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ORIGINAL STUDY

Effect of combined administration of Transforming Growth Factor-b1 and Insulin-like Growth Factor I on the mechanical properties of a patellar tendon defect model in rabbits

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The aim of this study was to test the hypothesis that combined administration of TGF-b1 and IGF-I in a patellar tendon defect model could enhance the mechanical properties of the healed tendon. Twenty four New Zealand white rabbits were used for this purpose. In each animal, the right knee was used for the application of the growth factors, whereas the left knee served as an untreated control. The growth factors were mixed with fibrin sealant as a delivery vehicle. Two groups of rabbits were sacrificed after 2 weeks and 6 weeks respectively. Application of the growth factors resulted in a significant increase in force at failure, ultimate stress, stiffness, and energy uptake at 2 weeks, whereas none of the parameters revealed any significant difference between the two groups at 6 weeks. This study provides valuable information on the effect of the two growth factors on this patellar tendon defect model.

Keywords : Growth factors ; TGF-b1 ; IGF-I ; patellar tendon ; rabbits.

INTRODUCTION

It is well known that the properties of healed tendons are inferior when compared to those of normal tissue (*3,4*). Therefore, various exogenous growth factors have been used, alone or in combination, in order to enhance the healing process (*5,7,20*). Nevertheless, to our knowledge, there are no studies in the international literature concerning the effect of combined administration of Transforming growth factor-b1 (TGF-b1) and Insulin-like growth factor I (IGF-I) on tendon healing.

TGF-b1 can stimulate mitogenic responses of cultured patellar tendon fibroblasts and mediate proteoglycan synthesis in tendon explants (*17,21*).

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Transforming Growth Factor-b1 can also modulate production of fibronectin (*19*), proteoglycan (*17*), collagen Type I, and collagen Type III (*6,18*). On the other hand, IGF-I is a major mediator in all stages of wound healing, including inflammation, and its absence dramatically impairs wound healing across multiple parameters (*13,22*).

We hypothesized that combined administration of TGF-b1 and IGF-I in a patellar tendon defect model could enhance the mechanical properties of the healed tendon. The aim of this study was to test this hypothesis.

MATERIALS AND METHODS

Animals

As a pre-trial study was performed, sample size was determined using an 80% power, significance level 0.05, an initial estimate of the standard deviation equal to 17 so as to detect a difference of 30 in the mean. The desired sample size for each group was equal to 6 at each time point (EpiInfo, version 6, Centers for Disease Control and Prevention, Atlanta, Georgia). Two groups, each with 12 rabbits, were used to evaluate tendon repair biomechanically (10 rabbits) and histologically (2 rabbits) ; they were sacrificed after 2 weeks and 6 weeks respectively. In each animal, the right knee was used for the application of the growth factors, whereas the left knee served as an untreated control. Tendons which received the growth factors formed the Growth Factor Group (GFG), whereas tendons which served as controls served as the Control Group (CG). A total of 24 adult old male New Zealand white rabbits, weighing 3 to 3.5 kg, were used in this study after approval from the regional ethics board of Democritus University of Thrace. Institutional guidelines for the care and treatment of laboratory animals were adhered to. The rabbits were housed one per cage with food and water available *ad libitum*.

Growth factors preparation

Human recombinant forms of TGF-b1 and IGF-I (R&D Systems, Minneapolis, Minnesota, USA), at a dose of 4 ng and 25 μg respectively, were obtained and reconstituted according to the manufacturer's instructions. Determination of doses was based on previously published data (*5,8*). Fibrin sealant (Baxter, Deerfield, Illinois, USA) was used as a growth factor delivery vehicle (*5,7*).

Surgical procedure

The rabbits were anaesthetized with an intramuscular injection of xylazine (Rompun® Injectable, Bayer) at a dosage of 5-7 mg/kg and 0.15 mg of atropine (DEMO S.A.) ; 10-15 minutes later, ketamine (Imalgene®, Rhone Mérieux, France) at a dosage of 12-15 mg/kg was injected intramuscu1arly. During surgery, supplemental sedation was given as required. Local anaesthesia was applied at regular intervals at the site of incisions with 1 ml of a 2% lidocaine-adrenaline solution (AstraZeneca, UK).

