OsteoArthritis and Cartilage (2006) **14,** 1272–1280

doi:10.1016/j.joca.2006.05.008

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Platelet-rich plasma stimulates porcine articular chondrocyte proliferation and matrix biosynthesis

K. Akeda M.D.†, H. S. An M.D.†, M. Okuma M.D., Ph.D.†, M. Attawia M.D.‡, K. Miyamoto M.D., Ph.D.†, E. J-M. A. Thonar Ph.D.†§‖, M. E. Lenz M.S.§, R. L. Sah M.D., Sc.D.¶ and K. Masuda M.D.†§*

† *Department of Orthopedic Surgery, Rush Medical College at Rush University Medical Center, Chicago, IL, USA*

‡ *DePuy Biologics, DePuy Spine Incorporation, Raynham, MA, USA*

§ *Department of Biochemistry, Rush Medical College at Rush University Medical Center, Chicago, IL, USA*

‖ *Department of Internal Medicine, Rush Medical College at Rush University Medical Center, Chicago, IL, USA*

¶ *Department of Bioengineering & Whitaker Institute of Biomedical Engineering, University of California-San Diego, La Jolla, CA, USA*

Summary

Objective: Platelet-rich plasma (PRP) is a fraction of plasma that contains high levels of multiple growth factors. The purpose of this study was to examine the effects of PRP on cell proliferation and matrix synthesis by porcine chondrocytes cultured in alginate beads, conditions that promote the retention of the chondrocytic phenotype, in order to determine the plausibility of using this plasma-derived material for engineering cartilage.

Design: PRP and platelet-poor plasma (PPP) were prepared from adult porcine blood. Adult porcine chondrocytes were cultured in the presence of 10% PRP, 10% PPP or 10% fetal bovine serum (FBS) for 3 days. Cell proliferation, proteoglycan (PG) and collagen synthesis were quantified, and the structure of newly synthesized PG and collagen was characterized.

Results: Treatment with 10% PRP resulted in a small but significant increase in DNA content (+11%, vs FBS; $P < 0.01$; vs PPP; $P < 0.001$). PG and collagen syntheses by the PRP-treated chondrocytes were markedly higher than those by chondrocytes treated by FBS or PPP (PG; PRP: +115% vs FBS; +151% vs PPP, both $P < 0.0001$, collagen; PRP: +163% vs FBS; +163% vs PPP, both $P < 0.0001$). Biochemical analyses revealed that treatment with PRP growth factors did not markedly affect the types of PGs and collagens produced by porcine chondrocytes, suggesting that the cells remained phenotypically stable in the presence of PRP.

Conclusion: PRP isolated from autologous blood may be useful as a source of anabolic growth factors for stimulating chondrocytes to engineer cartilage tissue.

Key words: Platelet-rich plasma, Chondrocyte, Growth factor, Proteoglycan, Collagen.

Introduction

Articular cartilage provides a resilient, low friction, wear resistant joint surface. Properties inherent to adult articular cartilage, such as a lack of vascularity and a limited numbers of chondrocytes, restrict the tissue's intrinsic capacity for healing after acute damage, especially if an injury does not penetrate into the subchondral bone[1]. In addition, the tissues formed by spontaneous repair are fibrocartilaginous[2], having an abnormal biochemical composition and inferior biomechanical function[3]. The implantation of tissue-engineered cartilage fabricated *in vitro* shows clinical promise as a method for delivering reparative cells and preformed tissue into articular cartilage defects[4–7].

Because human chondrocytes have a relatively low basal propensity for proliferating and producing extracellular matrix, methods to accelerate the formation of cartilaginous tissue during *in vitro* culture are of practical importance. The stimulation of cell proliferation and matrix deposition by exogenous growth factors during culture shortens the culture duration required for producing cell-laden tissue, and may also increase the mass of transplantable tissue.

Growth factors, applied singly or in combination with cells, have been shown to stimulate cell proliferation and matrix formation of tissue-engineered cartilage (reviewed by Hunziker[4]). However, in most of those studies, fetal bovine serum (FBS) has been used in combination with growth factors to enhance their stimulatory effects. Alternatively, without FBS, a mixture of growth factors, such as transforming growth factor-β (TGF-β), fibroblast growth factor-2 and platelet-derived growth factor (PDGF)[8], can be used to stimulate cell proliferation and matrix formation[9].

*Address correspondence and reprint requests to: Koichi Masuda, M.D., Departments of Orthopedic Surgery and Biochemistry, Rush Medical College at Rush University Medical Center, 1735 W. Harrison St., Cohn Building Suite, 720, Chicago, IL 60612, USA. Tel: 1-312-942-4661; Fax: 1-312-942-8828; E-mail: kmasuda@rush.edu

Received 3 February 2006; revision accepted 10 May 2006.

The use of autologous serum-derived stimuli for the cultivation of cells *in vitro* circumvents the possibility of disease transmission. Platelet-rich plasma (PRP) is a fraction of plasma that can be produced by centrifugal separation of whole blood. Because PRP contains multiple growth factors concentrated at high levels[10–12], PRP from patients is used as an autologous source of growth factors for soft tissue and bone repair in several clinical settings[13–16]. PRP contains multiple growth factors, such as TGF-β[17], PDGF[8], epidermal growth factor (EGF)[18] and insulin-like growth factor-1 (IGF-1), and these growth factors induce biological changes in cell proliferation and matrix metabolism of a variety of connective tissues[10–12,19]. Because most growth factors found in PRP have been demonstrated to independently enhance the cell proliferation and/or matrix production of articular chondrocytes, we postulate that autologous PRP, a highly concentrated natural combination of growth factors, has the potential to stimulate cell proliferation and extracellular matrix metabolism by articular chondrocytes.

The purpose of this study was to examine the effects of PRP on cell proliferation and matrix synthesis by porcine chondrocytes cultured in alginate beads in order to determine the plausibility of using this plasma-derived material for engineering cartilage.

