Platelet-rich plasma exhibits beneficial effects for rheumatoid arthritis mice by suppressing inflammatory factors

SHICHAO TONG[1], CHANGQING ZHANG[1] and JI LIU[2]

[1]Department of Orthopedics, Shanghai Sixth People's Hospital; [2]Department of Orthopedics, Shanghai Ruijin Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200233, P.R. China

Abstract. Platelet-rich plasma (PRP) is a multifunctional blood product containing highly concentrated platelets, and various cell growth factors which promote cell proliferation and differentiation. PRP exhibited benefits in injurious articular cartilage repair and the removal of inflammatory factors in clinical studies. Rheumatoid arthritis (RA) is an autoimmune disease manifesting primarily as inflammatory arthritis, which is associated with notable morbidity in humans. In the present study, the therapeutic effects and primary mechanism of PRP on a type II collagen-induced arthritis (CIA) mouse model was investigated. Inflammatory factors interleukin (IL)-6, IL-8, IL-17, IL-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ were analyzed in PRP and PBS-treated groups. Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)-β expression in peripheral whole blood was additionally analyzed. The therapeutic efficacy of PRP for RA mice was evaluated using clinical arthritis scores. The results of the present study demonstrated that treatment with PRP alleviated arthritis, and reduced humoral and cellular immune responses, leading to beneficial effects on histological parameters as observed using joint tissue histological staining. CIA mice treated with PRP exhibited downregulated expression of IL-6, IL-8, IL-17A, IL-1β, TNF-α, receptor activator for nuclear factor-κB and IFN-γ in inflammatory tissue. In addition, VEGF, PDGF, IGF-1 and TGF-β expression in peripheral whole blood was increased following treatment with PRP. The serum concentration of anti-collagen antibody was decreased in PRP-treated CIA mice. In conclusion, CIA mice treated with PRP exhibited beneficial effects, including decreased joint inflammation, cartilage destruction and bone damage, and increased repair of joint tissue. The results of the present study suggested that PRP may be an effective therapeutic agent for RA.

Introduction

Platelet-rich plasma (PRP) is a prominent biomedical agent that has demonstrated efficacy in the treatment of femoral head damage, cartilage disorders and rheumatoid arthritis (1). PRP is a blood product rich in cytokines, growth factors and other bio-active molecules, which is obtained from autologous peripheral blood mononuclear cells (PBMCs) and has been used in innovative treatment protocols (2). The use of PRP may not lead to the side effects and treatment-associated adverse events of classical drug products (3). Previous studies demonstrated that an intravenous injection of PRP was able to repair tendons and damaged articular bone, and contribute to inflammatory elimination, which may serve an important role in the morphology, collagen microarchitecture and subsequent mechanical properties of the injected vein (2,4,5). As demonstrated in previous studies, treatment with PRP results in marked clinical improvements in patients with symptomatic osteoarthritis of the knee (6-8). Rheumatoid arthritis (RA) is an intractable and chronic inflammatory autoimmune disease characterized by the release of inflammatory cytokines, leading to infiltration of autoimmune cells into the synovium and subsequent joint damage (9).

Innate and adaptive immune responses of autoantibodies have been demonstrated to serve a role in the dysregulated expression of inflammatory cytokines, which may target the synovium and subsequently result in joint disease (9,10). RA is an autoimmune disease characterized by chronic inflammation of the joints (11). RA is associated with inflammation of synovial joints, primarily the hands and feet, in addition to systemic extra-articular inflammation (12). Inflammatory cytokines are identified as a complex regulatory signaling network in RA, which is mediated by various intracellular kinase signaling pathways to regulate the recruitment, stimulation, activation and function of autoimmune cells (13). Although the causes of RA are not completely understood, laboratory and clinical evidence suggests that pro-inflammatory cytokines, particularly tumor necrosis factor (TNF), serve an important role in its pathogenesis (14,15). It has been hypothesized that inhibiting the TNF pathway using a specific anti-TNF-α antibody may interrupt the inflammatory process to break the cycle of

Correspondence to: Professor Changqing Zhang, Department of Orthopedics, Shanghai Sixth People's Hospital, Shanghai Jiaotong University, 600 Yishan Road, Shanghai 200233, P.R. China
E-mail: angcq@sjtu.edu.cn; zhangchangqingsci@163.com

inflammation and limit joint damage (16,17). A previous study has indicated that intracellular signaling pathways mediated by inflammatory cytokines may underlie the mechanism of initiation, development and aggravation of RA, and may represent a key interaction in the cytokine-induced RA network (18). Therefore, therapeutic agents targeted to inflammatory cytokines may be beneficial for RA treatment by modulating and neutralizing cytokines, leading to inhibition of inflammatory responses.

