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Evaluation of the anterior cruciate ligament, medial collateral ligament, achilles tendon and patellar tendon as cell sources for tissue-engineered ligament

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Abstract

This study investigated four different connective tissue cell types to determine which cell type should be the source for seeding a tissue-engineered anterior cruciate ligament (ACL) replacement. Cells derived from the ACL, medial collateral ligament (MCL), achilles tendon (AT), and patellar tendon (PT) of New Zealand White rabbits were isolated and cultured. Each cell type was cultured in vitro after seeding on three-dimensional (3-D) braided polymer scaffolds and on tissue culture polystyrene that served as a control. Samples were evaluated and compared for their morphology, proliferation, and gene expression of fibronectin, type I and type III collagen. Scanning electron microscopy (SEM) photomicrographs verified cell attachment of all four types of connective tissue fibroblasts to the scaffolds. Preliminary results comparing proliferation indicate that cells obtained from the PT and AT have the fastest proliferation. Whereas gene expression of the phenotypic markers measured using real-time reverse transcription polymerase chain reaction (RT-PCR) indicates ACL cells have the highest gene expression for the matrix markers. This leads to the question of which cell type should be the cell source for tissue-engineering of ligament, the highly proliferating cells or the differentiated matrix producing cells. This study would suggest that ACL differentiated matrix producing cells are the most suitable cells for further study and development of a tissue-engineered ligament. Published by Elsevier Ltd.

Keywords: Tissue engineering; Ligament; Tendon; Scaffold; Biomaterials; Real-time reverse transcription polymerase chain reaction (RT-PCR)

1. Introduction

One of the most common sports-related injuries occurs with the rupture or tear of the anterior cruciate ligament (ACL). In recent years, roughly 200,000 ACL repairs were performed in the US alone [1]. This is up from an average of 60,000 procedures per year during 1990–1995 an increase of 333% [2,3]. The reason for the rise in procedures is due in part to an increasing elderly population and physical activity.

Current ACL replacements involve two distinct types of grafts, biological and synthetic. Most commonly used are biological replacements. These include autografts and allografts, which are tendons taken from the patient's patellar tendon (PT), hamstring tendon, or a cadaver source. Both of these sources have unique disadvantages. Autografts are associated with pain, dysfunction and donor site morbidity. Additionally, there is an insufficient supply of tissue for a second donation if the graft needs to be replaced in the future. Allografts carry the risk of

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disease transmission and inflammatory response to the foreign tissue.

In order to reduce some of the adverse side effects patients may experience with non-degradable synthetic grafts, many researchers have looked to tissue engineering for the development of biodegradable materials that would provide immediate stabilization to the repaired ligament while also acting as a scaffold for the ingrowth and replacement by host cells [3]. Our laboratory is using a braided three-dimensional (3-D), porous, fiber scaffold composed of poly-L-lactic acid (PLLA) seeded with cells cultured in vitro for use as a tissue-engineered ligament graft [4,5]. In previous studies, we have shown that cellular response, mechanical and degradation properties of PLLA made it the most suitable polymeric composition to be used as a scaffold for the development of tissue-engineered ligaments [5].

The knee joint contains several large ligaments that serve to control motion by connecting the femur with the tibia and by bracing the joint against abnormal types of motion. The ACL and medial collateral ligament (MCL) are the major stabilizing ligaments of the knee. Injury to these ligaments results in the loss of function of the joint, which can lead to the early development of osteoarthritis. The PT is the gold standard for ACL replacement and achilles tendon (AT) would also be a good source because of its strength and accessibility for harvest without destabilization of the knee [6–10]. The MCL, due to its vascular supply, has the ability to heal. Whereas, the ACL has a poor healing capacity due to its low vascularity and envelopment by synovial fluid [3,11,12]. The differences in healing capacity are due to a combination of factors including intrinsic properties such as environment (intrasynovial or extrasynovial) and functional differences [10,13–15]. Therefore, most current clinical techniques have focused on the replacement of the ACL with biological grafts harvested from cadaver or autogenous grafts harvested from various donor locations.

In this study, we compared the cultures of rabbit PT, AT, ACL and MCL fibroblasts on 3-D braided structures to investigate the biological cellular responses that can be used to determine the cell source most appropriate for a tissue-engineered ligament. While the use of ACL fibroblasts would be ideal, the location of the ACL within the synovial capsule of the knee makes it difficult to obtain cells for culturing and eventual seeding of the scaffold. Other ligaments and tendons, such as the MCL, AT and PT are much more accessible, less invasive and less traumatic to the patient for obtaining cells. Also, in general the fate of living donor fibroblasts in allograft studies of canine, rabbit and goat models have shown that cells repopulating the graft originate from another source besides the native tissue [10,16–18]. In this study, we assess whether the determination of cell source should be solely based on proliferative capacity and collagen production or should the differentiated cells sending the proper matrix signals to guide the host cells be the source for the graft.

