Tendon Regeneration and Repair with Adipose Derived Stem Cells

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Abstract: Tendon, the crucial element of the musculoskeletal system, when damaged, never restores the biological and biomechanical properties completely. Recently, tissue engineering and regenerative medicine have enabled the differentiation of postnatal somatic stem cells or mesenchymal stem cells (MSCs) to different cell lineages and tissues including tendon. In addition, the MSCs, mainly bone marrow derived stem cells (BSCs) were proven to enhance tendon healing. Adipose derived stem cells (ASCs) were shown to be as effective as the other MSCs by their multipotency and proliferative efficiency. However, neither the differentiation of ASCs to tenocytes nor the tendon regeneration using ASCs have been described in literature. Recently, we have studied the effect of ASCs on primary tendon repair in in-vivo model. In this paper, we sought to discuss tendon tissue engineering by focusing on culture of tenocytes, biomaterials, scaffolds, mechanical loading, fibroblasts and mesenchymal stem cells and mainly on adipose derived stem cells. Tendon regeneration using ASCs might be one of the clinical remedies in near future. In addition, the enhancing effect of ASCs on tendon repair and tendon defects might enable better clinical outcomes in musculoskeletal system reconstruction. Advances in biomaterial technology will improve the methodology in tendon regeneration however, up to date, ASCs present an ideal cell source for experimental and clinical research on tendon engineering.

Keywords: Adipose derived stem cell, mesenchymal stem cell, tendon, regeneration, engineering, tendon repair.

INTRODUCTION

A tendon is a band of fibrous connective tissue that is connected, on the one hand, with the muscles, and, on the other hand, with the movable structures, as the bones, cartilages ligaments, and fibrous membranes are capable of withstanding tension. Tendons are composed of parallel arrays of collagen fibers mostly type I. These collagens are held together with other proteins, particularly the proteoglycan, decorin and, in compressed regions of tendon, aggrecan. The tenocytes produce the collagen molecules which aggregate end-to-end and side-to-side to produce collagen fibrils. Fibril bundles are organized to form fibers with the elongated tenocytes closely packed between them. Collagen fibrils coalesce into macroaggregates. Groups of macroaggregates are bounded by connective tissue endotenon and are termed fascicles. Groups of fascicles are bounded by the epitotenon and peritenon to form the tendon organ.

Tendon, the crucial element of the musculoskeletal system, when damaged, never restores the biological and biomechanical properties completely. The collagen fibrils remain thinner, with a reduction in the biomechanical strength of the tendon [1]. The tendons heal by way of both extrinsic and intrinsic mechanisms. The exact nature of tendon healing remains unknown. The weakest point of tendon healing and the most likely time for rupture after repair is 5 to 10 days postoperatively. There is also an increased rate of rupture 6 weeks postoperatively when strengthening exercises may exceed the tensile strength of the repair. The goal of rehabilitation after tendon repair is to achieve optimal function and gliding while preventing tendon rupture [2]. Thus, there have been different studies on how to increase the strength of the repaired tendon and the clinical and experimental researches mainly focused on the type of sutures and repair techniques [3].

In different clinical cases, tendon defects are ought to be repaired by tendon grafting thus necessitating a tendon donor side. Autologous cell culturing combined with appropriate scaffolds resulted in in-vitro and in vivo tendon engineering and became an alternative remedy for tendon defects [4, 5]. Recently, tissue engineering and regenerative medicine have enabled the differentiation of postnatal somatic stem cells or mesenchymal stem cells (MSCs) to different cell lineages and tissues including tendon. In addition, the MSCs, mainly bone marrow derived stem cells (BSCs) were proven to enhance tendon healing [6-9].

