Platelets and Plasma Proteins Are Both Required to Stimulate Collagen Gene Expression by Anterior Cruciate Ligament Cells in Three-Dimensional Culture

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Collagen-platelet (PL)-rich plasma composites have shown *in vivo* potential to stimulate anterior cruciate ligament (ACL) healing at early time points in large animal models. However, little is known about the cellular mechanisms by which the plasma component of these composites may stimulate healing. We hypothesized that the components of PL-rich plasma (PRP), namely the PLs and PL-poor plasma (PPP), would independently significantly influence ACL cell viability and metabolic activity, including collagen gene expression. To test this hypothesis, ACL cells were cultured in a collagen type I hydrogel with PLs, PPP, or the combination of the two (PRP) for 14 days. The inclusion of PLs, PPP, and PRP all significantly reduced the rate of cell apoptosis and enhanced the metabolic activity of fibroblasts in the collagen hydrogel. PLs promoted fibroblast-mediated collagen scaffold contraction, whereas PPP inhibited this contraction. PPP and PRP both promoted cell elongation and the formation of wavy fibrous structure in the scaffolds. The addition of only PLs or only plasma proteins did not significantly enhance gene expression of collagen types I and III but the combination, as PRP, did. Our findings suggest that the addition of both PLs and plasma proteins to collagen hydrogel may be useful in stimulating ACL healing by enhancing ACL cell viability, metabolic activity, and collagen synthesis.

Introduction

THE ANTERIOR CRUCIATE LIGAMENT (ACL) is one of the four major ligaments of knee and serves as the primary stabilizer of knee motion. The ACL is also the most commonly injured knee ligament and is susceptible to ruptures or tears that can cause pain and discomfort, joint instability, and eventually degenerative joint disease. The ACL fails to heal after suture repair, and for this reason, ACL injuries are commonly treated with ACL reconstruction, where the ACL is removed and replaced with a graft of tendon. Although this procedure is excellent at restoring gross knee stability, it does not restore normal knee kinematics,¹⁻⁴ and the majority of patients go on to have early premature osteoarthritis (as many as 78% of patients at 14 years after surgery).^{5,6} Therefore, there is a great interest in developing new techniques of ACL treatment.

Recently, enhancing healing of ligaments using bioactive substances has received increasing interest. For example, growth factors have been shown to influence chemotaxis, differentiation, proliferation, and synthetic activity of ACL cells and may potentiate the healing of ligaments.⁷⁻¹⁰ Platelet-rich plasma (PRP) is a fraction of plasma that can be pro-

duced by centrifugal separation of whole blood. It has been found to be a useful delivery system for growth factors important in application of tissue engineering. The growth factors released from platelets (PLs), such as PL-derived growth factor (PDGF-AA, AB, and BB), transforming growth factor (TGF)- β 1 and β 2, PL-derived angiogenesis factor, insulin growth factor-1 (IGF-1), and PL factor-4, have been noted to play a pivotal role in initiating and sustaining wound healing and tissue repair mechanisms.¹¹ Additionally, PRP contains plasma proteins such as fibrin, fibronectin, vitronectin, and thrombospondin, which are known to act as cell adhesion molecules important for osteoblast, fibroblast, and epithelial cell migration and viability.¹² Therefore, it is suggested that the bioactive substances included in PRP may activate several of the cell types involved in ACL healing.

Our previous studies have shown that collagen-PRP composites can stimulate ACL healing at early time points in large animal models.⁸⁻¹⁰ However, little is known about the cellular mechanisms by which the PRP components of these composites may induce healing. To begin to address this, an *in vitro* study was designed to evaluate the biological effects of PRP and its two components, PLs and PL-poor plasma (PPP), on ACL cell behavior. We hypothesized that the addition of

PRP, PLs, or PPP would significantly alter ACL cell proliferation and viability and would also induce collagen expression. To test this hypothesis, ACL cells were cultured in a three-dimensional (3D) scaffold with or without PRP, PPP, or PLs for 14 days. A collagen type I hydrogel was selected as scaffold because it has the ability to activate PLs to release growth factors and also because collagen type I is a major component of ligament tissue.^{13,14} ACL cell proliferation, viability, and morphology in the scaffold were investigated. Collagen expression was also assessed using quantitative real time-polymerase chain reaction (RT-PCR) and immunohistological staining.

