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The Different Effects of TGF-β1, VEGF and PDGF on the Remodeling of Anterior Cruciate Ligament Graft

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1. Introduction

Rupture of anterior cruciate ligament (ACL) is a common knee trauma which leads to anterior knee instability. The ACL has long been thought to have poor capacity for healing with suture repair. ACL reconstruction is the standard of care which can restore the knee stability of ACL deficit knee. Although ACL reconstruction is an excellent operation for restoring the sagittal plane stability of the knee, significant problems remain. Studies have demonstrated that remodeling of the graft usually takes longer than expected, and many patients develop arthritis after an ACL tear, even if they have a reconstruction, with rates as high as 78% reported at 14 years post-operatively (1).

In ACL reconstruction, inflammation and necrosis of the graft can occur immediately after transplantation. The graft then undergoes revascularization and cellular repopulation from an extrinsic origin, followed by a remodeling period. In the early phase, the properties of the grafted tendon are deteriorated and do not recover to physiological levels even at 18 months after surgery (2). Therefore, the main goals of ACL reconstruction are to prevent the deterioration of grafted tendon and accelerate mechanical restoration of the deteriorated graft. New solutions are needed to accelerate and improve remodeling of tendon grafts.

The ACL graft remodeling process is regulated by a complex growth factor network. Many growth factors have been evaluated for their ability to stimulate tendon and ligament healing in vitro and in vivo. In enhancing ligament repair, the most interesting candidates are insulin-like growth factor-I (IGF-I), transforming growth factor β1 (TGFβ1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), and basic fibroblast growth factor (bFGF). PDGF and VEGF appear in the early stage of reconstruction, during the stage of graft maturing, the granular tissue becomes ligament-like tissue and both PDGF and VEGF are not able to be detected gradually, as the replacement, TGFβ1and other cytokines are involving in the reconstruction process to mature the ligament. TGFβ1 is the main cytokine for the maturing of ligament. Studies have

demonstrated improved cellular proliferation and migration as well as increased collagen production rates with the addition of growth factors.

2. VEGF

Angiogenesis is an essential step in the process of tendon healing and tendon graft remodeling, in which neovascularization prompts delivery of inflammatory cells, fibroblasts and growth factors to the wound site. VEGF plays an essential role in angiogenesis, regulating the activation, migration, and proliferation of endothelial cells in various pathological conditions. It is well-known that angiogenesis is an essential step in the healing process of tendon and tendon graft remodeling. Two studies found that VEGF therapy can enhance revascularization in the graft, as well as promoting the infiltration of fibroblasts after ACL reconstruction; however, it did not affect the mechanical properties of the in situ freeze-thawed ACL model (3). Furthermore, it decreased the stiffness of the grafted tendon with increased knee laxity, 12 weeks after ACL reconstruction in a sheep model (4). Yoshikawa et al. examined the effects of an application of VEGF to a hamstring graft in a sheep ACL reconstruction model and demonstrated that the linear stiffness of the VEGF-treated graft was significantly lower than that of the PBS-treated group at 12 weeks (4). In this study, the stiffness of the VEGF165 group was higher than those of the control group at 12 weeks, although there was no significant difference ($P > 0.05$). There are two possible reasons for this difference.

In our study, the stiffness of the VEGF165 group was higher than those of the control group at 12 weeks, although there was no significant difference (5). There are two possible reasons for this difference. One is the method of applying this factor. In our study, the method was transfer of the VEGF165 gene, rather than the gene products. The secretion time and quantity of VEGF165 may be suitable for ACL graft remolding. The other possible reason is the use of BMSCs in our experiment. BMSCs have the capacity to differentiate into various mesenchymal lineages, including ligament, tendon, muscle, bone, cartilage and adipose tissue. Kanaya et al. reported that the intra-articular injection of BMSCs could accelerate the healing of partially torn knee ACLs in a rat model (6). Our study also demonstrated that the application of BMSCs is effective in enhancing collagen deposition and changing certain structural properties for improving tendon allograft remodeling after ACL reconstruction in a rabbit model (7). The exact reasons for the better healing observed in injured ACLs using BMSCs are uncertain. One possibility is that BMSCs directly differentiated into ligament fibroblasts within the healing environment. Alternatively, another possibility is that injected mesenchymal stromal cells directly contribute as a cellular source for ligament healing. BMSCs may also secrete a variety of growth factors, which may contribute to the activation and recruitment of local fibroblast precursors or enhance extracellular matrix synthesis.

