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ACL reconstruction in a rabbit model using irradiated Achilles allograft seeded with mesenchymal stem cells or PDGF-B gene-transfected mesenchymal stem cells

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Abstract The present study was conducted to develop a new strategy to accelerate reconstruction of the anterior cruciate ligament (ACL) by modifying the Achilles allograft with autogenous mesenchymal stem cells (MSCs) or PDGF-B transfected MSCs in a rabbit model. The allografts were first irradiated with Co^{60}, stored at –80°C, and then seeded with cells for implantation. Bilateral ACL reconstructions were performed. On the left, the allograft was either seeded with MSCs or PDGF-B transfected MSCs and acted as the experimental group. On the right, the graft without any cells seeded acted as control. At 3, 6 and 12 weeks after surgery, histological observation found that implatation of MSCs or PDGF-B transfected MSCs accelerated cellular infiltration into the ACL and enhanced collagen deposition in the wound. PDGF-B transfected MSCs could also lead to an initial promotion of angiogenesis. This gene transfer technique or cell implantion may be a potentially useful tool for improving ligament remodeling.

Keywords Mesenchymal stem cells · Allograft · Anterior cruciate ligament · PDGF-BB · Retrovirus

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Introduction

Anterior cruciate ligament (ACL) injuries are common, and surgical reconstruction is desirable for patients who participate in vigorous sports activities. There are two kinds of biological substitutes, autograft and allograft, which are commonly used for ligament reconstruction. The use of allogeneic tendons for ligament reconstruction has many advantages [22], including the retention of normal tissues, wider choice of graft size, and reduction in surgical and anesthetic time. In our study, we used Achilles allograft for ACL reconstruction.

Tendon grafts which stay in an intra-articular synovial environment proceed through some phases of biologic incorporation, such as necrosis, angiogenesis, cell repopulation and final maturation. 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. Therefore, we tried to develop a new strategy by enhancing angiogenesis to accelerate the remodeling of tendon graft.

With the application of knowledge gained from basic science and clinical research, we tried to use autogenous mesenchymal stem cells (MSCs) and PDGF-B gene to modify the allograft to accelerate angiogenesis. MSCs are pluripotential cells that are being investigated extensively in a wide variety of settings, including enhancement of healing of tendon injuries [2, 3, 30, 33]. PDGF-BB has been shown to play essential roles in wound healing. Recent investigations [7] suggest that PDGF-BB mediates many processes required for tissue repair, including chemotaxis (monocytes, neutrophils, fibroblast), proliferation (fibroblasts, smooth muscle cells, capillary endothelial cells), induction of several matrix molecules (fibronectin,

hyaluronic acid) and the production and secretion of collagenase by fibroblasts. Furthermore, in vitro and in vivo [4, 8, 12, 14, 16, 25, 27] application of PDGF to the tendon cells and ligament has been demonstrated to enhance the proliferation of fibroblasts and deposition of extracellular matrix.

Based on those findings, we investigated the biological effect of the introduction of PDGF-B gene and MSCs on the healing process of the rabbit anterior cruciate ligament.

Materials and methods

Experimental design

Bilateral ACL reconstructions with Achilles allografts were performed by a single surgeon in 36 skeletally mature female New Zealand white rabbits weighing 2.0–2.5 kg each. Autologous bone marrow-derived MSCs were harvested 3–5 weeks before ligament reconstruction surgery. During bilateral ACL reconstruction, on the left knee, the allograft was either seeded with MSCs or PDGF-B transfected MSCs and acted as the treatment group; on the right, the graft without any cells seeded acted as control. The animals were euthanized at 3, 6 and 12 weeks postoperatively for histologic analysis. At each time they were killed, 12 animals (six for MSCs group and six for MSCs-PDGF-BB group) were used for analysis. A further two rabbits were used as normal control. The experimental protocol, animal care and use procedures were in accordance with the policies of Peking University Health Science Center and the National Institute of Health.

Construction of retroviral vector

The human PDGF-B fragment was cut from psv7d-PDGF-B plasmid (kindly provided by Christer Betsholtz, Institute of Medical Biochemistry, University of Gothenburg, Sweden) and inserted into a retrovirus vector PLXRN (Clontech, USA) to get PLXRN-PDGF-B. The Rous sarcoma virus promoter controls expression of the Neo^r gene.

