Effects of Autologous Platelet-Rich Plasma on Cell Viability and Collagen Synthesis in Injured Human Anterior Cruciate Ligament

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Background: Platelet-rich plasma is a fraction of plasma in which platelets are concentrated. It is reported to represent a source of multiple growth factors that promote tissue repair. In anticipation of the eventual testing of platelet-rich plasma in anterior cruciate ligament (ACL)-deficient patients, we examined the effect of autologous platelet-rich plasma on human ACL cell function in vitro.

Methods: Fresh blood and ACL remnants were obtained from four patients who underwent ACL reconstruction surgery. Platelet-poor plasma and platelet-rich plasma were prepared from the blood samples. The concentrations of various growth factors in each preparation were tested with use of enzyme-linked immunosorbent assays. Isolated ACL cells were cultured in the presence of 5% fetal bovine serum, 5% platelet-poor clot releasate, 5% platelet-rich clot releasate, or 10% platelet-rich clot releasate. Platelet-rich plasma and platelet-poor plasma releasates were applied to the ACL cells from the same patient autologously. Cell viability and collagen synthesis in each group were analyzed, and semiquantitative gene-expression assays for type-I and III collagen were also performed.

Results: The concentrations of the main growth factors (transforming growth factor-beta, platelet-derived growth factor, epidermal growth factor, and vascular endothelial growth factor) were much higher in platelet-rich clot releasate than in platelet-poor clot releasate. In vitro treatment of ACL cells with platelet-rich clot releasate resulted in a significant increase in cell number compared with platelet-poor clot releasate. Total collagen production by the platelet-rich clot releasate-treated cells was significantly higher than that of the platelet-poor clot releasate-treated cells only because of enhanced cell proliferation. There was no significant effect of platelet-rich clot releasate treatment on gene expression for type-I collagen, but expression of type-III collagen was significantly enhanced by the treatment with platelet-rich clot releasate.

Conclusions: These results suggest that autologous platelet-rich plasma can enhance ACL cell viability and function in vitro.

A platelet contains the majority of biologically active molecules required for blood coagulation, such as adhesive proteins, coagulation factors, and protease inhibitors. In addition to the factors that coagulate blood, activated platelets are known to release many kinds of growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β1), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF)1-2, which are thought to play a key role in the healing process of many tissues3-5. Platelet-rich plasma was developed in the early 1970s as a fraction of plasma in which platelets are concentrated; thus, higher concentrations of the fundamental protein growth factors also exist in platelet-rich plasma6. These growth factors are known to induce biological changes in the cell proliferation and matrix metabolism of a variety of connective tissues7,8.

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Platelet-rich plasma has been used as an autologous source of the growth factors that are able to induce tissue regeneration in several clinical settings\textsuperscript{9,12}, such as wound-healing\textsuperscript{11,12}, bone regeneration in periodontal and maxillofacial surgery\textsuperscript{5,12-15}, otolaryngology\textsuperscript{24}, plastic surgery\textsuperscript{27}, and cardiovascular surgery\textsuperscript{18}. The same approach involving the use of a platelet-rich gel or platelet supernatant has been attempted in orthopaedic surgery\textsuperscript{19}, including basic research on tendon cells\textsuperscript{25-23}, articular chondrocytes\textsuperscript{25}, intervertebral disc cells\textsuperscript{24}, and anterior cruciate ligament (ACL) cells\textsuperscript{25-30}.

The use of platelet-rich plasma to enhance ACL healing has also received some attention, in part because of the relatively poor healing potential of this intra-articular structure compared with extra-articular tissues. For example, Murray et al. reported that collagen-platelet-rich plasma gel treated with thrombin enhanced bovine ACL cell proliferation and migration in the gel\textsuperscript{26} and that the use of a collagen-platelet-rich plasma scaffold stimulated healing of a defect in the canine ACL\textsuperscript{28-30}. For the clinical application of platelet-rich plasma to ACL-deficient patients, it is desirable to examine whether platelet-rich plasma has an enhancing effect on ACL cell metabolism with use of human samples. However, to our knowledge, no studies have clarified the effect of autologous platelet-rich plasma on adult human ACL cells. In the present study, we examined the effect of autologous platelet-rich plasma, as a reservoir of multiple growth factors, on viability and collagen synthesis of human ACL cells in vitro.