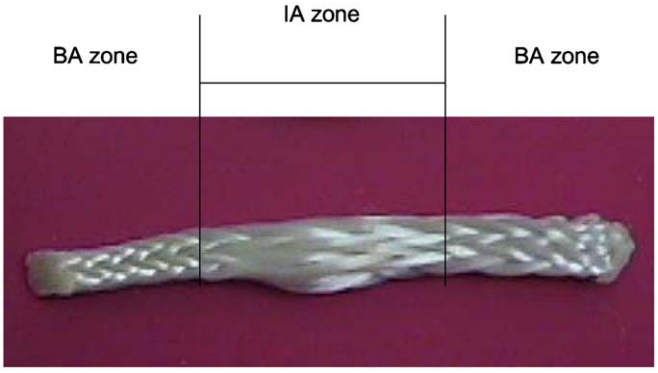

Fig. 1. Photograph of tissue-engineered ligament (3-D PLLA 5×5 square braid) developed for implantation in a rabbit model. For this particular cell study the scaffold was fabricated with a full sized intraarticular (IA) zone and reduced bone attachment (BA) zones. Cells were seeded within the IA zone.

Our study aims to determine if these other connective tissue cells may be used as a substitute to the ACL cells when seeding polymer scaffolds for ACL replacement using SEM, proliferation and RT-PCR to characterize cellular response.

2. Materials and methods[1]

2.1. Preparation of PLLA braided scaffold

The 3-D fibrous scaffolds were fabricated using a 3-D braiding machine [4,5]. Briefly, PLLA fiber (70 denier) (Albany International Research Corporation, Mansfield, MA, USA) was laced to produce yarns with a fiber density of 10 fibers per yarn. Yarns were then placed in a 3-D rectangular braider adapted to a 5×5 square carrier arrangement. Sequential motion of the tracks and columns along with manual compaction resulted in the formation of 3-D 24 yarn square braids. The 3-D square braids used for this study were approximately 2.7 mm × 23 mm × 2.7 mm taken from the intraarticular zone as shown in Fig. 1. The scaffold porosity of the samples was $(59 \pm 12)\%$, the surface area was $(70 \pm 9)\,cm^2$ and the average mode pore diameters were $(212 \pm 22)\,\mu m$ as measured by mercury porosimetry ($n = 6$) (Micromeritics Autopore III, Micromeritics, Norcross, GA, USA).

2.2. Cell culture

Primary ACL, MCL, AT and PT cells from New Zealand White rabbits weighing approximately 1 kg were harvested and cultured according to the method of Ross et al. [19] and Morgan and Yarmush [20]. Briefly, tissues were dissected and rinsed with Hank's buffer salt solution (HBSS, Invitrogen, Carlsbad, CA, USA). Cell isolation by explant technique was used as described by Ross et al. [19]. The growth medium consisted of alpha-modified Eagle's medium (α-MEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Mediatech, Herndon, VA, USA), 1% L-glutamine and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The cultures were grown to confluency at 37 °C and 5% CO_2. Cell culture medium was replaced every 3 d. Cells obtained at passage 4 were used in this study.

[1]Certain commercial materials and equipment are identified in this paper in order to specify adequately the experimental procedure. In no case does such identification imply recommendation by the National Institute of Standards and Technology nor does it imply that the material or equipment identified is necessarily the best available for this purpose.

Surgeries were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Drexel University.

2.3. Seeding and characterization of the scaffold

To determine the number of cells in culture and also the cell density for seeding of the polymer scaffolds, the cell culture flasks were washed with HBSS and the cells removed using trypsin EDTA (Invitrogen, Carlsbad, CA, USA). The number of viable cells was counted using a hemocytometer and the cell density determined.

The 3-D fibrous braided scaffolds were sterilized with 70% alcohol and UV irradiation prior to culture. These 3-D braided scaffolds were then seeded at a cell density of 250,000-cells/scaffold for SEM and cell proliferation analysis. In addition, scaffolds used for real-time reverse transcription polymerase chain reaction (RT-PCR) were seeded at a higher cell density of 500,000-cells/scaffold. This corresponds to an initial seeding density of approximately 3571 and 7143 cells/cm^2, respectively.

Using the cell density obtained from above, the volume of solution needed to obtain 250,000 cells and 500,000 cells was determined. This volume of solution was then injected into the intraarticular zone of the polymer scaffold and incubated for 30 min at 37 °C and 5% CO_2. Following the attachment period, the scaffolds and cells cultured on tissue culture polystyrene (control) were covered with growth media and incubated at 37 °C and 5% CO_2.