Materials and Methods

Experimental design

Soluble type I collagen was used as the basis of the provisional scaffold models used in this study. Four types of provisional scaffolds were studied: (1) collagen hydrogel (COL), (2) COL-PPP composite, (3) COL-PL composite, and (4) COL-PRP composite. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA) or Sigma (St. Louis, MO), unless otherwise specified.

Preparation of COL

Acid-soluble, type I collagen slurry was made by sterile harvesting of bovine knee capsular tissue, which was solubilized in an acidic solution as previously described.⁸ Collagen content within the slurry was adjusted to 8 mg/mL and neutralized with 0.1 M HEPES (Cellgro; Mediatech, Herndon, VA), 5× phosphate-buffered saline (PBS; HyClone, Logan, UT), and 7.5% sodium bicarbonate (Cambrex BioScience Walkersville, Walkersville, MD).

Preparation of blood fractions

Six-hundred milliliters of whole blood was drawn from the femoral vein of pigs into tubes containing sodium citrate. Additional blood was collected in a bag with 10% acid-citrate dextrose at Children's Hospital Boston (Boston, MA).

PRP fraction. To make the PRP fraction, 200 mL of whole blood was transferred into 15 mL centrifuge tubes (10 mL per tube), which were then centrifuged for 6 min at 150 g (GH 3.8 rotor, Beckman GS-6 Centrifuge; Beckman, Fullerton, CA). The supernatant was aspirated and collected as PRP in a 50 mL tube. The PL concentration in PRP was 801×10^6 /mL, whereas in systemic blood it was 277×10^6 /mL, and thus the process resulted in an enrichment of $2.9 \times$.

PPP fraction. To make the PPP fraction, 200 mL of whole blood was used to make PRP as described earlier. The collected PRP was then transferred into 15 mL tubes and centrifuged at 1000 g for 10 min. The top layer was removed and placed into a new 15 mL tube (the old tube with the pellet after the first centrifugation was saved to make the PL fraction) and centrifuged again for 10 min at 1000 g. The top layer was removed as PPP and placed in a new 50 mL tube. The PL concentration in the PPP was 19×10^6 /mL.

PL fraction. To make the PL-only fraction, the pellets contained in the 15 mL centrifuge tubes saved after the first centrifugation in the PPP procedure were resuspended in 1×

PBS (EMD Chemical, Gibbstown, NJ). The PL concentration was 733×10^6 /mL.

Saline fraction. To make the saline fraction, 10 mL of 1× PBS was used.

Preparation of ACL cells

Pig ACL explants were obtained from the knee using a sterile technique. After ligament harvest, explants were cultured in completed medium (Dulbecco's modified Eagle's medium [DMEM]) containing 4.5 g/L glucose, 10% fetal bovine serum, and 1% antibiotic/antimycotic [AB/AM]), which was changed two times per week. When primary outgrowth cells were 80% confluent, they were trypsinized and frozen. All cells used for this experiment were of fifth passage, and cell viability ranged from 86% to 94% according to trypan blue exclusion.

Construct preparation and cultivation

Cells were resuspended with PBS or blood fractions (PRP, PPP, and PL). Eight milliliters of each cell suspension was mixed with 13 mL COL. The final cell density in the mixture was 5×10^5 cells/mL, and the final collagen content in the mixture was 3 mg/mL. For each group, the hydrogel-cell mixture was delivered to 3-cm-long semicylindrical molds with a polyester mesh at each end to anchor the gels. Each construct was placed in culture, warmed in a humidified 5% CO₂/37°C incubator for 1 h to achieve gelation, and then cultured with completed DMEM. Medium was changed every 3 days during the culture period. The constructs were assessed on day 14.

Construct contraction

Digital pictures of the cultures ($n = 6$ for each group) were taken on days 0, 1, 4, 7, and 14, and construct area was measured using Image J software (NIH, Bethesda, MD). Contraction was measured for each construct as the percent decrease in area at days 1, 4, 7, and 14, with respect to the time zero value. Collagen gel without cells (cell-free COL) served as a control in this experiment.

DNA content

DNA content of the constructs was determined for $n = 5$ constructs per group by using Quant-iT™ PicoGreen dsDNA Assay Kit at day 14, with type 1 highly polymerized calf thymus DNA as a standard.