Production of retroviral vector particles

The PLXRN-PDGF-B plasmids were used to transfect PT-67 cells (Clontech, USA) to get retroviral particles. Clonal amphotropic packaging cells were derived as described in Clontech protocol. The retrovirus supernatants were filtered through a 0.45 μm syringe filter (Sartorius, German) and stored at –80°C before use.

Preparation of MSCs

Bone marrow samples were collected from the iliac crest of New Zealand white rabbits by adopting previously published methods [3, 5]. A volume of 1 ml of bone marrow was aspirated and put in complete Dulbecco's Modified Eagle Medium low glucose (DMEM-lg, Gibco, USA) containing 10% fetal bovine serum (FBS), penicillin 100 μg/ml, streptomycin 100 μg/ml (pH 7.35). Samples were washed twice with DMEM-lg and centrifuged at 2,000 rpm for 6 min. The supernatant was discarded and the precipitating cell pellet was suspended in 10 ml of MSCs growth media. Cells were counted and then plated at 17×10^6 to 22×10^6 cells per 100 mm dish. MSCs adhered to the plates and proliferated to form colonies between 5 and 8 days into primary culture. Once the colonies of MSCs reached confluency, the adherent MSCs were retrieved and subcultured again.

Transduction of cells

MSCs of Passage 1 were plated in six-well dishes. When the cells reached 50% confluence, transductions were performed by adding 1.6 ml fresh DMEM-lg, supplemented with 8μg/ml polybrene (Sigma, USA) to each well. A total of 400 μl retrovirus supernatant was added to the cells and they were incubated overnight at 37°C, the following morning the medium was replaced with 2 ml fresh DMEM-lg supplemented with 200μg/ml G418. After 10–14 days of selection in G418, the clonal cells (MSCs-PDGF-BB) were formed. They were trypsinized, transferred to T-75 flasks and cultured in DMEM-lg. When the cells became confluent, they were seeded onto graft for later implantation.

Graft preservation

A total of 36 Achilles tendons were harvested from donor New Zealand white rabbits and used as grafts. Each tendon was separated into at least two bundles of ligament and each was cut 2 mm wide and 3 cm long. Then these two ligaments were used in the ACL reconstruction of one animal. All the grafts were enclosed in tubes and placed in dry ice in a container for gamma irradiation by Co^{60}. The exposure time of 9 h yielded a calculated dose of 2.5 Mrad. Then the tendon graft was stored at –80°C for 2 to 5 weeks before use.

Seeding MSCs or MSCs-PDGF-BB on the Achilles graft

At the time of transplantation, the frozen grafts were thawed in saline gauze at room temperature. In the

experiment group, MSCs or MSCs-PDGF-BB were embedded in fibrin glue (Beixiu Company, China) by mixing the cell pellet with the fibrin part and then mixing this solution with the thrombin part. The final concentration was approximately 1.5×10^6 cells/ml. Of this mixture, a 0.5 ml sample was placed in an Achilles graft before it became gel and allowed to gel in a culture dish in a CO_2 incubator for 10 min at 37°C. In the control group, 0.5 ml of fibrin glue without cells was incubated in the same manner. The graft was washed gently three times in PBS solution, 20 min before implantation.

Surgical protocol

Bilateral ACL reconstructions were performed. A lateral parapatellar arthrotomy was used to expose the knee joint. The native ACL was divided and tibial and femoral tunnels were created with a 2 mm drill. The combined length of the tunnel from the anterior femoral surface, across the joint, to the anterior tibial surface was recorded. The graft was then inserted via the holding suture using a Beath pin from the tibial and femoral tunnels at the same time slowly. We tethered each end of the graft with the suture to a screw inserted into the bone, manually applying minimal graft tension in a direction aligned with the long axis of the bone tunnel with the knee flexed at 90°. The gap between the tunnel and the graft was filled with an additional 100μl of fibrin glue. Then the wound was closed in layers. After surgery, all the animals were allowed to move freely in their cages.

