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Amelogenins in Human Developing and Mature Dental Pulp

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Abstract

Amelogenins are a group of heterogenous proteins first identified in developing tooth enamel and reported to be present in odontoblasts. The objective of this study was to elucidate the expression and function of amelogenins in the human dentin-pulp complex. Developing human tooth buds were immunostained for amelogenin, and mRNA was detected by *in situ* hybridization. The effects of recombinant amelogenins on pulp and papilla cell proliferation were measured by Brd U immunoassay, and differentiation was monitored by alkaline phosphatase expression. Amelogenin protein was found in the forming dentin matrix, and amelogenin mRNA was localized in the dentin, presumably in the odontoblast processes. Proliferation of papilla cells was enhanced by recombinant human amelogenin rH72 (LRAP+ exon 4), while pulp cells responded to both rH72 and rH58 (LRAP), with no effect by rH174. These studies suggest that odontoblasts actively synthesize and secrete amelogenin protein during human tooth development, and that low-molecular-weight amelogenins can enhance pulp cell proliferation.

Keywords

amelogenin; alternative splicing; proliferation; dental papilla cells; dental pulp cells; odontoblasts; dentin

Introduction

In developing tooth enamel, amelogenins are the principal matrix proteins, comprising more than 90% of the extracellular matrix proteins in the secretory stage of enamel formation. Amelogenins are characterized by heterogeneity, which results in part from alternative splicing of mRNA (Den Besten and Li, 1992; Bonass *et al.*, 1994; Simmer *et al.*, 1994). Alternative splicing of amelogenins generates a group of protein variants whose proportions change during tooth formation (Yuan *et al.*, 1996).

Several early studies, using immunohistochemistry, identified amelogenins in endocytotic vesicles and lysosomes within odontoblasts before mineralization of mantle dentin in the mouse (Nakamura *et al.*, 1994), along the cell surfaces and processes of odontoblasts (Sawada and Nanci, 1995), and in young odontoblasts of the hamster (Karg *et al.*, 1997). Recently, amelogenin mRNA transcripts have been amplified from mouse dental mesenchyme and immortalized odontoblast-like cells (Papagerakis *et al.*, 2003) and porcine odontoblasts (Nagano *et al.*, 2003). Studies of rodent teeth by *in situ* hybridization failed to find amelogenin

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transcripts in odontoblasts (Karg *et al.*, 1997; Torres-Quintana *et al.*, 2005), suggesting that transcripts may be present at very low levels.

The isolation and identification of low-molecular-weight amelogenins in rat dentin, as a chondrogenic stimulating factor, brought rapid attention to the potential role of amelogenins in mesenchymal cell signaling (Veis *et al.*, 2000). The expression pattern of the alternatively spliced amelogenins in the human dentin-pulp complex has not yet been reported, and the role of amelogenins in human dentin formation and dental pulp repair remains unclear. The objective of this study was to elucidate the expression and function of amelogenins in the human dentin-pulp complex.

Materials & Methods

Immunohistochemical Localization of Amelogenin

Developing human tooth buds were obtained from approximately 22-week-old fetal tissue through the tissue-sharing program within the University of California at San Francisco, CA, USA, following approval by the institutional review board. The teeth were frozen and cryo-sectioned. An adult non-carious premolar, obtained following approval by the institutional review board and informed consent, was fixed by immersion in 10% neutral formalin for 24 hrs, decalcified in 17% buffered EDTA, and processed routinely for paraffin embedding. Sections were blocked with 10% fetal bovine serum (FBS) and 0.1% Triton X-100, and immunostained with anti-amelogenin antibody raised from recombinant H174 (Li *et al.*, 2001), or pre-immune rabbit serum (1:200) diluted in the same blocking solution. All sections were labeled by the secondary antibody, anti-rabbit IgG-Alexfluor594 (Sigma, St. Louis, MO, USA). Nuclei were counter-stained with 0.5 µg/mL Hoechst 33324 (Invitrogen, Carlsbad, CA, USA).

In situ Hybridization of Amelogenin mRNA

Incisor tooth buds were fixed in 4% paraformaldehyde and processed for *in situ* hybridization as previously described (Albrecht *et al.*, 1997), with ³⁵S-labeled human amelogenin and type I collagen riboprobes. We prepared the amelogenin probe by amplifying full-length amelogenin cDNA from a human ameloblast cDNA library. Sections were counter-stained with a nuclear stain (Hoechst Stain; Sigma, USA). Hybridization signals were detected by dark-field optics, and the nuclear stain was visualized by epifluorescence.

