Specific Amelogenin Gene Splice Products Have Signaling Effects on Cells in Culture and in Implants in Vivo*

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Arthur Veis‡, Kevin Tompkins, Keith Alvares, Kuiru Wei, Lin Wang§, Xue Song Wang, Anna G. Brownelly, Shure-Min Jengh, and Kevin E. Healy

From the Department of Basic and Behavioral Sciences, Northwestern University Dental School, Chicago, Illinois 60611

Low molecular mass amelogenin-related polypeptides extracted from mineralized dentin have the ability to affect the differentiation pathway of embryonic muscle fibroblasts in culture and lead to the formation of mineralized matrix in in vivo implants. The objective of the present study was to determine whether the bioactive peptides could have been amelogenin protein degradation products or specific amelogenin gene splice products. Thus, the splice products were prepared, and their activities were determined in vitro and in vivo. A rat incisor tooth odontoblast pulp cDNA library was screened using probes based on the peptide amino acid sequencing data. Two specific cDNAs comprised from amelogenin gene exons 2,3,4,5,6,7 and 2,3,5,6,7 were identified. The corresponding recombinant proteins, designated r[A+4] (8.1 kDa) and r[A−4] (6.9 kDa), were produced. Both peptides enhanced in vitro sulfate incorporation into proteoglycan, the induction of type II collagen, and Sox9 or Cbfai mRNA expression. In vivo implant assays demonstrated implant mineralization accompanied by vascularization and the presence of the bone matrix proteins, BSP and BAG-75. We postulate that during tooth development these specific amelogenin gene splice products, [A+4] and [A−4], may have a role in preodontoblast maturation. The [A+4] and [A−4] may thus be tissue-specific epithelial mesenchymal signaling molecules.

Members of the BMP/VGR family of proteins have the ability to induce osteogenesis when implanted in appropriate carriers at nonbone sites in vivo (1, 2). Demineralized bone matrix was the initial source of the BMPs. Addition of the proteins extracted from bone to nonbone cells in vitro led to the expression of proteins characteristic of the chondrogenic and/or osteogenic phenotype (3–10). Surprisingly, demineralized dentin matrix implants exhibited a stronger osteogenic inductive activity (3–10). Members of the BMP-VGR family (8–10) were not pure, and the amino-terminal sequence and composition data obtained could not be related to a single protein component. The very low content of the active peptides in rat incisor dentin made it impractical to continue using rat incisor dentin as the peptide source. With some modifications in isolation procedure but using the same in vitro assay systems (11), the principal fraction was isolated from bovine dentin in essentially homogeneous form, and its activity was verified in vitro and in vivo assays. The amino-terminal sequence and one internal tryptic peptide sequence were determined. Both sequences proved to be derived from the amino-terminal portion of bovine amelogenin (11, 12). This was a surprising result for two reasons. First, the active peptides had been isolated from both rat and bovine dentin cleaned as well as possible from enamel contamination. Second, the principal function of the amelogenins and their degradation products have been assigned to structural roles in creating the space and milieu for promoting enamel mineralization (13). Recently, however, a mixture of porcine enamel proteins has been used clinically (14) to induce cementogenesis along the tooth root surface, and the activity was attributed to amelogenin. Thus, it appeared of interest to explore the cell signaling activity of the amelogenin peptides.

The amelogenins present in the tooth at any stage are a complex mixture of gene isoforms and degradation products (13). The two peptides partially sequenced by Nebgen et al. (11) were the products of exon 2–3 and exon 5 transcription, respectively, both from the amino-terminal region of amelogenin. Every intact amelogenin molecule, most alternatively spliced isoforms, and the major amino-terminal region degradation product known as TRAP (tyrosine-rich amelogenin peptide) would have yielded these sequences. Amelogenin amino acid sequences are highly conserved across all species, although the human and bovine have amelogenin genes on the X and Y chromosomes, whereas rat and murine amelogenin genes reside only on the Y chromosome. These genes yield distinct sets of splice product isoforms (12, 15, 16). However, the larger amelogenins are specifically degraded in stepwise fashion and also yield a variety of smaller peptides during the process of enamel mineralization (17).

