Bioactive Nanofibers Instruct Cells to Proliferate and Differentiate During Enamel Regeneration

Zhan Huang,¹ Timothy D Sargeant,² James F Hulvat,² Alvaro Mata,² Pablo Bringas Jr,¹ Chung-Yan Koh,³ Samuel I Stupp,^{2,3,4} and Malcolm L Snead¹

ABSTRACT: During tooth development, ectoderm-derived ameloblast cells create enamel by synthesizing a complex protein mixture serving to control cell to matrix interactions and the habit of hydroxyapatite crystallites. Using an in vitro cell and organ culture system, we studied the effect of artificial bioactive nanostructures on ameloblasts with the long-term goal of developing cell-based strategies for tooth regeneration. We used branched peptide amphiphile molecules containing the peptide motif Arg-Gly-Asp, or “RGD” (abbreviated BRGD-PA), known to self-assemble in physiologic environments into nanofibers that display on their surfaces high densities of this biological signal. Ameloblast-like cells (line LS8) and primary enamel organ epithelial (EOE) cells were cultured within PA hydrogels, and the PA was injected into the enamel organ epithelia of mouse embryonic incisors. The expression of amelogenin, ameloblastin, integrin $\alpha 5$, and integrin $\alpha 6$ was detected by quantitative real-time PCR and immunodetection techniques. We performed cell proliferation assay using BrdU labeling and a biomineralization assay using Alizarin red S staining with quantitative Ca^{2+} measurements. In the cell culture model, ameloblast-like cells (LS8) and primary EOE cells responded to the BRGD-PA nanostructures with enhanced proliferation and greater amelogenin, ameloblastin, and integrin expression levels. At the site of injection of the BRGD-PA in the organ culture model, we observed EOE cell proliferation with differentiation into ameloblasts as evidenced by their expression of enamel specific proteins. Ultrastructural analysis showed the nanofibers within the forming extracellular matrix, in contact with the EOE cells engaged in enamel formation and regeneration. This study shows that BRGD-PA nanofibers present with enamel proteins participate in integrin-mediated cell binding to the matrix with delivery of instructive signals for enamel formation.

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INTRODUCTION

B IOMINERALIZATION IS THE process by which living cells synthesize proteins that undergo self-assembly to form a matrix that is competent to control the initiation, growth, and termination of a mineral phase under conditions compatible with cell physiology.^(1–3) Enamel is the outermost covering of vertebrate teeth and the hardest tissue in the vertebrate body, formed by ameloblast cells through a biomineralization process. Ameloblast cells synthesize and secrete a complex mixture of tissue-specific proteins into the extracellular space where the proteins self-assemble to form a matrix that patterns the hydroxyapatite as long and thin crystallites⁽⁴⁾ woven together by the cells to form a tough, wear-resistant composite material.⁽⁵⁾ The mature enamel composite contains almost no protein⁽⁶⁾ and is a

tough, crack-tolerant, and abrasion-resistant tissue⁽⁷⁾ that can withstand a lifetime of wear, unless destroyed by infection (caries) or trauma.⁽⁸⁾

Enamel is the only tissue of ectodermal origin that biomineralizes in mammals. During enamel biomineralization, the assembly of the protein matrix precedes mineral replacement. The dominant protein of mammalian enamel is amelogenin, a hydrophobic protein that self-assembles to form nanospheres that in turn influence the crystal habit and packing of the crystallites.^(9–11) Ameloblastin, also known as sheathelin,⁽¹²⁾ is the second most abundant enamel structural protein of the forming enamel matrix. During mineral maturation, ameloblastin is degraded, and a specific fragment has been shown to girdle the secretory route of the ameloblasts that defines the lateral cell boundaries.⁽¹³⁾ Creation of ameloblastin-null mice by homologous recombination results in the loss of ameloblast to matrix interaction, with the ameloblast re-entering the cell cycle

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¹The Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles, California, USA; ²Department of Materials Science and Engineering and The Institute for BioNanotechnology in Medicine, Northwestern University, Chicago, Illinois, USA; ³Department of Chemistry, Northwestern University, Evanston, Illinois, USA; ⁴Department of Medicine, Northwestern University, Chicago, Illinois, USA.

and becoming neoplastic,⁽¹⁴⁾ marking the importance of cell to matrix interactions. The differentiation of ameloblasts is achieved by the exchange of signals with the underlying neural crest-derived mesenchyme, with these signals taking the form of growth factors and/or extracellular matrix proteins that interact with receptors on the ameloblast surface.^(15,16) These cell signals result in changes to gene expression by activating or inhibiting specific transcription factors or cell division control factors that serve to regulate cell fate. Integrin cell receptors have been shown to participate in amelogenesis, interacting with several ligands in the enamel extracellular matrix, including ameloblastin, an enamel structural protein and laminin 5, a cell adhesion molecule.^(17,18) In contrast to the mesenchyme-controlled biomineralization of bone, which uses collagen and remodels both the organic and inorganic phases over a lifetime, enamel contains no collagen and does not remodel. Moreover, rodent enamel is highly patterned, a property that allows alterations to the cell-mediated process to be readily detected.⁽⁵⁾

