

Laboratory Investigations

Ameloblastin Fusion Protein Enhances Pulpal Healing and Dentin Formation in Porcine Teeth

Y. Nakamura,¹ I. Slaby,² A. Spahr,³ G. Pezeshki,³ K. Matsumoto,¹ S. P. Lyngstadaas⁴

¹Department of Clinical Cariology and Endodontology, School of Dentistry, Showa University, 2-1-1, Kitasenzoku, Ohta-ku, Tokyo 145-8515, Japan

²Biora AB, a company of Straumann AG, Medeon Science Park, Malmö, Sweden

³Department of Conservative Dentistry and Periodontology, University of Ulm, Ulm, Germany

⁴Department of Biomaterials, Faculty of Dentistry, University of Oslo, Oslo, Norway

Received: 13 June 2005 / Accepted: 21 February 2006 / Online publication: 9 May 2006

Abstract. Ameloblastin (Ambn, also named “amelin” or “sheathlin”) is a protein participating in enamel formation and mesenchymal-ectodermal interaction during early dentin formation in developing teeth. Experiments have demonstrated an association between Ambn expression and healing of acute pulp wounds. The purpose of this study was to investigate if local application of recombinant fusion Ambn (rAmbn) could influence reparative dentin formation in pulpotomized teeth. In this randomized, double-blinded study, pulpotomy was performed in 28 lower central incisors in 17 adult miniature pigs. Following the surgical procedure, the exposed pulp tissue was covered either with rAmbn or with calcium hydroxide. After 2, 4, or 8 weeks, the teeth were extracted and examined by histomorphometry and immunohistochemistry using antibodies against porcine ameloblastin, collagen type I, and dentin sialoprotein (DSP). In rAmbn-treated teeth, a substantial amount of newly formed reparative dentin was observed at the application site, completely bridging the pulpal wound. Dentin formation was also observed in calcium hydroxide-treated teeth; however, the amount of reparative dentin was significantly smaller ($P < 0.001$) than after rAmbn treatment. Immunohistochemistry confirmed that the new hard tissue formed was similar to dentin. This is the first time a direct link between ameloblastin and dentin formation has been made *in vivo*. The results suggest potential for rAmbn as a biologically active pulp-dressing agent for enhanced pulpal wound healing and reparative dentin formation after pulpotomy procedures.

Key words: Ameloblastin — Amelin — Dental pulp healing — Dentinogenesis — Pulpotomy

Following a trauma, the dental pulp has an inherent capacity to produce reparative dentin when the local environment is favorable to healing [1]. Recent advances

in the understanding of dentinogenesis have opened new vistas for pulp therapy [2–4]. Biologically active molecules, such as the bone morphogenic proteins (BMPs) [5], osteogenic protein-1 (OP-1) [6], and biomatrices such as demineralized dentin [7] and the amelogenins [8–11], have all been proposed to act as bioinducers of reparative dentin formation. When these substances are brought in close contact with wounded pulp tissue, they all show a positive influence on pulpal repair processes, as observed by rapid fibrodentin matrix formation and subsequent reparative dentinogenesis.

In recent studies, using amelogenins in the form of enamel matrix derivative (EMD), we have shown that EMD-enhanced reparative dentin formation was associated with a sequential expression of dentin-specific factors in the wounded pulp [9–11]. Notably, in these studies, expression of pig ameloblastin (also known as “sheathlin”) was detected in pulpal cells in the wound area shortly following EMD application.

During early odontogenesis, ameloblastin (Ambn, amelin) is known to be involved in the mesenchymal-ectodermal interaction that precedes dentin and enamel secretion. The protein and its corresponding gene (*Ambn*) have been identified in rat [12, 13], pig [14], and human [15]. Ambn expression was first detected in the enamel organ, where the protein is secreted from secretory-stage ameloblasts into the enamel, where it is located in the periphery of enamel prisms. In enamel extracellular matrix, Ambn is believed to participate in ameloblast attachment to the underlying enamel matrix [12] and in modulation of enamel crystal growth [16]. Later, Ambn expression was also detected in the Hertwig epithelial root sheath cells and in young differentiating odontoblasts [17, 18]. In these tissues, the sequential expression pattern of Ambn hints at its role as a signal molecule [17, 18]. These observations together with our previous finding that expression of

Correspondence to: Y. Nakamura; E-mail: yukio@senzoku.showa-u.ac.jp

Ambn is spatially and temporally associated with trauma-induced dentin formation [19] suggest a close link between Ambn expression and pulpal wound healing as well as dentin regeneration. To confirm a functional role for Ambn in dentinogenesis and to explore the potential use of Ambn for pulpal healing, we investigated if application of recombinant fusion Ambn (rAmbn) onto exposed pulp tissue could enhance pulpal healing and secondary dentin formation.