Analysis of PDGF-B mRNA and protein production

RT-PCR

Total RNA was extracted from confluent tissue culture flasks of MSCs or MSCs- PDGF-BB using Trizol (Invitrogen, USA), according to the manufacturer's instructions. 5-TCTGCTGCTACCTGCGTCTG-3 and 5-GCGTTGGT GCGGTCTATG-3 were used as primers for RT-PCR. Obtained PCR products were separated on 1% agarose gels and stained with ethidium bromide.

Arthrosis fluid puncture

Number 7 needles were introduced from the superior aspect into the upper compartment of the joint cavity of the knee. The knee joints on both sides were perfused with 1 ml saline and the perfusate samples were collected. Then they were centrifuged at 6,000 rpm at 4°C for 10 min to remove cells and other debris. Thereafter, the supernatant was transferred to another tube and was immediately frozen at –80°C for ELISA.

ELISA

The level of human PDGF-BB in cell culture medium and lavaged fluid were measured with ELISA kits (mouse anti-hPDGF neutralizing antibody, R&D Systems, USA) according to the manufacturer's instructions.

Histologic analysis

The femur and tibia were removed; the knee joints were left intact, and the specimens fixed in 4% paraformaldehyde. The samples of ACL were embedded in paraffin and cut into 5μm thick sections longitudinal to the bony tunnels. The slides were stained using hematoxylin and eosin, toluidine blue staining and immunohistochemical stain for collagens type I (Sigma, USA) and III (Oncogene, USA) and PDGF-BB (rabbit anti-serum, Lab Vision Corporation, USA). Four knees from two rabbits with normal ACLs were harvested and prepared in a similar manner for histologic analysis.

Results

RT-PCR

As described in Materials and methods, the MSCs-PDGF-BB contain the entire coding sequence of human PDGF-BB, while the MSCs do not (Fig. 1).

ELISA

MSCs-PDGF-BB were found to secrete PDGF-BB at a steady-state level of 1,100 ng/10^6cells/24 h, whereas the MSCs did not. The concentration of PDGF-BB from the perfusion of joint cavity in the gene treatment group is as shown in Table 1

Gross observations

The intra-articular knee gross examination showed intact grafts in all cases.

Histologic findings in the 3-week group

HE staining showed that 3 weeks after surgery, in the control group, host cells had already invaded into the graft and most of them were inflammatory cells; while in the graft seeded with MSCs, granulation was formed and vascularization could be observed. In the gene-transfected group, significantly more cells and hypervascularity could be found (Fig. 2).

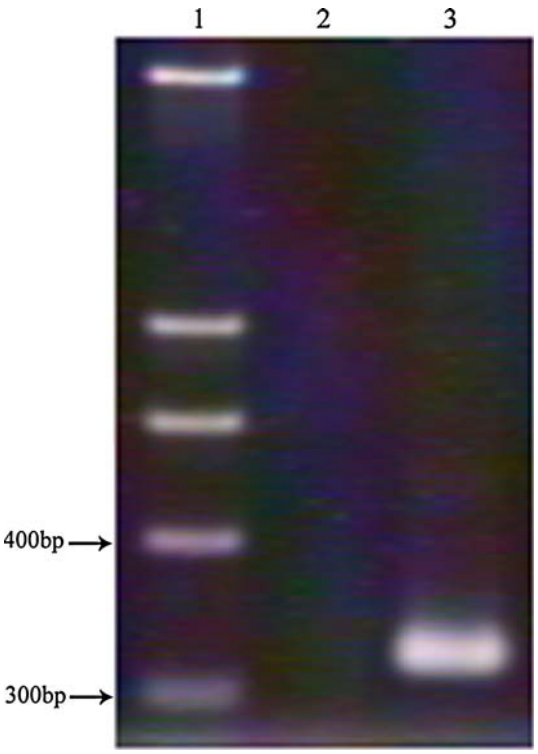

Fig. 1 Detection of mRNA of human PDGF-BB by RT-PCR in MSCs and MSCs-PDGF-BB. *Lane 1*, molecular weight markers; *lane2*, MSCs; *lane 3*, MSCs-PDGF-BB

Histologic Findings in the 6w-group

In the control group cells invaded further into the graft; while in both the MSCs group and gene-transfected group some chondrocyte-like cells appeared (Fig. 3).

