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Histopathological and biomechanical evaluation of tenocyte seeded allografts on rat Achilles tendon regeneration

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ABSTRACT

Tendon injuries in humans as well as in animals' veterinary medicine are problematic because tendon has poor regenerative capacity and complete regeneration of the ruptured tendon is never achieved. In the last decade there has been an increasing need of treatment methods with different approaches. The aim of the current study was to improve the regeneration process of rat Achilles tendon with tenocyte seeded decellularized tendon matrices. For this purpose, Achilles tendons were harvested, decellularized and seeded as a mixture of three consecutive passages of tenocytes at a density of 1×10^6 cells/ml. Specifically, cells with different passage numbers were compared with respect to growth characteristics, cellular senescence and collagen/tenocyte marker production before seeding process. The viability of reseeded tendon constructs was followed postoperatively up to 6 months in rat Achilles tendon by histopathological and biomechanical analysis. Our results suggests that tenocyte seeded decellularized tendon matrix can significantly improve the histological and biomechanical properties of tendon repair tissue without causing adverse immune reactions. To the best of our knowledge, this is the first long-term study in the literature which was accomplished to prove the use of decellularized matrix in a clinically relevant model of rat Achilles tendon and the method suggested herein might have important implications for translation into the clinic.

1. Introduction

Tendon is a highly specialized fibrous tissue whose function is to transfer mechanical forces between muscle and bone. Therefore, it has evolved to have toughness to resist tensile loads and elasticity to withstand both repetitive as well as constant loading. These extraordinary biomechanical properties of tendon are attributable to the highly organized extracellular matrix (ECM) components consisting dominantly type I collagen fibrils, type III and type V collagens, proteoglycans, elastin and fibronectin; and tenocytes embedded within this network of matrix [1–3].

The incidence of human Achilles tendon rupture was reported as 12–18/100,000/year in recent studies [4–6]. However, tendon injuries in humans as well as in veterinary medicine are problematic because tendon has a poor regenerative capacity and complete regeneration of the ruptured tendon is never achieved [7]. Despite remodeling, the histological and mechanical properties of naturally healed tendon tissue never match those of intact tendon because the remodeling of naturally healed tissue is not extensive but it is basically deposition of scar tissue at the site of injury. Current standard of care for tendon injury is either surgical or conservative route. Surgical treatment involves the removal of inflamed, devitalized or contaminated tissue from the lesion site by a number of incisions while conservative treatment only involves medical support accompanying exercise without any surgical protocol. In the literature, several synthetic and natural origin biomaterials including poly-glycolic acid (PGA), poly-lactic glycolic acid (PLGA), nylon, silicone, carbon fibers, Dacron grafts chitosan and collagen derivatives were evaluated as an augment for tendon regeneration [8–18]. However, most of these applications were reported to cause notable inflammatory responses and frequent complications leading to postoperative scar formation and adhesions between tendon and the surrounding tissues due to limited biocompatibility and/or functional compatibility [10,13].

Recently, regenerative medicine has been emerging as an alternative in tendon repair. Several approaches have been developed to improve tendon healing such as stem cell injection, gene

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transfer and growth factor injection into the site of injury [19–23]. Awad [22] reported that mesenchymal stem cells (MSCs) suspended in type I collagen gel may not be adequate for patellar tendon regeneration in rabbits because of the "non-aligned" structure of collagen matrix, which probably effected the histological disorganization of the recovering tendon. Furthermore, Doroski [21] reported that use of MSCs for their potential to differentiate into tenocytes may be hampered by the fact that they have the ability differentiate into other related cell types as well. Several growth factors including BMP, TGF-β, bFGF and PDGF have been shown to enhance wound healing process, increase cell proliferation and collagen production in rat tendon [23–25]. However, during the wound healing process, available growth factor concentration has to be precisely balanced so that it is neither insufficient to cause failure to induce repair nor excess to cause adhesion of the skin on tendon scar surface and the loss of normal tendon function [26]. *In vivo* gene transfer approach is also known to have major disadvantages such as the adversity of finding vectors with high transgenic activity, immune response development against vectors, and general safety concerns as in all gene therapy applications [27–29].

