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The effect of the coumarin-like derivative osthole on the osteogenic properties of human periodontal ligament and jaw bone marrow mesenchymal stem cell sheets

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ABSTRACT

Cell sheet engineering is a scaffold-free delivery concept that has been shown to improve mesenchymal stem cell-mediated regeneration of injured or pathologically damaged periodontal tissues in preclinical studies and several clinical trials. However, the best strategy for cell sheet production remains to be identified. The aim of this study was to investigate the biological effects of osthole, a coumarin-like derivative extracted from Chinese herbs, on the cell sheet formation and osteogenic properties of human periodontal ligament stem cells (PDLSCs) and jaw bone marrow mesenchymal stem cells (JBMMSCs). Patient-matched PDLSCs and JBMMSCs were isolated, and an appropriate concentration of osthole for cell culture was screened for both cell types in terms of cell proliferation and alkaline phosphatase (ALP) activity. Next, the best mode of osthole stimulation for inducing the formation of sheets by each cell type was selected by evaluating the amount of their extracellular matrix (ECM) protein production as well as osteogenic-related gene expression. Furthermore, both PDLSC and JBMMSC sheets obtained from each optimized technique were transplanted subcutaneously into nude mice to evaluate their capacity for ectopic bone regeneration. The results revealed that 10^{-5} M/L osthole significantly enhanced the proliferation of both PDLSCs and JBMMSCs ($P < 0.05$), although for JBMMSCs, there was no concentration-related change among the four established osthole groups ($P > 0.05$). In addition, 10^{-5} M/L osthole was the best concentration to promote the ALP activities of both cells ($P < 0.01$). Based on both the production of ECM proteins (collagen type I, integrin β1, and fibronectin) and the expression of osteogenic genes (ALP, Runt-related transcription factor 2 (RUNX2), and osteocalcin (OCN)), the provision of 10^{-5} M/L osthole throughout the entire culture stage (10 days) for PDLSCs or at the early stage (first 3 days) for JBMMSCs was the most effective osthole administration mode for cell sheet formation ($P < 0.05$). The results of *in vivo* transplantation showed that osthole-mediated PDLSC and JBMMSC sheets formed more new bone than those obtained without osthole intervention ($P < 0.001$). Our data suggest that a suitable concentration and mode of osthole stimulation may enhance ECM production and positively affect cell behavior in cell sheet engineering.

1. Introduction

Periodontitis is an inflammatory disease that causes pathological alterations in the tooth-supporting apparatus, consisting of alveolar bone, periodontal ligament (PDL) and root cementum [1]. It is a major cause of tooth loss in adults and is linked to multiple systemic conditions, such as diabetes mellitus, cardiovascular disease, and premature low birth weight [1–4]. Clinically available techniques, such as open flap debridement, which provides critical access to evaluate and detoxify root surfaces and improves

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periodontal form and architecture, are able to control periodontal inflammation and, to a certain degree, to stimulate the restoration or reconstitution of component periodontal tissues if combined with guided tissue regeneration (GTR) technology and/or the adjuctant use of bone substitutes, biological mediators, or specific genes/growth factors [5–8]. However, a complete and functional regeneration of injured or pathologically damaged periodontium remains a formidable challenge [5–7]. An improved understanding of periodontal biology coupled with current advances in stem cell research and biomaterials science has introduced advanced treatments based on the concept of tissue engineering to enhance periodontal tissue reconstruction and its biomechanical integration [9,10]. Particularly, there is a growing interest in the clinical application of stem cells as a novel therapeutic approach for the treatment of periodontal disease [11–16]. Transplanted stem cells improve periodontal regeneration through multiple mechanisms, which include but are not limited to replacing dead osteoblasts, fibroblasts, and cementoblasts within a diseased periodontium, promoting angiogenesis, and modulating the bone-PDL-cementum structure remodeling [11–14]. Most of the results obtained so far in animal studies using stem cells derived from PDL [17–21], bone marrow [20,22–24], adipose tissue [25], and alveolar periosteum [20,26] have indicated that the transplantation of stem cells can be an effective treatment for periodontal defects, spawning an increasing number of case studies as well as clinical trials in this field [15,27–29]. However, many problems remain to be resolved, such as the best cell(s) to use, the optimal dose and most effective mode of stimulation, and the issue of how to induce the transplanted cells to participate in the regeneration of periodontal tissue is of exceptional clinical importance [15,16,30].