Materials and Methods

Collection of Blood and Anterior Cruciate Ligament Tissue from the Patients

With the approval of our institutional ethics committee, we obtained informed consent from four ACL-deficient patients (two women and two men with a mean age of twenty-seven years [range, eighteen to thirty-five years]) who had sustained the injury an average of twelve months (range, nine to twenty-four months) previously. At the time of ACL reconstruction surgery, blood (54 mL) was drawn from the fore-arm vein of each patient into a 60-mL syringe and was treated with an anticoagulant, citrate dextrose solution (Boehringer, Ingelheim, Germany). Removed ACL remnant tissue was used as a source of ACL cells in the following experiments.

Plasma Preparation

Collected blood was brought immediately to the laboratory, and both platelet-rich plasma and platelet-poor plasma were isolated with use of a platelet concentration system (Symphony; DePuy Synthes Spine, Raynham, Massachusetts). Briefly, the blood that had been treated with anticoagulant was separated into plasma and hemocyte (erythrocyte and leukocyte) fractions, and the plasma was separated into platelet-rich plasma (containing a high number of platelets) and platelet-poor plasma (containing few platelets) by means of continuous two-step sedimentation. Isolated platelet-poor plasma and platelet-rich plasma were clotted with a 10% thrombin solution (v/v, 1000 U/mL in 100-mM CaCl\textsubscript{2}) to yield a final thrombin concentration of 100 U/mL, followed by centrifugation (1500 g for five minutes) and separation into fibrin clots and soluble supernatants. These final soluble releasates from platelet-rich clot and platelet-poor clot were frozen and stored at -80°C until used.

Concentration of Growth Factors in the Plasma Releasates

The concentrations of each of the basic growth factors (PDGF-AB, TGF-β1, VEGF, and EGF) in both platelet-poor clot releasate and platelet-rich clot releasate were determined with use of separate specific enzyme-linked immunosorbent assay kits according to the manufacturer’s instructions (Quantikine immunoassay; R&D Systems, Minneapolis, Minnesota). These kits contain specific antibodies directed to the targeted human growth factors. Readings of color absorbance were made with use of a multiple plate reader (Corona Electric, Tokyo, Japan) with wavelength absorption at 530 nm subtracted from readings at a 450-nm wavelength.

Cell Isolation and Culture

Cells were isolated from remnant tissues obtained during ACL reconstruction surgery by means of sequential enzyme digestion with 0.2% Pronase (EMD Bioscience, La Jolla, California) for one hour and 0.025% collagenase P (Roche Applied Science, Indianapolis, Indiana) for sixteen hours at 37°C. After several washes in Dulbecco modified Eagle medium and Ham F-12 medium (DMEM/F12; Mediatech, Herndon, Virginia), the isolated cells were cultured in monolayer in complete media containing 10% fetal bovine serum (FBS; HyClone, Logan, Utah), 25 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, Missouri), 360 mg/mL L-glutamine (Mediatech), and 50 mg/mL gentamicin (Invitrogen, Carlsbad, California) at 37°C and 5% CO\textsubscript{2} in air atmosphere. In all cases, the medium was changed every other day. Platelet-rich plasma and platelet-poor plasma releasates were applied to the ACL cells from the same patient autologously. The ACL cells were cultured in the same manner for each test.

Cell Culture Protocol for the Studies

The cells from the initial culture were used for a cell viability assay, whereas the ones from the second passage were used for collagen and gene-expression assays. After twenty-four to forty-eight hours of preculture in complete medium, the cells were placed in serum-free medium, which consisted of DMEM/F12 with supplements as described above, for twelve hours. The cells were then cultured in serum-free medium with either 5% fetal bovine serum, 5% platelet-poor clot releasate, 5% platelet-rich clot releasate, or 10% platelet-rich clot releasate in SFM (serum-free medium). The cultures were incubated for twenty-four hours, forty-eight hours, and four days for the cell viability assay and for seven days for the measurement of collagen content. ACL cells were also cultured in the medium with 5% fetal bovine serum, 5% platelet-poor clot releasate, or 5% platelet-rich clot releasate for forty-eight hours for gene expression assays. Because of the limited amounts of blood sample and ACL tissue collected from each patient, the
10% platelet-rich clot releasate cells were not assayed for gene expression. The medium was changed every other day for each group.

