Ectopic Expression of Dentin Sialoprotein during Amelogenesis Hardens Bulk Enamel*

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Dentin sialophosphoprotein (Dspp) is transiently expressed in the early stage of secretory ameloblasts. The secretion of ameloblast-derived Dspp is short-lived, correlates to the establishment of the dentinoenamel junction (DEJ), and is consistent with Dspp having a role in producing the specialized first-formed harder enamel adjacent to the DEJ. Crack diffusion by branching and dissipation within this specialized first-formed enamel close to the DEJ prevents catastrophic interfacial damage and tooth failure. Once Dspp is secreted, it is subjected to proteolytic cleavage that results in two distinct proteins referred to as dentin sialoprotein (Dsp) and dentin phosphoprotein (Dpp). The purpose of this study was to investigate the biological and mechanical contribution of Dsp and Dpp to enamel formation. Transgenic mice were engineered to overexpress either Dsp or Dpp in their enamel organs. The mechanical properties (hardness and toughness) of the mature enamel of transgenic mice were compared with genetically matched and age-matched nontransgenic animals. Dsp and Dpp contributions to enamel formation greatly differed. The inclusion of Dsp in bulk enamel significantly and uniformly increased enamel hardness (20%), whereas the inclusion of Dpp weakened the bulk enamel. Thus, Dsp appears to make a unique contribution to the physical properties of the DEJ. Dsp transgenic animals have been engineered with superior enamel mechanical properties.

Until recently, it was believed that ectoderm-derived ameloblasts and ectomesenchyme-derived odontoblasts only expressed matrix proteins that were exclusive to themselves. But it is now known that there is also a transient expression of dentin sialophosphoprotein (Dspp)2 in early ameloblasts, adjacent to the dentinoenamel junction (DEJ) (1–6). We propose that Dspp has a role in producing the specialized first-formed harder enamel adjacent to the DEJ. Such a localized spatio-temporal pattern of Dspp protein expression may contribute to remarkable crack-resistant properties of the DEJ.

Specialized highly mineralized first-formed enamel, and specialized tubule-rich mantle dentin have long been known to be present close to the DEJ interface (7–9). Although there is an abrupt transition between dentin and enamel tissues at the DEJ interface (10–13), it is now known that the DEJ area functions as a broad mechanical zone (14–17). Crack diffusion by branching and dissipation within the specialized first-formed enamel close to the DEJ prevents catastrophic interfacial damage and tooth failure (18, 19).

We tested the prediction that overexpression of Dspp proteins throughout enamel formation would produce altered enamel material properties. In this study, we have examined the mechanical properties of enamel of transgenic mice that overexpress either Dsp or Dpp in ameloblasts within their enamel organs.

EXPERIMENTAL PROCEDURES

Our strategy was to ascertain the functions of selected known proteins acting during enamel biomineralization by overexpressing them in quantity and time so that a localizable and quantifiable functional change could be measured and related to the specific protein. Expressed under the control of the amelogenin promoter (5), the relationship between overexpression of specific proteins, Dsp and Dpp, and the biomechanical function of enamel and the DEJ was evaluated by measuring and comparing specific DEJ failure modalities and interfacial fracture toughness, and bulk enamel properties among normal nontransgenic (wild-type) mice, transgenic mice overexpressing Dsp, and transgenic mice overexpressing Dpp. The null hypothesis is that there is no difference in the DEJ interfacial fracture toughness, DEJ failure mechanisms, bulk enamel hardness, bulk enamel fracture toughness, and in bulk dentin hardness among these three mouse types. The null hypothesis was tested quantitatively by microindentation using analysis of variance (p < 0.05) to compare group means (n = 14 specimens with the mean of 10 repetitions to describe each tested parameter per specimen) with the Tukey multiple range test being applied to those situations where differences were revealed among groups discerned by the analysis of variance. Qualitatively comparisons were made using light, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images.

