Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/aob

Inhibitory effects of ameloblastin on epithelial cell proliferation

Noriko Saito [a,b], Wataru Ariyoshi [a,*], Toshinori Okinaga [a], Mariko Kamegawa [c], Miho Matsukizono [c], Yasuo Akebiyama [c], Chiaki Kitamura [b], Tatsuji Nishihara [a]

[a] Division of Infections and Molecular Biology, Department of Health Promotion, Kyushu Dental University, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu, Fukuoka 803-8580, Japan
[b] Division of Pulp Biology, Operative Dentistry and Endodontics, Department of Cariology and Periodontology, Kyushu Dental University, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu, Fukuoka 803-8580, Japan
[c] Genenet Co., Ltd, 5-22-8, Tanotsu, Higashi-Ku, Fukuoka 813-0034, Japan

ARTICLE INFO

Article history:
Accepted 5 May 2014

Keywords:
Ameloblastin
Protein purification
Periodontitis
Epithelial cell proliferation
Cell cycle
Apoptosis

ABSTRACT

Objective: Ameloblastin is an enamel matrix protein expressed in several tissues. Many potential mechanisms have been identified by which ameloblastin functions as an extracellular matrix protein. However, the biological effects of ameloblastin on gingival epithelial cells remain unclear. In the present study, we established a novel system to purify recombinant human ameloblastin and clarified its biological functions in epithelial cells *in vitro*.

Design: Recombinant human ameloblastin was isolated from COS-7 cells overexpressing HaloTag®-fused human ameloblastin by the HaloTag® system and then purified further by reverse-phase high-performance liquid chromatography. SCC-25 cells, derived from human oral squamous cell carcinoma, were treated with recombinant ameloblastin and then cell survival was assessed by a WST-1 assay. Cell cycle analysis was performed by flow cytometry.

Results: The novel purification system allowed effective recovery of the recombinant ameloblastin proteins at a high purity. Recombinant ameloblastin protein was found to suppress the proliferation of SCC-25 cells. Flow cytometric analysis showed that ameloblastin treatment induced cell cycle arrest G1 phase.

Conclusions: We developed a procedure for production of highly purified recombinant human ameloblastin. Biological analyses suggest that ameloblastin induces cell cycle arrest in epithelial cells and regulates the progression of periodontitis.

1. Introduction

Periodontitis is one of the most common chronic inflammatory diseases characterized by the destruction of tooth-supporting structures.[1] It is a complex disease resulting from the combination of the direct effects of microbial virulence factors and the host response to microbial challenge.[2,3] Although numerous microbial aetiologies have been reported for periodontitis, its pathogenesis remains to be elucidated because of the complexity of host–microbial interactions.

* *Corresponding author*. Tel.: +81 93 285 3052; fax: +81 93 581 4984.
E-mail address: arikichi@kyu-dent.ac.jp (W. Ariyoshi).
http://dx.doi.org/10.1016/j.archoralbio.2014.05.010
0003–9969/

The gingiva is covered by a stratified squamous epithelium that constantly receives local stimuli. The gingival epithelium can serve as the first line of defense against bacterial invasion.[4] In addition to its function as a physical and chemical barrier, the gingival epithelium plays a crucial role in the immune response against infectious inflammation in periodontal tissue by expression of a large variety of cytokines and antimicrobial peptides.[5–8] Therefore, the gingival epithelium plays a critical role in maintaining mucosal homeostasis, and the loss of the epithelial barrier function is thought to contribute towards periodontitis progression.

Ameloblastin, a matrix adhesion protein also known as sheathlin and amelin, was first detected in secretory stage ameloblasts.[9,10] It is also expressed by osteoblasts,[11] cementoblasts,[12] and epithelial rests of Malassez in the periodontal ligament.[13] Ameloblastin contains a fibronectin interaction sites,[14] several heparin-binding domains,[15,16] a potential $\alpha 2\beta 1$ integrin-binding domain, and a thrombospondin cell adhesion motif,[17] which might be responsible for the interaction of ameloblastin with the surface of dental epithelial cells.

