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Effects of enamel matrix proteins on multi-lineage differentiation of periodontal ligament cells in vitro

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ABSTRACT

The adult periodontal ligament (PDL) is considered to contain progenitor cells that are involved in the healing of periodontal wounds. Treatment with enamel matrix derivative (EMD), a heat-treated preparation derived from enamel matrix proteins (EMPs), has been shown to be of some clinical benefit in eliciting periodontal regeneration in vivo. Although there is extensive information available about the effects of EMD on periodontal regeneration, the precise influence of this material on alveolar bone and the formation of blood vessels and proprioceptive sensory nerves, prominent features of functionally active periodontal tissue, remain unclear. The aim of the present study was therefore to examine the effects of EMD on the ability of human periodontal ligament cells (HPCs) to undergo multi-lineage differentiation in vitro. Our results showed that HPCs treated with EMD under non-selective growth conditions did not show any evidence of osteogenic, adipogenic, chondrogenic, neovasculogenic, neurogenic and gliogenic "terminal" differentiation. In contrast, under selective lineage-specific culture conditions, EMD up-regulated osteogenic, chondrogenic and neovasculogenic genes and "terminal" differentiation, but suppressed adipogenesis, neurogenesis and gliogenesis. These findings thus demonstrate for the first time that EMD can differentially modulate the multi-lineage differentiation of HPCs in vitro.

1. Introduction

The periodontal ligament (PDL) that supports the teeth within the alveolar socket is comprised primarily of osteoblasts, osteoclasts, cementoblasts and fibroblasts, and has recently been reported to contain cells capable of expressing stem cell-associated genes such as CD29, CD44, CD105, CD106, CD146, STRO-1, Oct3/4 and nanog [1–4]. Such cells have been shown to have prolonged proliferative ability (approximately 60 population doublings before cell senescence) and the ability to differentiate into a number of different mesenchymal lineages (osteoblasts, adipocytes and chondrocytes), as previously reported [3], although they do not necessarily express the characteristic STRO-1 mesenchymal stem cell marker [3,51]. Homeostasis, repair and regeneration of the PDL are considered to be dependent on the proliferation and differentiation of such progenitor cells [5,6]. This complex periodontal regenerative process involves not only formation of new extracellular matrix (ECM) and connective tissue architecture, but also the production of new bone, blood vessels (BVs) and proprioceptive sensory nerve innervations [7]. Although a number of exogenous growth factors (platelet-derived growth factor, insulin-like growth factor, transforming growth factor-beta (TGF-β), fibroblast growth factor), bone replacement grafts and recently developed biomaterials (calcium phosphate and ceramic-based materials as well as deproteinized bovine bone mineral [5,8–11,58]) have been used for the clinical remodelling of periodontal tissue, these therapies have had only limited and variable outcomes [5,8–12]. There is therefore a need for materials capable of eliciting the reproducible, predictable and effective differentiation of resident or transplanted precursor cells into mature functional ligament tissue.

Enamel matrix derivative (EMD; Institut Straumann, Basel, Switzerland), a heat-treated heterogeneous mixture comprising mainly amelogenin-derived porcine enamel matrix proteins (EMPs) secreted during development of tooth-supporting tissues, has been widely used in attempting to rebuild periodontal tissues [5,13–18,55,57], although the precise mechanism(s) by which EMD may promote tissue regeneration is still unclear. Moreover, although EMD has been shown to promote PDL and bone regeneration in animals and humans [16,17], a number of inconsistencies have been reported for the effects of this material on osteogenesis in vitro and bone remodelling in vivo [18–20]. For example, in vitro, EMD has been shown to promote osteogenesis by up-regulating bone-associated genes such as alkaline phosphatase (ALP),

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osteopontin (OP), osteocalcin (OC) and bone sialoprotein (BSP), and by stimulating the production of bone-like mineralized nodules of PDL cells [21]. In contrast, other studies have shown that EMD down-regulated these bone-associated markers and terminal osteogenic differentiation of periodontal ligament cells, osteoblasts and bone marrow-derived mesenchymal stem cells [22–24]. Similarly, a study evaluating the osteoinductive properties of EMD in the muscle of rats reported that EMD did not stimulate bone formation [20]. Furthermore, it is now recognized that BV formation, the complex process of neovasculogenesis, involves both vasculogenic and angiogenic differentiation during adult wound healing as well as in developing microenvironments [25,26], but investigations of the effects of EMD have been limited to angiogenesis in vitro [28,36,59,60]. In addition, although periodontal disease has been shown also to affect PDL proprioceptive nerves and sometimes cause debilitating pain [27], the effects of EMD on PDL cell neurogenesis and gliogenesis in vitro have hitherto not been examined.

The present study has therefore investigated the effects of lyophilized EMD (Institut Straumann, Basel, Switzerland) on multi-lineage differentiation of adult human periodontal ligament cells (HPCs) in vitro.