The “active” peptide described by Nebgen et al. (11) was characterized only by amino-terminal sequencing. It was not determined whether it was an amelogenin degradation product or an intact polypeptide transcribed and translated as a specific enamel gene splice product. This is a very important distinction relative to the function and regulation of the potential in vivo
activity of the peptide. Thus, the objective of the work reported here was to determine whether the message corresponding to the specific gene splice product was present and, if so, to prepare the peptide and determine whether it could express the cell inductive activities equivalent to the peptide isolated by Nebgen et al. (11).

Because the protein isolation work (7, 8, 10, 11) had focused on dentin extracts, our approach was to examine a rat incisor odontoblast pulp-based cDNA library for the presence of an amelogenin-related cDNA. The rationale for choosing the rat incisor cDNA library was 3-fold. First, there is high conservation of the amelogenin sequences between rat and bovine species (13). Second, our cDNA library has been verified (18–20) to contain the cDNAs for the dentin matrix proteins, DMP1,
DMP2, and DMP3 (dentin sialophosphoprotein, DSPP). Third, the mRNAs for these three dentin proteins are transiently expressed in mouse molar enamel organs during fetal and immediately post-natal tooth development (21–23), suggesting that there might be a reciprocal transient expression of particular splice products of the amelogenin gene in developing odontoblasts.

**MATERIALS AND METHODS**

Cloning and Sequencing of the Amelogenin Peptides

Freshly extracted rat incisors were cleaned to remove the soft enamel. The odontoblasts and pulp cells were retained. Poly(A)+ RNA was isolated from these cells using the Oligotex mRNA kit (Qiagen). The mRNA was converted to first strand cDNA using a 18-mer oligo(dT) and Superscript II reverse transcriptase (Life Technologies, Inc.). The first strand cDNA was then used in PCR.1 The forward primer (P1) ATGCTCTTACACCT was based on the amelogenin amino-terminal peptide sequence MPPPP, and the reverse primer (P2) TATCATGCTCCTGGTACCA corresponded to the tryptic peptide sequence WYQISMI (11). Fig. 1A shows the rat amelogenine gene intron-exon constitution and the specific location of the primers. The PCR conditions were 25 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Two PCR product bands differing in size by 42 nucleotides were obtained. These were further amplified by another round of PCR and then cloned in pGEMT vector (Promega, Madison, WI) and sequenced.

Each of the amplified bands was used to screen a previously prepared λgt11 rat incisor odontoblast cDNA library (18). Positive clones were picked and plaque purified through three successive rounds of screening. Finally, pure plaques were then amplified, and phage DNA was prepared (24), digested with EcoRI, and then cloned into the EcoRI site of pBluescript KS (Stratagene, La Jolla, CA) and sequenced.

Because two amelogenin amino-terminal domain PCR products were obtained initially, two new primers were designed to examine the possibility of differentially spliced products. Forward primer (P3) TTCGGATTCCATGCCCTACCACCTCA contained a unique EcoRI site (underlined) and included the first fifteen nucleotides of the secreted form of the protein. The reverse primer (P4) GCCGCCTCGAGTTAATCCCTACCTCCCA contained a unique EcoRI site (underlined) and included the last 15 nucleotides and the stop codon TAA. These primers (see Fig. 1A) were used in a PCR reaction under the conditions described above using the phage DNA obtained from amplification of the same λgt11 odontoblast library as template. The PCR amplified bands were cloned in pGEMT vector and sequenced.

Expression of the Cloned Amelogenins

The cloned amelogenins were expressed as the GST fusion proteins. The inserts in pGEMT were reamplified by PCR using the primers P3 and P4 and conditions described above. The PCR products were digested with EcoRI and Xhol, purified on a 1% agarose gel, and cloned in frame into the EcoRI/Xhol site of the GST expression vector pGEXT4 (Amersham Pharmacia Biotech). The resulting plasmid was introduced into the Escherichia coli strain BL21(DE3). For preparation of the fusion protein, a single colony was inoculated into 100 ml of LB and grown overnight. An additional 900 ml of LB was added, and growth continued for 4 h, after which isopropyl-β-D-thiogalactoside (Amersham Pharmacia Biotech) was added to a final concentration of 1 μM. Incubation was carried on for an additional 4 h. The expressed protein was then passed over and collected on a glutathione-Sepharose affinity column (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. For different purposes, either the fusion proteins were directly eluted from the column with reduced glutathione or the bound protein was treated with thrombin to release the recombinant peptide.