In the present study, the efficacy and mechanism of action of PRP was investigated in a type II collagen-induced arthritis (CIA) mouse model. Inflammatory factors were analyzed in PRP and PBS-treated groups. The therapeutic efficacy of PRP for RA mice was evaluated using clinical arthritis scores. The effects of PRP on the tyrosine protein kinase JAK (JAK) signaling pathway, and on immune and inflammatory responses in RA mice, were analyzed.

Materials and methods

Ethics statement. The present study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MA, USA) (19). The protocol was approved by the Chinese Association for Laboratory Animal Sciences (Beijing, China), Animal Health Products (Beijing, China), Committee on the Ethics of Animal Experiments Defense Research (Beijing, China) and Development China (Beijing, China), and Animal Experiments of Shanghai Sixth People's Hospital (approval no. SCXK-2014-1243). All surgery and euthanasia was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Cells and reagents. Hela cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Mouse RA synoviocytes were donated by Harbin Medical University (Harbin, China). Culture flasks, medium, chemicals and reagents were purchased from recognized commercial suppliers.

Detection of cytokine gene expression. Mouse RA synoviocytes were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with 10% fetal bovine serum (FBS) in 6-well plates. At 90% confluence, cells were stimulated with 50 μl/ml PRP (Sigma-Aldrich; Merck KGaA), or 50 μl/ml, dexamethasone (DEX; Sigma-Aldrich; Merck KGaA) or PBS for 12 h at 37°C in a 5% CO_2 humidified atmosphere. RA synoviocytes without antigen and antibody were used as negative controls. Hela cells incubated with DEX, PRP or PBS for 12 h were used as positive controls. The cells were collected following 12 h of incubation, and total RNA was extracted to detect the expression of mRNA for IL-6 and IL-8 by using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays. PMBCs were prepared by Ficoll-Hypaque density gradient (Sigma-Aldrich; Merck KGaA) centrifugation (2,000 x g

at 4°C for 10 min). ADCC and CDC activities of PRP were measured by lactate dehydrogenase (LDH) assay (Merck KGaA, Darmstadt, Germany), which measures the activity of LDH released from the cytosol of damaged cells. Hela cells stably expressing transmembrane TNF-α were incubated with different concentrations (0.2-35 μg/ml) of anti-human TNF antibody for 1 h in assay medium (DMEM + 5% FBS) in a 5% CO_2 incubator at 37°C, followed by the addition of human PBMCs (Shanghai Sixth People's Hospital, Shanghai, China) as effector cells (ADCC assay, effector to target ratio = 20:1) or human complement-rich serum (Quidel Corporation, San Diego, CA, USA) (CDC assay, 1.25% v/v). Following an additional incubation at 37°C for 16 h for the ADCC assay and 5 h for the CDC assay, 100 μl supernatant from each well was transferred into a flat-bottomed 96-well plate. LDH substrate (100 μl) was added to each well and incubated for 30 min at room temperature in the dark. The absorbance of the samples was measure at 490 nm with a microplate reader.

