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Increased Young's Modulus and Hardness of *Col1a2*^{oim} Dentin

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Abstract

Mice harboring the *Col1a2*^{oim} mutation (*oim*) express dentinogenesis imperfecta. To determine the effect of *Col1a2* genotype on tissue mechanical properties, we compared Young's modulus and hardness of dentin in the 3 *Col1a2* genotypes. Upper incisors were tested by nanoindentation. Genotype had a significant effect on Young's modulus, but there was not a simple mutant allele dosage relationship. The effect of genotype on hardness did not reach significance. Hardness and Young's modulus were greater near the dento-enamel junction than near the pulp chamber. Greater hardness and Young's modulus values near the dento-enamel junction reflected continued mineralization of the dentin following its initial synthesis. Analysis showed the mechanical data to be consistent with Fourier transform infrared and backscattered electron microscopy studies that revealed increased mineralization in *oim* bone. Analysis of the data suggests that clinical fragility of teeth in *oim* mice is not due to deficiencies of hardness or Young's modulus, but may be due to defects in post-yield behavior or resistance to fatigue damage.

Keywords

collagen type I; dentinogenesis imperfecta; osteogenesis imperfecta; biomechanics; tooth calcification

Introduction

Osteogenesis imperfecta, also known as brittle bone disease, is an inherited clinical syndrome marked by skeletal fragility and associated abnormalities (reviewed by Rowe and Shapiro, 1998; Byers and Cole, 2002; Whyte, 2003). Nearly all cases are caused by mutations of the genes encoding type I collagen. In the most severe cases, osteogenesis imperfecta is lethal *in utero*, while in the mildest cases it is subclinical (Dalgleish, 1997, 1998, 2004). The associated abnormalities include dentinogenesis imperfecta, skin fragility, hearing loss, scleral discoloration, and other manifestations of connective tissue fragility. These varied clinical manifestations reflect the abundance of type I collagen in various connective tissues, including teeth, skin, ligaments, fasciae, and tendons.

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Classic features of dentinogenesis imperfecta include discoloration of teeth, increased tooth fragility, and obliteration of the pulp chambers. Histological and ultrastructural abnormalities reported in human dentinogenesis imperfecta include a decreased number of irregularly shaped dental tubules, remnants of capillary inclusions, obliterated pulps, morphological abnormalities of dental collagen fibers, and mineralization defects (Lukinmaa *et al.*, 1987; Lygidakis *et al.*, 1996; Waltimo *et al.*, 1996; Hall *et al.*, 2002). Not all dentinogenesis imperfecta is associated with osteogenesis imperfecta, however, since mutations in dentin sialophosphoprotein can also cause a syndrome with similar clinical manifestations (Xiao *et al.*, 2001; Zhang *et al.*, 2001).

The *Col1a2*^{oim} (*oim*) mutation is a widely studied model of chain deficiency osteogenesis imperfecta. The mutation is a 1 base deletion in the gene encoding the α2 chain of type I procollagen (Chipman *et al.*, 1993), resulting in premature termination of the nascent peptide and nonsense-mediated degradation of the message. Homozygous mutant (*oim/oim*) animals display marked skeletal fragility, suffering spontaneous fractures with usual cage activity and husbandry-related handling (Chipman *et al.*, 1993; Camacho *et al.*, 1999). Heterozygous (*oim*/+) animals have a more subtle biomechanical impairment, appearing grossly normal but with diminished performance on three-point bend and torsion testing (Saban *et al.*, 1996; Camacho *et al.*, 1999). Moreover, homozygous *oim/oim* teeth are fragile, so that our standard husbandry conditions for these animals include the provision of powdered food.

Type 1 collagen is the major structural protein in dentin as well as bone, so we reasoned that mice harboring the *oim* mutation should exhibit a dental phenotype. We previously described the morphological and ultrastructural features of *oim/oim* and *oim*/+ teeth (Lopez Franco *et al.*, 2005). In this study, we extend our previous work by performing nanoindentation of teeth from animals having the +/+, *oim*/+, and *oim/oim Col1a2* genotypes, addressing the question of whether the mutation causes a measurable alteration of dentin material properties. We hypothesized, based on previous experimental evidence of an increased mineral:matrix ratio and decreased post-yield performance in *oim/oim* bone, that Young's modulus would be increased in the mutant teeth in a gene-dosage-dependent fashion. Further, we expected that dentin near the dento-enamel junction would have greater Young's modulus than that near the pulp chamber, reflecting the longer time since its synthesis and deposition.

