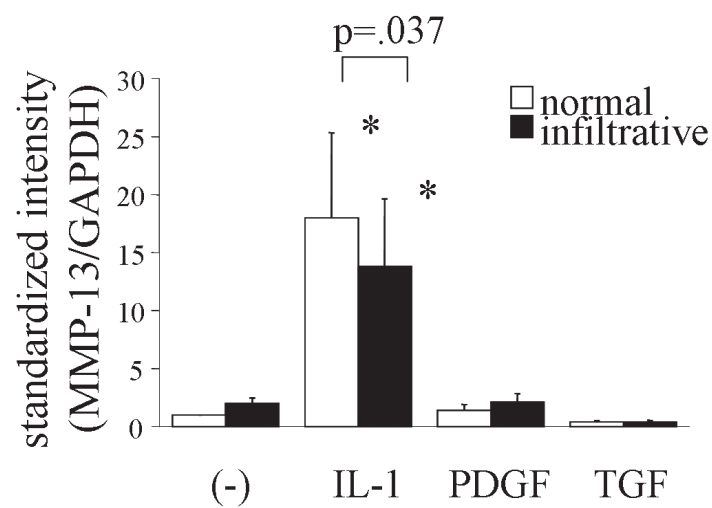


Fig. 2. Cellular proliferation (a), migration (b) and responsiveness to IL-1beta (c) of infiltrative fibroblasts (IFs) and normal fibroblasts (NFs) (From ref. Ikema (2005) and ref. Tohyama (2007)).

The authors then found that the cellular proliferation, migration, and responsiveness of infiltrative fibroblasts to IL-1 β , which is one of the major inflammatory cytokine, are quite inferior to those of normal fibroblasts (Fig. 2)(Ikema, 2005; Tohyama, 2007). The slow remodeling process in the tendon graft may be attributed to these inferior potentials of infiltrative fibroblasts. Therefore, we may be able to accelerate the remodeling process of the grafted tendon after ligament reconstruction if we restore the potentials of infiltrative fibroblasts to the levels of normal tendon fibroblasts with regard to cellular proliferation, migration, and responsiveness to cytokines.

3. Growth factor application to the graft after ACL reconstruction

As described above, previous studies have demonstrated that intrinsic fibroblasts in the tendon grafted across the knee joint to reconstruct the ACL are necrotized immediately after transplantation, and that cellular repopulation from an extrinsic origin and revascularization sequentially occur (Arnoczky, 1982; Kleiner, 1986). In this process, the mechanical properties of tendon autografts deteriorated after ligament reconstruction surgery, and they remain inferior even at 8 months after surgery (Beynon, 1997). Also, the cell infiltration into the grafted tendon occurs very slowly after ACL reconstruction (Delay, 2002). Recently, a number of studies have shown that application of various growth factors stimulates cellular proliferation, angiogenesis, and synthesis of extra-cellular matrix in tendon and ligament tissues (Deie, 1997; DesRosiers, 1996; Kobayashi, 2000; Marui, 1997; Scherping, 1997; Schmidt, 1995; Zachary, 1998). Therefore, there are two approaches in the application of growth factor to the graft after ACL reconstruction. The first approach is to enhance angiogenesis and cellular repopulation in the grafted tendon after the necrosis. The second one is to improve tissue quality of the grafted tendon via remodelling of the collagen matrix after ACL reconstruction.

3.1 Growth factor application for enhancement of angiogenesis

Angiogenesis is a biological mechanism of new capillary formation and involves the activation, migration, and proliferation of endothelial cells from preexisting venules. Angiogenesis can be influenced by many factors including hypoxia, growth factors, and matrix components. The angiogenic activation of endothelial cells probably plays a role in promoting and regulating other biological events, such as inflammation, fibroblast proliferation, and extracellular matrix synthesis in the remodeling process of the grafted tendon after ACL reconstruction. Vascular endothelial growth factor (VEGF) is considered to be a potent mediator of angiogenesis in various pathological conditions (Ferrara and Davis-Smyth, 1997). Recently, our study in the rabbit ACL reconstruction model clarified that infiltrative cells produced VEGF before revascularization in the grafted tendon (Fig. 3) (Yoshikawa, 2006a). This has suggested that VEGF mediates angiogenesis in the intra-articular tendon graft for the ACL reconstruction.

Based on our finding, an administration of VEGF may significantly enhance angiogenesis in the grafted tendon after ACL reconstruction and then may accelerate the remodeling process of the grafted tendon after necrosis. On the other hand, there is also a possibility that the revascularization induced by VEGF deteriorates the mechanical strength of the grafted tendon. Newly formed vessels in the graft may weaken the grafted tendon as soft-tissue "flaws" (Shrive, 1995). Therefore, we examined the effect of an application of VEGF in

