Investigation of pH regulation mechanisms and the formation of tight junctions important in amelogenesis in a functional *in vitro* model

Doctoral thesis

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INTRODUCTION

Dental enamel is the hardest structure in the human body, with a degree of mineralization of nearly 97%. Its main inorganic component is hydroxyapatite, a form of calcium phosphate. It is formed in a multistep process during tooth development due to reciprocal interactions of epithelial and ecto-mesenchymal cells. The process of amelogenesis is well known at morphological and histological level, but the regulation of crystal formation and the exact molecular mechanisms underlying it are still largely unknown.

Highly specialized ameloblast cells differentiated from the inner enamel epithelium control enamel maturation. Their main tasks are the secretion of matrix proteins and the transport of enamel-forming minerals, especially the transcellular transport of calcium. **Amelogenesis can be divided into two main functional stages**. In the **secretory stage**, a less mineralized, protein-rich matrix structure is formed at the full height of the enamel. In the **maturation stage**, the organic matter is almost completely absorbed, and intensive transport processes result in a high level of mineralization, thus an increase in the thickness of the enamel crystals take place.

In addition to calcium and phosphate transport, **pH regulation is also important in enamel maturation**, as the formation of hydroxyapatite crystals generates a large quantity of protons, which must be neutralized by the secretion of bicarbonate ions (Racz et al., 2018). The important role of some transporters involved in pH regulation is also evidenced by the fact that certain transporter knock-out (KO) mouse models show significant enamel developmental defects.

Furthermore, the phenomenon of **pH modulation** is of paramount importance at the maturation phase, during which two different maturation ameloblast cell types, the smooth and ruffled surface ameloblasts form, transform cyclically into each other resulting in neutral and acidic enamel zones in the extracellular, apical space. During the cycle, the rearrangement of tight junctions (TJ) from a proximal location to a distal one (and vice versa) between the two cell forms is crucial (Varga et al., 2018).

Tight junctions do not seal the intercellular space hermetically, allowing passive flow of certain ions selectively (paracellular transport). According to the findings of the last decade, it is not a static structure, but rather acts as a kind of dynamic ion channel that allows some selectivity according to the charge and size of ions. This is primarily determined by the composition of claudin

proteins, but very little information is yet available on paracellular transport in ameloblasts.

Very little is known about the **regulation of secretory processes** in amelogenesis. A couple reports suggest the importance of steroid hormone (e.g. androgen and estrogen) receptors, extracellular ATP (purinergic receptors), calcium-sensing receptor, and certain G-protein coupled receptors (e.g., receptors of CCK, GRP, PACAP) (Varga et al., 2018); however, to explore their actual role, functional studies would be necessary.

Genetic (such as amelogenesis imperfecta) and environmental **disorders of amelogenesis** not only provide important information on the role of transporters, ion channels, and regulatory molecules essential in enamel formation, but our better understanding of molecular processes may open up new ways in the prevention and defence against various enamel hypomineralization disorders (e.g., dental fluorosis, or molar incisor hypomineralization).

Therefore, in our department **we developed an *in vitro* functional model** in recent years to explore the epithelial ion transport processes which are important in amelogenesis. Central to this are HAT-7 cells seeded and differentiated on a permeable polyester support (Transwell). HAT-7 is a dental epithelial cell line derived

from the cervical loop epithelium (the stem cell reservoir of ameloblast cells) of a rat incisor.

The model meets the requirements that a model studying epithelial transport processes must satisfy: the formation of a tightly closed cell layer, and the establishment of tight junctions that enable paracellular and directed transcellular transport processes to take place, as they prevent apical (AP) and basolateral (BL) mixing of membrane domains.

The functional polarization of our model was demonstrated in our publication published in 2016 (Bori et al., 2016). We have shown by immunocytochemistry and PCR studies that HAT-7 cells exhibit several ameloblast characteristics, including the expression of amelogenin and ameloblastin, the expression of kallikrein-4 and amelotin maturation-stage ameloblast markers, and also important ion transporters and tight junction proteins. HAT-7 cells grown on permeable supports reach a high transepithelial resistance, are functionally polarized, and can mediate vectorial HCO_3^- transport (Bori et al., 2016).