Histologic findings in the 12-week group

In the control group, a large amount of fibrocytes were found in the graft; while in the MSCs group and gene-transfected group, the grafts had structure similar to normal ACL, especially in the MSCs group. However, the number of cells was still more than normal (Fig. 4).

Table 1 The PDGF-BB expression of perfusate sample from knee joint in the gene-transfected group (mean deviation)

Group	PDGF-BB (pg/ml)
0	0
3 weeks	522.76
6 weeks	728.848
12 weeks	412.09

Immunohistochemical staining of collagen type I and III

After transplantation, collagen type III increased in the grafts along with the growth of cells. Collagen deposition increased significantly in the PDGF-B gene-introduced wound at 3 weeks. After 6–12 weeks, collagen type I gradually increased to replace type III in the MSCs group and gene-transfected group (data not shown).

Immunohistochemical staining of PDGF-BB

The graft treated with MSCs-PDGF-BB demonstrated intense staining cells at 3 weeks. After 6–12 weeks, the staining was gradually decreased (Fig. 5), whereas the wounds treated with cells or without cells had virtually no staining (data not shown).

Discussion

This is the first report known to us that evaluates the effect of cell therapy and gene therapy technique on ACL allograft reconstruction. The aim of our experiment was to develop a new strategy to accelerate the remodeling of allograft and to build an allograft tendon bank for ligament reconstruction.

Although ACL reconstruction with allogeneic tendon has many advantages, its use has not been chosen widely. There are many reasons, such as marked inflammatory rejection responses and the risks of disease transmission, which prevent its use. As for the rejection response, Minami et al. [17] showed that normal MHC antigens are in the tendon cell component of the graft and numerous studies [1, 6, 10, 11, 17, 21, 28] have demonstrated that deep freezing at –80°C, by destroying these cells, can reduce graft antigencity. To reduce the risks of disease transmission, gamma irradiation [22] is now widely used as a safe and effective secondary sterilization technique. It can inhibit HIV transmission in T lymphocytes. Graft sterilization by gamma irradiation was also reported to produce less deleterious effects on healing.

After dealing with the allograft to reduce its side effect, we tried to find a way to modify the allograft to accelerate its ligamentation. For this, we used the MSCs and hPDGF-B gene. Many researches [5, 9, 15, 29] were done to find suitable plant cells to accelerate the remodeling of the allograft. In vitro study on MSCs in tendon healing by Van et al. [29] found that MSCs seemed to be the most suitable candidate for the development of tissue-engineered ligament, with the highest cell proliferation and highest collagen production, compared with ACL cells or skin fibroblasts, when the cells were seeded onto a resorbable suture material, respectively.