We are interested in developing cell-based strategies to regenerate enamel and other dental tissues in adulthood. In this context, we studied here the possibility of using synthetic and bioactive nanostructures to influence biological events involved in enamel formation. The nanostructures used in this study form by self-assembly of molecules known as peptide amphiphiles (PAs). These molecules were developed by Stupp and colleagues^(19–21) and are designed to self-assemble into high aspect ratio cylindrical nanofibers that measure 6–8 nm in diameter and micrometers in length. PAs consist of three molecular segments: a hydrophobic segment that is typically a fatty acid tail, a peptide sequence containing amino acids with a propensity to form β -sheets, and a terminal hydrophilic peptide sequence that can be altered depending on the application. In this study, this terminal sequence contained an epitope for cellular receptor signaling. The nanoscale fibers display their signaling information on their surfaces at exceedingly high densities that can be tuned by co-assembly with molecules that do not bear the same peptide signal. Two recent examples were the display of the laminin epitope IKVAV, which led to the unprecedented rapid and selective differentiation of neural progenitor cells into neurons⁽²²⁾ and the display of the fibronectin RGD epitope for signaling biological adhesion.⁽²³⁾ In other recently reported systems, the nanofibers were designed to display heparin-binding segments to activate angiogenic growth factors.⁽²⁴⁾ In the context of this work, the enamel extracellular matrix contains proteins with pertinent cell adhesion properties that affect ameloblast behavior; examples include ameloblastin,^(13,14,18,25,26) amelogenin,^(5,27) and laminin.^(17,28–30) Thus, ameloblasts or their progenitor cells were predicted to interact with our artificial nanostructures bearing this RGD peptide signal.

In this work, we used an *in vitro* model consisting of mouse ameloblast-like LS8 cells^(31,32) that express enamel genes.^(31–35) To guard against confounding properties of an immortalized cell line, we also used mouse primary enamel organ epithelial cells to characterize the response of enamel forming cells to the PAs. In addition, the peptide amphi-

phile nanostructures were directly injected into the enamel organ epithelial cells of developing mouse incisor teeth along the rostral-caudal gradient of ameloblast differentiation, where these cells are engaged in enamel formation. Here we document the influence of branched RGD-peptide amphiphile nanostructures (BRGD-PA) on ameloblast behavior in cell culture and in organ culture.

MATERIALS AND METHODS

Peptide amphiphile synthesis and preparation

A branched peptide amphiphile containing the RGD peptide epitope (BRGD-PA) was used for this study, and its structure is shown in Fig. 1A. The molecule was synthesized using methods previously described.^(36,37) Briefly, Fmoc-Lys(Mtt)-OH (EMD Biosciences) was first attached to the Rink amide MBHA resin. Trifluoroacetic acid in methylene chloride (3 wt%) selectively deprotected the epsilon amine of lysine and the palmitic acid tail was coupled to it, followed by synthesis of the remaining peptide sequence using the α amine of lysine with standard 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids in *N,N*-dimethylformamide (DMF) with diisopropylethylamine (DIEA) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) coupling reagents. The branched peptide amphiphile architecture was selected for this study based on prior work^(23,36) showing improved accessibility of the epitope when the RGD sequence was attached to the epsilon amine of a lysine side-chain rather than to the α amine (as in a conventional peptide), and the density of the epitope has been described.⁽²³⁾

PAs were synthesized on an automated peptide synthesizer (CS Bio Co. 136XT). After synthesis, the PA was cleaved from the resin using TFA/triisopropylsilane (TIS)/water (95:2.5:2.5), precipitated with ice-cold ether, solubilized in water, and dried by lyophilization. The product was purified using a Varian ProStar Model 210 preparative-scale reverse-phase HPLC on a Waters Atlantis column and characterized using a PE Biosystems Voyager-DE PRO MALDI-TOF mass spectrometer and a Hewlett-Packard 1050 RP-HPLC. Samples were lyophilized and stored at -20°C until use.

Two different control molecules for BRGD-PA were used in these experiments. For one control PA, the “RGDS” amino acid sequence was “scrambled” to RSDG. This was done to preserve the charge distribution of the side-chains while disrupting the normal biological recognition of this amino sequence. In a separate control, the same branched “RGD” structure was synthesized only in the dextrorotary form that mimics the levorotary groups but is biologically inactive. In addition, for the purposes of visualization, a fluorescent-tagged PA molecule was prepared in which the terminal “RGD” sequence was replaced with the rhodamine dye. Here, 5-rhodamine carboxylic acid (Anaspec, San Jose, CA, USA) was activated with neat thionyl chloride (Sigma-Aldrich) and coupled to the epsilon-amine of the terminal lysine residue. In all other respects, these control PAs were synthesized and purified as described above.

FIG. 1. Schematic chemical structure of the branched RGD peptide amphiphile (BRGD-PA) and its effect on calcium deposition and cell proliferation. (A) Schematic chemical structure of the branched RGD peptide amphiphile (BRGD-PA). (B–D) Alizarin red S staining for calcium salts in ameloblast-like LS8 cells cultured on tissue culture plates (B), control PAs (C), and BRGD-PA (D). (E) Quantification of calcium concentration measured by means of dye absorption at 612 nm for ameloblast-like LS8 cells cultured on tissue culture plates (TC), control PA, and BRGD-PA. ^aOne-way ANOVA: $p(\text{CALCIUM}) = 0.0005$. (F) BrdU incorporation into ameloblast-like LS8 cells cultured on tissue culture plates (TC), control PA, and BRGD-PA by FACS sorting. ^aOne-way ANOVA: $p(\text{BRDU}) = 0.016$.

Cell culture

The ameloblast-like cell line, LS8, was maintained in DMEM (Invitrogen, GIBCO) supplemented with 10% FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin, as previously described.^(31,32) To identify the cells within the assembled PA, the LS8 cells were transiently transfected with green fluorescent protein vector pEGFP-N1 (Clontech Laboratories), whose expression was driven by the Cytomegalovirus immediate early promoter (CMV), excited at 488 nm, and visualized at 510 nm. The transfected LS8 cells were mixed and co-cultured with 1 wt% peptide amphiphiles.

For all cell culture experiments (e.g., real-time PCR, BrdU labeling, Western blot analysis), the LS8 or primary enamel organ epithelial cells were mixed and co-cultured within the selected PA by first coating the dish with the selected PA, growing the cells on the assembled PA surface for 24 h, and covering the cells with the same PA to enmesh the cells in the assembled PA for 4 h before harvesting. The cells were grown on the surfaces of tissue culture plates, in 1 wt% self-assembled BRGD-PA, or in 1 wt% control PA.