Ameloblastin was initially reported to involved in the regulation of ameloblasts.[18] Many potential mechanisms have been identified by which ameloblastin functions as an extracellular matrix protein, including the regulation of enamel biomineralisation[19–21] and osteoblast differentiation.[22] Furthermore, recent studies have suggested that ameloblastin may act as a signalling molecule[23,24] and possesses growth factor activity.[25]

In our previous study, we purified bioactive fractions from enamel matrix derivative (EMD) and revealed that ameloblastin is a candidate component that inhibits epithelial cell proliferation (unpublished data). However, the detailed mechanism by which ameloblastin inhibits epithelial cell proliferation has not because of the difficulty of discriminating the responses induced by ameloblastin from those caused by other proteins that often contaminate ameloblastin preparations. In the present study, we purified recombinant ameloblastin protein using a novel fusion tag system and clarified its biological functions in epithelial cells *in vitro*.

2. Materials and methods

2.1. *Cell culture*

SCC-25 cells, derived from human squamous cell carcinoma of the tongue, were obtained from DS Pharmaceutical (Osaka, Japan) and maintained in a 1:1 mix of Dulbecco's modified Eagle's medium and Ham's F-12 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, USA), penicillin G (100 U/ml) and streptomycin (100 μg/ml).

2.2. *Expression and purification of recombinant ameloblastin*

The expression vector pFNA21A (FHC21950M; Promega KK, Tokyo, Japan) was used to express HaloTag®-fused human ameloblastin protein. Expression plasmids were transfected into COS-7 cells by electroporation using a NEPA21 Super Electroporator (Nepa Gene, Chiba, Japan). After 24 h, the transfected cells were lysed using a lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, and 0.1% sodium deoxycholate, pH 7.5). HaloTag® fusion recombinant protein was then partially purified by the HaloTag® Mammalian Protein Purification System (Promega KK) according to the manufacturer's instructions. The isolated fraction was lyophilised and then the recombinant ameloblastin was further purified by reverse-phase high-performance liquid chromatography (HPLC) using a Waters system (Medford, MA, USA) and C_{18} column (4.6 × 150 mm; Vydac, Hesperia, CA, USA) equilibrated with 0.1% trifluoroacetic acid. The fraction with one major peak was collected, diluted in culture medium, sterilised using a surfactant cellulose acetate membrane filter (0.20 μm pore size; Corning, NY, USA), and then used as purified recombinant ameloblastin in bioassays. Protein concentrations were measured using a DC™ protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. *Preparation of a monoclonal antibody (mAb) against human ameloblastin*

Animal immunisation was performed by a standard procedure using recombinant ameloblastin as the immunogen. Ameloblastin (1.0 ml, 15 μg) was emulsified in an equal volume of Freund''s complete adjuvant (Difco Laboratories, Detroit, MI, USA) and then injected intraperitoneally into 7-week-old female Balb/c mice (Charles River Laboratories Japan, Yokohama, Japan). Then, at about 2-week intervals, Balb/c mice were administered with a booster injection of ameloblastin prepared in the same manner. After the second immunisation, the titre of antiserum was tested by an enzyme linked immunosorbent assay (ELISA).

Recombinant ameloblastin was diluted in coating buffer (50 mM $NaHCO_3$) to 1 μg/ml, added to the micro-titre plates (50 μl/well) as the coating antigen, and then incubated overnight at 4 °C. Plates coated with recombinant ameloblastin were washed three times with PBST (PBS containing 0.05% Tween-20) and then blocked with 1% Block Ace (DS Pharma Biomedical, Osaka, Japan) at room temperature for 2 h. After three washes with PBST, serial dilutions of antiserum (50 μl/well) were added to the plates, followed by incubation at room temperature for 2 h. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBST was added to the reaction wells, followed by incubation at room temperature for 30 min. After five washes with PBST, SigmaFast OPD tablet set (Sigma–Aldrich, St. Louis, MO, USA) was added to the wells for colour development, and then 10% H_2SO_4 was added to stop the reaction. The absorbance was measured at 490 nm by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

The immunised mouse with a high serum titre was given an intravenous injection with of 5 μg ameloblastin. After 4 days, the B cells were isolated from spleen and fused with P3U1 myeloma cells at a ratio of 1:5 by polyethylene glycol 1500. Then, the cells were cultured in 96-well plates containing HAT medium at 37 °C with 5% CO_2. After about 10 days, the HAT medium was changed to HT medium. After 2 days, the culture supernatants were tested by ELISA and positive clones