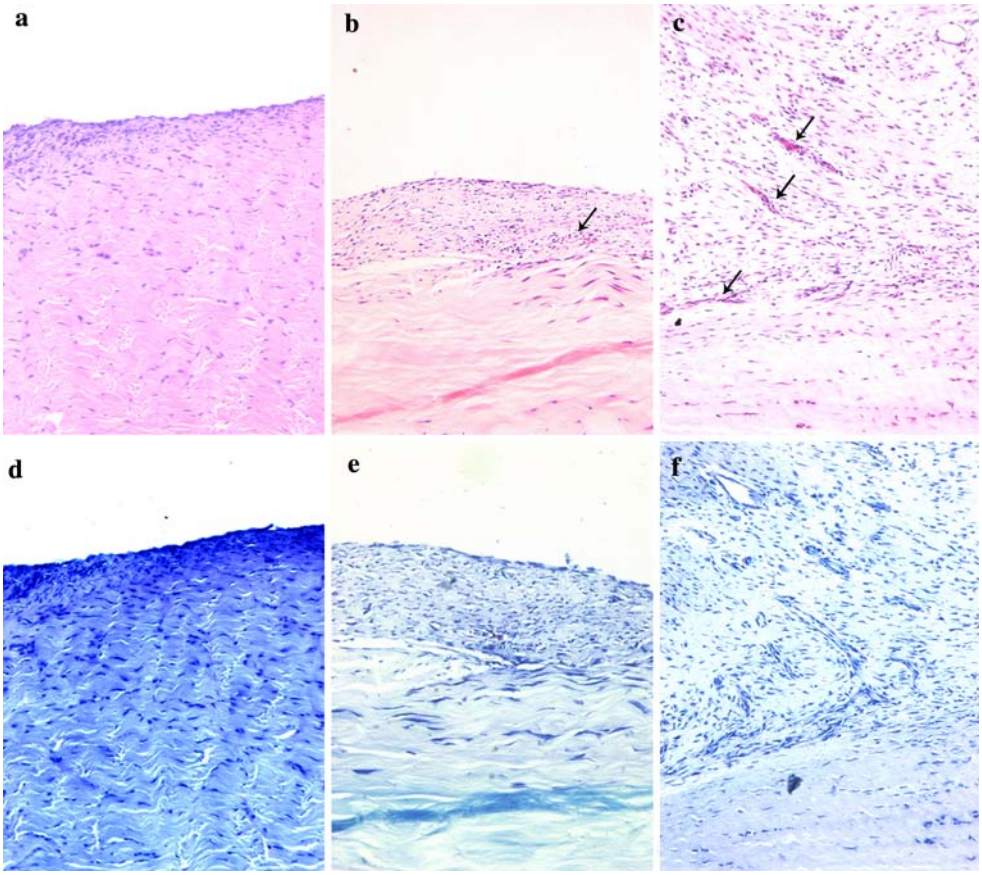

Fig. 2 Photomicrographs of 3-week ACL reconstruction. **a–c** H&E stain, original magnification ×100; **d–f** toluidine blue stain, original magnification ×100; **a**, **d** control group; **b**, **e** MSCs group; **c**, **f** gene-transfected group. In the control group, 3 weeks after surgery, host cells had already invaded into the graft, while in the MSCs group, granulation was formed and vascularization could be observed. In the gene-transfected group, significantly more cells and hypervascularity could be found. **b**, **c** The *arrows* show blood vessels)

Histological findings of our study suggested that implantation of autologous cells accelerated the cellular infiltration into the ACL and enhanced the maturation of collagen. We noticed that administration of cultured autologous cells in MSCs group and gene-transfected group caused an influx of cells into the ACL graft. But we are not sure where the cells were from: the MSCs applied to the surface of tendon grafts may have served as a source of additional recruitable fibroblast-like cells for tendon repopulation, or they may have been involved in activation and recruitment of local fibroblast precursors, and bone marrow-derived cells may have contributed to the graft that was placed in a bone tunnel.

MSCs also have the potential to migrate and transform into certain cells. In another study [19], MSCs, which were obtained from green fluorescent protein (GFP) transgenic SD rat and cultivated, were injected into normal SD rats in which multiple tissues had been injured including the anterior cruciate ligament. GFP-positive cells could mobilize into the injured ACL. Watanabe et al. [31] also demonstrated that when the autogenous MSCs were explanted into the injured MCL of the animal, the cells could migrate and survive for a certain time.

In our study, some fibroblast-like cells, fibrocartilage-like cells and endothelium of blood vessels were also found from 3 to 12 week during the remodeling process in cell plant groups. It was hypothesized that MSCs from bone marrow would differentiated into certain cells in the healing ligament following adaption to the specific environmental conditions of the tendon, and it may be reasonable to expect that MSCs could differentiate as well as promote the regeneration and maturing of the graft.

Many studies [4, 8, 12, 14, 16, 25, 27] 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 (data not shown). Similar to our findings were the results of a study by Nakamura et al. [20], which described an increased vascularity and enhanced collagen deposition in the wound of a patellar ligament after PDGF-B gene transfer in rats.