3. PDGF

Many studies have also attempted to determine the effects of PDGF-BB on a ligament engineering system. It has been demonstrated that it promoted fibroblast proliferation, matrix synthesis, neovascularization, and mechanical properties. In our studies in the gene-transfected group, more cells and blood vessels could be found at 3 weeks, and more collagen was synthesized in the ACL as compared with the control and MSCs group (7).

Similar to our findings were the results of a study by Nakamuraetal, which described an increased vascularity and enhanced collagen deposition in the wound of a patellar ligament afte rPDGF-B gene transfer in rats (8).

The mechanisms by which PDGF affects ligament healing are complex. Kuroda et al. studied immunohistochemically the presence and the level of bFGF, TGF-ß, PDGFAA and PDGF-BB expression in a model of ACL reconstruction using a free patellar tendon autograft (9). They found that all tested growth factors were upregulated with a maximum expression at 3 weeks and up to 60% of all cells were stained PDGF positive. PDGF is one of the most effective growth factors during tendon graft remodeling.

Tendon grafts are exposed to the reduced PO2 of the intra-articular environment, Petersen et al. (10) showed that PDGF as well as hypoxia strongly enhanced VEGF secretion from tenocytes. Besides this VEGF-mediated angiogenetic effect, PDGF further more induces the synthesis of other growth factors, including IGF, and regulates the presence of other receptors (11). Therefore, it could be concluded that the expression of PDGF-BB by the small number of transfected cells may activate a cascade of PDGF-BB throughout the wound. Some researches (12) showed that there are some growth factors receptors such as PDGFR on the surface of MSCs. MSCs from bone marrow might have a better response to PDGF as compared with those from the ACL or MCL regarding proliferation and migration. Thus, it might be suggested that a tendon graft seeded with MSCs-PDGF-BB is more likely to promote MSCs or fibroblast proliferation and migration and accelerate graft tissue remodeling.

The observations of Kuroda et al. also imply that if a growth factor is administered to the tendon graft, its tissue concentration should be highest around the third week to enhance the effect of the other intrinsical growth factors (9). In our study at 3 weeks, we found significantly higher vascularity in the PDGF-transfected grafts. The concentration of PDGF-BB inarticular fluid from gene-transfected rabbit increased at 3 week, got to the highest point at 6 week and then dropped down. This rapid reduction in the level of their localization indicates that once the extrinsic cells infiltrate to the graft and revascularization is complete, these growth factors may have less significance for subsequent remodeling. Therefore, it is very important to find the appropriate time point for the administration of growth factors to promote healing.

In vivo study by Hildebrand et al. demonstrated that the improvements in the MCL structural properties were dose-dependent to PDGF-BB (13).That is, a higher dose of PDGF-BB improved more structural properties of the femur–MCL–tibia complex than a lower dose of PDGF-BB did. In both the MSCs group and gene-transfected group, although there are much more differences in morphology at the early stages, the structure of the ACL in the two groups have less significant differences at the later stages, especially at 12 weeks. For an ACL reconstruction model, we don't know if the dosage we used was appropriate to maximally enhance graft remodeling, or this indicates that the growth fact or PDGF may have early effect on the ACL reconstruction and have less significance for subsequent remodeling. It is essential to find an appropriate dosage for ACL reconstruction.

4. TGFβ1

Many studies indicate that TGFβ1 plays a key role in the healing process of ligaments. TGFβ1 increased both collagen and non-collagenous protein synthesis by the introduction of

ACL fibroblasts (14, 15). Furthermore, it has also been shown to increase ACL fibroblast proliferation. TGFβ1 enhanced graft remolding in ACL reconstruction by inhibiting mechanical deterioration (16).

