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Amelogenin binds to both heparan sulfate and bone morphogenetic protein 2 and pharmacologically suppresses the effect of noggin

Kan Saito, Ikuri Konishi, Miyuki Nishiguchi, Tomonori Hoshino *, Taku Fujiwara

Division of Pediatric Dentistry, Nagasaki University Graduate School of Biomedical Sciences, 1–7–1 Sakamoto, Nagasaki 852–8588, Japan

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ABSTRACT

Enamel matrix derivative (EMD) is widely considered useful to promote tissue regeneration during periodontal treatment. It has been reported that the main constituent of EMD is amelogenin and that the BMP-like and TGF-β-like activity of EMD promotes osteogenesis. However, it remains unclear whether those activities are dependent on amelogenin or another growth factor contained in EMD. We performed two-dimensional SDS-PAGE analysis of EMD, as well as Western blot analyses using anti-amelogenin, anti-BMP2/4, and anti-TGF-β1 antibodies, and amino acid sequencing. Our results revealed that a large number of splicing forms of amelogenin, BMP2/4, and other unknown molecules were involved in EMD, though TGF-β1 was not. In addition, we have evaluated intracellular signaling of ERK1/2 and Smad1/5/8, binding potential and alkaline phosphatase activity and have explored the potential regulatory relationship between amelogenin and BMP. Amelogenin bound to BMP2 as well as heparin/heparan sulfate. Thus, it was suggested that BMP2/4 carried over in EMD during processing promote binding activity and phosphorylate Smad1/5/8 in osteoblasts. On the other hand, amelogenin did not phosphorylate Smad1/5/8, but rather ERK1/2. Further, high-density amelogenin reduced the inhibition of alkaline phosphatase activity by noggin, though amelogenin did not have antagonistic properties against BMP. Together with the above findings, our findings suggest that the BMP2/4 contaminated during the purification process of EMD because of the avidity of amelogenin plays an important role in signaling pathway of calcification.

Introduction

Enamel matrix derivative (EMD) is derived from developing porcine teeth. A recent clinical review reported that EMD promotes both cementogenesis [1] and osteogenesis [2], while it has also been shown that EMD has bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) activities [3,4], while *in vitro* experiments demonstrated that it stimulates osteoblast proliferation and differentiation [5,6]. EMD consists of amelogenin at nearly 90%, along with other enamel matrix proteins, such as enamelin, tuftelin, amelin, and ameloblastin [7,8]. Amelogenin is expressed in a tissue-specific manner by ameloblast, of which the origin is ectoderm. Immature enamel contains a complex mixture of amelogenin polypeptides, primarily due to the combined effects of alternative splicing [9,10]. Numerous mutations have been found in the genes encoding amelogenin in patients with amelogenesis imperfecta, the most common genetic disorder affecting enamel [11,12]. Thus, it is thought that amelogenin plays a crucial role in enamel formation. On the other hand, though there is no known clinical report showing that amelogenin causes bone diseases, *in vitro* experiment results have provided some indications of its function in bone formation. For example, leucine-rich amelogenin peptide (LRAP) is expressed in cementoblasts/periodontal ligament cells and regulates osteoclastogenesis [13], and was shown to down-regulate osteocalcin, a marker of bone turnover [14,15]. In another study, amelogenin decreased the levels of RANKL, M-CSF, and fibronectin in osteoblasts [16]. However, it remains unknown whether the activity of amelogenin is equal to that of EMD in bone formation. In the present study, we investigated EMD using two-dimensional SDS-PAGE assays and Western blot analyses to determine the differences between EMD and amelogenin. Our findings revealed that BMP2/4 contaminated EMD during processing. We also examined ERK1/2 and Smad1/5/8 intracellular signaling, the binding properties of amelogenin for BMP2, heparin, and heparan sulfate, and the alteration of alkaline phosphatase activity by amelogenin during calcification, to determine the relationship between amelogenin and BMP.