Since a pioneer study published in 2004 that demonstrated the presence of stem cells in human PDL, generally termed PDL stem cells (PDLSCs), there is mounting evidence that cells residing in the periodontium are the best candidates for periodontal cytotherapy [11–21,26]. Meanwhile, with promising outcomes, bone marrow-derived mesenchymal stem cells (BMMSCs) are also used in preclinical models [20,22–24] as well as humans [27,29] to treat periodontal disease. Alveolar BMSCs may be more useful than iliac crest BMSCs for regenerative medicine because marrow aspiration from alveolar bone can be performed with minimal pain [31]. Analysis of the data published to date suggests that stem cells isolated from periodontal supporting tissues, i.e., PDLSCs or alveolar or jaw BMMSCs (JBMMSCs), are the best candidates for clinical application in future periodontal regenerative therapies. In addition to considerations of specific cell types that may favor periodontal repair, the delivery strategy also plays an essential part in the design of cell-based periodontal therapy [15]. Therapies with the potential to become routine in the clinic will only be possible if these methods ensure efficient engraftment and the survival of a therapeutically relevant number of cells [32]. In this regard, cell sheet technology has been established as a promising concept for cell delivery that allows for a sheet of interconnected cells and cells in full contact with their natural extracellular environment to be delivered [33,34]. Although cell sheet technology has been widely used as a clinically relevant method for the delivery of therapeutic cells to the periodontium [17,19,20,35–37], concerted efforts have been and still are being made to harvest the living cell sheet more easily and effectively to avoid a relatively complicated, time-consuming sheet grafting procedure [33,34]. Wei et al. (2012) recently developed a new, simple, and practical approach to generate vitamin C (Vc)-induced PDLSC sheets, leading to up-regulated expression of extracellular matrix (ECM) proteins and typical osteogenic markers in the resultant cell sheets [38]. In the present study, we investigate the biological effects of osthole, a small-molecule compound of traditional Chinese medicine (TCM), on the cell sheet formation and osteogenic properties of patient-matched human PDLSCs and JBMMSCs.

Functional ingredients derived from TCM have been shown to be powerful tools for the activation of adult stem cells for tissue regeneration, leading to the use of some of these molecules as useful adjuvants in cell processing technology and periodontal tissue engineering applications, sometimes as a substitute for recombinant human growth factors [39,40]. Particularly, osthole is a fundamental ingredient of coumarin compounds that are the main pharmaco-components of *Fructus cnidii* [41]. Osthole exhibits estrogen-like effects, and in ovariectomized rats, it can prevent osteoporosis and reduce bone loss [42]. Although the cellular effects of osthole on osteoblast proliferation and differentiation have been documented [43,44], the value of osthole as an adjuvant for cell sheet formation remains to be determined. We hypothesized that osthole administration would facilitate cell sheet formation, increase cell matrix production, and positively influence the cellular behavior of the sheet-grafted cells in terms of differentiation and maturation, and hence enhance tissue regeneration and functional integration following *in vivo* transplantation.

2. Materials and methods

2.1. Cell isolation and culture

The experimental protocols were approved by the ethics committee (Institutional Review Board for Human Subjects Research) of the School of Stomatology, Fourth Military Medical University (FMMU), and all donors or guardians provided written informed consent for the donation of their removed tooth and bone chips and their subsequent use in this research project. Following informed consent, 6 third impacted molars with healthy PDL tissue were removed from 6 donors (18–28 years), and jaw bone chips were also harvested from the same 6 donors because bone-removing surgery was required during the extraction of their impacted third molars. All samples were used for patient-matched cell culture within 2 h of extraction.

PDL primary cell culture was carried out as described previously [45,46] with minor modification. Briefly, teeth were washed in sterile phosphate-buffered saline (PBS), and the PDLs were gently separated from the middle part of the root surface and then digested with 3 mg/mL of collagenase type I and 4 mg/mL of dispase (Sigma–Aldrich, St. Louis, MO, USA) for 15 min. Upon completion of digestion, the tissue explants from different individuals were pooled and plated into 6-well culture dishes (Corning, Lowell, MA, USA) with basal medium, i.e., α-minimum essential medium (α-MEM; Gibco BRL, Gaithersburg, MD, USA), containing 10% fetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. Zhejing, China), 0.292 mg/mL of glutamine (Invitrogen Life Technology, Carlsbad, CA, USA), 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Gibco BRL). Then, the cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air until cells grew out from the tissue patch and approached confluence.

For jaw bone primary cell culture, the obtained bone chips were aseptically rinsed twice in PBS, and then the bone marrow stromal cells were flushed out from the bone marrow cavity by using α-MEM. Next, the resultant medium was centrifuged at 1000 revolutions per minute (rpm) for 5 min. Finally, the deposits (containing cells) were resuspended with basal medium and cultured in 6-well plates. Three days later, floating cells were removed, and then the medium was replaced by fresh α-MEM for continuing culture until the cells approached confluence. Although the primary cells may be obtained from both the alveolar bone marrow and the bone chips, we generally termed the resultant stem cells as JBMMSCs in the present study.

To further isolate and purify the stem cells, single-cell suspensions of primary cells were cloned using the limiting-dilution technique, and passage 0 (P_0) cells were cultured, as previously reported [45,46]. All colonies were then pooled and expanded to obtain PDLSCs or JBMMSCs, which were then cultured separately in basal medium. To avoid cell behavioral changes that are associated with prolonged culture, the cells at passages P3–P5 (P_3–P_5) were used for contrastive investigation in the present study.

2.2. Characterization of human PDLSCs and JBMMSCs

2.2.1. Colony-forming unit-fibroblast (CFU-F) assays

A total of 1×10^3 of PDLSCs or JBMMSCs (P_3) were suspended in basal medium and cultured in 10-cm-diameter culture dishes (Corning, Lowell, MA, USA) for CFU-F assays. These cells were fixed on day 14 with 4% paraformaldehyde and stained with 0.1% toluidine blue. Aggregates of 50 or more cells viewed under the microscope were counted as a colony. The number of colonies per well was counted for contrastive analysis between the two types of cells. The experiment was repeated at least three times for each cell line.