RNA isolation and RT-qPCR. Total RNA was extracted from RA synoviocytes or synovial tissue using RNAzol (Sigma-Aldrich; Merck KGaA), and RNase-free DNase (Sigma-Aldrich; Merck KGaA) was used to digest total RNA at 37°C for 15 min. An RNeasy kit (Sigma-Aldrich; Merck KGaA) was used to purify RNA and adjust its concentration to 1 μg/μl. A total of 1 μg RNA was reverse-transcribed using the Thermo Script RT-qPCR system (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The concentration of RNA was quantified using spectrophotometry at 260 nm (Smart Spec TM 3000; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total RNA in the RA synoviocytes and synovial tissue of CIA mice was denatured by incubating for 5 min at 70°C with 4 μM oligo(dT) primer and reverse-transcribed using a final concentration of 0.5 mM dNTP, 40 U/μl RNaseOUT (Sigma-Aldrich; Merck KGaA), 0.01 M dithiothreitol, and 10 U/μl Thermo Scriptreverse transcriptase (mRNA expression in RA synoviocytes or synovial tissue). Reverse transcription was performed by incubating at 42°C for 180 min. The obtained cDNA was diluted 10-fold with distilled H_2O and 10 μl was used for amplification. Specific primer sets for interleukin (IL)-6, IL-8, matrix metalloproteinase (MMP)-3, IL-17A, IL-1β, TNF-α, receptor activator for nuclear factor-κB ligand (RANKL), VEGF, PDGF, IGF-1, TGF-β and interferon-γ (IFN-γ) were generated. All PCR primers are listed in Table I. The PCR was performed using the Light Cycler Fast Start DNA SYBR Green I kit (Roche Diagnostics, Basel, Switzerland) according to the protocol provided with the parameter-specific kits (45 amplification cycles, denaturation at 96°C, primer annealing at 68°C with touchdown to 58°C, and applicant extension at 72°C) (20).

Animal study. DBA/1J male mice at 8-10 weeks of age (30-35 g) were purchased from Shanghai SLAC Laboratory Animals Co., Ltd. (Shanghai, China). Mice were maintained in a climate-controlled 20-25°C environment with a 12-h light/dark cycle. The induction of type II CIA was achieved as previously described (21), by the subcutaneous injection of 2 mg collagen (ModiQuest Research, Oss, Netherlands) in each mouse (n=9/group) (22). Clinical arthritis scores

Table I. Sequences of primers used.

Gene name	Sequence	
	Reverse	Forward
TNF-α	5'-TCCAGACTTCCTTGAGACA-3'	5'-GGCGATTACAGACACAACT-3'
IL-1β	5'-GGCTGCTTCCAAACCTTTGA-3'	5'-GAAGACACGGATTCCATGGT-3'
IL-6	5'-GTGAGGAACAAGCCAGAG-3'	5'-TGACCAGAAGAAGGAATGC-3'
IL-17A	5'-ATGCACAGCCACCGCGACTT-3'	5'-CTTCATGACTGCCTCCAAGTAG-3'
MMP-3	5'-GCCCTGGAACTCACACGACA-3'	5'-TTGGAAACTCACACGCCAGAAG-3'
RANKL	5'-AAGGCGAGAGATTCTTTCCCT G'-3'	5'-ACTGGGGACAATTCACTAGAGC-3'
VEGF	5'-TGCATTCACATTGTGCTGCTGTAG-3'	5'-GCAGATTATGCGGATCAAACC-3'
IGF-1	5'-ATCCATGTGTGACCATGAGGAAATG-3'	5'-TCGGCTAGTTAGGGTACACTTC-3'
TGF-β	5'-ATCCATGTGTGACCATGAGGAAATG-3'	5'TCGGCTAGTTAGGGTACACTTC-3'
PDGF	5'-ATGAGAGTGAGATCGAAGGCA-3'	5'-CGGCAAGGTATGATGGCAGAG-3'
IFN-γ	5'-CACCCTCTGTCACCTGCTCAA-3'	5'-ATGGCGCTGAGAAGACTTGGT-3'
β-actin	5'-CGGAGTCAACGGATTTGGTC-3'	5'-AGCCTTCTCCATGGTCGTGA-3'

TNF, tumor necrosis factor; IL, interleukin; MMP, matrix metalloproteinase; RANKL, nuclear factor-κB ligand; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; TGF, transforming growth factor; PDGF, platelet-derived growth factor; IFN, interferon.

were evaluated using a scale of 0-2 for each paw, for a total score of 8. Paws were assigned a clinical score based on the index (ModiQuest Research scoring method): 0=normal; 0.25=1-2 swollen toes; 0.5=3-4 swollen toes; 0.75=slightly swollen footpad/ankle; 1=swollen footpad/ankle; 1.25=1-2 swollen toes and swollen footpad/ankle; 2.0=swollen toes, footpad and ankle. At days 30 and 70 following the first injection, blood samples were taken from each mouse and anti-CII immunoglobulin (Ig)G antibodies in sera were detected using a mouse anti-type II collagen IgG assay kit (CDT31102; Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. The treatment was started on day 30 following the initial injection. Mice were administered 10 mg/kg PRP or PBS by subcutaneous injection every 4 days for a total of 9 doses, and were sacrificed on day 72 (14).