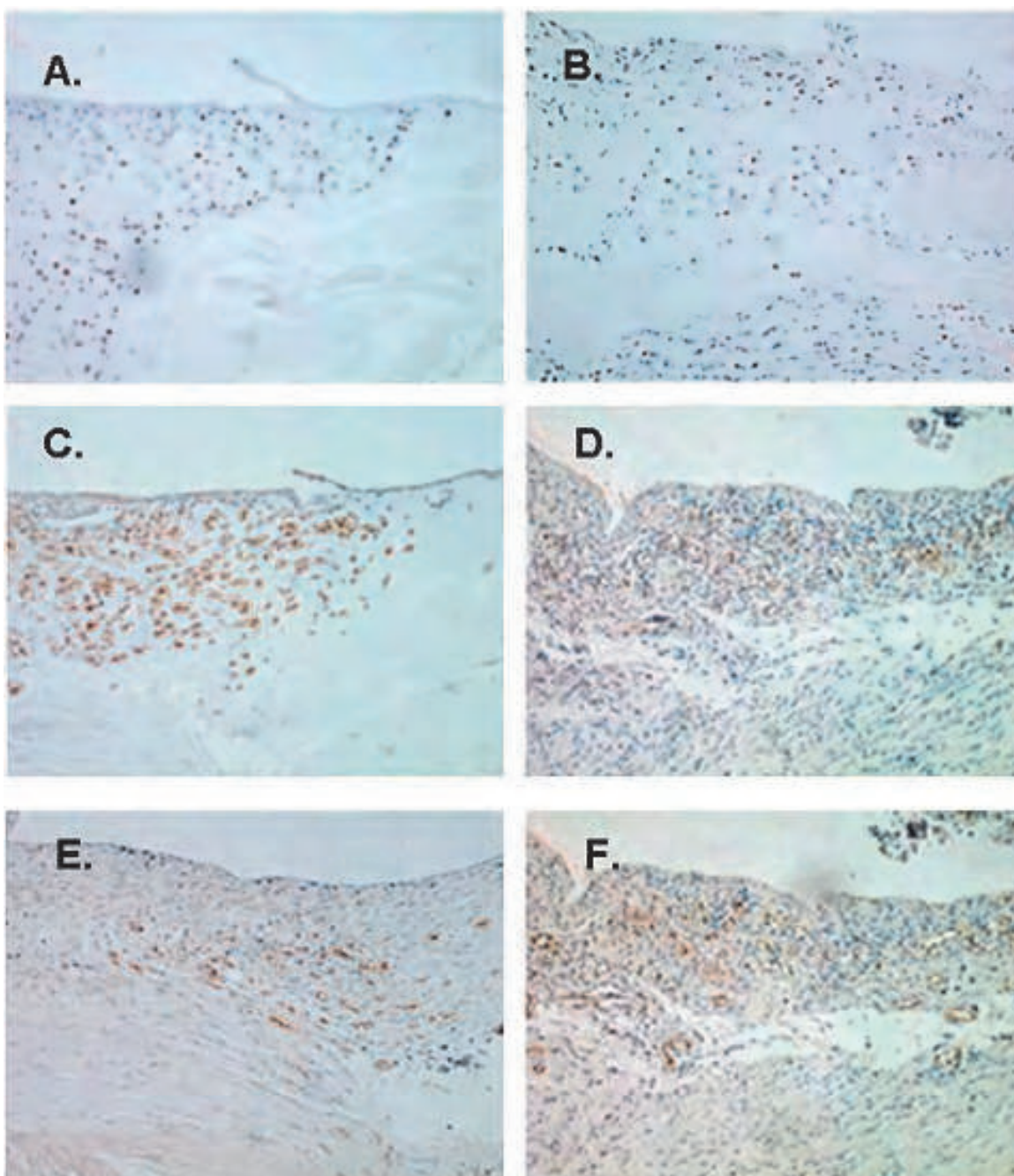


Fig. 3. Immunohistologies for proliferative cells (PCNA stain)(A: 2 weeks, B: 8 weeks), VEGF (C: 2 weeks, D: 8 weeks), and vascular endothelial cells (CD31 stain) (E: 3 weeks, F: 8 weeks) of the patellar tendon graft after ACL reconstruction in the rabbit model. A: Proliferative cells were frequently found at the superficial portion of the tendon graft at 2 weeks. B: At 8 weeks, few proliferative cells were observed in the patellar tendon graft. C: At 2 weeks, VEGF-positive cells scattered at the similar area where proliferative 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 (From ref. Yoshikawa (2006a)).

the rabbit in situ frozen-thawed ACL and the sheep ACL reconstruction models. The in situ frozen-thawed ACL, which is anatomical but acellular, has been established as an idealized ACL graft model (Jackson, 1991; Katsuragi, 2000; Sakai, 2002). In the rabbit model, we performed the freeze-thaw treatment for the right ACL (Fig. 4) and then injected 30- μ g VEGF with 0.2-ml phosphate-buffered saline in the right knee joint. Several vessels formed by endothelial cells were observed at the superficial portion of the ACL 3 weeks after the in situ freeze-thaw treatment and VEGF injection, while few vascular endothelial cells were found in the ACL at 3 weeks after the in situ freeze-thaw treatment alone (Fig. 5) (Ju, 2006). The number of vessels with endothelial cells was significantly higher in the ACLs after the in situ freeze-thaw treatment and VEGF injection than in the ACLs after the in situ freeze-thaw treatment alone (Fig. 5). This finding implied that recombinant VEGF therapy may be used to enhance graft remodeling in ACL reconstruction. However, the in situ frozen-thawed ACL was not a true model of ACL reconstruction by use of a free tendon graft. Biological differences must exist between the frozen-thawed ACL and the intra-articular grafted tendon after ACL reconstruction, since bone marrow-derived cells contribute to a graft that is placed in a bone tunnel. Therefore, we conducted a following large animal model study to clarify if recombinant VEGF application affects the mechanical properties of the grafted tendon after ACL reconstruction before its clinical application of recombinant VEGF therapy to ACL reconstruction.

In this experiment, we used mature female Suffolk sheep (Yoshikawa, 2006b). We harvested the semitendinosus tendon from the right leg and then soaked the tendon in recombinant human VEGF with 10-ml phosphate buffered saline (PBS) for 15 minutes and then performed ACL reconstruction using this semitendinosus tendon in the same leg (Fig. 6). These animals were killed 12 weeks after ACL reconstruction for the histological and biomechanical evaluations. Concerning mechanical evaluation, the antero-posterior (A-P) drawer tests were performed in 30°, 60°, and 90° of flexion and neutral rotation with load application. The knee was mounted to a custom-made adjusting device with 3 degrees of freedom (translations in the anterior-posterior, medial-lateral, and proximal-distal directions) in a materials testing machine. An A-P force of ± 100 N was applied 15 times with a load displacement rate of 50 mm/min and the A-P displacement between ± 100 -N A-P forces was quantified. After A-P drawer testing, all soft tissue including the menisci was removed, leaving only the grafted tendon. The cross-sectional area of the graft was measured at the middle level of intra-articular portion of the graft by a non-contact optical method with video dimension analyzer. The femur-graft-tibia (FGT) complex underwent tensile testing at the cross head speed of 50 mm/min until the FGT complex failed.

The A-P translation of the tibia relative to the femur in the experimental group was significantly larger than that in the control group, in which the knee underwent identical procedures to those of the experimental group except that the harvested tendon was soaked in 10-ml PBS instead of recombinant VEGF with 10-ml PBS (Fig. 7) (Yoshikawa, 2006). At the failure tests to determine the structural properties of the femur-graft-tibia complex, all grafts failed at the midsubstance portion in the graft during tensile testing, while normal ACL specimens had avulsion fractures at the tibial insertion sites to the ACLs. The linear stiffness of the femur-graft-tibia complex in the experimental group was significantly lower than that in the control group, while there were no significant differences in the ultimate failure load or the energy absorbed at failure between the experimental and the control group (Fig. 8).