Identification of Alternatively Spliced Amelogenins in Papilla and Pulp Cells *in vitro*

A dental papilla was dissected from a 23-week-old fetal tooth bud. The papilla was further digested with 4 mg/mL collagenase/dispase (Cell Culture Facility, UCSF, San Francisco, CA, USA) for 1 hr at 37°C, followed by digestion with 0.05% trypsin for 5 min. Cells were plated at 2×10^5 cells/plate on 10-cm dishes (Primaria, Falcon, Franklin Lakes, NJ, USA) in alpha-modified Eagle's Medium (Cell Culture Facility), supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO₂. Adult pulp cells were obtained from adult human pulp tissues as previously described (Gronthos *et al.*, 2000).

Both papilla and dental pulp cells were grown on Lab-Tek chamber slides (Nalge Nunc Int., Rochester, NY, USA), fixed in 95% methanol and 5% acetic acid for 30 min at -20°C, and immunostained by, first, incubation with anti-amelogenin antibody (1:1000), followed by anti-rabbit IgG-FITC antibody (Sigma).

Alternatively spliced amelogenin mRNA expressed by these cells was identified by reverse-transcription of 2 µg total RNA of cells (extracted with use of an RNAase Mini kit [Qiagen,

Valencia, CA, USA] with SSR reverse-transcriptase [Invitrogen]). Two primers—Amg U01 (5'-TG GGGACCTGGATTTTATTTG-3') and Amg D02 (5'-CTCTTCCTCCCGCTTGGTC-3')—located at the 5' and 3' termini of amelogenin, were used to amplify the alternatively spliced amelogenins. A PCR reaction of 30 cycles, with annealing temperature of 55°C for 45 sec, was performed. The PCR products were separated on 1.5% agarose gels, sub-cloned into the TOPO-Blunt II vector (Invitrogen), and sequenced.

Cell Proliferation in the Presence of Alternatively Spliced Amelogenins

Human full-length amelogenin minus exon 4 (rH174) was prepared as previously described (Li *et al.*, 2003). Human LRAP+E4 (rH72) and LRAP (rH58) were prepared as described in Appendix 1. These proteins were purified by HPLC and confirmed by mass spectrometry (data not shown).

Cells were grown in clear-bottomed black 96-well plates (Falcon, USA) at a density of 2×10^3 cells/well until 60% confluence and serum-starved for 24 hrs. Recombinant human alternatively spliced amelogenins (rH174, rH72, and rH58) were added in triplicate in cell culture media at concentrations of 0 to 1000 ng/mL, without FBS. Cells were maintained in this medium for 24 hrs, and cell proliferation was measured with the use of an ELISA BrdU kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Statistical analysis between groups was performed by ANOVA with Dunnett's post-test analysis, with GraphPad Prism version 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA).

Cell-cycle Gene Superarray

A cell-cycle Gene SuperArray A GEArray Q series human cell-cycle gene array kit was obtained from SuperArray Inc. (Bethesda, MD, USA). Dental pulp cells were plated at a density of 2×10^5 cells/dish, cultured until 60% confluence, and serum-starved for 24 hrs. The cells were divided into two groups, an experimental group with 10 nM rH58, and a control group. After 24 hrs, total RNA was isolated with an RNeasy Mini Kit (Qiagen), and 3 µg total RNA was used as a template to generate Biotin-16-dUTP-labeled cDNA probes, which were hybridized to the SuperArray membrane according to the manufacturer's instructions (SuperArray Corp., <http://www.superarray.com>). Duplicated assays were analyzed with ScanAlyze software (shareware, <http://rana.lbl.gov/EisenSoftware.htm>), and the signal intensity from the membranes was compared with the GEarray analyzer program, as previously described (Liu *et al.*, 2004).

Cell Differentiation Assay

Cells were cultured and grown to confluence. Alternatively spliced amelogenins (10 nM)—rH174, rH72, and rH58—were added to the culture dishes for 48 hrs in triplicate assays. Relative levels of alkaline phosphatase (ALPase) and dentin sialoprotein (DSP) were determined by Western blots and RT-PCR, and compared with control, as described in Appendix 2.

Results

Amelogenins are Expressed in the Dental Papilla and Adult Dental Pulp

Immunostaining of a developing tooth organ showed amelogenin protein in the developing dentin in a tooth bud where enamel matrix formation had not yet been initiated (Fig. 1B). Similar diffuse staining of the odontoblast layer was found in the adult human dentin (results not shown).

Amelogenin mRNA was localized in the dentin matrix, and in the secretory ameloblasts (Fig. 1D). The pattern of amelogenin mRNA localization in the dentin matrix, opposing the unlabeled acellular enamel matrix, was in contrast to the Type I collagen-positive control (Fig. 1E) mRNA, which was localized in the odontoblast cell bodies lining the forming dentin.