Primary enamel organ epithelial tissue was recovered from newborn mouse lower incisors. The incisors were dissected free of the mandible in ice-cold PBS and incubated in 2 mg/ml dispase (Invitrogen, Carlsbad, CA, USA) at 37°C for 1 h. The sheets of enamel organ epithelial cells were separated from the underlying extracellular matrix and mesenchyme and cultured in supplemented keratinocyte media KGM-2 (Clonetics BioWhittaker, Walkersville, MD, USA).^(38–40) The dissociated primary enamel organ

epithelial cells were co-cultured with the selected PAs as described for the LS8 cells above. Cell culture experiments were performed in triplicate.

Organ culture

Animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee. Timed pregnant Swiss Webster mice were killed at day 18 of gestation (vaginal plug = day 0). Embryos were transferred to Hank's balanced salt solution (HBSS) and decapitated. Mandibular incisors were microdissected free of surrounding tissue with cataract knives and electro-polished tungsten wires as previously described^(41,42) and placed on a pre-cut Millipore filter disc (0.8- μm pore size; Millipore) overlying a stainless steel grid that is just in contact with the BGJ_b culture medium (Invitrogen, GIBCO) plus 100 $\mu\text{g}/\text{ml}$ ascorbic acid (Sigma) and penicillin-streptomycin (100 U/ml, 100 $\mu\text{g}/\text{ml}$) in a Grobstein culture dish.⁽⁴³⁾ The embryonic incisors were cultured at the air-media interface at an atmosphere of 5% CO_2 -95% air at 37°C. The incisors were injected with the various PAs 1 day after dissection and maintained for 2 days after injection. The culture medium was renewed every 2 days.

Injection of peptide amphiphiles into incisor primordia

After 24 h of culture, the incisor primordia was injected with 100 nl of selected peptide amphiphiles at a concentration of 1 wt% through a heat-drawn borosilicate glass capillary (PB-7; Narishige, Tokyo, Japan). The injections were

performed with a micromanipulator (Leitz, Weitzlar, Germany) used to position the micropipette over the incisor organ. A pneumatic pump (PV 830; World Precision Instrument) calibrated for the desired volume was used to deliver the peptide amphiphile. The fine tip of the drawn-glass micropipette was inserted into the enamel organ among the ameloblasts using oblique illumination of the incisor organ to provide visual clues from the inherent contrast of the tissue and forming dental matrix. The site of injection was at the interface between dentine and enamel, into the space of the enamel organ, at a zone containing differentiated and undifferentiated ameloblasts along the rostral-caudal gradient of development. Initially, a nontoxic chromogen, 0.1% trypan blue, was added to the PA to aid in localization of the glass tip and the injection site. However, all experimental conditions shown herein were made without inclusion of a chromogen to the selected PA. Sham injections were performed using PBS identical in composition to the solvent used to suspend and promote assembly of PA. The same operator (ZH) made all injections described in these experiments to ensure uniformity.

Histochemistry

To detect calcium salts, von Kossa staining was performed by adding 5% silver nitrate solution (Merck), exposed to UV light for 1 h, and stopped by treatment with 5% sodium thiosulfate. von Kossa staining was used to detect bivalent cations presumed to be deposits of calcium or calcium salt, and this was confirmed using Alizarin red S staining to detect calcium deposits in tissue as previously described.⁽⁴⁴⁾

Calcium assay

LS8 cells were mixed and co-cultured in the selected PAs or on plastic surfaces of a 12-well culture plate for 24 h. Quantification of calcium concentration was measured by means of the intensity of blue color at 612 nm (Quantichrom Calcium Assay kit; BioAssay Systems). The total amount of protein in each sample was used as a standard to normalize the calcium data.⁽⁴⁵⁾

Proliferation assay

LS8 cells were mixed and co-cultured in the selected PAs or on plastic surfaces of a 12-well culture plate for 24 h. Ameloblast-like LS8 cells were exposed to the thymidine-analog BrdU (Sigma) at a concentration of 10 μ M or in the case of PA-injected primordia at a concentration of 100 μ M for 2 h to label cells undergoing DNA replication. BrdU was detected using a monoclonal antibody (Sigma) followed by secondary antibody coupled to a chromogenic substrate following the directions of the manufacturer. LS8 cells grown on selected surfaces were harvested and counted using fluorescence-activated cell sorting (FACS) as previously described.^(31,32) For PA-injected primordia, BrdU-labeled cells were manually counted in a 320 $\times$ 75 μ m cube that defined the injection site.

Immunohistochemistry

Immunostaining was performed as described previously.⁽⁴⁶⁾ Briefly, the PA-injected primordia were fixed overnight in freshly prepared 4% paraformaldehyde in

PBS, pH 7.4, at 4°C. Tissue sections of 6 μ m in thickness were prepared and blocked for nonspecific absorption. After incubation with the selected primary and secondary antibody, the localization of the antibody-antigen was visualized with suitable chromogen. In the case of the anti-mouse amelogenin polyclonal antibody (1:1000 dilution) raised in chicken and recovered from the egg yolk as IgY, an anti-chicken horseradish peroxidase (HRP)-conjugated secondary antibody (1:1500; Invitrogen) and a peroxidase substrates (AEC) was used for detection by local precipitation of the visible chromogen. For ameloblastin, polyclonal rabbit antibodies (a gift from Dr Paul Krebsbach, University of Michigan) against recombinant rat ameloblastin were prepared, purified, and used as previously described at a dilution of 1: 2000.⁽⁴⁷⁾ The primary monoclonal antibody against integrin α 6 was used at a dilution of 1:500 (Santa Cruz) with an Alexa fluor 488 goat anti-rat IgG (H+L) at 1:100 dilution (Invitrogen) with detection by fluorescent microscopy.