**Cell Viability Testing**

A WST-8 assay (Doujin Laboratories, Kumamoto, Japan) was used to quantify cell numbers after twenty-four hours, forty-eight hours, and four days in culture. Ten microliters of tetrazolium salt WST-8 was added to each well in a ninety-six-well plate, and light absorbance was detected with use of an EZS-ABS microplate reader (Iwaki, Tokyo, Japan) at 450 nm after a two-hour incubation. The amount of chromophore that is generated is directly proportional to the number of living cells.

**Measurement of Collagen Content**

After the incubation with the corresponding media for a week, the cell layer in a 100-mm culture plate was collected with use of a cell scraper and was digested with 0.05-M acetic acid including pepsin (1 mg/mL) (Wako, Osaka, Japan) at 4°C with rotation for three days. After pH was brought to 8.0 with NaOH, each sample was treated with pancreatic elastase (1 mg/mL in 1×TBS [Tris-buffered saline]) (Sigma-Aldrich) at 4°C for two days. The soluble collagen in the supernatant was precipitated with 2.7-M NaCl and then was dissolved again in NaOH, each sample was treated with pancreatic elastase (1 mg/mL in 1×TBS) and was digested with 0.05-M acetic acid. To measure the concentration of soluble collagen, the Sircol assay (Biocolor, Belfast, Northern Ireland) was performed according to the manufacturer's protocol, followed by reading color absorbance at 540 nm with a micro-well plate colorimeter (Corona Electric). The collagen content was normalized by dividing by the DNA content of each sample as measured with use of the bisbenzimidazole fluorescent dye method (Hoechst 33258; Polysciences, Warrington, Pennsylvania).

**Gene Expression of Collagen Types I and III**

Following a forty-eight-hour culture in the corresponding media in culture flasks, the cells were collected with use of a cell scraper into micro-centrifuge tubes and were washed with saline solution twice. Total RNA was isolated with use of TRIzol (Invitrogen). The purified RNA was dissolved in DEPC (diethylpyrocarbonate)-treated water, and its concentration was measured with a spectrophotometer (JENWAY, Genova, United Kingdom). One microgram of total RNA was reverse-transcribed into complementary DNA (cDNA) with use of the SuperScript First-Strand cDNA Synthesis system (Invitrogen). All of the required incubations were done with use of a PCR (polymerase chain reaction) thermal cycler (TP600; Takara, Kyoto, Japan). Amplification of the target gene was carried out in triplicate with use of a 7500 Real Time PCR System (Applied Biosystems, Foster City, California) with a pair of primers for human type-I collagen α1 chain and human type-III collagen α1 chain (TaqMan Gene Expression Assays, Inventoried; Applied Biosystems) and a primer for human beta-actin (TaqMan Endogenous Control; Applied Biosystems). Thermal cycling and fluorescence detection with use of SYBR Green PCR Mix (Applied Biosystems), and the quantitative results of real-time PCR were assessed with a cycle threshold (Ct) value, which was normalized to the Ct value of the endogenous gene (beta-actin). The results then were calculated with use of ΔCt of the control (5% fetal bovine serum) as a calibrator.

**Statistical Analysis**

The experiment was performed in triplicate for all four donor samples. The results for collagen content were assessed by means of relative quantification with use of the fetal bovine serum group as the control group. The values were reported as the mean and the standard deviation (SD) of the results of the four separate cultures. The Kruskal-Wallis H test with Dunnett test as a post hoc test was used to assess the effects of the treatments on cell viability, collagen content, and gene expression of type-I and III collagen. The level of significance was set at p < 0.05 for the comparison of 5% platelet-rich clot releasate versus 5% platelet-poor clot releasate and the comparison of 5% platelet-rich clot releasate versus 10% platelet-rich clot releasate.

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**Results**

**Concentration of Growth Factors in the Plasma Releasates**

The concentrations of all of the targeted growth factors in platelet-rich clot releasate were much higher than those in platelet-poor clot releasate in all four samples (Fig. 1). The mean concentrations (and standard deviations) of PDGF-AB and TGF-β1 in the platelet-rich clot releasate samples obtained from the four donors were 24.9 ± 5.7 and 48.6 ± 2.5 ng/mL, respectively. These values were more than thirteenfold higher than those in the platelet-poor clot releasate samples. VEGF and EGF were also detected in all four platelet-rich clot releasate samples (mean, 0.7 ± 0.5 and 9.3 ± 3.5 ng/mL, respectively). However, the concentrations of VEGF and EGF in three of the four platelet-poor clot releasate samples were below the detection limit.

