Use of Human Placenta-Derived Cells in a Preclinical Model of Tendon Injury

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Background: Emerging data suggest that human cells derived from extraembryonic tissues may have favorable musculoskeletal repair properties. The purpose of this study was to determine whether the injection of human placenta-derived mesenchymal-like stromal cells, termed *placental expanded cells (PLX-PAD),* would improve tendon healing in a preclinical model of tendinopathy.

Methods: Sixty male Sprague-Dawley rats underwent bilateral patellar tendon injection with either saline solution (control) or PLX-PAD cells (2×10^6 cells/100 μL) 6 days after collagenase injection to induce tendon degeneration. Animals were killed at specific time points for biomechanical, histological, and gene expression analyses of the healing patellar tendons.

Results: Biomechanical testing 2 weeks after the collagenase injury demonstrated better biomechanical properties in the tendons treated with PLX-PAD cells. The load to failure of the PLX-PAD-treated tendons was higher than that of the saline-solution-treated controls at 2 weeks (77.01 ± 10.51 versus 58.87 ± 11.97 N, p = 0.01). There was no significant difference between the 2 groups at 4 weeks. There were no differences in stiffness at either time point. Semiquantitative histological analysis demonstrated no significant differences in collagen organization or cellularity between the PLX-PAD and saline-solution-treated tendons. Gene expression analysis demonstrated higher levels of interleukin-1β (IL-1β) and IL-6 early in the healing process in the PLX-PAD-treated tendons.

Conclusions: Human placenta-derived cell therapy induced an early inflammatory response and a transient beneficial effect on tendon failure load in a model of collagenase-induced tendon degeneration.

Clinical Relevance: Human extraembryonic tissues, such as the placenta, are an emerging source of cells for musculoskeletal repair and may hold promise as a point-of-care cell therapy for tendon injuries.

Tendon injuries are common and affect individuals of all ages. While activity modification, nonsteroidal anti-inflammatory drugs, and physical therapy are the mainstay treatments, many cases are refractory to these standard modalities. In those instances, corticosteroid injections have traditionally produced good short-term results. However, data have shown a lack of sustained therapeutic benefit and possible deleterious effects on several musculoskeletal tissues[1-4]. Therefore, there is growing interest in biological therapies that may augment tendon healing.

An emerging source of cells with favorable musculoskeletal regenerative characteristics is human extraembryonic tissue[5-9]. Unlike adipose-derived cells or bone marrow, cells isolated from extraembryonic tissues, such as the placenta, are not associated with the morbidity caused by harvesting procedures since the placenta is typically discarded. Cells isolated from human extraembryonic tissues have been shown to have attractive healing properties[5,10,11]. Human placenta-derived mesenchymal-like stromal cells display typical mesenchymal stem cell (MSC) markers (CD105, CD73, and CD29) and do not express hematopoietic markers (CD45, CD19, CD14, and HLA-DR) or the endothelial cell marker CD31[12,13]. In vitro and in vivo studies have suggested that placental expanded cells (PLX-PAD; Pluristem Therapeutics) display immunomodulatory and

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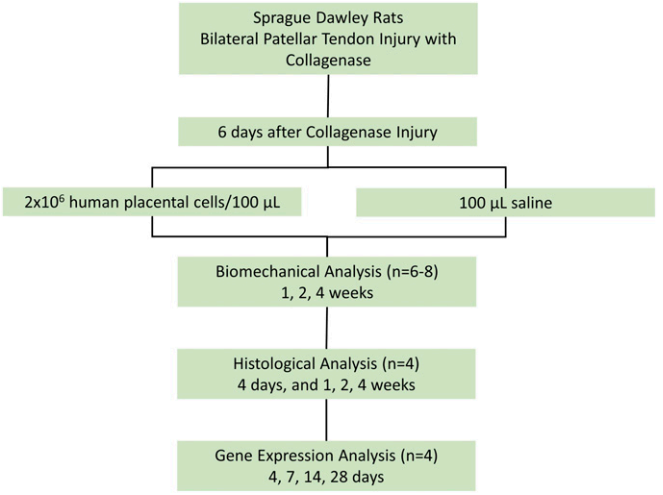

Fig. 1
Experimental layout.

pro-angiogenic properties via secretion of growth factors and cytokines such as vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6)[12,14]. As a result, these cells have been used in completed clinical studies for treating critical limb ischemia and gluteal muscle regeneration after total hip arthroplasty[15,16].

This study was conducted to evaluate the use of PLX-PAD cells for the treatment of tendinopathy. We are not aware of any previous preclinical studies of the application of cells derived from human placenta for tendon injuries. Our hypothesis was that placenta-derived cell therapy would have a beneficial therapeutic effect on tendinopathy as demonstrated by biomechanical and histological analyses following treatment.