Materials and methods

ANIMALS—BLOOD AND CARTILAGE COLLECTION

Eight adult (1.0–1.5 years old) minipigs (Sinclair Research Center, Inc., Columbia, MO) were used to isolate PRP, platelet-poor plasma (PPP), and articular cartilage with the approval of the authors' institutional Animal Care and Use Committee. Animals were first anesthetized by subcutaneous injections of Telazol (4.4 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and Xylazine (2.2 mg/kg, Xyla-Ject®, Phoenix Pharmaceuticals, Inc., St. Joseph, MO). Blood (55 ml) was drawn from the popliteal vein with a 14G needle into a 60 ml syringe treated with an anticoagulant, citrate dextrose solution (Boehringer Laboratories Inc., Norristown, PA). After collecting peripheral blood, the animals were sacrificed by intravenously injecting supersaturated pentobarbital (Euthanasia B solution, Henry Schein Inc., Washington Port, NY) and the bilateral elbow joints were dissected *en bloc* without opening the joint capsule.

PREPARATION OF PRP AND PPP

PRP and PPP were isolated from fresh porcine blood (about 55 ml) using the SYMPHONY 2 Platelet Concentration System (DePuy Spine, Raynham, MA) according to the manufacturer's protocol. Briefly, the blood treated with anticoagulant was separated into plasma and hemocyte (erythrocyte and leukocyte) fractions, and then the plasma was separated into PRP (containing a high number of platelets) and PPP (containing few platelets) by a continuous two-step sedimentation. PRP and PPP were clotted by adding a 10% thrombin solution (v/v, 1000 U/ml in 100 mM $CaCl_2$) to yield a final thrombin concentration of 100 U/ml. Soluble PRP and PPP releasates from the clotted preparations were isolated by centrifugation (1500 *g* for 5 min) and cleared by ultrafiltration (0.22 μm). These final soluble releasates preparations of PRP and PPP were frozen at −80°C until used.

PLATELET COUNT ANALYSIS AND QUANTIFICATION OF A GROWTH FACTOR

The number of platelets in miniature swine whole blood (before centrifugation) and in isolated PPP and PRP fractions was assessed using the Advia 120 Bayer Hematology Analyzer (Bayer Diagnostics, Tarrytown, NY). As an indicator for the efficacy of the concentration of growth factors in PRP, the level of TGF-β in serum made from the whole blood used to prepare PPP and PRP fractions and in PPP and PRP releasates was assessed. The PPP and PRP isolated fractions were clotted with thrombin and centrifuged to obtain releasates as described above. The level of TGF-β in serum and in PPP and PRP releasates was determined using an enzyme-linked immunosorbent assay method according to the manufacturer's instructions (RD Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA Kit, R&D Systems, Inc., Minneapolis, MN).

CHONDROCYTE CULTURE IN ALGINATE BEADS

Following blood collection, articular cartilage was obtained from the elbow joints and pooled from two or three minipigs for each cell preparation. Chondrocytes were isolated from tissues by sequential enzyme digestion with 0.2% pronase (EMD Bioscience, La Jolla, CA) for 1 h, and 0.025% collagenase P (Roche Applied Science, Indianapolis, IN) for 16 h at 37°C, as previously described[7]. After several washes in Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12: Mediatech, Herndon, VA), the cells were resuspended in 1.2% low-viscosity sterile pharmaceutical grade alginate (Keltone LV-(HM), a gift from ISP Alginate Inc., San Diego, CA) solution at 4 million cells/ml[20]. The cultures, containing nine beads per well in a 24-well plate, were maintained in triplicate culture in 0.4 ml of complete medium containing 10% FBS (Hyclone, Logan, UT), 25 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), 360 μg/ml L-glutamine (Mediatech) and 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA)] in a 5% CO_2:95% air incubator at 37°C. The medium in all cases was changed daily.

CULTURE PROTOCOL FOR THE STUDY OF THE EFFECTS OF PRP RELEASATE, PPP RELEASATE AND FBS ON CELL PROLIFERATION AND PROTEOGLYCAN (PG) AND COLLAGEN SYNTHESIS

After 7 days of culture in complete medium, the cells were precultured in serum-free medium (SFM), which consisted of DMEM/F12 with supplements as described above, for 24 h. The cells were then cultured for another 72 h under three different conditions: FBS (10% FBS in SFM), 10% PPP (10% PPP releasate in SFM) or PRP (10% PRP releasate in SFM). The cultures were incubated in 0.8 ml of the respective medium for 72 h without changing the medium during the treatment period. A preliminary experiment demonstrated that the rate of incorporation of ^{35}S-sulfate into PGs by cells cultured under these conditions was constant throughout the 72-h treatment period (data not shown).

MEASUREMENT OF DNA CONTENT

To evaluate cell proliferation during the 72-h treatment period, the DNA content of alginate beads was determined using the bisbenzimidazole fluorescent dye (Hoechst 33258: Polysciences, Warrington, PA) method[21] with calf thymus DNA (Sigma-Aldrich) used as a standard. These DNA values were used to normalize the values obtained from the following analyses.

MEASUREMENT OF PG SYNTHESIS

The incorporation of radiolabeled ^{35}S into sulfated PGs was measured as an indicator of PG synthesis. During the last 4 h of the 72-h treatment period, the cells were cultured in the presence of ^{35}S-sulfate (final concentration: 20 μCi/ml; Perkin–Elmer Life and Analytical Sciences, Boston, MA). After the 72-h treatments, the beads and culture media were collected and the cell-associated and further removed matrix compartments (CM and FRM, respectively) were separated by dissolving the beads with sodium citrate followed by mild centrifugation, as previously described[20]. The CM fractions were extracted for 48 h at 4°C with 4 M guanidine-HCl in the presence of proteinase inhibitors[20]. The amount of radiolabeled ^{35}S-PGs in the CM and FRM fractions and in the medium was quantified by a rapid filtration assay following precipitation of the glycosaminoglycans with alcian blue[22].

CHARACTERIZATION OF ^{35}S-LABELED PGS

The size of newly synthesized PGs was assessed using Sepharose CL-2B sieve chromatography under dissociative conditions, as previously described[20]. Briefly, CM and FRM fractions extracted with 4 M guanidine-HCl were chromatographed on a Sepharose CL-2B column. The ^{35}S-PGs in each fraction were quantified by liquid scintillation counting. The void volume (V_o) and total volume (V_t) of the column were determined using a high-molecular-weight glucose polymer containing covalently bonded Reactive Blue 2 dye, blue dextran (Sigma-Aldrich), and free ^{35}S-Sulfate, respectively. The partition coefficient (Kd) of PGs in each column fraction was calculated as follows; $(V_e - V_o)/(V_t - V_o)$ where V_e represents the elution volume for each column fraction. Proportions of large and small PGs were calculated by using a Peak Fit v4.12™ software (SeaSolve Software. Inc., Framingham, MA) that performs nonlinear least squares curve-fitting using the Marquardt–Levenberg algorithm to find the minimum value of the sum of the squared deviations.