The mechanisms by which PDGF affect ligament healing are complex. Kuroda et al. [24] studied immunohistochemically the presence and the level of bFGF, TGF-ß, PDGF AA and PDGF-BB expression in a model of ACL reconstruction using a free patellar tendon autograft. They

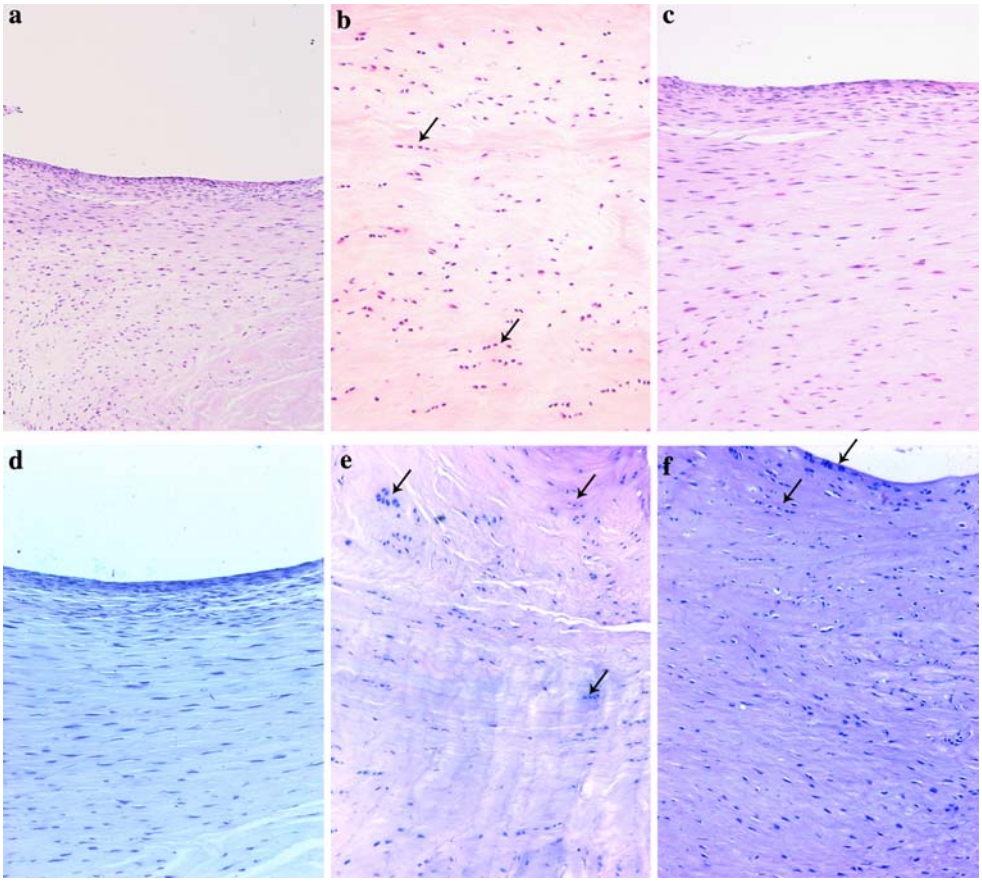

Fig. 3 Photomicrographs of 6-week ACL reconstruction. **a–c** H&E stain, original magnification ×100; **d**, **e**, **f** toluidine blue stain, original magnification ×100; **a**, **d** control group; **b**, **e** MSCs group; **c**, **f** gene-transfected group. In the control group, cells invaded further into the graft; while in both the MSCs group and in the gene-transfected group, some chondrocyte-like cells appeared. **b** The *arrows* show chondrocyte-like cells; **e**, **f** the *arrows* show extracellular matrix around the cells positive with toluidine blue stain.)

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 PO_2 of the intra-articular environment, Petersen et al. [23] showed that PDGF as well as hypoxia strongly enhanced VEGF secretion from tenocytes. Besides this VEGF-mediated angiogenetic effect, PDGF furthermore induces the synthesis of other growth factors, including IGF, and regulates the presence of other receptors [32]. 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 [18] 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. [24] 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. In our study at 3 weeks, we found significantly higher vascularity in the PDGF-transfected grafts. The concentration of PDGF-BB in articular 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. [12] demonstrated that the improvements in the MCL structural properties were dose-dependent to PDGF-BB. 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