Materials & Methods

Mice

Mice used in this study were male and female offspring of *oim*/+ parents and were genotyped at *Col1a2* at weaning by direct sequencing (Camacho *et al.*, 1998). Animals were maintained in a 12-hour light/12-hour dark photoperiod, were fed powdered, irradiated PICO 5058 rodent chow (Purina, St. Louis, MO, USA) and autoclaved tap water *ad lib*, and were housed in groups of up to 5 mice/500 cm^2 cage between weaning and death at 11 wks of age. Following death by CO_2 asphyxiation, heads were stored frozen at -20°C until teeth were dissected free of alveolar bone for study. Three teeth of each genotype, each taken from a different mouse, were studied. This work satisfied the Hospital for Special Surgery's requirements for the ethical use of laboratory research animals.

Nanoindentation

We studied 3 mice *per* genotype and 1 tooth *per* mouse. Upper incisors were fractured at the level of the junctional epithelium, and nanoindentation was performed at 2 sites (Fig.). The “far from pulp” region was chosen to be in proximity to the dento-enamel junction, while the “near the pulp” site was chosen to be in proximity to the pulp chamber, but beyond the border of the predentin. Following polishing, we performed at least 3 indents/site/tooth. Loading was

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applied with the Hysitron (Minneapolis, MN, USA) triboscope apparatus and a diamond Berkovitch tip to a maximum of 3000 μN, with a five-second hold before unloading. We analyzed the data according to the Oliver and Pharr method (Oliver and Pharr, 1992), as applied by the instrument software to the 95%-50% unloading limb of the load-deformation curves. We calibrated the instrument and determined machine compliance with a fused quartz standard. Young's modulus (E) and Meyer hardness (H) were calculated from the raw data.

Statistics

Data are shown as mean ± standard error of the mean, with ranges given in parentheses. We averaged the hardness and Young's modulus values for each location within a single tooth and performed statistical analysis by two-way (genotype and location) repeated-measures ANOVA. Genotype had 3 strata, +/+, *oim*/+, and *oim/oim*; while location had 2 strata, near the dento-enamal junction and near the pulp chamber. We used Tukey's test to perform *post hoc* comparisons between groups.

Results

Regardless of genotype, hardness was greater near the dento-enamel junction than near the pulp chamber, with $F = 23.33$ and $p = 0.0029$ (Table 1). Genotype was not significant, with $F = 1.08$. *Post hoc* analysis by Tukey's test revealed that only the *oim*/+ genotype displayed a significant difference between locations ($p = 0.0052$). The same qualitative pattern held for the other genotypes, but fell short of statistical significance. Similarly, greater hardness in mutant teeth apparently did not reach significance.

A somewhat different pattern held for Young's modulus, where both genotype and location were significant, with $F = 5.72$, $p = 0.041$ and $F = 7.94$, $p = 0.030$, respectively (Table 2). There was no evidence of a significant interaction between genotype and location, with $F = 3.41$. *Post hoc* testing revealed a significant difference between locations only for the *oim*/+ genotype ($p = 0.0096$). Near the dento-enamel junction, the +/+ and *oim*/+ genotypes differed significantly ($p = 0.016$). Near the pulp chamber, Young's modulus did not differ significantly among genotypes.

Discussion

Although it may seem counterintuitive that Young's modulus should be greater in animals harboring the *oim* mutation than in wild-type controls, the observed behavior is nevertheless consistent with our understanding of pathophysiology. In bone, the principal defect in osteogenesis imperfecta is an increase in brittleness. The brittleness aspect of mechanical performance was not directly evaluated in nanoindentation testing, because there were no cracks around the indents that might otherwise have helped us to estimate the fracture toughness (Lawn, 1993). Instead, the nanoindentation experiments were restricted to examination of hardness and Young's modulus. Higher hardness is usually associated with greater brittleness, especially when a comparison is made among composites that differ in the amount of the hard, brittle “reinforcement” phase. When the hard phase also has a higher modulus than the soft “matrix” phase, then there should be a correlation between the brittleness and Young's modulus as well.

Young's modulus in a composite material (E_c) such as bone or dentin is estimated to lie between the sum of the component materials' Young's moduli (E_i),

$$E_c = \sum (E_i)(V_i)$$

and the reciprocal of the sum of the reciprocals of the component materials' Young's moduli,

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$$1 / E_c = \Sigma (1 / E_i)(V_i)$$

weighted according to the volume fraction (V_i) of each component in the composite.

