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ABSTRACT

Enamel biomineralization results in a release of protons into the enamel matrix, causing an acidification of the local microenvironment. This acidification, which may be enhanced by more rapid mineral deposition in the presence of fluoride, must be neutralized by the overlying ameloblasts. The electrogenic sodium bicarbonate co-transporter NBCe1 has been localized in mouse ameloblasts, and has been proposed to have a role in matrix pH regulation. In this study, transcript analysis by PCR showed NBCe1-A present in human ameloblasts, whereas mouse ameloblasts expressed NBCe1-B. *In situ* hybridization and qPCR in mouse and fetal human incisors showed that NBCe1 mRNA was up-regulated as ameloblasts differentiated. Ingestion of 50 ppm fluoride resulted in an up-regulation of NBCe1 mRNA in maturation-stage mouse ameloblasts *in vivo*, as compared with controls. NBCe1 expression was up-regulated by low pH, but not by fluoride, in human ameloblast-lineage cells *in vitro*. The up-regulation of NBCe1 *in vivo* as enamel maturation and mineralization progressed provides evidence that NBCe1 participates in pH modulation during enamel formation. Up-regulation of NBCe1 in fluorosed maturation ameloblasts *in vivo*, with no effect of fluoride *in vitro*, supports the hypothesis that fluoride-enhanced mineral deposition results in acidification of the mineralizing enamel matrix.

KEY WORDS: ameloblast, enamel, fluoride, pH regulation, sodium bicarbonate co-transporter.

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NBCe1 in Mouse and Human Ameloblasts may be Indirectly Regulated by Fluoride

INTRODUCTION

Ameloblasts, which differentiate from dental epithelial precursor cells, form the mineralized enamel matrix. Ameloblast differentiation progresses from the pre-ameloblast stage, to presecretory, secretory, transition, and maturation ameloblasts (Nanci and Ten Cate, 2003). Enamel mineralization is initiated in the matrix at the secretory stage, and rapidly increases to final completion in the maturation stage of enamel formation.

During enamel mineralization, hydroxyapatite is formed by incorporation of Ca^{2+} and HPO_4^{2-} . Formation of every unit of hydroxyapatite crystal releases $\sim 8 \text{ H}^+$ protons in the enamel matrix and lowers local pH (Smith, 1998). However, the pH of the enamel fluid in the secretory stage is kept neutral (pH 7.2), indicating the presence of an effective pH regulation mechanism. Abundant amelogenins generated by secretory ameloblasts may be a potent contributor to controlling pH (Simmer and Fincham, 1995; Bronckers *et al.*, 2009b), but not necessarily the sole mechanism.

Mineralization increases and peaks in the maturation stage (Simmer and Fincham, 1995), where the final organic matrix is removed and enamel crystal formation is completed. In the maturation stage, pH in the enamel matrix changes periodically between acidic (pH 5.8) and neutral (pH 7.2) (Sasaki *et al.*, 1991; Smith, 1998). This modulation of pH in the maturing enamel matrix requires effective pH regulation. Bronckers and co-workers (Bronckers *et al.*, 2009b) have hypothesized that fluoride in the enamel matrix may enhance mineralization, which could further influence pH modulation by the overlying ameloblasts. This fluoride effect in the mineralizing matrix could contribute to enamel fluorosis by altering ameloblast modulation in the maturation stage.

Several ion transporters are considered to be involved in pH regulation of the mineralizing enamel matrix (Bronckers *et al.*, 2009b; Josephsen *et al.*, 2010). Immunohistochemical staining for NHE1 and $\text{H}^+-\text{K}^+-\text{ATPase}$ is detected in the basolateral membrane of the ameloblast layer throughout amelogenesis (Sasaki *et al.*, 1988; Josephsen *et al.*, 2010). CFTR, a chloride channel involved in $\text{Cl}^-/\text{HCO}_3^-$ exchange, has been detected in the apical membrane of maturation-stage ameloblasts (Bronckers *et al.*, 2009a), and with a deletion of either the *Cftr* gene or the anion exchanger *Ae2*, mouse incisors are softer, with a chalky white appearance (Wright *et al.*, 1996; Lyaruu *et al.*, 2008), implying the importance of pH regulation during enamel formation.