Cells were seeded onto the 3-D square braided scaffolds or on tissue culture polystyrene and cultured for 3, 7, 14 and 21 d in growth medium. Cellular morphology characterization was accomplished using scanning electron microscopy (SEM) (Amray 3000, MA, 20 kV) (time points—7, 14 and 21 d) ($n = 1$). At the end of each time point, the sample was fixed in 1% glutaraldehyde for 1 h and 3% glutaraldehyde overnight at 4 °C. Samples were then dehydrated through a series of ethanol dilutions and in order to minimize charging effects, the samples were sputter-coated with gold (Denton Desk-1 Sputter Coater, NJ, USA). Explanted cells from each cell type used for the proliferation assay were cells on tissue culture polystyrene (control) and 3-D scaffolds that were lysed. The cell proliferation was determined using a commercially available cell proliferation DNA assay, Molecular Probes PicoGreen dsDNA quantification Assay ($n = 3$) (Invitrogen, Carlsbad, CA, USA). The assay was measured using a Tecan SpectroFluo Plus Plate reader (TECAN USA, Boston, MA) whose excitation wavelength was set at 460 nm and with an emission wavelength of 520 nm. Cell proliferation was determined using DNA standard curve ($r^2 = 0.99$). RT-PCR was used to examine the cellular gene expression after culturing for 14 d ($n = 3$).

2.4. RNA isolation and quantitative RT-PCR

RNA was isolated and used to characterize the gene copy number within the cells with RT-PCR. Briefly, media was removed from the tissue culture polystyrene and scaffold wells and replaced with HBSS for 10 min. The HBSS was removed and scaffolds placed into 15 mL centrifuge tubes. Trypsin was added to the centrifuge tubes and control wells. Scaffolds were placed on a shaker in a hot room at 37 °C for 30 min. After 30 min, trypsinized cell media was placed in 15 mL tubes and 7 mL of media were added to all of the centrifuge tubes to stop the reaction. The scaffolds were removed from the original centrifuge tubes and moved to microtubes to lyse the cells left on the scaffolds in 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The centrifuge tubes containing trypsinized cells and media from the scaffolds and wells were centrifuged at 157 rad/s for 5 min. The supernatant was removed and the cell pellets resuspended in 2 mL of media. The centrifuge tubes were centrifuged again at 157 rad/s for 5 min and the supernatant discarded, the cell pellets resuspended in 1 mL of media and transferred to 2 mL micro-centrifuge tubes. The samples in the microtubes were centrifuged at 209 rad/s for 5 min and media was discarded with the cells being resuspended in 1 mL of TRIzol solution (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The scaffolds were removed from the microtubes with TRIzol and the cell lysate for all samples was then passed through a pipette to ensure break up of the cell membrane. The samples were then incubated at 20–22 °C to allow for complete dissociation of any nucleoprotein complexes. Added to the samples was 0.2 mL of chloroform and the tubes were shaken for 15 s. After incubating the samples for an additional 2–3 min at 20–22 °C the samples were centrifuged at 1257 rad/s for 15 min at 4 °C. The upper aqueous phase, which contained the RNA, was removed and placed in new microtubes. The samples were then stored at −70 °C for future use.

The RNA was treated with RNA Secure (Ambion, Austin, TX, USA) and stored at −20 °C. Standard spectrophotometric measurements were taken and a 2% by mass agarose gel stained with 10 μg/mL ethidium bromide (Sigma, St. Louis, MO, USA) was used to image the RNA. Densitometry was performed using the Versa Doc imaging system (Bio-Rad, Hercules, CA, USA). The primers were designed using Primerfinder (Whitehead Institute for Biomedical Research). The primers generated were used in quantitative RT-PCR experiments (MWG-Biotech, High Point, NC, USA). The "housekeeping" gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is present in all cells was used as an internal control to normalize the data, and utilized as a marker that has an unchanged or constant level of expression in all samples. Quantitative RT-PCR was conducted to determine the gene expression levels of fibronectin, type I and type III collagen. These genes were identified as phenotypic markers for the explanted cell types utilized in this study, as all are connective tissue lineage [12,13,21]. Primers specific for type I collagen, type III collagen, fibronectin and GAPDH are shown in Table 1. The amplicons generated from these primers are 227 base pairs, 160 base pairs, 186 base pairs and 177 base pairs, respectively. DNA sequencing was performed using the Big Dye Terminator Kit (ABI, Foster City, CA, USA) on a 310 DNA Genetic Analyzer (ABI, Foster City, CA, USA).

