Archives of Oral Biology 85 (2018) 1–9

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Research paper

Role of PDGF-BB in proliferation, differentiation and maintaining stem cell properties of PDL cells *in vitro*

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ARTICLE INFO

Keywords:
Platelet-derived growth factor
Periodontal ligament
Mesenchymal stem cells

ABSTRACT

Objective: Platelet-derived growth factor-BB (PDGF-BB) is one of the most abundant growth factors in platelet derived products and has been shown to stimulate regeneration after tissue injury. There is a population of mesenchymal stem cells (MSC) in human periodontal ligament (PDL) which can contribute to tissue regeneration under appropriate conditions.

Design: PDL cells were isolated and characterized using stem cell and differentiation markers via immunofluorescence and flow cytometry and then cultured *in vitro* and treated with different concentrations of PDGF-BB. The effect of PDGF-BB on cell proliferation, stem cell and differentiation markers expression, soluble collagen production, lysyl oxidase (LOX) activity, alkaline phosphatase (ALP) activity and calcium nodules formation was assessed.

Results: PDGF-BB stimulated the proliferation of cells with the maximum effect at 50 ng/mL. The growth factor increased the expression of stem cell markers and SPARC; Col1a2 expression was decreased, whereas the expression of Col3a1 remain unchanged. Soluble collagen production, ALP activity and calcium nodules formation were also significantly decreased by PDGF-BB; LOX activity was significantly increased.

Conclusions: PDGF-BB is a powerful promoter of cell proliferation and increases the expression of stem cell markers; inhibites collagen production and mineraliration but accelerates the maturation of collagen chains through increased LOX activity and SPARC expression.

1. Introduction

Tooth periodontium consists of periodontal ligament (PDL), cementum, alveolar bone and gingiva. These soft and hard tissue components provide the tooth stability, nutrition and resistance to mastication. PDL is a specialized fibrous connective tissue located between the cementum, covering the tooth root and the alveolar bone (Hassell, 1993). The destruction of PDL due to severe periodontitis, followed by tooth loss is one of the main issues in periodontology (Mihaylova, Stanimirov, Mitev, & Ishkitiev, 2015). Lack of healthy intact PDL is associated with poor prognosis and compromised clinical outcomes.

Central to tissue engineering is the ability to regenerate naturally occurring structures using an appropriate combination of stem/progenitor cells, extracellular matrix (ECM) or biomaterials such as scaffolds and appropriate growth factors (Langer & Vacanti, 1993). Stem/progenitor cells are able to differentiate into various cell types and to initiate ECM synthesis at the side of injury, which is essential to the process of tissue regeneration (Beertsen, McCulloch, & Sodek, 1997; Ishkitiev et al., 2010). The differentiation potential of stem cells is manifested by exposing non-differentiated cells to diverse tissue culture conditions *in vitro* (Ishkitiev et al., 2013).

The presence of mesenchymal stem cells (MSC) in human PDL was first reported in 2004 by Seo et al. (2004). They have identified the presence of populations possessing typical stem cell-like characteristics – small size, response to stimulating factors, slow cellular cycle. PDL stem cells are localized paravascularly (Sant'Ana, Marques, Barroso, Passanezi, & Rezende, 2007) and are able to retain their stem cell properties, and to differentiate along mesenchymal cell lineages giving rise to adipocytes, cementoblast-like cells, collagen-rich tissue *in vitro* etc. (Seo et al., 2004). These cells have since become a subject of interest for a wide range of studies.

Cell proliferation, migration, differentiation and ECM synthesis are the main events providing tissue recovery regulated by the growth factors (William, 1996). A vast number of studies have focused on the

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http://dx.doi.org/10.1016/j.archoralbio.2017.09.019
Received 7 November 2016; Received in revised form 10 August 2017; Accepted 24 September 2017

role of polypeptide growth factors in the regulation of periodontal regenerative processes (Rutherford, Niekrash, Kennedy, & Charette, 1992).

In recent years many clinicians apply autologous platelet derived products to enhance healing process and regeneration of PDL with mixed success. Growth factors contained in human platelets are crucial for blood clot formation and tissue regeneration (Mihaylova et al., 2017). PDGF is one of the main active morphogens in the platelet concentrates which have been increasingly used to stimulate tissue healing at the injury site (Schär, Diaz-Romero, Kohl, Zumstein, & Nesic, 2015). Recombinant PDGF is already applicable in various medical and dental disciplines including oral and maxillofacial surgery, periodontology, dental implantology etc. The growth factor plays a significant role in orchestrating mesenchymal cell activity. It is involved in angiogenesis and neutrophil- and macrophage- activation-key cell types for tissue repair at the early stage of wound healing (Deuel, Senior, Huang, & Griffin, 1982). Multiple studies have demonstrated that PDGF is a potent mitogen which enhances cell proliferation and chemotaxis in cells of periodontal origin (Boyan et al., 1994; Dennison, Vallone, Pinero, Rittman, & Gaffesse, 1994; Matsuda, Lin, Kumar, Cho, & Genco, 1992; Oates, Rouse, & Cochan, 1993; Ojima, Mizuno, Kuboki, & Komori, 2003; William, 1996). It is a dimeric molecule with several isoforms: PDGF-AA, −BB, −AB, −CC, −DD, comprised of A, B, C, D polypetide chains and two well-known types of receptors: alpha and beta-receptors (Alvarez, Kantarjian, & Cortes, 2006).

The effect of PDGF on stem cell markers expression, enzymatic activity, ECM synthesis and mineralization in PDL, however, has not yet been fully elucidated.

