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ABSTRACT

Patients with amelogenesis imperfecta (AI) have defective enamel; therefore, bonded restorations of patients with AI have variable success rates. To distinguish which cases of AI may have good clinical outcomes with bonded materials, we evaluated etching characteristics and bond strength of enamel in mouse models, comparing wild-type (WT) with those having mutations in amelogenin (*Amelx*) and matrix metalloproteinase-20 (*Mmp20*), which mimic 2 forms of human AI. Etched enamel surfaces were compared for roughness by scanning electron microscopy (SEM) images. Bonding was compared through shear bond strength (SBS) studies with 2 different systems (etch-and-rinse and self-etch). Etched enamel surfaces of incisors from *Amelx* knock-out (*AmelxKO*) mice appeared randomly organized and non-uniform compared with WT. Etching of *Mmp20KO* surfaces left little enamel, and the etching pattern was indistinguishable from unetched surfaces. SBS results were significantly different when *AmelxKO* and *Mmp20KO* enamel surfaces were compared. A significant increase in SBS was measured for all samples when the self-etch system was compared with the etch-and-rinse system. We have developed a novel system for testing shear bond strength of mouse incisors with AI variants, and analysis of these data may have important clinical implications for the treatment of patients with AI.

KEY WORDS: enamel, acid-etching, amelogenesis imperfecta, shear bond strength, amelogenin, matrix metalloproteinase-20.

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The Use of Mouse Models to Investigate Shear Bond Strength in Amelogenesis Imperfecta

INTRODUCTION

Amelogenesis imperfecta (AI) is a group of inherited developmental disorders that affects the structure and appearance of dental enamel. Mutations in 4 genes, amelogenin (*AMELX*, OMIM 300391), enamelin (*ENAM*, OMIM 606585), kallikrein-4 (*KLK4*, OMIM 603767), and matrix metalloproteinase-20 (*MMP-20*, OMIM 604629), that encode proteins involved in enamel development have been found to cause AI (Collier *et al.*, 1997; Hart *et al.*, 2002, 2004; Kim *et al.*, 2005a,b; Stephanopoulos *et al.*, 2005; Wright *et al.*, 2009). Mutations in distal-less homeobox 3 (*DLX3*, OMIM 600525) (Dong *et al.*, 2005), FAM83H (Kim *et al.*, 2008), and WDR72 (El-Sayed *et al.*, 2009) have also been linked to AI. These enamel proteins alter the quantity and/or quality of the enamel during the transition from soft to mineralized structure. Fourteen forms of AI have been described based on the specific dental abnormality and the pattern of inheritance (Witkop and Sauk, 1976). While alterations in the *AMELX* gene are responsible for X-linked AI, mutations in *ENAM*, *KLK4*, and *MMP-20* genes cause AI with an autosomal pattern of inheritance (Hart *et al.*, 2004; Kim *et al.*, 2005a,b; Stephanopoulos *et al.*, 2005).

Clinically, the AI phenotype results in enamel that is hypoplastic, hypomineralized, or hypomature. There is a broad spectrum of clinical presentation, from thin enamel with normal mineral, to teeth that are discolored, sensitive, and prone to disintegration (Witkop, 1988; Wright *et al.*, 1993; Crawford *et al.*, 2007). Hypoplastic defects result from deficiencies in the amount of enamel, characterized by thin enamel with pits or grooves, or smooth enamel, while hypomineralized AI results from defects in crystallite formation and growth (Witkop, 1988; Wright *et al.*, 1993). Hypomaturation is the result of insufficient processing of the enamel organic matrix, and enamel is soft and brown (Kim *et al.*, 2005a).

