Effects of Platelet-rich Plasma and Cell Coculture on Angiogenesis in Human Dental Pulp Stem Cells and Endothelial Progenitor Cells

Xinzhu Li, MS,[*†] *Jin Hou, PhD,*[*†] *Buling Wu, PhD,*[*†] *Ting Chen, PhD,*[*†] *and Aoxiang Luo, MS*[*†]

Abstract

Introduction: Platelet-rich plasma (PRP) has been described as platelet concentrate. Growth factors released by activated platelets can improve wound vasculogenesis and enhance wound healing. In this study, we used PRP instead of serum to culture human dental pulp stem cells (hDPSCs) and endothelial progenitor cells (EPCs) and investigated revascularization ability. The effect of hDPSC and EPC coculture on vasculogenesis was also studied. **Methods:** PRP was prepared by secondary centrifugation. Real-time polymerase chain reaction and Western blotting were used to determine the expression of vasculogenesis-related factors vascular endothelial growth factor, platelet-derived growth factor, fetal liver kinase 1 (Flk-1), and stromal cell-derived factor 1 (SDF-1) in cultured hDPSCs and EPCs. The cells were divided into 4 groups: EPCs + 10% fetal bovine serum (FBS), EPCs + 10% PRP, EPCs + hDPSCs + 10% FBS, and EPCs + hDPSCs + 10% PRP. Then, the formation of vessel-like structures was tested by the tube formation assay. **Results:** On day 3, the expression levels of all the markers in the coculture groups were much higher than in the single-culture groups and were also higher in the PRP groups compared with the FBS groups ($P < .05$), except for SDF-1. Expression levels were significantly higher in the experimental groups (EPCs + 10% PRP, EPCs + hDPSCs + 10% FBS, and EPCs + hDPSCs + 10% PRP) than in the control group (EPCs + 10% FBS) and in the PRP groups/coculture groups compared with the FBS groups/single-culture groups ($P < .01$). The tube formation assay showed the area of vessel-like structures formed by the PRP group to be larger than in the FBS group ($P < .05$). **Conclusions:** PRP and coculture can both promote vasculogenesis, and PRP can promote EPCs to form vessel-like structures. *(J Endod 2014;40:1810–1814)*

Key Words

Coculture, endothelial progenitor cells, human dental pulp stem cells, platelet-rich plasma, vasculogenesis

Platelet-rich plasma (PRP) separated from whole blood has been described as platelet concentrate. Activated PRP includes many growth factors such as platelet-derived growth factor (PDGF), transforming growth factor beta, insulin-like growth factor, and vascular endothelial growth factor (VEGF) (1–3). When platelets are activated, they release these growth factors almost immediately and continue to synthesize additional growth factors for several days (4).

This suggests that PRP might promote tissue repair. PRP has been reported to alter the biomechanical and histologic properties of rotator cuff repair during acute injury response (5). Many investigations have shown that growth factors released by activated platelets can improve wound angiogenesis and enhance skin wound healing (6–10). The application of PRP in oral clinical research began in the late 1990s. In the field of oral medicine, PRP has been used to study bone tissue regeneration. The use of PRP in oral surgical practice could have beneficial outcomes such as reduced bleeding and enhanced soft tissue healing and bone regeneration (10, 11). It could also eliminate concerns of immunogenic reactions and disease transmission. On the other hand, the bioactive factors released by PRP also participate in anabolism, catabolism, and proinflammatory and anti-inflammatory responses, some of which also underlie the immune response (12). Therefore, PRP has potential in new applications in tissue engineering.

In this study, we investigated the synergistic effects of 2 kinds of cells: human dental pulp stem cells (hDPSCs) and endothelial progenitor cells (EPCs). We also used PRP instead of serum to culture cells so that we could minimize the immune response and investigate its vasculogenic potential.