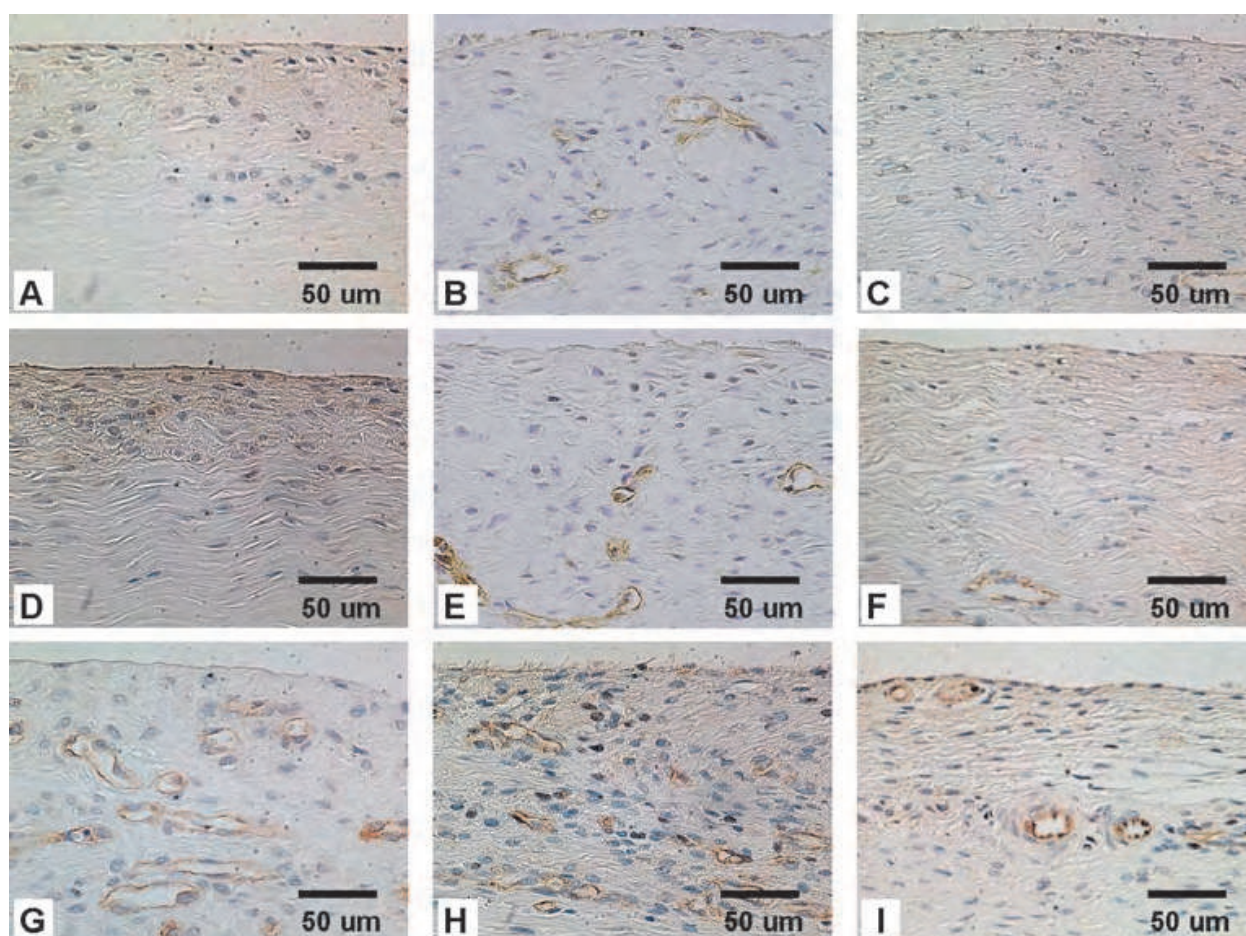


Fig. 4. Immunohistologies for vascular endothelial cells to evaluate the effects of local VEGF application on vessel formation in the ACL at 3 weeks (A,B,C), 6 weeks (D,E,F), and 12 weeks (G,H,I) after the in situ freeze-thaw treatment in the rabbit model (CD31 stain). At 3, 6, and 12 weeks after surgery, we did not find any obvious differences in angiogenesis between the ACLs with (C,F, and I) and without intra-articular injection of 2-ml phosphate-buffered saline (A, D, G). On the other hand, several vessels formed by endothelial cells were observed at the superficial portion of the ACL 3 weeks after the in situ freeze-thaw treatment and VEGF injection (B) (Ju, 2006). The number of vessels with endothelial cells was significantly higher in the ACLs after the in situ freeze-thaw treatment and VEGF injection (B,E,H) than in the ACLs after the in situ freeze-thaw treatment alone (B,E,H)(Fig. 5).(From ref. Ju (2006)).



Fig. 6. Anterior cruciate ligament reconstruction procedure in the sheep model. A: semitendinosus tendon graft, B: a radiographic lateral view immediately after the surgery (From ref. Kondo (2011)).

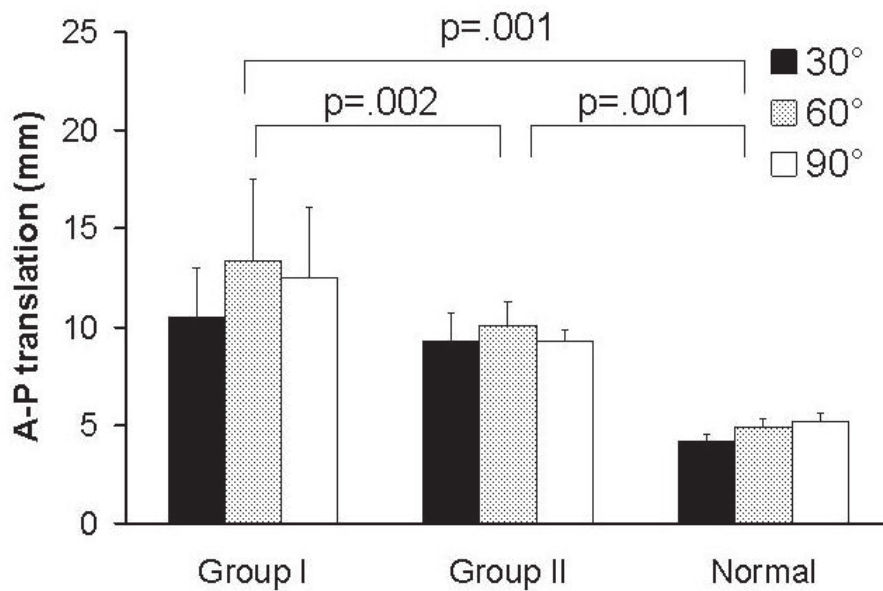


Fig. 7. The effects of VEGF application on A-P displacement between ± 100 -N A-P forces (From ref. Yoshikawa (2006b)). Group I: the knee 12 weeks after ACL reconstruction with semitendinosus tendon graft soaked in phosphate buffered saline, Group II: the knee 12 weeks after ACL reconstruction with semitendinosus tendon graft soaked in VEGF solution, Normal: normal knee with no treatment.