RT-PCR was carried out using the QuantiTect SYBR Green RT-PCR Kit and protocol (Qiagen, Valencia, CA, USA) on an iCycler instrument (Bio-Rad, Hercules, CA, USA). Our protocol utilizes the following thermal parameters: reverse transcription: 30 min at 50 °C; activation step:

Table 1
Oligonucleotide sense and antisense primers used to amplify the genes listed

mRNA template	Primer sequence (sense/antisense)	PCR product size (bp)
Type I collagen	5′-CCTGGCACCCCAGGTCCTCA-3′ 5′-TCGCTCCCAGGGTTGCCATC-3′	227
Type III collagen	5′-AAGCCCCAGCAGAAAATTG-3′ 5′-TGGTGGAACAGCAAAAATCA-3′	160
Fibronectin	5′-CTCACCCGAGGCGCCACCTA-3′ 5′-TCGCTCCCACTCCTCTCCAACG-3′	186
Glyceraldehyde-3-phosphate dehydrogenase	5′-TCGGCATTGTGGAGGGGCTC-3′ 5′-TCCCGTTCAGCTCGGGGATG-3′	177

15 min at 95 °C; and three-step cycling: denaturation for 30 s at 95 °C, annealing for 1 min at 58 °C, extension for 1 min at 72 °C for 50 cycles [22,23]. A melt curve was subsequently performed to analyze the products generated, which began at 50 °C and increased to 95 in 0.5 °C increments. The RT-PCR products were run on a 2% by mass agarose gel stained with 10 μg/mL ethidium bromide (Sigma, St. Louis, MO, USA) to image for the specificity of the gene markers (not shown).

2.5. Statistical analysis

Data is represented in the form of mean ± standard deviation. An unpaired student's *t*-test was used to determine significant differences between two means. In the case of multiple comparisons, one-way analysis of variance (ANOVA) was performed on sample means to determine statistical differences between study groups. P values < 0.05 were considered statistically significant.

3. Results

The results concerning the morphology, proliferation and RT-PCR analysis are shown in Figs. 2–5. The cells proliferated gradually with incubation time and presented an upregulation of the phenotypic markers for tendon and ligament. Cell cultures grown on tissue culture polystyrene attached with PT and AT having the greatest proliferation. Cultures grown on the PLLA 3-D braided scaffolds attached at a lower number than on tissue culture polystyrene and the cells remained viable with PT and AT having the greatest proliferation. However, the cellular responses of the ACL fibroblasts were significantly different as compared to the AT, PT and MCL fibroblasts as characterized by SEM and RT-PCR.

3.1. Morphology

Observation of the cells on the 3-D braided structures by SEM showed cell attachment and coexistence of cells in different stages of spreading. The attachment morphology of cells derived from connective tissues usually progresses from spherical, spindle, sheath to confluent cell sheath [24–26].

The photomicrographs of the connective tissue fibroblasts show very different morphologies after culturing between 7 and 21 d on the 3-D braids (Fig. 2). This was