Animals—Dsp and Dpp transgenic mice were engineered as described previously (5, 20, 21). Breeding of the transgenic lines...
Dentin Sialoprotein Hardens Enamel

was not affected by the enamel phenotype. The diet was constant for both nontransgenic and transgenic animals. Transgenic animals developed normally, and there was no evidence of malnutrition.

All transgenic animals used in these studies were taken from the ninth or higher generation after inbreeding and were assumed homozygotes for the transgene. For all experimental procedures, age-matched, nontransgenic animals were taken from the same breeding stock (earlier generations) and used as controls. No gross abnormalities were detected in the dentition of any of the Dsp transgenic mouse lines at the time of the eruption of the incisor or molar teeth. No gross abnormalities were observed for the molar or the incisor teeth of Dsp transgenic animals at six weeks of age. All Dsp mice included in this study were from animal lines 214, 244, or 245 (5) with equivalent sampling taken from each.

Our previous studies (5) have shown that the Dpp transgenic animal line 47 had an order of magnitude greater copy number of the transgene incorporated into the genome; this when compared with lines 10, 13, 46, and 53. As a result, common traits in these Dpp line 47 transgenic animals (traits not observed in Dpp lines 10, 13, 46, and 53) were delayed molar tooth eruption, pitted enamel, excessive enamel wear, and frequent enamel fracture (5). The Dpp mice included in this study were primarily from animal lines 46 and 53 with equivalent sampling from each. A few representative specimens from Dpp line 47 were collected, and the erupted, mature enamel region was selected for analysis. The images shown are representative of all samples analyzed.

Macrostructural Anatomic Form—The wild-type and Dsp overexpressing mice were indistinguishable in anatomic form (Fig. 1). However, the Dsp overexpressing mice tended to exhibit slightly fewer wear facets on their mesial surfaces when viewed in cross-section (Fig. 1). In contrast, the Dpp overexpressing mouse teeth showed substantial disruption in macrostructure and some disruption in microstructure (Fig. 1). Often, the Dpp enamel was grossly thinner and of an irregular form.

To determine whether potential enamel changes were localized to specific areas, or they were uniformly distributed, microhardness profiles from outer enamel to the pulp chamber area were created. Microindentations were made at 100 sites across the profiles of 10 teeth of each type from the outer enamel surface of each specimen to the dentin adjacent to the pulp chamber area. Hardness (y) was plotted against distance from the DEJ (x) and best-fit lines for bulk enamel and bulk dentin identified using linear regression. Data from indentations centered within 15 μm of the DEJ interface were plotted, but was excluded from regression analysis, because these indentations represented both dentin and enamel tissues.

The DEJ zone toughness indentations were made close to the optical DEJ, so that the characteristic radial-medial cracks would be oriented parallel and perpendicular to the interface, but oblique to the enamel rods and dentin tubules, and that the distance from the center of the indentation impression to the optical interface was less than the characteristic impression dimension. The DEJ zone fracture toughness, Kz, was calculated as

\[ K_z = \frac{\gamma P}{e^{1.5}} \]  

(Eq. 1)

where γ is a fitting constant, P is the indenter load, and e is half the length of the interfacial de-bond, and/or cracks within the DEJ zone measured in a plane parallel to the DEJ (18, 23). The solution represented by this equation only represents an approximation of a complex situation in a non-uniform anisotropic bimaterial substrate, and its use here is intended for comparative purposes rather than absolute values (24, 25). This method provided identification of different failure mechanisms, with cracks of comparable scale to the intrinsic dental microstructure on a cellular scale, as well as quantitative measurement of DEJ zone toughness.

Microscopy—The light microscopy techniques described above were used to view indentations, cracks, and structure in teeth from 25 animals of each type.

Thin sections from four days postnatal lower incisor teeth from the two independent lines of Dsp transgenic mice were prepared for TEM analysis. Age-matched nontransgenic control animals taken from the same breeding stock were also prepared for TEM analysis. Methodology for sample preparation and imaging by TEM was previously reported and was followed without modification (5, 26, 27). For each sample, at least ten representative fields were viewed and photographed for analysis. The images shown are representative of all samples analyzed.