Cell metabolic activity

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed on $n = 5$ constructs per group to obtain the index of cell metabolic activity. In brief, constructs were rinsed with PBS and incubated in a solution of 0.5 mg/mL MTT in DMEM for 4 h in a 5% CO₂/37°C incubator. Constructs were then incubated in a solution of 0.1 N HCl in isopropyl alcohol for an additional 4 h, and the optical density of the resulting supernatant was measured at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Histological and immunohistological analyses

To identify the collagen deposited in the hydrogel, immunohistochemical staining for collagen was performed on

TABLE 1. REAL-TIME POLYMERASE CHAIN REACTION PRIMER SEQUENCES

Gene	Forward primer sequences	Reverse primer sequences
Collagen I	5'-CAGAACGGCCTCAGGTACCA-3'	5'-CAGATCACGTCATCGCACAAC-3'
Collagen III	5'-CCTGGACTTCCTGGTATAGC-3'	5'-TCCTCCTTCACCTTTCTCAC-3'
GAPDH	5'-GGGCATGAACCATGAGAAGT-3'	5'-GTCTTCTGGGTGGCAGTGAT-3'

$n = 4$ constructs per group. In brief, constructs were rinsed in PBS and cryosectioned at 16 μ m. The sections were incubated for 1 h at 37°C with monoclonal type I collagen (ab6308; Abcam, Cambridge, MA) or type III collagen antibody (MAB3392; Millipore, Billerica, MA), diluted 1:150 in PBS containing 0.5% Tween 20 and 1.5% horse serum. Subsequently, sections were incubated for 30 min at room temperature with a secondary antibody (horse anti-mouse

IgG, Standard Elite ABC kit; Vector, Burlingame, CA), diluted 1:200, and then incubated with an avidin-biotin complex agent for 30 min at room temperature and with 3,3'-diaminobenzidine (D0426; Sigma) for 15 min at room temperature. The sections were counterstained with Harris hematoxylin and coverslipped.

For histological analysis, constructs ($n = 4$ per group) cultured for 14 days were rinsed in PBS, fixed for 24 h in 10% neutral-buffered formalin, and embedded in paraffin. Serial longitudinal sections (6 μ m) of the constructs were cut, and sections at 150 μ m interval from outer surface to the center were selected for staining. Cell morphology and distributions were evaluated with hematoxylin and eosin (H&E) staining. Collagenous extracellular matrix (ECM) was evaluated with Masson's trichrome staining.

Immunofluorescent staining for the measurement of apoptotic cells and nuclear aspect ratio was performed on $n = 4$ constructs per group. The sections were stained with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using a commercial available kit (Roche, Indianapolis, IN), according to the manufacturer's instructions. Subsequently, the sections were treated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Carlsbad, CA) to quantify the total number of nuclei. The apoptotic (TUNEL positive) and total (DAPI positive) cells were manually counted for each group, and the fraction of apoptotic cells were expressed as a percentage of the total cells. Average nuclear aspect ratio was measured to characterize ACL cell morphology.¹⁵ The major axis of cell nucleus identified by DAPI fluorescence was divided by the minor axis to

FIG. 1. Cell apoptosis and metabolic activity in 14-day constructs. (A) Apoptotic (TUNEL-positive) cells expressed as a fraction of total (4',6-diamidino-2-phenylindole dihydrochloride-stained) cells. (B) Cell metabolic activity, assessed by the MTT assay. Data are the mean $\pm$ standard deviation of five to six measurements. *Significantly different from the COL group ($p < 0.05$). COL, collagen hydrogel; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

FIG. 2. Construct size as a function of time in culture, evaluated as the percent decrease of a particular construct's area at days 1, 4, 7, and 14, with respect to that construct's area at day 0. Data are the mean $\pm$ standard error of six measurements.

get nuclear aspect ratio. Three regions were selected from each test specimen and 150–600 cells were measured in each group.

Transmission electron microscopy

Constructs cultured for 14 days in the PRP and COL groups were fixed with 10% buffered neutral formalin for 24 h, postfixed in 2% osmium tetroxide for 1 h, dehydrated in ethanol, and then embedded in BEEM® vials (BEEM Inc.,

Bronx, NY) with fresh LR white resin (Ted Pella Inc., Redding, CA). Sections were cut at 60 nm, stained with uranyl citrate and lead citrate, and viewed under a JEM 2011 transmission electron microscopy (TEM) instruments (Jeol, Tokyo, Japan).