The surgical procedure was performed according to the animal model described by Anaguchi *et al* (*1*). The skin of the right knee was shaved and the operation was performed under aseptic conditions. A longitudinal incision was then made in the skin overlying the middle of the patellar tendon. The superficial surrounding fascia was incised longitudinally to expose the patellar tendon. Thereafter, the deep fascia overlying the tendon was opened and a full thickness, 3 mm wide, and 10 mm long tendon substance was excised from the central portion of the patellar tendon with a specially designed knife with two stainless-steel surgical blades. The 3-mm width of the defect is approximately equal to one-third of the width of the tendon. Thereafter, the growth factors were mixed in a dish with 2 ml of fibrin sealant. The whole patellar tendon defect was then filled with the sealant. The sealant did not flow out from the defect because it was a gel. The overlying fascia was closed with a running suture of 4-0 nylon and the skin was closed with clips. The same procedure was performed in the left knee, with the application of 2 ml of pure sealant without the growth factors. No immobilization was applied after surgery, and the rabbits were allowed unrestricted daily activities in their cages.

Sample harvesting

After 2 or 6 weeks, the animals were sacrificed with an overdose of intracardiac injection of 10% KCl solution under general anaesthesia. Immediately after sacrifice, each complex, consisting of the patella, patellar tendon and proximal tibia was dissected free from other tissues. For mechanical testing, each hind limb was wrapped in gauze moistened with physiologic saline solution, covered with an airtight plastic film, and stored at -80° C until testing. Before mechanical testing, each limb was thawed overnight at 4° C. Biomechanical testing was performed using a materials testing machine (Instron, Canton, Massachusetts). The patella and the

tendon's tibial attachment were fixed in the clamps. Thus both ends of the tendon were fixated via their bone attachments, so that tendon sliding could be avoided. The tendon was pulled at a constant speed of 1 mm/s until failure. Peak force at failure, stiffness (the resistance of tendons to deformation by the applied force) and energy uptake to failure were recorded. Area and stress were calculated.

The tendons designated for histological examination were dissected free from other tissues and prepared with routine methods for paraffin sections. The specimens were sectioned transversely to the longitudinal direction of the tendon. From each tendon, 6 paraffin sections were stained with haematoxylin and eosin for the histological evaluation. All sections were analyzed by a single pathologist, who was blinded to the treatment groups.

Statistical analysis

All results are expressed as mean ± SD. Significant differences among groups were evaluated using the Mann-Whitney U test. Statistical significance was set at $p < 0.05$ (SPSS 12.0, Chicago, Illinois, USA).

RESULTS

Histological analysis

At 2 weeks after injury, the gap was bridged by a synovium-like tissue (fig 1) in both groups.

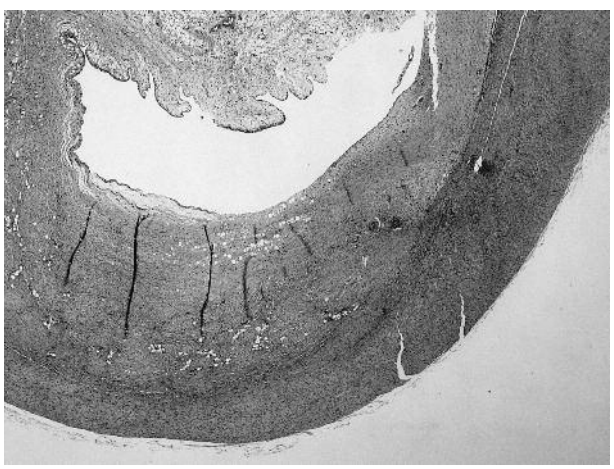

Fig. 1. — Patellar tendon from GFG group at 2 weeks. Low-power field (×20 magnification) showing thickening of the epitenon cell layer which fills the wounded site.

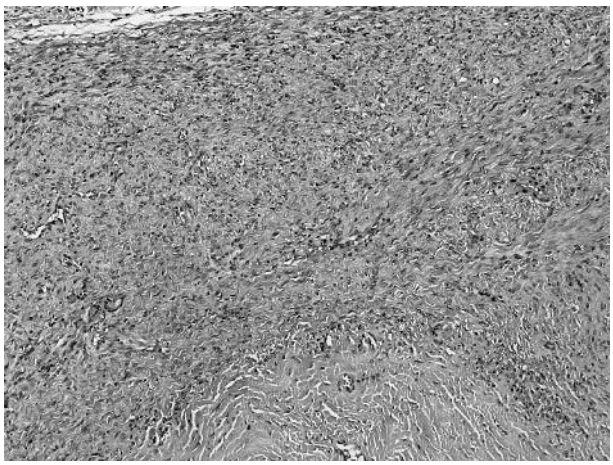

Fig. 2. — Patellar tendon from the CG group at 2 weeks (×200 magnification). Fibroblasts were randomly oriented, whereas an increased cellularity with changes in cell morphology was also demonstrated.