RNA extraction and reverse transcription

Total RNA was extracted using the RNA-Bee reagent (TEL-TEST, Friendswood, TX, USA). First-strand cDNA was synthesized with 100 ng random oligodeoxynucleotide decamers using a RETROscript reverse transcription kit (Ambion).^(39,40)

Real-time PCR analysis

Real-time PCR was performed with an iCycler IQ multicolor real-time PCR detection system (BioRad). Detailed primer sequences for each target gene are as follows: amelogenin, forward primer, 5'-GGGGACCTGGATTTT-GTTTG-3', reverse primer, 5'-AACCATAGGA AG-GATACGGC TG-3'; β -actin (internal control), forward primer, 5'-GGGAAATCGTGCGTGACATC-3', reverse primer, 5'-GCGGCAGTGGCCATCTC-3'; integrin α 5, forward primer, 5'-TGCAGTGGTTCGGAGCAAC-3'; reverse primer, 5'-GGGTCATTCT GTGGGTCCTT TT-3'; ameloblastin, forward primer, 5'-CCAGTATG-GCAAGGGGTCC-3', reverse primer, 5'-GGGGTGTG-GTAACATCAGCAC-3'. The IQ SYBR Green supermix kit (BioRad) was used in PCR assays according to the manufacturer's protocol in a volume of 20 μ l with 3–4 mM MgCl₂ and 0.2–0.4 mM each primer (final concentration). Taqman methods were used for quantitation of integrin and ubiquitin mRNA. For integrin α 6 and ubiquitin C (internal control), the Taqman primer and probe sets were used (Mm00434375_m1, Mm01201237_m1; Applied Biosystems). Taqman Universal PCR Master Mix (Applied Biosystem) was used in PCR assays in a volume of 20 μ l with 0.4 μ M each primer (final concentration). All real-time RT-PCR reactions were performed in triplicate and conducted with an initial denaturing interval (95°C, 15 min) and then 40 cycles: 95°C (10 s), 55°C (45 s). The iCycler iQ real-time PCR detection system software version 3.1 was used to analyze results, and the PCR baseline subtraction curve fit function was used to determine threshold cycle (C_T) values.^(39,40) Gene expression level was quantified with the comparative C_T method employing software provided by the manufacturer (BioRad).

Electron microscopy

The PA-injected incisors were prepared in Karnovsky's fixative (6% glutaraldehyde and 4% paraformaldehyde in 0.2 M cacodylate buffer) for 2 h. The specimens were post-fixed with 2% osmium tetroxide in 0.2 M cacodylate buffer for 1–2 h at 4°C and dehydrated with ascending ethanol concentration. After an increasing concentration gradient of EPON in propylene oxide medium, specimens were embedded in 100% EPON at 65°C for 36–48 h. The embedded incisors were prepared for semiultra-thin sections, stained with toluidine blue, and examined until a suitable area for transmission electron microscopy study was identified. The specimen blocks were transferred to a Reichert-Jung ultramicrotome for ultra-thin sections (6 nm). Sections were double-stained with uranyl acetate and lead citrate and examined with an electron microscope at 80 kV.

Western assay

Protein recovery, resolution to size and detection by a specific antibody was performed as previously described.⁽⁴⁸⁾ Cells were washed twice in ice-cold PBS followed by 1 ml of ice-cold RIPA buffer (1× PBS, 1% Nonidet P-40, 0.1 mg/ml phenylmethylsulfonyl fluoride, 30 µl/ml aprotinin [Sigma], and 1 mM sodium orthovanadate). The lysate was cleared by centrifugation at 15,000 rpm for 15 min at 4°C, and the protein concentration of the supernatant was measured using a BioRad protein assay kit (BioRad) with BSA as standard. Samples were electrophoretically resolved using 4–12% SDS-PAGE (Invitrogen) and transferred to Immobilon-P membrane (Millipore). The membranes were incubated in blocking buffer overnight at 4°C, followed by primary antibody for 1 h and an HRP-conjugated secondary antibody for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence (Amersham).

Statistical analysis

Each experiment was repeated at least three times. Statistical analysis used ANOVA for statistical significance. Multiple incisor primordia were injected, and representative images are shown.

RESULTS

Cell culture manipulations

When a 1-wt% solution of branched RGD-peptide amphiphile (BRGD-PA) is mixed with LS8 cells in media, these molecules self-assembled into nanofibers, and a gel is formed, encapsulating cells in three dimensions (Supplementary Fig. 1A). We synthesized a derivative of the BRGD-PA conjugated with rhodamine to visualize the material under fluorescence microscopy (Supplementary Fig. 1A1). We used a construct composed of the pCMV-EGFP to drive expression of a green fluorescent protein (Supplementary Fig. 1A2) that marks the LS8 cells within the BRGD-PA gel, so that we observed LS8 cells as green spheres enmeshed within the rhodamine-tagged nanofiber gel, seen as a red-colored matrix in this fluorescence microscopy image. Suspending cells within the PA uses excessive amounts of PA, and we elected instead to grow the cells on a self-assembled PA surface and then recoated

these cells with the same PA, encasing them within self-assembled nanofibers. This method was intended to provide biological readouts from the effect(s) of the PA on ameloblast-like cell lines or on primary enamel organ epithelial cells, serving to mimic the PA injections into whole dental primordia.

The LS8 cells grew to confluency and formed single layer structures with little mineralization shown by the absence of Alizarin red S staining when grown on tissue culture plates or within the control PA, (Figs. 1B and 1C, respectively). Interestingly, when the LS8 cells grew within the BRGD-PA gel, they formed focal multilayered structures and produced a matrix that accumulated minerals as shown by Alizarin red S staining (Fig. 1D). The reduced mineral deposition was corroborated using von Kossa staining of LS8 cells grown on conventional tissue culture dishes compared with LS8 cells grown in the BRGD-PA (Supplementary Figs. 1B and 1C, respectively). Using a sensitive calcium dye, we measured the amount of Ca^{2+} deposited by the LS8 cells and identified that cells grown within the BRGD-PA deposited significantly more mineral than cells grown on the tissue culture plates or cells grown within control PA gels (Fig. 1E).