Real-time PCR

Total RNA was extracted from constructs using an RNeasy mini kit (Qiagen, Valencia, CA). Briefly, constructs

FIG. 3. Cell distribution and morphology in 14-day constructs, as evaluated with hematoxylin and eosin staining (longitudinal section). (A–D) Low-magnification images from the (A) COL, (B) PL, (C) PPP, and (D) PRP groups. Scale bars: 100 μ m. (E–H) High-magnification images from the (E) COL, (F) PL, (G) PPP, and (H) PRP groups. Scale bars: 50 μ m. PL, COL-platelet composite; PPP, COL-platelet-poor plasma composite; PRP, COL-platelet-rich plasma composite. Color images available online at www.liebertonline.com/ten.

that had been cultured for 14 days were rinsed in PBS, cut into small pieces, lysed with supplied buffer (Qiagen), and transferred to RNeasy spin columns. RNA concentration and purity were determined at 260 and 280 nm, respectively. The RNA samples were reverse transcribed into cDNA using RETROscript Kit (Ambion, Austin, TX), following the supplier's instructions. Real-time PCR was performed in ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBRGreen PCR Master Mix Kit (Applied Biosystems). Targeted genes were types I and III procollagens (*COL1A1* and *COL3A1*), and *GAPDH* was selected as a reference gene. The primer sequences of selected genes for real-time PCR are listed in Table 1. The transcript level of target genes normalized to *GAPDH* was calculated using the $2^{-\Delta Ct}$ formula.

Statistical analyses

Data were calculated as means $\pm$ standard deviation and analyzed using two-way analysis of variance. Values of $p < 0.05$ were considered significant.

Results

Cell viability and metabolism

The amount of DNA in the constructs was similar in all the groups (4.42 ± 1.34 , 4.69 ± 1.14 , 5.46 ± 0.65 , and 5.95 ± 0.89 g/construct in the COL, PL, PPP, and PRP groups, respectively; $p > 0.6$). The apoptotic rate in the COL group was more than double that in the PL, PPP, and PRP groups, with the percentage of apoptotic (TUNEL positive) cells in the COL group at $10.8\% \pm 2.5\%$, whereas the PL, PPP, and PRP groups had $5.2\% \pm 0.9\%$, $4.1\% \pm 1.7\%$, and $3.2\% \pm 0.5\%$, respectively (Fig. 1A) ($p < 0.01$). There was no significant difference in apoptosis among the PL, PPP, and PRP groups ($p > 0.3$). The cellular metabolic activity of the PL, PPP, and PRP groups were all significantly higher than the COL group ($p < 0.05$), and there was no significant difference among these three groups ($p > 0.5$) (Fig. 1B).

Construct contraction

The cell-free COL gel contracted $14.1\% \pm 2.6\%$ over 14 days of culture (Fig. 2). The contraction rate of the ACL cell-seeded COL gels were significantly higher than that in the cell-free COL group after 7 days culture ($18.9\% \pm 2.7\%$, $p < 0.05$) and also after 14 days ($39.7\% \pm 5.7\%$, $p < 0.01$). The contraction rate in the PL group was significantly higher than that in the COL group at all time points ($p < 0.05$). The contraction rates in the PPP and PRP groups after 14 days were $17.1\% \pm 9.8\%$ and $25.8\% \pm 3.9\%$, respectively, which were significantly lower than that in the cell-seeded COL group ($p < 0.05$).

Histological and TEM analysis

Cell distribution and morphology were evaluated by H&E staining and the representative images are shown in Figure 3. ACL cells were distributed throughout the entire volume of 14-day constructs from all the groups (Fig. 3A–D). ACL cell morphology was very similar in each group. In the PRP and PPP groups, most of the cells appeared elongated and contained centrally positioned elongated nuclei, suggestive

FIG. 4. Average nuclear aspect ratio of ACL cell in 14-day constructs. *Significantly higher than the COL and PL groups ($p < 0.001$). #Significantly higher than the PPP group ($p < 0.001$). ACL, anterior cruciate ligament.