**Cell Viability Assay**

The result of the WST-8 assay in the 5% platelet-rich clot releasate group showed significantly higher color absorbency in comparison with the 5% platelet-poor clot releasate group at Days 2 and 4 of culture, which reflects an increase in the number of viable cells over this time. There was significantly greater ACL cell viability at Days 2 and 4 in the 10% platelet-rich clot releasate group as compared with the 5% platelet-rich clot releasate group (p < 0.05) (Fig. 2).
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Fig. 1
Histograms showing the concentrations of four basic growth factors (PDGF-AB, TGF-β1, VEGF, EGF) in platelet-poor clot releasates (PPCRs) and platelet-rich clot releasates (PRCRs) of four blood samples measured with enzyme-linked immunosorbent assay. ND = not detected.

Total Collagen Assay
Although the baseline amount of total collagen produced over seven days varied among the four different samples in the fetal bovine serum group (mean, 3.69 μg/plate; range, 1.67 to 5.6 μg/plate), ACL cells cultured in the platelet-rich clot releasate groups accumulated significantly more collagen compared with the platelet-poor clot releasate group in all four samples (p < 0.05) (Fig. 3). However, the differences in collagen production were not statistically significant between the different platelet-rich clot releasate groups.

Fig. 2
Histogram showing anterior cruciate ligament cell viability (at twenty-four hours, forty-eight hours, and four days) as determined with WST-8 assay, which indirectly reflects the function of platelet-rich plasma treatment in promoting ACL cell viability. The results are expressed in optical density values read at the 450-nm wavelength as the mean (and standard deviation) of triplicate analyses. FBS = fetal bovine serum, PPCR = platelet-poor clot releasate, and PPRC = platelet-rich clot releasate. *P < 0.05. N = 4 for each group.
content were not significant when the data were corrected for cell number (i.e., per µg DNA).

There was no significant difference in type-I collagen gene expression between the platelet-rich clot releasate and platelet-poor clot releasate groups (p > 0.05) (Fig. 4), but the platelet-rich clot releasate group showed significantly higher type-III collagen gene expression than the platelet-poor clot releasate group (p < 0.05) (Fig. 5).

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**Fig. 3**
Histogram showing the total amount of collagen accumulated in the ACL cell layer during one week of monolayer culture with four different media: 5% fetal bovine serum (FBS), 5% platelet-poor clot releasate (PPCR), 5% platelet-rich clot releasate (PRCR), and 10% platelet-rich clot releasate. The results are expressed as the mean (and standard deviation) of triplicate analyses. *P < 0.05, N = 4 for each group.

**Fig. 4**
Histogram showing the gene expression levels of type-I collagen in ACL cells after two days of cultivation with 5% fetal bovine serum (FBS), 5% platelet-poor clot releasate (PPCR), and 5% platelet-rich clot releasate (PRCR). The y axis represents the gene expression level in terms of the relative quantity, which was calculated by normalizing the cycle threshold (Ct) value of each sample with the Ct value of the endogenous control (human beta-actin) and finally dividing by the ΔCt of the control (5% fetal bovine serum) as the calibrator. The values are expressed as the mean (and standard deviation) of triplicate analyses. N = 4 for each group.
In the present in vitro study, we demonstrated the effects that platelet-rich clot releasate can have on the viability and matrix synthesis of adult human ACL cells. We are aware of two published studies analyzing the effect of platelet-rich clot releasate on human tendon cells. Anitua et al. showed that platelet-rich clot releasate promoted proliferation and induced the production of VEGF and hepatocyte growth factor by semitendinous tendon cells obtained from a patient who underwent ACL reconstruction surgery. De Mos et al. reported that platelet-rich clot releasate stimulated cell proliferation and total collagen production and slightly increased the expression of matrix-degrading enzymes and endogenous growth factors in hamstring tendon cells. However, in both of those studies, blood from healthy volunteers was used as a source of the platelet-rich plasma. The results presented here are the first to show the effect of human autologous platelet-rich plasma on ACL cells with use of materials from the same patient.