The electrogenic sodium bicarbonate co-transporter NBCe1 is another candidate for pH regulation during enamel formation (Paine *et al.*, 2008). Enamel-mineralization-related defects, along with proximal tubular acidosis, are detected in NBCe1 mutant mice (Dinour *et al.*, 2004; Gawenis *et al.*, 2007). Deletion of NBCe1^(-/-) in mice results in hypomineralized enamel and a weak prismatic architecture, though the amelogenin expression profile remains unchanged (Lacruz *et al.*, 2010).

NBCe1 is a submember of the SLC4 superfamily that mediates base (HCO_3^- , CO_3^{2-}) transport, regulating both intracellular and extracellular pH in various cells and tissues (Romero *et al.*, 2004). NBCe1 encoded by the SLC4A4 gene consists of 3 transcripts derived from alternate promoter usage in the SLC4A4 gene, including NBCe1-A (transcript 1), NBCe1-B (transcript 2), and NBCe1-C (transcript 3) (Romero *et al.*, 2004). These transcripts distribute in a cell-type- and localization-specific fashion. NBCe1-B has been identified in mouse ameloblasts *in vivo*, and *in vitro* is up-regulated when the pH of the culture medium is reduced (Paine *et al.*, 2008).

In this study, we localized NBCe1 mRNA in human and mouse teeth, and characterized expression levels as ameloblasts differentiated. We investigated the effects of the dental matrix environment on NBCe1 expression by comparing expression in mice exposed to fluoride during enamel formation, and in human ameloblast-lineage cells exposed to fluoride or reduced pH *in vitro*.

MATERIALS & METHODS

Characterization of NBCe1 in Human Tooth Organs

Human tooth buds were obtained from 18- to 22-week-old fetal cadavers within 3 hrs after legal abortion, under the guidelines of the University of California Committee on Human Research. Teeth were dissected from the mandibles under a laminar flow hood, embedded in OCT compound (Sakura Finetek, Torrance, CA, USA), and cryosectioned at 10- μm thickness. These sections were used for laser capture microdissection (LCM) and immunofluorescence. Immunofluorescence staining was done with a rabbit polyclonal antibody to NBCe1 (Abcam Inc., Cambridge, MA, USA) (see Appendix for details).

Relative mRNA Expression in Human Ameloblasts

Epithelial cells from the cervical loop, pre-ameloblasts, presecretory ameloblasts, and secretory ameloblasts were separately isolated by LCM (see Appendix for details). The expression of human Proliferating Cell Nuclear Antigen (PCNA) (Moldovan *et al.*, 2007), pituitary homeobox 2 (Pitx2) (Thesleff and Mikkola, 2002), amelogenin, and both human and mouse SLC4A4 was examined by quantitative real-time PCR in an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). mRNA expression was compared by $\Delta\Delta\text{Ct}$ (Schmittgen and Livak, 2008) (see Appendix for detailed explanation of $\Delta\Delta\text{Ct}$ analysis). Data were compared by one-way ANOVA followed by the *post hoc* Tukey's test.

Specific primers for NBCe1-A and NBCe1-B were designed to identify each isoform. PCR amplification was completed on the micro-dissected enamel organ epithelial cells as compared

with mRNA from fetal mandibular tissue (see Appendix for primers and PCR conditions).

In situ Hybridization (ISH)

Tooth organs were fixed in 4% PBS-buffered paraformaldehyde, and then paraffin-embedded and sectioned at 5- μm thickness. ISH was done with probes amplified from cDNA derived from human fetal mandibles, and from a mouse tooth cDNA library (see Appendix for probe sequences and details).

Generation of Human Ameloblast-lineage Cells for in vitro Analyses

Human tooth organs were digested with collagenase/dispase, and epithelial cells were selectively grown in KGM-2 media to generate ameloblast-lineage cells. These cells were transfected with SV40 to extend their growth, and a colony-amplifying NBCe1 was selected (see Appendix for further details).

Clonally selected cells were grown in DMEM and 10% FBS either at pH 7.2 with 0, 10, and 50 μM fluoride or at pH 6.2 without fluoride for 72 hrs. The cells were collected, and relative expression of NBCe1 was determined by qPCR. Treatment and control groups were compared by Student's *t* test.