Isolation of the Recombinant Peptides

In most preparations, the thrombin released peptides were a heterogeneous mixture. Therefore, the eluted thrombin cleaved protein was passed over a C-18 reverse phase column (Vydac, Sep/pak/trations Group, Hesperia, CA) developed by an increasing gradient of acetonitrile, 1%

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1 The abbreviations used are: PCR, polymerase chain reaction; GST, glutathione S-transferase; embryonic rat muscle fibroblast(s); BSA, bovine serum albumin; PBS, phosphate-buffered saline; FBS, fetal bovine serum; pen/strep, penicillin/streptomycin; nt, nucleotide(s); bp, base pair(s); HPLC, high pressure liquid chromatography; H&E, hematoxylin-eosin; DAPI, 4',6-diamidino-2-phenylindole hydrochloride.

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**Fig. 2. Identification of the amelogenin-related products obtained by PCR from rat incisor tooth cDNA.** A, demonstration that amelogenin mRNA was present in the odontoblast pulp-derived mRNA, using the primers P1 and P2 for detection of the amino-terminal message sequence common to all amelogenin gene splice products. These data show the unequivocal presence of two messages (right arrowheads), with nucleotide sizes 90 and 132 bases. B, amelogenins detected by screening the gt11 rat odontoblast pulp cDNA library with primers P3 and P4 to obtain all potential splice products. The products obtained initially were reamplified by PCR using the same primers. The amplified products are [A–4] (PCR200, lane 2), A4 (PCR250, lane 4), [B–4] (PCR600, lane 5), and B4 (PCR650, lane 3). The PCR products were run on a 1% agarose gel and visualized by staining with ethidium bromide. Lane 1 was loaded with a 1-kilobase DNA ladder. In both panels, the marker DNA sizes are indicated on the left.

trifluoroacetic acid as described (11) for the final step of purification of the protein extracted from dentin matrix.

**Assays for Biological Activity**

**In Vitro 35S]SO4 Assay for Chondrogenic Activity—**The purified recombinant proteins were tested for biological activity by the assay for enhanced incorporation of [35S]SO4 into proteoglycan (8, 11) by embryonic rat muscle fibroblasts (EMF). Recombinant human BMP2 (a kind gift from the Genetics Institute, Boston, MA) and the bioactive crude S100 fractions from rat incisor dentin (8) and/or bovine dentin (11) were used as the positive controls. Bovine serum albumin (BSA) in phosphate-buffered saline (PBS) was the negative control. A commercial preparation of purified porcine amelogenins, known as Endogain® (BIORA AB, Malmö, Sweden) (14) was also tested.

In Vitro Assay for Expression of Chondrogenic/Osteogenic Activity via Production of Marker mRNAs—The expression of Sox9 protein is necessary but not sufficient for the induction of chondrogenesis and type II collagen (25–32), whereas expression of Cbfa1 protein is necessary but not sufficient for osteoblast differentiation (33–41). EMF cultures at passage 2 were seeded into type I collagen-coated T-150 flasks (Corning, Corning, NY) according to Nebgen et al. (11) and grown to ~80% confluence in 10% fetal bovine serum (FBS), 1% pen/strep. The cells were trypsinized and passed into T75 flasks and grown again to ~80% confluence. The medium was removed, and the cells were washed with PBS. Conditioning medium (0.5% FBS in α-minimal essential medium, 1% pen/strep) was added, and the cells were held for 24 h. The conditioning medium was replaced with fresh conditioning medium containing various concentrations of the test factors or no additions for the controls. At selected time periods of incubation, the cells were washed in PBS, detached with trypsin. An equal volume of 10% FBS was added, and the cells were pelleted. The pelleted cells were resuspended in PBS, repelleted, and stored at ~80 °C.