MEASUREMENT OF TOTAL PG CONTENT

After dissolving the alginate beads, the matrix compartments (CM and FRM) were separated and digested with papain (Sigma-Aldrich) at 60°C for 16 h[23]. The total sulfated PG content was measured in each compartment using a modified dimethylmethylene blue dye-binding method[24] in the presence of 0.03% alginate[25]. The sum of the total sulfated PG contents of the CM and FRM was used to represent the total content of PG.

MEASUREMENT OF COLLAGEN SYNTHESIS

The incorporation of radiolabeled proline into pepsin-resistant protein was measured as an indicator of collagen synthesis. During the last 16 h of the 72-h treatment period, the cultures were radiolabeled with L-[2,3,4,5-^{3}H]-proline (final concentration: 50 μCi/ml; Amersham Biosciences Corp., Piscataway, NJ) in the presence of β-aminopropionitrile (100 μg/ml, Sigma-Aldrich). After separation of the CM and FRM, as described above, the CM, FRM and medium fractions were digested with 0.5 M acetic acid including pepsin (Sigma-Aldrich; 100 μg/ml) at 4°C for 16 h. An aliquot (25 μl) of each sample and 125 μl of 30% trichloroacetic acid (TCA; final concentration 25%; Sigma-Aldrich) were pipetted into a well of a 96-well MultiScreen filtration plate assembly with gentle agitation for 1 h at 4°C[26]. The formed precipitate, which contained pepsin-resistant ^{3}H-labeled protein, was collected on the filter membrane, and unincorporated ^{3}H-proline was removed by washing each well three times with 200 μl of 10% TCA followed by vacuum filtration through the membrane. The membrane in each well was punched out into a scintillation vial with 500 μl of dissolving buffer (2% Sodium Dodecyl Sulfate (SDS) in 0.1 N NaOH) followed by gentle shaking overnight at 37°C. The radioactivity of the samples was counted after adding 2.5 ml of Hydrofluor scintillation fluid (National Diagnostics, Atlanta, GA). Although ^{3}H-proline can be incorporated into other proteins, a major portion of the pepsin-resistant radiolabeled molecules (greater than 80%) was precipitated with ammonium sulfate at 30% saturation and identified as the α1 chain of collagen molecules when analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and fluorography as described below in the "characterization of ^{3}H-labeled pepsin-resistant proteins." In addition, to assess collagen synthesis more specifically, representative samples were analyzed with separation of ^{3}H-hydroxyproline and ^{3}H-proline on reverse-phase high performance liquid chromatography equipped with an online radioactivity detector using previously described conditions[27]. This analysis showed that PRP treatment stimulated formation of ^{3}H-hydroxyproline to an extent similar to that assessed as ^{3}H-proline-labeled pepsin-resistant protein (data not shown).

CHARACTERIZATION OF ^{3}H-LABELED PEPSIN-RESISTANT PROTEINS

The same samples prepared for collagen synthesis were also used for the analysis of the types of newly synthesized pepsin-resistant protein. After pepsin digestion, as mentioned above, each sample was centrifuged at 12,000 *g* for 30 min at 4°C. The supernatants, containing the pepsin-resistant collagens, were precipitated by the addition of ammonium sulfate (176 mg/ml) and incubated overnight at 4°C. The samples were then centrifuged at 12,000 *g* for 30 min at 4°C and the pellet was recovered and resuspended in 0.4 M NaCl, 50 mM Tris. The samples were then dialyzed for 48 h at 4°C against 10 mM Tris containing CHAPS at 1 μg/ml and protein inhibitors (1 mM *N*-ethylmalemide, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA). The radiolabeled collagens were separated by SDS-PAGE (Mini-gel, Bio-Rad) in a 7.5% acrylamide gel (reducing conditions) and detected by fluorography[27].

STATISTICAL ANALYSES

All values are reported as the mean ± standard deviation of the results of analyses of three separate cultures of nine beads. Experiments were repeated on at least three separate occasions and representative data are presented. Analysis of variance with Fisher's Protected Least Significant Difference (PLSD) test as a *post hoc* test was used to assess the effect of treatment on DNA content, matrix synthesis and accumulation.

Results

PLATELET COUNT ANALYSIS OF PRP

Platelet count analysis was performed on whole blood and PPP and PRP releasates from eight miniature swine. The concentration of platelets of whole blood (baseline)

was $359 \pm 83 \times 10^3$/ml (range: $210-506 \times 10^3$/ml); compatible with the range of normal values for miniature swine ($217-770 \times 10^3$/ml)[28]. The platelet concentration system resulted in a 3.9-fold increase in the platelet count of the PRP fraction when compared to whole blood (PRP: $1399 \pm 174 \times 10^3$/ml, $P < 0.0001$, vs whole blood; PPP: $157 \pm 80 \times 10^3$/ml, $P < 0.05$, vs whole blood). These data were compatible with the data obtained from the manufacturer for human serum (4.7 fold).

QUANTIFICATION OF GROWTH FACTOR IN PRP

The level of TGF-β in whole blood and in PPP and PRP releasates was quantified in eight animals. The level of TGF-β was significantly higher (two fold) in the PRP releasates compared to the serum and/or PPP releasates (serum: 13.8 ± 0.9 ng/ml, PPP: 2.1 ± 0.3 ng/ml, $P < 0.001$ vs serum; PRP: 29.1 ± 8.0 ng/ml, $P < 0.00$-1, vs serum). The efficiency of concentration was lower than that reported in the human (six fold).

DNA CONTENT

To study the effects of PRP on cell proliferation, the cultures were treated with FBS, PPP or PRP for 72 h and the DNA content of the alginate beads was measured. Treatment with PRP resulted in a small but significant increase in DNA content when compared to the FBS and PPP groups (PRP: +11%, $P < 0.01$; vs FBS, +18%, $P < 0.001$; vs PPP, Fig. 1).