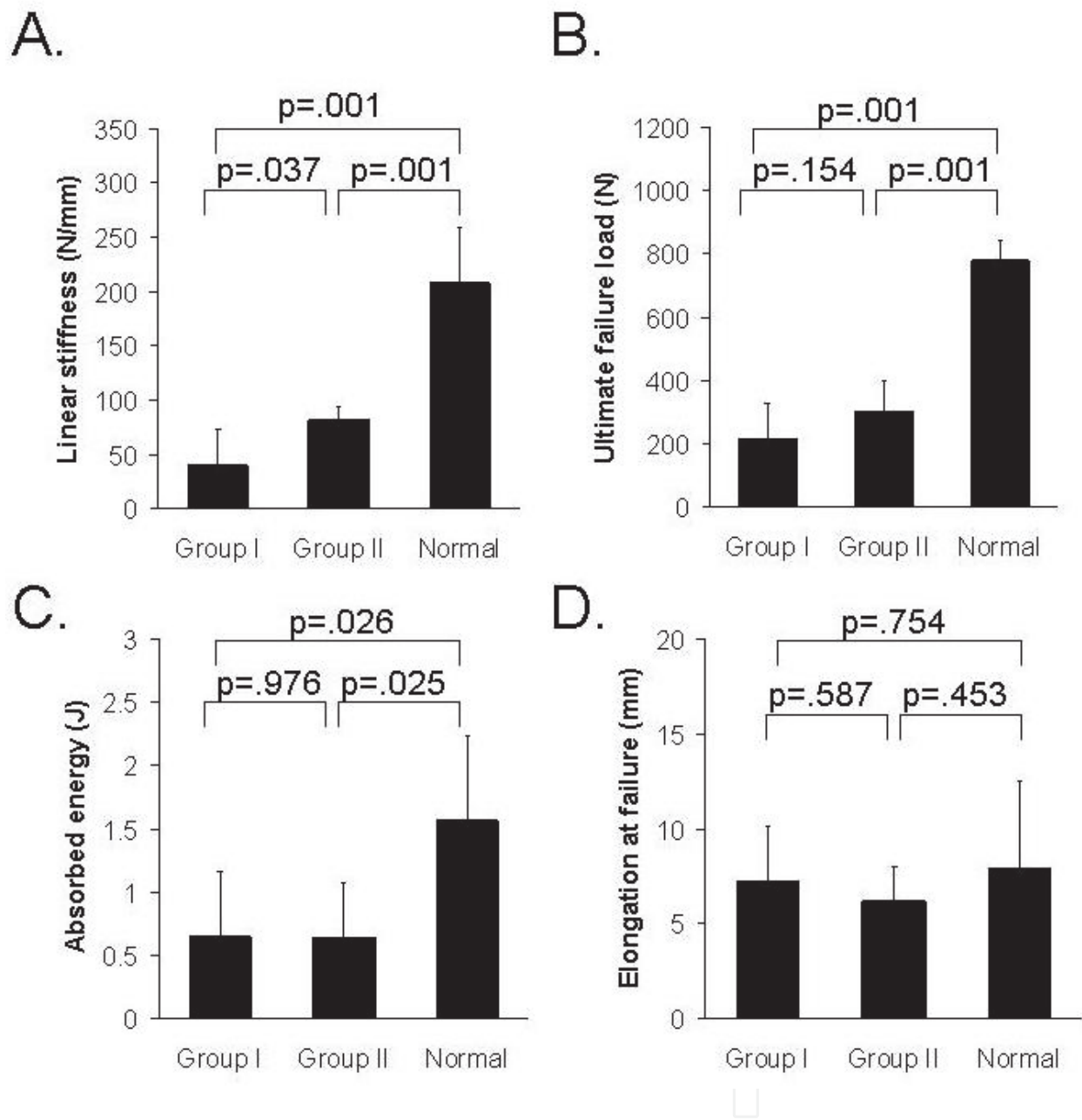


Fig. 8. The effects of VEGF application on structural properties of the femur-graft-tibia complex after ACL reconstruction (A: The linear stiffness; B: The ultimate failure load; C: The absorbed energy; D: Elongation at failure) (From ref. Yoshikawa (2006b)). Group I: the femur-graft-tibia complex 12 weeks after ACL reconstruction with semitendinosus tendon graft soaked in phosphate buffered saline, Group II: the femur-graft-tibia complex 12 weeks after ACL reconstruction with semitendinosus tendon graft soaked in VEGF solution, Normal: normal the femur-graft-tibia complex with no treatment.