Adipose derived stem cells (ASCs) recently presented by Zuk et al. [10] were shown to be as effective as the other MSCs by their multipotency and proliferative efficiency [11-15]. ASCs were used in different experimental and clinical circumstances to evaluate their effects in addition combination of ASCs with scaffolds has culminated inappropriate models that might be utilized in clinical problems. Kryger et al. [16] have used acellularized allogeneic tendon as scaffold material with epitotenon tenocytes, tendon sheath fibroblasts, BSCs and ASCs to determine the ideal cell type. After in-vitro and in-vivo evaluation of these different cell-scaffold complexes, they have concluded that these four types of cell lineages might be successfully used to engineer tendon. In addition, they have mentioned that ASCs proliferated faster when compared to other cell lineages which is a well known fact for most of researchers. Thus ASCs has much more potential in the clinical application especially in tendon regeneration and repair. Recently, we have studied the effect of ASCs on primary tendon repair in in-vivo model. In this paper, we sought to discuss tendon tissue engineering.

THE CULTURE OF TENOCYTES

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ [17]. Tissue engineering is a technique developed for generating biological tissue replacement using autologous cells; thus, the engineered tissue can be transplanted in vivo without causing immune rejection. The role of tendon cells, mainly epitotenon cells and tenocytes, were studied and tenocytes were found out to secrete larger and more matured collagen fibers than epitotenon cells [18]. Different studies revealed that the generation time of the cultured tenocytes after 36 passages were not changed and they retained the expression of tenocyte differentiated functions, synthesis of type I collagen and decorin. Standard cell
culturing methods were adopted for in-vitro culture of tenocytes. Tenocyte isolation and culturing has already been described and feasible in different instances [19].

**BIOMATERIALS, SCAFFOLDS, MECHANICAL LOADING**

Tenocytes are the crucial cellular factor among tendon morphology; however, biomaterial that is used as a scaffold to form the three dimensional shape of the tendon is the main key element in tendon regeneration. The constructs are customized to mimic the natural tendon composed of collagen. To date, three major categories of scaffolding materials have been employed: polymers, polysaccharides, and collagen derivatives. Furthermore, with these materials as a base, a variety of specialized methodologies have been developed or adopted to enhance neo-tendogenesis. These strategies include cellular hybridization, interfacing improvement, and physical stimulation [20-22]. When determining the success of a three-dimensional scaffold seeded with cells that can be directed to form tendon/ligament tissue, the viability and proliferation of the cells in the construct, as well as extracellular matrix production and structure should be taken into account. Histology and histochemistry, microscopy, colorimetric assays, and real-time reverse transcriptase-polymerase chain reaction (RT-PCR) are techniques that are employed to assess these biological characteristics [23].

The main problem in these constructs are to possess the appropriate initial stiffness and at the same time, the feasibility of cell seeding into. Nirmalanandhan et al. [24] has found out that the longer, stimulated, collagen sponge constructs showed the highest in vitro linear stiffness when compared to shorter, non-stimulated and collagen gel constructs. In another study by the same group, they have described that the different cell to collagen ratios showed different results indicating that the amount of cell seeding should be determined depending on the type and amount of the collagen content [25]. Cao et al. [26] demonstrated the possibility of neo-tendon regeneration using synthetic polymers seeded with tenocytes. In vitro studies of the tendon regeneration without any mechanical stimulus to the construct cell structure resulted in tendon tissue that was inappropriate for the clinical application. Thus, investigations were focused on mechanical stimulus, bioreactors and conditioning of the cell-construct. Nabeshima et al. [27] had indicated that uniaxial tension inhibited collagen degradation by collagenase, increased the stiffness of the tendon significantly and the tendons exposed to mechanical stimulus failed at significantly higher elongations and maximum forces than the slack tendons. In vitro cyclic loading significantly increases the biomechanical properties of cell-seeded constructs. The stiffness of the cell-seeded constructs is increased by mechanical loading cycles directly. In addition, the initial stiffness of the cell-seeded, cyclically loaded constructs was found to be a strong predictor of the change in construct stiffness [28]. While some of the changes occur in the absence of mechanical loading, appropriate regeneration requires the mechanical stimulus provided by cyclic bioreactor activity [29]. The necessity for mechanical stimulus is a result of the characteristic of the tenocyte and the construct structure. As described, tenocytes are specialized fibroblast however, the collagen production, deposition and organization depend on the mechanical stimulus. The construct structure enables collagen deposition, however, mechanical stimulus organizes the composition and the strength of these collagen fibers. Mechanical stimulation of cell-sponge constructs increases the expression of collagen type I and type III structural genes, as well as linear stiffness and linear modulus [30]. In vitro mechanical stimulation of tissue-engineered tendon constructs significantly increases both construct stiffness and the biomechanical properties of the repair tissue. When optimized using response surface methodology, Nirmalanandhan et al. [31] indicated that a mechanical stimulus with three components (2.4% strain, 3000 cycles/day, and one cycle repetition) produced the highest in vitro linear stiffness.