Materials and methods

Two-dimensional SDS-PAGE (2-D PAGE) and Western blot analyses

The commercial enamel matrix derivative Emdogain®, extracted from developing porcine teeth, was purchased from Seikagaku-kougyou Corporation (Tokyo, Japan) and used in the experiments. Approximately 5 μg of protein was added to 155 μL of sample rehydration buffer and absorbed overnight onto 7-cm nonlinear immobilized pH gradient (IPG, pH 3–10) ZOOM strips (Invitrogen Corp., Carlsbad, CA). Isoelectric focusing was carried out using a ZOOM® IPG Runner system (Invitrogen) and a MAJOR SCIENCE MP-3500/250P power supply (Invitrogen) with the following voltage step protocol: 200 V for 15 min, 450 V for 15 min, 750 V for 15 min, and 2000 V for 60 min. For the second dimension, focused IPG strips were equilibrated in NuPAGE® LDS sample buffer

* Corresponding author. Fax: +81 95 819 7676.
E-mail address: thoshino@nagasaki-u.ac.jp (T. Hoshino).

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(Invitrogen) in the presence of NuPAGE® Sample Reducing Agent (Invitrogen) for 15 min, and then further incubated in LDS sample buffer in the presence of 125 mM iodoacetamide for 15 min. Next, the strips were placed on NuPAGE® 4–12% Bis–Tris gels (Invitrogen) and embedded in 0.5% agarose (wt/vol).

Coomassie brilliant blue (CBB) staining of the gels was performed using SimplyBlue SafeStain™ (Invitrogen). For immunoblotting, proteins were separated using 2-D PAGE and transferred to 0.2-μm polyvinylidene fluoride (PVDF) membranes (Invitrogen) for 30 min at a constant 200 V. After blocking with 5% nonfat dry milk and 0.2% Tween 20 in Tris borate saline (TBS) at 4 °C overnight, the membranes were incubated with rabbit anti-amelogenin (anti-AMEL; HOKUDO CO., LTD, Sapporo, Japan), goat anti-BMP2/4 (R&D systems, Minneapolis, MN), or mouse anti-human TGF-β1 (R&D systems) antibodies in TBS containing 1% bovine serum albumin for 1 h at room temperature. The antibodies were used at a dilution of 1:1000. The membranes were washed 5 times with TBS containing 0.2% Tween and then incubated with secondary antibodies at a dilution of 1:3000 in TBS with 1% bovine serum albumin for 1 h at room temperature. The membranes were then washed 5 times with TBS and signals were detected using an ECL kit (Amersham Pharmacia Biosciences, Uppsala, Sweden).

Analyses of 2-D gel and Western blotting results

CBB-stained gels were scanned at 500 dpi and the images analyzed using Progenesis PG200 Software (Nonlinear Dynamics, Newcastle upon Tyne, UK) to determine the number, molecular weight, isoelectric point, and relative volume ratio of the molecules in EMD. After normalization based on total spot density was performed, the relative volume of individual spots was calculated and quantified by the intensity of staining. After Western blotting images were also scanned at 500 dpi, the signals on the ECL film were merged and calibrated to the spots on gels stained with SimplyBlue SafeStain™.

Preparation of recombinant mouse amelogenin

To construct a plasmid expressing amelogenin, cDNA was amplified by PCR. The amplified DNA fragment was cloned into the BamHI–XhoI region of pET22b (+) (Novagen, Darmstadt, Germany), which allowed the expressed amelogenin protein to fuse to the poly-histidine tag at the C-terminus. Recombinant amelogenin (rAMEL) was expressed as an insoluble inclusion body in *Escherichia coli* BL21 harboring the plasmid by treatment with isopropylthio-β-D-thiogalactoside, and was solubilized in 20 mM sodium phosphate buffer (NaPB) containing 0.5 M NaCl and 6 M guanidine hydrochloride. The solubilized rAMEL was purified by Ni^{2+}-chelate affinity chromatography using a ProBond™ resin column (Invitrogen) according to the manufacturer's instructions. Briefly, crude rAMEL was applied to the column and eluted with 20 mM NaPB containing 8 M urea and 0.1% Triton X-100. The purified fractions were dialyzed against 20 mM NaPB (pH 4.0) containing 0.1% Triton X-100 to remove the urea. Removal of lipopolysaccharide (LPS) was accomplished by Triton X-114 (SIGMA, St. Louis, MO) [17]. The rAMEL used in this study contained less than 1 pg of LPS per 1 μg of protein.