RNA was isolated from the cells using the Rneasy Mini kit (Qiagen) according to the manufacturers instructions. Reverse transcription was carried out using the Promega RT system with reaction at 49 °C for 50 min. The gene-specific primers were used in every case for the reverse transcription reaction, except for the type I collagen. In that case a nonspecific oligo(dT) primer was used, as well as the gene-specific primer noted below. PCR was carried out using 45 μl of Life Technologies, Inc. PCR Platinum Tag Supermix, to which 1 μl of each primer (40 mm) and 3 μl of cDNA template was added. The primers and conditions were as follows: (i) for Sox9 (42) forward, CGGAAGACACT-CACATCTCTCTATGTC (nt 878–906); reverse, CGAGGAGGTCAAT-GTGGGAGATGACCA (nt 1142–1170), denaturation 3 min at 94 °C, followed by 30 cycles: 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, followed
by extension at 72 °C for 10 min; product, 292 bp; (ii) for Cbfa1 (34) forward, CCGCACGACAACCGCACCAT (nt 511–530); reverse, CGCTCCGGCCCACAAATCTC (nt 781–800), denaturation 3 min at 94 °C, followed by extension at 72 °C for 10 min; product, 289 bp; (iii) for collagen II (rat type II, GenBank™ accession number L48440) forward, CACACCGGT AAGTGGGGCAAGACC (nt 4258–4281), reverse, CT-GCGGTTAGAAAGTATTTGGGTC (nt 4444–4468), denaturation 3 min at 94 °C, followed by 30 cycles: 30 s at 94 °C, 30 s at 65 °C; 50 s at 72 °C, followed by extension for 10 min at 72 °C, product, 210 bp; (iv) for collagen I (rat type I, pro a2(I), GenBank™ accession number AF121217), forward, GCTCAGCTTTGTGGATACGCG (nt 3–24), reverse, GTCAGAATACTGAGCAGCAAA (nt 243–267), denaturation 3 min at 94 °C, followed by 30 cycles: 30 s at 94 °C, 30 s at 58 °C, 50 s at 72 °C, followed by extension at 10 min at 72 °C; product, 264 bp; and (v) for glyceraldehyde-phosphate dehydrogenase (43, 44), forward, CCAACCAGAAGGGAAGG (nt 276–293), reverse, CTTACTCCTTG-GAGCCCAT (nt 944–963), denaturation 3 min at 94 °C, followed by 30 cycles: 30 s at 94 °C, 30 s at 58 °C, 50 s at 72 °C, followed by extension for 10 min at 72 °C; product, 687 bp. All PCR products were run on ethidium bromide-containing 3% agarose gels at 75 volts for 60 min.

In Vivo Activity

Implant Protocols—The recombinant proteins, Emdogain and BSA controls were each included in a bioabsorbable polymer matrix of poly(D,L-lactide-co-glycolide) (45). The polymer scaffolds were cast as 2.5-cm discs containing a total of 1 mg of recombinant protein, 1 mg of BSA, 1 mg of rhBMP2, or 1.5 mg of Emdogain. Each disc was cut into six equal wedges. A wedge was then placed into the right hind thigh muscle of a 4-week-old, 100-g Long-Evans rat. A negative control wedge of bovine serum albumin in PBS was placed in the contralateral left thigh. Four animals were used for each test condition. All surgical implant protocols and animal care procedures were reviewed and approved by the Northwestern University Animal Care and Use Committee. The implants were followed radiographically with a measurement every week.

Vascularization and Mineralization—The matrices were removed at 4 or 6 weeks after implantation and processed for histology. The implant blocks were fixed in 10% formalin, radiographed, and then embedded in paraffin. Serial sections were cut and examined following staining with standard hematoxylin-eosin (H&E), von Kossa, Alizarin Red, and Goldner’s Trichrome stains.

Immunodetection of Bone-specific Matrix Proteins—The sections
were deparaffinized with xylene washes three times for 3 min each time and rehydrated by passage through decreasing concentrations of alcohol. The tissue was then fixed in 10% formalin for 15 min and washed 1 min with PBS. The cells were permeabilized by exposure to acetone for 5 min, washed 1 min with PBS, blocked for 1 h in phosphate-buffered saline plus 0.5% BSA, and then washed with PBS. Primary antibody was added to each section directly without dilution from stock buffer. The sections were incubated in the dark for 1 h and then washed three times for 1 min with PBS. The secondary antibody was applied at 1:50 dilution (10–20 μl/section). Sections were incubated for 1 h in the dark and then washed three times with PBS (1 min/wash). The sections were mounted and viewed immediately using either a Zeiss Axiovert 100 microscope with a ZVS-3C75DE digital camera or a Leitz Dialux 20 microscope with a RT SPOT slider camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

The primary antibodies were anti-bone sialoprotein (BSP, antibody WVID1(9C5), Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and anti-bone acidic glycoprotein 75 (BAG-75), a generous gift from Dr. Jeffrey P. Gorski, University of Missouri, Kansas City. Secondary antibodies were Texas Red conjugated to anti-mouse IgG for BAG-75. These antibodies were all from Jackson ImmunoResearch Laboratories, West Grove, PA. Nuclei were labeled with DAPI reagent (Pierce).