We next examined the proliferation of the LS8 cells maintained on plastic tissue culture dishes versus LS8 cells maintained in a BRGD-PA gel or a gel formed from the control PA, which has the same amino acid composition as BRGD-PA but lacks the sequence information of the “RGD” motif. Using FACS sorting of cells labeled with BrdU, the LS8 cells showed a relatively low proliferation rate when grown on plastic (25%), as shown by the few cells that incorporated BrdU into their DNA under such culture conditions (Fig. 1F). The number of LS8 cells incorporating BrdU was greater when the cells are grown within control PA nanofibers (47%). However, when the LS8 cells grew within the bioactive PA showing the RGD epitope, the cell proliferation rate increased significantly to 59%. Similar trends in LS8 cell proliferation were observed in independent labeling experiments in which BrdU-labeled cells were counted manually on either plastic tissue culture dishes (39%), control PA (47%), or BRGD-PA (52%) (Supplementary Figs. 1D, 1E, and 1F, respectively).

Because amelogenin is the most abundant enamel protein of the developing enamel matrix, we used immunohistochemistry to show amelogenin protein accumulation into the extracellular matrix. LS8 cells express relatively low levels of amelogenin protein when maintained on plastic culture dishes (Supplementary Fig. 1G), whereas they increase their amelogenin protein expression when grown on BRGD-PA (Supplementary Fig. 1I). Changes to amelogenin protein expression were corroborated using reverse transcription coupled to real-time PCR to measure amelogenin transcript abundance. Amelogenin transcripts were approximately twice as abundant in ameloblast-like LS8 cells grown on BRGD-PA compared with the expression levels of LS8 cells maintained on plastic or control PA (Fig. 2A). To ensure that the observed effects of the BRGD-PA on cell behavior were not restricted to a cell line, primary enamel organ epithelial cells were identically assayed. Primary enamel organ epithelial cells also enhanced their am-

FIG. 2. Changes to gene expression measured by real-time PCR amplification for ameloblast-like LS8 cells and primary enamel organ epithelial cells after growth on tissue culture plates (TC), control PA, and BRGD-PA. (A and B) Amelogenin mRNA expression. ^aOne-way ANOVA: ameloblast-like LS8 cells $p(\text{AMELOGENIN}) = 0.003$; primary enamel organ epithelia $p(\text{AMELOGENIN}) = 0.00001$. (C and D) Integrin $\alpha 6$ mRNA expression. ^aOne-way ANOVA: ameloblast-like LS8 cells $p(\text{INTEGRIN } \alpha 6) = 0.0003$; primary enamel organ epithelia $p(\text{INTEGRIN } \alpha 6) = 0.01$. (E and F) Integrin $\alpha 5$ mRNA expression. ^aOne-way ANOVA: ameloblast-like LS8 cells $p(\text{INTEGRIN } \alpha 5) = 0.002$; primary enamel organ epithelia $p(\text{INTEGRIN } \alpha 5) = 0.04$.

elogenin transcript abundance >2-fold when grown on the BRGD-PA compared with transcript abundance for these same cells maintained on plastic or control PA (Fig. 2B).

Real-time PCR was used to measure ameloblastin mRNA abundance, because ameloblastin is the second most abundant protein in the forming enamel protein matrix.⁽¹⁴⁾ Ameloblastin mRNA abundance was only marginally increased by the ameloblast-like LS8 cells grown within BRGD-PA nanofiber gels compared with either control PA gels or tissue culture surfaces (Supplementary Fig. 1K). In contrast to LS8 cells, ameloblastin mRNA expression was significantly enhanced by primary enamel organ epithelial cells grown within the BRGD-PA gels compared with these cells grown on tissue culture surfaces or within control PA gels (Supplementary Fig. 1L). Similarly, Western blot analysis showed slightly increased steady-state levels of ameloblastin expressed by these cells when grown within the BRGD-PA (Fig. 3A).

Based on data described above, the ameloblast-like LS8

cells and the enamel organ epithelial cells seem to respond to the presence of the biologically active “RGD” motifs shown on the surface of nanofibers, prompting us to characterize their integrin expression patterns. When cultured at low density on the BRGD-PA, the LS8 cells grew as multilayered cell aggregates (Supplementary Fig. 1H) and expressed the $\alpha 6$ integrin protein on their cell surface as detected by monospecific antibody (Supplementary Fig. 1J). We measured the abundance of $\alpha 6$ integrin transcripts in these cells using reverse transcription with real-time PCR. We confirmed a modest but significant increase for integrin $\alpha 6$ transcripts abundance for ameloblast-like LS8 cells grown on BRGD-PA compared with control PA or tissue culture plates (Fig. 2C). Primary enamel organ epithelial cells cultured within the BRGD-PA showed more than a doubling in integrin transcript expression compared with identical cells grown on plastic culture dishes (Fig. 2D).

To corroborate the integrin protein expression levels,

FIG. 3. Western blot analysis of ameloblastin or integrin $\alpha 6$ protein from primary enamel organ epithelial cells grown on selected substrates. (A, top) Ameloblastin protein. (Bottom) GAPDH used to control for loading. Bar graph to the right of each panel is the densitometry measurement of the specific protein after being normalized to GAPDH. Lane 1, tissue culture plate; lane 2, control PA; lane 3, BRGD-PA; lane 4, mouse incisor (positive control for ameloblastin detection). Ameloblastin is rapidly degraded by proteinases leading to multiple bands.⁽⁶⁷⁾ (B, top) Integrin $\alpha 6$ protein. (Bottom) GAPDH used as a control for loading. Bar graph to the right of each panel is the densitometry measurement of the specific protein after being normalized to GAPDH. Lane 1, tissue culture plate; lane 2, control PA; lane 3, BRGD-PA.