Cell culture and signaling assay

The mouse osteoblast cell line MC3T3-E1 was obtained from RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained in α-MEM (SIGMA) containing 10% fetal bovine serum (FBS; Invitrogen) and 100 U/mL of penicillin-G at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cultured MC3T3-E1 cells were treated with 5 μg/mL of EMD, 5 μg/mL of rAMEL and 100 ng/mL of recombinant BMP2 (Genzyme/Techne, Cambridge, MA) for 0 to 60 min. Treated cells were washed with phosphate buffered saline (PBS) containing 1 mM Na_3VO_4, and then solubilized in 200 μL of lysis buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 units/mL aprotinin]. The lysed cell solution was centrifuged at 12,000× *g* for 10 min and the supernatants recovered were used as samples to assay cell signaling. The protein concentration of each sample was measured using Lowry's method. After the samples were separated on NuPAGE® 4–12% Bis–Tris gels (Invitrogen), the gels were transferred onto PVDF membranes. To assay cell signaling during phosphorylation, the membranes were analyzed by Western blotting using anti-phospho ERK1/2 (p-ERK1/2), anti-ERK1/2, anti-phospho Smad1/5/8 (p-Smad1/5/8), and Smad5 antibodies (Cell Signaling Technology, Beverly, MA).

Amino acid sequence of characteristic spots on 2-D gels

Separated EMD samples on 2-D gels were blotted onto PVDF membranes. Then, characteristic spots on the 2-D gels were excised from the blotted membranes and

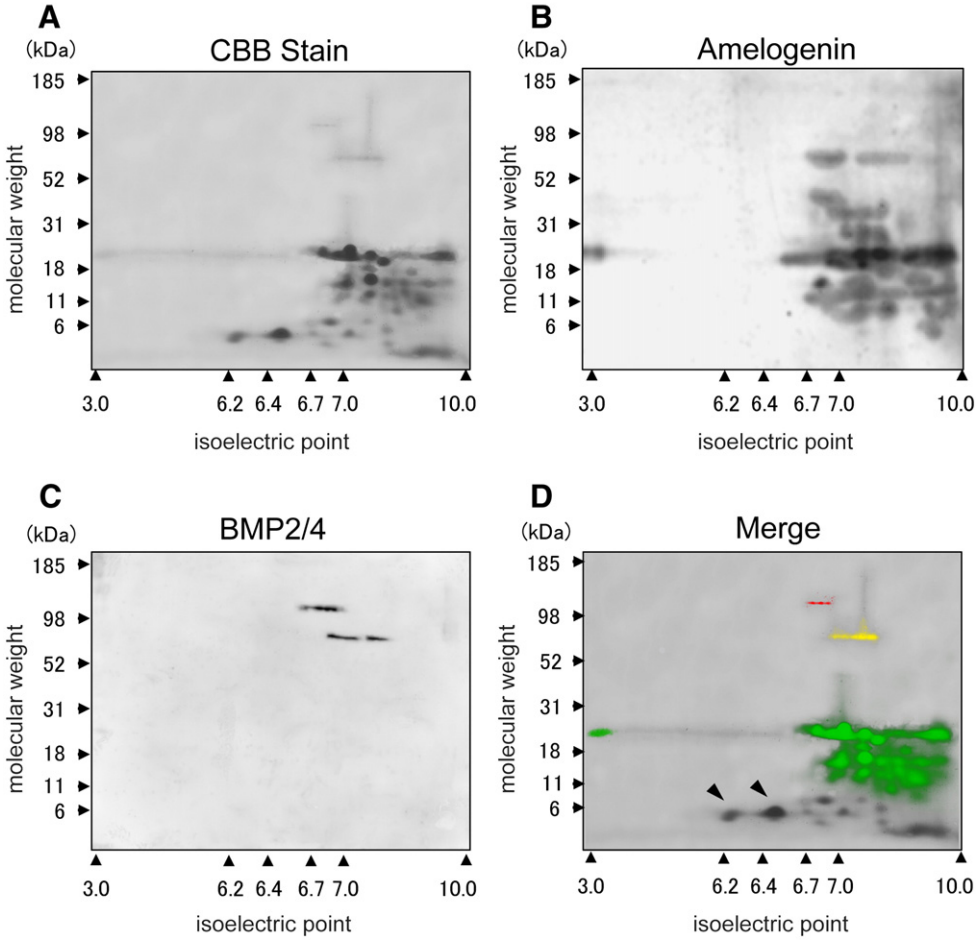

Fig. 1. Two-dimensional electrophoresis analysis of EMD. CBB staining of EMD separated on 2-D PAGE gel (A). The gel was transferred to a PVDF membrane and immunostained with anti-AMEL (B) and anti-BMP2/4 (C) antibodies. The images were matched using Progenesis PG200 software. Merged image (D) shows *BMP2/4* (*red*) and *amelogenin* (*green*), with overlapping regions shown in yellow.