Rehabilitation of patients with AI represents a challenge from both a functional and an esthetic standpoint (Sabatini and Guzmán-Armstrong, 2009). Management of AI in young patients requires maintaining the maximum amount of dental hard tissues until patients reach an age that allows for complex prosthodontic approaches. Because of advances in adhesive resin materials, the improvement of poor esthetics and function can be achieved by the use of bonded restorations. In some patients with AI, bonded restorations have been successfully used to restore teeth to acceptable form and function with favorable esthetics (Sabatini and Guzmán-Armstrong, 2009). However, in

many other patients, adhesive restorations show high failure rates in areas of poorly mineralized and friable enamel. Histological, morphological, and micromorphological differences among the types of AI are thought to be responsible for failures (Seow and Amaratunge, 1998; Gemalmaz *et al.*, 2003; Yip and Smales, 2003). These differences can be related to uncertain acid-etch patterns of enamel and consequently weak bond strength of resin material to tooth tissue (Ng and Messer, 2009). It is difficult in a clinical situation to distinguish which cases of AI will prove to be successful when bonded materials are used, and which will ultimately fail.

Mice lacking amelogenin protein (*AmelxKO*) develop hypoplastic enamel lacking prismatic structure (Gibson *et al.*, 2001), similar to X-linked AI in humans. There is a loss of discernible prismatic architecture apparent in both mice and humans having mutations that cause a loss of amelogenin (Wright *et al.*, 2009). Mice lacking *Mmp20* develop hypoplastic, hypomature enamel that separates from the dentin, presumably from a defect in the dentin-enamel junction (Caterina *et al.*, 2002), similar to human AI with *MMP20* mutations (Wright *et al.*, 2009). Analysis of developing incisor enamel from *AmelxKO* and *Mmp20KO* mice suggests that both genes are essential for the generation of the full enamel thickness and decussating pattern of the enamel rods (Bartlett *et al.*, 2006). The aims of this study were to: (1) evaluate the appearance and etching patterns of teeth in wild-type mice (normal controls) and mice with 2 genetically different forms of AI, *AmelxKO* and *Mmp20KO*, to observe differences in etching patterns that may affect bonding; and (2) compare microshear bond strengths of 2 different types of bonding systems (etch-and-rinse and self-etch) which differ significantly in the manner by which they prepare the tooth tissues, using different acid-etching strategies.

MATERIALS & METHODS

Animal Models

Amelogenin null (*AmelxKO*) and matrix metalloproteinase-20 null (*Mmp20KO*) mice were generated by introduction of a deletion into the coding region as described previously (Gibson *et al.*, 2001; Caterina *et al.*, 2002). Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility, and procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

DNA Analysis of Animals

We isolated high-molecular-weight genomic DNA from mouse tails (Gibson *et al.*, 2001) to determine *AmelxKO* or *Mmp20KO* vs. wild-type (WT) genotype. The Qiagen multiplex PCR kit (Valencia, CA, USA) was used for PCR reactions, and products were analyzed on 4% Nusieve 3:1 agarose gels. The PCR primers used have been described (Li *et al.*, 2008) and result in a wild-type PCR product of 640 bp and an *AmelxKO* product of 190 bp. For genotyping of *Mmp20KO* mice, PCR primers and conditions described by Caterina *et al.* (2002) were used.

Acid Etching of Enamel Surfaces

Mice were sacrificed by CO₂ administration. After dissection of mandibles from 4-week-old *AmelxKO*, *Mmp20KO*, and WT mice (n = 5 for each mouse type and treatment), incisors were removed from the mandibles and secured to acrylic blocks (Appendix Fig. 1). The facial surfaces of mandibular incisors were rinsed in double-deionized water (DD H₂O) and pumiced (Benco Dental, Wilkes Barre, PA, USA) gently to standardize the surfaces to be etched. Right incisors were etched with 37% phosphoric acid gel (Pulpdent Corp., Watertown, MA, USA) for 45 sec and rinsed with DD H₂O. Left incisors from the same jaw were rinsed with double-deionized H₂O, and were considered unetched controls.