Materials and Methods

Sixty male Sprague-Dawley rats (weight, 300 to 350 g; Harlan Laboratories) were used under an Institutional Animal Care and Use Committee-approved protocol (Fig. 1). An immunocompetent species was selected because previous experiments demonstrated that human placenta-derived cells do not elicit a relevant immunohistocompatibility response in other species[13]. All animals underwent bilateral intratendinous injection of bacterial collagenase (250 U of type-II collagenase [Sigma-Aldrich Chemical] in 0.3 mL of normal saline solution[17]) into the patellar tendon via a mini-open incision to induce tendon degeneration (Fig. 2). Six days after the collagenase injection, the control treatment (100 µL of 0.9% normal saline solution) was randomly assigned to 1 limb of an animal and the contralateral knee received the experimental treatment (2×10^6 PLX-PAD cells/100 µL).

Preparation of PLX-PAD Cells

Human PLX-PAD cells are adherent stromal cells isolated from full-term human placentas. They are cultured and undergo a 3-dimensional growth phase in a closed bioreactor system[13]. The PLX-PAD cells are then cryopreserved in liquid nitrogen as an allogenic "off-the-shelf" product until use. PLX-PAD cells are of maternal origin, share the classic MSC membrane markers, and are limited in their differentiation potential in vitro[18].

For this study, PLX-PAD cells were prepared in accordance with the manufacturer-recommended protocol prior to injection[13]. A cell suspension was created with PLASMA-LYTE A solution (Baxter) to achieve a final concentration of 2×10^6 human PLX-PAD cells/100 µL. The dosage of PLX-PAD cells per animal was based on prior work using a smaller mouse model (1×10^6 cells/animal)[13] and the range of dosages used in previous rat tendon healing studies (1 to 3 $\times 10^6$ cells per animal)[19-23].

Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-SE) Labeling of PLX-PAD Cells for Fluorescent Microscopy

To track them after injection, the PLX-PAD cells were labeled with 3 µg of CFDA-SE (Biotium [catalog #30050]) dissolved in 1 mL of dimethyl sulfoxide (DMSO) and 999 µL of phosphate-buffered saline solution. The PLX-PAD cells were then resuspended in 2 mL of the CFDA-SE solution and incubated for 10 minutes at 37°C. A cell pellet was then isolated through centrifugation and resuspended with PLASMA-LYTE A solution to achieve a final cell concentration of 2×10^6 CFDA-SE-labeled PLX-PAD cells/100 µL.

Biomechanical Analysis

After euthanasia, the patella as well as the patellar tendon and its proximal tibial attachment were isolated. Specimens were mounted onto a custom materials-testing system that ensured that tension was aligned along the long axis of the tendon. The

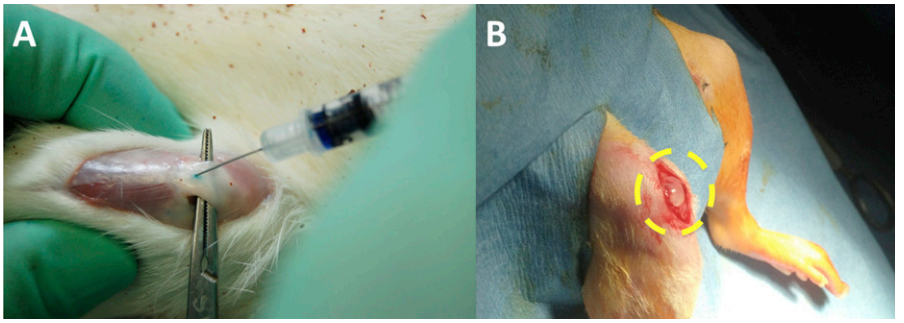

Fig. 2
Fig. 2-A Intratendinous injection of collagenase. **Fig. 2-B** Injection of the patellar tendon with PLX-PAD cells (yellow circle).

specimens were preconditioned with a preload of 1 N for 5 cycles and then loaded in uniaxial tension at a rate of 1 mm/min until failure. Load to failure (N) and stiffness (N/mm) were determined from the linear portion of the load-displacement curve.

Histological Analysis

The patellar tendons were embedded in Optimum Cutting Temperature (O.C.T.) compound (Tissue-Tek) and frozen in liquid nitrogen for fluorescent microscopy. Cryostat sections were visualized under fluorescent microscopy (Nikon Instruments). In order to discern tissue architecture, sections were fixed with 0.4% paraformaldehyde and stained with hematoxylin and eosin. Immunohistochemical staining for human anti-CD29 was also performed to localize the PLX-PAD cells within tendon tissue. The specimens were treated with 3% H_2O_2 and then incubated with anti-CD29 antibody (BioLegend) for 1 hour at room temperature.

