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The effects of human platelet lysate on dental pulp stem cells derived from impacted human third molars

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ABSTRACT

Human platelet lysate (PL) has been suggested as a substitute for fetal bovine serum (FBS) in the large-scale expansion of dental pulp stem cells (DPSCs). However, the biological effects and the optimal concentrations of PL for the proliferation and differentiation of human DPSCs remain unexplored. We isolated and expanded stem cells from the dental pulp of extracted third molars and evaluated the effects of PL on the cells' proliferative capacity and differentiation potential *in vitro* and *in vivo*. Before testing, immunocytochemical staining and flow cytometry-based cell sorting showed that the cells derived from human dental pulp contained mesenchymal stem cell populations. Cells were grown on tissue culture plastic or on hydroxyapatite-tricalcium phosphate (HA/TCP) biomaterials and were incubated with either normal or odontogenic/osteogenic media in the presence or absence of various concentrations of human PL for further investigation. The proliferation of DPSCs was significantly increased when the cells were cultured in 5% PL under all testing conditions ($P < 0.05$). However, this enhancement was inconsistent when the cells were cultured in 1% PL or in 10% PL; 10% PL significantly inhibited cell proliferation and was therefore excluded from further differentiation testing. Culture medium containing 5% PL also significantly promoted the mineralized differentiation of DPSCs, as indicated by the measurement of alkaline phosphatase activity and calcium deposition under mineral-conditioned media ($P < 0.05$). Scanning electron microscopy and modified Ponceau trichrome staining showed that the cells treated with 5% PL and mineralizing media were highly capable of integrating with the HA/TCP biomaterials and had fully covered the surface of the scaffold with an extensive sheet-like structure 14 d after seeding. In addition, 5% PL showed significantly positive effects on tissue regeneration in two *in vivo* transplantation models. We conclude that the appropriate concentration of PL enhances the proliferation and mineralized differentiation of human DPSCs both *in vitro* and *in vivo*, which supports the use of PL as an alternative to FBS or a nonzoonotic adjuvant for cell culture in future clinical trials. However, the elucidation of the molecular complexity of PL products and the identification of both the essential growth factors that determine the fate of a specific stem cell and the criteria to establish dosing require further investigation.

1. Introduction

Human dental pulp stem cells (DPSCs) are a unique mesenchymal stem cell (MSC) type that is present in the cell-rich zone and core of the dental pulp [1,2]. Upon isolation and culture in high-serum-containing medium, rapidly proliferating DPSCs are demonstrated to be capable of differentiating into mesenchymal-derived odontoblasts, osteoblasts, adipocytes, and chondrocytes, depending on the types of regulatory molecules that are present [3–5]. This characteristic facilitates the *ex vivo* expansion and enhances the translational potential of these cells for cell-based therapeutics and tissue engineering applications [6,7]. Human

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dental pulp is arguably one of the most accessible sources of postnatal stem cells because more than 80% of human beings have at least one impacted "third molar" that has to be removed surgically, and a large number of teeth are routinely extracted due to caries, periodontitis, or orthodontic reasons [1–3]. DPSCs can be obtained with ease from these extracted teeth that are generally discarded, which makes them a potent surrogate for the traditional use of bone marrow MSCs in regenerative medicine [8–11]. However, the translational usage of DPSCs requires large-scale expansion to produce the quantity that is needed for therapeutics. The media for cell culture is traditionally supplemented with fetal bovine serum (FBS; alternatively termed "fetal calf serum," FCS). Unfortunately, FBS is an undesirable additive to cell cultures because the translation of stem cells expanded in FBS-containing medium is impeded by the risk of a xenogeneic response and exposure to zoonoses [12,13].

To make stem cell therapy more safe and practical, there is a growing interest in avoiding the use of FBS and in the development of synthetic culture media. Although several products are now commercially available, their precise composition is often undisclosed, and they often include other xenogeneic components, such as recombinant growth factors [14]. Recently, several groups have demonstrated that human platelet lysate (PL) represents an attractive alternative to FBS that permits the scale-up of MSCs, including dental pulp stromal cells, for clinical applications [15–19]. Human PL can be generated by subjecting common platelet units to several freeze/thaw cycles, which damages the platelet membranes and releases the growth factors into the plasma. To avoid extensive aggregate formation and to deplete potential antigens, the platelet fragments are removed by centrifugation [14–19]. It is also possible to use human PL from the same donor to further minimize the risk of immunological side effects and viral infections [20,21]. Although much of the past literature has focused on MSCs cultured in human PL [15–18] and recent data have demonstrated the positive effects of platelet-rich plasma (PRP) [22] and PL [19] on the proliferation and mineralized differentiation of human dental stem cells (e.g., DPSCs and periodontal ligament stem cells), the appropriate concentration of PL for cell manufacturing on a clinical scale remains unknown.

Platelet-rich products, which have received much attention for their use as additives for *in vitro* cell manipulation and as adjuvants for augmenting *in vivo* wound healing and regeneration, represent a cost-effective, clinically relevant therapeutic regimen [23–25]. Platelets contain a rich cocktail of bioactive molecules and proteins present in physiologic proportions to each other within one universal mix [23]. These factors can interact with cells during the wound healing cascade and support the diverse processes that promote tissue re-vascularization and repair. Proteomic dissection has identified transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) as highly ranked effectors of human PL activity, which reveals a paradigm of healing that underlies PL adjuvancy [14]. Although platelet-rich preparations can boost the healing processes, the risk of disease transmission that is associated with the use of potentially toxic substances, such as exogenous bovine thrombin in PRP preparation, has largely hampered their application [26,27]. The development of the 'preparation rich in growth factors (PRGF)' technology has addressed this issue with the use of calcium chloride instead of animal derivatives to activate the platelet preparations [27,28]. However, the optimized protocol has not yet been established; the platelet concentration can vary from batch to batch. In this regard, the use of PL makes concentration control much easier. Furthermore, PL excludes the cellular elements of platelets. Thus, the immunological reactions in relation to allogeneic products, if used, can be obviated. Due to the complexity of wound healing and the obvious contribution of platelets to this delicate process, PL may constitute an endogenous regenerative tool that can employ the body's inherent capacities to boost the healing and regenerative processes [25,29]. PL clearly has a very important role to play in future developments in regenerative medicine.