Histopathological analysis. The RA mice were sacrificed on day 72, and the knee joints (condyle) were obtained and subsequently fixed in 10% formalin for 2 h at 37°C. The joints were decalcified and embedded in paraffin. The ankle joints were stained with hematoxylin and eosin (1%) for 10 h at 37°C to analyze the efficacy of PRP for CIA RA mice. The severity of arthritis in the joints was scored on a scale of 0-5 as described in a previous study (23).

Statistical analysis. All date were expressed as mean and standard deviation of triplicate dependent experiments. Statistical significance was determined using the Student's t-test and Microsoft Excel software (2010; Microsoft Corporation, Redmond, WA, USA). One-way analysis of variance (ANOVA), two-way ANOVA, Kaplan-Meier estimators and log rank statistical analyses were performed using SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Functional analysis of PRP in synovial cells in vitro. The *in vitro* function of PRP in regulating inflammatory factors in synovial cells was assessed. Gene expression of IL-6 and IL-8 in Hela cells and human fibroblast-like synovial cells was measured by RT-qPCR. The results in Fig. 1 demonstrated that PRP stimulated Hela and fibroblast-like synovial cells to increase gene expression of IL-6 and IL-8, while IL-1β, IL-17A, TNF-α and IFN-γ mRNA expression was downregulated compared with the DEX and control groups. The results of the present study indicated that treatment with PRP decreased cytokine production *in vitro*.

ADCC and CDC mediated by PRP. A previous study demonstrated that inflammatory factors may be able to affect the progression of RA through ADCC or CDC mechanisms, and that this may lead to various clinical effects (24). In the ADCC assay, the results presented in Fig. 2A demonstrated that ~34% of the IL-1R-expressing Hela target cells were lysed by PRP at a concentration of 10 µg/ml. In the CDC assay, the result in Fig. 2B demonstrated that PRP was also capable of lysing IL-1R-expressing Hela cells in the presence of human complement. These data indicated that PRP was able to mediate ADCC and CDC effects upon binding to transmembrane IL-1R expressed on the cell surface, and therefore exhibits the potential to be developed into a more effective IL-1R-neutralizing agent in RA therapy. In addition, all synovial specimens in RA mice treated by PRP, DEX or PBS were analyzed. The results demonstrated that hypertrophy and leukocyte infiltration were suppressed following 4 weeks of treatment with PRP, compared with DEX- and PBS-treated groups (Fig. 2C and D).