For light and polarized light microscopy, specimens were harvested and were fixed in 10% formalin. Samples were decalcified (Immunocal; StatLab) and embedded. Sequential sagittal 5-μm sections of the entire patellar tendon were stained with hematoxylin and eosin and picrosirius red. The slides were examined using light and polarized light microscopy (Eclipse E800; Nikon). Digital images were captured (Diagnostic Instruments). Collagen organization was evaluated using picrosirius-red-stained sections under polarized light microscopy; these images were assessed for tissue birefringence[14,15]. Semiquantitative analysis of picrosirius-red-stained photomicrographic slides was performed with MATLAB (MathWorks).

Photomicrographs made under 2× and 10× magnification were then imported into ImageJ (National Institutes of Health). Histological measurements were performed within the tendinosis region. The illumination and detection parameters of the microscope were kept constant between specimens to allow direct comparisons. Quantitative measurements were made for total cell number (10× magnification), the area of abnormal tendinosis tissue (2×), and change in polarization brightness following bidirectional rotation (10×) as a measure of tendon collagen organization[24,25]. The number of cells was quantified using manual thresholding with ImageJ and conversion of images of hematoxylin and eosin-stained specimens to binary. Particle analysis was then performed to count the cells within the area of tendinosis. The tendon healing areas were measured after thresholding of each image. Collagen fibril organization was evaluated with picrosirius red staining under polarized light. A bidirectional analysis was performed using a custom-made stage rotator. Photographs were made in the 0° position (defined as a position in which the longitudinal axis of the patellar tendon was horizontal) and in −45° and +45° of rotation under 10× magnification[24,25]. Mean signal intensity (brightness) was measured in grayscale in each position, and brightness change as a measure of collagen organization was calculated. A greater brightness change indicates superior collagen organization[24,25].

Gene Expression Analysis

At 4, 7, 14, and 28 days following treatment, the entire patellar tendon was harvested. Total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific). Equal concentrations of mRNA (messenger RNA) were reverse transcribed using the Bio-Rad iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's suggested protocol. The cDNA (complementary DNA) products were amplified and quantified through reverse transcription polymerase chain reaction (RT-PCR) using iQ SYBR Green Supermix on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). All reactions were cycled 40 times in triplicate. Relative expression levels were

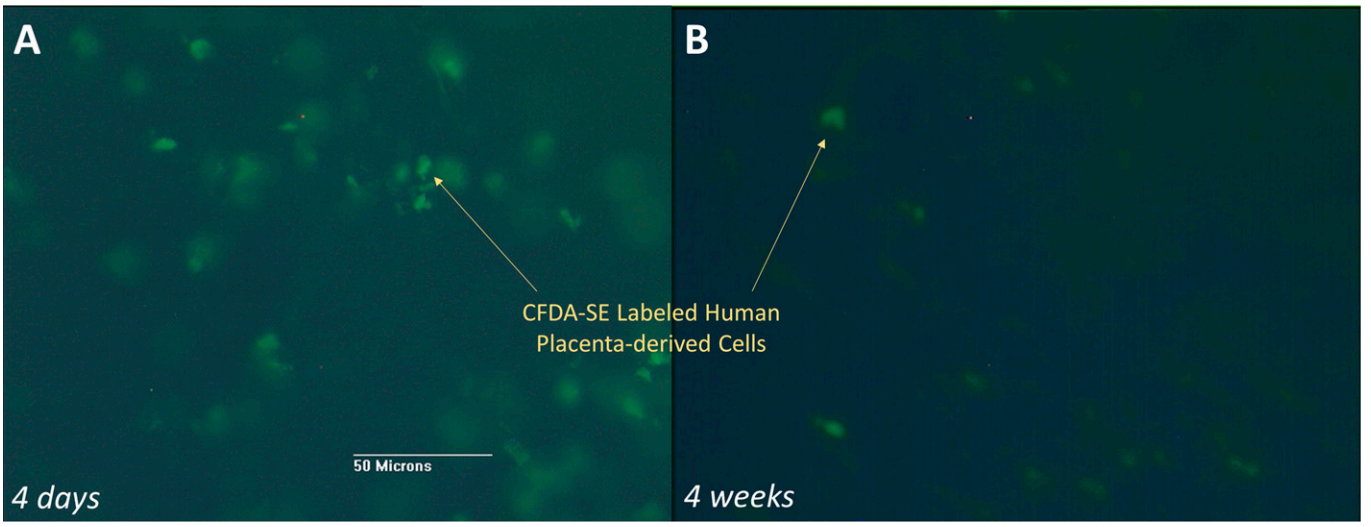

Fig. 3

Fluorescent microscopy of CFDA-SE-labeled PLX-PAD cells (green circles) in patellar tendon tissue. There was a greater prevalence of fluorescent cell signals at 4 days (**Fig. 3-A**) than at 4 weeks (**Fig. 3-B**).