Materials and Methods

PRP Preparation and Activation

The study was approved by the institutional research ethics committee of Southern Medical University, Guangzhou, China. Human umbilical cord blood was collected from healthy volunteers with informed consent in sterile tubes with anticoagulant. Platelets were separated from umbilical cord blood by secondary centrifugation; 14 mL blood was centrifuged at 360*g* for 20 minutes at room temperature. After the upper layer was collected, the sample was further centrifuged at 500*g* for 10 minutes. The upper layer was discarded carefully using a pipette, and the remnants of 1 mL liquid comprised PRP. The platelet number in the PRP was counted by a hematology analyzer (XE-2100; Sysmex, Kobe, Japan). The PRP was then activated by repetitive freeze thawing after the addition of heparin. Next, centrifugation was performed at 3000*g* for 20 minutes at room temperature to remove platelet membrane fractions. The resulting supernatant was used in our *in vitro* study.

From the *Department of Stomatology, Nanfang Hospital, Guangzhou, China; and †College of Stomatology, Southern Medical University, Guangzhou, China.
Address requests for reprints to Dr Buling Wu, Department of Stomatology, Nanfang Hospital, Guangzhou 510515, China. E-mail address: wubuling623@163.com
0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2014.07.022

Cell Culture

hDPSCs were isolated and cultured using the method described by Ma et al (13). Dental pulp was separated from healthy premolars or third molars that had been extracted for orthodontics or impaction from 12- to 25-year-old patients. The hDPSCs were passaged until 80% confluent. Cell phenotype analysis was performed by flow cytometric analysis for CD90/PE, CD105/FITC, CD29/PE, CD44/FITC, and CD14/PE (PharMingen-BD Biosciences, San Diego, CA). After cultured until passage 3, hDPSCs were used for subsequent experiments.

EPCs were isolated and cultured from human umbilical cord blood obtained from healthy volunteers with informed consent. Mononuclear cells were obtained by density gradient fractionation as described by Reinhold et al and Noriko et al (14, 15). The cells were resuspended in EBM-2-MV medium (Lonza, Basel, Switzerland) supplemented with 20% fetal bovine serum (FBS) and plated on 6-well plates at a density of 1×10^7 cells/mL that were coated with fibronectin. The EPCs were passaged until more than 80% confluent.

In this study, the cells were grouped as follows: EPCs + 10% FBS (EF), EPCs + 10% PRP (EP), EPCs + hDPSCs + 10% FBS (EDF), and EPCs + hDPSCs + 10% PRP (EDP). In the coculture group, the proportion of the 2 kinds of cells and 2 types of medium was 1:1 each.

Quantitative Real-time Polymerase Chain Reaction

Total isolated messenger RNA (mRNA) served as the template to generate complementary DNA through reverse transcription using a reagent kit (Invitrogen, Life Technologies, Grand Island, NY). For evaluation of the gene expression levels of VEGF, PDGF, Flk-1, SDF-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the primer sequences were designed as follows:

1. VEGF: sense, 5′-CTA CCT CCA CCA TGC CAA GT-3′, and antisense, 5′-CAC ACA GGA TGG CTT GAA GA-3′
2. PDGF: sense, 5′-ACG TCA GGA AGA AGC CAA AA-3′, and antisense, 5′-TCT GGT TGG CTG CTT TAG GT-3′
3. Flk-1: sense, 5′-GGT ATT GGC AGT TGG AGG AA-3′, and antisense, 5′-ACA TTT GCC GCT TGG ATA AC-3′
4. SDF-1: sense, 5′-GCA TTG ACC CGA AGC TAA AG-3′, and antisense, 5′- ACA CAC ACA CCT GGT CCT CA-3′
5. GAPDH: sense, 5′-TCA CCA GGG CTG CTT TTA AC-3′, and antisense, 5′-GAC AAG CTT CCC GTT CTC AG-3′

The quantitative real-time polymerase chain reactions (qRT-PCRs) were performed using the SYBR Select Master Mix (Life Technologies) on an ABI 7500 real-time polymerase chain reaction system (ABI, Carlsbad, CA). Relative quantization was performed by determining the difference between the threshold cycle (Ct) of GAPDH and the Ct of each transcript and computing ΔΔCt. Amplification proceeded as per manufacturer instructions.