Characterization of NBCe1 mRNA Expression in Mouse Incisors

Three-week-old female A/J strain mice were given drinking water containing either 0 (control) or 50 ppm NaF (Sigma-Aldrich, St. Louis, MO, USA) *ad libitum*. After 4 wks, the animals were sacrificed, and mandibles were removed, fixed in 4% PBS-buffered paraformaldehyde, and then decalcified in Morse's solution (22.5% formic acid, 10% sodium citrate) overnight. Mandibles were embedded and cryosectioned, and secretory- and maturation-stage ameloblasts were isolated by LCM. mRNA from the laser-captured cells was analyzed in triplicate to identify the NBCe1 transcripts and to determine the relative quantity of NBCe1 in fluoride-exposed secretory- and maturation-stage mouse ameloblasts as compared with controls.

RESULTS

NBCe1 Isoforms Differed in Humans and Mice

PCR analysis showed that NBCe1-A, but not NBCe1-B, was expressed by human fetal incisor dental epithelia cells (Fig. 1A), though both NBCe1-A and NBCe1-B were expressed by cells contained within the human mandible (Fig. 1C). NBCe1-B was the only transcript identified by PCR amplification of mRNA from mouse secretory and maturation ameloblasts (Fig. 1B).

NBCe1 Up-regulation Paralleled Ameloblast Maturation

ISH showed that NBCe1 was up-regulated in both human and mouse ameloblasts as cells differentiated and matrix formed (Figs. 2A, 2C). In human incisors, positive signal for NBCe1 was first detected in presecretory ameloblasts and then became more intense in secretory ameloblasts (Fig. 2A, boxes c, d).

Figure 1. Determination of the NBCe1 transcripts in fetal human and adult mouse dental epithelial cells. **(A)** In 4 different stages of human dental epithelial lineage cells captured by LCM (hCL, human cervical loop; hPAB, human pre-ameloblasts; hPSAB, human presecretory ameloblasts; hSAB, human secretory ameloblasts), NBCe1-A (N-A) was the only transcript detected (N-B, NBCe1-B). **(B)** In secretory (mSAB) and maturation (mMAB) stages of adult mouse ameloblasts, NBCe1-B was the only detectable transcript. **(C)** Human fetal mandibular cDNA was used to amplify PCR products specific to both hNBCe1-A and -B transcripts (HWM, human whole mandible). **(D)** GAPDH was used as a loading control (h, human; m, mouse). 99 × 105mm (300 × 300 DPI).

However, in neonatal mouse incisors, NBCe1 mRNA signal was detected at all stages of ameloblast differentiation, beginning at the cervical loop proliferating cells and extending to differentiated ameloblasts (Fig. 2C). Before the secretory stage, mRNA signals were detected at the basal region of the inner enamel epithelial cells (Fig. 2C, boxes a, b). Beginning at the secretory stage (Fig. 2C, box c), the signal was detected in cell cytoplasm and became more intense as development progressed up through the transition stage. Maturation-stage ameloblasts are not yet fully differentiated in newborn mouse incisors.

To confirm ISH results, and to further investigate maturation-stage ameloblasts, we completed qPCR analysis on micro-dissected cells from human and adult mouse incisors. In human developing incisors, NBCe1 transcript could be detected in all cells from the cervical loop to secretory ameloblasts. PCNA and Pitx2 were down-regulated, while amelogenin and NBCe1 mRNA were up-regulated as the cells differentiated (Figs. 3A-3C). In adult mouse incisors, NBCe1 was significantly up-regulated as ameloblasts differentiated from secretory to maturation stage in ameloblasts from both control and fluoride-treated animals (Fig. 3D).

NBCe1 Protein was Immunolocalized to Ameloblasts and Odontoblasts in Human Incisors

The distribution of NBCe1 was clearly demonstrated in human incisors where immunopositive signals were detected starting from presecretory ameloblasts (Fig. 4b), and became more intense in secretory ameloblasts (Fig. 4c). Positive signals were detected in secretory odontoblasts and dentinal tubules. The negative control (NC) stained with secondary antibody only was immunonegative.

Fluoride Exposure Resulted in a Significant Up-regulation of NBCe1 in Mouse Ameloblasts *in vivo*

Analysis of triplicate samples of mRNA isolated from secretory- and maturation-stage ameloblasts showed a small (1.5 X) but significant up-regulation of NBCe1 in fluorosed maturation ameloblasts as compared with controls (Fig. 3D).

NBCe1 Expression by Human Ameloblast-lineage Cells *in vitro*

NBCe1 expression was up-regulated in ameloblast-lineage cells cultured at low pH (pH 6.2) for 72 hrs. However, fluoride (up to 50 μ M) had no effect on NBCe1 expression at neutral pH (Fig. 3E).