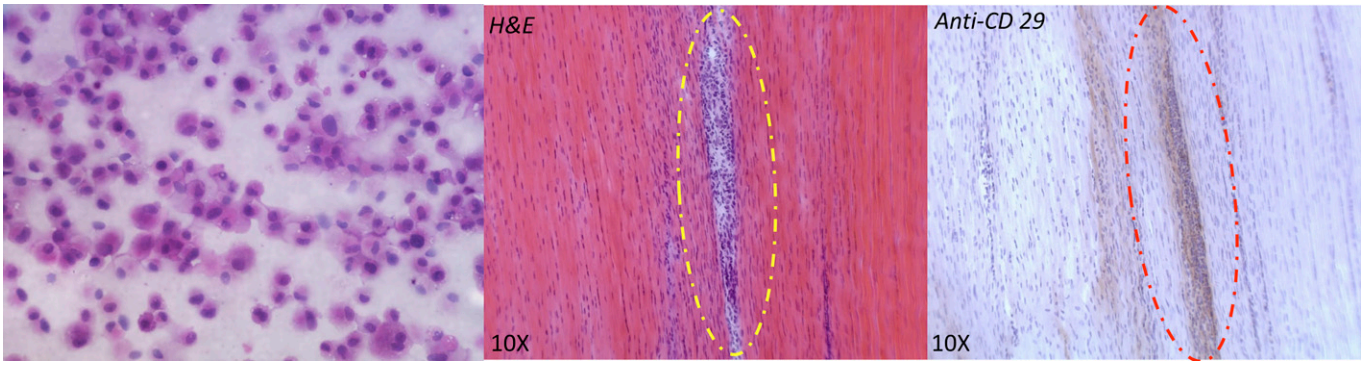

Fig. 4

PLX-PAD cells in isolation (**left**). Note the cluster of PLX-PAD cells within patellar tendon collagen fibers at 4 weeks (**middle**, yellow oval). The cluster of cells was further confirmed with anti-CD29 immunostaining identifying it as human PLX-PAD cells (**right**, red oval). H&E = hematoxylin and eosin.

calculated on the basis of ΔCT values (difference between the cycle threshold of the gene of interest and the housekeeping gene GAPDH [glyceraldehyde 3-phosphate dehydrogenase]). Primers were designed for rodent GAPDH and the following markers of interest: type-I collagen (Col1a2; GenBank NM_053356.1), type-III collagen (Col3a1; GenBank NM_032085.1), IL-1β (GenBank NM_031512.2), IL-6 (GenBank NM_012589.1), basic fibroblast growth factor (bFGF; GenBank NM_019305.2), VEGF (GenBank NM_031836.2), and transforming growth factor (TGF)-β1 (GenBank NM_021578.2).

Statistical Analysis

Biomechanical, semiquantitative histological, and gene expression data were expressed as means and standard deviations. Biomechanical testing results (load to failure and stiffness) were compared between the experimental group and the control group at each time point using a 2-sided Wilcoxon rank sum test. Within-group comparisons (1 week versus 2 weeks and 4 weeks) were also made. A 2-way analysis of variance (ANOVA) with Tukey multiple comparison analysis was performed on cell count, area of tendinosis, and brightness change. A Student t test was performed on the PCR data. The level of significance was set at $p < 0.05$.

Results

Gross Inspection of Tendons

The patellar tendons in both groups appeared inflamed at 1 and 2 weeks. They appeared thickened and fibrotic, consistent with degeneration and an early healing response. The gross appearance and caliber of the tendons began to resemble normal at 4 weeks in both the control and the experimental group but did not return to the baseline appearance of an uninjured patellar tendon in either group. No gross differences