Six-week (42-day postnatal) lower incisor teeth were collected, and the erupted, mature enamel region was selected for analysis. SEM images were made on coronally fractured, gold palladium sputter-coated teeth from six transgenic and six wild-type (control) animals as published previously (5, 28). The images shown are representative of all samples analyzed.

RESULTS

Macrostructural Anatomic Form—The wild-type and Dsp overexpressing mice were indistinguishable in anatomic form (Fig. 1). However, the Dsp overexpressing mice tended to exhibit slightly fewer wear facets on their mesial surfaces when viewed in cross-section (Fig. 1). In contrast, the Dpp overexpressing mouse teeth showed substantial disruption in macrostructure and some disruption in microstructure (Fig. 1). Often, the Dpp enamel was grossly thinner and of an irregular form.
Dentin Sialoprotein Hardens Enamel

Especial care was taken during extraction and preparation so as not to cause damage before testing or imaging. It is likely that routine mastication altered the enamel from teeth of transgenic Dpp animals prior to extraction. Some of the Dpp teeth had insufficient enamel to permit microindentation, so additional specimens were added; thus the Dpp mechanical data (Table 1) were based upon necessarily biased samples. Likewise, the irregular and variable thickness precluded meaningful microhardness profiling.

Fine Structure—The wild-type and Dsp overexpressing mice were indistinguishable in structural organization as measured by representative light, SEM, and TEM microscopy (Figs. 1–3). Both types of mice exhibited clearly defined crystallites, rods, and normal decussation patterns produced by alternating layers of rods. In contrast, as described previously (5), the Dpp overexpressing mice exhibited less clearly defined microstructural elements, including large areas where rods and decussation patterns were not clearly defined and the most severely affected areas exhibiting an amorphous appearance (Fig. 1).

Enamel Hardness and Toughness—Differences were discerned in enamel hardness among all three mouse types (Table 1). Remarkably, overexpression of Dsp produced enamel harder than that of the wild-type mouse. Wild-type and Dsp overexpressing enamel were of equivalent fracture toughness. However, wild-type and Dsp overexpressing specimens were significantly tougher than Dpp overexpressing specimens (Table 1).

![FIGURE 1. Polarized light micrographs of mature four-week-old wild-type and transgenic mouse lower incisor cross-sections.](Image)

**TABLE 1**

| Mouse type | Enamel hardness (GPa) | ENAMEL TOUGHNESS (MPa) | DENTIN HARDNESS (GPa) | DEJ zone toughness (MPa) |
|------------|------------------------|-------------------------|-----------------------|-------------------------|
| Wild type  | 2.1 ± 0.3              | 1.3 ± 0.1               | 0.42 ± 0.04           | 0.9 ± 0.1               |
| Dsp        | 2.5 ± 0.2              | 1.4 ± 0.2               | 0.44 ± 0.04           | 0.9 ± 0.2               |
| Dpp        | 1.8 ± 0.3              | 1.0 ± 0.2               | 0.41 ± 0.04           | 0.4 ± 0.2               |

ANOVA:

| Mouse type | p value | ANOVA p value |
|------------|---------|--------------|
| Wild type  | <0.0001 | <0.0001      |
| Dsp        |         |              |
| Dpp        |         |              |

* Similar data are linked by vertical lines of *s, as determined by Tukey multiple comparisons testing (p ≤ 0.05).

**Dentin Hardness**—Differences in dentin hardness were not discerned among mouse types (Table 1). This finding was consistent with normal Dspp expression occurring through all stages of dentinogenesis; the wild-type mouse serving as an appropriate control; and the overexpression in enamel being driven by the amelogenin gene promoter (5).

**Hardness Profiling**—The best-fit lines for wild-type and Dsp dentin hardness profiles respectively were

\[ y = 0.0013x + 0.67 \]  
(Eq. 2)

and

\[ y = 0.0012x + 0.66 \]  
(Eq. 3)

Their \( R^2 \) values were 0.22 and 0.26, respectively. These profiles were almost indistinguishable with respect to slope, intercept and scatter (Fig. 4). Overall, dentin hardness tended to decrease from DEJ toward pulp.