Therapeutic effects of PRP for CIA RA mice. In order to assess the therapeutic effects of PRP on RA development, the CIA mouse model was used. The experimental mice were
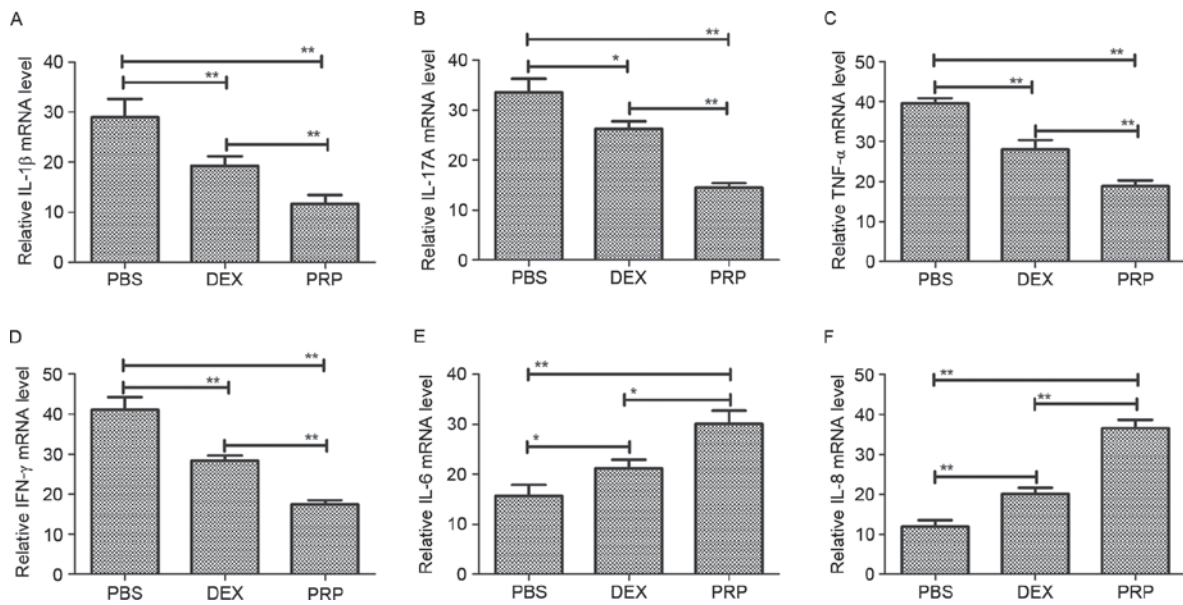
Figure 1. RT-qPCR analysis of the *in vitro* biological activity of PRP. Gene expression of (A) IL-1β, (B) IL-17A, (C) TNF-α, (D) IFN-γ, (E) IL-6 and (F) IL-8 was measured by RT-qPCR. Expression of each gene was calculated relative to the expression of housekeeping gene β-actin. Data are presented as the mean ± standard error of the mean of triplicate samples. Student's paired two-tailed t-test; *P<0.05, **P<0.01. PRP, platelet-rich plasma; DEX, dexamethasone; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
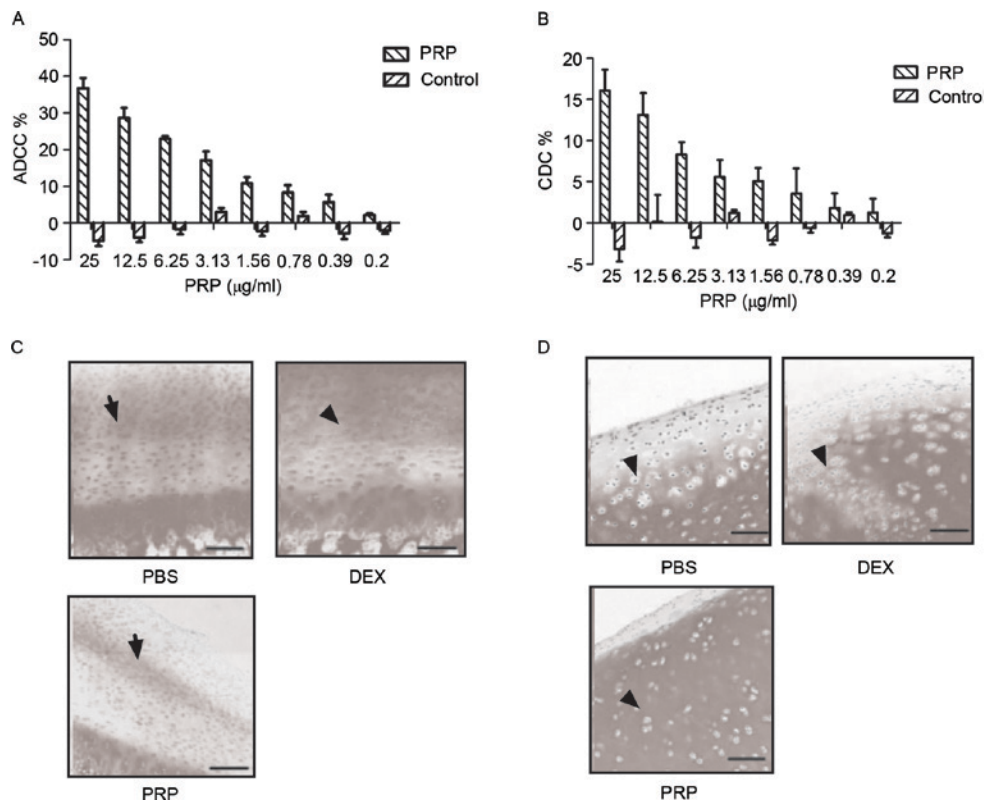