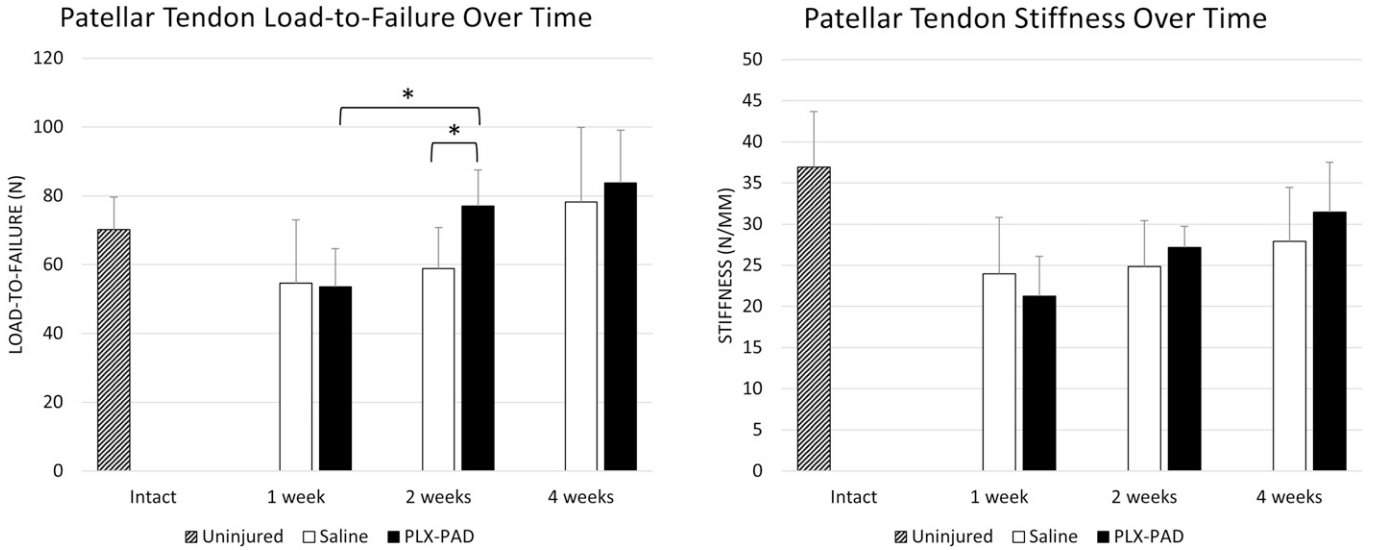

Fig. 5

Biomechanical analysis of patellar tendons treated with PLX-PAD demonstrated greater load-to-failure properties at 2 weeks compared with saline-solution-treated controls. The difference dissipated by 4 weeks. The mean load to failure of uninjured patellar tendons is represented on the left. The error bars represent the standard deviation. *$P < 0.05$.

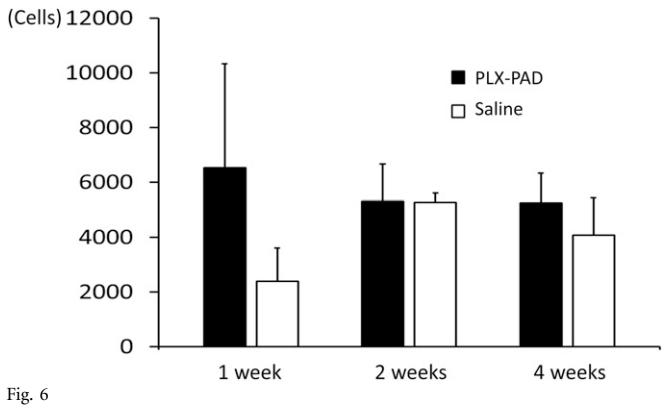

Fig. 6

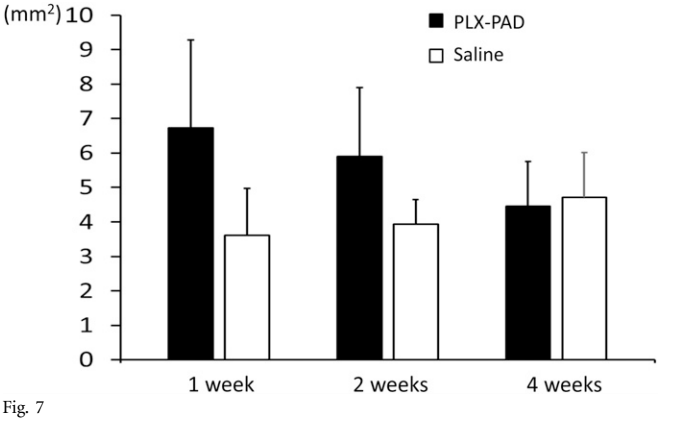

Fig. 7

Fig. 6 Total cell counts within the affected area of tendons treated with PLX-PAD or saline solution. The error bars represent the standard deviation. **Fig. 7** Quantitative measurement of the area of tendinosis (mm^2) in injured patellar tendons after PLX-PAD or saline solution injection. The error bars represent the standard deviation.

in terms of tissue inflammation severity or adverse soft-tissue reaction were seen between the 2 groups at any time point.

Viability of Placenta-Derived Cells After Injection

Fluorescent signals indicative of CFDA-SE-labeled PLX-PAD cells were abundant in the tendons at 4 days after the injection and were still seen at 4 weeks. The prevalence of signals decreased over time (Fig. 3). Stained sequential frozen sections demonstrated darkly nucleated cells interspersed between the collagen fibers of the patellar tendon. Human anti-CD29 immunostaining of the tendon tissues further confirmed the presence of PLX-PAD cells at 4 weeks after the injection (Fig. 4).