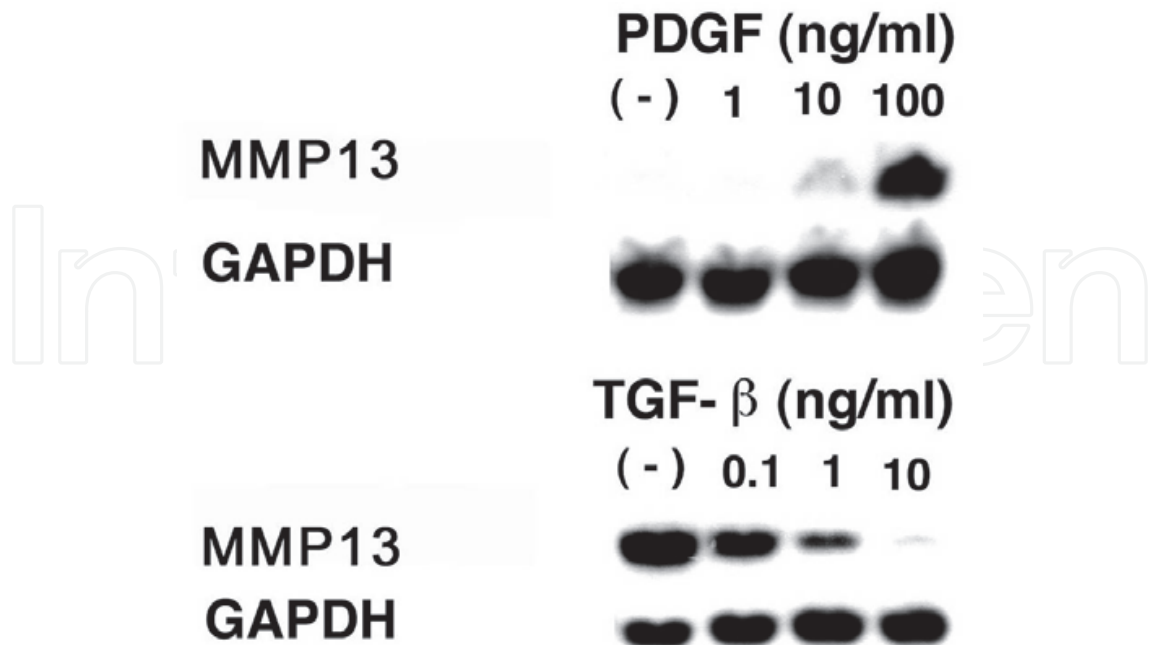
We did not know exactly why our VEGF application reduced the stiffness of the grafted tendon after ACL reconstruction. Shrive et al. (1995) reported that in the medial collateral ligament injury model in the rabbit, the area of newly formed vessels, infiltrative cells and disorderly arranged collagen fibers in the scar tissue was reversely correlated with mechanical strength of the scar tissue and that a number of newly formed vessels and infiltrative cells might act as "flaws" and enhance the deterioration of the mechanical property of the grafted tendon. Therefore, a number of newly formed vessels and infiltrative cells which VEGF administration induced in the ACL graft might deteriorate mechanical properties of the ACL graft as soft tissue flaws. In addition, it was reported that VEGF promotes collagenase production by some types of cells (Ferrara, 1997; Munaut, 2003; Pufe, 2004; Zachary, 1998). Therefore, VEGF-induced collagenase directly might digest the matrix of the graft. VEGF was widely used for patients with extensive tissue ischemia in whom primary vascular reconstruction procedures were not feasible or had previously failed in clinical trials (Kusumanto, 2003). Early clinical data provide evidence that the VEGF application can achieve beneficial angiogenesis, with minimal side-effects. Our findings imply that an application of the recombinant VEGF therapy can supposedly enhance revascularization in the graft as well as cellular infiltration after ACL reconstruction. On the other hand, our biomechanical results have indicated 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 as a treatment to accelerate angiogenesis and cellular infiltration in the tendon graft for ACL reconstruction, we should take into account this adverse effect of exogenous VEGF application on the mechanical characteristics of the grafted tendon.

3.2 Growth factor application for collagen synthesis in fibroblasts

Numerous studies have shown that various types of cells can over-express growth factors such as transforming growth factor-beta (TGF-beta), basic fibroblast growth factor (b-FGF), and platelet-derived growth factor (PDGF), epidermal growth factor (EGF) during healing process of the tissue. In addition, these factors regulate the synthesis and degradation of collagen by the fibroblasts of tendons and ligaments. Therefore, the effects of growth factors on mRNA expression of MMP-13, which is main collagenase in the rat, were evaluated in the rat model using Northern blot analysis. At 6 hours after the challenge with PDGF-BB, up-regulation of MMP-13 mRNA became apparent at the dose of 100 ng/ml, while slight up-regulation of MMP-13 mRNA was observed at the dose of 10 ng/ml (Fig. 9A). In contrast, down-regulation of MMP-13 mRNA was found at 6 hours after the stimulation with TGF-beta1. The suppression of MMP-13 mRNA by TGF-beta1 was dose-dependent in the range less than 10 ng/ml (Fig. 9A). We also found that TGF-beta1 significantly increases the ratio of type I collagen mRNA to type III collagen mRNA in extrinsic fibroblasts infiltrative fibroblasts (Fig. 9-B). It is well known that EGF stimulates fibroblast proliferation in vitro (Schmidt, 1995). A combined application of these two growth factors enhances these effects (DesRosiers, 1996). Therefore, we conducted following animal experimental studies for the application of TGF-beta1 and EGF to ligament reconstruction.

First, we investigated the effects of a combined application of TGF-beta and EGF on the rabbit in situ frozen-thawed ACL (Sakai, 2002). In this study, a low and a high doses of combinations (low dose: 4-ng TGF-beta1 and 100-ng EGF, high dose: 2-microgram TGF-beta1 and 50-microgram EGF) mixed with the fibrin sealant were applied to rabbit ACLs after the in situ freeze-thaw treatment, compared with in situ frozen-thawed ACLs without any other treatment and with fibrin sealant alone. These ACLs were evaluated at 12 weeks

A.



B.