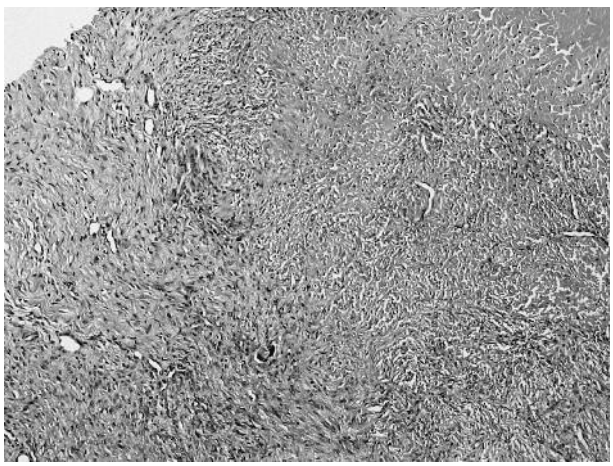

Fig. 3. — Patellar tendon from GFG group at 2nd week (×200 magnification). As in Fig. 3, the fibroblasts were also randomly oriented. Tenocytes were plump and a mix of spindle-shaped fibroblastic cells and mononuclear cells were also present. The number of vessels was increased in comparison with the controls.

Randomly oriented fibroblasts were also present. The differences between the 2 groups were the more plump shape of the tenocytes and the increased number of newly formed vessels in the Growth Factor group (GFG) (fig 2 & 3).

At 6 weeks, histostological evaluation revealed no notable differences between the 2 groups. There was no visible border between the healed site and

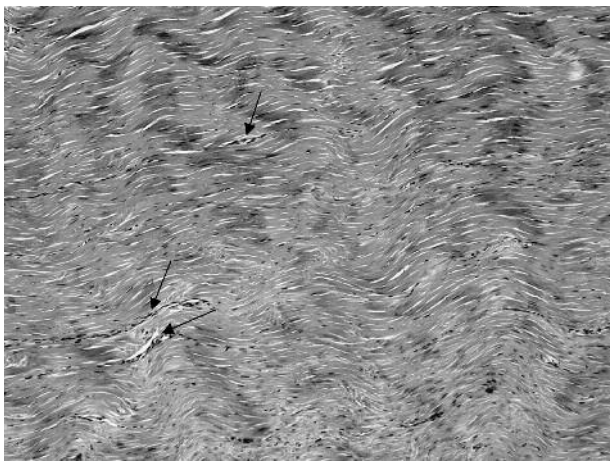

Fig. 4. — Patellar tendon from CG group at 6 weeks (×200 magnification). A healed tendon with fine architecture and rare vessels and inflammatory cells (arrows) was demonstrated at this time point.

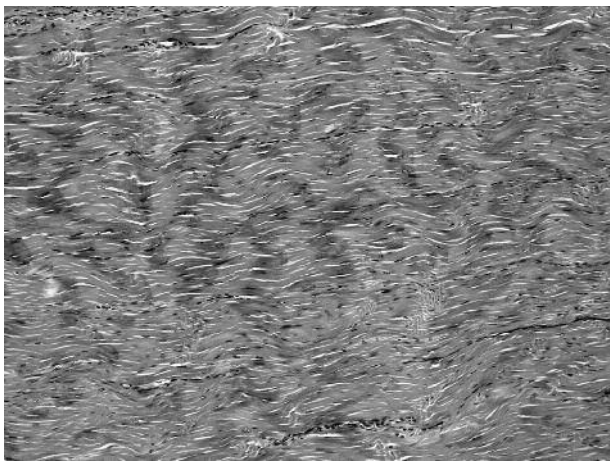

Fig. 5. — Patellar tendon from GFG group at 6nd week (×200 magnification). No differences were noted in comparison with controls at this time point, besides the slightly better orientation of the fibroblasts.

the proximal tendon in both groups. A fibrous repair tissue which was bridging the gap and an increased number of distinct oriented fibroblasts were demonstrated. A slightly better orientation of the repair tissue was noted in the GFG group. Poor vascularization was also demonstrated, indicating the completion of healing process (fig 4 & 5).