Western blot analysis was performed on primary enamel organ epithelial cells with detection of $\alpha 6$ integrin protein by a monospecific antibody. The 132-kDa integrin $\alpha 6$ protein was significantly increased when primary enamel organ epithelial cells were grown within nanofibers bearing the RGD motif compared with levels expressed by primary enamel organ epithelial cells grown within control PA or on tissue culture dishes (Fig. 3B).

We measured the abundance of the mRNA transcript for the integrin $\alpha 5$ receptor that can interact with the RGD domain. The ameloblast-like cell line LS8 showed a significant increase in $\alpha 5$ mRNA levels compared with these cells grown on tissue culture dishes or within a control PA (Fig. 2E). Similarly enhanced transcript abundance was observed for primary enamel organ epithelial cells grown within the BRGD-PA gel compared with primary enamel organ epithelial cells grown on tissue culture plastic or within control PA nanofibers (Fig. 2F).

Incisor manipulations

We next examined the effects of RGD peptide amphiphiles nanostructures on ameloblasts engaged with enamel formation in whole organs of mouse incisor teeth. The tooth organs were maintained in Grobstein dishes (Fig. 4A).^(41,45) Mouse lower incisors were chosen because they display a gradient of ameloblast differentiation along their rostral-caudal longitudinal axis, with a stem cell population at their caudal base (growing end) through to fully mature enamel at the most rostral end (biting end) (Fig. 4B).^(49–51) Here, we placed the PAs directly within the enamel organ epithelia by microinjection, incorporating the PA around the ameloblasts and associated cells, in situ, similar to their distribution in the preceding cell culture experiments. Immediately after injection, the site bearing the BRGD-PA injection shown in Fig. 4B (box) was made more evident by the inclusion of trypan blue mixed with the PA. Histological sections of the injected incisor showed the trypan blue and PA to be localized within the enamel organ epithelia (Fig.

4C), an area that includes the ameloblast cells. The inclusion of PA into the enamel organ epithelia was corroborated using rhodamine-labeled BRGD-PA (Fig. 4D), showing that the assembled PA was positioned correctly to include the ameloblasts. Transmission electron microscopic examination of teeth 5 days after injection showed that the RGD-PA assembled into nanofibers within the enamel organ epithelia (Fig. 4E), with cytoplasm extension of enamel organ epithelial cells into the assembled nanofibers, suggesting that the cells surrounding the injection site were in direct contact with the peptide amphiphile showing the functional motif. Mineral deposition at the site of BRGD-PA injection was confirmed using either Alizarin red S or von Kossa staining of histological sections from injected teeth (Figs. 4F and 4G, respectively).

Guided by the cell culture results from the ameloblast-like LS8 cell line and from primary enamel organ epithelial cells, we examined cell proliferation and differentiation in whole tooth organs injected with selected PAs. Here, incisors were injected with a PA bearing a biologically active epitope, RGD, or a control PA, and processed for detection of BrdU incorporation into DNA after a 24 h culture period. Incorporation of BrdU was used to assess cell division and showed that sham injection resulted in >6 of 100 cells dividing among the cells of the enamel organ (Figs. 5A and 5D). Similar to the observations from the cell culture manipulations reported above (Fig. 1F), incisors injected with the control PA showed a low number of cells entering the cell cycle after injection, with ~13 of 100 cells incorporating BrdU (Figs. 5B and 5D). However, incisors injected with the BRGD-PA elicited a dramatic increase in proliferation in which the cells making contact with the BRGD-PA had undergone at least one cell division resulting in >40 of 100 cells being marked with BrdU (Figs. 5C and 5D). Some cells, likely from the stratum intermedium, seemed to be labeled more intensely than other labeled cells from among the enamel organ, suggesting multiple rounds of cell division. Cell proliferation was confined to the enamel organ

FIG. 4. Organ culture of mouse incisor primordia injected with BRGD-PA. (A) Incisor tooth organs from embryonic day 18 maintained in a Grobstein tissue culture dish. (B) Incisor tooth organ after microinjection of BRGD-PA mixed with trypan blue among the undifferentiated and differentiated ameloblasts within the enamel organ epithelia along the rostral-caudal longitudinal axis. (C) Histological section from BRGD-PA with trypan blue injected into the enamel organ epithelia surrounded by ameloblast cells from the enamel organ epithelia. (D) Histological section from an enamel organ epithelia injected with a mixture of rhodamine-conjugated PA and BRGD-PA. (E) Transmission electron microscopic examination of BRGD-PA-injected incisor primordia 5 days after injection. BRGD-PA assembled into nanofibers within enamel organ epithelia, with cytoplasm extension into the nanofibers. (F) Alizarin red S staining of a representative tissue section from an embryonic mouse incisor primordia injected with BRGD-PA. Mineral deposits were stained red. (G) von Kossa staining of a representative tissue section from embryonic mouse incisor primordia injected with BRGD-PA. Calcium deposits were stained black.

epithelia adjacent to the injected BRGD-PA, and no labeling was observed within the odontoblast compartment, an ectomesenchyme-derived cell type found immediately adjacent to the site of injection. Intense BrdU incorporation occurred in the caudal stem cell compartment of each incisor that is unrelated to injection of the PA.

The cells of the enamel organ adjacent to the sites of PA injection were evaluated for expression of select cell receptor using a specific antibody directed against integrin $\alpha 6$. The integrin protein expression pattern for the sham-treated incisors showed localization to the ameloblast layer and to cells of the dental papillae (Fig. 5E). In the case of the control PA injection, integrin $\alpha 6$ protein expression was found localized to enamel organ cells in a punctuate staining pattern (Fig. 5F). The pattern for $\alpha 6$ integrin protein expression for incisors injected with the BRGD-PA was distinctive (Fig. 5G), with integrin localized to the ameloblasts surrounding the PA. Cells in this same location were previously found to be proliferating (Fig. 5C) in response to the BRGD-PA. Cells from among the stratum intermedium were also identified as expressing integrin $\alpha 6$ protein.