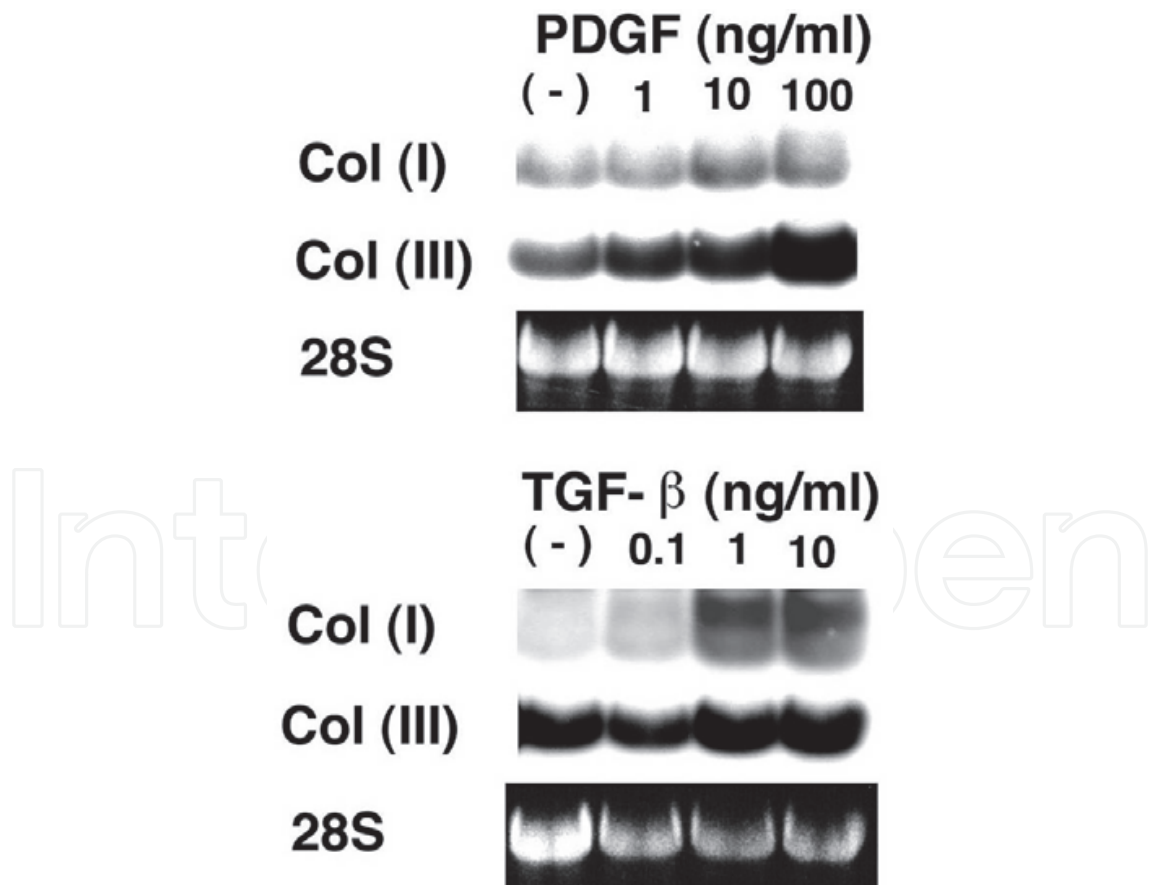


Fig. 9. The effects of growth factors on gene expression of extrinsic infiltrating fibroblasts into the patellar tendon after the necrosis (A. MMP-13 mRNA; B. type-I and type-II collagens mRNAs).

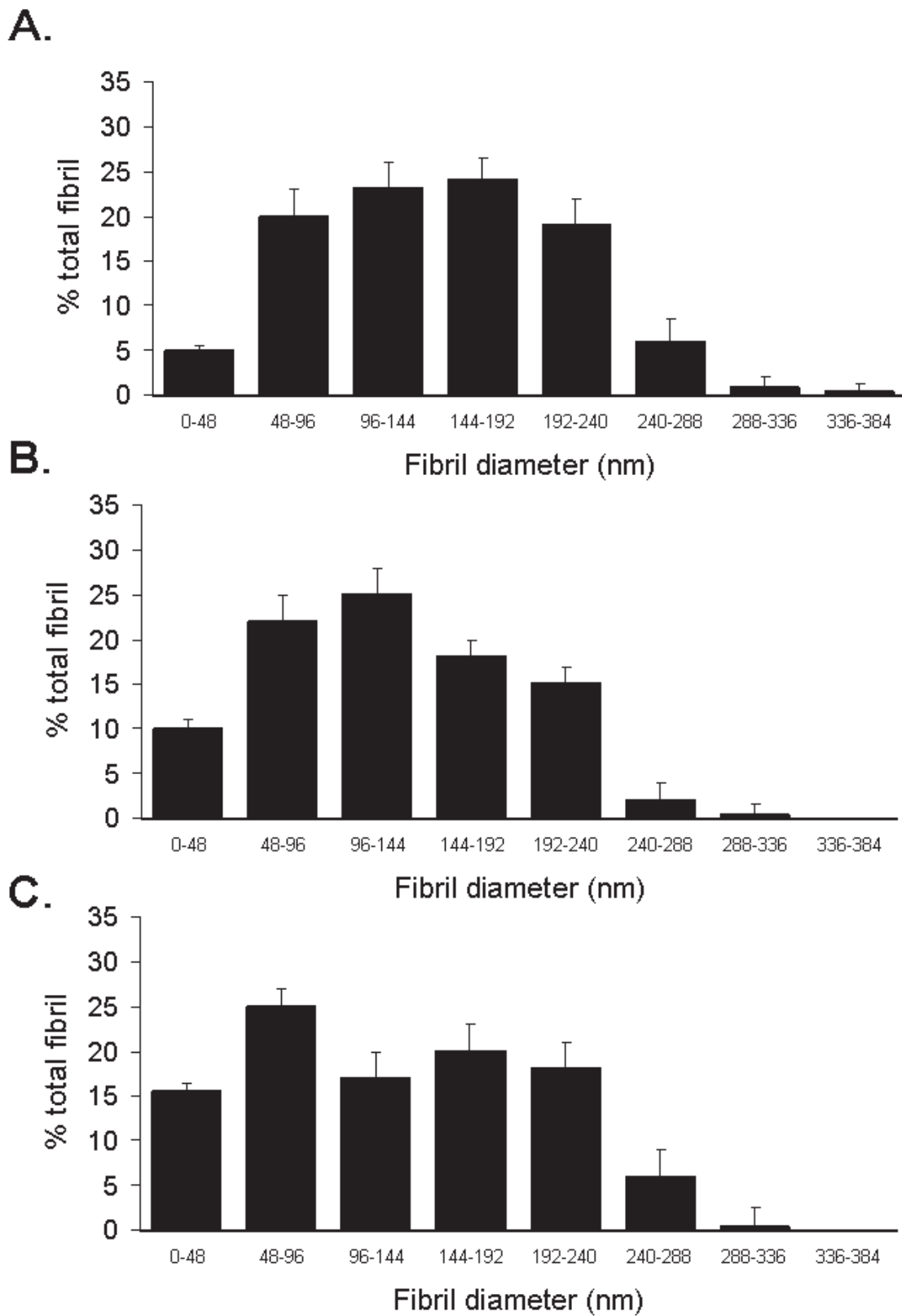
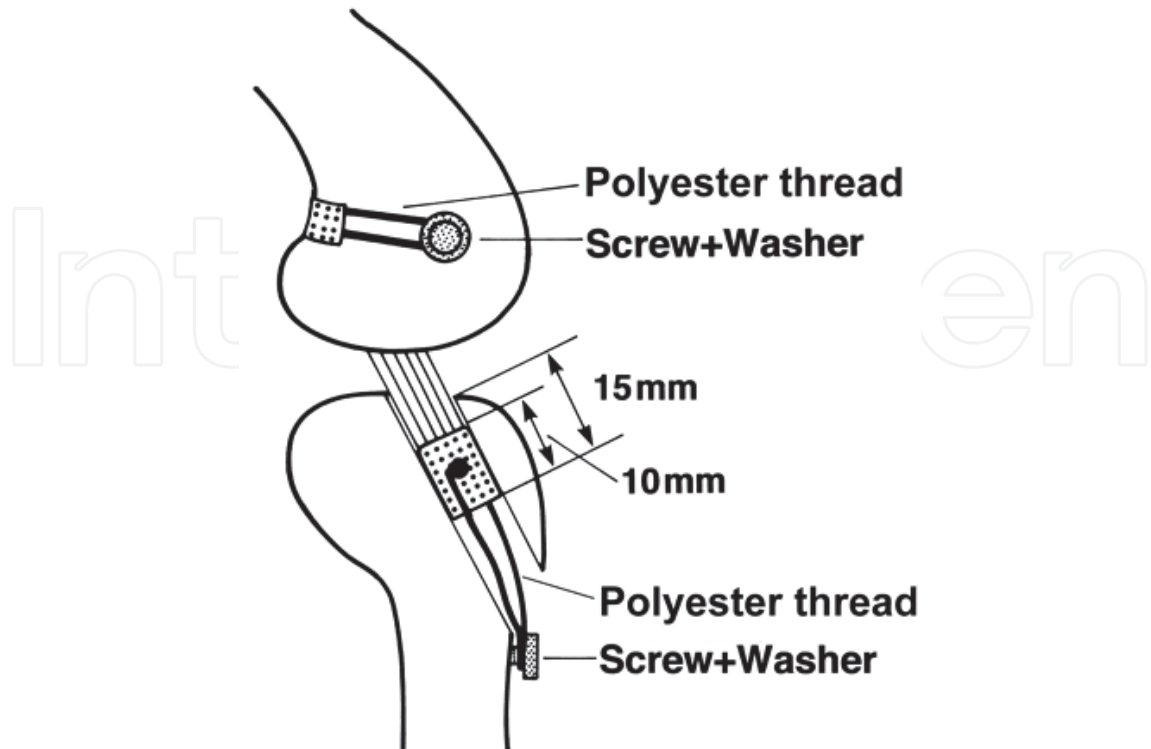


