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Different effects of 25-kDa amelogenin on the proliferation, attachment and migration of various periodontal cells

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ABSTRACT

Previous studies have assumed that amelogenin is responsible for the therapeutic effect of the enamel matrix derivative (EMD) in periodontal tissue healing and regeneration. However, it is difficult to confirm this hypothesis because both the EMD and the amelogenins are complex mixtures of multiple proteins. Further adding to the difficulties is the fact that periodontal tissue regeneration involves various types of cells and a sequence of associated cellular events including the attachment, migration and proliferation of various cells. In this study, we investigated the potential effect of a 25-kDa recombinant porcine amelogenin (rPAm) on primarily cultured periodontal ligament fibroblasts (PDLF), gingival fibroblasts (GF) and gingival epithelial cells (GEC). The cells were treated with 25-kDa recombinant porcine amelogenin at a concentration of 10 μg/mL. We found that rPAm significantly promoted the proliferation and migration of PDLF, but not their adhesion. Similarly, the proliferation and adhesion of GF were significantly enhanced by treatment with rPAm, while migration was greatly inhibited. Interestingly, this recombinant protein inhibited the growth rate, cell adhesion and migration of GEC. These data suggest that rPAm may play an essential role in periodontal regeneration through the activation of periodontal fibroblasts and inhibition of the cellular behaviors of gingival epithelial cells.

1. Introduction

Many case reports and clinical studies have reported the therapeutic effect of EMD in periodontal tissue healing and regeneration. The amelogenin protein family comprises approximately 90% of the EMD and numerous research groups have proposed that amelogenin isoforms are the main effective components of EMD [2].

Amelogenin isoforms are derived from a single gene by alternative splicing or post-secretory degradation of full-length amelogenin (25 kDa), which gives rise to a range of heterogeneous hydrophobic proteins and peptides [3]. After secretion, the original 25-kDa amelogenin proteins are rapidly cleaved into 20-kDa fragments and other low molecular weight amelogenins by proteolysis, which subsequently migrate to the inner layer of the secretory enamel. It is assumed that at different stages of periodontal tissue development, the various isoforms of amelogenin have distinct activities [4,5]. However, the precise functions of the amelogenin peptides derived from the splice variants and degradation products on the regeneration of periodontal tissues have not yet been clearly determined.

Periodontal tissue regeneration is a complex process that involves several cell types and depends on a sequence of associated cellular events including cell attachment, migration and proliferation [6]. It has been previously demonstrated that Emdogain®, a commonly used device containing low molecular weight amelogenin peptides [1], can successfully enhance the proliferation and migration of PDLF and GF, and induce bone turnover and regeneration [3]. The 25-kDa isoform of amelogenin has been reported to be more important than the various amelogenin cleaved products in the directed growth of enamel crystals [7,8]. However, the mechanism by which the native or recombinant full-length 25-kDa amelogenin isoform modulates cellular activity of periodontium-related cells, especially GEC, is largely unexplored.

In this study, we investigated the effects of a 25-kDa amelogenin on cellular attachment, proliferation and migration of cultured primary human PDLF, GF and GEC.

2. Materials and methods

2.1. The expression and purification of a 25-kDa recombinant porcine amelogenin

The expression and purification of a 25-kDa recombinant porcine amelogenin (rPAm) was performed according to previously

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described protocols [9]. Following the manufacturer's protocol, RNA was extracted from the dental germ of a nascent pig using Trizol reagent (Invitrogen, Life Technologies Corporation, California, USA). The cDNA fragment of the porcine amelogenin gene was obtained from total RNA by RT-PCR. This fragment was inserted into pGEX4T1 (Novagen, Madison, WI) to express glutathione-*S* transferase (GST) fusion amelogenin protein. After confirming the right sequence, the resulting plasmid was transformed into *Escherichia coli* BL21. The GST-tagged recombinant protein was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) and then purified using a GSTrap 4B purification system (GE Healthcare Bio-Sciences, Uppsala, Sweden) according to the manufacturer's instructions. The purified recombinant protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie brilliant blue (CBB) staining or Western blotting and liquid chromatography–mass spectrometry (LC–MS) as described below.

2.2. Porcine EMD preparation

Porcine EMD was isolated from un-erupted fourth and fifth mandibular molars of 6-month-old pig jaws obtained fresh from a local slaughterhouse. The pooled scrapings were dissolved in 10% acetic acid and natural porcine EMD was extracted according to a previously reported procedure [10,11].