Scanning Electron Microscopy Analysis of Enamel Surfaces

Surfaces of etched and unetched incisors were coated with gold. Scanning electron microscopy (SEM) analysis of tooth surfaces of incisors was completed at 15 kV and 2000X magnification, in secondary mode (JEOL JSM T330A; JEOL, Inc., Peabody, MA, USA). Images were obtained of the facial surface of each etched or unetched incisor. We fractured the pre-etched incisors through the center of each unetched tooth to view the thickness of the enamel layer in cross-section by SEM at 2000X magnification in 3 random areas.

Quantification of Enamel Surface Roughness

We analyzed SEM images at 2000X magnification quantitatively with the Image J (Rasband, 1997-2011) roughness calculation plug-in module to calculate the relative roughness averages (Ra) of each image, based on relative grayscale value. Three randomly spaced images were obtained, and three 10- μ m by 10- μ m areas were randomly chosen on each image for roughness measurements. Ra calculations of WT, *AmelxKO*, and *Mmp20KO* unetched and etched enamel surfaces were tested for significant differences (p < 0.05) by one-way ANOVA and the Bonferroni *post hoc* test (GraphPad Prism, GraphPad Software, Inc., La Jolla, CA, USA).

Microshear Bond Strength Test

Mandibular incisors were dissected from 4-week-old WT, *AmelxKO*, and *Mmp20KO* mice (n = 30 mice for each genotype and treatment except for *AmelxKO* self-etch, where n = 40). The sample mounting and bonding method is detailed in Appendix Fig. 1. The flat portion of the facial surface of incisor enamel was polished with 800-grit SiC paper for 2 sec to create a uniform, flat bond area. Composite inlay sticks (1.0 mm x 0.4 mm) were prepared with Clearfil Majesty Anterior (Kuraray, Tokyo, Japan) and bonded to enamel surfaces by either: (1) etch-and-rinse (ER) with 35% phosphoric acid gel and Adper Scotchbond Multipurpose (3M ESPE, St. Paul, MN, USA); or (2) self-etch (SE) with Clearfil SE Bond (Kuraray). The materials and their components are listed in the Appendix Table. For ER, the acid-etch time was reduced from the recommended time of 20 sec to

Figure 1. SEM images of mouse incisors and cross-sections of unetched fractured incisors. (A-C) Low-resolution images (35x) of (A) WT, (B) *AmelxKO*, and (C) *Mmp20KO* incisors, showing the etched incisor on the left and the unetched incisor on the right. Both *AmelxKO* and *Mmp20KO* had blunted, worn incisors compared with those of WT; and *Mmp20KO* incisors showed large areas of delaminated enamel, leaving a thin enamel layer (F). (D-F) Cross-section images (2000x) of unetched fractured incisors from (D) WT, (E) *AmelxKO*, and (F) *Mmp20KO* mice, showing the enamel layer (E) and dentin (D). Both *AmelxKO* and *Mmp20KO* had noticeably thinner enamel.

5 sec for all 3 groups, to reduce the excess removal of remaining enamel in the mutant mice.

SBS was measured by means of a Micro-shear Tester (Bisco, Schaumburg, IL, USA), with a crosshead speed of 0.5 mm/min. We analyzed data by 2-way ANOVA to determine statistical significance ($p < 0.05$). We evaluated de-bonded surfaces and cross-sections by SEM and light microscopy to determine the location or mode of bond failure. Bond failure was classified as adhesive if the bond appeared to fail within the adhesive layer, cohesive if the bond failed within the enamel or dentin tissue, or mixed if the bond failure appeared to be a mixture of adhesive and cohesive.

RESULTS

The phenotypic differences between the WT mice and the 2 mutants are shown in Fig. 1. Mandibular incisors of adult *AmelxKO* (Fig. 1B) and *Mmp20KO* (Fig. 1C) mice were shorter and more blunted than those in WT (Fig. 1A) incisors. Furthermore, unetched *Mmp20KO* incisors had sections of enamel missing from the incisor surfaces (Fig. 1C); however, as shown in Fig. 1F, the *Mmp20KO* incisors have a thin layer of enamel remaining prior to etching. As compared with WT (Fig. 1D), fractured cross-sections of *AmelxKO* incisors (Fig. 1E) indicated that, prior to etching, there was a thin layer of enamel covering dentinal tissue, revealing the DEJ or underlying dentin in some areas, and adjacent areas had some remaining enamel tissue.