Load to Failure and Stiffness

The experimental group demonstrated significantly greater load to failure at 2 weeks (77.01 ± 10.51 N) compared with the control group (58.87 ± 11.97 N; p = 0.01) (Fig. 5), and it was also greater than that of an uninjured tendon (70.23 ±

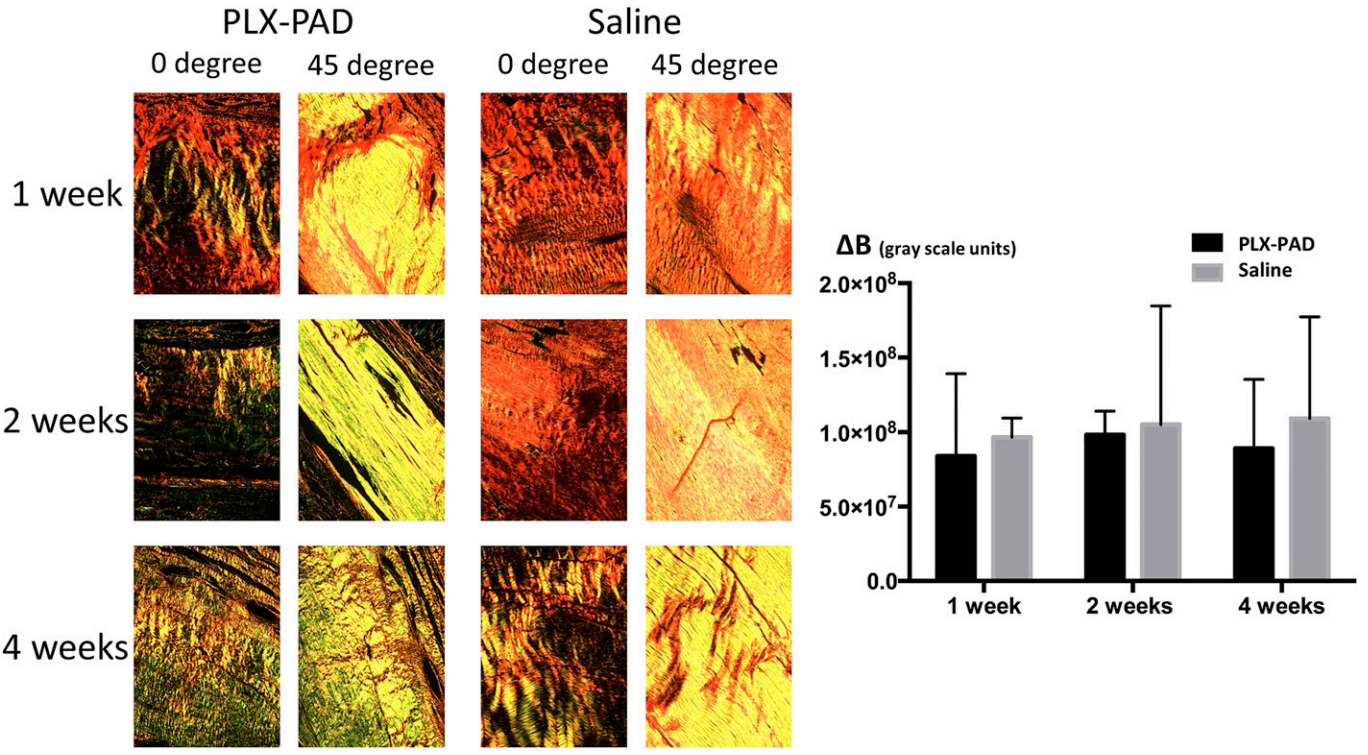

Fig. 8

Polarized light microscopy of patellar tendon specimens at 0° and 45° of rotation at 10× magnification. There were no significant differences in collagen organization as measured by brightness changes (ΔB) between the PLX-PAD and saline solution groups during the experiments. The error bars represent the standard deviation.