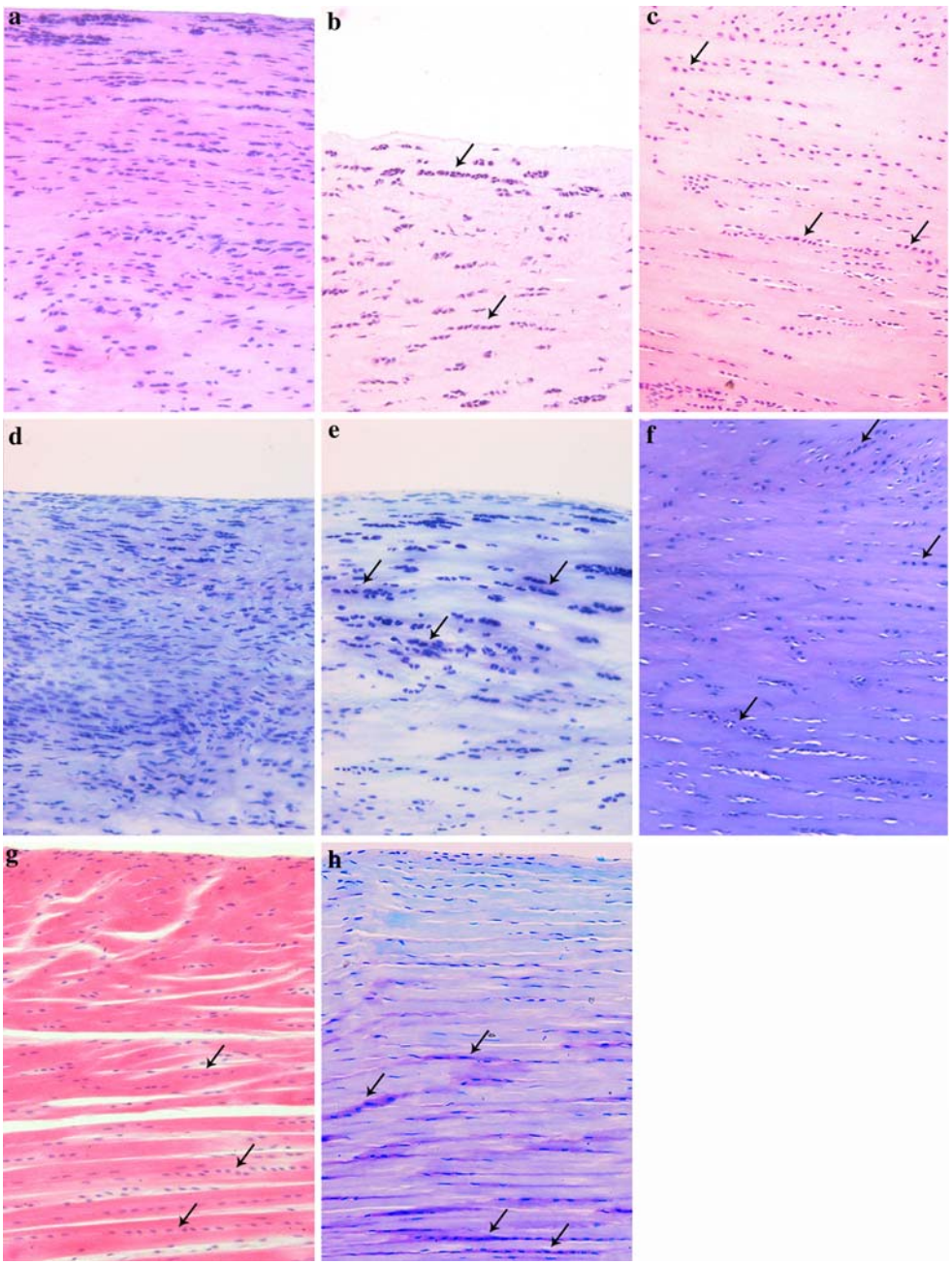

Fig. 4 Photomicrographs of 12-week ACL reconstruction and normal ACL. **a–c**, **g** H&E stain, original magnification ×100; **d–f**, **h** toluidine blue stain, original magnification ×100; **a**, **d** control group; **b**, **e** MSCs group; **c**, **f** gene-transfected group; **g**, **h** normal ACL. In the control group, a large amount of fibrocytes were found in the graft; while in the MSCs group and gene-transfected group grafts were similar to normal ACL structure. The normal ACL (**g**, **h**) demonstrated more heterogenous cell types and arrangements. Most cells were enclosed within lacunae. The ACL (**h**) is positive to toluidine blue stain; the nucleus is blue while the extracellular matrix is red-purple, especially adjacent to the cells. **b**, **c**, **g** The *arrows* show cell arrangements within lacunae. **e**, **f**, **h** The *arrows* show the extracellular matrix around cells positive to toluidine blue stain)

the growth factor 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.