PG SYNTHESIS

The rate of PG synthesis (per μg DNA) by the PRP-treated chondrocytes was significantly higher than that by the FBS- or PPP-treated chondrocytes (PRP: +115% vs FBS; +151%, vs PPP; both $P < 0.0001$, Fig. 2). When the data were expressed per nine beads, essentially the same effects were apparent (data not shown). There were no significant differences between PG synthesis by the FBS- or PPP-treated chondrocytes (PPP vs FBS, $P = 0.27$). When the distribution of newly synthesized ^{35}S-PGs between medium and CM was analyzed, no significant treatment-related differences were observed. However, treatment with PRP significantly increased the proportion (41% vs 30%) of newly synthesized ^{35}S-PGs in the FRM compared to the treatment with FBS ($P < 0.05$, Fig. 2).

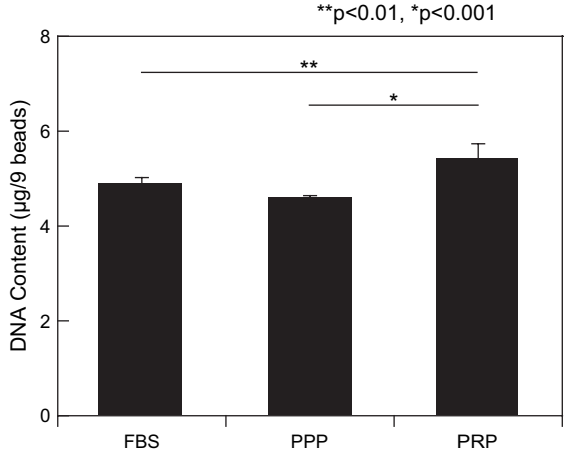

Fig. 1. Effect of PRP on cell proliferation of articular chondrocytes cultured in alginate beads. Isolated chondrocytes encapsulated in alginate beads were cultured in DMEM/F12 medium containing 10% FBS for 7 days. After serum starvation, the cells were treated for an additional 72 h with SFM containing 10% FBS, 10% PPP or 10% PRP. The content of DNA was measured using the Hoechst dye 33258 method as described in the Materials and methods section. The increase in DNA content in alginate beads containing PRP-treated cells was significantly higher than in those containing PPP-treated cells.

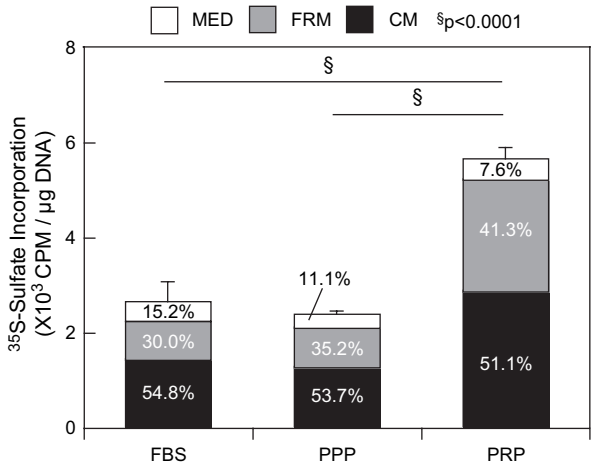

Fig. 2. Effect of PRP treatment on PG synthesis by articular chondrocytes cultured in alginate beads. During the last 4 h of the 72-h treatment period, the cells were radiolabeled with ^{35}S-sulfate (20 μCi/ml). The amount of radiolabeled ^{35}S-PGs in the CM extracts, the FRM fractions and the media were quantified (see the Materials and Methods section). The rate of PG synthesis by the PRP-treated chondrocytes was significantly faster than that by the PPP- or FBS-treated cells. The numbers presented in each column indicate how much PG was present in the CM, FRM and medium as a percentage of the total ^{35}S-PG in the beads.

To assess the phenotypic stability of chondrocytes, newly synthesized ^{35}S-PGs in the CM and FRM fractions were purified and subjected to sieve chromatography on Sepharose CL-2B under dissociative conditions. ^{35}S-PGs extracted both from the CM and FRM consisted predominantly of large molecular weight sulfated PGs (CM, average Kd = 0.27; FRM, average Kd = 0.30), which exhibited a profile similar to that of aggrecan, the major PG synthesized by mature chondrocytes cultured in alginate[20]. Small nonaggregating PGs (such as decorin, biglycan etc. [CM; average Kd = 0.65/FRM; average Kd = 0.70]) were present in much smaller amounts (Fig. 3). The size of the large molecular weight PGs and the proportion of small nonaggregating PGs in the PRP group were similar to those in the FBS or PPP groups in either the CM or FRM.

PG ACCUMULATION

Chondrocytes cultured in alginate beads for 3 days in the presence of PRP accumulated significantly more sulfated PGs than chondrocytes in beads cultured in FBS or PPP (PRP: +15%, vs FBS, $P < 0.001$; +26% vs PPP; $P < 0.0001$, Fig. 4). However, when the data were expressed per μg DNA, there was no significant difference in PG accumulation among the three experimental groups (data not shown). No significant differences in the distribution of PGs within the CM or FRM were observed (Fig. 4).