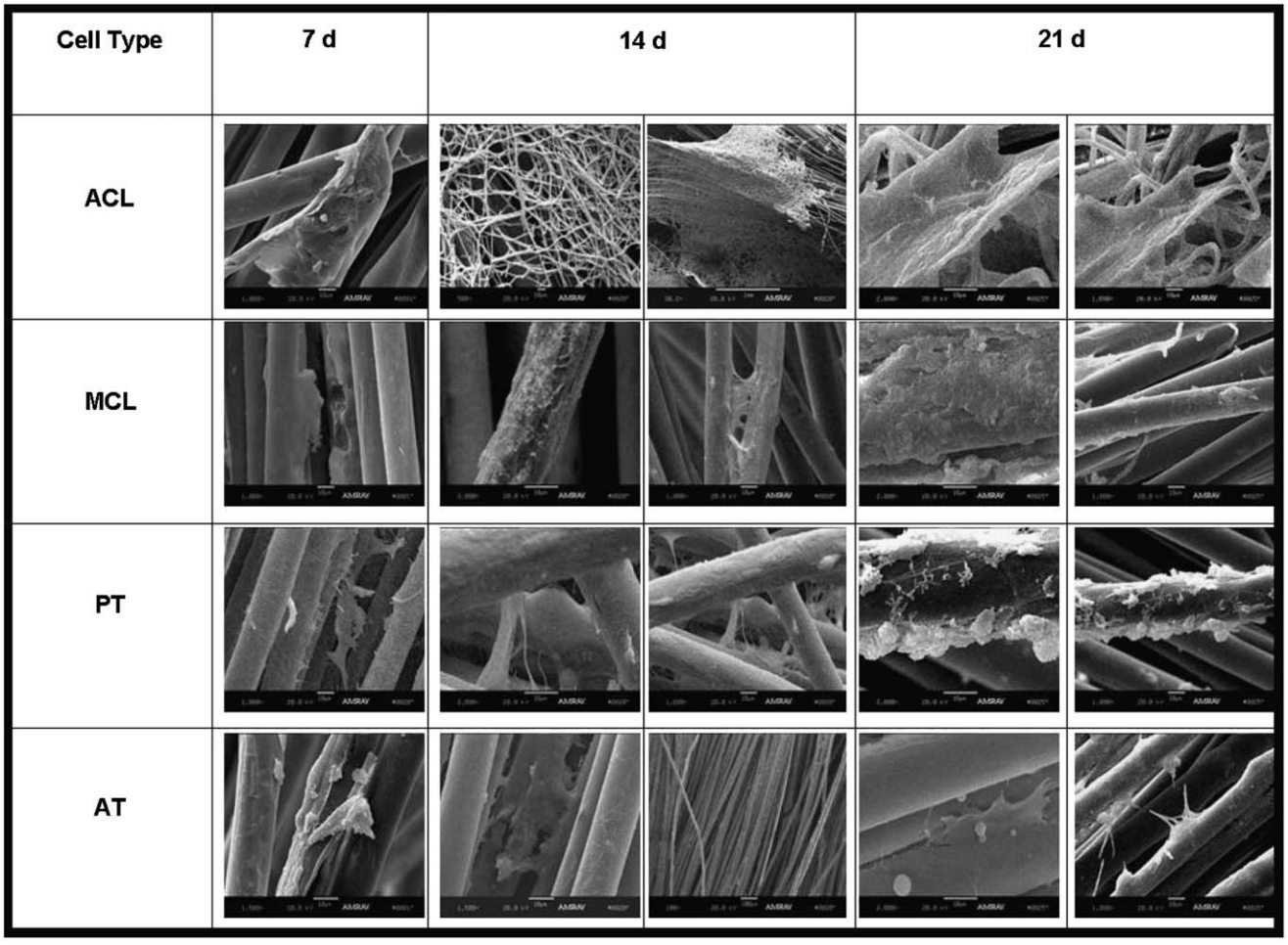

Fig. 2. SEM photomicrographs showed matrix deposited on PLLA 3-D braided scaffold by rabbit anterior cruciate ligament (ACL), medial collateral ligament (MCL), patellar tendon (PT) and achilles tendon (AT) cells after 7, 14 and 21 d in culture. The SEM photomicrographs show the random orientation of the deposited fibrous matrix because no stress was applied to the scaffold. Non-oriented matrix was also deposited on the scaffolds (ACL 14 d). Cells are also shown bridging spaces between the polymeric fibers (MCL 14 d) and wrapping around the PLLA fibers of the 3-D braids (PT 14 d).

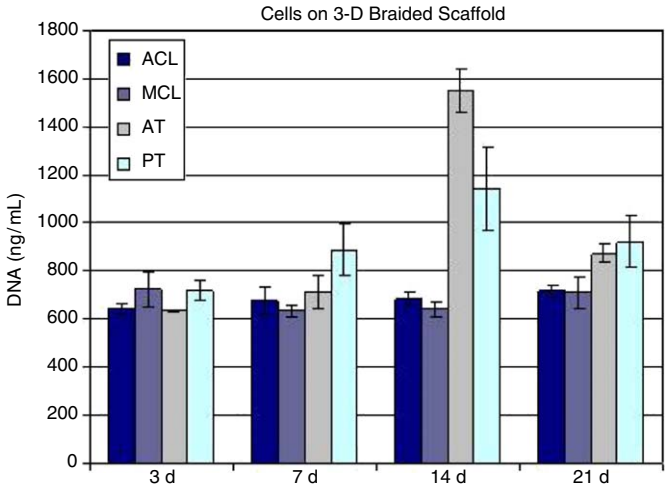

Fig. 3. The cellular proliferation after culturing for 3, 7, 14 and 21 d on 5×5 PLLA 3-D square braided scaffolds. Error bars are representations of the standard deviation from the mean of triplicate samples harvested from two separate populations and are an estimate of standard uncertainty. The temporal cell growth of the ligament cells was slower as compared to the tendon cells. In addition, the data also suggest that the initial attachment and growth of cells was much less on the 3-D PLLA scaffolds as compared to the tissue culture polystyrene (Fig. 4).

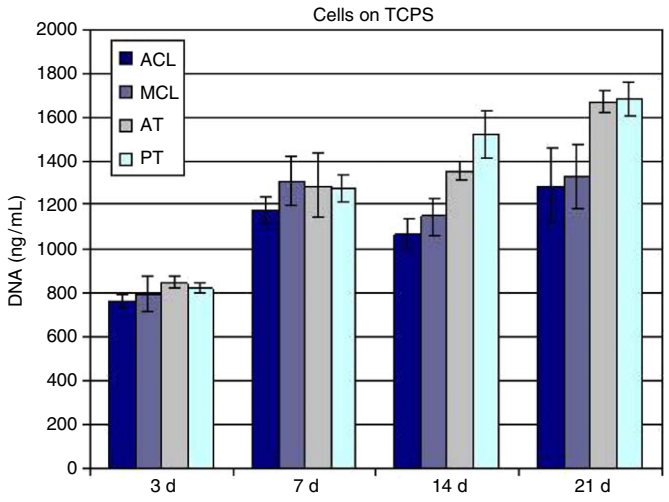

Fig. 4. The cellular proliferation after culturing for 3, 7, 14 and 21 d on tissue culture polystyrene (TCPS). Error bars are representations of the standard deviation from the mean of triplicate samples and are an estimate of standard uncertainty. The temporal cell response of ligament cell growth was slower as compared to the tendon cells.