RESULTS

Preparation of Amelogenin Peptides from Rat Incisor cDNA—When the PCR primers P1 and P2 were used to probe the mRNA isolated from fresh rat incisor odontoblast pulp complex (Fig. 1A), two PCR products were detected (Fig. 2A) and sequenced. Their nucleotide sequences corresponded to the amino acid sequences, MPLPPHPGHPGYINFSEYVLTPKWEYQSMI (PCR90) (primers P1 and P2 underlined), and MPLPPHPGHPGYINFSEYKHSQINTDRTALVTTPKWWYQSMI (PCR132). The band corresponding to PCR90 was much more intense than that for PCR132. PCR90 corresponded exactly to the secreted protein amino-terminal sequence encoded by rat amelogenin gene exons 2, 3, and 5. PCR132 included exon 4 (sequence in italics above) (46). These data established that differentially spliced amelogenin mRNAs, containing exons 2, 3, and 5 and 2–5, respectively, were indeed present in the presumed odontoblast pulp tissue. Based on the higher intensity of PCR90 on the gels, it is likely that there was a higher concentration of its mRNA than for the PCR132 transcript containing exon 4, although this could also signify that the two mRNAs require different conditions for optimal reverse transcription.

When the established odontoblast pulp rat incisor Agt11 cDNA library (18) was screened with forward and reverse primers P3 and P4, four PCR product bands were amplified from the template phage DNA. The PCR bands at approximately 600 and 200 bp were strong, and PCR 650 and PCR250 were weak. All four bands were reamplified (Fig. 2B) and cloned in pGEMT vector and sequenced. These data showed that mRNAs for four specific amelogenin gene splice products had been present when the rat incisor odontoblast pulp cDNA library was created: [PCR650] exons 2,3,4,5,6,7; [PCR600] exons 2,3,5,6,7; [PCR250] exons 2,3,4,5,6,7 (73 amino acids, 8135 Da); and [PCR200] exons 2,3,5,6,7 (59 amino acids, 6697 Da). These are shown diagrammatically in Fig. 1A and designated as [B,+4], [B–4], [A,+4], and [A–4], in order of decreasing size.

Screening of the Agt11 cDNA library (18) using PCR132 as probe identified several plaques. Two positive clones were picked and plaque purified through three successive rounds of screening. The phage DNA was digested with EcoRI. The inserts were cloned into the EcoRI site of pBluescript KS and sequenced. The nucleotide and derived amino acid sequence of one proved to be those of rat incisor amelogenin [B,+4], from the signal peptide through to the poly(A)– tail, corresponding in detail to the rat incisor amelogenin data of Bonass et al. (47).
except for the inclusion of the exon 4 sequence. The second clone corresponding to the splice product [A+4] with the deletion of exons 6a,b,c yielded the sequence shown in Fig. 1B.

Because the in vitro chondrogenic activity of the dentin extract correlated with rat and bovine peptides in the Mf 6,000–10,000 range (7, 8, 11), attention was focused on the plasmids corresponding to [A+4] and [A−4] (Fig. 1A). These were amplified by PCR, using the primers described above. The PCR products were digested with EcoRI and XhoI and cloned into the EcoRI/XhoI sites of the GST expression vector pGEX-4T. The resulting plasmids were transfected into E. coli BL21. Following isopropyl-β-D-thiogalactoside induction the expressed fusion proteins were collected on glutathione-Sepharose affinity columns. The [A+4] and [A−4] were cleaved from the bound GST with thrombin and eluted. Gel electrophoresis showed the eluted proteins to be rich in the desired full-length polypeptides in both cases, but some lower mass, incompletely elongated peptides were present along with other protein impurities. The eluted proteins were therefore fractionated by reverse phase HPLC using the same system as the final step in the isolation of the tissue extracted peptides (11), yielding the pure recombinant peptides, as illustrated for both r[A+4] and r[A−4] in Fig. 3.

