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Oral colonization by *Streptococcus mutans* and caries development is reduced upon deletion of carbonic anhydrase VI expression in saliva

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ABSTRACT

Carbonic anhydrase VI (CA VI), encoded by type A transcripts of the gene *Car6*, is a secretory product of salivary glands and is found in the enamel pellicle. Because higher caries prevalence is associated with lower salivary concentrations of CA VI in humans, we tested whether CA VI protects enamel surfaces from caries induced by *Streptococcus mutans*, using *Car6*$^{-/-}$ mice, in which salivary CA VI expression is absent. We detected aberrant *Car6* type A transcripts in *Car6*$^{-/-}$ mice, likely targets for nonsense-mediated mRNA decay. Expression of the intracellular stress-induced isoform of CA VI encoded by type B transcripts was restricted to parotid and submandibular glands of wild type mice. The salivary function of *Car6*$^{-/-}$ mice was normal as assessed by the histology and protein/glycoprotein profiles of glands, salivary flow rates and protein/glycoprotein compositions of saliva. Surprisingly, total smooth surface caries and sulcal caries in *Car6*$^{-/-}$ mice were more than 6-fold and 2-fold lower than in wild type mice after infection with *S. mutans* strain UA159. Recoveries of *S. mutans* and total microbiota from molars were also lower in *Car6*$^{-/-}$ mice. To explore possible mechanisms for increased caries susceptibility, we found no differences in *S. mutans* adherence to salivary pellicles, *in vitro*. Interestingly, higher levels of *Lactobacillus murinus* and an unidentified *Streptococcus* species were cultivated from the oral microbiota of *Car6*$^{-/-}$ mice. Collective results suggest salivary CA VI may promote caries by modulating the oral microbiota to favor *S. mutans* colonization and/or by the enzymatic production of acid within plaque.

1. Introduction

As the portal for ingestion of water and food nutrients, the oral cavity is constantly under attack by microbiota from the external environment. The flow of saliva combined with salivary microbial-interacting constituents and with factors that can buffer and remineralize tooth surfaces function together to help protect the hard oral surfaces from mineral dissolution (i.e., caries) by acidogenic microorganisms such as *Streptococcus mutans*. *S. mutans* is the organism most consistently associated with dental caries worldwide, but is further linked to bacterial endocarditis [1] and to atheromatous plaques [2], suggesting an interconnection between oral infections and cardiovascular diseases. Hence, efforts to understand and treat oral diseases have potential ramifications to serious systemic disease processes.

First discovered in ovine saliva [3], carbonic anhydrase VI (CA VI) is the only secretory isozyme of the CA gene family. It is also found in other secretory systems such as lacrimal glands [4,5], tracheobronchial glands [6] and nasal glands where it may function in olfaction [7]. It is also found in high concentrations in colostrum, suggesting a role in the development of the alimentary tract [8]. In the diverse system of salivary glands CA VI is produced in the parotid and submandibular glands [9] as well as minor salivary glands of the tongue, including von Ebner's glands [10]. Although many carbonic anhydrase isoforms are key enzymes for pH regulation in tissues and biological fluids, CA VI does not appear to regulate the pH of whole saliva, but instead may function in oral microenvironments [11]. For example, CA VI within von Ebner's gland secretions bathing taste receptors of the circumvallate and foliate papillae [10] may function in the growth and development of taste buds [12–14].

CA VI is also a component of the enamel pellicle, a thin layer of proteins between teeth enamel and overlying bacterial plaque [15].

Abbreviations: CA VI, carbonic anhydrase alpha, isozyme 6; *car6*, carbonic anhydrase 6 gene; CHOP, ER stress-related transcription factor C/EBP homologous protein; *neo*, *neomycin*; TK, thymidine kinase; SMG, submandibular gland; SLG, sublingual gland

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A higher prevalence of caries is associated with lower concentrations of CA VI in the saliva of human subjects, thus raising the hypothesis that CA VI serves to protect enamel surfaces from caries, possibly through the removal of bacterial derived hydrogen ions within the microenvironment near the enamel surface by catalyzing the interaction of hydrogen ions with salivary bicarbonate ions to form CO_2 and H_2O [16]. An attractive model to test this hypothesis are mice in which targeted deletion of the gene encoding CA VI, *Car6*, display an absence of CA VI expression in salivary glands as confirmed in Western blots and by immunohistochemistry [17,18]. The targeted deletion in *Car6*$^{-/-}$ mice removes *Car6* exon 3 and part of exon 4, leaving the 3′-end of this latter exon. Both exons are normally incorporated into the two known isoforms of CA VI expressed by the *Car6* gene, the secreted enzyme (type A) and an intracellular form (type B) [19]. Type B transcripts use a promoter within intron 1, are stress-induced in mouse NIH 3T3 fibroblasts and were initially detected in salivary tissue, although the type of salivary tissue was not specified [19]. Expression of the type B isoform by the three different major salivary glands in mice is therefore unclear, as is whether its deleted expression alters salivary function. Moreover, it is not known whether the transcriptional machinery in *Car6*$^{-/-}$ mice reads through the inserted *neomycin* cassette to reach the remaining exon 4 splice site and, if so, whether it is utilized during pre-mRNA splicing to create an aberrant translated message that may disrupt salivary function.

In the current study, we assessed whether the loss of *Car6* gene expression has a significant impact on the cellular structure of the major salivary glands and on salivary constituents and flow. Furthermore, consequences from the absence of CA VI on the functions of saliva related to protection against caries development were tested, both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

Unless indicated, all materials were from Sigma Chemical Co., St. Louis, MO. All kits were used according to manufacturer's instructions.