Currently, little information is available on the effect of human PL on the performance of DPSCs in normal or odontogenic/osteogenic differentiation culture media. In this study, we report the biologic influences of various concentrations of PL on the proliferation and differentiation of human DPSCs in plastic culture dishes or in biomaterials; the most effective concentration was selected. Finally, the regenerative potential of PL-treated DPSCs was assessed by *in vivo* transplantation models.

2. Materials and methods

2.1. The isolation of human DPSCs

The isolation of human DPSCs was performed according to the previously reported methods, with minor modification [30]. Briefly, 12 third molars from 12 donors (19–23 years of age) were used for cell isolation with written permission. The ethics committee of the Fourth Military Medical University School of Stomatology approved the experimental protocols (permission number 002). Freshly extracted teeth were stored in serum-free alpha minimum essential medium (α-MEM; Hyclone, Logan, UT, USA) and transported to the laboratory within 60 min of extraction. The pulp tissues were removed from the teeth, minced, and digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase (both from Sigma–Aldrich, St. Louis, MO, USA) for 30–60 min at 37 °C. The digested mixtures were passed through a 70 μm cell strainer to obtain single-cell suspensions. Afterward, the cells were seeded in six-well plates, cultured with α-MEM supplemented with 20% fetal bovine serum (FBS; Hyclone), 100 U/mL penicillin-G (Roche, Basel, Switzerland) and 100 mg/mL streptomycin (Roche), and maintained in an atmosphere of 5% CO_2 at 37 °C. When the cell colony formation units reached 80% confluence, the cells were collected to isolate single cell clones by limiting dilution. The cells from these clones were observed and passage 0 (P0) cells were cultured and expanded for the following experiments. Cells at passages four or five were used for this study.

2.2. Immunocytochemical staining and flow cytometry

To identify the putative stem cells of the dental pulp, single cell suspensions were seeded on top of a 22 × 22 mm cover slip at 2×10^4 cells/well in a six-well plate and cultured for 24 h at37 °C in an atmosphere of 5% CO_2. After the cells were washed in phosphate-buffered saline (PBS, pH 7.4) and fixed in 4% phosphate-buffered paraformaldehyde, they were permeabilized for 5 min on ice with 0.1% Triton X-100 in PBS and blocked with 1% bovine serum albumin overnight at 4 °C. The cells were incubated with primary antibodies (1:200 dilution) overnight at 4 °C. The following antibodies were used: anti-STRO-1 (R&D Systems Inc., Minneapolis, MN, USA), anti-dentin sialoprotein (DSP; Santa Cruz Biotechnology, Inc., CA, USA), anti-mouse IgG-Cy3, anti-mouse IgG-FITC anti-vimentin, and anti-Keratin (Boster, Wuhan, China). The 3, 3-Diaminobenzidine tetrahydrochloride (Zsgb-Bio, Beijing, China) and broad-spectrum immunoperoxidase ABC kit (Boster) were used according to the manufacturer's protocol. The cells were counterstained with hematoxylin and DAPI.

The percentages of STRO-1-, CD29-, CD34-, CD44-, CD45-, CD90-, CD105- and CD146 positive cells were analyzed by flow cytometry. The fourth generation of isolated DPSC aliquots (>2×10^5 cells) were washed and resuspended in PBS supplemented with 3% FBS that contained saturating concentrations (1:100 dilution) of the following fluorescein isothiocyanate-conjugated anti-human monoclonal antibodies (Becton & Dickinson, Mountain View, CA, USA): anti-STRO-1- allophycocyanin (APC), anti-CD29-phycoerythrin (PE), anti-CD34-PE, anti-CD44-fluorescein isothiocyanate (FITC), anti-CD45-PE, anti-CD90-PE, anti-CD105-FITC, and anti-CD146-PE for 1 h at room temperature in the dark. As a negative control, FITC-, PE-, and APC-conjugated non-specific mouse IgG1 (Falcon, BD Bioscience, FranklinLakes, NJ) were substituted for the primary antibodies. The cell suspensions were washed twice, resuspended in 3% FBS/PBS and analyzed with a flow cytometry cell sorting Vantage cell sorter (Becton & Dickinson). The data were analyzed with a Mod-Fit 2.0 cell cycle analysis program (Becton & Dickinson).

2.3. Multiple lineage differentiation

For odontogenic/osteogenic induction, cells were seeded in 6-well plates, grown to 80% confluence, and incubated in differentiation medium containing 10 nM dexamethasone, 10 mM β glycerophosphate, 50 mg/mL ascorbate phosphate (all from Sigma-Aldrich), and 10% FBS for 2–3 weeks. The cultures were fixed in 4% paraformaldehyde in PBS, and the mineral deposits in the extracellular matrix (ECM) were stained with 40 mM Alizarin Red S (Sigma-Aldrich) (pH = 4.1). For adipogenic