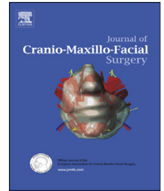
Journal of Cranio-Maxillo-Facial Surgery 45 (2017) 845–854

Contents lists available at ScienceDirect

Journal of Cranio-Maxillo-Facial Surgery

journal homepage: www.jcmfs.com



Microcarrier culture enhances osteogenic potential of human periodontal ligament stromal cells☆

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ARTICLE INFO

Article history:
Paper received 6 March 2016
Accepted 20 March 2017
Available online 29 March 2017

Keywords:
Microcarrier
PLSC
Osteogenic differentiation
Periodontitis

ABSTRACT

Regeneration of periodontal tissue represents a major challenge to modern tissue engineering, since cell-based therapies require large amounts of periodontal ligament stromal cells (PLSC), which can be obtained only by *in vitro* expansion. Ideally, the period of the *in vitro* expansion should be optimized for the generation of large enough numbers of pre-specified progenitor cells ready to contribute to the restoration of periodontal tissues.

In the present study, we used a commercially available, three-dimensional culturing platform and alginate microcarrier cell culture system for the propagation of human PLSCs, which were derived using the explant outgrowth method. Induction of osteogenic differentiation resulted in rapid and robust mineralization of the extracellular matrix in PLSCs grown on microcarriers, but not in PLSCs grown under standard culture conditions. Gene expression studies revealed upregulation of osteogenesis-related genes, *BMP2*, *ALP*, *RUNX2*, *MSX2*, *cementum protein 23*, *bone sialoprotein*, *osteopontin* and *periostin*, in undifferentiated and differentiating microcarrier cultures of PLSCs. In addition, the microcarrier culture enhanced the expression of β-catenin, intermediate filament protein vimentin and focal adhesion proteins vinculin and paxillin.

Our study shows that microcarrier culture allows rapid generation of large numbers of PLSCs pre-specified towards an osteogenic-like phenotype. This method may be useful for the development of new tissue engineering protocols for the reconstruction of periodontal tissues.

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1. Introduction

Periodontitis is a chronic infectious periodontal tissue disease characterized by progressive destruction of the tooth-attachment structures, leading to the tooth loss in adults. Current treatments are directed towards prevention or slowing the progression of the disease, but that is usually insufficient for the regeneration of a tooth-supporting apparatus (Wang et al., 2005). Accordingly, several experimental strategies for the regeneration and (or) stimulation of endogenous regenerative properties of periodontal tissues have been proposed (Chen and Jin, 2010). Among them, bone replacement grafts, use of various growth factors, and strategies for guided tissue regeneration have been used with some clinical success. However, clinical outcomes are variable depending on the defect size and configuration, amount of remaining healthy periodontal ligament (PDL), patient age and other factors (Wang et al., 2005).

Cell-based therapies represent another promising strategy for periodontal regeneration (Trofin et al., 2013). Chronic inflammation may lead to the exhaustion of endogenous pools of stem cells in periodontium; therefore grafting of expanded autologous stem cells could be critical for the regeneration of new functional tissue. PDL comprises specialized connective tissue fibres that join the teeth to the alveolar bone. PDL tissue contains heterogeneous populations of fibroblasts, osteoblasts and cementoblasts and also periodontal

☆ This research was supported by the European Social Fund under the Global Grant measure (No. VP1-3.1-ŠMM-07-K-03-016).

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http://dx.doi.org/10.1016/j.jcms.2017.03.009
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vessels and nerves (Chen and Jin, 2010). Tissue homoeostasis and regeneration are supported by a small population of PDL stem cells (Nagatomo et al., 2006) capable of differentiating into osteoblasts, cementoblast-like cells, adipocytes, and collagen-forming cells *in vitro*. Even more important, when transplanted into immunocompromised animals using hydroxyapatite/tricalcium phosphate (HA/TCP) as a carrier, PDL stem cells showed the capacity to generate cementum/PDL-like structures and to contribute to periodontal tissue repair (Seo et al., 2004). Periodontal ligament stromal cell (PLSC) cultures share certain similarities such as fibroblastoid morphology, immunophenotype and, to some extent, differentiation potential with mesenchymal stem cell–like (MSC-like) cells derived from other tissues (Zhu and Liang, 2015). Moreover, the PLSCs demonstrated tissue-specific differentiation capacities *in vivo*, because the cementum/PDL-like structures appeared to be totally different from those generated by bone marrow MSCs and dentin/pulp-like structures generated by dental pulp stem cells (DPSCs) (Seo et al., 2004). From a practical standpoint, it is important, that PLSCs can be readily expanded *in vitro* providing sufficient amounts for cell therapy protocols. However, similarly to other MSC-like cells, PLSC *in vitro* cultures yield a mix of functionally different heterogeneous subpopulations (Sanz et al., 2015).