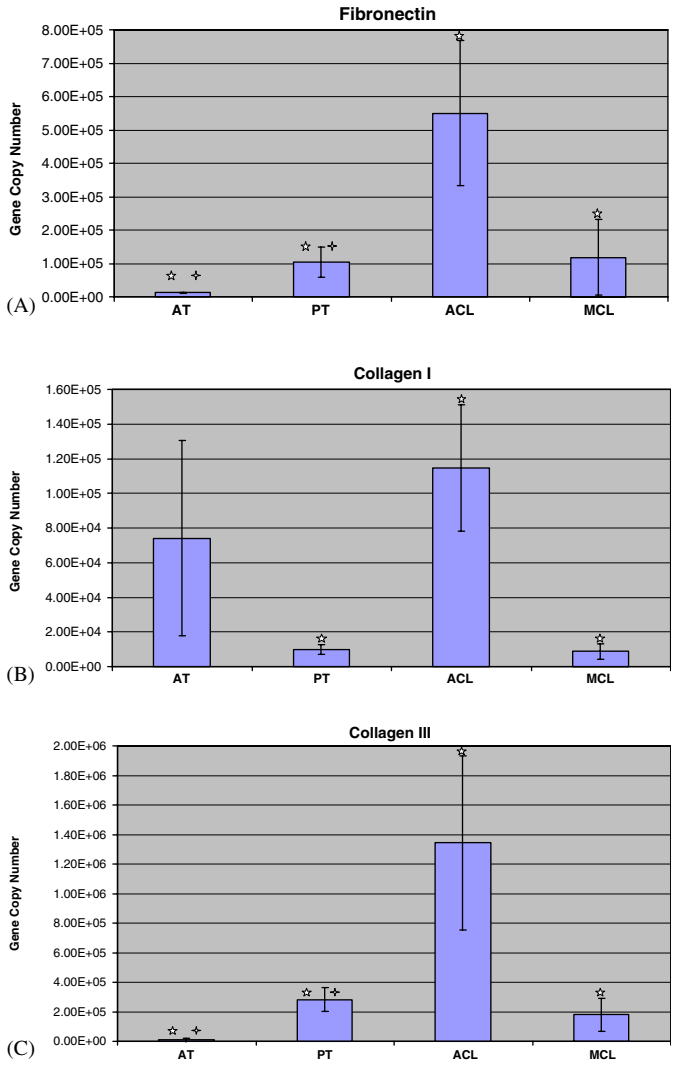

Fig. 5. Gene copy numbers for fibronectin (A), type I collagen (B) and type III collagen (C) were quantified from primary rabbit achilles tendon (AT), patellar tendon (PT), anterior cruciate ligament (ACL), and medial collateral ligament (MCL) cells. Error bars are representations of the standard deviation from the mean triplicate of cells harvested from cell populations on 3-D scaffolds after 14 d in culture and are an estimation of the standard uncertainty. Samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for the specific cell type. Results showed the significantly greater upregulation of all genes within the ACL fibroblasts as compared to the other fibroblast types (☆-significant difference with ACL and ✦-significant difference between AT and PT ($p < .05$)).

partly due to the manner in which the samples were seeded by injecting the cells approximately 1 mm below the outside surface of the 3-D braided scaffold, a technique to be used later when seeding the scaffolds for in vivo implantation. As a result, the cells are required to proliferate from the inner scaffold toward the outside surface to be readily visible with the SEM. Initial observation of cell morphologies on the surface of the filaments was not easily achieved for the early time points. The fibroblast morphology that was observed at the surface of the scaffold was spindle and sheath cells along the filaments [24–26]. At the 14 d time point the majority of the cells continued to attach to the scaffolds with spindle and sheath cells wrapped around the filaments with cellular projections bridging spaces between filaments (Fig. 2). In addition, at the 14 d time point the ACL fibroblasts are producing filamentous structures along the geometry of the scaffold (Fig. 2). These unstressed fibrils show a random orientation on the 3-D braided structures. The SEM photomicrographs at 21 d show spherical (PT and AT cells), spindle (PT, AT and MCL) and sheath (ACL and MCL) cells along the filaments of the scaffolds (Fig. 2). The ACL fibroblasts

continued to form filamentous structures with random orientation. The connective tissue cells in general exhibited semi-ovoid, fibroblast-like morphology with specific growth orientations which was attributed to the cell type and 3-D geometry of the braided scaffold.

