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Research article

Assessment of cellular materials generated by co-cultured 'inflamed' and healthy periodontal ligament stem cells from patient-matched groups

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ABSTRACT

Recently, stem cells derived from the'inflamed' periodontal ligament (PDL) tissue of periodontally diseased teeth (I-PDLSCs) have been increasingly suggested as a more readily accessible source of cells for regenerative therapies than those derived from healthy PDL tissue (H-PDLSCs). However, substantial evidence indicates that I-PDLSCs exhibit impaired functionalities compared with H-PDLSCs. In this study, patient-matched I-PDLSCs and H-PDLSCs were co-cultured at various ratios. Cellular materials derived from these cultures were investigated regarding their osteogenic potential *in vitro* and capacity to form new bone following *in vivo* transplantation. While patient-matched I-PDLSCs and H-PDLSCs could co-exist in co-culture systems, the proportion of I-PDLSCs tended to increase during *in vitro* incubation. Compared with H-PDLSC monoculture, the presence of I-PDLSCs in the co-cultures appeared to enhance the overall cell proliferation. Although not completely rescued, the osteogenic and regenerative potentials of the cellular materials generated by co-cultured I-PDLSCs and H-PDLSCs were significantly improved compared with those derived from I-PDLSC monocultures. Notably, cells in co-cultures containing either 50% I-PDLSCs plus 50% H-PDLSCs or 25% I-PDLSCs plus 75% H-PDLSCs expressed osteogenesis-related proteins and genes at levels similar to those expressed in H-PDLSC monocultures ($P > 0.05$). Irrespective of the percentage of I-PDLSCs, robust cellular materials were obtained from co-cultures with 50% or more H-PDLSCs, which exhibited equivalent potential to form new bone *in vivo* compared with sheets generated by H-PDLSC monocultures. These data suggest that the co-culture of I-PDLSCs with patient-matched H-PDLSCs is a practical and effective method for increasing the overall osteogenic and regenerative potentials of resultant cellular materials.

1. Introduction

Periodontitis is a chronic inflammatory and infectious disease that affects humans on all continents and leads to the progressive damage of the tooth-supporting apparatus, including alveolar bone, the periodontal ligament (PDL) and cementum. Once advanced periodontitis occurs, periodontal defects cannot be restored to their innate organization and function due to the complex structure of the periodontium, even if decontaminating treatments, such as the removal and continuous control of dental plaque, are well performed in clinical practice [1]. Over the past 30 years, numerous techniques, from guided tissue regeneration technology to the use of bone-grafting materials and a wide range of biological agents, have been introduced and evaluated for the regeneration of periodontal structures lost due to periodontitis. Although all these therapies have been shown to be effective to certain degrees, the clinical outcomes remain largely equivocal and variable, particularly with respect to true periodontal regeneration [2]. Biologically, the attainment of predictable reconstruction of damaged periodontium requires a combination of several critical elements, including, but not limited to, a sufficient number of progenitor/stem cells, an appropriate level and sequence of cell-signaling molecules and an artificial environment that mimics the native extracellular matrix (ECM) and favors cell viability and differentiation [3,4].

With the understanding that the basic functioning unit of any wound-healing cascade is the reparative cells, stem cell-based therapy is entering a new era in periodontics, and the potential of

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this approach is expanding to include reconstructing the entire periodontal organ system [5,6]. The relative ease of harvesting stem cells from dental soft tissues renders teeth a valuable and easily accessible source of mesenchymal stem cells (MSCs) and stromal cells for therapeutic application [7–9]. In particular, stem cells derived from PDL tissue (PDLSCs) are the best and first choice for use in periodontal regenerative therapies [10]. For more than 10 years, MSCs have been recognized to reside within human PDL tissue [11], and it is now clear that those cells are responsible for both the physiological remodeling and therapeutic regeneration of the periodontium [10]. Of note, the transplantation of PDLSCs has delivered beneficial periodontal regeneration outcomes in a set of preclinical animal studies, irrespective of the defect type and animal model [10]. Indeed, there is substantial evidence supporting the advancement of PDLSC therapy from animal studies (preclinical) to human trials (clinical) to examine its safety, feasibility and efficacy [6,12]. Although recent attempts have focused on using PDLSCs from the same patient [13,14], teeth for use in cell production are not readily available because orthodontic treatments and the extraction of wisdom teeth generally do not occur in conjunction with the need for cell-based treatments. In addition, the number of stem/progenitor cells localized in human PDL tissue is too small to be conveniently and routinely obtained from extracted teeth. Therefore, researchers have been attempting to scout periodontally diseased teeth for sources of PDLSCs that are suitable for cytotherapy [15–21].