It is instructive to consider the ranges of moduli that would be predicted in *oim* based on these simple assumptions. In previous work, we demonstrated a 20% decrease in *oim/oim* bone collagen content relative to +/+ and an intermediate collagen content in heterozygotes (Camacho *et al.*, 1999), and regardless of genotype, mineral comprised approximately 65% to 70% of the dry mass. Young's modulus of apatite is ~110 GPa (Walsh and Christiansen, 1995), while that of collagen in compression is about 2 orders of magnitude less (Zioupos *et al.*, 1999), thus contributing negligibly to the Young's modulus of the composite. The theoretical maximum Young's modulus of dry dentin is therefore approximately 78 GPa, and the minimum Young's modulus is approximately 0.3 GPa. The measured values in our study fell in the middle of this range. Both wetting and the presence of other minerals with Young's moduli lower than that of apatite will lower measured Young's modulus of the composite dentin. In particular, calcium carbonate's Young's modulus is ~35 to 70 GPa, and it makes up approximately 1 to 2% of bone mineral. In tibiae, we previously found that carbonate in *oim/oim* animals was ~23% less than in either *oim*/+ heterozygotes or +/+ mice (Camacho *et al.*, 1999). Moreover, Phillips and colleagues, using neutron activation analysis, measured the concentrations of various minerals in femora and incisors of mice harboring the *oim* mutation (Phillips *et al.*, 2000), finding a mutant allele dosage-dependent decrease in Mg in teeth and an increase in F in heterozygotes relative to both homozygotes. The $CaCO_3$ and Mg concentrations were both low enough that their quantitative impact on Young's modulus would be small, but the qualitative impact of substituting Mg for Ca or carbonate for phosphate in the apatite crystal is unknown. In our earlier description of the *oim* dental phenotype, we observed a reduction in the number of dentinal tubules in a mutant-allele-dependent manner (Lopez Franco *et al.*, 2005). These contribute “empty space” to the dentin matrix, whose loss would be expected to increase Young's modulus. Notably, empty space in the matrix is not reflected in quantitative analyses of the matrix's composition. Reduced collagen and carbonate content are also possible contributing reasons for our observation that the *oim* mutation increased Young's modulus. In reality, behavior is still more complex, since each mechanism of loading —*i.e.*, compression, bending, tension, and torsion—will have an impact on the measured material properties of the composite.

Our results agree with those of Grabner *et al.* (2001), who found that Vickers hardness increased in embedded cortical bone in an *oim* dosage-dependent manner, ranging from ~0.6 GPa for +/+ to ~0.9 GPa for *oim/oim*. They also measured mineralization by backscattered electron microscopy, reporting that the mutation also increases mineralization, agreeing with our prior observation that the Fourier transform infrared (FTIR)-spectroscopy-determined mineral-to-matrix ratio was increased by the *oim* mutation (Camacho *et al.*, 1996). Our mechanical results also agree with our previous observation of greater Young's modulus in femoral cortical bone (Misof *et al.*, 2005). This is at variance with observations at larger scales (*e.g.*, by dual-energy x-ray absorptiometry or ash percentage) that reveal lower mineral density in osteogenesis imperfecta. This apparent discrepancy is most readily explained by the inclusion of non-mineralized tissue space in the larger-scale measurements, reflecting a decrease in mineralized tissue mass rather than a decrease in its degree of mineralization.

Our results also agree in part with those of Kinney and co-workers (Kinney *et al.*, 2003). These investigators studied human dentin in normal teeth and teeth from patients with a clinical diagnosis of dentinogenesis imperfecta type 2 (no evidence of osteogenesis imperfecta). Unlike our data, they found that the dentinogenesis imperfecta dentin had a lower, not a higher, Young's modulus than did normal dentin. The difference in the molecular lesions underlying dentinogenesis imperfecta in their samples and ours may account for this disparity. Their values

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for normal-dentin Young's modulus were consistent with ours and were greater at the dento-enamel junction and smaller close to the pulp chamber. They demonstrated that this reflected the degree of dentin mineralization as determined by synchrotron radiation CT scanning. Our observations regarding hardness were generally consistent with those of Kinney *et al.*, but went beyond them in considering the location dependence of hardness. The dentin at the dento-enamel junction has a greater tissue age than that adjacent to the pulp chamber, and therefore has had a longer period to undergo mineralization.