Fig. 10. Histograms of the collagen fibril diameter in the normal control ACL (A) and the ACL after the in situ freeze-thaw treatment without TGF-beta/EGF application (B) and with a high dose of TGF-beta and EGF ((From ref. Sakai (2002)).

A.



B.

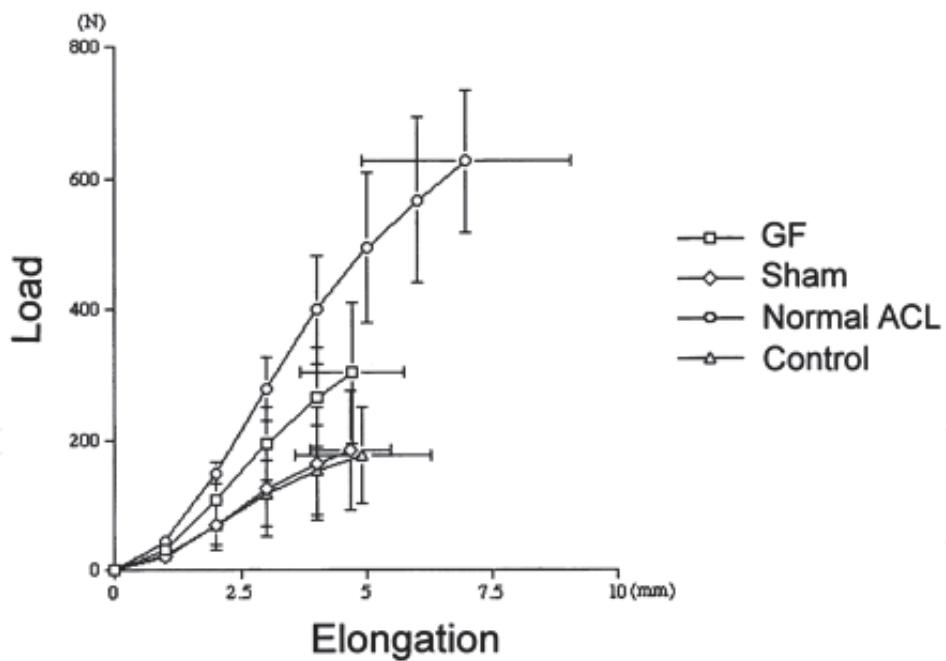


Fig. 11. The effects of low-dose application of TGF-beta and EGF on structural properties of the femur-graft-tibia complex after ACL reconstruction (From ref. Yasuda (2004)). A. ACL reconstruction procedure with the bone-patellar tendon-bone graft, B. The load-elongation curves of the femur-graft-tibia complexes in the knees with growth factor application (GF), with fibrin sealant alone (Sham), and without growth factor or fibrin sealant (Control) groups and the normal femur-ACL-tibia complex (Normal ACL).

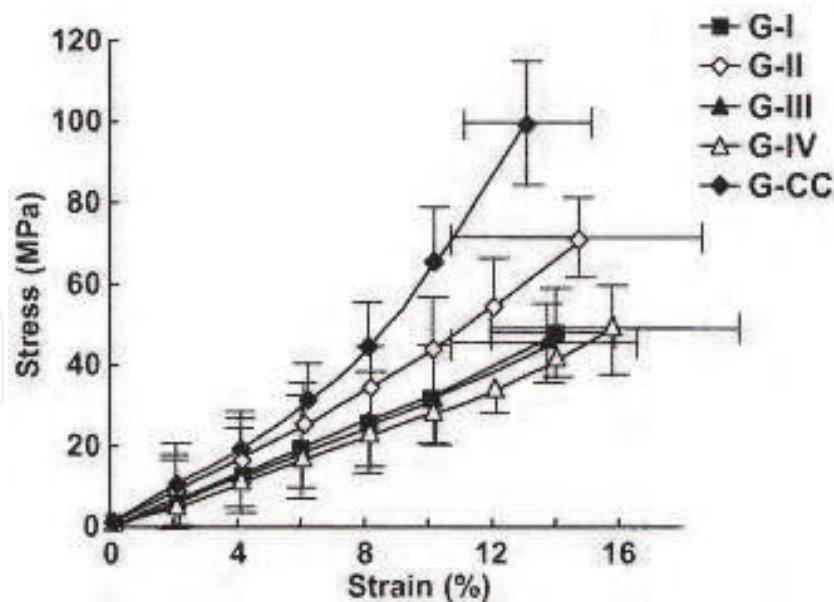


Fig. 12. The effects of a separate application of TGF-beta, EGF, and PDGF-BB on the material properties of the ACL 12 weeks after the in situ freeze-thaw treatment (From ref. Nagumo (2005)). The stress-strain curves of the anteromedial bundle of the ACL in Group I (G-I, only 0.2 ml fibrin sealant was applied), Group II (G-II, 4 ng TGF-beta1 mixed with 0.2 ml fibrin sealant was applied), Groups III (G-III, 100 ng EGF mixed 0.2 ml fibrin sealant was applied) Group IV (G-IV, 4 µg PDGF-BB mixed with 0.2 ml fibrin sealant was applied) and Group “contralateral control” (G-CC).