Recently, we isolated and characterized MSCs from the 'inflamed' and healthy PDL tissues (denoted as I-PDLSCs and H-PDLSCs, respectively) of patient-matched groups [22]. Similar to previously published findings, I-PDLSCs exhibited an increased proliferative capacity and greater migratory potential, whereas the functionally compromised immunomodulation and differentiation of I-PDLSCs largely limited the direct use of these cells in cytotherapy and tissue engineering [15–21]. Therefore, effective strategies to improve the therapeutic potential of cellular materials derived from I-PDLSCs are necessary [22]. Notably, in a previous study, we found that the impaired osteogenic differentiation of I-PDLSCs could potentially be rescued by incubating these cells in media conditioned by patient-matched H-PDLSCs [23]. In the present study, we hypothesized that the co-culture of I-PDLSCs directly with patient-matched H-PDLSCs would be a simple and effective strategy to obtain robust cellular materials for future therapeutic applications. If this approach is practical, then I-PDLSCs could be used as an adjuvant cell source for regenerative therapies when the number of available H-PDLSCs is inadequate to generate sufficient cellular material.

2. Materials and methods

2.1. Patient-matched I-PDLSCs and H-PDLSCs

Patient-matched I-PDLSCs and H-PDLSCs were obtained from 6 donors as described in our previous study [22]. The entire experimental process in this research was verified and supervised by the Fourth Military Medical University (FMMU) Ethics Committee, Xi'an, China. Before co-culturing, the stem cell surface markers of the I-PDLSCs and H-PDLSCs were determined with immunofluorescence staining. Briefly, single-cell suspensions of I-PDLSCs and H-PDLSCs (P_3) were separately seeded into 96-well plates (Corning, Lowell, MA, USA) at a density of 5×10^3 cells/well. After being cultured for 24 h to allow the cells to adhere, the cells were fixed, blocked, incubated in the corresponding primary and secondary antibodies, and then counterstained with a nuclear staining agent (Hoechst 33342, Sigma-Aldrich, St. Louis, MO, USA). Then, the cells were immediately observed and imaged using an immunofluorescence microscope (Leica Microsystems, Heerbrugg, Switzerland). The following antibodies were used: mouse anti-human primary antibodies, including anti-STRO-1 (R&D Systems, Minneapolis, MN, USA), anti-CD146 (Abcam, Cambridge, UK) and anti-CD105 (Abcam); rabbit anti-human primary antibodies, including anti-CD90 (Abcam), anti-CD31 (Abcam) and anti-CD34 (Abcam); and Alexa Flour 488-conjugated (green) goat anti-mouse IgG or Cy3-conjugated (red) goat anti-rabbit IgG secondary antibodies (Zhuangzhi Biotech, Xi'an, China).

2.2. Co-culture designs

For co-culturing I-PDLSCs and H-PDLSCs in various combinations, patient-matched cells at a total density of 1×10^5 cells/well (for 6-well plates) or 2×10^3 cells/well (for 96-well plates) were seeded into culture plates. In the present study, we established 3 co-culture test groups and 2 controls. The co-culture test groups included 75% I-PDLSCs plus 25% H-PDLSCs (75% IC+25% HC group), 50% I-PDLSCs plus 50% H-PDLSCs (50% IC+50% HC group), or 25% I-PDLSCs plus 75% H-PDLSCs (25% IC+75% HC group). Monocultures of I-PDLSCs (100% IC) or H-PDLSCs (100% HC) served as the 'inflamed' or healthy cell controls.

2.3. I-PDLSC tracing in a co-culture system

To explore the cell growth capability of I-PDLSCs in cell co-cultures, I-PDLSCs that had been previously labeled with extrinsic fluorescent material were traced via flow cytometry at defined time points. Briefly, a single-cell suspension of I-PDLSCs (P_4, 2×10^6 cells) was labeled with 2 μl of carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, 1000x) in 2 ml of cell marker diluent and incubated at 37 °C for 10 min in the dark, according to the instructions of the CFDA-SE Cell Proliferation Assay and Tracking Kit (Beyotime, Shanghai, China). The excess dye was washed out with basic medium, *i.e.*, α-minimum essential medium (α-MEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 0.292 mg/ml glutamine (Invitrogen Life Technology, Carlsbad, CA, USA), 100 U/ml penicillin (Gibco BRL), 100 mg/ml streptomycin (Gibco BRL) and 10% fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials, Zhejiang, China). The labeling efficiency of the I-PDLSCs was analyzed immediately with a flow cytometer (Beckman Coulter, Fullerton, CA, USA) and observed with an immunofluorescence microscope (Leica) after a 24-h culture for cell adhesion. Following 1, 3 and 5 days of incubation in 6-well plates, co-cultured cells (1×10^5 cells/well) were harvested, and the percentage of I-PDLSCs in each group was determined to reflect the cell growth of I-PDLSCs in the co-culture systems.

2.4. Proliferation of co-cultured cells in vitro

Based on the groups described above, patient-matched I-PDLSCs (P_4) and H-PDLSCs (P_4) were co-cultured in 96-well plates (Corning) in various combinations at a total density of 2×10^3 cells/well and were incubated for 24 h for cell adhesion. Then, the proliferation of all cell cultures was analyzed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays according to the method reported in our previous study [22].

2.5. Osteogenic potential of co-cultured cells in vitro

The *in vitro* osteogenic potential of co-cultured I-PDLSCs and H-PDLSCs was evaluated in terms of their production and activity of alkaline phosphatase (ALP), their expression levels of the collagen type-1 (COL-1), osteocalcin (OCN) and runt-related transcription factor-2 (RUNX-2) proteins and the *COL-1, OCN* and *RUNX-2* genes, as well as the ability of these cells to form