3.2. Proliferation

The goal of the cell proliferation assay within this study was to evaluate cell proliferation over 21 d to gain a preliminary assessment of the cellular response to the 3-D geometry of the braided scaffold. Results of the proliferation assay show that all four of the connective tissue fibroblast types seeded on tissue culture polystyrene showed higher proliferation when compared to those seeded on the scaffold. At day 3, there were a greater number of cells on the tissue culture polystyrene samples than on the scaffold. This may be due to the porous nature of the scaffold and the hydrophobicity of the polymer. The proliferation of fibroblasts on the 3-D braids showed a significant increase in cell number for ACL ($p<0.002$), MCL ($p<0.002$), AT ($p<0.0001$) and PT ($p<0.0001$) between the 3 and 21 d time points (Fig. 3). The PT and AT had the greatest proliferation followed by the ACL and MCL fibroblasts. In addition, there was a significant decrease in PT ($p<0.004$) and AT ($p<0.0001$) fibroblasts on the 3-D braid between 14 and 21 d as compared to the other fibroblast types which may be due to the cells not receiving enough nutrients. The study of fibroblasts cultured on the tissue culture polystyrene showed the significant proliferation of ACL ($p<0.0001$), MCL ($p<0.0001$), AT ($p<0.0001$) and PT ($p<0.0001$) fibroblasts between the 3 and 21 d time points (Fig. 4). The PT and AT fibroblasts had the greatest proliferation followed by the MCL and ACL fibroblasts. Overall the tendon fibroblasts proliferated faster than the ligament fibroblasts regardless of the material and geometry.

3.3. RT-PCR

RT-PCR analysis of ACL RNA, MCL RNA, AT RNA and PT RNA were performed to identify gene expression profiles. The bar graphs denoting the gene expression of fibronectin, type I and type III collagen are shown for AT, PT, ACL and MCL cells after 14 d of culturing on 3-D braided scaffolds (Fig. 5A–C). The RT-PCR demonstrated significant gene expression profile differences between the fibroblast types for the gene expression of fibronectin ($p<0.001$), type I collagen ($p<0.009$) and type III collagen ($p<0.003$). The gene expression profile for fibronectin showed that ACL fibroblasts were upregulated significantly greater than AT ($p<0.02$), PT ($p<0.03$) and MCL ($p<0.02$) fibroblasts. The gene expression profile for type I collagen showed that ACL fibroblasts were upregulated significantly greater than PT ($p<0.003$) and MCL ($p<0.003$) fibroblasts while there was no significant difference in gene expression between ACL and AT fibroblasts. The gene expression profile for type III collagen showed that ACL fibroblasts were upregulated significantly greater than PT ($p<0.04$), AT ($p<0.02$) and MCL ($p<0.04$) fibroblasts. The only other significant differences between fibroblast types were shown for PT and AT fibroblasts for the gene expression of fibronectin ($p<0.01$) and type III collagen ($p<0.006$). These findings indicate that ACL fibroblasts as compared to the PT, AT and MCL fibroblasts showed significantly greater upregulation of gene expression for the ligament differentiation markers needed for the development of a tissue-engineered ligament.

4. Discussion

Critical barriers to the development of tissue-engineered ligament grafts in vitro are cell source, limited transport of nutrients, reduced cell viability and activity within 3-D scaffolds. In the present study, a 3-D PLLA braided scaffold was used to investigate cell activity and proliferation of four possible cell sources for tissue engineering ligament. It was observed that the fibroblast cell source had a significant effect on the cellular response on the 3-D braided scaffold. These fibroblast cell sources resulted in differences in cell attachment, morphology, proliferation and gene expression. Findings from this study highlight the importance of cell source selection in tissue-engineering applications and its potential impact on the success of an implant.

In this study, primary rabbit connective tissue fibroblasts (ACL, MCL, AT and PT) were found to attach and proliferate on the 3-D PLLA 5×5 biodegradable braided scaffolds. The 3-D square braided scaffold design promoted the adhesion and growth of all fibroblast types along the longitudinal axis of the fibers. The fibroblasts appeared to attach with spherical shaped morphology initially, and later as the cells spread showed a spindle shaped and sheath morphology with a greater number of sheath cells present at 21 d. This is consistent with results previously obtained by Ricci et al. who studied connective tissue fibroblast attachment on non-biodegradable fibers [24–26]. This study showed that tendon fibroblasts had the highest proliferation on the 3-D PLLA scaffolds as compared to the ligament fibroblast types. The ACL fibroblasts produced filamentous structures that have been identified as unstressed fibers by Nordin et al. at the 14 d time point [27]. There have been previous studies that have shown ACL proliferation and migration rates are slower than that of MCL fibroblasts under various conditions [28–30]. Our results confirmed the cellular proliferation behaviors of ACL and MCL fibroblasts on tissue culture polystyrene, but the ACL and MCL fibroblasts show a reversed cellular response when proliferating on 3-D braided scaffolds. The cellular response showed a significantly increased proliferation of ACL fibroblasts as compared to MCL fibroblasts at 7 and 14 d, whereas no significant difference is found for the DNA concentration

at 21 d. All the primary connective tissue fibroblasts on the 3-D braided geometry expressed genes associated with ligament differentiation [11,12]. There is no generally established principle to explain or predict the extent of adhesion, proliferation and spreading of cultured fibroblasts on 3-D scaffold structures. Therefore more studies are necessary to investigate the various cellular types and the cellular responses to various shaped scaffolds before any conclusions can be drawn. The information obtained from this study has shown that all of the connective tissue fibroblasts that are candidates for tissue-engineered ACL replacement remain viable and proliferate on the 3-D braided structures in vitro.