Figure 2. Efficacy of PRP on ADCC, CDC, proteoglycan and type II collagen content. The efficacy of PRP was analyzed by (A) ADCC and (B) CDC assays. Transmembrane TNF-α-expressing Hela cells were incubated in the presence of different concentrations of PRP for 30 min. Subsequently, peripheral blood mononuclear cells for ADCC, or human complement-rich serum for CDC, were used as effector cells and the source of complement, respectively, and transmembrane TNF-α-expressing cells were used as targets. (C) Recovery of the loss of cartilage-specific protein (arrowheads) following treatment with PRP demonstrated by staining of cartilage from the joints of rheumatoid arthritis mice (magnification, x200). (D) Alterations in type II collagen at the arthritis induction site (arrowheads) following treatment with PRP in RA mice. ADCC, antigen-dependent cell-mediated cytotoxicity; CDC, complement-derived cytotoxicity; PRP, platelet-rich plasma; TNF, tumor necrosis factor; DEX, dexamethasone.

recorded, and the arthritis score was evaluated every 4 days. As presented in Fig. 3A, treatment with PRP ameliorated

the clinical symptoms and hind paw swelling, with reduced arthritis scores compared with DEX- and PBS-treated CIA
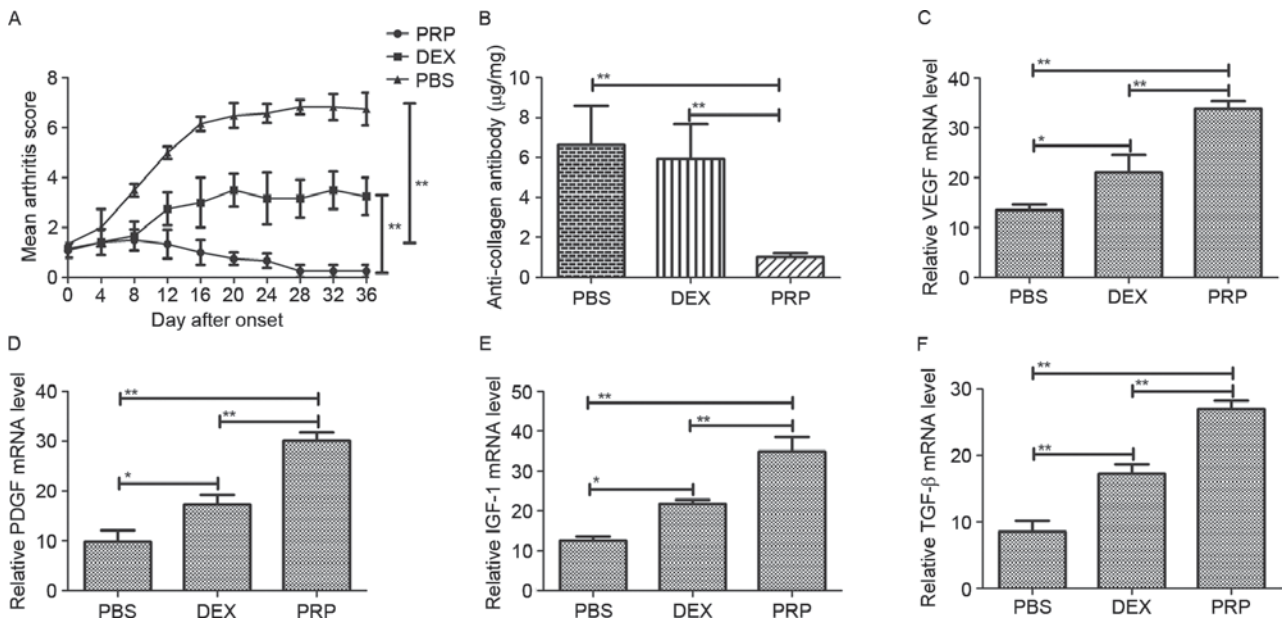
Figure 3. Assessment of the therapeutic effects of PRP in CIA mice. (A) Mice were given a subcutaneous injection of PRP (10 mg/kg), DEX or an equal volume of PBS. The arthritis scores were compared among non-treated (n=9), DEX (n=9) and PRP-treated (n=9) groups during treatment. (B) Serum concentrations of anti-collagen immunoglobulin G in experimental mice were determined using ELISA. The expression of (C) VEGF, (D) PDGF, (E) IGF-1 and (F) TGF-β was detected in synovial tissue in mice with CIA. Data are presented as the mean ± the standard deviation of triplicate samples. *P<0.05; **P<0.01. PRP, platelet-rich plasma; DEX, dexamethasone; PDGF, platelet-derived growth factor; IGF, insulin-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; CIA, collagen-induced arthritis.
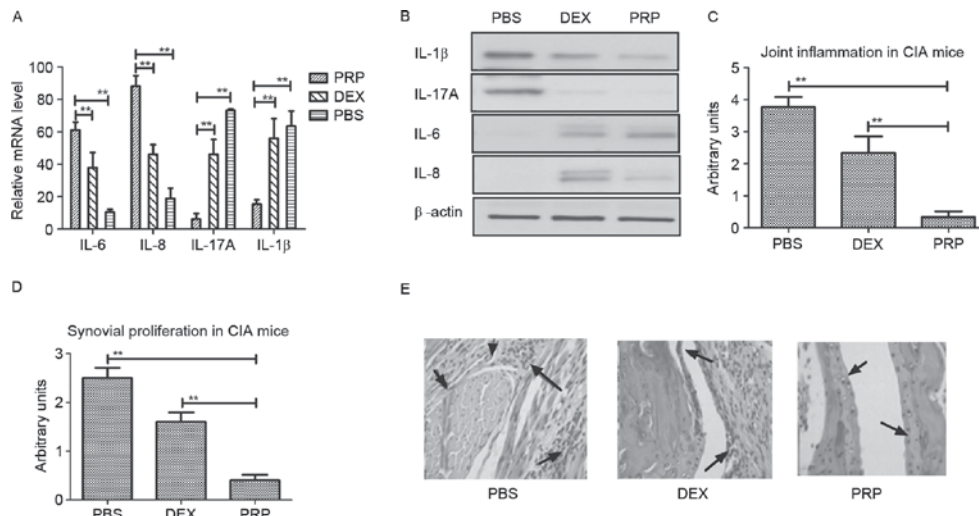