Materials and Methods

Surgical Procedure

All animal procedures were in accordance with Swedish and European Union regulations and approved by the local experimental animal board and the local ethics committee for animal experiments at Malmö Academic Hospital (Malmö, Sweden). Animals were kept in separate rooms during the experimental period and received daily veterinary surveillance. Room temperature and humidity were standardized at $19 \pm 1^\circ\text{C}$ and $55 \pm 10\%$, respectively, according to local regulations. A total of 17 female adult miniature pigs (Göttingen Minipig™, *Sus scrofa*; Møllegaard, Ejby, Denmark) with a mean age of 3 years (ranging 18–42 months) were used. Animals were randomly divided into three groups. Observation time was 2 weeks for the first group ($n = 5$), 4 weeks for the second group ($n = 6$), and 8 weeks for the third group ($n = 6$).

The surgical procedures were performed as previously described by Nakamura et al. [10, 11]. The animals were anesthetized with an injection of 10 mL Ketalar® (Park Davis, Barcelona, Spain) i.m. and 8 mL pentobarbital i.v. The surgical sites were anesthetized with Xylocain® (20 mg/mL; AstraZeneca, Göteborg, Sweden). Traumatized teeth and teeth that showed excessive occlusal attrition were excluded from the study. This left a total of 28 lower central incisors. In order to facilitate a calibrated cavity preparation and pulpotomy, the coronal two-thirds of the tooth crowns were removed using a high-speed diamond bur. Subsequently, the viable pulp tissue was exposed through a central cavity prepared with a cylindrical diamond bur. Finally, at the bottom of the prepared cavity, pulpotomy was performed using a sterile, round steel bur, 2 mm in diameter. The pulp tissue beneath the cavity floor was removed to a depth of 2 mm, generating a calibrated, cylindrical pulp defect of approximately 2×2 mm for application of the test materials. During all steps of the operative procedure, the tooth and cutting instruments were irrigated with ample amounts of sterile saline. Bleeding after pulpotomy was controlled with sterile cotton pellets. After the bleeding had stopped, the test materials, rAmbn or calcium hydroxide (Dycal®; Dentsply, Konstanz, Germany), were applied directly onto the exposed pulp tissue using a syringe or Dycal applicator, respectively. After a 1-minute waiting period, the cavities were sealed with a glass-ionomer cement (GC Fujii II®; GC Corporation, Tokyo, Japan). Special care was taken to avoid displacement of the test material during the filling procedure and to keep the experimental teeth out of occlusion. All procedures were performed by the same surgeon. The test materials were applied in a blind fashion; i.e., teeth were randomly assigned either to the test group (rAmbn) or to the control group (calcium hydroxide). The randomization code for each tooth was revealed only after the cavity was prepared and the pulpotomy had been performed. Teeth were scheduled for extraction according to the following scheme: at 2 weeks, four rAmbn-treated and four control teeth were extracted; at 4 weeks, four rAmbn-treated and five control teeth were extracted; and at 8 weeks, four rAmbn-treated and seven control teeth were extracted.

Preparation of Ameloblastin Fusion Protein (rAmbn)

Rat Ambn was cloned and overproduced as a fusion protein with *Escherichia coli* thioredoxin at the N terminus and a 6His tag at the C terminus, using the pTrxFus vector (Invitrogen, Carlsbad, CA) and *E. coli* G1698 cells [18]. Transformed cells containing the overproduced fusion protein were harvested by centrifugation and resuspended in 3 volumes of 100 mM Naphosphate buffer (pH 7.5), 50 mM NaCl, 10 mM imidazole, and Pefabloc (2 mg/mL; Boehringer Mannheim, Mannheim, Germany). After sonication (3×10 -second bursts), the lysate was treated with Dnase I (10 µg/mL, 30-minute incubation) and centrifuged at $12,000g$ for 30 minutes. The supernatant was then loaded on a Ni-nitrilotriacetic acid column (Qiagen, Chatsworth, CA). The column was extensively washed with lysis buffer containing 30 mM imidazole, and the protein retained in the column was eluted using a 200 mM imidazole solution. rAmbn appeared as a single band at about 68 kDa on polyacrylamide gel electrophoresis (PAGE) stained with Coomassie blue (Fig. 1). The protein solution was then desalted on a Sephadex G-25 column in 0.05 M acetic acid and lyophilized. Prior to application, the recombinant protein was dissolved in 4% propylene glycol alginate (PGA; Biora, Malmö, Sweden) to a final concentration of 2 mg/mL.

The rAmbn protein was also coupled to Sepharose 4B and used for affinity purification of a rabbit antibody. PAGE followed by Western blotting using this antibody revealed the main band of rAmbn at 68 kDa together with several less prominent bands in the range of 50–15 kDa, representing shorter rAmbn products, probably from proteolytic cleavage of the full-length product during processing (Fig. 1).