AIMS / OBJECTIVE

We have developed an *in vitro* model, using the HAT-7 rat ameloblast cell line, to study epithelial ion transport processes during amelogenesis (Bori et al., 2016). We have shown by immunocytochemistry and PCR studies that HAT-7 cells express ameloblast markers. When HAT-7 cells are grown on permeable membranes, they develop tight junctions, are functionally polarized, and can mediate vectorial HCO_3^- transport (Bori et al., 2016). We were also interested in the components of the HCO_3^- transport and pH regulatory mechanisms in ameloblast cells.

1) Therefore, we aimed to functionally identify the **localized activity of transporters** affecting pH regulation in polarized HAT-7 cells:

a) Is there any sodium/proton exchange activity in the cells that ensures the intracellular accumulation of bicarbonate?
b) Can the activity of electrogenic sodium-bicarbonate cotransporter be detected, and which symporter is capable of the direct transport of bicarbonate?
c) Is there any sodium-potassium-chloride cotransporter activity in the cells, a symporter that can provide chloride ion replenishment for the transport of bicarbonate?

d) Can anion exchange activity be detected in cells, an electroneutral antiporter of bicarbonate and chloride ions?

Our in vitro monolayer model may offer a tool to investigate some of the mechanisms associated with fluorosis: the direct cytotoxic effects of fluoride, its effect on the formation of tight-junctions, and its direct inhibitory effect on vectorial HCO_3^- transport. Therefore, we also aimed to examine

2) whether acute **fluoride** exposure interferes with the ability of transporters to ensure **basolateral-apical HCO_3^- transport**, and

3) to investigate the effect of fluoride on the **viability** of HAT-7 cells, furthermore

4) whether fluoride has any effect on the **transepithelial resistance** of polarized cells, on formation of **tight junctions**, and on the gene expression of tight junction proteins.

METHODS

Cell culture and establishment of the polarized cellular model

To obtain monolayers, HAT-7 cells were seeded on permeable polyester Transwell membranes (Corning) and HAT-7 cells were cultured in DMEM/F12-Ham medium supplemented with 10% HyClone FBS (fetal bovine serum), 100 U/ml penicillin and 10 μg/ml streptomycin in a humified atmosphere containing 5% CO_2 at 37°C. The basic medium was supplemented with $CaCl_2$ (2.1 mM final concentration) and 10^{-5} mM dexamethasone to differentiate and grew the cells on Transwell permeable supports.

Measurement of transepithelial electrical resistance

The development of tight junctions was evaluated by measurement of the transepithelial electrical resistance (TER) of HAT-7 cells grown on Transwells. TER values give an indication of tight junction formation and the paracellular permeability to electrolytes, key characteristics of secretory epithelia, were measured daily using an epithelial volt-ohmmeter (EVOM) on five consecutive days prior to microfluorometric measurements and during NaF treatments. For fluoride treatment experiments, media were changed at 24h after cell seeding to 0 (control), 0.3, 0.6 and 1 mM sodium-fluoride containing differentiation medium.

Mikrofluorometry

Intracellular pH (pH_i) changes were monitored real-time by microfluorometry using the pH sensitive fluorescent indicator, BCECF-AM dye. The dye was used in the ratiometric way, i.e. we detected the pH-dependent (F490) and pH-independent (F440) fluorescence intensities measured at 530 nm using two distinct (490 and 440 nm) excitation wavelengths. Using the ratio of the two fluorescence signals, we could eliminate artefacts originated from differences in dye loading, dye concentration, dye leakage or photobleaching. F490/F440 ratio values were converted to pH using calibration data obtained by the nigericin/high potassium method.

We perfused the measurement chamber with different solutions at the two sides of monolayer cells on Transwells, and particular transporter activities could be identified by controlling the extracellular solutions (e.g. specific ion withdrawal, application of transporter inhibitors). The so-called ammonium pulse technique was applied when an alkali/acid load was necessary to examine the transporters involved in compensation of these pH_i changes.

The solutions used for perfusion were standard HEPES-buffered solution, standard HCO_3^- buffered solution. For Na^+ withdrawal,

Na^+ was replaced by equimolar N-methyl-D-glucamine ($NMDG^+$). For Cl^- withdrawal, Cl^- was replaced with equimolar $gluconate^-$.