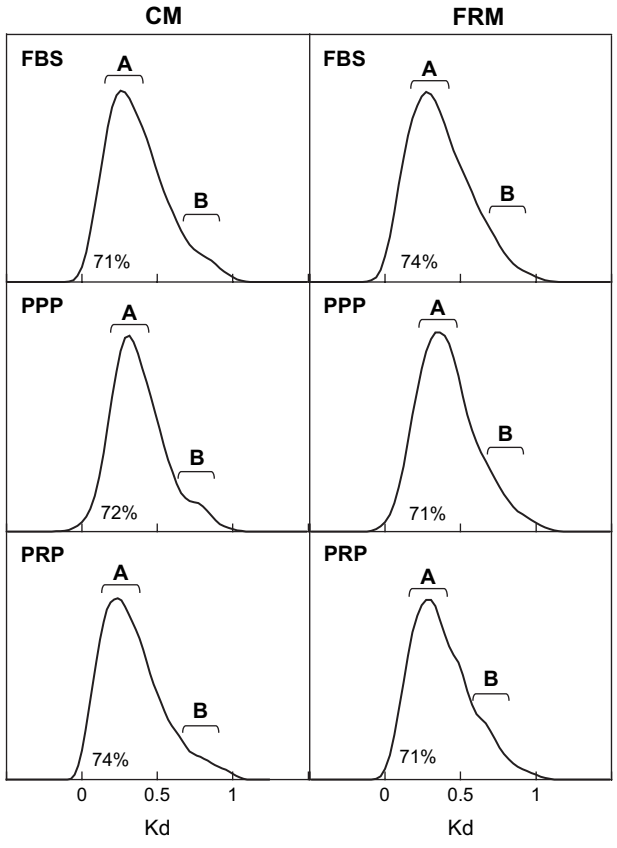

Fig. 3. Characterization of newly synthesized PGs in the CM and FRM fractions. Sepharose CL-2B molecular sieve chromatography of ^{35}S-PGs isolated from beads cultured under three different conditions: 10% FBS in SFM (FBS), 10% PPP releasate in SFM (PPP) or 10% PRP releasate in the SFM (PRP). The chromatography was performed using dissociative conditions (see Materials and Methods section). Percentages for each peak represent the proportion of [A] large (similar to aggrecan) and [B] small (decorin, biglycan, etc.) ^{35}S-PGs in each group. In addition, the average partition coefficient of the two molecules (peak A and B) is shown as follows: (CM; [A] average Kd = 0.27, [B] average Kd = 0.66/FRM; average Kd [A] = 0.30, average Kd [B] = 0.70).

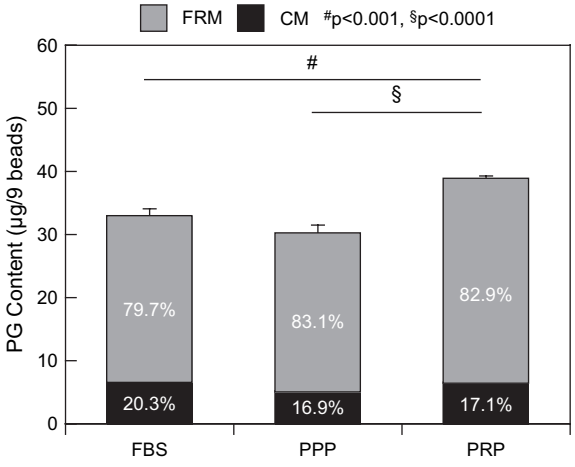

Fig. 4. Effect of PRP treatment on PG accumulation by articular chondrocytes cultured in alginate beads. After the 72-h treatment period, the total sulfated PG content was measured in each compartment using a modified dimethylmethylene blue dye-binding method. Articular chondrocytes cultured in alginate beads treated with 10% PRP accumulated significantly more PGs than those treated with FBS ($P < 0.001$)) or PPP ($P < 0.0001$). The numbers presented in each column indicate how much PG was present in the CM and FRM as a percentage of the total PG content in the beads.

COLLAGEN SYNTHESIS

The amount of newly synthesized pepsin-resistant ^{3}H-protein (per µg DNA), as a measure of collagen synthesis by PRP-treated cells, was remarkably higher than that by FBS- or PPP-treated cells (PRP: +163% vs FBS; +163% vs PPP; both $P < 0.0001$, Fig. 5). Measurement of pepsin-resistant ^{3}H-protein synthesis per nine beads yielded essentially the same results as the data expressed per µg DNA (data not shown). There were no significant differences in synthesis between FBS- and PPP-treated cells (PPP vs FBS; $P = 0.1$). The proportion of newly synthesized pepsin-resistant ^{3}H-protein within the medium, CM or FRM did not change as a result of treatment (data not shown).

The profile of newly synthesized pepsin-resistant protein was evaluated by separation of the ^{3}H-proline-labeled, pepsin-resistant proteins incorporated into the CM and FRM compartments using SDS-PAGE. The results of SDS-PAGE fluorography revealed that both compartments in each experimental group contained a similar prominent band corresponding to the α1 chain of the collagen molecules at 95 kDa (Fig. 6). An α2 chain, that would have indicated the presence of type I collagen, was not detected. This relative prominence of α1 chains suggests that the majority of the collagen synthesized in the presence of FBS, PPP or PRP was type II collagen, as previously described[29,30]. This 95 kDa band was confirmed to be immunopositive to an anti-type II collagen antibody (data not shown) and did not react with an anti-type I collagen antibody (data not shown).

Discussion

The study presented here is the first to show that PRP, a "natural cocktail" of growth factors obtained using a point of care apparatus, has the potential to stimulate cell proliferation and matrix metabolism of porcine articular chondrocytes cultured in alginate beads. At the same concentration (10% in SFM), the stimulatory effects of PRP were stronger than those of FBS or PPP. Furthermore, the major profiles of PGs and collagens synthesized by chondrocytes in the presence of PRP were similar to those seen in cells cultured in FBS.

A platelet contains the vast majority of biologically active molecules required for blood coagulation, such as adhesive proteins, coagulation factors and protease inhibitors, within cytoplasmic α-granules[31]. In addition to the factors that coagulate blood, growth factors such as TGF-β, PDGF, EGF, vascular endothelial growth factor and IGF-1 are released from α-granules[31,32] when platelets are activated. These growth factors are known to stimulate collagen deposition, accelerate epithelial regeneration, promote angiogenesis, and ultimately improve wound healing in skin and periodontal tissue[33–36].

Recently, the use of patient autologous PRP, which serves as a source of growth factors able to induce tissue regeneration, has gained wide acceptance in several surgical applications, such as bone regeneration in periodontal and maxillofacial surgery[37–39], orthopedic surgery[40],

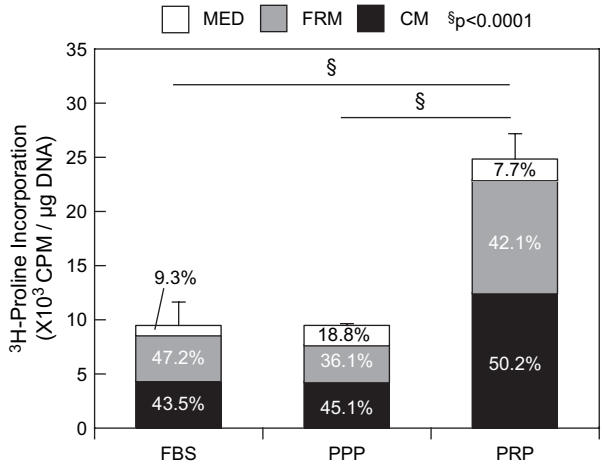

Fig. 5. Effect of PRP treatment on collagen synthesis by porcine articular chondrocytes cultured in alginate beads. The incorporation of ^{3}H-labeled proline into pepsin-resistant protein was measured as an indicator of collagen synthesis. During the last 16 h of the 72-h treatment period, the cells were radiolabeled with L-[2,3,4,5-^{3}H]-proline (50 µCi/ml). Radioactivity in the pepsin-resistant radiolabeled proteins was measured as described in the Materials and Methods section. The rate of collagen synthesis of FBS-treated cells was similar to that of PPP-treated cells. However, the stimulatory effect on the rate of collagen synthesis was greater in the case of PRP-treated cells than of cells treated with FBS or PPP. The numbers presented in each column indicate how much collagen was present in the CM, FRM and medium as a percentage of the total ^{3}H-labeled collagen in the beads.