In Vitro Activity of the Recombinant Peptides—Because the basic assay that led to the isolation of the amelogenin peptides was their ability to induce an enhancement of sulfate incorporation into proteoglycan by the EMF cells, the in vitro 35S[S]SO4 incorporation assay was used to determine whether the recombinant peptides had comparable activities. The parameters of this assay were developed on the basis of the activity of the crude S-100 fraction at 100 μg/ml, which produces a maximal 4-fold increase in sulfate incorporation/cell. The second positive control, rhBMP2, yields a 3-fold increase at 10 ng/ml. [A+4] and [A−4] at 10 ng/ml were comparable in activity to rhBMP2 (Fig. 4). The [A−4] showed a maximum in activity between 1 and 5 ng/ml (∼140–700 pM) as compared with concentrations >10 ng/ml. The r[A+4] did not show the low concentration maximum seen with [A−4]. A distinct difference in behavior was that in vitro the r[A−4] did not act as a growth factor, whereas r[A+4] and rhBMP2 did. Even after 5 days in culture, following a 24 h exposure to r[A−4], the cell number did not increase as it did in the presence of rhBMP2 and r[A+4]. Thus, although similar, the effects of r[A−4] and r[A+4] were distinguishable. The commercial preparation of porcine amelogenins known as Endogain® was not effective in this assay at such low concentrations, but activity could be seen at concentrations greater than 500 μg/ml (data not shown). The standard deviations shown in Fig. 4 were based on five independent assays in each case.

The transcription factor Sox9 (25–32, 48, 49) is a regulator of the type II collagen gene and is required for the expression of the chondrogenic phenotype. The transcription factor Cbfa1 (33) is similarly required for induction of the osteogenic phenotype, but it has wider functions. It is expressed in the early stages of tooth formation in the dental mesenchyme and, later, in the maturation phase ameloblasts, clearly having a role in the epithelial mesenchymal interactions involved in tooth morphogenesis (41). Cbfa1 also plays a role in chondrocyte differentiation and maturation (50, 51). PCR was used to determine the appearance of these messages in EMF cultures treated with r[A+4] and r[A−4] for several time periods. PCR was also used to determine the induction of the messages for type II collagen (52), as well as changes in the level of type I collagen message. These data are shown in Fig. 5, along with the expression of the message for housekeeping gene GAPDH (Fig. 5A), which remained essentially constant for all cultures, indicating that comparable amounts of total mRNA had been used. Sox9 message was detected only in the r[A+4] treated cultures, induced at between 8 and 24 h (Fig. 5B, lanes 12 and 13). Type II collagen (COL2) message (Fig. 5C, lanes 12 and 13) appeared in...
concert with the Sox9 message in the r[A-4]-treated cultures. The COL2 message also appeared very early after addition of r[A-4] at 1–4 h and then diminished but persisted through 48 h (Fig. 5C, lanes 14–18). Cbfa1 transcription also rose sharply immediately after addition of r[A-4] to the cultures but then diminished over the 48-h period examined (Fig. 5D, lanes 14 and 15). The EMF expressed a background of COL1 transcription at all conditions (Fig. 5E). These data support the sulfate incorporation data noted above in showing that the two amelogenin peptides do not act identically on the cells.

**In Vivo Implants**—The in vivo assay for activity was the ectopic induction of mineralization in implants of the recombinant protein in bioabsorbable matrices in muscle. As shown in Fig. 6, after 4 weeks, implants containing r[A-4] stained strongly with Alizarin Red and von Kossa, showing the presence of mineral deposits. The in vivo assay also distinguished between r[A+4] and r[A-4]. The r[A+4] implants were mineralized to a lesser extent, with restricted and more focal mineral deposits than seen with r[A-4], but they were clearly more strongly mineralized than the BSA negative control. Treatment of the r[A+4] and r[A-4] sections with EGTA eliminated the Alizarin Red and von Kossa staining in the implants (Fig. 6, panels 3 and 6), verifying that the radio-opaque areas seen in panel 9 of Fig. 6 represented calcium phosphate deposits in the implants. In data not shown, the r[A-4] implants were positive for alkaline phosphatase, another marker of mineralizing systems. Emdogain® implants were virtually identical to the BSA implants.