of early differentiation of these cells (Fig. 3G, H). Most of the cells were oriented along the longitudinal axis of the constructs. In contrast, most of the cells in the COL and PL groups appeared round (Fig. 3E, F). ACL cell morphology was further evaluated by the measurement of average nuclear aspect ratio (Fig. 4). The PRP group had the highest average nuclear aspect ratio among all the groups (2.74 ± 1.13 ; $p < 0.001$). The average nuclear ratio in the PPP group (2.51 ± 0.85) was significantly higher than that in the COL and PL groups (1.47 ± 0.27 and 1.58 ± 0.31 ; $p < 0.001$), and the ratio was similar in the COL and PL groups ($p > 0.2$). Collagenous ECM was evaluated with Masson's trichrome staining. Hydrogels in the PRP group showed a wave fiber-like structure of collagenous ECM aligned with the longitudinal axis of the constructs (Fig. 5D), as is typical for ligament tissue (Fig. 5E). Hydrogels in the PPP group also showed a similar wave fiber-like structure but with less density and alignment when compared with the PRP group (Fig. 5C). In contrast, the aligned wave-like structure was not seen in the COL and PL groups, which exhibited only a homogenous structure (Fig. 5A, B). Ultrastructure of the constructs from the PRP and COL groups was evaluated by TEM. Classic collagen fibrils were observed in both groups (Fig. 6). Collagen fibrils were prevalent in the PRP group (Fig. 6B, D), and most of them were aligned with the cells and packed into collagen fibers. In contrast, the collagen fibrils in the COL group were sporadic and less oriented (Fig. 6A, C).

Real-time PCR

mRNA transcript expressions of types I and III collagen were evaluated by real-time PCR after 14 days in culture (Fig. 7). The analysis indicated that hydrogels in the PRP group had the highest transcript level of both types I and III collagen among all the groups. The transcript level of type I collagen in the PRP hydrogels was 9.4 times higher than that in the COL group ($p < 0.01$). The transcript level of type III collagen in the PRP group was 11.2 times higher than that in the COL group ($p < 0.01$). The PL and PPP groups showed slightly increased transcript levels of both types I and III

FIG. 5. Collagenous extracellular matrix in 14-day constructs, as evaluated with Masson's trichrome staining (longitudinal section). (A) COL group, (B) PL group, (C) PPP group, (D) PRP group, and (E) intact pig ACL tissue. Scale bars: 100 μ m. Color images available online at www.liebertonline.com/ten.

collagen when compared with the COL group, but there was no significant difference among these three groups.

Immunohistochemical analysis

Deposition of types I and III collagen in the scaffold was evaluated by immunohistochemical staining (Fig. 8). For each antibody, cell-free constructs were stained as control, which in both cases showed similar staining intensity to the constructs from the COL group. Hydrogels in the PRP group showed a more intense staining for collagen types I and III when compared with the other groups (Fig. 8). The PL and PPP groups had similar staining intensity for collagen types I and III, and both of them were more intense than the COL group.

Discussion

PRP is thought to facilitate successful wound healing and is likely to be useful in stimulating healing of tissues that have an impaired ability to heal, like the ACL.⁸⁻¹⁰ Understanding the influence of PRP and its components, PL and PPP, on fundamental ACL cell behaviors is important for optimizing the use of these stimulatory cells in translational applications of these materials.

Many previous studies have reported that PLs can stimulate the proliferation of various cells, for example, osteoblast, gingival fibroblast, endothelial cells, and stromal stem cells.¹⁶⁻²⁰ Most of these studies were carried out in two-dimensional culture systems. In this study, ACL cells were

FIG. 6. Microstructure in 14-day constructs, as evaluated with transmission electron microscopy. Representative images from the (A, C) COL and (B, D) PRP groups. Longitudinal section: (A) COL group and (B) PRP group; Cross section: (C) COL group and (D) PRP group. Scale bars: 500 nm. ACL cells and collagen fibrils are indicated by asterisks and arrows, respectively.

cultured in 3D collagen type I hydrogel, and no significant difference of cell proliferation was observed among all the four groups after 14 days culture. According to previous studies, the stimulating effect of PL on cell proliferation is in a dose-dependent manner.²¹ Arpornmaeklong *et al.* reported that when cells were cultured in a 3D collagenous composite, only highly concentrated PRP (250×10^6 PL in a carrier at 5 mm diameter $\times$ 3 mm thick, about 4400×10^6 /mL) showed a stimulation of cell proliferation, whereas lower concentrations of PLs (62.5×10^6 /carrier and 16×10^6 /carrier) did not result in increased levels of cell proliferation. Therefore, the relatively low PL concentration (300×10^6 /mL) used in this study might be the reason for the lack of significant cellular proliferative response in our PL hydrogels.