2.2. Animals and collection of glands

The University of Florida IACUC committee approved all animal procedures. Construction of the *Car6*$^{-/-}$ mouse model in the C57BL/6 genetic background has been described previously [17,18]. Animals were anesthetized by CO_2 inhalation and killed by sectioning the aorta. Mice were genotyped as described previously [18]. Unless indicated, tissues were excised, blotted on filter paper, flash frozen in liquid nitrogen and stored at −80 °C.

2.3. Caries experimental protocol and scoring

The caries protocol was similar to that reported previously [20] with modifications. Briefly, timed pregnancies were established using twenty breeding pairs of heterozygous (+/−) *Car6* mice. Females were negative for indigenous *S. mutans* as determined by streaking oral swabs on Mitis Salivarius agar (Becton, Dickinson and Co., Sparks, MD) with 1% Tellurite solution (Becton, Dickinson and Company), 20% sucrose, and 0.2 units/ml bacitracin (MSB) [21]. Pups were marked for identification with ear clips at 14 days of age and genotyped. At 16–17 days of age, each dam with pups were transferred to a BSL2 suite of the vivarium in microisolator cages containing a wire bottomed insert and a thin layer of corn-cob bedding underneath. *S. mutans* UA159 from a frozen low-passage aliquots were grown overnight in Brain Heart Infusion medium + 0.5% glucose (BHI; Becton, Dickinson and Co.) and concentrated to approximately 10^{10} CFU/ml by centrifugation. The dams and pups were then inoculated by oral swabbing, which delivers about 10 μl (10^8 CFU) of the concentrated solution. The diet was converted to powdered Diet 2000 (56% sucrose) with 5% sucrose water. Pups and dams were re-inoculated each of the next two days. At 21 days of age, pups were weaned and caged in pairs with non-littermates of the same sex. Pups were screened for *S. mutans* colonization 5 days after the initial infection by plating oral swabs on MSB. Non-infected mice were inoculated and subsequently tested as before. All were positive for *S. mutans* colonization. Mice were weighed weekly. Mice were sacrificed seven weeks after the initial inoculation. The left and right mandibles from twelve mice in each group were removed aseptically to assay for bacterial colonization of the molars (see below). The skulls and remaining mandibles were defleshed over an 18–24 h period by Dermestid beetles (*Dermestes maculates*) as described by Tanzer [22]. Buccal, lingual and proximal surfaces of all molars were scored for smooth surface caries with the aid of an Olympus dissecting microscope (Model SZX16). The teeth were then stained with murexide (0.024% w/v in 70% ethanol) and prepared for sectioning along the midline in order to score sulcal caries and to determine severity scores of proximal surfaces. Skulls and mandibles were prepared for sectioning by embedding in Embed-It Low Viscosity Epoxy (Polysciences, Warrington, PA) to a level about 1 mm below the enamel crown. Epoxy blocks were mounted in a custom made vice attached to a Narishige micromanipulator (Narishige International USA, Inc, East Meadow, NY) and the exposed teeth sectioned with a double-sided diamond-coated abrasive disk (0.15 mm thick, Abrasive Technology, Lewis Center, OH) attached to a hand piece (Foredom model 44T, Foredom Electric Co., Bethel, CT) and motor unit (Foredom Model SM; 18,000 RPM). The hand piece is mounted on a ball-bearing slider that restricts movement of the abrasive disk along the aligned midline of the three molars. The teeth were sectioned under a light flow of water.

Scoring was according to Larson's modification of the Keyes' scoring system and was conducted by a single calibrated and blinded examiner [23,24]. The linear evaluations of enamel lesions were expressed as E, while severities were expressed as Ds, Dm and Dx (slight dentin involvement, moderate dentin involvement or penetrates the dentin, respectively). To stabilize variances, caries scores were expressed as proportions of their maximum possible values (124 for smooth surface caries and 56 for sulcal surface caries) and then the arcsine of the square root of the proportions calculated, as described previously [20].

2.4. Recoveries of S. mutans and total bacteria from molars

The mandibles from twelve mice in each caries group (wild type and *Car6*$^{-/-}$) were aseptically extracted and underwent dissection to remove loose tissue on the bone near the molars. The bone was sectioned about 2 mm anterior to the first molar and 2 mm posterior to the third molar. Each pair of prepared mandible/molars from each mouse were sonicated on ice in 5 ml of ice-cold sterile 0.9% saline using three pulses of 10 s with 30 s intervals, at 50% power with a Branson Digital Sonifier 250. Sonicates from two non-cagemates were pooled. From each 10 ml we removed 200 μl to prepare 10-fold serial dilutions that were plated (50 μl per 60 mm plate) onto MSB agar and onto blood agar to estimate colonization of the molars by *S. mutans* (MSB agar) and by total bacteria (blood agar). Colony forming units (CFU) were then determined from 48 h cultures.

2.5. Preparation of cDNA

Preparation was as described previously [25]. Briefly, frozen tissues or growing cell cultures were homogenized directly in TRIzol® Reagent (Invitrogen, Carlsbad, CA), total RNA isolated using standard protocols and then treated with DNase I using Ambion's DNA-*free*™ Reagent Kit (Applied Biosystems, Foster City, CA). Removal of genomic DNA was verified by the inability of the sample to amplify the mouse