The sites of BRGD-PA injection were evaluated for expression of amelogenin, a protein specific for enamel matrix production and biomineralization.^(52–54) Because the isolation and purification of intact amelogenin isoforms in quantity from the heterogeneous mixture of enamel matrix polypeptides is extremely challenging, recombinant amelogenins are often used for biochemical analysis.^(55,56) We

used a polyclonal antibody made in chicken eggs against a recombinant mouse 180 amino acid protein. The sham-injected incisors showed amelogenin protein expression in ameloblasts and the forming of enamel extracellular matrix (Fig. 5H) as expected. The incisors injected with the control PA showed disruption of the palisade ameloblasts, with amelogenin protein localized among the nonpolarized cells (Fig. 5I). The incisor organs injected with the BRGD-PA showed substantial amelogenin protein expression among the ameloblasts in contact with the PA (Fig. 5J). At the injection site, cells that express amelogenin appeared to be poorly polarized, with these same cells previously identified as proliferating from their BrdU incorporation (Fig. 5C). We also examined ameloblastin expression by the cells approximating the injection site and found that enamel organ epithelial cells in contact with the BRGD-PA expressed greater amounts of ameloblastin (Fig. 5M) than did those cells surrounding the sham (Fig. 5K) or control PA (Fig. 5L) injection sites.

DISCUSSION

It has been suggested that extracellular matrix proteins such as fibronectin,^(50,53) laminin,^(11,51) and ameloblastin^(19,26) not only function as a mechanical scaffold for cell attachment and survival but also provide a microenvironment for guiding cell growth and differentiation. In this work, we used peptide amphiphiles that self-assemble into a nanofiber network to mimic the extracellular matrix that

FIG. 5. Branched RGD-peptide amphiphiles (BRGD-PA) influencing ameloblast cell behavior after injection into the enamel organ epithelia (solid arrow) of incisor primordial maintained for 2 days in organ culture. The complete incisors is shown at low power in A', B', and C' with the injection site marked by a dashed line box. (A–M) Higher-magnification images of representative tissue sections from the site of injection (solid arrow). Od, odontoblasts; Am, ameloblasts; SI, stratum intermedium. A–D show cell proliferation using BrdU incorporation into cellular DNA. The stem cell compartment at the caudal end of the incisor is marked with an open arrow and corresponds to sites of high cell division.⁽⁵¹⁾ (A) Sham treatment consisting of incisor primordia injected with PBS showed little BrdU incorporation into the DNA of enamel organ epithelial cells at the injection site. (B) Incisor primordia injected with control PA showed but a few BrdU-labeled cells at the injection site. (C) Incisor primordia injected with BRGD-PA showed cells have incorporated BrdU as a consequence of increased cell division. (D) Bar graph of the total number of cells incorporating BrdU from the injections sites shown in A–C. (E–G) Immunodetection of integrin $\alpha 6$ protein expression from representative sections taken from incisor primordia at the site of PA injection (solid arrow). (E) Relatively low levels of integrin $\alpha 6$ protein expression localized to enamel organ cells and part of dental papillae in sham treated incisor primordia. (F) Integrin $\alpha 6$ protein expression at the injection site of the control-PA. (G) Increased $\alpha 6$ integrin protein expression among the cells at the injection site of the BRGD-PA. The cells at the injected site are shown to be proliferating in C. (H–J) Immunodetection of amelogenin protein for cells around the injection site (solid arrow). (H) Sham-injected incisor primordia. (I) Incisor primordia injected with control PA. (J) Incisor primordia injected with BRGD-PA shows amelogenin expression among the ameloblasts of the enamel organ epithelia. Similar cells at the injection site are shown to be proliferating (C) and expressing integrin $\alpha 6$ (G). (K–M) Immunodetection of ameloblastin protein for cells around the injection site (solid arrow). (K) Sham-injected incisor primordia. (L) Incisor primordia injected with control PA. (M) Incisor primordia injected with BRGD-PA shows substantial ameloblastin expression among the ameloblasts of the enamel organ epithelia. Similar cells at the injection site are shown to be proliferating (C), expressing integrin $\alpha 6$ (G), and expressing amelogenin (J).

surrounds the ameloblasts and provides it with a biological signal shown on the synthetic nanostructures. The BRGD-PA was chosen for this study for several reasons. First, the fibronectin-derived RGD epitope has been shown to signal cells and promote formation of focal adhesions.⁽²³⁾ During tooth development, fibronectin is proposed to regulate terminal differentiation of odontoblasts, mediated by ameloblasts, and to accelerate cell differentiation of primary rat ameloblast lineage cells.^(57,58) Second, ameloblastin, the main nonamelogenin enamel matrix protein synthesized by secretory stage ameloblasts, contains a cell adhesion motif

that plays an essential role in supporting and regulating secretory ameloblasts for initial enamel formation.^(18,25) Last, extracellular matrix molecules critical for ameloblast differentiation also possess integrin binding capacity.^(17,59) Inner enamel epithelial cells, the precursors to ameloblasts, and presecretory ameloblasts have been shown to express on their cell surfaces several integrin molecules, among which, integrins containing $\alpha 6$ subunit, the major receptors for laminins, were reported to be expressed in dental epithelium during early tooth development.^(14,28,60) Inner enamel epithelial cells have also been shown to express the

$\alpha 5$ integrin subunit during their differentiation into secretory ameloblasts.⁽²⁶⁾