In addition, the culture environment is known to significantly affect cell viability and metabolic activity. In general, *in vitro* statically cultured constructs rely on diffusion to

supply oxygen and nutrients. Within the body, most cells are found no more than 100–200 μ m from the nearest capillary, with this spacing providing sufficient diffusion of oxygen.²² *In vitro*, likewise, sufficient oxygenation of cells by diffusion is limited to a distance of 100–200 μ m.^{23,24} It has been well recognized that oxygen concentration gradient exists in statically cultured tissue constructs that are larger than few hundred micrometers in thickness.^{25,26} Previous studies also showed that hypoxia existed in sizable constructs seeded with cells.^{27,28} In this work, the constructs 2–5 mm in thickness were statically cultured for 14 days *in vitro*. Although oxygen concentration was not measured, we believe that hypoxia existed in the statically cultured constructs. Many previous studies have shown that hypoxia is a sufficient trigger for cell apoptosis *in vitro* and *in vivo*,^{29,30} and future studies to correlate the degree of hypoxia and the apoptotic mechanism in ACL cells are needed. In this work, cell apoptosis was found in all the

FIG. 7. Transcript level of collagen gene in 14-day constructs, as measured with real-time polymerase chain reaction. **(A)** Procollagen I gene and **(B)** procollagen III gene. Data are the means $\pm$ standard deviation of three measurements. *Significantly higher than the COL group ($p < 0.05$).

groups, and we speculate that hypoxia might be one of the major reasons for cell apoptosis. PLs are a biological reservoir of various growth factors, and high concentrations of some growth factors, such as PDGF, IGF-I, and TGF, are found in PLs. Our previous studies showed that the release of these growth factors from PL can be triggered by contact with type I collagen.¹³ Some of the released growth factors, such as PDGF and IGF, can suppress cell apoptosis and enhance cell viability.^{31,32} Some plasma proteins, such as fibronectin, were also reported to have a protective effect against apoptosis.³³ In this study, as we expected, PL, PPP, and PRP all significantly reduced apoptosis in the day-14 constructs.

Fibroblasts are also known to induce collagen type I hydrogel contraction, which was originally reported by Bell *et al.*³⁴ In a 3D collagen gel system, the ability of fibroblasts to contract the gels is dependent on a variety of factors including fibroblast strain, cell density, collagen concentration, and the presence of soluble factors.^{35,36} The contraction rate of the COL group in this study is much lower than that in previous reports,³⁷ which may be due to the higher collagen

concentration of the gel used here than that of previous reports (3 mg/mL here vs. 1.95 mg/mL previously). In addition, PLs are known to potentiate fibroblast-mediated contraction of collagen gels.³⁸ Our results are consistent with these reports, in that the contraction rate of the PL group was higher than the COL group at all time points evaluated. This is also consistent with our previous results.³⁹ In contrast, the PPP hydrogels showed an inhibitory effect on collagen gel contraction. The contraction rate of hydrogels in the PPP and PRP groups was significantly lower than that in the COL and PL groups. One of the possible reasons for this inhibition of contraction in the PRP and PPP groups is the high concentration of fibrinogen in collagen gel, which contained pig plasma (PRP and PPP groups). Fibrinogen is a soluble protein in pig plasma at concentrations of 2–3 mg/mL.⁴⁰ The final concentration of fibrinogen in the PRP and PPP groups was 0.75–1.15 mg/mL, whereas no plasma fibrinogen was contained in the PL and COL groups. Previous studies have reported that fibrinogen decreases the contraction of fibroblast-populated collagen gels in a dose-dependent manner, and the contraction of collagen gel was partially inhibited by fibrinogen at 0.5 mg/mL and was completely blocked at 3 mg/mL.⁴¹ However, the mechanism remains unknown, and the principal factor behind the observed differences requires further study.