Rat Model

To exclude possible biological side effects from the *E. coli* thioredoxin on pulpal cells, a separate control study in a rat model was performed. The rat model was chosen as a pilot for testing thioredoxin because of the smaller pulp cavity and the fact that apical closure is better for retaining the applied thioredoxin long enough to allow possible biological or toxic effects to the pulp tissue. Pulpotomy was performed through occlusal cavities prepared in lower first molars of four adult Sprague-Dawley rats. At one side, pure thioredoxin (2 mg/mL in sterile saline) was applied; on the contralateral side, only sterile saline was applied as control. The small size of the rat teeth and the viscosity of PGA made the use of PGA as carrier practically impossible. Therefore, to ensure dosage control and good cavity sealing, we decided not to use PGA in this pilot. All cavities were closed with an occlusal filling (IRM®; DENTSPLY DeTrey GmbH, Konstanz, Germany). The animals were killed after 2 weeks, and histological examination was performed.

Histological Examination

At 2, 4, or 8 weeks after surgery, animals were killed by an intracardial bolus injection of 50 mL sodium pentobarbital in ethanol. Experimental teeth were removed *in toto* and fixed in cold 4% neutral buffered formaldehyde for 24 hours. The teeth were then demineralized in 12.5% ethylenediaminetetraacetic acid (EDTA) and subsequently embedded in paraffin. Following longitudinal serial sectioning (6-µm-thick sections), every fifth section was stained with hematoxylin and eosin. All stained sections containing pulp tissue were then observed under a light microscope equipped for histometric analysis (Olympus Microimage®; Media Cybernetics, Silver Spring, MD). Unstained sections were submitted to immunohistochemistry.

Quantitative Analyses of Newly Formed Hard Tissues

The amount of new hard tissue formed subjacent to and within 4 mm from the prepared cavity floor was assessed in

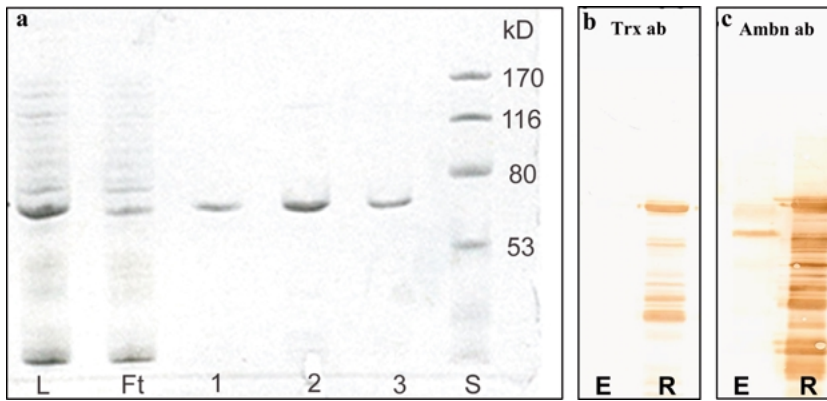


Fig. 1. (a) Coomassie blue-stained PAGE gel of samples from steps in the production of the rAmbn-thioredoxin fusion protein. *L*, crude *E. coli* cell lysate; *Ft*, from the first column wash; 1–3, samples from the column eluate containing the full-length recombinant protein; *S*, molecular weight standard. (b) Western blot of enamel epithelial cells (*E*) and the pooled eluates with the recombinant fusion protein (*R*) immunostained with an antibody against bacterial thioredoxin. (c) Western blot of enamel epithelial cells (*E*) and the pooled eluates with the recombinant fusion protein (*R*) immunostained with a rabbit anti-rAmbn antibody. Protein extraction from enamel epithelial cells of 4-day-old rats was performed using PeqGold Trifast reagent (Peqlab, Schwalbach, Germany). The protein pellet was dissolved in 60 mM Tris-HCl containing 2% sodium dodecyl sulfate (SDS) and 10% sucrose additionally supplemented with phenylmethylsulfonyl fluoride (2 mM), sodium

orthovanadate (1 mM), and aprotinin (50 µg/mL). Lysates were denatured at 95°C for 5 minutes and stored at –70°C until further use. Protein samples (enamel epithelial cells and rAmbn) were diluted 1:1 in sample buffer (250 mM Tris-HCl, pH 6.8, containing 4% SDS, 10% glycerol, and 2% β-mercaptoethanol) and boiled for 5 minutes. Proteins were separated by SDS-PAGE (10%) and transferred to nitrocellulose membrane. Nonspecific binding sites were blocked with 5% nonfat milk for 45 minutes, and blots were then incubated overnight at 4°C with one of the following antibodies: rabbit anti-rAmbn (1:1,000), sheep anti-*E. coli* thioredoxin (IMCO Corp., Stockholm, Sweden; 1:1,000). The immunoreaction was detected by incubation with appropriate horseradish peroxidase-labeled secondary antibodies (Dako Cytomation, Glostrup, Denmark; 1:1,000) for 1 hour at room temperature and

the five most central hematoxylin and eosin-stained sections from each experimental tooth. These sections represent the region with maximal defect width and cover about one-fifth of the actual defect volume. All other sections were left out from the histometric analysis, to avoid positive bias from narrowing walls of the cylindrically shaped pulp chamber. The areas covered by newly formed hard tissue in these central sections were calculated using digital histometry equipment (Olympus Microimage). Mean differences and standard deviations (SDs) were calculated for the measured variables. Statistical analyses were performed with the statistical program SPSS 10.0 for MS Windows (SPSS, Chicago, IL). Student's *t*-test was used to compare the rAmbn-treated and Dycal-treated groups. $P < 0.05$ was considered to indicate a statistically significant difference between test and control.