Tissue engineering is a promising alternative in tendon repair when applied in conjunction with decellularized extracellular matrices (DCM). However, donor site scarcity and morbidity limit the extensive use of autografts as DCM in tendon repair [30]. Alternatively, xenogenic DCM has been reported to improve tendon regeneration [31–33]. Nevertheless, current experience in the preparation of xenogenic grafts has shown that these grafts may instigate inflammatory responses and require a longer recovery period to integrate into native tissue [34]. In clinical practice, use of cadaveric DCM may therefore be a more viable alternative than auto − or xenografts.

Use of allograft DCM started attracting attention in several tissue engineering applications, including kidney, liver and lung reconstruction [35–38]. In allogenic tendon tissue engineering, current approaches involve seeding dermal fibroblasts, tenocytes, mesenchymal and bone marrow (BMSC) stem cells onto DCM as allogeneic tendon grafts, which has shown an improved healing [22,34,39]. In these cases, it is reported that the functional recovery of ruptured tendon is established earlier and the healing is accelerated with rapid remodeling.

In this study, it was hypothesized that DCM with seeded tenocytes improve regeneration process during Achilles tendon healing in rats. For this purpose, Achilles tendons were harvested, decellularized and seeded with tenocytes. Specifically, tenocytes were passaged 3 times to gain sufficient amount of cells to construct DCM + Tenocyte composites that were further transplanted to DCM + Tenocyte group. The viability of reseeded tendon constructs was followed postoperatively up to 6 months using histopathological and biomechanical analysis. To the best of our knowledge, this is the first long term study in the literature which investigated the effects of DCM seeded with previously characterized tenocytes both phenotypically and genotypically on tendon regeneration in rats.

2. Materials and methods

Adult male *Rattus norvegicus (Wistar albino)* rats (250 ± 50 g) (n = 50 as tenocyte and decellularized tendon source and n = 30 for *in vivo* experiments) were used. All experimental procedures and the use of animals were approved by Hacettepe University Animal Ethical Committee (Approval Number: 2009/8). Animals were reared on a basal diet with water *ad libitum* and maintained in an air-conditioned room at 22.4 ± 1.6 °C, in a relative humidity of 47.2 ± 1 and 12 h light/dark cycle.

2.1. Tenocyte isolation and culture

The rats were sacrificed by CO_2 asphyxiation. The tendon tissue was removed and rinsed in sterile phosphate-buffered saline (PBS) containing penicillin. Tendon segments were placed in six-well culture plates for explant culture in DMEM/F12 growth medium (SID8437) supplemented 10% fetal bovine serum (FBS) (SIF6178), 1% glutamine-penicillin-streptomycin (SIG6784).

Tenocytes were obtained and cultured in accordance with the literature [39,40]. Tenocytes from three consecutive passages were mixed together to obtain sufficient number of cells for immunofluorescence staining and qPCR studies and to construct DCM + Tenocyte composites to be transplanted to DCM + Tenocyte group (n = 30 and 1 × 10^6 cells/animal).

2.2. Cell proliferation and senescence

Viable cells from the first, second, and third passage (P1, P2, P3, respectively) were counted using digital cell counter (Beckmann, Vicell, USA). The number of viable cells was plotted against culture day to generate a growth curve for each passage.

Before seeding tenocytes on DCM surface, cell senescence of P1, P2 and P3 tenocytes was determined using the β-galactosidase activity staining assay with Senescence Cell Histochemical Kit (Sigma) according to the manufacturer's guidelines to obtain if passaging caused any senescence. Digital images were captured with AXIO Vision.4B Software Olympus IX70 (Olympus Corporation, USA).