ACL cell morphology was characterized by H&E staining and average nuclear aspect ratio (Figs. 3 and 4). Most ACL cells in the collagen-only and PL-only hydrogels cultured for 14 days exhibited a rounded-cell phenotype. The rounded-cell phenotype might be due to the method of obtaining cells where freshly digested cells were mixed with collagen gel and then cultured in the gel for 14 days. During digestion, cells undergo extensive morphological and biochemical changes including the loss of normal morphology and reduced marker protein synthesis.⁴² Compared with other scaffolds, this gel inoculation method enables rapid and spatially uniform seed at high cell density but may restrict cell elongation. In the PL group, many growth factors, such as PDGF, IGF-I, and TGF- β , are released by PLs; however, the growth factors released by the PLs in these constructs did not appear to influence cell morphology, and there was no significant difference in average aspect ratio between the COL and PL groups. Interestingly, most cells in the PPP and PRP groups appeared elongated, and the average nuclear aspect ratio in these groups were much higher than that in the COL and PL groups. Also most cells in the PRP and PPP groups were oriented along the longitudinal axis of the constructs. One possible reason for the cell morphology change in the PPP and PRP groups is the presence of cell adhesion proteins in plasma. For example, fibronectin is one of the major adhesive proteins in plasma that can mediate cell attachment and spreading on various substrates. Collagen molecules have specific binding sites for this protein,⁴³ and thus COL has the ability to bind high amounts of fibronectin. Previous studies have shown that when exposed to medium containing 10% bovine fetal serum, 3D type I collagen scaffolds can absorb high amounts of fibronectin, and cell adhesion to the collagen scaffold is partially blocked by specific antifibronectin receptor antibodies.⁴⁴ In this work, the PRP and PPP groups contained pig blood plasma, whereas the COL and PL groups did not. Based on previous studies, the presence of adhesive proteins in the plasma is one possible reason for ACL cell attachment and spreading in the PRP and PPP groups.

FIG. 8. Immunohistochemical analysis of 14-day constructs. (A–E) Images of collagen I staining from the (A) cell-free COL, (B) COL, (C) PL, (D) PPP, and (E) PRP groups. (F–J) Images of collagen III staining from the (F) cell-free COL, (G) COL, (H) PL, (I) PPP, and (J) PRP groups. Scale bars: 100 μ m. Color images available online at www.liebertonline.com/ten.

Intrinsic ACL cells are believed to be an important cell in tissue remodeling during stimulated repair as well as during the incorporation of a tendon graft after ACL reconstruction. In the PRP group, the ECM had a wavy appearance, which was aligned with the longitudinal axis of the constructs. This newly deposited ECM was populated by ACL cells, which had cytoplasmic extensions that were also aligned with the longitudinal axis. The PPP group had some similar regions, which occupied less area of the constructs. This structure was not observed in the COL and PL groups. As types I and III are the major matrix components of the natural ligament, we hypothesized that PRP has a stronger stimulating effect on collagen synthesis of ACL cells than either PPP or PL. Real time-polymerase chain reaction and immunohistochemical analyses confirmed this hypothesis. Cell culture environment is an important factor that can affect cell functions, including protein synthesis. Many studies reported that some growth factors, such as PDGF and TGF- β , have the ability to promote the synthesis of collagen types I and III by fibroblasts.^{45,46} In the PL group, PDGF and TGF were released from the PLs, and its collagen synthesis was increased when compared with the COL group, with the transcript level of type III collagen 180% higher than that in the COL group ($p > 0.05$). This increase was even more notable in the group combining PLs and plasma proteins (the PRP group): a significant increase in collagen synthesis was observed in the PRP group; specifically, the transcript level of type I collagen was 9.4 times and that of type III collagen was 11.2 times higher than the COL groups, and also much higher than both PL and PPP groups ($p < 0.05$). Immunohistochemical staining also revealed the increased deposition of collagen types I and III in the scaffold when compared with the other groups. As the PRP group contains PLs and plasma proteins that allowed the cells to obtain a more elongated shape, we suggest that both the cytokines and the ECM composition are able to influence collagen synthesis by ACL cells, and therefore, for ACL cells.

In conclusion, this study demonstrated that PRP and its two components, PLs and plasma proteins, can enhance the viability and metabolic activity of ACL cells in 3D culture. However, only the combination in PRP has significant beneficial effect on the collagen synthesis of ACL cells.

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Disclosure Statement

Dr. Murray is a paid consultant, founder, and stockholder in Connective Orthopaedics. No other competing financial interests exist.

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