Based on an *in vitro* cell culture model, BRGD-PA nanofibers induced proliferation of cells as shown by BrdU incorporation rates, as well as enhanced their differentiation into ameloblasts as shown by their synthesis and mineralization of the enamel extracellular matrix. Both the ameloblast-like cell line, LS8, and primary enamel organ epithelial cells showed similar responses to the BRGD-PA, characterized by increased expression of integrin $\alpha 5$ mRNA, increased $\alpha 6$ mRNA and protein, and increased cell proliferation with enhanced expression of amelogenin and ameloblastin mRNA and protein (Figs. 1–5) compared with the control-PA in which the RGD sequence had been “scrambled” to disrupt its bioactivity or using abiotic D-amino acids for the motif. The matrix accumulated by the proliferating cells on BRGD-PA gels further supported biomineralization *in vitro*, although the actual structure of this matrix is not known at this time. The finding that the LS8 cell line and the primary enamel organ epithelial cells responded similarly suggests that the response is not an artifact attributable to the use of an immortalized cell line. Rather, the response of the heterogeneous enamel organ epithelial cell population *in vitro* is useful in assessing the response of this same population of cells that is exposed to BRGD-PA injected into whole incisor organs. The *in vitro* cell responses showed the design and interpretation of the more complex whole incisor organ studies.

Based on the *in vitro* organ culture model using whole mouse embryonic incisors to ascertain the response of the inner enamel epithelia to peptide amphiphiles, we observed responses similar to the findings from cell lines. The dental epithelia of rodent incisors contain a linear gradient of distinct differentiation stages including presecretory, secretory, and maturation stage ameloblasts.⁽⁶¹⁾ Secretory stage ameloblasts retain the functions of synthesizing and secreting enamel matrix proteins that are replaced by calcium and phosphorus as hydroxyapatite during enamel biomineralization. It was into these whole organs that we injected a small volume of the PA into the secretory zone of ameloblast differentiation near the site of growing end to enamel organ epithelia of mouse embryonic lower incisor.⁽⁴⁹⁾ The PA underwent self-assembly into nanofibers in the tissue space as shown by transmission electron microscopy and participated in mineral deposition (Fig. 4), outcomes that were also supported by the *in vitro* cell culture experiments (Fig. 1).

Injection of BRGD-PA into the enamel organ epithelia of incisors resulted in proliferation of the cells adjacent to the site of injection but also supported continued enamel gene expression shown by the accumulation of amelogenin and ameloblastin protein into the extracellular space (Fig. 5). Whereas it is difficult to map amelogenin expression to the exact same cells that undergo proliferation, these findings suggest that both proliferation and commitment to a terminal phenotype are taking place after the PA injection process. The data showed that, after injection, the number of cells increased, as did the number of cells engaged in enamel formation. Thus, the presence of the bioactive PA possibly promoted enamel formation through its effect on

cells by increasing their proliferation and their differentiation into an amelogenin/ameloblastin-expressing phenotype. It has been previously shown that ameloblasts simultaneously secrete amelogenin and ameloblastin.^(5,14,62)

We have used several control peptide amphiphiles in this study. One of these consisted of the BRGD-PA synthesized with D-amino acids. The D-amino acid RGD epitope is not anticipated to exert an integrin-signaling response from the cells, given that it is an abiotic stereoisomer that is not recognized by the cells RGD receptors.⁽⁶³⁾ Storrie et al.⁽²³⁾ recently showed that this stereoisomer does not promote formation of focal adhesions. In this series of experiments, we also used a peptide amphiphile with a terminal amino acid sequence that was rearranged to display the same charge density as the bioactive motif, but without the critical amino acid sequence RGD. Finally, we have used a sham control by injecting PBS into the enamel organ of the mouse incisors. In all control experiments, the response of the cells was statistically different from that observed for the bioactive BRGD-PA. For either the LS8 cells or the primary enamel organ epithelial cells, the differences observed between control and experimental values for amelogenin mRNA expression (Fig. 2) and for integrin mRNA expression (Figs. 2 and 3) were statistically significant. Whereas it is more difficult to quantify these outcomes in the injected incisors, because we cannot isolate, recover, and characterize these cells from the whole organ, the data shown in Figs. 4 and 5 provide descriptive evidence of the differences in control versus experimental samples.

Morphological hallmarks of odontogenic epithelium include polarization and palisading, and both are believed to be required for the production of the enamel extracellular matrix. Whereas useful to these initial studies into enamel regeneration, disrupting the delicate enamel organ epithelia by injection of a regenerative peptide amphiphile will need to be optimized. An alternative approach would use atomic force microscopic dip pen lithography using the BRGD-PA to produce a patterned template that would promote epithelial patterning and polarization, events needed to organize the enamel matrix so that it can be converted to mineral.^(5,64)

Although this study was conducted over short intervals of time and in organ culture, it suggests that the enamel organ epithelial cells participate in repair and regeneration with the BRGD-PA scaffold delivering key integrin signals. However, maintenance of the organ over a longer period of time is needed to fully ascertain the quality and quantity of the regenerated enamel. The physical properties of the enamel structural hierarchy are dependent on the sequential and spatial expression of matrix protein networks. It has been found that changes in the abundance of amelogenin may alter its ability of self-assembly into a matrix that can direct biomineralization, thus compromising the structural organization of mature enamel.^(65,66) At this time, it is not clear if the interweaving of the enamel prisms, the fundamental units of enamel synthesized by one ameloblast and essential for its unique material properties, has been retained in the regenerated enamel. We are currently engaged in transplanting these injected incisors into animal hosts where they can be engrafted for the longer periods

required for this analysis. In these studies, it will also be important to screen additional bioactive peptides that might provide a more favorable regenerative response.

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Address reprint requests to:
Malcolm L Snead, DDS, PhD
The Center for Craniofacial Molecular Biology
The University of Southern California
HSC, CSA 142
2250 Alcazar Street
Los Angeles, CA 90033, USA
E-mail: mlsnead@usc.edu

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