Our study has some important limitations that readers should keep in mind. First, we examined dentin only in compression, so material properties in other loading regimes were not addressed. Second, we examined only dry specimens in these experiments. Third, our sample sizes were small, limiting our ability to detect differences among genotypes and sites. This is particularly apparent with regard to hardness, where the observed differences among genotypes fell short of significance, as did the differences between locations in the homozygous genotypes. Fourth, we examined only a single age and a single osteogenesis imperfecta model, so the generality of our results to abnormal chain (in distinction to chain deficiency) forms of osteogenesis imperfecta cannot be assumed. The same limitation also holds for dentinogenesis imperfecta unassociated with osteogenesis imperfecta, and it is imperative that both osteogenesis imperfecta and dentinogenesis imperfecta be considered as clinical syndromes representing multiple disease states.

These limitations notwithstanding, we have demonstrated that both hardness and Young's modulus increase as a function of distance from the pulp chamber in all genotypes, consistent with continued mineralization following initial synthesis and secretion. It is well-established that the volumes of intertubular and peritubular dentin vary as a function of location in the tooth, with dentin near the dento-enamel junction containing a greater fraction of intertubular dentin (Pashley, 1989). Furthermore, intertubular dentin is more heavily mineralized than peritubular dentin. In this regard, it is interesting to note that recent evidence shows that collagen is present only in the intertubular dentin (Gotliv *et al.*, 2006), so that a change in the relative amounts of inter- and peritubular dentin as a result of the mutation could contribute to our observations. In animals harboring the *oim* mutation, Young's modulus was increased, but without a simple mutant allele dosage relationship. Tooth fragility in mice harboring the *oim* mutation cannot be explained by deficiencies of hardness or Young's modulus, and so defects in plastic behavior should be sought. Future progress in our understanding of the mechanical properties of dentin will benefit from the application of mechanical testing protocols that can address fatigue damage and the plastic regime.

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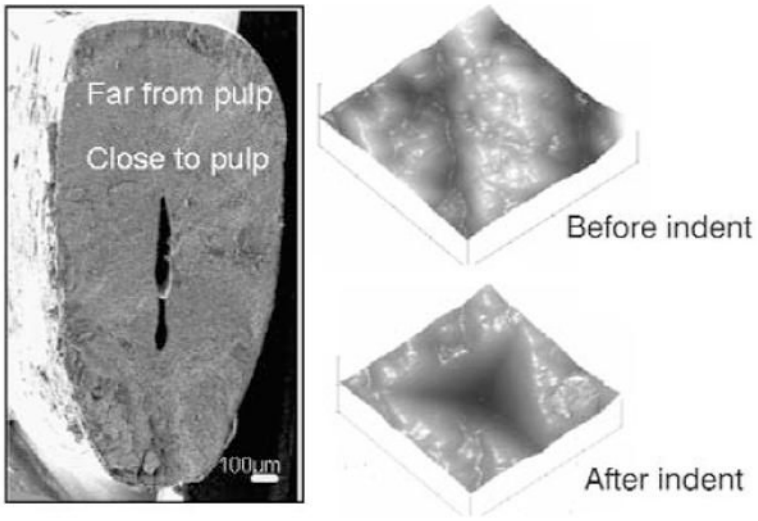

Figure.
Upper incisor showing nanoindentation sites and typical indent. 100-µm scale bar is shown in scanning electron micrograph of incisor. Sides of indent details are 2.0 µm.

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Table 1

Hardness of Dentin (GPa)

	+/+	*oim*/+[†]	*oim/oim*
Near dento-enamel junction[*]	1.5 ± 0.1 (1.3-1.8)	2.2 ± 0.6 (1.1-3.0)	1.7 ± 0.2 (1.5-2.1)
Near pulp chamber[*]	0.6 ± 0.1 (0.5-0.8)	0.5 ± 0.1 (0.4-0.6)	1.0 ± 0.1 (0.9-1.1)

[*] There is a significant difference according to location, F = 23.33 and p = 0.0029.

[†] Within the *oim*/+ genotype, hardness differs significantly by location, with p = 0.0052.

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Table 2

Young's Modulus of Dentin (GPa)

	+/+[†]	*oim*/+[†‡]	*oim/oim*[†]
Near dento-enamel junction[*§]	23 ± 1 (22-23)	37 ± 7 (24-45)	29 ± 1 (27-31)
Near pulp chamber[*]	19 ± 3 (17-24)	18 ± 2 (16-21)	26 ± 1 (25-27)

[*] There is a significant difference according to location, F = 7.94 and p = 0.030.

[†] There is a significant difference according to genotype, F = 5.72 and p = 0.041.

[‡] Within the *oim*/+ genotype, Young's modulus differs significantly by location, with p = 0.0096.

[§] Near the dento-enamel junction, *oim*/+ dentin has greater Young's modulus than +/+ dentin, with p = 0.016.