otolaryngology[41] and plastic surgery[42]. The same approach, using a platelet-rich gel, was recently applied to wound healing[16,36] and cardiovascular surgery[43]. Those growth factors identified in PRP independently stimulate either cell proliferation or matrix metabolism by articular chondrocytes[8,17,18,44,45]. This study focused on the effect of PRP on matrix synthesis and accumulation to test the practicality of using PRP as a tissue culture supplement.

Adult minipigs were used in this study as a source of both articular cartilage and blood. Due to the limitation in the number of cells that could be obtained from the elbow joint of a single minipig, cells from different animals were pooled

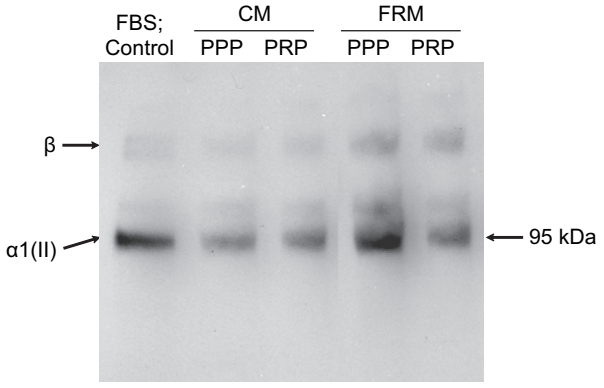

Fig. 6. Characterization of newly synthesized collagens in CM and FRM fractions. A fluorogram of pepsin-resistant ^{3}H-proline-labeled proteins in the CM fractions of FBS-, PPP- and PRP-treated groups. The radiolabeled collagens were separated by SDS-PAGE in a 7.5% acrylamide gel (reducing conditions) and detected by fluorography. The CM fractions in each group contained a similar prominent band at 95 kDa by SDS-PAGE fluorography.

to perform the experiments. Therefore, it was not possible to use autologous PRP, which would have been preferable. This notwithstanding, it should be noted that four different batches of PRP from individual animals were tested and yielded essentially similar results. Although our study to determine the concentration of growth factors was limited to TGF-β, a significant concentration of TGF-β (two fold, 29.1 ng/ml) was achieved. Using human blood, further studies on the effects of donor age, length and other conditions of storage of PRP, and biological characteristic variability should be performed. Thrombin, which has been shown to have some biological effects on cell metabolism, was used to form a clot in the preparation of PRP[46–49]. Because PPP was also treated with thrombin in order to control for the effects of thrombin, it is unlikely that thrombin itself contributed to the differences between the effects of PRP and PPP on cell proliferation and matrix metabolism.

Although the PG accumulation per beads and PG synthesis per DNA were significantly upregulated by the PRP treatment, the PG accumulation per DNA was not significantly different among the treatment groups. This suggests that the increased sulfated PG accumulation in alginate beads containing chondrocytes cultured with PRP was mainly a result of increased cell proliferation induced by the PRP treatment for 3 days. In addition, incubation in the three different experimental conditions for a period of 3 days might not be long enough to induce a significant difference in the PG content in alginate beads that had been precultured for 6 days under identical conditions.

In porcine chondrocytes, the effects of PRP were more pronounced on collagen synthesis than PG synthesis, especially when compared to cells treated with FBS. The essential role of growth factors released from platelets is to accelerate the wound-healing process of soft tissues (such as blood vessel walls, muscle and skin)[14] or hard tissues such as bone after injury to those tissues[15]; these tissues all contain large amounts of collagen. Thus, this 'natural cocktail' of growth factors included in PRP might have the ability to preferentially promote collagen synthesis. This is especially important because most tissue-engineered cartilage lacks sufficient collagen when formed *in vitro*[7,50].

Several investigators have previously reported that platelet lysates stimulate the cell proliferation of chondrocytes[51–53]. However, the results on the effects of platelet lysates on matrix accumulation differ from our results with PRP, probably because of differences in isolation procedures[51–53]. In the study by Choi *et al.*[51], the "platelet lysate" was isolated by centrifugation and repeated freeze–thawing, while the "PRP releasate" used in the present study was purified by a "point of care" procedure followed by clot formation with thrombin. These differences in isolation may result in differences in the proportions and/or concentrations of growth factors and other plasma components present in "platelet lysates" and "PRP". The technical advances in the isolation of the PRP releasate may promote its acceptance as a simple procedure to obtain culture supplements that stimulate the metabolism of chondrocytes.

For clinical use, autologous chondrocyte transplantation (ACT) has been used to repair articular cartilage defects[54]. The most popular and commercially available cell-based therapy for autologous chondrocyte implantation in the United States is Carticel® (Genzyme Biosurgery, Boston, MA), which uses FBS in the culture process. When utilizing FBS in culture systems for clinical use, there is a potential risk for the transmission of animal diseases. To avoid the risk of zoonotic transmission, the use of autologous serum from patients is preferable in *in vitro* cell-based culture

systems[55,56]. Because PRP has the potential to stimulate cell proliferation and matrix synthesis by articular chondrocytes, the application of autologous PRP from patients may be an attractive supplement for ACT, as well as tissue-engineered cartilage. Although the identification of factors involved in the effect of PRP on matrix metabolism of chondrocyte was outside the scope of this study, a study aimed at determining the concentration of growth factors responsible for these effects and changes of concentration with aging will provide important information about the limitations of this approach.