As we know, there are limitations of topically applied growth factor proteins, including rapid degradation of the protein and inefficient action at the cellular level. Delivery of PDGF by gene therapy is potentially a much more efficient method of delivering active proteins with resultant sustained activity; From the perspective of clinical utility, BMSCs can be easily accessed in most patients without significant additional surgery or the risk of immune reaction, and these cells can be rapidly expanded in a cell culture media. Therefore, such combined therapy of gene therapy and cell therapy may be an effective approach for ACL allograft reconstruction.

We chose to use a rabbit model for ACL reconstruction. This is a model that has been validated in previous reports. Although we have designed special tools for the surgery in order to minimize injury to tissues during the operation, it is necessary to recognize some limitations concerning the experimental model used in this study. Rabbit is too small for surgery and the model is not similar to a human being. There were also significant differences between experi-

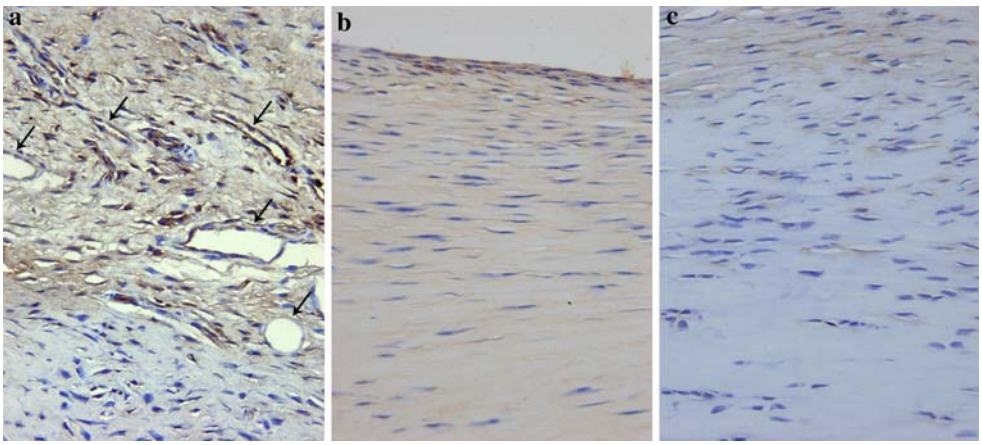

Fig. 5 Photomicrographs of ACL reconstruction in gene-transfected group in immunohistochemical staining of PDGF-BB (original magnification ×200). The graft treated with MSCs-PDGF-BB demonstrated intense staining cells at 3 weeks (**a**), after 6–12 weeks the staining was gradually decreased (**b**, **c**)

mental and control limbs in histology and other tests. More accurate and reliable experiment such as biomechanical testing is needed with a large animal model, such as sheep or dog, to obtain the results for judging the possibility of the use of gene therapy to enhance the process of ligament reconstruction in human beings.

Conclusions

We have demonstrated that the application of PDGF-B gene to a tendon graft is effective in changing certain structural properties during the early phase of tendon graft remodeling after ACL reconstruction. PDGF-B gene leads to an initial promotion of angiogenesis and subsequent enhanced collagen deposition in the wound. Therefore, this gene transfer technique may be a potentially useful tool for improving tendon repair.

In our study, we have also shown that MSCs can be transplanted to serve as a source for healing tissues or used as a molecular vehicle for therapeutic purposes. The application of MSCs is effective in enhancing collagen deposition and changing certain structural properties.

We have only used one growth factor to improve the structural properties of remodeling ligament. On finding an appropriate dosage, or combining with other growth factors, this effect might further be improved. This should encourage further research.

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