2. Materials and methods

2.1. Isolation of primary HPCs

HPCs were obtained from periodontal tissue of healthy patients undergoing routine tooth extraction. The participants signed informed consent, in accordance with the protocol approved by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital. Briefly, ligament tissue from the middle portion of the root of the tooth was digested with 3 mg ml^{-1} of collagenase type I and 4 mg ml^{-1} of dispase (both from Sigma) for 1 h at 37 °C, as previously described [3]. Single-cell suspensions of the HPC were obtained by passing the cells through a 70 μm stainless steel filter (Falcon, Becton Dickinson, Cowley, UK) and cultured in growth medium (GM) containing α-modified Eagle's medium (α-MEM) (Gibco Life Technologies Ltd, Paisley, UK) and 10% fetal calf serum (FCS) (PAA Laboratories, Yeovil, UK), and supplemented with 200 U ml^{-1} penicillin, 200 μg ml^{-1} streptomycin, 2 mM L-glutamine (all from Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Three separate HPC populations from three different healthy donors (all male, ages between 18 and 25) were used between passages 3 and 5 in all the experiments.

2.2. Treatment of cells with EMD

Lyophilized EMD (batch No. 9112) was dissolved at a concentration of 10 mg ml^{-1} in 0.1% acetic acid and added directly to the cells when they reached approximately 90% confluence, at a final concentration of 100 μg ml^{-1} as used in previous studies [28–30] and as found by dose–effect experiments as described in the Supplementary Material. As a control for the presence of 0.001% acetic acid, which was the concentration added when using the highest concentration of EMD (100 μg ml^{-1}), all genes and terminal differentiation responses were tested using 0.001% acetic acid alone (in the absence of EMD). This acetic acid concentration was found to have no significant effect on the values obtained using each respective medium only (GM and differentiation media (DM) alone, with no EMD or acetic acid) (data not shown). In addition, the same batch of EMD was used throughout the in vitro experiments reported here, although it is notable that all batches of this material are prepared by Institut Straumann from developing pigs according to a highly standardized and rigidly controlled FDA-approved commercial process to try and ensure as much as is feasible a very similar qualitative and quantitative composition between different batches.

2.3. Quantitative polymerase chain reaction (Q-PCR) analysis of lineage associated genes

Q-PCR was used to measure quantitatively the effects of non-selective conventional growth condition (GM), of specific differentiation-inducing media and of EMD on the levels of lineage-associated genes expressed by the HPCs. Total RNA was isolated from HPCs using the RNeasy Mini Kit (Qiagen, Crawley, UK), after culture in GM and in several DM each specific for a different cell lineage, as described below. For reverse transcription first-strand cDNA was synthesized using 1 μg of total RNA, as previously described [31], with primers obtained from Applied Biosystems (Applied Biosystems, Foster City, CA). The following genes were analyzed as key "markers" of the different lineages which have previously been reported to be expressed by precursor cells at "early" and "late" stages of their differentiation [62]: the osteogenic-associated genes, Runt-related transcription factor 2 (Runx2) (early), alkaline phosphatase (ALP) (early), osteopontin (OP) (early) and osteocalcin (OC) (late) (another late osteogenic marker, bone sialoprotein (BSP), was not included because of practical limits of time and cost); the adipogenic genes peroxisome proliferator activator receptor γ2 (PPARγ2) (early) and lipoprotein lipase (LPL) (late); the chondrogenic genes Sox-9 (early) and aggrecan (late); the vasculogenic/angiogenic genes angiopoeitin-1 (Ang-1) (early) and von Willeband Factor (vWF) (late); the neuronal differentiation marker microtubule-associated protein (MAP2) (late) and glial-associated marker glial fibril acidic protein (GFAP) (late); and a housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Q-PCR analysis was carried out using an ABI Prism® 7300 sequence detector (Applied Biosystems), the Taqman® Gene Expression Assay consisting of the unlabelled specific PCR primers and Taqman® MGB probes with FAM™ dye labelling in a 96-well plate format. Thermal cycler conditions were used as recommended by the manufacturer and the data were collected and analyzed by the SDS 1.2 software (Applied Biosystems). All PCR reactions were performed in triplicate and each of the gene cycle threshold (ct) values were normalized to the GAPDH ct value detected simultaneously on the same plate.

2.4. Differentiation of HPCs

2.4.1. Osteogenic differentiation

HPCs were seeded into 24-well plates at a density of 2.5×10^4 cells $well^{-1}$ and cultured in GM for 2–3 days, then osteogenic medium (OM) added, consisting of GM supplemented with 0.1 mM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate and 10 nM dexamethasone (all from Sigma). EMD was added to half of the cultures and the OM and EMD changed every 3–4 days. On days 7 and 10, total RNA was extracted and Q-PCR carried out to measure the "early" marker genes Runx2, ALP and OP gene expression (on day 7) and the "late" marker gene OC on day 10, as previously described [31].

The formation of mineralized nodules, a characteristic feature of terminal osteogenic differentiation, was determined by Alizarin Red S staining of calcium-containing deposits, as follows. Cells were cultured in OM with and without EMD for 21 days, fixed with 10% formaldehyde for 15 min and washed with distilled water. The samples were incubated with 2% Alizarin Red S (pH 4.2) (Sigma) for 15 min at room temperature, then washed, air-dried and photographed. The level of Alizarin Red staining was quantified by absorbance at 562 nm (A_{562}), after eluting the stain for 2 h with 10% cetylpyridinium chloride in 10 nM sodium phosphate buffer, pH 7.0.