Antibodies

Polyclonal antibodies raised in rabbits against human collagen type I cross-reacting with human, pig, mouse, and rat type I collagen were obtained from Calbiochem (San Diego, CA). The rabbit polyclonal anti-rat DSP antibody was raised against DSP peptide QGLETEGSSTGNKSSITKES coupled to keyhole limpet hemocyanin (KLH) [20, 21]. Four polyclonal antibodies specific for pig Ambn peptides were raised in rabbits. Animals were immunized with one of four different polypeptides based on the pig *Ambn* gene sequence. Their amino acid sequences were as follows: (1) RPREHETQQY-EYSL, (2) QVAPSEKPPEAELPG, (3) ARGPAGRSRGPV, and (4) QPQIKRDAWRFQEP. The polypeptides were produced by Eurogentec (Seraing, Belgium) and purified by reverse-phase high-performance liquid chromatography. The peptides were coupled to KLH, emulsified with Freund's complete adjuvant, and used for immunization of rabbits

(three rabbits for each peptide). After the first immunization, rabbits were boosted four times with the peptide preparations before blood was harvested. The total immunoglobulin G (IgG) fractions were purified from the sera by protein-A affinity chromatography and high-performance liquid chromatography and diluted in phosphate-buffered saline (PBS) to a final concentration of 1 mg/mL. A mixture of the four anti-Ambn antibodies was used for immunohistochemistry (equal parts of each at a concentration of 1 mg/mL in PBS).

Immunohistochemistry

For immunohistochemistry, the avidin-biotin peroxidase complex (ABC) method was used [22]. Sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 20 minutes. To avoid nonspecific staining, the sections were incubated with 20% normal goat serum before overnight incubation at 4°C with primary antibodies, which were diluted in PBS as follows: anti-DSP 1/200; anti-type I collagen 1/100, and anti-Ambn mix 1/2,000. After being rinsed in PBS, sections were further incubated with biotinylated goat anti-rabbit IgG (DaKo Sweden AB, Solna, Sweden; 1/400 dilution) for 60 minutes at room temperature. The stained sections were incubated with peroxidase-conjugated goat anti-rabbit IgG (1/200 dilution). Slides were then rinsed in PBS. The stained slides that were incubated with a peroxidase-conjugated secondary antibody were then treated with ABC/horseradish peroxidase complexes (DaKo) for 60 minutes. Following a final rinse in PBS, all sections were incubated with 3,3'-diaminobenzidine (DAB) substrate (DaKo) for 10–20 minutes at room temperature. Directly after DAB incubation, the sections were dehydrated and cover slides were mounted using Pertex (Histolab Products, Göteborg,