2.3. Immunofluorescence staining

Before seeding, cells from consecutive passages were compared for their collagen/tenocyte marker production by immunofluorescence staining and qPCR. P1, P2, and P3 tenocytes were fixed with 2.5% glutaraldehyde. For antigen retrieval, tenocytes were incubated with Triton X − 100 (Merck, New Jersey, USA). Cells were incubated with primary antibodies (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) against collagen type I/type II (sc25974 − sc7764), collagen type III/type V (sc8781 − sc9856), tenomodulin/tenascin − C (sc49325 − sc9871) and biotin-labeled secondary antibodies (Vectorlabs, California, USA) for double staining. Detection was performed using the Vectastain ABC Kit, FITC (A2001) and Texas Red (A2002) fluorochromes (Vectorlabs, California, USA). Digital images were acquired with AXIO Vision.4B Software and Olympus IX70 (Olympus Corporation, USA).

2.4. Gene expression analysis with quantitative, real-time PCR (qPCR)

Total RNA from 1.5 × 10^6 tenocytes was extracted with Tri Reagent according to the manufacturer's directions (Sigma, UK). First-strand complementary DNA (cDNA) was synthesized with cDNA kit (Roche, Applied Science, Germany) according to the manufacturer's protocol.

Primers were designed with Perl-Primer software (Sourceforge.Net) and obtained from Metabion International, Martinsried, Germany. Primer sequences are given in Table 1. Specificity of all primers was confirmed by a single product amplification using melting curve analysis.

qPCR assays were performed in triplicate to obtain relative expression levels for each gene. Expressions of target genes in cell samples belonging to each passage (P1, P2 and P3) were compared to *Gapdh* expression -as internal control- and data analysis was performed according to a previously reported formulation [41].

Gene expression patterns were obtained by using Roche 480 Sybr Green I Master Kit with Light Cycler 480 (Roche, Applied Science, Germany). The reaction mixture consisted of 2 μL cDNA, 0.5 μmol/l of each primer, 200 μmol/l each dNTP, 1.5–2 mmol/l $MgCl_2$, 2.5 μL 10× reaction buffer, 0.05 IU/μL *Taq* DNA polymerase and 17.3 μL distilled water. In addition, SYBR Green, a double-stranded DNA dye, was used. The cDNA was denatured by heating to 95 °C for 1 min. The template was amplified for 40 cycles of denaturation for 30 s at 95 °C, annealing of primers at 60–65 °C for 1 min and extension at 72 °C for 30 s. PCR products were identified by generating a melting curve. The melting protocol consisted of heating the samples to 95 °C for 10 min, followed by cooling to 65 °C for 1 min and slowly heating to 95 °C while monitoring SYBR Green fluorescence.

Table 1
Sequence of primers used in qPCR.

Target gene	Primer	Sequence	GC%	Tm °C
Col1	Forward	5′-AGT CGA TTC ACC TAC AGC AC-3′	50	58
	Reverse	5′-GCC AAT GTC CAT TCC GAA-3′	50	54
Col2	Forward	5′-CAG CAG GTT CAC GTA CAC T-3′	52	64
	Reverse	5′-GAG GTC TTC TGT GAT CGG T-3′	52	64
Col3	Forward	5′-CAA ATT CAC TTA CAC AGT TCT A-3′	31.80	55
	Reverse	5′-ATG TCA TAG GGT GCG ATA-3′	44.4	52
Col5	Forward	5′-AGT ATC CAC TCT TCC CTG-3′	50	54
	Reverse	5′-GAG GAT CAA GGT GAC ATT-3′	44.4	50
Tnsc	Forward	5′-GTG GCT GCA TTG ATG GTT G-3′	60	63
	Reverse	5′-TCT CAG CAT GGT CAC CTC C-3′	50	58
Tnmd	Forward	5′-CAA AGA ATC CTC CAG AGA A-3′	42.1	53
	Reverse	5′-CAG GAC AAT TAG AGT TAA GG-3′	40	54
Gapdh	Forward	5′-TAT GAC TCT ACC CAC GGC AA-3′	50	58
	Reverse	5′-GAC TCC ACG ACA TAC TCA GCA-3′	52.4	61