Alternatively Spliced Patterns of Amelogenin in Human Tooth

PCR amplification of amelogenin mRNA from dental papilla cells generated 2 transcripts, H175 and H72. H175 lacked exon 4, whereas the splice variant H72 was the same as the leucine-rich amelogenin peptide (LRAP) plus exon 4 (LRAP+E4). Amplification of amelogenin from dental pulp cells resulted in 3 variants: H175, H72, and H58. H58 corresponded to the LRAP splice pattern.

Effects of Alternatively Spliced Amelogenins on Cell Proliferation

Dental papilla cell proliferation was enhanced with the addition of 100-500 ng/mL rH72 (Fig. 2B), but not rH58. Both rH72 and rH58 showed stimulatory effects on the proliferation of dental pulp cells at an optimal concentration of 200 ng/mL for rH58 and rH72 (Figs. 3A, 3B), while rH174 had no significant effects on the proliferation of these cells ($P > 0.05$) (data not shown).

Cell-cycle gene array with dental pulp cells showed that 3 genes—including cyclin-dependent kinase 6 (CDK6), cullin-4 (CUL4), and Neural Precursor Cell Expressed Developmentally Down-regulated Gene 8 (NEDD8)—were up-regulated two-fold by 10-nM rH58 (Fig. 3C).

Effects of Amelogenins on Cell Differentiation Characterized by ALPase and DSPP mRNA Expression

Dental-pulp-derived cells were positive for alkaline phosphatase (ALP) and dentin sialoprotein (DSP) at both mRNA and protein levels, whereas papilla cells were positive only for ALP. Addition of recombinant amelogenins into the culture media had no effect on the initial expression of either of these differentiation markers in either cell type (Fig. 4).

Discussion

Immunohistochemical localization of amelogenin at the interface of the inner enamel epithelium and the mesenchymal cells of the dental papilla is similar to reports from studies of the rat (Bronckers *et al.*, 1993; Janones *et al.*, 2005). Some previous studies have suggested that amelogenins secreted by developing ameloblast-lineage cells may diffuse into the predentin matrix (Uchida *et al.*, 1989; Inai *et al.*, 1991). However our studies, with both *in situ* hybridization and immunohistochemistry, indicated that the odontoblasts synthesized amelogenins and secreted these proteins at the epithelial/enamel interface.

It is interesting that amelogenin mRNA localization in the dentin layer containing the odontoblastic processes is different from Type I collagen mRNA localization to the odontoblast cell bodies. The localization of amelogenin mRNA by *in situ* hybridization differs from the negative results reported for rodent teeth (Karg *et al.*, 1997; Torres-Quintana *et al.*, 2005), possibly related to differences between rodents and humans, or to differences in sensitivity of the *in situ* hybridization assay. These results suggest that amelogenins may not directly interact with pulp cells during development, but may have a role in epithelial/mesenchymal interactions related to enamel and dentin formation.

The incisal end of the dental papilla adjacent to the amelogenin-positive epithelium contained more differentiated columnar, polarized cells, which was expected, since tooth development progresses with an incisal to apical gradient. These cells were amelogenin-immunopositive,

while the more apical ones were negative. A similar stage-specific expression of amelogenin was found in mouse molars, where amelogenin was expressed by young odontoblasts (Papagerakis *et al.*, 2003).

Amelogenin transcripts identified in our study were similar to those found in dentin tissues in other species: H175 and H58 are counterparts of P173 and P56, found in porcine odontoblasts (Nagano *et al.*, 2003), while H72 and H58 are identical with [A+4] and [A-4], found in the rat (Veis *et al.*, 2000). We did not find the human amelogenin transcript corresponding to the P41 in porcine odontoblasts, which could be species-specific, or H185 (including all of exon 1 to exon 7), previously reported (Salido *et al.*, 1992).

It is interesting that we found H72 (which contains exon 4) in cells from both developing and mature pulp. mRNA splice forms of amelogenin containing exon 4 have been reported to be in relatively low abundance in other species (Brookes *et al.*, 1995), suggesting that the amelogenin protein amplified from the H72 mRNA may have a specific role in early dentin formation.

Papagerakis *et al.* (2003) have reported that only odontoblast cell lines express amelogenin transcripts, with no expression in dental pulp and mature odontoblast cell lines. Our *in vivo* results support this finding; however, we did find amelogenin expressed in pulp cells grown *in vitro*. Since pulp cells are known to differentiate into odontoblast-like cells *in vitro*, it is possible that the amelogenin transcripts were specific to these differentiated cells.

Repeated analyses of the effect of rH58 on dental pulp cells showed a specific effect on the up-regulation of the cell-cycle-related genes: CDK6, CUL4, and NEDD8. CDK6 can promote cell-cycle progression by accelerating G1-S transition (Malumbres *et al.*, 2004). CUL4 is a component of E3 ubiquitin ligase complexes, which mediate the ubiquitination and the degradation of short-lived regulatory proteins, including cyclins and other cell-cycle regulators, such as the p27^{KIP1}, and regulates the cell cycle and signaling (Nakayama *et al.*, 2001). The NEDD8-modifying pathway, which is essential to E3 ubiquitin ligase complexes, plays a key role in the Ub-mediated pathway with respect to cell-cycle regulation (Hochstrasser, 1998). Up-regulation of these 3 genes had a positive effect on cell-cycle progression, while the mechanism by which H58 alters expression of these proteins to affect the cell cycle is not known, and requires further study.