In situ freeze-thawed ACL tissues, which were established as an autograft model, transfected with TGFβ1 and EGF, significantly reduced the increase in water content and cross-sectional area and reduced the deterioration of the ACL (17). However, the results were dose- and time-dependent, and of high cost. Furthermore, the clinical application of growth factors is hampered by delivery problems. Amiel et al. reported that a high dose of TGFβ1 inhibits proliferation of rabbit ACL fibroblasts (18). One study reported that an application of low-dose TGFβ1 mixed with fibrin sealant enhanced the remodeling process of the in situ freeze-thawed ACL, whereas a high dose of TGFβ1 had no effect (17). We demonstrated that TGFβ1 gene-modified BMSCs implanted within the Achilles allograft in ACL reconstruction significantly affects the biomechanical properties of the graft. Our results suggest that the transfer of the TGFβ1 gene, rather than the gene products, may be the most expeditious method of harnessing this factor for the purposes of accelerating ACL graft remolding (5).

Our results showed that the ultimate failure load and the stiffness of the grafted tendon were significantly increased in the TGFβ1 treatment group compared with the VEGF165 and control groups at 6, 12 and 24 weeks after surgery. Meanwhile toluidine blue staining of the grafts appeared positive only in the TGFβ1 group at 12 weeks. ACL has different histological characteristics from medical collateral ligament (MCL), which is more cartilage-like in nature. The normal ACL is positive to toluidine blue staining, so we hypothesized that TGFβ1 may promote early maturation of the graft. Histological examination and immunohistochemistry showed significantly enhanced cell infiltration and revascularization at 3, 6, and 12 weeks in the VEGF165 and the TGFβ1/VEGF165 groups. However, VEGF165 cDNA-transduced BMSCs exhibited no significant effects on the mechanical properties of the ACL graft, while TGFβ1/VEGF165 cDNA-transduced BMSCs showed the best biomechanical properties of the ACL graft at 24 weeks after surgery. This work shows that the use of TGFβ1 and the co-expression of TGFβ1/VEGF165 of gene therapy may be useful for accelerating the remolding of the graft after ACL reconstruction.

Previous study by Lee et al. demonstrated that VEGF and TGFβ1 were both expressed in ACL healing (18). We demonstrate here that the combination of TGFβ1 and VEGF165 gene has a synergistic effect on accelerating the remolding of the ACL graft, but few studies have defined the exact regulating mechanisms of these two growth factors during ligament healing. Several studies have demonstrated that together VEGF and TGFβ1 can enhance each the synthesis of one another (19, 20). These studies revealed that the mechanism of synergy is probably mediated through Smad3, HIF-1α/β, Smad3/4, PI3K/Akt or ERK1/2 signaling pathways.

5. The Effects of TGF-β1, VEGF165 transfer on achilles tendon healing

The Achilles tendon itself is a dense, regular connective tissue consisting primarily of type I collagen and interspersed specializedmesenchymal cells (tenocytes) responsible for the maintenance of collagen structure. With respect to reconstruction of the tendon, there are three key factors which must be addressed: the cell, the fiber, and their arrangement. The

properties of the tendon matrix and its regulation by growth factors are largely uncharacterized. We examined the effects of implantation of BMSCs transduced with either the TGF-β1, VEGF165, or both on experimentally injured Achilles tendons in a rabbit model in vivo (21).

