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In vitro studies on human periodontal ligament stem cell sheets enhanced by enamel matrix derivative

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ABSTRACT

Numerous preclinical and clinical studies have focused on the periodontal regenerative functions of enamel matrix derivative (EMD), a heat-treated preparation derived from enamel matrix proteins (EMPs) of developing porcine teeth. In this study, periodontal ligament (PDL) stem cells (PDLSCs) were isolated, and the effects of EMD on the extracorporeal induction process and the characteristics of PDLSC sheets were investigated for their potential as a more effective stem-cell therapy. EMD-enhanced cell sheets could be induced by complete medium supplemented with 50 μg/mL vitamin C and 100 μg/mL EMD. The EMD-enhanced cell sheets appeared thicker and more compact than the normal PDLSC sheets, demonstrated more layers of cells (3–7 layers), secreted richer extracellular matrix (ECM), showed varying degrees of increases in mRNA expression of periodontal tissue-specific genes (COL I, POSTN), calcification-related genes (RUNX2, OPN, OCN) and a cementum tissue-specific gene (CAP), and possessed a better mineralization ability in terms of osteogenic differentiation in vitro. These EMD-enhanced cell sheets may represent a potential option for stem-cell therapy for PDL regeneration.

1. Introduction

Periodontitis is a widespread infectious disease in humans that causes the progressive destruction of the tooth-supporting tissues comprising alveolar bone, periodontal ligament (PDL) and root cementum [1]. Periodontitis is the main cause of tooth loosening and loss and is associated with systemic diseases such as diabetes mellitus, cardiovascular disease, and stroke [2,3]. Conventional clinical treatment strategies, including tooth scaling and open flap debridement, and other regenerative therapies such as guided tissue regeneration (GTR) and bone grafting have been routinely applied to guide regeneration in clinical practice; nevertheless, the success of these strategies has been limited [4]. Recently, stem-cell therapy, a nascent but fast-growing field, has become a hotspot in the field of periodontics. Stem-cell therapy could potentially overcome the limitations of traditional regenerative therapies and lead to more effective and robust treatment outcomes for predictable periodontal regeneration [5,6]. In animal models, the local administration of dissociated stem cells exerted anti-inflammatory and immunomodulatory effects and repaired defects caused by periodontitis [7]. In the clinic, in vitro-expanded cell populations derived from autologous PDL tissue have already been utilized for the treatment of human periodontitis [8,9]. Although stem cell-based periodontal regeneration has been rapidly developed, the traditional delivery models (e.g., cell suspension injection) are associated with poor survival of the transplanted cells, which might impede the expected therapeutic effects in humans [10]. The standard protocol in terms of cell populations and delivery mode remains to be optimized [9].

Recently, cell sheet engineering has emerged as a novel approach for effective delivery of seeding cells with good preservation of intact cell–cell junctions and in vitro-secreted extracellular matrix (ECM) [11]. Cell sheets have routinely been induced based on a vitamin C (Vc) induction method because of increased cell matrix production and deposition. This cell processing approach has been shown to effectively preserve cellular microenvironments comprising various biological and mechanical properties and to increase the cell survival rate and reduce cell loss during cell sheet implantation [12]. This technique is also effective for treating myocardial infarction [13], corneal dysfunction [14], and esophageal ulcerations [15]. Thus, producing higher-quality cell sheets with more in vitro-secreted extracellular matrix and an improved in vitro-induced multi-lineage differentiation capacities, including differentiation toward the PDL, cementum and alveolar bone, may result in superior biological performance for periodontal regeneration.

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Meanwhile, for effective periodontal wound healing and regeneration, numerous preclinical and clinical studies have focused on the functions of enamel matrix derivative (EMD), a heat-treated preparation derived from enamel matrix proteins (EMPs) of developing porcine teeth [16]. EMD was initially introduced in 1996 as a commercial product (Emdogain, Straumann, Basel, Switzerland). The major component of EMD is amelogenin, a family of hydrophobic proteins that accounts for over 95% of the total protein content. Other proteins in EMD include enamelin, ameloblastin, amelotin, apin, and various proteinases [17]. Histological findings in monkeys and subsequent observations from human case reports have demonstrated the capacity of EMD to stimulate periodontal regeneration and to reconstitute lost periodontal structures (i.e., the formation of new root cementum, periodontal ligament and alveolar bone) [18–20]. In contrast, other studies demonstrated that EMD exhibited limited or no obvious therapeutic effects for PDL regeneration compared to the effects observed in the control group not receiving EMD [21]. Similar discrepancies have also been observed in some clinical and histological studies, as previously discussed in reviews by Venezia et al. [22] and Sculean et al. [18]. However, more and more studies have found that the proteins found in EMD significantly influenced the behaviors of many types of cell by mediating cell migration, spreading, proliferation, and differentiation, as well as the expression of signaling molecules, growth factors, and extracellular matrix constituents [23,24]. According to a recent study, the addition of EMD enhanced the differentiation potential of PDL stem cells (PDLSCs) toward mesenchymal and non-mesenchymal lineages such as osteogenic, chondrogenic and adipogenic cells, neurogenic precursors that form neuronal cell types, and endothelial precursors that give rise to new blood vessels [25].