Figure 4. Analysis of RA-associated cytokines and histopathology of RA synovial grafts in CIA mice. (A) mRNA and (B) protein expression levels of IL-6, IL-8, MMP-3, IL-17, IL-1β and IL-17A in cartilage tissue were quantified using reverse transcription-quantitative polymerase chain reaction. The results are expressed as the n-fold difference relative to β-actin. PRP treatment reduced (C) joint degeneration and (D) synovial proliferation in mice with CIA. (E) Healthy mouse and RA synovial tissue samples were stained with hematoxylin and eosin (magnification, x200). Data are presented as the mean ± standard error of the mean. Tukey Kramer test; **P<0.01. RA, rheumatoid arthritis; IL, interleukin; MMP, matrix metalloproteinase; PRP, platelet-rich plasma; CIA, collagen-induced arthritis; DEX, dexamethasone.

mice. In addition, the therapeutic effects of PRP were assessed by analyzing the humoral anti-collagen response. The serum concentration levels of anti-collagen type II IgG on day 72 were measured. The results in Fig. 3B demonstrated that a significant decrease in the serum level of anti-CII IgG was exhibited in PRP-treated RA mice compared with PBS- and DEX-treated groups (P<0.01 vs. control groups). Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)-β expression was assessed

at the mRNA level in whole blood samples from RA mice, using RT-qPCR. The results of the present study indicated that VEGF, PDGF, IGF-1 and TGF-β expression in peripheral whole blood was upregulated following treatment with PRP compared with PBS and DEX groups (Fig. 3C-F).