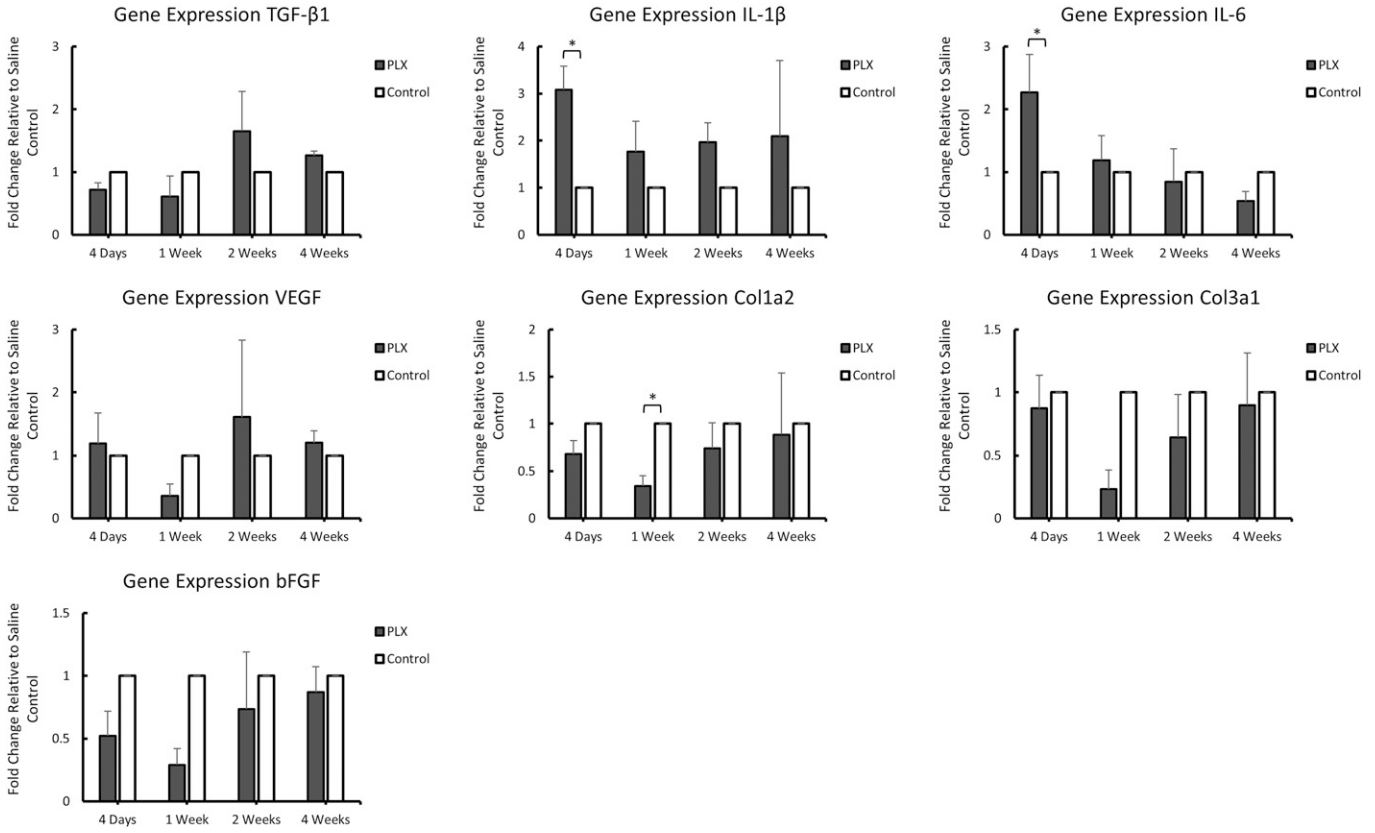

Fig. 9

Gene expression in injured patellar tendons after treatment with PLX-PAD therapy or saline solution. *P < 0.05. The error bars represent the standard deviation.

9.36 N), although that difference was not statistically significant. The tendons treated with PLX-PAD cells exhibited a significant increase in the load to failure between 1 and 2 weeks (53.52 ± 11.11 versus 77.01 ± 10.51 N, $p < 0.002$), suggesting greater interval healing during this period; the saline solution-treated tendons did not display similar improvement between 1 and 2 weeks (54.64 ± 18.38 versus 58.87 ± 11.97 N, $p = 0.57$). While tendons treated with PLX-PAD cells had a higher mean load to failure (83.74 ± 15.34 versus 78.19 ± 21.74 N in the control group) and stiffness (31.44 ± 6.06 versus 27.91 ± 6.57 N/mm) at 4 weeks, the differences were not significant.

Histological Analysis

The patellar tendons became hypercellular and disorganized as a result of the collagenase-induced degeneration. While there was higher cellularity in the tendons treated with PLX-PAD than in the controls at 1 week, the cell counts did not differ significantly between the 2 groups at any time point (Fig. 6). There was a trend toward a greater area of tendinosis in the PLX-PAD-treated tendons at 1 and 2 weeks, but this also did not reach significance at any time point (Fig. 7). Finally, there was no significant difference in collagen organization as measured by brightness change on polarized light birefringence between the groups at any time point (Fig. 8).

Gene Expression Analysis

Patellar tendons treated with PLX-PAD cells demonstrated a different early inflammatory gene expression profile, including increased levels of IL-1β and IL-6 at 4 days, compared with the controls (Fig. 9). At 1 week, the control tendons had greater type-I collagen gene expression ($p = 0.02$) and a trend toward greater type-III collagen gene expression ($p = 0.06$). We did not observe significant differences in bFGF, VEGF, or TGF-β1 expression between the 2 groups.

Discussion

The human placenta is an emerging source of reparative cells. In this study, we found that PLX-PAD had a modest effect on early tendon healing following collagenase-induced tendinopathy. We believe that this is a notable finding, as a robust healing response is expected in this model. A unique consideration is that the collagenase injection is essentially an acute tendon injury that initiates a vigorous healing response in rats. The presence of an active biological healing process would make it harder to demonstrate acceleration of healing. The animal model that we used in our study has been widely utilized to evaluate various tendon repair therapies; however, it should be viewed as a model of acute tendon injury and repair rather than a simulation of chronic tendinopathy[26-30].