The best-fit lines for wild-type and Dsp enamel hardness profiles, respectively, were

\[ y = 0.0023x + 1.74 \]  
(Eq. 4)

and

\[ y = 0.0024x + 2.12 \]  
(Eq. 5)

Their \( R^2 \) values were 0.008 and 0.009, respectively; enamel indentations produced substantially more scatter than dentin indentations (Table 1, Fig. 4). The enamel profiles were almost indistinguishable with respect to slope and scatter. However, consistent with the data displayed in Table 1, they differed substantially with respect to intercept, a surrogate for overall hardness, or by 0.38 GPa (Equations 4 and 5). The Dsp enamel was uniformly harder than the wild-type enamel throughout its thickness, consistent with steady levels of overexpression during maturation.

**DEJ Zone**—Wild-type and overexpressing Dsp mouse DEJ zones were of equivalent fracture toughness. However, they were significantly tougher, over 2-fold, than that of the Dpp overexpressing mouse (Table 1). Mouse DEJ zone toughness tended to be less than that of their respective bulk enamel toughness (Table 1). This finding does not represent interfacial delamination, but toughness of the broader DEJ zone, notably including the first-formed enamel. The dominant DEJ zone failure mechanism involved damage distribution within the first-formed enamel close to the DEJ interface in all three animal types, as described previously in humans (3, 4). Enamel cracks generally approached the DEJ obliquely. Most enamel cracks were arrested at the DEJ interface. Many enamel cracks were arrested at the DEJ interface.
cracks appeared to follow rod/interrod organization. Interfacial DEJ delamination was an extremely rare event.

DISCUSSION

Increased enamel hardness produced by the overexpression of Dsp has intriguing clinical implications. Hardness is a commonly used surrogate for wear resistance and for caries resistance. Overexpression of Dsp in an artificial reparative enamel, in engineered bulk enamel, or in complete engineered teeth may have the potential to produce more disease-resistant teeth. Caries ranks only second to periodontal disease as a cause of tooth loss. Likewise, caries is the most common infectious disease to affect humanity. Even if affected teeth are not lost, caries is the dominant reason for restorative interventions and the dominant cause of pulpal pathology. Enamel wear is generally considered to be a physiologic, not a pathologic process. However, undue enamel wear in itself is considered to be unesthetic, and exposed dentin tends to become stained much more quickly than when protected by enamel. Many patients seek dental treatment to replace worn enamel. Others affected by disorders such as bruxism and acid regurgitation are profoundly affected by severe dental wear and erosion.

Murine enamel is only half as hard as its primary component, crystalline hydroxyapatite (22). Therefore, like other mammalian enamels, it has the potential to be much harder. The hardening of Dsp enamel could be a manifestation of increased mineral density or of decreased plasticization. The measurement of the sizes, aspect ratios, and packing densities of crystallites in mature wild-type and Dsp enamel or of the quantification of plasticizers such as water and proteinaceous remnants is fraught with difficulty and has not yet been attempted.

During evolution of rodent enamel, it is possible that a harder enamel could be achieved by altering the timing and position of Dsp expression, for example, or other proteins affecting enamel hardness. Mice thus have the potential to have harder enamel by regulating the expression of Dspp-derived gene products. However, harder enamel may not produce an evolutionary advantage in mice. Interestingly, the mouse incisor appears to

FIGURE 2. SEM images of mature lower incisor enamel close to the DEJ for six-week-old wild-type and Dsp transgenic animals. The enamel (E), dentin (D), and DEJ (***) of wild-type (panels A and C) and Dsp transgenic (transgenic animal line 244; panels B and D) teeth were fractured in cross-sections and were studied by SEM. Wild-type and Dsp transgenic teeth showed identical morphology. Scale bar for panels A and B (as shown in panel B) is 100 μm and for panels C and D (as shown in panel D) is 20 μm.