We have demonstrated that 3-D geometry of the scaffold can affect growth and differentiation of fibroblasts from four types of connective tissue sources. We show how the preferred differentiation paths of non-ACL fibroblasts diverged from those of the ACL. ACL fibroblasts formed fibers on the 3-D scaffolds which were absent in the matrix of the other fibroblast types. The fibers secreted by the ACL fibroblasts were non-oriented because the scaffolds are cultured under conditions with no dynamic stress applied on the scaffolds. This was important to show that the cells could produce a fibrous matrix due to 3-D geometry without the need of mechanical cues. This would be necessary in cases where a bioreactor is not used for cell seeding the scaffold before implantation. The role of 3-D fibrous architecture clearly has an effect on the differentiation of cells. It was demonstrated through the matrix produced and the genes expressed to foster differentiation. Based on the higher upregulation of gene expression of type III collagen as compared to type I collagen we know that the ACL fibroblasts are responding to the 3-D geometry of the braided scaffold. During the early phases of ligament healing and remodeling more type III collagen is produced than type I collagen [14,31,32]. Therefore the greater upregulation of type III collagen as compared to type I collagen is a positive indicator that the cells are secreting signals for the healing and remodeling process. The greater upregulation of type III collagen as compared to type I collagen is not surprising because we placed no mechanical loads on the scaffold. Within native ACL tissue the presence of type I collagen produced by the fibroblast is closely related to tensile strength [33–35]. The cellular response is totally due to the 3-D geometry of the scaffold, in which the cells have recognized that their environment is not under loading conditions and so they have upregulated the genes to compensate for an unstressed environment. It is reasonable to speculate that the 3-D geometry sends signals that may trigger cell-surface receptors and adhesion sites that upregulate the genes responsible for the synthesis and secretion of the key ligament extracellular matrix components. Also the rates of mass transfer of nutrients and oxygen can be affected by the geometry and porosity of the scaffold. Therefore, more studies associated with chemical, topographical and mechanical signals as well as porosity for nutrient transport are needed to discover the sequence of events that allow for differentiation and tissue formation.

Jackson et al. have reported the use of DNA-probe analysis in a goat model which has shown that fresh allografts implanted for ACL reconstruction had the donor DNA completely replaced by host DNA [18]. The donor cells in these allografts used to reconstruct the ACL did not survive and they reported that the host cells that repopulated the allografts assumed histologic similarity to the fibroblasts they replaced. In another study by Bellincampi et al. the donor cells in fibroblast seeded ligament analogs placed in the intrasynovial joint of a rabbit model decreased dramatically after 2 weeks [36]. Therefore, an assessment of the fate of donor cells should be characterized for any seeded tissue engineering application so that researchers can fully understand the source of the new matrix production.

The mRNA expression of fibronectin, type I and type III collagen were all expressed by the fibroblast types as the typical markers for differentiation, but the ACL fibroblasts had a statistically significant upregulation of gene expression as compared to the other fibroblast types. Donor cells significantly decline within 2 weeks of implantation and are replaced by host cells [18,37]. Therefore, proliferation and collagen production should not be the main criteria for cell source selection in tissue engineering ligament as has been suggested by Van Eijk et al. [38]. Differentiation should also be included as a specific criterion for cell source selection because differentiated cells will secrete the matrix and cellular signals to guide the infiltrating host cells as host fibroblasts and macrophages will replace any cells on the seeded scaffolds after implantation.

5. Conclusion

In this study, achilles tendon (AT), patellar tendon (PT), medial collateral ligament (MCL) and anterior cruciate ligament (ACL) fibroblast cellular response to a novel braided scaffold for ACL tissue engineering was found to be dependent on cell source. Although the PT, AT, ACL and MCL fibroblasts show similar appearance in morphology in culture on tissue culture polystyrene, the cellular growth in vitro was different on 3-D braided structures. The scaffold had an effect on the matrix production by ACL fibroblasts by causing them to produce a filamentous matrix which was not seen in our previous studies on the PLLA scaffolds [5].

Based on cell morphology, matrix production and gene expression, the primary ACL fibroblasts are most suited for ACL tissue engineering as compared to PT, AT and MCL fibroblasts. The differences in intrinsic properties of the fibroblast types in vitro might contribute to the differential healing potentials of these ligaments and tendons in vivo. In addition, there may be other factors during in vivo implantation such as donor cell survival, synovial fluid and dynamic stress that could contribute to different cellular responses. Therefore, our future studies

will focus on using ACL cells as the source for seeded tissue engineered ligaments for ACL reconstructions in vivo in a rabbit model.

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