Transplantation of PDL derived stem cells could serve as a promising tool for regenerative therapy. The purpose of the present study is to determine the presence of cells in human PDL capable of expressing stem cell and differentiation markers and to determine the effect of PDGF-BB on cell proliferation, markers expression, collagen synthesis, enzymatic activity and mineralization.

2. Materials and methods

2.1. Cells isolation

Research was approved by Ethics Committee of Medical University – Sofia, Bulgaria. Cells were obtained from intact surgically extracted third molars (n = 10) of healthy patients (n = 10) between 18 and 40 years old after signing informed and written consent. All the surgical extractions were conducted in the department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Medical University – Sofia. The tissue explants used in this study presented biological material waste obtained after routine oral surgical procedures. Immediately after the extraction teeth were rinsed with sterile saline solution and placed in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Eugene, OR, USA) supplemented with 1% antibiotic-antimycotic (Sigma-Aldrich, St. Louis, MO, USA) and pH 7,4 and kept at 4 °C. Within the next 12–24 h teeth were transferred to the laboratory and washed with Phosphate-buffered saline (PBS) (Lonza, Verviers, Belgium). Tissue explants were scraped with sterile scalpel blades from the middle third part of the tooth roots and enzymatically digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase (Sigma-Aldrich) for 1 h at 37 °C. The cell suspension was centrifuged and the supernatant was removed. Cells were resuspended in DMEM. Single-cell suspension was obtained after passing the cells through 70 μm-cell strainers (BD Falcon, Heidelberg, Germany). Cells were seeded in 2 cm^2 petri dishes (Greiner Bio-One, Frickenhausen, Germany) containing DMEM supplemented with 1% antibiotic-antimycotic and 10% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich). Cell cultures were incubated at 37 °C in humidified atmosphere of 5% CO_2 and 95% air for a period of 2 to 4 weeks.

2.2. Cell culture and treatment with PDGF-BB

The medium was replaced every 2nd or 3rd day thereafter until a sub-confluent monolayer was established. Cell growth was monitored by phase contrast microscopy (Leica DMRE, Leica Microsystems GmbH, Germany). The cells were detached by trypsinization (0,05% trypsin/EDTA, (Lonza)) for 10–20 min and transferred to 75 cm^2 tissue culture flasks for further culturing. Experiments were conducted with cells between 2nd and 4th passage and cells of the same passage were used within each experiment. Each experiment was carried out at least 3 times.

The experiments in the present study were mainly conducted in serum-free media. Cell culture medium was supplemented with 1% insulin-trasferrin-selenium (ITS) (Gibco Life Technologies Inc., Grand Island, NY, USA) as FBS substitute. The aim was to eliminate the influence of all active substances present in FBS that could confound the experiment.

Recombinant human PDGF-BB (Santa Cruz, Santa Cruz, CA, USA) was dissolved in sterile filtered PBS supplemented with 0,1% bovine serum albumin (BSA) (Miltenyi Biotec, Bergisch Gladbach, Germany) to achieve 50 μg/mL final concentration of the stock solution and stored at −20° to −80 °C, according to the manufacturer's instructions. Immediately before use the stock solution was diluted in DMEM to obtain the following concentrations: 1 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL. Cells were cultured with PDGF-BB for a period of 48 h to three weeks. At least 3 parallel cell batches were used for each dosage of PDGF-BB.

2.3. Immunofluorescence

Expression of the following markers: CD49f, CD117, CD146, CD271, STRO-1, alkaline phosphatase (ALP), collagen type 1α2 (COL1a2), collagen type 3α1 (COL3a1) and osteonectin/Secreted protein acidic and rich in cysteine (SPARC) was assessed. Five thousand cells/cm^2 were seeded in 96-well plates (TPP®, Trasadingen, Switzerland). After reaching sub-confluence cells were fixed with 4% paraformaldehyde for 30 min, washed 3 times with PBS and incubated with 1% BSA for another 30 min. For intracellular markers staining cells were permeabilized with 0,05% Tween20 (ICN Biomedicals Inc, Aurora, OH, USA) for 10 min and with 0,05% Triton X-100 (Calbiochem – Merck, Darmstadt, Germany) for 30 min, right after the fixation step. Cells were incubated with mouse anti-human CD117 (Santa Cruz), mouse anti-human STRO-1 (Santa Cruz), mouse anti-human ALP (R & D Systems, Minneapolis, MN, USA), mouse anti-human COL1a2 (Santa Cruz), mouse anti-human COL3a1 (Santa Cruz), mouse anti-human SPARC (Santa Cruz) primary non-conjugated antibodies (dilutions 1:250) for 1 h at room temperature. Cells were then washed with PBS 3 times and saturated with Alexa Fluor 488-conjugated goat anti-mouse (Invitrogen) used as a secondary antibody (1:1000) for 1 h in the dark. Cells were also incubated with the following conjugated antibodies: rat anti-human CD49f conjugated with PE (Thermo Fisher Scientific Inc. Waltham, MA, USA), mouse anti-human CD146 (FITC) (Thermo Fisher) and anti-human CD271 (APC) (Miltenyi Biotec) at dilutions 1:1000, protected from light for 60 min. After washing with PBS three times, cultures were incubated with DAPI (4,6-diamidino-2-phenylindole) (Invitrogen) for 15 min to stain the nuclei. Cell cultures were observed with two different instruments: the first one is a confocal scanning laser fluorescence microscope (Leica Microsystems GmbH) and the second one is IN Cell Analyzer 6000 imaging system-laser confocal slit system (GE Healthcare, Pittsburgh, PA, USA). With the second instrument we managed to identify and quantify cell markers expression. We were able to check the entire plates with two-channel color recording. The first channel (blue) identified fluorescently stained nuclei; the other channel (green) recorded the specific fluorescent protein used for receptor tracking.