H&E staining showed that r[A-4] and r[A+4] implants became vascularized and filled with extracellular matrix within 4 weeks (Fig. 7). Relative to the BSA control implants (Fig. 7, panel 1), capillary invasion was most prominent in the r[A-4] implants (Fig. 7, panel 2), as was the formation of extracellular matrix. The formation of islands of osteoid/bone-like extracellular matrix surrounding the capillaries was clearly revealed by both H&E and Goldner’s Trichrome stains and was especially prominent in the focal mineralization regions of the r[A+4] implants (Fig. 7, panels 3 and 4).
The matrices of the r[A 1 4] and r[A 2 4] implants showed the presence of typical bone matrix proteins, BSP, and BAG-75 (Fig. 8), upon staining with their respective antibodies. Fig. 8 (panel 1) shows a r[A 1 4] matrix containing region comparable with that in Fig. 7 (panels 3 and 4) stained with anti-BAG75 (green) and DAPI (blue) to show the cell nuclei. The intense green marked the red blood cells within the capillaries. Regions immediately surrounding the cell nuclei in areas where the matrix had not yet formed showed abundant BAG-75 staining. A typical area of BAG-75 staining, shown in Fig. 8 (panel 2) at higher magnification, also showed the presence of BSP (red, Fig. 8, panel 3). The BAG-75 and BSP were co-localized (Fig. 8, panel 4). The r[A 4] implants (Fig. 8, panels 5–8), which had been more heavily mineralized, showed a more abundant cellularity but similar co-localization of BSP and BAG-75. The control BSA loaded implants did not show the presence of these proteins, and the sections stained only with the second antibodies were also negative (data not shown).

The in vitro and in vivo assays for biological activity thus showed that the two specific recombinant small amelogenin splice products, [A 4] and [A 4], have the ability to interact with immature mesenchymal cells, both in culture and in in vivo implants and initiate a change in cell phenotype and maturation pathway. In the EMF culture system interaction [A 4] up-regulates transcription factor Cbfa1, whereas [A 4] more prominently up-regulates Sox9. Both amelogenins, at concentrations of 10 ng/ml, induce a 3-fold enhancement of sulfate incorporation into proteoglycan and lead to the subsequent production of type II collagen, markers

DISCUSSION

Two distinctly different points can be made from the data presented above. First, the specific low molecular mass amelogenin gene splice products, [A 4] and [A 4], have the ability to interact with immature mesenchymal cells, both in culture and in in vivo implants and initiate a change in cell phenotype and maturation pathway. In the EMF culture system interaction [A 4] up-regulates transcription factor Cbfa1, whereas [A 4] more prominently up-regulates Sox9. Both amelogenins, at concentrations of 10 ng/ml, induce a 3-fold enhancement of sulfate incorporation into proteoglycan and lead to the subsequent production of type II collagen, markers
of the chondrogenic phenotype. In vivo [A−4] containing implants become profusely mineralized within a 4-week period, [A+4] implants are mineralized more focally, but both types of implants are infiltrated by cells, become vascularized, and form islands of extracellular matrix. The matrix developed after 4 weeks shows the presence of BSP and BAG-75, proteins characteristic of mineralized tissues. Thus, the cell signaling activities of the amelogenin peptides relate to the formation of mineralized tissues. The second point to be made, based on the demonstration of their mRNAs in the odontoblast pulp complex cells, is that the messages for the amelogenins may be transiently expressed within the odontoblasts during tooth morphogenesis, just as the messages for several supposedly dentin specific proteins are transiently expressed by ameloblasts (21–23).