**FIBROBLASTS, MESENCHYMAL STEM CELLS**

For further clinical applications, the culture of tenocytes needs a tendon donor site and also the in-vitro doubling time is longer when compared to other cell sources. Thus, in an attempt to substitute tenocyte Liu et al. [32] demonstrated that dermal fibroblasts and tenocyte engineered tendons shared similar tensile strength, about 75% of natural tendon strength suggesting that dermal fibroblasts may have the potential as seed cells for tendon engineering. The experimental research in and the clinical application of mesenchymal stem cells, mainly bone marrow derived stem cells (BSCs), have been promising since the historical pioneering of successful bone marrow transplants following the first description by Freidenstein [33]. In the in-vitro and in-vivo tendon regeneration and repair using MSCs, mainly BSCs have been described in different studies [34-38].

**ADIPOSE DERIVED STEM CELLS**

Since the description of ASCs by Zuk et al. [10], it has been well known that ASCs are as efficient as BSCs among MSCs. One of the pioneer studies about tendon engineering by Stanford Group [16] have indicated that ASCs were as efficient as the other cell types: epitelen tenocytes, tendon sheath fibroblasts, BSCs. They found out that all cell types had similar collagen expression and cell proliferation was higher in ASCs in late passage compared with early passage and in ASCs compared with epitelen tenocytes at late passage. They have shown that in vitro assessment of reseeded constructs showed the presence of ASCs on the construct surface, as well as the other cell types. Recently, we have studied the effect of ASCs on primary tendon healing. We have performed a primary incision to and then repaired Achilles tendon in rabbit model (Fig. 1). The left side was applied platelet rich plasma (PRP) gel and the right side was autologous ASCs mixed PRP (Fig. 2). The tensile stiffness was measured on the 4th week (Fig. 3). The samples were taken for immunohistochemical evaluation of collagen type I, transforming growth factor beta (TGF-β) 1, 2, 3, fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF).

**BIOMECHANICAL EVALUATION**

There are some variations in the methodology used in biomechanical testing of tendons in experimental and clinical circumstances. However, there are studies carried out to determine the muscle tendon forces generated by various active and passive functions. The information from these studies is of considerable value when attempting to equate the forces applied to repaired tendons with the strength of those repairs during the healing process [2, 3, 29]. In clinical instances, the environment and the clinical situation of the muscle tendon complex present important factors, however, in experimental situations, standard biomechanical testing has been the gold standard. The main point in biomechanical testing is the stabilization of the tendon complex to the biomechanical testing machine. The common method of fixation depends on the properties of the machine. The jaw screw vise of the machine has to be strong enough for the tendon ends to be fixed all throughout the testing. The tendon ends are usually preferred to be excised with the bone and muscle attachments to exclude the detachment. The second point in the biomechanical testing is the assessment of gap formation at the repair site. Gap formation might hinder the accurate detection of the actual force applied at the repair site thus should be eliminated by consecutive measurements and statistical evaluations. In strong repair techniques and with appropriate head speed, the gap formation might overcome. Thus, the objective evaluation of the biomechanical testing might be accomplished [39, 40].