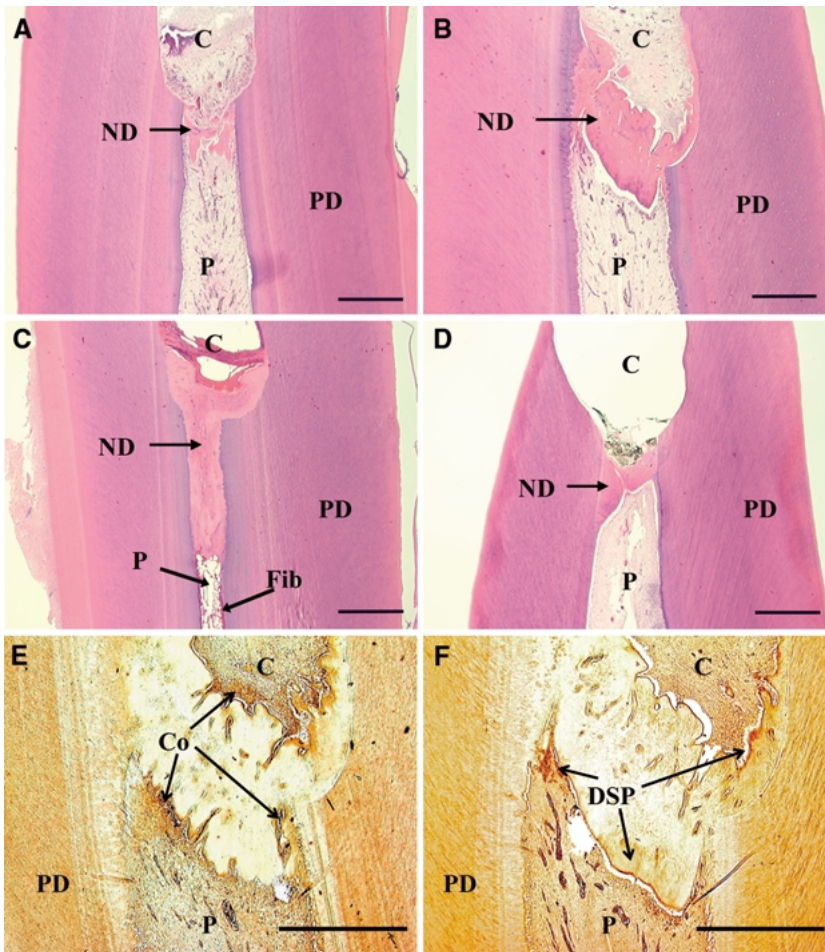


Fig. 2. Micrographs showing incisor teeth 2, 4, and 8 weeks after treatment with rAmbn (A, B, C, E, F) or calcium hydroxide paste (D). (A) Micrograph of amputated pulp observed 2 weeks after application of rAmbn. A necrotic layer overlaying a zone with a moderate inflammatory cell infiltrate can be observed along the wound surface. Osteodentin-like tissue nodules outlined by odontoblast-like cells are formed along the wound margin. (B) Micrograph of amputated pulp 4 weeks after application of rAmbn. No sign of irreversible pulp damage or partial necrosis was observed in rAmbn-treated teeth at this stage. Also, at this early time, ample amounts of pre-dentin-like tissue were observed forming beneath the surgical site, sometimes even completely bridging the cavity. (C) Micrograph of amputated pulp 8 weeks after application of rAmbn. Extensive formation of reparative dentin completely closing the wounds is observed. There are no signs of internal resorption, but signs of fibrosis are evident in the deeper parts

of the pulp. (D) Micrograph of amputated pulp 8 weeks after wounding and capping with calcium hydroxide. The dentin bridge, where formed, is significantly thinner than in the rAmbn-treated teeth. No signs of adverse effects were observed in these teeth. (E) Photomicrograph of anti-type I collagen staining 4 weeks after rAmbn application. Anti-type I collagen staining was prominent in and around regions where reparative dentin was formed. (F) Photomicrograph of anti-DSP staining at 4 weeks after rAmbn application. Anti-DSP staining was prominent in and around regions where reparative dentin had formed. The line of heavy staining between the newly formed dentin and the pulp is regarded as an edge effect and is probably not an effect of intense DSP expression here. Scale bar = 1.0 mm. C, prepared cavity; PD, primary dentin; P, pulp tissue; ND, new dentin; Fib, fibrotic pulp tissue; Co, collagen I expression; DSP, DSP expression. A–D are stained with hematoxylin and eosin.

Sweden). All dilutions were made in PBS. Control sections were treated simultaneously and in all ways identically to the test sections except that the primary antibodies were substituted with PBS only.

Results

No fillings or experimental teeth were lost during the study period, nor were any adverse effects recorded. All animals behaved normally, and there was no need to

administer analgesics or antibiotics during the study period. When killed, all animals had the same or higher body weight compared to the time of surgery, indicating that feeding habits and metabolism were unaffected by the experimental procedure.

Histology

In rAmbn-treated teeth extracted 2 weeks after surgery, a necrotic layer overlaying a zone with a mild to mod-

Table 1. Immunohistochemistry score

Antibody Treatment	Anti-pig ambn		Anti-collagen type I		Anti-DSP	
	rAmbn	Ca(OH) ₂	rAmbn	Ca(OH) ₂	rAmbn	Ca(OH) ₂
2 weeks	±	±	++	++	±	-
4 weeks	-	+	+++ Fig.2E	+++	++ Fig. 2F	+
8 weeks	-	++	+++	+++	++	++

-: No staining detected, ±: Weak staining, +: Weak to moderate staining, ++: Moderate staining, +++: Strong staining