FIGURE 3. TEM images of transition zone incisor enamel close to the DEJ for four days postnatal wild-type and Dsp transgenic animals. The forming enamel, dentin, and DEJ of wild-type (panel A) and Dsp transgenic teeth (panel B) were studied by TEM and showed an identical microstructure. Rod (R) and interrod (IR) enamel is labeled, as is the DEJ (***). An enamel rod sectioned longitudinally is identified with arrows in each panel; all other labeled rods are seen in cross-section. Image shown for Dsp tooth is from line 244. Scale bar is 2 μm.

FIGURE 4. Hardness profiles of the mature regions of four-week-old wild-type, and Dsp teeth. Enamel indentation profiles were almost indistinguishable with respect to slope and scatter; however, they differed substantially with respect to intercept or hardness. This pattern was indicative of even levels of Dsp expression throughout enamel formation. Overall, enamel hardness tended to increase from the DEJ toward the outer surface. Enamel produced substantially more scatters than dentin. Dentin profiles were almost indistinguishable with respect to slope, intercept, and scatter. Overall, dentin hardness tended to decrease from DEJ toward pulp.
be designed to wear rapidly as part of a self-sharpening mechanism. Sheets of rods approach the incisal edge at such an angle so as to be sheared off to produce a chisel-like blade (28, 29). Harder enamel might actually interfere with this self-sharpening mechanism. Continuous eruption of rodent incisors compensates for incisal wear. Additionally, the life spans of mice are fleeting by brief in comparison to those of primates. Wild mice are unlikely to eat much refined carbohydrate, a necessary condition for caries.

Mouse teeth differ from human teeth in many ways. Notably, human enamel possesses both large and small angles of decussation as well as Hunter-Schreger bands, whereas murine enamel possesses just a single large angle of decussation. The larger scale of fiber interweaving in human enamel may be designed to better arrest long crack growth in thick human enamel, whereas the more abrupt differences in rod interweaving between adjacent layers may be designed to better resist short crack growth in the much thinner murine enamel. Profiling of human teeth has discerned the presence of specialized harder first-formed enamel and of softer first-formed mantle dentin on either side of the DEJ interface (16). The presence of specialized DEJ-adjacent enamel and dentin has also been supported by microscopy, microradiography, and by Moire and laser interferometric analyses (14, 15, 17). However, such specialized near-DEJ features were not obvious in the microhardness profiles of murine teeth in this study (Fig. 4). However, the TEM images do not exclude the presence of a thin layer of specialized enamel directly adjacent to the DEJ interface (Fig. 3). Identification of proportionately thin specialized murine tissues would have been beyond the resolution by the microrindentation technique used here. Certainly, DEJ interfacial delamination was an extremely rare occurrence in both wild-type and Dsp overexpressing animals, and the broad DEJ zone was of comparable toughness to the humans (18).

Overexpression of Dpp produced an enamel that was slightly softer than wild-type, 0.9-fold, but much more brittle, 0.4-fold. This suggests that the difference between the wild-type and the Dpp overexpressing enamels was not simply because of decreased mineral content, but also because of enamel organization. Certainly, the overexpressing Dpp enamel had less clearly defined rod organization (5) (Fig. 1), but defects at a scale smaller than that of rod organization may also have been present but were not observed. Microrindentation of the last-formed outer enamel was fraught with technical difficulty and was not attempted in this study. The very thin outer enamel is difficult to test because of the confounding effects of nearby bulk enamel and nearby mounting medium. Similarly, the proteinaceous matrix of the last-formed enamel and its genetic control is little understood and has received little attention. What is apparent from animal studies is that Dsp expression in ameloblasts is silent during bulk enamel formation and is not known to be expressed during formation of the outer enamel (3, 4, 30).