Biomechanical testing

The application of the growth factors resulted in a significantly increased force at failure, ultimate stress, stiffness, and energy uptake at 2 weeks in comparison with the controls ($p < 0.05$) (table I, fig 6). On the other hand, neither of the parameters

Table I. — Results of biomechanical evaluation at 2 weeks

BIOMECHANICAL DATA				
Parameters	**n**	**Mean**	**SD**	**p***
Force (N)**				
Growth Factor Group	10	144	4.56	< 0.001
Control Group	10	69	3.79	
Stiffness (N/mm)**				
Growth Factor Group	10	56	13.74	0.006
Control Group	10	34	11.56	
Energy (N m)**				
Growth Factor Group	10	362	17.28	< 0.001
Control Group	10	209	15.43	
Area (mm^2)				
Growth Factor Group	10	61.3	14.78	0.866
Control Group	10	58.8	12.29	
Stress (Mpa)**				
Growth Factor Group	10	3.5	0.43	< 0.001
Control Group	10	2.7	0.48	

*$p < 0.05$ indicates significant difference between the 2 groups.

**Force : Peak force at failure, Stiffness : The resistance of tendons to deformation by the applied force, Energy : Energy uptake to failure, Stress : The average amount of force exerted per unit area.

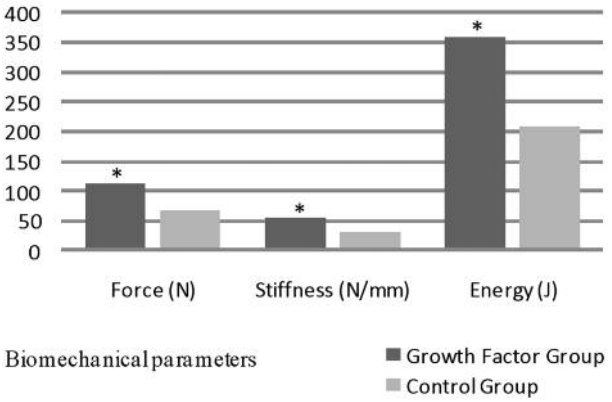

Fig. 6. — Biomechanical evaluation at 2nd week after injury. *indicates a significant difference between the 2 groups ($p < 0.05$).

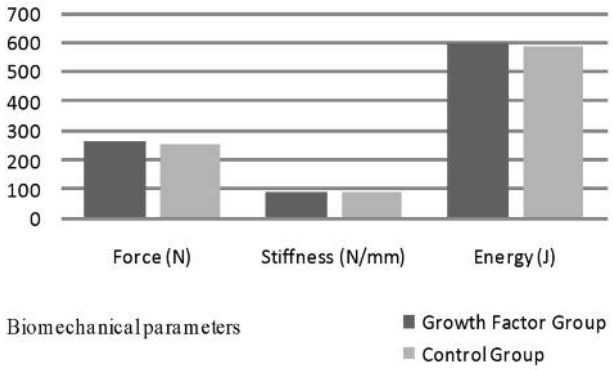

Fig. 7. — Biomechanical evaluation at 6th week after injury. *indicates a significant difference between the 2 groups ($p < 0.05$).

Table II. — Results of biomechanical evaluation at 6 weeks

BIOMECHANICAL DATA				
Parameters	**n**	**Mean**	**SD**	**p***
Force (N)**				
Growth Factor Group	10	265	32.91	0.23
Control Group	10	253	35.64	
Stiffness (N/mm)**				
Growth Factor Group	10	94	13.85	0.54
Control Group	10	92	11.53	
Energy (N m)**				
Growth Factor Group	10	601	19.39	0.30
Control Group	10	593	17.24	
Area (mm^2)				
Growth Factor Group	10	81	3.83	0.312
Control Group	10	80	3.95	
Stress (MPa)**				
Growth Factor Group	10	4.1	0.34	0.088
Control Group	10	3.9	0.31	

*$p < 0.05$ indicates significant difference between the two groups.

**Force : Peak force at failure, Stiffness : The resistance of tendons to deformation by the applied force, Energy : Energy uptake to failure, Stress : The average amount of force exerted per unit area

revealed any significant difference between the two groups at 6 weeks ($p > 0.05$) (table II, fig 7).