In recent years, different strategies have been proposed for the regeneration of periodontal tissues using PLSCs, indicating that the optimal protocol has yet to be established. To date, use of HA/TCP ceramic particles as scaffolds for the PLSCs represents one of the most promising approaches. Transplantation of autologous PLSCs seeded onto HA/TCP scaffolds were capable of regenerating periodontal tissues in minipigs (Liu et al., 2008). In another study, cellular sheets of autologous or allogeneic PLSCs were produced *in vitro* in the presence of HA/TCP and then transplanted to the periodontitis lesions of minipigs (Ding et al., 2010).

All current cell-therapy protocols require considerable amounts of PLSCs, which can be obtained only by *in vitro* expansion (Zhu and Liang, 2015). In this respect, microcarrier technology may represent a useful alternative with a number of advantages over conventional cell culture systems (Chen et al., 2013). Microcarriers provide a large surface area for cell growth during propagation in suspension culture, allowing for scaling-up cell production in a relatively small volumes of medium (Malda and Frondoza, 2006), as well as periodic analysis of small samples of cell-seeded microcarriers during the experiment. Nutrient and gas exchange are improved, and cells are exposed to the shear stress, which might facilitate differentiation along certain lineages (Yourek et al., 2010). Cells cultured on microcarriers more closely resemble *in vivo* phenotype (Martin et al., 2011). This technology also allows direct delivery of cell-seeded microcarriers into the damaged tissue (Martin et al., 2011) being incorporated into the secondary vehicles. These advantages make the microcarrier technology potentially useful for the regeneration of periodontal tissues using PLSCs.

In the present study, we demonstrate that microcarrier culture allows rapid generation of large numbers of PLSCs pre-specified towards an osteogenic-like phenotype. These findings may potentially be exploited for the development of new tissue engineering protocols.

2. Materials and methods

2.1. Isolation and characterization of periodontal ligament stromal cells (PLSCs)

Primary PLSC cultures were isolated from healthy periodontal tissues of 2 donors using explant outgrowth method. Material was collected under the approval of the Lithuanian Bioethics Committee (No. 6B-08-173). Two intact premolars were extracted from two healthy Caucasian females (18 and 21 years old) for orthodontic reasons. Periodontal ligament tissue was obtained from the middle third of the tooth root using surgical blades, minced using surgical blades, cut into 2-mm^3 pieces and then placed in the 35-mm-diameter culture dishes and cultured in a low-glucose Dulbecco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 10% foetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (all from Biochrom, Berlin, Germany) (hereafter this formulation will be referred to as basal medium). Explant cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 with medium change routinely twice a week, until cultures reached confluency (typically between 2 and 3 weeks). For passaging cells, were washed three times with phosphate-buffered saline (PBS), harvested with 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) solution (Gibco, Life Technologies), resuspended in culture medium and plated onto cell culture flasks for expansion. Flow cytometry revealed that these cells expressed characteristic antigens of MSC-like cells (Supplementary Table 1).

2.2. Standard and microcarrier cell culture of PLSCs

PLSCs grown under standard conditions were cultivated in basal medium. Before the experiments, cells from the third passage were seeded in six-well culture dishes coated with a 2% solution of gelatin at the concentration 5 μg/cm^2 (Sigma, St. Louis, MO, USA).

For the microcarrier culture of PLSCs, we used BioLevitator (Hamilton Bonaduz AG), which is a commercially available, three-dimensional culturing platform, and an alginate microcarrier cell culture system (Global Cell Solutions, Charlottesville, VA). The BioLevitator system uses protein-coated magnetic microcarriers for cell attachment and is gently rotated to provide suspension-like cell culture conditions. The Global Eukaryotic Microcarriers (GEMs; Global Cell Solutions) are composed of an alginate core embedded with paramagnetic particles and coated with adhesion molecules. Cells attach to the GEMs and are simultaneously moved in the vertical direction by a magnet and in the horizontal direction by rotating the culture vessel, providing a suspension-like culture system that promotes gas exchange and maintains a homogeneous medium composition.

In our experiments, we used gelatin-coated GEMs. Inoculation and culture of PLSCs on GEMs in the Biolevitator was performed according to the manufacturer's recommendations with some modifications. Briefly, 500 μL of pre-washed, gelatin-coated GEMs was added to the LeviTube (Global Cell Solutions) containing 1.5 mL basal medium. Then single cell suspension of PLSCs (8×10^5 cells in 3 mL basal medium) was injected into the GEM/medium preparation in the LeviTube and the inoculation program was initiated for 4 h. After inoculation, GEMs were checked microscopically, and 15 mL basal medium was added to the each LeviTube before the program for cell culture was initiated. Medium changes were performed twice a week. Imaging and cell counting was performed periodically by taking of small amounts of cell-seeded microcarriers from the suspension cultures. PLSCs on microcarriers were stained with 0.1 mg/mL Hoechst 33342 (Applichem, Darmstadt, Germany), and images were captured under UV light and DAPI filter by a Motic AE31 microscope equipped with a Moticam 2500 camera and Motic Images Plus 2.0 software. Harvesting, counting and detachment of PLSCs were performed according to the manufacturer's instructions (Global Cell Solutions).

2.3. Induction of osteogenic differentiation

For differentiation experiments, PLSCs from the third passage were seeded at a density of $5 \times 10^3/cm^2$ in 6-well

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