2.3. SDS–PAGE and Western blot analysis

The protein concentrations of the samples were determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratory, Hercules, CA, USA). Fractions of proteins were denatured in SDS sample buffer and subjected to a 15% SDS–PAGE. The resulting gels were stained with SimplyBlue™ SafeStain (Invitrogen, Life Technologies Corporation, California, USA). The molecular weights of the proteins were estimated by comparison with PageRuler® Prestained Protein Ladder markers (Fermentas International, Inc., CAN). Duplicate SDS–PAGE gels were transblotted onto a polyvinylidene difluoride (PVDF) membrane (0.45 μm, Millipore, Bedford, MA). The membranes were blocked with 5% skim milk in Tween Tris-buffered saline (TTBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM K_2HPO_4, 0.1% Tween-20) at room temperature on a shaker for 1 h. The blocked membranes were incubated with goat polyclonal antibody against the C-terminus of amelogenin (Santa Cruz Biotechnology, Inc., CA, USA), diluted to 1:200 with TTBS containing 5% skim milk overnight. After washing with TTBS, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare Bio-Sciences, Uppsala, Sweden) at a dilution of 1:3000 for 1 h. ECL Plus Western Blotting detection reagent (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used for the detection of immunoreactive products.

2.4. Liquid chromatography–mass spectrometry (LC–MS)

After expression and purification, rPAm was subjected to LC–MS analysis. The samples were fractionated on a CTI C8 reverse-phase column (Column Technology, Inc., USA) at a flow rate of 50 mL/min, and eluted with a gradient of water/acetonitrile/trifluoroacetic acid on an Agilent HPLC 1100. The fractions were characterized on a Platform II mass spectrometer with the use of an electrospray source, and the data were analyzed using MassLynx (Fisons Instruments, Manchester, UK).

2.5. Cell culture

Human PDLF cells were isolated and cultured following previously published procedures [12]. Briefly, clinically healthy premolar teeth or third molars that were extracted for orthodontic reasons were washed several times with phosphate buffered saline (PBS). The periodontal ligament fragments were curetted from the middle third of the roots using a scalpel. After washing, the fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 23 mM $NaHCO_3$ (Gibco Biocult, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Biocult, Paisley, UK) and antibiotics (50 μg/mL of streptomycin sulfate, 100 U/mL of penicillin). When the cells surrounding the different explants were confluent, they were harvested with 0.25% trypsin (Gibco BRL, California, USA) and 0.1% ethylenediaminetetraacetic acid (EDTA, Gibco BRL, California, USA) in PBS and used for the second culture.

Both GF and GEC were obtained from healthy gingival tissue isolated from clinically healthy individuals at the time of the third molar extraction. GEC were isolated and cultured according to the method described by Oda et al. [13]. In brief, after overnight incubation of gingival tissue with dispase, surface epithelium was removed from the underlying connective tissue and then trypsinized to prepare a single cell suspension. The cells were pelleted by centrifugation, collected and resuspended in keratinocyte serum-free medium (KSFM, Gibco Biocult, Paisley, UK) to obtain the GEC. The GEC were maintained in KSFM during *in vitro* cultivation. The remaining connective tissue was washed with PBS and cut into small pieces (<2 mm), which were placed in culture plates and maintained in DMEM as described above. After 8–10 days, the confluent monolayer of cells surrounding the tissue explants were trypsinized and re-seeded.

All the cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every 2–4 days. In this study, fibroblasts between the 3rd and 5th passage, and GEC between the 2nd and 3rd passage were used for all the experiments. An informed consent was obtained from each patient prior to participation.

2.6. Cell attachment analysis

Rat tail tendon type I collagen was purified according to the previously established procedures [14,15].

For cell attachment assays, 24-well culture plates (Corning, Inc., NY, USA) were first coated with 10 μg/mL rPAm. Rat tail tendon type I collagen (2 μg/cm^2) was used as a positive control and the plates were incubated overnight at 4 °C. Before seeding the cells, PDLF and GF were cultured in the presence of serum-free medium for 12 h. After the culture plates were washed with PBS 3 times, PDLF and GF (2×10^4 cells per well) were incubated for 30, 60, 120, 180 and 240 min, whereas GEC were incubated for 4, 8, 12, 16 and 20 h. At each time point, unattached cells were gently removed with PBS. The washing solution was centrifuged and the mean number of unattached cells was counted using a Z2™ Coulter Counter® (Beckman Coulter, Inc., CA, USA) (data not showed). The attached cells were harvested from the plates using trypsin and cell counts were performed as described above.

2.7. Cell proliferation assay

Prior to adding the cells, 24-well culture plates were coated with rPAm at a concentration of 10 μg/mL, incubated overnight at 4 °C and then washed with culture medium 3 times. PDLF and GF were seeded on the coated dishes at a density of 1.2×10^4 cells per well and cultured in DMEM containing 10% FBS. After the cells had adhered to the plate and spread out over 24 h, the fibroblasts were washed with PBS and then cultured in fresh serum-free DMEM for an additional 24 h prior to treatment with 10 μg/mL rPAm. For GEC, the culture medium KSFM was removed and 10 μg/mL of recombinant amelogenin was added to the plates.