DISCUSSION

In this study, the combined administration of TGF-b1 and IGF-I via a fibrin sealant delivery tool in a patellar tendon defect model resulted in enhanced mechanical properties of the healed tendon 2 weeks after injury, whereas no influence was demonstrated at 6 weeks. To our knowledge, this is the first study that has used the particular combination of growth factors in a tendon defect model.

Previously published studies have demonstrated the influence of growth factors such as TGF-b1, IGF-I, basic Fibroblast Growth Factor (bFGF), Platelet Derived Growth Factor (PDGF), and Epidermal Growth Factor (EGF) on wound heal-

ing (*2,9,11,12,15,16*). Their ability to promote fibroblast proliferation and migration as well as the production of extracellular matrix proteins offers the possibility to use these substances to accelerate healing of other soft tissues such as the connective tissue (*24*).

The influence of the administration of exogenous TGF-b1 or IGF-I on the mechanical properties of tendons and ligaments has been demonstrated in previous studies. Anagushi *et al* reported that injection of 5 ng TGF-b1 in a patellar tendon defect model in rabbits resulted in a significantly greater tangent modulus and tensile strength of the regenerated tissue at 6 weeks after injury. Tangent modulus is the ratio of the change in stress to the corresponding change in strain (the slope of the stress-strain curve) at a specified point on the curve (*1*). Conversely, Hildebrand *et al* demonstrated that adding TGF-b1 via fibrin sealant in a ruptured medial collateral ligament, did not lead to any further increase in the structural properties in comparison with treatment with PDGF-BB at 6 weeks after injury (*5*). In other studies, administration of TGF-b1 via fibrin sealant in autografts used in ACL reconstruction, revealed a positive influence on tensile strength and bonding strength 3 and 12 weeks postoperatively (*14,20,23*). Finally, concerning IGF-I, Kurtz *et al* reported that administration of 25 µg IGF-I via fibrin sealant in transected Achilles tendons resulted in a significantly greater stiffness and maximum load to failure at 12 weeks after injury (*8*).

Overall, our findings diverge from those in the above mentioned studies. No differences were noted in our study 6 weeks after injury, whereas other studies that used the same dose of TGF-b1 and IGF-I, and the same delivery method demonstrated superior mechanical properties of the groups treated with growth factors after 6 and 12 weeks (*1, 8,14,23*). Surprisingly, we reported superior mechanical properties of the healed tendons treated with growth factors at 2 weeks after injury. We believe that this is an important finding, because in clinical practice a stronger tendon in an earlier phase of healing could allow safe mobilization.

There are some limitations in the present study. The first limitation is that we did not perform immunohistochemical staining of the growth factors to demonstrate how long the growth factors had been present *in situ.* This could possibly explain the differences between the findings at 2 weeks and at 6 weeks. The second limitation is that we did not perform immunohistochemical analysis of collagen type I and III to evaluate the quality of the healed tendons.

It is difficult to directly infer clinical conclusions from this experimental study. Beyond the limitations, however, we believe that this study provides valuable information on the effect of the two growth factors in this patellar tendon defect model. Due to the fact that the healing process includes a cascade of interactions between many growth factors, further studies are needed to determine the doses and the delivery methods of growth factors and establish a therapeutic approach for tendon healing enhancement.

REFERENCES

1. **Anaguchi Y, Yasuda K, Majima T *et al.*** The effect of transforming growth factor-beta on mechanical properties of the fibrous tissue regenerated in the patellar tendon after resecting the central portion. *Clin Biomech* 2005 ; 20 : 959-965.
2. **Bartold PM, Raben A.** Growth factor modulation of fibroblasts in simulated wound healing. *J Periodontal Res* 1996 ; 31 : 205-216.
3. **Frank C, McDonald D, Shrive N.** Collagen fibril diameters in the rabbit medial collateral ligament scar : a longer term assessment. *Conn Tiss Res* 1997 ; 36 : 261-269.
4. **Gomez M.** *Rehabilitation of the Injured Knee*. 2nd ed, Mosby Company, St. Louis, 1995, pp 34-44.
5. **Hildebrand K, Woo SL-Y, Smith DW *et al.*** The effects of Platelet-Derived Growth Factor-BB on healing of the rabbit medial collateral ligament. *Am J Sports Med* 1998 ; 26 : 549-554.
6. **Klein MB, Yalamanchi N, Pham H *et al.*** Flexor tendon healing in vitro : Effects of TGF-beta on tendon cell collagen production. *J Hand Surg* 2002 ; 27-A : 615-620.
7. **Kondo E, Yasuda K, Yamanaka M *et al.*** Effects of administration of exogenous growth factors on biomechanical properties of the elongation-type anterior cruciate ligament injury with partial laceration. *Am J Sports Med* 2005 ; 33 : 188-196.
8. **Kurtz CA, Loebig TG, Anderson DD *et al.*** Insulin-like growth factor I accelerates functional recovery from Achilles tendon injury in a rat model. *Am J Sports Med* 1999 ; 27 : 363-369.