Analysis of the mechanism of PRP-associated benefits in RA mice. The therapeutic mechanism of PRP in RA synovial cells was analyzed in the present study. Target mRNA and protein expression levels were measured in synovial tissue from

independent experiments following treatment with PRP. The results in Fig. 4A and B demonstrated that IL-6, IL-8, IL-17A, IL-1β mRNA expression was decreased in synovial tissue in PRP-treated mice compared with PBS-treated and DEX-treated groups. In addition, in order to observe alterations in RA synovial tissue following treatment with PRP, histological staining was used to analyze the therapeutic effects of PRP in mice with RA. As presented in Fig. 4C-E, the PRP-treated mice with RA exhibited marked reduction of synovial hyperplasia, inflammatory cell infiltration and destruction of cartilage and bone. In addition, it was observed that fewer infiltrating cells presented in the joints of the PRP-treated mice compared with PBS-treated and DEX-treated mice with CIA. The pathological symptoms of the PRP-treated CIA mice were attenuated. These results indicated that PRP may be an effective drug for RA therapy.

Discussion

The first description of the applications of PRP in biomedicine was reported in 1974 (25) and demonstrated differential alterations in the potency of platelet-aggregation inhibitors following pre-incubation in PRP. Further studies aimed to elucidate the biological mechanism underlying the effects of PRP, and potential applications as a therapeutic strategy for musculo-skeletal disorders, including ligament and tendon pathologies, and various cartilage pathologies, including RA and avascular necrosis (26,27). PRP has been reported to be associated with numerous human diseases; in joint diseases, the application of autologous PRP has been observed to lead to decreased healing times in various clinical cases (28-30). In addition, a previous report suggested that the efficacy and feasibility of PRP treatment was notable in RA and avascular necrosis (31). However, although the application of PRP in the laboratory and in clinical practice has exhibited positive outcomes, the use of PRP has remained controversial, particularly its application as a treatment for RA and ankle cartilage pathology (32).

RA is an autoimmune inflammatory disorder which results in persistent synovitis with cartilage phlegmonosis and severe synovium destruction (33). Treatment with dexamethasone, golimumab, infliximab and adalimumab is frequently used in RA patients to reduce bone osteoporosis and manage synovium destruction. However, few reports have investigated the effects of treatment with PRP on RA. Lippross *et al* (34) demonstrated that inhibition of inflammatory mediators by PRP in RA led to symptomatic remission through modern molecular interventions and synovial repairmen (35). In addition, inhibition of inflammation and increasing advantageous cytokines by PRP has been evidenced to be a treatment for RA and osteoarthritis (26).

The present study investigated the therapeutic effects of PRP in CIA mice with RA. It was observed that treatment with PRP alleviated arthritis, reduced humoral and cellular immune responses, and led to beneficial effects on histological parameters as demonstrated by joint tissue histological staining. CIA RA mice treated with PRP exhibited downregulated expression of IL-6, IL-8, MMP-3, IL-17, IL-1β, TNF-α, RANKL and IFN-γ in inflammatory tissue (36,37). In addition, the serum concentration of anti-collagen was decreased in the

PRP-treated CIA mice. As the effect of PRP in the articular environment derives from an interaction with the pre-existing environment and other cells, and as certain surgical protocols involve the application of platelets and cells, several studies have also investigated the effect of PRP on mesenchymal stem cells of various origins (38-41). The stimulatory effects of treatment with PRP have been well-documented, originating from the action of promoting proliferation and chondrogenic differentiation, and thereby increasing the production of beneficial molecules for the maintenance of articular cartilage (23,42). The results of the present study indicated that hypertrophy and leukocyte infiltration were suppressed following 4 weeks of treatment, which suggested that PRP treatment is beneficial for RA therapy.

Although a previous study has investigated the direct effects of different drugs on chondrocytes (43), further research is required to investigate the role of treatment with PRP in maintaining the cytokine homeostasis of the joint. The present study demonstrated that PRP markedly enhanced the activity of synovial cells and improved synovial angiogenesis. In conclusion, the results of the present study indicate that CIA mice treated with PRP exhibit beneficial effects on joint inflammation, cartilage destruction and bone damage.

Acknowledgements

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