The use of PRP has several advantages over the use of recombinant growth factors or products of animal origin. First, as a point of care approach, the autologous preparation of PRP avoids the complex regulatory pathway. In addition, safety issues, such as immunologic reactions or carcinogenesis, are of much less concern. Finally, the costs of using PRP would be considerably less than those associated with the use of recombinant proteins. If 800 ml of autologous blood was collected from a patient, this would translate into 600 ml of culture medium containing 10% PRP and 1500 ml of 10% PPP-containing medium, making this a feasible approach to *in vitro* cell-based cartilage tissue engineering. For the application of PRP growth factors to human tissue-engineered cartilage, further studies on the effects of PRP on matrix metabolism by human primary and passaged chondrocyte should be performed.

The results of this study provide evidence for the plausible use of PRP to stimulate the matrix metabolism of articular cartilage engineered *in vitro*. Further studies to establish the efficacy and reproducibility of PRP prepared from human blood, especially from donors of different ages, to stimulate chondrocyte metabolism should be performed to extend this simple and safe new approach to the engineering of cartilage constructs.

Acknowledgments

This work was supported by a research grant from DePuy Spine Inc. and in part by National Institute of Health (NIH) grants 2-P50-AR39239, 1-P01-AR48152 and 1-R21-EB-004905 and a Howard Hughes Medical Institute Professor Award to University of California, San Diego (RLS). The authors would like to thank Dr Thomas Schmid, Department of Biochemistry, Rush Medical College, and for the help in analyzing collagen profiles and Dr Cynthia Lee, DePuy Biologics, for fruitful discussions.

References

1. Buckwalter JA. Integration of science into orthopaedic practice: implications for solving the problem of articular cartilage repair. J Bone Joint Surg Am 2003;85-A(Suppl 2):1–7.
2. Hjertquist SO, Lemperg R. Histological, autoradiographic and microchemical studies of spontaneously healing osteochondral articular defects in adult rabbits. Calcif Tissue Res 1971;8:54–72.
3. Wei X, Messner K. Maturation-dependent durability of spontaneous cartilage repair in rabbit knee joint. J Biomed Mater Res 1999;46:539–48.
4. Hunziker EB. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. Osteoarthritis Cartilage 2002;10: 432–63.
5. Adkisson HD, Gillis MP, Davis EC, Maloney W, Hruska KA. In vitro generation of scaffold independent neocartilage. Clin Orthop 2001;391(Suppl):S280–94.
6. Kandel RA, Boyle J, Gibson G, Cruz T, Speagle M. In vitro formation of mineralized cartilagenous tissue by articular chondrocytes. In Vitro Cell Dev Biol Anim 1997;33:174–81.
7. Masuda K, Sah RL, Hejna MJ, Thonar EJ. A novel two-step method for the formation of tissue-engineered cartilage by mature bovine chondrocytes: the alginate-recovered-chondrocyte (arc) method. J Orthop Res 2003;21:139–48.
8. Weiser L, Bhargava M, Attia E, Torzilli PA. Effect of serum and platelet-derived growth factor on chondrocytes grown in collagen gels. Tissue Eng 1999;5:533–44.
9. Barbero A, Ploegert S, Heberer M, Martin I. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum 2003;48:1315–25.
10. Weibrich G, Kleis WK, Hafner G, Hitzler WE. Growth factor levels in platelet-rich plasma and correlations with donor age, sex, and platelet count. J Craniomaxillofac Surg 2002;30:97–102.
11. Okuda K, Kawase T, Momose M, Murata M, Saito Y, Suzuki H, *et al*. Platelet-rich plasma contains high levels of platelet-derived growth factor and transforming growth factor-beta and modulates the proliferation of periodontally related cells in vitro. J Periodontol 2003;74:849–57.
12. Dugrillon A, Eichler H, Kern S, Kluter H. Autologous concentrated platelet-rich plasma (cPRP) for local application in bone regeneration. Int J Oral Maxillofac Surg 2002;31:615–9.
13. Anitua E. Plasma rich in growth factors: preliminary results of use in the preparation of future sites for implants. Int J Oral Maxillofac Implants 1999;14:529–35.
14. Anitua E, Andia I, Ardanza B, Nurden P, Nurden AT. Autologous platelets as a source of proteins for healing and tissue regeneration. Thromb Haemost 2004; 91:4–15.
15. Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998;85:638–46.
16. Crovetti G, Martinelli G, Issi M, Barone M, Guizzardi M, Campanati B, *et al*. Platelet gel for healing cutaneous chronic wounds. Transfus Apher Sci 2004;30:145–51.
17. Grimaud E, Heymann D, Redini F. Recent advances in TGF-beta effects on chondrocyte metabolism. Potential therapeutic roles of TGF-beta in cartilage disorders. Cytokine Growth Factor Rev 2002;13:241–57.
18. Kato Y, Hiraki Y, Inoue H, Kinoshita M, Yutani Y, Suzuki F. Differential and synergistic actions of somatomedin-like growth factors, fibroblast growth factor and epidermal growth factor in rabbit costal chondrocytes. Eur J Biochem 1983;129:685–90.
19. Landesberg R, Roy M, Glickman RS. Quantification of growth factor levels using a simplified method of platelet-rich plasma gel preparation. J Oral Maxillofac Surg 2000;58:297–300.
20. Mok SS, Masuda K, Hauselmann HJ, Aydelotte MB, Thonar EJ. Aggrecan synthesized by mature bovine chondrocytes suspended in alginate. Identification of two distinct metabolic matrix pools. J Biol Chem 1994;269:33021–7.
21. Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using hoechst 33258. Anal Biochem 1988;174:168–76.