During the embryonic period of organ development, complex sets of signals are passed in both directions between epithelial tissues and their adjacent mesenchyme. These inductive, regulatory signals determine the course of tissue differentiation and can lead to highly specialized meristic structures such as hair follicles, kidney tubules, and teeth (53). A key aspect of such interactions is that they take place as a chain of sequential and reciprocal events (54) throughout the course of development. Odontogenesis is a particularly interesting process because individual tooth epithelium and mesenchyme can be separated at specific stages of embryonic development and then recombined with tissues at other stages or from other organs (55–57). The stage-specific progress of development can then be observed in the recombined tissues. In tooth development, the oral epithelium first thickens and then forms a bud growing into the underlying neural crest mesenchyme. The bud grows to form a "cap" that enfolds part of the mesenchyme. Sox9 (42) and Cbfa1 (33, 41) exert their actions on such mesenchymal cell condensations. In the tooth, those mesenchymal cells condense to form the dental papilla. The papillary cells immediately in contact with the inner enamel epithelium differentiate to become odontoblasts and form dentin. Subsequently the epithelial cells in contact with the mesenchyme differentiate to ameloblasts and produce enamel. In heterotypic recombination experiments, Mina and Kollar (57) showed that in the mouse embryo, the mandibular arch epithelia at embryonic day 12 could elicit formation of a dental papilla in nonodontogenic neural crest-derived cells. Conversely, the cells of the dental papilla could induce nonodontogenic epithelia to become committed to odontogenesis but only after the papilla cells had become odontogenic at E−12. Thus, signals instructive or permissive for differentiation pass between the two tissues at different developmental stages (2, 54, 58, 59) during tooth morphogenesis. The elements of specificity that direct the programming of the differentiating cells remain undefined at this time.

We believe that the data presented here are pertinent to this problem of epithelial mesenchymal signaling. Our earlier biochemical studies (8, 11) showed that dentin does indeed contain small amounts of amelogenin-related protein closely associated with the dentin matrix. Others (60–62) have shown by immunostaining that the mantle dentin contains amelogenin-related peptides. Sawada and Nanci (62) postulated that low molecular size amelogenin degradation products diffuse through the basement membrane separating preameloblasts and predentoblasts and become trapped between odontoblasts in the forming dentin. Karg et al. (63) found amelogenin immunostaining in developing hamster teeth in the early predentin and adjacent partially polarized preameloblasts before any overt deposition of enamel. Young odontoblasts stained weakly with anti-amelogenin antibodies before they formed the first layer of dentin. Wurtz et al. (38) specifically examined the presence of amelogenin mRNA in growing rat molars using in situ hybridization with a probe encoding the exon 5–6d boundary (as in [A+4] and [A−4]). They reported that the mRNA was exclusively limited to cells of the inner enamel epithelium. Inspection of Fig. 3 in their paper, in the light of our present results, suggests that there was specific digoxigenin labeling in the preodontoblast layer. However, the labeling was substantially weaker than that in the adjacent preameloblasts and hence was treated as background. It is worth noting, as well, that in that study there was a clear difference in the pattern of expression of the mRNAs for the “short” and “full-length” amelogenins in teeth of the same age.

Our data provide direct evidence that the r[A+4] and r[A−4] have specific biological activities, equivalent in many ways to rBMP2, in directing the change in phenotype of the embryonic rat muscle fibroblasts in vitro and inducing development of a mineralized matrix in muscle implants in vivo. The induction may operate via up-regulation of Sox9 and/or Cbfa1. Cbfa1 is required for induction of the osteogenic phenotype (33), but it is
expressed in the early stages of tooth formation in the dental mesenchyme and, later, in the maturation phase ameloblasts. Clearly, Cbfa1 has a role in the epithelial mesenchymal interactions involved in tooth morphogenesis (41). Cbfa1 is also involved in chondrocyte differentiation and maturation (50, 51). Sox9 (25–32, 48, 49) is a regulator of the type II collagen gene and required for the expression of the chondrogenic phenotype. Thus, one may postulate that these specific amelogenin gene splice products may be among the sought after epigenetic signaling factors operating during odontogenesis. Their effect may depend upon the local environment and the presence of additional cytokines and growth factors. As suggested by the literature cited above, the [A−4] and [A+4] peptides could originate in the preameloblast layer of the inner enamel epithelium and, because of their small size, diffuse into the preodontoblast layer. The peptides could then trigger the maturation of the preodontoblasts and initiate dentinogenesis. However, it is likely that the appearance of the amelogenin peptides in dentin is a programmed event because the epithelial mesenchymal signaling process is such a crucial aspect of tooth development. If that is the case, it is also likely that the amelogenin peptides in dentin are probably the specific gene splice products, rather than degradation peptides. An alternative scenario to the diffusion of the peptides into the mantle dentin comparable to the transient, very early expression of dentin matrix proteins in preameloblasts (21, 22) is the transient expression of [A−4][A−4] and [A+4] in the in the preodontoblasts. A study of that possibility is underway. We are now in position to evaluate the cell-specific expression and mechanisms of action of these hitherto unrecognized differentiation-instructive/permissive agents.

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