on the basis of mechanical properties, water content, and histological and ultrastructural observations. As a result, the cross-sectional area and the water content of ACLs with a low dose of TGF-beta1 and EGF were significantly less than those of ACLs with other treatments at 12 weeks. The tensile strength of ACLs with a low dose of TGF-beta1 and EGF was significantly greater than those of ACLs with other treatments at 12 weeks. In addition, the average tangent modulus of with a low dose of TGF-beta and EGF was 96% of the average value in the normal ACLs, while that with a low dose of TGF-beta and EGF was 68% of the normal ACLs. A unimodal distribution of collagen fibril diameters was noted in ACLs without TGF-beta/EGF application, while a bimodal pattern was found in ACLs with a low dose of TGF-beta1 and EGF (Fig. 10). These findings revealed that low-dose application of TGF-beta and EGF significantly inhibited not only the increased water content and cross-sectional area, but also the decreased tensile strength caused by the freeze-thaw treatment, while a high dose of TGF-beta and EGF does not have the same beneficial effects.

Second, we conducted a canine model study to clarify if low-dose application of TGF-beta and EGF enhances the mechanical properties of the grafted tendon after ACL reconstruction (Yasuda, 2004). In this study, 20 dogs underwent ACL reconstruction with the autogenous bone-patellar tendon-bone graft, which is a standard graft for ACL reconstruction, in bilateral knees. A combination of 12 ng TGF-beta and 300 ng EGF mixed with fibrin sealant was applied to the left knee and compared to the right knee without any treatment after identical ACL reconstruction procedure to the left side. In the remaining 10 dogs, fibrin sealant alone was applied to the left knee. We then found that combined application of TGF-beta and EGF increased the stiffness and maximum failure load of the femur-graft-tibia complex at 12 weeks, while the application of fibrin sealant alone did not significantly affect

them (Fig.11). Our findings suggest that application of transforming growth factor-beta and epidermal growth factor improves the structural properties of the femur-graft-graft complex after ACL reconstruction. Therefore, application of growth factors is a possible strategy to prevent graft deterioration in ACL reconstruction.

Third, we evaluated effects of a separate application of TGF-beta, EGF, and PDGF-BB on the material properties of the in situ frozen-thawed ACL (Nagumo, 2005). In this study, we applied 4 ng TGF-beta, 20 ng EGF, and 4 microgram PDGF-BB to the ACL after the in situ freeze-thaw treatment, separately. We also applied only fibrin sealant to the ACL after the in situ freeze-thaw treatment as a control. At 12 weeks after growth factor application, the tensile strength and the tangent modulus of the ACL with TGF-beta application was significantly higher than in the control group (Fig. 12). On the other hand, there were no significant differences in the strength and the modulus among the ACLs with EGF application, PDGF-BB application and the controls. These findings suggests that that the effect of TGF-beta was significant, but the effect of EGF not. Therefore, there is the possibility that the application of TGF-beta enhances maturation of the graft after ligament reconstruction.

4. Conclusion

After ligament reconstruction, the cell infiltration into a core portion of the graft is considered to occur very slowly (Delay, 2002). The slow graft maturation may result in graft failure during the postoperative rehabilitation period. In this chapter, the authors showed the recent experimental findings suggesting that an administration of growth factors, in particular, TGF-beta can inhibit the deterioration of mechanical properties of the grafted tendon after ACL reconstruction. Therefore, application of growth factors, in particular TGF-beta, is a possible strategy to enhance maturation of the graft after ligament reconstruction. However, a few recent studies reported that TGF-beta induced arthritic changes of the articular cartilage in the knee joint (Hulth, 1996; van Beuningen, 1994). Therefore, intraarticular administration of TGF-beta may be unsuitable for clinical application with an ACL reconstruction procedure. The cell-based therapy with cellular activation by growth factors may be a potential solution against this problem (Kondo, 2011; Okuizumi, 2004). The recent advancement in biology about ligament reconstruction can bring new strategies in additional therapeutic options to accelerate the remodeling of the graft and enhance mechanical strength of the grafted tendon after ACL reconstruction.

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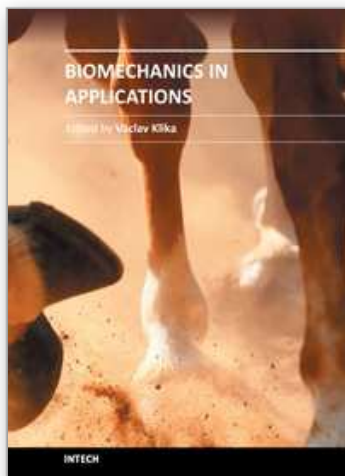
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During last couple of years there has been an increasing recognition that problems arising in biology or related to medicine really need a multidisciplinary approach. For this reason some special branches of both applied theoretical physics and mathematics have recently emerged such as biomechanics, mechanobiology, mathematical biology, biothermodynamics. The Biomechanics in Application is focusing on experimental praxis and clinical findings. The first section is devoted to Injury and clinical biomechanics including overview of the biomechanics of musculoskeletal injury, distraction osteogenesis in mandible, or consequences of drilling. The next section is on Spine biomechanics with biomechanical models for upper limb after spinal cord injury and an animal model looking at changes occurring as a consequence of spinal cord injury. Section Musculoskeletal Biomechanics includes the chapter which is devoted to dynamical stability of lumbo-pelvi-femoral complex which involves analysis of relationship among appropriate anatomical structures in this region. The fourth section is on Human and Animal Biomechanics with contributions from foot biomechanics and chewing rhythms in mammals, or adaptations of bats. The last section, Sport Biomechanics, is discussing various measurement techniques for assessment and analysis of movement and two applications in swimming.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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