22. Masuda K, Shirota H, Thonar EJ. Quantification of 35s-labeled proteoglycans complexed to alcian blue by rapid filtration in multiwell plates. Anal Biochem 1994;217:167–75.
23. Chiba K, Andersson GB, Masuda K, Thonar EJ. Metabolism of the extracellular matrix formed by intervertebral disc cells cultured in alginate. Spine 1997;22: 2885–93.
24. Chandrasekhar S, Esterman MA, Hoffman HA. Microdetermination of proteoglycans and glycosaminoglycans in the presence of guanidine hydrochloride. Anal Biochem 1987;161:103–8.
25. D'Souza AL, Masuda K, Otten LM, Nishida Y, Knudson W, Thonar EJ. Differential effects of interleukin-1 on hyaluronan and proteoglycan metabolism in two compartments of the matrix formed by articular chondrocytes maintained in alginate. Arch Biochem Biophys 2000;374:59–65.
26. Koyano Y, Hammerle H, Mollenhauer J. Analysis of 3h-proline-labeled protein by rapid filtration in multiwell plates for the study of collagen metabolism. Biotechniques 1997;22:706–8, 710–2, 714 passim.
27. Masuda K, Takegami K, An H, Kumano F, Chiba K, Andersson GB, *et al*. Recombinant osteogenic protein-1 upregulates extracellular matrix metabolism by rabbit annulus fibrosus and nucleus pulposus cells cultured in alginate beads. J Orthop Res 2003;21:922–30.
28. Radin MJ, Weiser MG, Fettman MJ. Hematologic and serum biochemical values for Yucatan miniature swine. Lab Anim Sci 1986;36:425–7.
29. Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, Aydelotte MB, *et al*. Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J Cell Sci 1994;107(Pt 1):17–27.
30. Petit B, Masuda K, D'Souza AL, Otten L, Pietryla D, Hartmann DJ, *et al*. Characterization of crosslinked collagens synthesized by mature articular chondrocytes cultured in alginate beads: comparison of two distinct matrix compartments. Exp Cell Res 1996; 225:151–61.
31. Harrison P, Cramer EM. Platelet alpha-granules. Blood Rev 1993;7:52–62.
32. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. J Biol Chem 1983;258: 7155–60.
33. Declair V. The importance of growth factors in wound healing. Ostomy Wound Manage 1999;45:64–8, 70–2, 74 passim.
34. Ross R. Platelet-derived growth factor. Annu Rev Med 1987;38:71–9.
35. Kawase T, Okuda K, Wolff LF, Yoshie H. Platelet-rich plasma-derived fibrin clot formation stimulates collagen synthesis in periodontal ligament and osteoblastic cells in vitro. J Periodontol 2003;74:858–64.
36. Carter CA, Jolly DG, Worden CE Sr, Hendren DG, Kane CJ. Platelet-rich plasma gel promotes differentiation and regeneration during equine wound healing. Exp Mol Pathol 2003;74:244–55.
37. Anitua E. The use of plasma-rich growth factors (PRGF) in oral surgery. Pract Proced Aesthet Dent 2001;13:487–93,
38. Carlson NE, Roach RB Jr. Platelet-rich plasma: clinical applications in dentistry. J Am Dent Assoc 2002;133: 1383–6.
39. Tsay RC, Vo J, Burke A, Eisig SB, Lu HH, Landesberg R. Differential growth factor retention by platelet-rich plasma composites. J Oral Maxillofac Surg 2005;63:521–8.
40. Li H, Zou X, Xue Q, Egund N, Lind M, Bunger C. Anterior lumbar interbody fusion with carbon fiber cage loaded with bioceramics and platelet-rich plasma. An experimental study on pigs. Eur Spine J 2004;354–8.
41. Lozada JL, Caplanis N, Proussaefs P, Willardsen J, Kammeyer G. Platelet-rich plasma application in sinus graft surgery: part I—background and processing techniques. J Oral Implantol 2001;27:38–42.
42. Man D, Plosker H, Winland-Brown JE. The use of autologous platelet-rich plasma (platelet gel) and autologous platelet-poor plasma (fibrin glue) in cosmetic surgery. Plast Reconstr Surg 2001;107:229–37, discussion 238–9.
43. Hiramatsu T, Okamura T, Imai Y, Kurosawa H, Aoki M, Shin'oka T, *et al*. Effects of autologous platelet concentrate reinfusion after open heart surgery in patients with congenital heart disease. Ann Thorac Surg 2002; 73:1282–5.
44. Tyler JA. Insulin-like growth factor 1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to cytokines. Biochem J 1989;260:543–8.
45. McQuillan DJ, Handley CJ, Campbell MA, Bolis S, Milway VE, Herington AC. Stimulation of proteoglycan biosynthesis by serum and insulin-like growth factor-I in cultured bovine articular cartilage. Biochem J 1986;240:423–30.
46. Fujii N, Kaji T, Akai T, Koizumi F. Thrombin reduces large heparan sulfate proteoglycan molecules in cultured vascular endothelial cell layers through inhibition of core protein synthesis. Thromb Res 1997;88: 299–307.
47. Chan B, Merchan JR, Kale S, Sukhatme VP. Antiangiogenic property of human thrombin. Microvasc Res 2003;66:1–14.
48. Cizmeci-Smith G, Carey DJ. Thrombin stimulates syndecan-1 promotor activity and expression of a form of syndecan-1 that binds antithrombin iii in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 1997;17:2609–16.
49. Haralabopoulos GC, Grant DS, Kleinman HK, Maragoudakis ME. Thrombin promotes endothelial cell alignment in matrigel in vitro and angiogenesis in vivo. Am J Physiol 1997;273(1 Pt 1):C239–45.
50. Grogan SP, Rieser F, Winkelmann V, Berardi S, Mainil-Varlet P. A static, closed and scaffold-free bioreactor system that permits chondrogenesis in vitro. Osteoarthritis Cartilage 2003;11:403–11.
51. Choi YC, Morris GM, Sokoloff L. Effect of platelet lysate on growth and sulfated glycosaminoglycan synthesis in articular chondrocyte cultures. Arthritis Rheum 1980;23:220–4.
52. Castor C, Ritchie J, Williams C, Scott M, Whitney S, Myers S, *et al*. Connective tissue activation. Xiv. Composition and actions of a human platelet autacoid mediator. Arthritis Rheum 1979;22:260–72.
53. Kaps C, Loch A, Haisch A, Smolian H, Burmester GR, Haupl T, *et al*. Human platelet supernatant promotes proliferation but not differentiation of articular chondrocytes. Med Biol Eng Comput 2002;40:485–90.
54. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889–95.

55. Harrison PE, Pfeifer PM, Turner SL, Richardson JB, Jones PW, Ashton BA. Serum from patients anesthetized with opiates less effective in the support of chondrocyte growth in vitro. Tissue Eng 2003;9: 37–9.

56. Litzke LE, Wagner E, Baumgaertner W, Hetzel U, Josimovic-Alasevic O, Libera J. Repair of extensive articular cartilage defects in horses by autologous chondrocyte transplantation. Ann Biomed Eng 2004; 32:57–69.