DISCUSSION

NBCe1 is encoded by the SLC4 gene, which functions in the absorption or secretion of HCO_3^- or H^+ by epithelia (Romero *et al.*, 2004), and it has a cell- or tissue-specific distribution pattern (Pushkin and Kurtz, 2006). In this study, we investigated the localization and expression pattern of NBCe1 in human and mouse incisors, and also determined whether NBCe1 expression was affected by fluoride-related effects on enamel formation and biomineralization.

NBCe1 is required for the development of normal enamel (Lacruz *et al.*, 2009), and has been hypothesized to have a role in enamel matrix pH regulation (Paine *et al.*, 2008; Josephsen *et al.*, 2010). We found that NBCe1 was present in both human and mouse incisors and was up-regulated as ameloblasts differentiated. However, different transcripts and patterning were found in the two species. Similar to Paine and co-workers (Paine *et al.*, 2008), we found NBCe1-B in mouse ameloblasts. NBCe1-B was also present in the human tooth/mandible, but was not found in human ameloblasts. This suggests that, in humans, NBCe1-B is related to cells of mesenchymal origin (e.g., odontoblasts), with NBCe1-A specific to human dental epithelial cells. It is not clear why the transcripts would differ in mouse and human ameloblasts. However, future studies of the differences between the two isoforms are needed to better understand the differential expression of these transcripts.

ISH of human incisors first showed the presence of NBCe1 mRNA in secretory-stage ameloblasts, whereas with qPCR, NBCe1 could be identified as early as in the cells of the cervical loop and was progressively up-regulated as the cells differentiated. In mouse incisors, NBCe1 could be detected by ISH beginning

in the cervical loop. The differences between ISH in mouse and human enamel organs is most likely due to a relative increase in abundance of the NBCe1 transcript in mice as compared with humans, allowing for detection by the relatively less-sensitive technique of ISH as compared with PCR amplification.

It is not clear why NBCe1 would be expressed so early in human and mouse incisor enamel organs, but the identification of NBCe1 protein first beginning at the secretory stage of human incisors is similar to what was found by Paine and co-authors (Paine *et al.*, 2008) in the mouse incisor. These results suggest that although mRNA is present early in enamel organ differentiation, NBCe1 protein becomes functional at the secretory stage when enamel mineralization is initiated. The presence of NBCe1 in secretory odontoblasts and dentinal tubules also supports the hypothesis that NBCe1 is related to mineralization that also takes place in dentin.

As the enamel matrix mineralizes, increasing numbers of protons are released, requiring neutralization of the matrix by the ameloblasts (Lacruz *et al.*, 2009). In the kidney, pH modulation occurs by an efflux of bicarbonate across the cellular apical membrane (Pushkin and Kurtz, 2006), and this same mechanism may buffer the acidification of the enamel matrix. In addition, it is possible that secretion of bicarbonate into enamel matrix may increase the local pH in the matrix adjacent to ameloblasts, forming a matrix microenvironment that favors hydroxyapatite formation to enhance continuing enamel crystal formation (Lacruz *et al.*, 2010).

In a mouse ameloblast-like cell line, NBCe1 was up-regulated when pH was reduced (Paine *et al.*, 2008). We found this same effect in human ameloblast-lineage cells when pH was reduced for 72 hrs. This supports the hypothesis that NBCe1 functions as a regulator of pH in ameloblasts.

To investigate the possible role of NBCe1 in mineral-mediated changes in matrix pH *in vivo*, we used a fluorosed mouse model. Rodents are considered good models for the study of enamel fluorosis, and serum levels of 2.33 μM F in A/J mice given 50 ppm fluoride in drinking water (Carvalho *et al.*, 2009) are similar to those in humans

ingesting 2-5 ppm ($\mu\text{g/mL}$) fluoride in their drinking water. Fluoride is known to enhance mineral deposition, which has been hypothesized to further acidify the enamel matrix

Figure 2. *In situ* hybridization of NBCe1 in developing human (A) and mouse (C) incisors. (A) NBCe1 mRNA was detected in human presecretory (PSAB) and secretory ameloblasts (SAB) (boxes c, d), with more intense signal in SAB. No signal was detected in the cervical loop (CL) and pre-ameloblast region (PAB) (boxes a, b). The sense control (NC) for human (box e), and (B) mouse incisors showed no positive signal. (C) Signal for NBCe1 mRNA was detected in the basal region of all ameloblast lineage cells in mouse incisors, beginning at the cervical loop (a), and including presecretory (b) and secretory ameloblasts (c). At the secretory stage, the signal became more intense, and was also detected in the cytoplasm. Scale bar: 100 μm . 170 $\times$ 267 mm (300 $\times$ 300 DPI).