Prather et al. demonstrated that human PLX-PAD cells have pro-angiogenic effects that result in greater blood flow and capillary formation[13]. Furthermore, these cells appear to be immunoprivileged and require no histocompatibility matching prior to administration, which is important in allogenic cell therapies. The combination of pro-angiogenic properties and lack of histocompatibility concerns makes PLX-PAD cells an attractive treatment option for tendon disorders. To our knowledge, this study is the first to evaluate the application of such cells in tendon injuries.

On the basis of our findings, we hypothesize that PLX-PAD cells modulate tendon healing via their effect on the inflammatory cascade, which is necessary to start the injury-repair continuum. At early time points, we found higher levels of IL-1β and IL-6 in PLX-PAD-treated tendons. Pro-inflammatory cytokines such as IL-1β are important in promoting prostaglandin synthesis and vasodilation, which play a role in inflammatory cell chemotaxis and initiation of the tendon reparative process[31,32]. It is well-established that inflammation can lead to new collagen formation (i.e., fibrosis). IL-6 has an important role in activating the immune system, has both pro-inflammatory and anti-inflammatory properties[33], and promotes collagen synthesis[31,32,34]. The importance of IL-6 in tendon healing has been demonstrated in IL-6 knockout mice, which have inferior tendon properties after repair[35,36]. Successful tissue healing after injury likely requires a complex interplay between varying levels of pro-inflammatory and anti-inflammatory mediators.

Differences in the early inflammatory cascade may have resulted in the early improvement in the load to failure of the tendons treated with PLX-PAD cells. Combined with the histological data that demonstrated an early trend toward greater areas of tendinosis in PLX-PAD-treated tendons, the cumulative data may indicate a more exuberant fibrotic scar response in those tendons. The fibrotic tissue likely therefore accounts for the early modest biomechanical difference at 2 weeks after treatment with PLX-PAD cells. Future work to measure the cross-sectional area of the treated tendons may further corroborate these findings.

The observed differences between the PLX-PAD and saline solution groups in this study dissipated by 4 weeks after treatment. The lack of sustained benefit at 4 weeks likely reflects both the rat's innate exuberant healing response and the decreasing presence of PLX-PAD cells within the tendon. The decrease in fluorescent signals over the study period likely reflects the cells' lack of capacity for self-renewal. This finding appears consistent with previous biodistribution data that demonstrated that PLX-PAD cells were present 3 weeks after implantation in mice[13]. The role of additional injections of PLX-PAD cells to maintain the early observed therapeutic effects is one potential area for future investigation.

While we believe that our study is the first to evaluate the use of human PLX-PAD cells for treatment of tendon injuries, other preclinical studies have shown the regenerative properties of extraembryonic tissues in musculoskeletal applications[11,37]. Mesenchymal-like cells isolated from human umbilical cords and placentas have demonstrated favorable chondrogenic properties[38-44]. Gene expression analyses support this by showing higher type-II collagen and glycosaminoglycan synthesis relative to mesenchymal-like cells harvested from the bone marrow[38,39,43]. Placenta-derived mesenchymal-like stromal cells have also shown potential for cartilage tissue engineering[40,42].

This study has limitations inherent to the use of animal models. While injuries induced with collagenase are an accepted experimental model for tendinopathy[45], a chemically induced injury does not truly replicate the human condition of a chronic overuse condition. We believe that it should be viewed as a model of acute tendon injury and repair rather than a simulation of chronic tendinopathy. A tendon overuse model, with daily treadmill running, may better approximate the human clinical condition; however, such models result in subtle microscopic structural changes that may not be consistently produced in each laboratory animal[46], which is not ideal for evaluating novel pharmacotherapies[45-48]. Our study also used 1 cell concentration; therefore, future studies of alternative dosages will be important to optimize the treatment. Furthermore, the results of a particular therapy may also be affected by the specific animal model and species. Rodents are routinely used for laboratory studies because of the cost and hardiness of the species. However, the rat's innate robust healing response may limit the ability to detect differences, particularly at later time points. Finally, results of laboratory animal studies, such as the current investigation, should be viewed as proof of concept. Translating treatment from preclinical studies to human clinical conditions requires further validation.

In summary, we found that a single injection of PLX-PAD cells resulted in transient early improvement in tendon load-to-failure properties in an experimental model of tendinopathy. Human extraembryonic tissues are readily available and represent another source of musculoskeletal reparative cells that may have promise for tissue repair. Additional preclinical investigations are necessary to understand the interaction between PLX-PAD cells, the postinjury inflammatory cascade, and tendon healing. ■

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