Although EMD has been widely studied for its biological effects on cellular proliferation and differentiation in vitro [21,25] and for its clinical effects in periodontal applications [22]. the effects of EMD on PDLSC cell sheets in vitro have not been thoroughly assessed. Therefore, the present study investigated the effects of lyophilized EMD on the extracorporeal induction process and characteristics of PDL cell sheets to develop a more effective stem-cell therapy.

2. Materials and methods

2.1. Isolation of PDLSCs

Human PDLSCs were isolated based on reported protocols [26]. Briefly, human PDL was isolated from extracted premolars from orthodontic patients (20–26 years of age, $n = 3$) with written informed consent and IRB approval from the Stomatological Hospital of FMMU. The PDL tissues in the middle part of the root surfaces were carefully scraped, cut into small blocks and then subjected to digestion in 5 mL of α-minimum essential medium (α-MEM; Hyclone, MA, USA) containing 1% collagenase type I and 1% dispase (both from Sigma–Aldrich, St Louis, MO, USA) for 40 min at 37 °C. The digested tissues were cultured in complete medium containing α-MEM supplemented with 10% fetal bovine serum (FBS, Hyclone), 0.292 mg/mL glutamine (Sigma–Aldrich), 100 U/mL penicillin (Sigma–Aldrich), and 100 mg/mL streptomycin (Sigma–Aldrich). Finally, the plates were incubated in a humidified atmosphere at 37 °C in 5% CO_2, and the media were refreshed every 3 days until the cells successfully migrated from the PDL tissue blocks. As previously described [27], the limiting dilution technique was used to obtain clones derived from single cells (P0). PDLSCs at passages P3–P5 were used in the subsequent study.

2.2. Colony-forming assay

Colony-forming unit fibroblast (CFU-F) assays were employed to determine the colony-forming abilities of stem cells. Briefly, human PDLSCs (P3) were seeded into 10-cm-diameter culture dishes at a density of 1×10^3 cells per dish and cultured in complete medium. After 12 days of incubation, the cells were fixed in 4% paraformaldehyde and then stained with 0.1% crystal violet (Sigma–Aldrich) for 15 min. The dishes were then washed twice with PBS and observed under an inverted microscope. Aggregates of 50 or more cells were viewed as colonies and were included in the final statistical analysis; smaller aggregates were excluded [12,26].

2.3. Flow cytometric analysis of cell surface markers

Flow cytometry analysis was adopted to determine the cell surface markers of PDLSCs, as previously described [27]. Briefly, after each cell line cultured in 10-cm dishes reached 90% confluence, the cells were digested with trypsin, divided into sterile microtubes and incubated with 0.1% antibodies against human STRO-1 (BD Bioscience, San Jose, CA, USA), CD146 (eBioscience, San Diego, CA, USA), CD34 (Biolegend, San Diego, USA), and CD45 (Biolegend) at 37 °C in the dark for 40 min; cell suspensions in PBS without added antibodies served as controls. The cells were then washed three times with PBS to remove excess antibodies and finally resuspended with 300 μL PBS supplemented with 3% FBS and analyzed with the flow cytometer vantage cell sorter (Becton Dickinson, Mountain View, USA).

2.4. Osteogenic/adipogenic differentiation assays in vitro

PDLSCs (P3) were seeded into 6-well dishes at 5×10^5 cells/well and incubated in complete medium until they reached 90% confluence. To assess the osteogenic ability of the cells, osteoinductive medium (complete medium supplemented with 1.8 mM KH_2PO_4, 50 μg/mL vitamin C and 10 nM dexamethasone) was provided and refreshed at 3-day intervals. After a 4-week osteogenic induction, the cells were fixed in 4% paraformaldehyde and then stained with Alizarin Red staining solution (Sigma–Aldrich) for 3 min at room temperature. To assess adipogenesis, cells were cultured in adipogenic medium (complete medium supplemented with 100 nM dexamethasone, 10 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 50 mM indomethacin). After two weeks, the cells were fixed in 4% paraformaldehyde and stained with Oil Red O (Sigma–Aldrich) staining solution for 15 min. The dishes were then washed twice with PBS and observed in PBS under a microscope (IX70, Olympus, Tokyo, Japan).

2.5. Cytotoxicity assay

Lactate dehydrogenase (LDH) activity was used as an index of cytotoxicity in the culture medium with α-MEM/10% FBS (control group) and α-MEM/10% FBS plus EMD (enamel matrix derivative, Straumann, Malmo, Sweden) at concentrations of 25, 50 and 100 μg/mL. According to the product instructions, EMD was dissolved in 0.1% acetic acid to make a stock solution, and different doses of that stock solution were added to culture media to make EMD-enhanced culture media with different concentrations. After 24 h of culture, the culture media was collected and centrifuged, and the supernatant was used for the LDH activity assay. LDH activity was determined spectrophotometrically, according to the manufacturer's instructions. Three parallel experiments were conducted in each group.