Several recent investigations of the DEJ have used microrindentation and nanoindentation to measure dental hardness and fracture toughness while circumventing problems associated with conventional macromechanical testing (10–13, 16, 18, 19, 22, 31, 32). The indentation fracture technique probes the process of subcritical damage evolution and crack propagation at the scale of the microstructural components of enamel, rods. Unlike many prior investigations that used fracture toughness techniques appropriate for homogenous materials, the present investigation used a technique specifically designed to evaluate interfaces (18, 23). Microhardness measurements discerned substantially more scatters in brittle enamel than in tougher dentin (Fig. 4). This could be because of substantive differences in behavior between mechanically dissimilar substrates, intrinsically larger microstructural variation in enamel, slightly larger indentation footprints in dentin averaging local differences, or to experimental error; however, a similar pattern was previously found in human incisor profiles (16). It appears from the scatter in the microhardness profiles (Fig. 4) that mouse enamel shows the greatest variation near its inner and outer surfaces and the least variation in its central bulk areas. This may be because of intrinsic properties of specialized first and last-formed enamels or to confounding effects of nearby dentin and mounting resin.

The dominant DEJ zone failure mode was by cracking, with deflection and branching, distributed within the first-formed enamel, consistent with prior work (18). In this current study, a murine DEJ zone fractures toughness of $0.9 \text{ MPa m}^{1/2}$ was measured. It is important to note that this value is approximate because the multiple and complex cracking modes and non-uniform substrates render the application of Equation 1 imprecise (23–25). However, it is consistent with human DEJ zone data using similar microrindentation methodology (18) and with near-DEJ human enamel nanoindentation toughness of $0.6–0.9 \text{ MPa m}^{1/2}$ (11). Macromechanical tests have reported higher values from $1.5 \text{ MPa m}^{1/2}$ to $3.4 \text{ MPa m}^{1/2}$, but some of these higher values may actually have described dentin adjacent to the DEJ, rather than the DEJ itself (33–36). Study of DEJ strain energy release rates suggest that the actual DEJ interface toughness is much higher than that of enamel, but lower than that of dentin (19).

In this current study, a mean murine incisal enamel fracture toughness of $1.3 \text{ MPa m}^{1/2}$ was measured for 14 wild-type incisor teeth (Table 1). This value is broadly consistent with the value of $0.95 \text{ MPa m}^{1/2}$ reported for pooled data from four assorted human teeth (37), the range of 0.52 to 1.30 MPa m$^{1/2}$ reported for axial sections of four human third molars (31), and a range of 0.9 to 1.3 MPa reported for human incisors (22). However, direct comparison should only be made with the erupted mature incisal enamel of 6-week mice.

The murine Dspp gene produces at least two structurally and functionally distinct proteins following posttranslational proteolytic cleavage, Dsp and Dpp. The N- and C-terminal regions of Dsp and Dpp have been defined previously in rats (38) and extrapolated for mice (5). More recently, a third unique product from the DSPP gene has been described in pig (6). This third region is glycosylated and has been called dentin glycoprotein (6). In our transgenic animal lines described in this and a previous paper (5), the entire dentin glycoprotein region would be included as the C-terminal 81 amino acids of the Dsp transgene. These protein regions for rodents compared with pig are discussed and illustrated in Yamakoshi et al. (6).

Unique proteins encoded by the Dspp gene could be used to produce specialized dentins as well as enamels. Currently, dif-
ferences among dentin types are generally attributed to differing patterns of tubule orientation, density, and organization, as well as to localized changes in the collagenous matrix. However, the expression profiles for minor proteins may also play roles in defining specific dentin types and their materials properties.

In conclusion, Dsp and Dpp contributions to enamel formation greatly differed. The inclusion of Dpp in bulk enamel significantly weakened enamel. The inclusion of Dsp in bulk enamel significantly and uniformly increased enamel hardness. Dsp is normally only expressed in the inner first-formed enamel layer while the DEJ is being formed. Dsp thus appears to make a unique contribution to the physical properties of the DEJ. In this study, we demonstrated that ectopic expression of Dsp hardened bulk enamel. Transgenic animals have not previously been used to engineer hard dental tissues superior to those found in comparable wild-type animals.

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