ALP and DSP expression increases when dental pulp cells differentiate into odontoblasts (Couble *et al.*, 2000; Veis *et al.*, 2000; Yokose *et al.*, 2000). In our study of human dental pulp cells, there were no significant effects of 10-nM recombinant amelogenins on this process of cell differentiation. This is in contrast to previously reported findings that A+4 (counterpart of rH72) stimulated the production of type I collagen by odontoblasts in cultured mouse tooth germs, while A-4 (counterpart of rH58) did not (Tompkins *et al.*, 2005). Likewise, Veis and co-workers showed that embryonic muscle fibroblast differentiation into chondrocytes was enhanced by recombinant rat 72AA amelogenin (rR72, A+4/LRAP+E4) (Nebgen *et al.*, 1999). In a mouse cementoblast cell line, Viswanathan *et al.* (2003) found that murine full-length amelogenin promoted BSP expression at 0.1 µg/mL and decreased BSP expression at 10 µg/mL. The reasons for the discrepancies between our results, showing no effect on cell differentiation, and these reported studies are not immediately apparent, but may be species-related.

In summary, our studies are the first to identify specific alternatively spliced amelogenin fragments and their effects on developing and adult human dental pulp cells grown in culture. Amelogenin mRNA transcripts and amelogenin proteins are present in the developing human dentin matrix, though the role of amelogenin in dentin matrix formation is not known. Cells from the dental papilla and dental pulp can increase proliferation in response to rH72, though

it is not apparent whether sufficient amounts of protein are present in the dental papilla and pulp to modify or direct dentin formation. However, the effects of low-molecular-weight amelogenins on dental pulp cell proliferation suggest the potential applications of these proteins to be used as agents to promote proliferation of dental pulp tissue in the presence of injury, resulting in the formation of reparative dentin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Amelogenin in human dental pulp tissue. (A) Trichrome stain section of a late bell stage of tooth organ. (B) Amelogenin immunolocalization on a frozen section of an early-bell-stage tooth organ shows amelogenin (red) in the forming dentin matrix (d). (C) Dental pulp cells grown *in vitro* show positive amelogenin immunostaining (green) in the cytoplasm. (D) *In situ* hybridization of amelogenin mRNA on a section of the same tooth as shown in panel A. Positive signal is correlated to the ameloblast cell layer, and the dentin matrix (d), separated by the negative (black) enamel matrix (e). (E) Type I collagen *in situ* hybridization shows positive signal in the odontoblast layer lining the forming dentin matrix.

Figure 2.

Brd U incorporation into amelogenin-treated dental papilla cells. **(A)** Proliferation as measured by BrdU incorporation and quantitated as relative light units *per* second (r/u/s), was not altered in the presence of rH58. **(B)** rH72 significantly enhanced dental papilla cell proliferation at concentrations between 100 and 500 ng/mL. Results represent the means $\pm$ SE. Asterisks show significant differences. * $P < 0.05$ vs. control. N (for each cell of data) = 3.

Figure 3.

Brd U incorporation into amelogenin-treated dental pulp cells. **(A)** Proliferation as measured by BrdU incorporation was increased in the presence of rH58 at concentrations from 50 to 200 ng/mL, with 200 ng/mL as the optimal concentration. **(B)** rH72 enhanced dental pulp cell proliferation at concentrations from 20 to 1000 ng/mL, with 200 ng/mL as optimal. **(C)** Cell-cycle superarray results showed that CKD6, CUL4, and NEDD8 were up-regulated more than 2 times by rH58 by dental pulp cells. Results represent the means $\pm$ SE. Asterisks show significant differences. * $P < 0.05$ vs. control. N (for each cell of data) = 3.

Figure 4.

Pulp cell differentiation in the presence of amelogenins as measured by alkaline phosphatase (ALP) and dentin sialoprotein (DSP) expression. In dental papilla cells, ALP mRNA expression (**A**) or ALP protein synthesis (**B**) was not altered in the presence of 10 nM rH58 (lane 1), rH72 (lane 2), or rH174 (lane 3). Control with no added amelogenins (lane 4). In dental pulp cells, ALP mRNA and DSP expression (**C**) or protein synthesis (**D**) was similarly not altered in the presence of 10 nM rH58 (lane 1), rH72 (lane 2), or rH174 (lane 3). Control with no added amelogenins (lane 4). $p > 0.05$.