ENAMEL ACID ETCHING

SEM analysis of etched and unetched surfaces of WT, *AmelxKO*, and *Mmp20KO* incisor enamel is shown in Fig. 2. Unetched *AmelxKO* enamel had a non-uniform, rough appearance (Fig. 2C), while etched enamel from incisors of *AmelxKO* (Fig. 2D) and *Mmp20KO* (Fig. 2F) mice lacked the etching pattern seen in etched WT enamel (Fig. 2B). Unetched *Mmp20KO* incisors had most of the enamel missing due to delamination of the layer (Fig. 1F, 2E); thus etching did not noticeably alter the enamel surface (Fig. 2F).

Surface appearances were quantified with grayscale values analyzed by the roughness Image-J plug-in (Appendix Fig. 2). Measurements indicated that unetched *AmelxKO* enamel was significantly ($p < 0.05$) rougher than etched *Mmp20KO* enamel, and etched *Mmp20KO* was significantly less rough than etched WT enamel ($p < 0.05$). Analysis of these data validates the observed appearances of the etched and unetched surfaces shown in Fig. 2.

Microshear Bond Testing

Shear bond strength (SBS) tests indicated that the SE bonding system resulted in significantly improved SBS for all 3 groups (WT, *AmelxKO*, *Mmp20KO*) ($p < 0.05$). Two-way ANOVA indicated no significant interaction between the 2 experimental factors (ER vs. SE and WT vs. mutant). *AmelxKO* had the lowest SBS for both ER and SE test groups. Interestingly, for the ER group, *Mmp20KO* SBS was slightly higher than WT (Fig. 3).

SEM was not used to determine the failure modes of *Mmp20KO* teeth because of their fragility and the difficulty in removing them from the testing molds. Therefore, light microscopy was used to determine the failure mode for *Mmp20KO* teeth, but we could determine only cohesive vs. adhesive failure, due to lack of magnification. WT had a similar percentage of adhesive failures (~80%) for both ER and SE. Both *AmelxKO* (50% for SE compared with 68% for ER) and *Mmp20KO* (63% for SE compared with 90% for ER) had less adhesive failure in the SE group. For both *AmelxKO* and *Mmp20KO*, the cohesive failures in the SE group were mostly within the dentin (Table), presumably due to enamel loss from bonding procedures.

DISCUSSION

We have developed a unique, reliable system for evaluating etching patterns and bond strengths of resin materials to AI-affected enamel through the use of mouse models with specific AI mutations. Shear bond strengths of 17–24 MPa are generally required to effectively resist the polymerization contraction forces of composite resin materials in humans. Such bond strengths provide routinely successful retention of resins for a variety of clinical applications (Saroglu *et al.*, 2006). Since we were able to obtain similar shear bond strength values (10–24 MPa) in these murine teeth, we can perhaps gain greater insight into how restorative materials perform on human AI-affected enamel.

Surface irregularities in the enamel increase the surface area for mechanical bonding and wettability, to enhance the flow of resin onto the tissue. In our study, *AmelxKO* unetched enamel was naturally rougher and more disorganized than unetched WT enamel. Furthermore, etching *AmelxKO* and *Mmp20KO* enamel removed much of the remaining enamel, leaving some of the dentin surface or DEJ exposed (Fig. 2). These findings were not surprising, given the aprismatic architecture of the *Amelx* and *Mmp20* null mice. Since the unetched enamel from *AmelxKO* and *Mmp20KO* teeth had similar roughnesses compared with that of the etched WT teeth, our clinical approach for bonding would suggest elimination of traditional aggressive etching in patients with these genetic variants. The knowledge of the acid-etching patterns of the different types of AI could influence the types of restorative materials used and may possibly decrease failure rates in bonded restorations.