Figure 3. Characterization of the different stages of micro-dissected human dental epithelial cells. **(A)** PCNA and Pitx2 expression levels were down-regulated as cells differentiated. **(B)** qPCR showed that amelogenin expression was up-regulated when cells differentiated. The amelogenin expression level of secretory ameloblasts (SAB) was 327-fold higher as compared with that of cervical loop cells (CL). **(C)** Secretory ameloblasts (SAB) had the highest NBCe1 expression level relative to earlier stages of ameloblast differentiation. **(D)** In adult mouse incisors, maturation ameloblasts had higher NBCe1 expression than secretory ameloblasts, in both control (CS compared with CM, $p \leq 0.001$) and fluoride-treated groups (FS compared with FM, $p \leq 0.01$). Maturation ameloblasts in fluoride-treated mouse incisors (FM) had significantly higher NBCe1 expression than control mouse incisors (CM) ($p \leq 0.025$), as determined by one-way ANOVA test followed by the *post hoc* Tukey's test. **(E)** *In vitro*, fluoride had no effect on NBCe1 expression, whereas NBCe1 expression was significantly up-regulated at pH 6.2 as compared with pH 7.2 after 72 hrs of culture. Y axis: fold change of gene expression. CS, control secretory ameloblasts; CM, control maturation ameloblasts; FS, fluoride secretory ameloblasts; FM, fluoride maturation ameloblasts. 150 × 163mm (150 × 150 DPI).

(Bronckers *et al.*, 2009b). The up-regulation of NBCe1 expression in maturation ameloblasts, where rapid mineral formation occurs in the absence of the protein buffering available in the secretory-stage matrix (Lyaru *et al.*, 2008), supports the role of NBCe1 in modulating pH changes secondary to enamel mineral formation.

Although NBCe1 was significantly up-regulated in maturation-stage ameloblasts of mice ingesting fluoride, when fluoride was

added to ameloblast-lineage cell culture medium, NBCe1 expression was not affected. These results suggest that the effect of fluoride on NBCe1 expression is not a direct effect on ameloblasts, but rather supports the hypothesis that fluoride-related enhanced mineral deposition and matrix acidification up-regulate NBCe1 expression. Of course, it is possible that, with prolonged exposure to fluoride, there could be a direct effect on NBCe1 expression, which could further amplify potential pH-related effects of fluoride. Studies to investigate this possibility, including the use of mineral-inducing media, are warranted.

A fluoride-related enhanced mineral deposition resulting in an acidification of the maturation-stage enamel space could, in turn, trigger a wide range of compensating responses in ameloblasts. It is therefore possible that this proposed mechanism may, in part, account for our previous findings that exposure to high levels of fluoride during tooth development can result in retention of amelogenin proteins at the transition/early maturation stage (DenBesten and Crenshaw, 1984), and a reduced number of ameloblast modulations (DenBesten *et al.*, 1985; Smith *et al.*, 1993).

In conclusion, these studies support a role for NBCe1 in mediating matrix pH during enamel formation. They also show differences in NBCe1 expression in human as compared with mouse ameloblasts, and point to a role of pH modulators in compensating for fluoride-related changes in enamel mineralization.

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Figure 4. Cryosections of a human developing incisor stained with H&E (overview), with parallel sections immunostained for NBCe1 (green). **(a)** NBCe1 immunopositive signal was not detectable in the cervical loop (CL) and pre-ameloblast region (PAB) (box a). **(b)** Immunopositive signal was detected in presecretory ameloblasts (PSAB) (box b, arrows) and secretory odontoblasts (od) and dentinal tubules (triangles) (boxes b, c) (p, pulp tissue). **(c)** Immunopositive signal increased at the secretory ameloblast region (SAB) (box c, arrows). **(d)** Signal was not detected in the negative control (NC) human fetal incisor. Scale bar: 50 μ m. 170 $\times$ 110 mm (300 $\times$ 300 DPI).

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