In our study, the exposed tendons were subjected to biomechanical testing with uniaxial compression testing machine SGE-2033B (Maruto Testing Machine CO. Ltd. Tokyo, Japan) with NTS load cell (50KS, Nippon Tokushu Sokki Co. Ltd, Tokyo, Japan).
The free ends of the tendon were clamped in the three jaw screw vice of this machine. The head speed on the machine was set at a speed of 100mm/min. The specimens were tested to mechanical failure and the maximum load applied to each specimen was recorded. A satisfactory method of assessing gap formation during dynamic testing could not be found, and therefore this was not assessed. The golden standard for the evaluation of the efficacy of any technique for the evaluation of tendon regeneration or repair is still the biomechanical testing of the tensile strength [39, 40]. In our study, we have found out that ASCs increased the tensile strength of the repaired tendon (Fig. 4).

**IMMUNOHISTOCHEMICAL EVALUATION**

Tendons heal by way of both extrinsic and intrinsic mechanisms. Extrinsic healing occurs by means of the fibroblastic response of the sheath and surrounding tissues, and adhesion formation is essential for healing to occur. Flexor tendons have an intrinsic ability to heal by means of nutrients supplied by diffusion from the synovial fluid. Intrinsic healing does not involve adhesion formation [2]. Collagen type I synthesis is the crucial step in determination of the tensile strength. However, collagen type III is the initiator in the tendon healing process supplied by the fibroblasts and the tenocytes [41]. Ericson et al. [42] have indicated that the in...
creased content of type III collagen caused thinner collagen fibers and decreased the tensile strength. Tang et al. [43] have described that the expression of the type I collagen gene increased remarkably at later periods of in vivo tendon healing. In an attempt to construct an ideal scaffold for tendon regeneration, Butler et al. [44] have investigated that augmenting the gel scaffold with a type I collagen sponge further increased repair stiffness and maximum force, and resulted in the repair tangent stiffness matching normal stiffness up to peak in vivo forces. Juncosa-Melvin et al. [45] have shown that mechanical stimulation increased collagen type I and type III gene expressions in vitro culminating in higher linear stiffness and modulus.

Transforming growth factor beta is a cytokine that plays a key role in acute inflammation and wound healing. Chang et al. [46] have observed that both tendon parenchymal cells and tendon sheath cells exhibited upregulation of TGF-β1 mRNA, providing evidence that dual (intrinsic and extrinsic) mechanisms of tendon repair existed. The upregulated TGF-β following injury to the tendon was reported to increase the collagen synthesis leading to increase in tensile strength, however; this excessive collagen deposition was shown to be responsible for the adhesions in the tendon repair. This TGF-β induced collagen production was inhibited by TGF-beta neutralizing antibody [47]. Thus, the postoperative range of motion was increased [48]. On the other hand, PDGF and TGF-β application resulted in enhanced healing in ligaments and tendons regardless of the adhesions [49, 50].

Normal tenocytes and tendon sheath cells were described to be capable of FGF production and FGF mRNA was reported to be upregulated in the tendon wound environment [51]. Hsu et al. [52] have indicated the importance of FGF and VEGF in tendon healing as these growth factors were involved in cell differentiation and growth, including the normal processes of development and tissue repair.

The immunohistochemical evaluation of the samples revealed a statistically significant increase in collagen type I specific for tendon, FGF and VEGF in the ASCs group when compared to control group. However, there was a statistical decrease in TGF-β subfamily groups.

CARRIER FOR CELL THERAPY: Platelet Rich Plasma

There are various methods and solutions including direct injection to the site as stem cell carrier. The ideal cell carrier should possess the property of being absorbable with low immunological reaction at the environment. The natural elements that might be harvested from the organism itself have been popular with their low cost and low donor site morbidity. Scaffolds as described above might be acceptable cell carriers with their modulating factors including collagen gels, sponges, hyaluronic acids, fibrin compositions. Scaffolds still yield the immunological reaction problem in spite of the advanced technology. Fibrin gels might be an ideal carrier for stem cells to the regeneration and repair site however, in the tendon cases, the tendon adhesions depending on the immobilization might be a problem. Platelet rich plasma (PRP) is an ideal carrier with low immunological reaction, low cost and donor site morbidity. The growth factors and cytokines also enable the cells to be more effective in tendon regeneration and repair.