Current adhesive bonding systems interact with enamel and dentin by 2 different strategies. Etch-and-rinse (ER) uses an aggressive acid-etching step, while the self-etch (SE) approach uses non-rinsed weak acidic monomers to condition and prime the

Figure 2. Etched vs. unetched murine enamel. SEM images (2000x) of unetched (A,C,E) and etched (B,D,F) murine mandibular incisors from wild-type (WT) (A,B), *AmelxKO* (C,D), and *Mmp20KO* (E,F) mice. WT etched surfaces appeared rougher than unetched, while both *AmelxKO* and *Mmp20KO* unetched surfaces appeared rougher than etched surfaces.

Figure 3. Shear bond strength of 2 AI mouse models and WT controls ($n = 30$ except for *AmelxKO* SE, where $n = 40$). WT, *AmelxKO*, and *Mmp20KO* incisors were bonded with 2 bonding systems: etch-and-rinse and self-etch. Samples were de-bonded, and the shear bond strength was calculated in MPa. * indicates a statistically significant difference by ANOVA ($p < 0.05$).

Table. Shear Bond Failure Mode Results of Two AI Mouse Models and Wild-type Controls (n = 30, except for *Amelx*KO SE, where n = 40), Shown as Percentage (%) Adhesive, Cohesive, or Mixed

	Etch-and-Rinse Bonding System			Self-etching Bonding System		
	Adhesive	Cohesive	Mixed	Adhesive	Cohesive	Mixed
Wild-type	77	5	18	80	10	10
<i>Amelx</i> KO	68	20	12	50	30	20
<i>Mmp20</i> KO	90	10	N/A	63	37	N/A

Cohesive failure occurred either in the enamel or dentin layer.

tooth (Breschi *et al.*, 2008). We compared bonding to AI-affected enamel with the ER system to the SE system using SBS testing. Our study confirmed that applying milder etching agents (SE) to these 2 types of AI-affected enamel surfaces resulted in higher SBS of the adhesive resin. Analysis of failure mode data showed that adhesive fractures were prevalent in all WT and AI affected teeth with *Amelx* or *Mmp20* mutations. The adhesive failures were dramatically increased in *Mmp20*KO enamel in the ER testing group, probably as a result of the aggressive etching of thin and less mineralized enamel surfaces. It has been suggested that pre-treatment of hypocalcified enamel with sodium hypochlorite produced more favorable etch patterns and increased bond strengths by removing the excess proteins surrounding the crystals (Venezie *et al.*, 1994; Saroglu *et al.*, 2006). This deproteinization has been shown to enhance enamel bonding in hypocalcified AI enamel. Since *MMP20* mutations lead to an excess of protein, perhaps pre-treatment with sodium hypochlorite would be beneficial before bonding in patients with this mutation. Additionally, since the enamel layer is so thin, it is important to understand the potential impact of the *Mmp20*KO phenotype in dentin, since it normally contains matrix metalloproteinases (MMPs). The collagenolytic and gelatinolytic activities of MMPs in partially demineralized WT dentin treated with etch-and-rinse adhesives may cause disruption of the hybrid layer, and chlorhexidine, a protease inhibitor, may be used prior to bonding to preserve collagen integrity (Carrilho *et al.*, 2007). Additional studies are needed to investigate whether sodium hypochlorite or chlorhexidine would affect bonding in *Mmp20* null mice.

The clinical features of the different types of AI are similar, making distinguishing between them difficult in the clinic (Wright, 2006). Patients with AI are usually treated without knowledge of the causative gene mutation, since this is often unknown. Acid-etching patterns, etching time, and different interactions of bonding agents with various types of AI enamel could influence the outcome of restorative treatments, so understanding of the genetic mutations may provide insight into the management of patients with AI.

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