9. **Lepisto J, Laato M, Niinikoski J *et al.*** Effects of homodimeric isoforms of platelet-derived growth factor (PDGF-AA and PDGF-BB) on wound healing in rat. *J Surg Res* 1992 ; 53 : 596-601.
10. **Lynch SE, Nixon JC, Colvin RB *et al.*** Role of platelet-derived growth factor in wound healing : synergistic effects with other growth factors. *Proc Natl Acad Sci USA* 1987 ; 84 : 696-700.
11. **Lynch SE, de Castilla GR, Williams RC *et al.*** The effects of short-term application of a combination of platelet-derived and insulin-like growth factors on periodontal wound healing. *J Periodontol* 1991 ; 62 : 458-467.
12. **McGee GS, Davidson JM, Buckley A *et al.*** Recombinant basic fibroblast growth factor accelerates wound healing. *J Surg Res* 1988 ; 45 : 145-153.
13. **Mueller R, Spencer E, Sommer A *et al.*** *Modern Concepts of Insulin-like Growth Factors.* EM Spencer, New York, 1991, pp 185-192.
14. **Nagumo A, Yasuda K, Numazaki H *et al.*** Effects of separate application of three growth factors (TGF-beta1, EGF, and PDGF-BB) on mechanical properties of the in situ frozen-thawed anterior cruciate ligament. *Clin Biomech (Bristol, Avon*) 2005 ; 20 : 283-290.
15. **Pierce GF, Mustoe TA, Lingelbach J *et al.*** Platelet-derived growth factor and transforming growth factor-beta enhance tissue repair activities by unique mechanisms. *J Cell Biol* 1989 ; 109 : 429-440.
16. **Pierce GF, Vande Berg J, Rudolph R *et al.*** Platelet-derived growth factor-BB and transforming growth factor beta 1 selectively modulate glycosaminoglycans, collagen, and myofibroblasts in excisional wounds. *Am J Pathol* 1991 ; 138 : 629-646.
17. **Robbins JR, Evanko SP, Vogel KG.** Mechanical loading and TGF beta regulate proteoglycan synthesis in tendon. *Arch Biochem Biophys* 1997 ; 342 : 203-211.
18. **Saed GM, Zhang W, Chegini N *et al.*** Alteration of type I and III collagen expression in human peritoneal mesothelial cells in response to hypoxia and transforming growth factor-beta1. *Wound Repair Regen* 1999 ; 7 : 504-510.
19. **Saika S, Yamanaka O, Kawashima Y *et al.*** OPC-15161 suppresses the proliferation of Tenon's capsule fibroblasts and the production of Type I collagen and fibronectin stimulated by TGF-beta 1 in vitro. *Curr Eye Res* 1998 ; 17 : 933-940.
20. **Sakai T, Yasuda K, Tohyama H *et al.*** Effects of combined administration of transforming growth factor-beta1 and epidermal growth factor on properties of the in situ frozen anterior cruciate ligament in rabbits. *J Orthop Res* 2002 ; 20 : 1345-1351.
21. **Spindler KP, Imro AK, Mayes CE *et al.*** Patellar tendon and anterior cruciate ligament have different mitogenic responses to platelet-derived growth factor and transforming growth factor beta. *J Orthop Res* 1996 ; 14 : 542-546.
22. **Steenfos H, Spencer E, Hunt T.** Insulin-like growth factor has a major role in wound healing. *Surg Forum* 1989 ; 40 : 68-70.
23. **Yamazaki S, Yasuda K, Tomita F *et al.*** The effect of transforming growth factor-beta1 on intraosseous healing of flexor tendon autograft replacement of anterior cruciate ligament in dogs. *Arthroscopy* 2005 ; 21 : 1034-1041.
24. **Woo SL, Hildebrand K, Watanabe N *et al.*** Tissue engineering of ligament and tendon healing. *Clin Orthop Relat Res* 1999 ; 367 suppl : S312-S323.