We used platelet rich plasma (PRP) as a carrier for the ASCS to the repair site. Numerous proteins are contained within the alpha-granules of platelets that strongly influence wound healing, including PDGF, TGF-β, platelet factor 4 (PF4), interleukin (IL)-1, platelet-derived angiogenesis factor (PDAF), VEGF, EGF, platelet-derived endothelial growth factor (PDEGF), epithelial cell growth factor (ECGF), insulin-like growth factor (IGF), osteocalcin, osteonectin, fibrinogen, vitronectin, fibronectin, and thrombospondin (TSP)-1 [53-55]. These growth factors and cytokines do not only promote any wound healing but may also help the differentiation of multipotent cells [56]. The gel property of PRP could serve as a cell
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Platelet Rich Plasma (PRP) preparation was performed as described previously [12, 57] Thus, 10mL of whole blood from each 10-week-old white male Japanese rabbit was drawn preoperatively with a 23-gauge needle (TERUMO, Tokyo, Japan) into tubes containing 3.8% sodium citrate. The blood was first centrifuged in a standard laboratory centrifuge machine (Kubota 3740, Tokyo, Japan) for 10 min at 2400 rpm (450 g). The supernatant plasma was collected along with the buffy coat, which consists of platelets and leukocytes, into a neutral tube with a long cannula. A second centrifugation at 3600 rpm (850 g) was performed for 15 min to concentrate the platelets. The infranatant containing the buffy coat was resuspended with 1.3mL of the residual plasma to prepare the final PRP product. PRP gelation was activated with a 10% calcium chloride solution immediately before the administration in vivo. Autologous PRP preparations were made from each rabbit and applied to the same rabbit. In our study, we have used PRP mainly for the cell delivery carrier for ASCs, in addition; the cytokine and growth hormone content would have probably enhanced the tendon repair. However, the statistical differences in tensile strength, collagen type I, FGF and VEGF levels between the control and experimental groups revealed the positive effect of the ASCs.

CONCLUSION

Tendon regeneration and repair using the tissue engineering techniques might lead to advances in surgical procedures in addition to complicated cases in clinical situations. Tenocyte isolation and culturing have positive effects on tendon repair and tendon regeneration is feasible however, the harvest and culturing of tenocytes might not be applicable in clinical cases. The long period of culturing when compared to MSCs especially BSCs and ASCs might hinder the choice of cell type for the utilization. BSCs have been the gold standard for MSCs since first described. However, recent advances in ASCs have shown that ASCs might be a more preferred cell type for tendon regeneration and repair with their low donor site morbidity and high rate of growth during culturing. In addition, ASCs as being a part of lipo-aspirate cells might be a well tolerated cell type in clinical application.

ASCs utilization for tendon regeneration and repair has recently been taken into consideration [16, 58]. In our study, we have found out that ASCs could differentiate into tenocytes in-vivo. This differentiation, numerically 11.53%, could be described as the direct effect of the ASCs. However, the indirect effect that could be described as the control and secretion of the growth hormones and cytokines in the environment might be the main enhancer during primary tendon repair. The direct differentiation of ASCs to fibroblast and endothelial cells was previously described. The differentiation of fibroblasts to tenocytes also has been under the control of growth hormones and cytokines. The inhibitory effect of ASCs on TGF-beta subfamily group seems to be a conflict when compared to previous studies [15], however, ASCs might change their properties depending on the environment and the necessities at the healing site (Fig. 5). How the complex interaction and the cascades of ASCs could increase collagen type I, FGF and VEGF and decrease TGF-β levels should further be investigated.

ASCs have been popular not only because of their multipotency but also minimal donor site morbidity. Tendon regeneration using ASCs might be one of the clinical remedies in the near future. In addition, the enhancing effect of ASCs on tendon repair and tendon defects might enable better clinical outcomes in musculoskeletal system reconstruction. Advances in biomaterial technology will
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