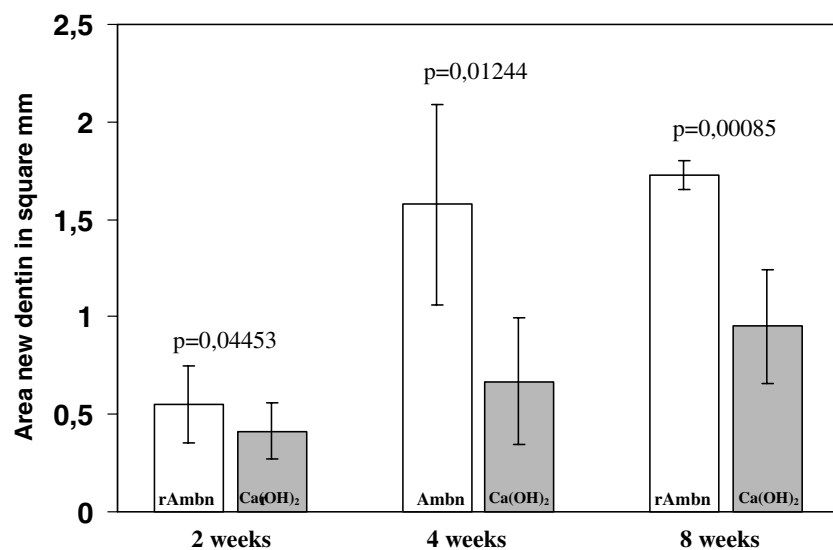


Fig. 3. Box plot with area of new dentin formed in teeth treated with rAmbn or Ca(OH)₂. Area is given in square millimeters. Error bars indicate SD. *P* values are calculated using Student's *t*-test comparing the two groups at each time point.

erate inflammatory cell infiltrate was observed adjacent to the experimental cavities (Fig. 2A). Moreover, small nodules resembling osteodentin outlined by a distinct layer of odontoblast-like cells could be seen forming in the pulp tissue along the wound margin. At this stage, in control teeth treated with calcium hydroxide, a distinct liquefaction necrosis of the tissue close to the capping material was observed. Here, only small amounts of predentin were observed, formed by the odontoblasts onto the primary dentin walls directly below the necrotic layer.

After 4 weeks, a marked increase of newly formed capillary vessels was evident in the deeper part of the pulp tissue below the application sites in rAmbn-treated teeth. At this stage, a moderate amount of new dentin-like tissue was observed, bridging the pulpal wound at the interface between the wounded and the unaffected pulp tissue. Several small hard-tissue islets in close contact with odontoblast-like cells were also observed, forming close to the newly formed dentin-like tissue (Fig. 2B). In calcium hydroxide-covered pulps, observed 4 weeks after surgery, new hard tissue resembling secondary dentin was formed onto and along the preexisting dentin walls, but no bridging was observed.

Eight weeks after wounding and rAmbn application, all pulpal defects were completely bridged by ample amounts of reparative dentin. There were no signs of internal resorption in these teeth, but signs of degenerative changes (fibrosis) were observed in the deeper parts of the pulp of some teeth (Fig. 2C). In comparison, only five of the seven control defects covered with calcium hydroxide paste showed complete closure of the pulpal wound with dentin-like tissue (Fig. 2D) (Table 1).

When the thickness of the dentin bridges formed after 8 weeks was assessed by histomorphometry, the mean thickness of the reparative dentin bridges was significantly larger in the group treated with rAmbn than in the calcium hydroxide paste control (Table 1). Also, when calculated as total reparative dentin area from the five most central sections from each defect, the total amount of reparative dentin was significantly larger in rAmbn-treated teeth (Fig. 3).

Evaluation of histological slides in the rat model showed no difference between the thioredoxin test and the saline control. In both groups, there were no signs of reparative dentin formation bridging the exposed pulp cavity. Only rudimentary signs of pulpal healing and dentin formation were observed.

Immunohistochemistry

The results of immunohistochemistry are summarized in Table 1. Only weak anti-pig Ambn (sheathlin) staining was detectable 2 weeks after application of rAmbn. At this stage, Ambn expression was detected only along the wound surface. No staining with anti-pig Ambn could be detected at 4 and 8 weeks. In calcium hydroxide-treated teeth, only weak anti-pig Ambn staining was detectable after 2 weeks of healing. After 4 and 8 weeks of healing, however, moderately strong staining with anti-pig Ambn was observed. In the early healing stages, staining occurred directly adjacent to visible calcium hydroxide paste at the surgical site. In later stages, Ambn expression shifted into the deeper parts of the wounded pulp; and at 8 weeks, anti-pig Ambn staining was observed in regions with newly formed dentin.

Staining with anti-type I collagen was detected in the pulpal stroma adjacent to the wound after 2 weeks of healing in rAmbn-treated teeth. This collagen expression was restricted to the surgical site. However, after 4 weeks, expression of type I collagen shifted to a more diffuse pattern in the deeper parts of the pulpal wound. After 8 weeks, type I collagen expression was concentrated in and around regions where reparative dentin was forming (Fig. 2E). The expression pattern of type I collagen in calcium hydroxide-treated teeth mimicked the pattern observed in rAmbn-treated teeth.

Two weeks after rAmbn application, weak anti-DSP staining was detectable in close connection with islets of newly formed dentin-like hard tissue. At later healing stages, anti-DSP staining was stronger and detected within the newly formed reparative predentin and dentin (Fig. 2F). The anti-DSP staining was detectable throughout the 8-week observation period. In calcium hydroxide-treated teeth, the onset of DSP expression was delayed when compared to the rAmbn counterpart. Here, anti-DSP staining could not be detected before 4 weeks of healing. As in rAmbn-treated teeth, DSP expression lasted to the end of the observation period. The distribution of DSP expression was, however, similar in both treatments.