Western Blot Analysis

The total protein was extracted from the cultured cells and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and transferred onto a PVDF membrane (Millipore, Billerica, MA). The membrane was incubated with rabbit monoclonal anti-VEGF (1:1000; Epitomics, Burlingame, CA) overnight at 4°C. Proteins were visualized using IRDye 800CW goat antirabbit immunoglobulin G (1:15000; Li-cor, Lincoln, NE) secondary antibody. The membrane was scanned on an Odyssey V3.0 scanner (Li-cor).

Tube Formation Assay

Growth factor–reduced basement membrane matrix (356231 Matrigel, BD Biosciences) was placed in 24-well plates per manufacturer's instructions. After Matrigel polymerization, 2×10^5 EPCs with corresponding medium were seeded in the wells. In the coculture group, the proportion of the 2 kinds of the cells was 1:1 in the medium (supplemented with 10% FBS). hDPSCs were seeded on Matrigel 24 hours after EPCs were seeded. After 48 hours, cells were stained with 2 μmol/L calcein AM (Ebioscience, San Diego, CA). After 7–48 hours, all the cells were assessed for the presence of vessel-like structures and imaged using an inverted microscope (Olympus, Tokyo, Japan). The percent tube area was ascertained using ImageJ2x software (NIH, Bethesda, MD) and calculated using the following formula:

$$\frac{\text{Tube area in different groups}}{\text{Single well area of 24 - well plates}} \times 100\%$$

Statistical Analysis

Statistical analysis was performed using 1-way analysis of variance using SPSS 13.0 software (SPSS Inc, Chicago, IL). First, we used the homogeneity test for variance using the Levene test, and if variances were unequal, we used the Welch test. For multiple comparisons between groups, we performed statistical analysis with the Fisher's Least Significant Difference (LSD) test or the T3 test when variances were equal or not, respectively. The RQ values ($2^{-\Delta\Delta CT}$) of the same factor in different groups were compared with those for the DF/EF groups (RQ value was 1) by using the 1-sample *t* test. Statistical significance was set at $P < .05$.

Results

PRP Characterization

The platelet count range in whole blood was 116–230 $\times 10^9$/L, whereas PRP averaged 1306–1949 $\times 10^9$/L, which was significantly higher compared with the umbilical cord blood ($P < .01$).

Effect of PRP on mRNA Levels of Vasculogenesis-related Factors

The expression levels of VEGF, PDGF, Flk-1, and SDF-1 mRNA were detected by qRT-PCR on days 3, 7, and 14. The results of qRT-PCR are shown in Figure 1. On day 3, the expression of all the markers in the coculture groups (EDF and EDP) were higher than in the single-culture groups (EF and EP); similarly, all the markers (except SDF-1) had higher expression levels in the PRP groups (EP and EDP) than in the FBS groups (EF and EDF) ($P < .05$) (Fig. 1*A*). The expression levels were significantly higher in the experimental groups (EP, EDF, and EDP) than in the control group (EF) ($P < .01$) on days 7 and 14 (Fig. 1*B* and *C*). In addition, the expression levels of 4 markers were significantly higher in the PRP groups/coculture groups than in the FBS groups/single-culture groups ($P < .01$) (Fig. 1*B* and *C*).

Effect of PRP on Vasculogenesis-related Proteins

The expression level of VEGF was the highest among the 4 markers. Therefore, we chose VEGF and detected its protein level by Western blotting on days 3, 7, and 14. The results of Western blotting are shown in Figure 2. The expression of VEGF had a rising trend in the EP, EDF, and EDP groups compared with the EF group (Fig. 2*A*–*C*). On day 3, there was no significant difference in the expression of VEGF between the EDF and EDP groups (Fig. 2*A*). In addition, our data showed higher VEGF expression in the PRP groups than in the FBS groups (Fig. 2*B* and