The ACL has long been thought to have a limited capacity for healing. Even with primary repair, the ACL still fails to heal in the majority of patients, which makes it different from extra-articular ligaments, including the medial collateral ligament, that heal readily with nonoperative therapy. This poor healing potential has been reported to be due to the intrinsic ACL properties and the intra-articular environment as well as the lack of blood clot formation in the gap between the parts of the injured ACL. The clot is thought to play two important roles: as a physical bridge between the separated parts of the injured ACL and as a source of growth factors. Using canine and porcine models, Murray et al. reported that treating an intra-articular ACL wound with a collagen-platelet-rich plasma hydrogel resulted in filling of the wound site with repair tissue similar to that seen with extra-articular ligament wounds.

In the present study, the effect of 5% platelet-rich clot releasate on ACL cells was assessed by comparing it with the effect of 5% platelet-poor clot releasate. Previous reports have shown the dose-dependent pattern of different growth factors in terms of their stimulating effect on tendon and ligament tissue in animal models. According to our method of measuring the concentrations of growth factors in adult human platelet-rich clot releasate, the culture medium with 5% platelet-rich clot releasate contained approximately 1 ng/mL of PDGF-AB and 2 ng/mL of TGF-β1, which is thought to be sufficient to induce direct biological effects on human ACL cells, whereas the concentrations of EGF and VEGF in 5% human platelet-rich clot releasate were relatively lower. Murray et al. showed that TGF-β1 and PDGF-AB enhanced the proliferation and collagen production of human ACL cells in a three-dimensional culture system. Although the combination of these growth factors could have effects similar to those of platelet-rich clot releasate, the use of autologous platelet-rich plasma has several advantages over the use of recombinant growth factors or products of animal origin, including simplicity, the cost of preparation, and safety issues such as immunologic reactions.

In the present study, human platelet-rich clot releasate promoted total collagen accumulation in an ACL cell layer, but this reflected mainly an increase in cell number rather than any real increased production per cell. Platelet-rich clot releasate stimulated the expression of type-III collagen. Since type-III collagen has been reported to play an important role in the early healing of ligament tissue, enhancing type-III collagen expression by ACL cells may have favorable implications for ACL repair. Although the expression of matrix metalloproteinases was not examined in the present study, the result of increased collagen accumulation indicated that it overcame the catabolic effect of matrix metalloproteinases whose expression might be...
also enhanced by platelet-rich clot releasate treatment. It has been reported that ACL injury itself induces an increase in the activity of matrix metalloproteinases. Matrix metalloproteinases are well known to play an important role in the remodeling of the extracellular matrix of injured ligaments, while too great an increase in the matrix metalloproteinase activity could be one of the reasons behind poor ACL healing. Thus, further investigation will be needed to clarify the effect of platelet-rich plasma on the activity of matrix metalloproteinases. Another limitation of the present study is that we measured the amount of collagen entrapped in the cell layer, but not the collagen released into the culture medium. Measuring the collagen content in both fractions could have shown a different effect of platelet-rich clot releasate on collagen production by ACL cells.

Among other studies involving the use of human tendon or ligament cells, the results regarding collagen metabolism in the study by de Mos et al. differ from ours, probably because of the differences in preparation procedures. De Mos et al. showed no significant difference in either total collagen or collagen expression between groups treated with platelet-rich clot releasate or platelet-poor clot releasate, which were prepared from heterologous blood by means of manual stepwise centrifugation followed by clotting with CaCl₂ at 37°C for one hour, whereas our procedure to prepare the platelet-rich plasma fraction was completely automated with use of the platelet concentration system (Symphony; DePuy Spine). In addition, the plasma clot releasates used in our study were purified from autologous blood followed by immediate clot formation with thrombin and CaCl₂. These differences in preparation may result in differences in the proportions and concentrations of active growth factors present in the platelet-rich clot releasate and differences in contamination by heterologous leukocytes.

As platelet-rich plasma is easy to prepare and utilize as a both a soluble platelet releasate and a fibrin gel, human autologous platelet-rich plasma has been proposed for clinical use in several tissues, and intra-articular injection of platelet-rich plasma may be useful for nonoperative treatment of ACL injury. The recent technical advances in the isolation of the platelet-rich plasma may promote its acceptance as a procedure to enhance tissue repair. However, more investigation is needed to confirm the effect of platelet-rich plasma on the metabolism of ACL cells before supporting its usefulness in clinical applications.

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