Discussion

Ambn expression has previously mainly been associated with odontogenesis and enamel formation. However, we recently reported that Ambn expression could be induced by traumatizing the odontoblast layer lining the dentin walls of developing rat molars [19]. The onset of Ambn expression was rapid and proficient and directly preceded, both spatially and temporally, the formation of reparative dentin in these teeth, suggesting a link between Ambn expression and reparative processes in the pulp.

The present study is the first to demonstrate *in vivo* a putative functional role for Ambn in pulpal healing and dentin formation. In rAmbn-treated teeth, both pulpal wound healing and reparative dentin formation were enhanced compared to calcium hydroxide-treated teeth. It seems that application of rAmbn provides a strong direct signal for dentin formation, a signal that usually is provided by pulpal cells as a response to trauma. We have previously also reported on the sequential onset of Ambn expression and dentin markers following treatment with EMD or calcium hydroxide [10, 11]. In the case of normal pulpal healing or healing induced by EMD, the healing event starts with the induction of Ambn expression, peaking during the first week of healing. Providing the Ambn signal directly by application of rAmbn seems to allow cells to proceed directly with dentin formation, visualized by collagen type I and DSP expression, cutting short the time needed to heal and close the wound.

Moreover, not only the rate but also the amount of reparative dentin formation was significantly larger in rAmbn-treated teeth than in calcium hydroxide-treated teeth at all observation times. After 8 weeks, thick dentin bridges effectively closed the cavities in all rAmbn-treated teeth, whereas in calcium hydroxide-treated teeth the wound was at best covered only by a thin dentine bridge. The presence of the dentin-specific markers collagen type I and DSP in the newly formed hard tissue confirms that the newly formed hard tissue resembles ordinary dentin [23]. This is also true for the new dentin forming after calcium hydroxide application, albeit here the onset of expression of these marker molecules was delayed relative to rAmbn-treated teeth.

The biological role of thioredoxin in our experiments cannot completely be ruled out without additional work. So far, we have not been able to overproduce Ambn without a fusion tag at its N terminus in a sufficient quantity. Also, taking away the thioredoxin part resulted in difficulties because the turnover number of the recommended cleavage enzyme, enterokinase, was very low and its cleavage sites were quite unspecific.

Nevertheless, the fusion protein rAmbn was unambiguously shown to be biologically active, and there are several observations suggesting that Ambn is the active part. Firstly, in our experiments in rats, thioredoxin alone did not enhance reparative dentin formation. Secondly, it has previously been reported that EMD induces pulpal healing through expression of Ambn in pulpal cells, hinting at a central role for this molecule [9–11]. Thirdly, Ambn expression has been shown to precede trauma-induced reparative dentin formation in rats [19]. Furthermore, the thioredoxin used for the fusion is of bacterial origin. The similarity between mammalian and bacterial thioredoxin molecules is so weak that antibodies against them do not cross-react. It is thus not likely that bacterial thioredoxin has any

activity in mammals. Finally, the fusion thioredoxin-Ambn-6His construct allows Ambn to be preferentially bound to the Ni column via the 6His tag at the C terminus. Degradation rAmbn products, washed out from the Ni column before elution, thus contain mainly the N-terminal thioredoxin.

A limited number of available adult animals restricted the number and type of controls included in this study. Ideally, a control group containing PGA alone should also be included. However, we knew from earlier studies with EMD that PGA does not have any significant biological effects on pulpal healing and regeneration [9]. Therefore, we chose to use calcium hydroxide as control as this is the most widely used pulp-capping material in the clinic.

The observed fibrosis in two rAmbn-treated teeth was a side effect that cannot be explained on the basis of this experiment. Over time, this side effect could reduce the life span of the pulp; and if rAmbn is to be used clinically, this effect will have to be closely evaluated. The effect might be an artifact related to chronic inflammation caused by the xenobiotic nature of *E. coli* toxins from the recombinant production, invading microbes or necrotic tissue remnants. On the other hand, the effect could be a consequence of overdose of rAmbn, which would trigger rapid reparative dentin formation that could overload the regenerative capacity of the remaining pulp tissue. If so, finding the best dosage and carrier will be crucial before rAmbn can be used as a biologically active endodontic material.

The indication that Ambn can enhance reparative dentin formation suggests that this protein plays an important role during pulpal healing and links it firmly to odontoblast differentiation and function. Ambn thus might have a potential clinical use in inducing reparative dentin formation in traumatized teeth as a biological alternative to conventional calcium hydroxide-based materials.

Acknowledgments. The DSP antibody was a kind gift from Dr. H. H. Ritchie. We also thank E. Lundberg, H. Ekdahl, and U.-B. Carlsson for excellent assistance during the animal procedures and B. Fabi for kind help with photographs and figures. The work was sponsored by EU grant QLK3-CT-2001-00090 MATRIX and Japan Society for Promotion of Science (JSPS) grant in scientific research (C) 16591921.