The maximum failure load, the tendon stiffness, and the elastic modular of the healing tendons were significantly increased in the TGF-β1 and the co-expression treatment groups compared with the other treatment groups at one, two, four, and eight weeks after surgery. Moreover, there was evidence of accelerated remodeling of the lesion in response to TGF-β1 and co-expression TGF-β1/VEGF165 treatment, while the size of the ruptured callus was increased in the presence of VEGF165. Histological examination showed a much more organized and homogeneous pattern of collagen fibers at all time points in the lesions of the TGF-β1 and the co-expression treatment groups. Both single fibrils and the collagen fibers had a greater diameter, with a higher degree of collagen crimp than the collagen of the other treatment groups. This was confirmed by Sirius red staining in conjunction with polarized light microscopy, which showed a higher shift of small yellow-green fibers to strong yellow-orange fibers after two, four, and eight weeks in the TGF-β1 and the co-expression treatment groups. Immunohistochemistry showed more vessels after one, two, four, and eight weeks in the VEGF165 treatment group, but only a few vessels in the co-expression treatment group. There was also an earlier shift from fibroblasts to fibrocytes within the healing tendon, with fewer fat cells present in the tendons of the TGF-β1 and the co-expression treatment groups compared with intact tendon. Thus treatment with TGF-β1 transduced BMSCs resulted in a promising acceleration and improvement of tendon healing, particularly influencing early tissue regeneration, leading to quicker recovery and improved biomechanical properties of the Achilles tendon. However, VEGF165 transduced BMSCs exhibited a negative role. The angiogenesis effects of VEGF165 were diminished by TGF-β1, while the collagen synthesis effects of TGF-β1 were only slightly affected by VEGF165. The TGF-β1 and VEGF165 synthesized of BMSCs with gene transfer were evaluated by ELISA. However, the stimulation effect is very weak with heterologous gene transfer in contrast with homologous gene transfer. In terms of the fibroblasts, the mRNA expression of Collagen type I, type III and Fibronectin were evaluated. All showed significant increasing under the stimulation of TGF-β1 or TGF-β1/VEGF165 co-existing. Nevertheless, the fibroblasts stimulated by VEGF165 showed no difference with control.

We demonstrated that gene modified BMSCs implanted within the tendon-repair site contributed to early tendon-healing following primary repair, and that growth factor signaling can regulate the mechanical properties of the tendon matrix by affecting tendon mass and architecture. These data provide a basis for future application of supplementary therapy of surgery for tendon and ligaments. Future studies determining the optimal growth factor(s), dose, and timing and site of administration are required, along with the interactions between different growth factors.

6. Summary

The effects of TGF-β1, VEGF and PDGF on the remodeling of anterior cruciate ligament graft are different. PDGF, VEGF and TGFβ1transfected MSCs accelerated cellular infiltration

and enhanced collagen deposition in the graft. PDGF and VEGF promoted the angiogenesis of the graft, but did not show any improvement of the biomechanical nature of the graft. Only TGFβ1accerated the maturing of graft and improved the biomehchanical nature. TGFβ1 co–expression with VEGF165 in gene-transfected BMSCs could accelerate the remodeling of the reconstructed ligament. The cross-talk between TGFβ1 and VEGF165 has positive consequences, with TGFβ1/VEGF165-gene-transfected BMSCs could significantly promote angiogenesis of the reconstructed ligament, while achieving the best mechanical properties of the reconstructed ligament. However, the molecular mechanism that regulates these two growth factors during ligament healing still needs to be fully elucidated. The effects of other growth factors on the remodeling of ACL graft, such as IGF-1, bFGF, need further study.

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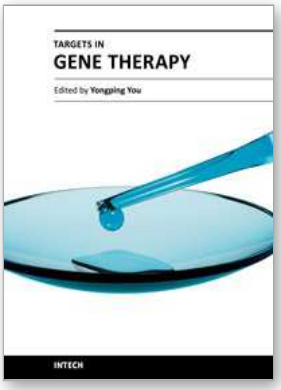

Targets in Gene Therapy

Edited by Prof. Yongping You

ISBN 978-953-307-540-2
Hard cover, 436 pages
Publisher InTech
Published online 23, August, 2011
Published in print edition August, 2011

This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Changlong Yu, Lin Lin and Xuelei Wei (2011). The Different Effects of TGF-β1, VEGF and PDGF on the Remodeling of Anterior Cruciate Ligament Graft, Targets in Gene Therapy, Prof. Yongping You (Ed.), ISBN: 978-953-307-540-2, InTech, Available from: http://www.intechopen.com/books/targets-in-gene-therapy/the-different-effects-of-tgf-1-vegf-and-pdgf-on-the-remodeling-of-anterior-cruciate-ligament-graft

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