References

1. Yamamura T (1985) Differentiation of pulpal cells and inductive influences of various matrices with reference to pulpal wound healing. *J Dent Res* 64:530–540
2. Ranly DM (1994) Pulpotomy therapy in primary teeth: new modalities for old rationales. *Pediatr Dent* 16:403–409
3. Ranly DM, Garcia-Godoy F (2000) Current and potential pulp therapies for primary and young permanent teeth. *J Dent* 28:153–161

4. Tziafas D, Smith AJ, Lesot H (2000) Designing new treatment strategies in vital pulp therapy. *J Dent* 28:77–92
5. Nakashima M (1994) Induction of dentin formation on canine amputated pulp by recombinant human bone morphogenetic proteins (BMP)-2 and -4. *J Dent Res* 73:1515–1522
6. Rutherford RB, Wahle J, Tucker M, Rueger D, Charette M (1993) Induction of reparative dentin formation in monkeys by recombinant human osteogenic protein-1. *Arch Oral Biol* 38:571–576
7. Nakashima M (1989) Dentin induction by implants of autolyzed antigen-extracted allogeneic dentin on amputated pulps of dogs. *Endod Dent Traumatol* 5:279–286
8. Termine JD, Belcourt AB, Christner PJ, Conn KM, Nysten MU (1980) Properties of dissociatively extracted fetal tooth matrix proteins. I. Principal molecular species in developing bovine enamel. *J Biol Chem* 255:9760–9768
9. Nakamura Y, Hammarström L, Lundberg E, Ekdahl H, Matsumoto K, Gestrelus S, Lyngstadaas SP (2001) Enamel matrix derivative promotes reparative processes in the dental pulp. *Adv Dent Res* 15:105–107
10. Nakamura Y, Hammarström L, Matsumoto K, Lyngstadaas SP (2002) The induction of reparative dentin by enamel proteins. *Int Endod J* 35:407–417
11. Nakamura Y, Slaby I, Matsumoto K, Ritchie HH, Lyngstadaas SP (2004) Immunohistochemical characterization of rapid dentin formation induced by enamel matrix derivative. *Calcif Tissue Int* 75:243–252
12. Cerny R, Slaby I, Hammarström L, Wurtz T (1996) A novel gene expressed in rat ameloblasts codes for proteins with cell binding domains. *J Bone Miner Res* 11:883–891
13. Krebsbach PH, Lee SK, Matsuki Y, Kozak CA, Yamada KM, Yamada Y (1996) Full-length sequence, localization, and chromosomal mapping of ameloblastin. A novel tooth-specific gene. *J Biol Chem* 271:4431–4435
14. Hu CC, Fukae M, Uchida T, Qian Q, Zhang CH, Ryu OH, Tanabe T, Yamakoshi Y, Murakami C, Dohi N, Shimizu M, Simmer JP (1997) Sheathlin: cloning, cDNA/polypeptide sequences, and immunolocalization of porcine enamel sheath proteins. *J Dent Res* 76:648–657
15. MacDougall M, Simmons D, Gu TT, Forsman-Semb K, Mardh CK, Mesbah M, Forest N, Krebsbach PH, Yamada Y, Berdal A (2000) Cloning, characterization and immunolocalization of human ameloblastin. *Eur J Oral Sci* 108:303–310
16. Uchida T, Murakami C, Wakida K, Dohi N, Iwai Y, Simmer JP, Fukae M, Satoda T, Takahashi O (1998) Sheath proteins: synthesis, secretion, degradation and fate in forming enamel. *Eur J Oral Sci* 106(suppl 1):308–314
17. Fong CD, Slaby I, Hammarström L (1996) Amelin: an enamel-related protein, transcribed in the cells of epithelial root sheath. *J Bone Miner Res* 11:892–898
18. Fong CD, Cerny R, Hammarström L, Slaby I (1998) Sequential expression of amelin gene in mesenchymal and epithelial cells during odontogenesis in rats. *Eur J Oral Sci* 106(Suppl 1):324–330
19. Spahr A, Lyngstadaas SP, Slaby I, Haller B, Boeckh C, Tsoulfidou F, Hammarström L (2002) Expression of amelin and trauma-induced dentin formation. *Clin Oral Investig* 6:51–57
20. Ritchie H, Wang L-H (1998) The expression of DSP protein from a baculovirus construct containing rat DSP-PP cDNA. *J Dent Res* 77:157
21. Van Ittersum J, Ritchie H (2002) Post-translational analysis of rat dentin phosphoporyn from dentin matrix and a DSP-PP construct. *J Dent Res* 81:A278
22. Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 29:577–580
23. Butler WT (1995) Dentin matrix proteins and dentinogenesis. *Connect Tissue Res* 33:59–65