For inhibiting specific transport processes 100 μM DIDS was used to block anion-exchangers, 300 μM amiloride was used to block Na^+/H^+exchange, 500 μM H2DIDS for Na^+-HCO_3^- cotransport, and 100 μM bumetanide for NKCC. For stimulation of transport, 50 μM ATP was used to elevate intracellular calcium and 10 μM forskolin, in combination with 500 μM IBMX (3-isobutyl-1-methylxanthine), were used to elevate intracellular cAMP.

Cell viability assay

Cells were plated in 96-well plates, and fluoride treatments were started 24h after seeding. After 24 and 48 h, cell metabolic activity was evaluated by alamarBlue Cell Viability Assay (Thermo Scientific) by reading the plates for fluorescence at 590 nm with a PerkinElmer LS50B luminescence spectrometer.

Quantitative PCR

The expression of tight junction proteins was investigated by quantitative real-time PCR. Total RNA was isolated from cells grown in control and fluoride-containing media, and cDNA was reverse transcribed from 1-2μg. Amplification was performed by the ABI StepOne System and using predesigned primers (Applied

Biosystems). Acidic ribosomal protein P0 (Rplpo) was used as internal control and the ΔΔCt method was used to quantify gene expression using the ABIPrism software.

Statistical analysis

Unpaired t-test was used where only two groups had to be compared. For comparing multiple groups, we used one-way or repeated-measures ANOVA followed by Dunnett's post hoc test. In the transepithelial resistance experiments the non-parametric Kruskal-Wallis test and the Dunn's post hoc test were used to compare values.

RESULTS

Activity of the major basolateral transporters participating in pH regulation in polarized HAT-7 cells

In our 2016 publication we provided evidence for that HAT-7 cells differentiated on Transwell membrane can form polarized monolayers, and are capable of a vectorial (apical to basolateral) HCO_3^- secretion (Bori et al., 2016), but the components of the HCO_3^- transport and pH regulatory mechanisms, in particular regard to their localized activity, have not been identified yet.

a) Sodium-proton exchanger activity at the basolateral side

Significant intracellular acidification can be achieved in HAT-7 cells by the ammonium pulse technique, i.e. the short-term perfusion of a measuring solution containing 20 mM NH_4Cl. Thus the rate of pH_i recovery from acid load can be measured by microfluorometry to examine the transporters involved in pH compensation.

Removal of Na^+ after acid load (ammonium pulse) from the *HCO_3^-/CO_2-free* HEPES buffered solution in both side completely blocked the compensation of intracellular pH, suggesting the role of sodium-dependent transporters in pH regulation. Na^+ restoration on the basolateral side caused a steep increase in pH_i, a rapid recovery of pH. This pH compensation mechanism on the basolateral side was amiloride (300μM) sensitive ($p<0.5$ vs control) indicating sodium-proton exchanger activity. The presence of NHE1 isotype was further verified using a more specific inhibitor, the cariporide.

b) Sodium-dependent bicarbonate transport activity at the basolateral side

Removal of Na^+ after acid load (ammonium pulse) from the *HCO_3^-/CO_2 containing* solution in both side also blocked the compensation of intracellular pH, suggesting that bicarbonate transporters acting in pH regulation are sodium-dependent. Na^+ restoration on the basolateral side caused a sharp increase in pH_i, and this pH compensation was largely amiloride sensitive ($p<0.5$ vs control). This inhibition was significantly enhanced by using 500μM H_2DIDS ($p<0.5$ vs amiloride alone) indicating basolateral NBC activity. Thus pH compensation mechanisms in polarized HAT-7 cells might involve both bicarbonate uptake by NBCe1, and proton extrusion by NHE1.

c) Sodium-potassium-chloride cotransporter activity at the basolateral side

It is known from the literature that sodium-potassium-chloride cotransporter (NKCC1) can transport NH_4^+ ions instead of K^+, while NH_3 freely passes through membranes. These features can be exploited to study the activity of this transporter by microfluorometry using the alkaline phase of ammonium pulse technique. The pH_i compensation after alkali load (20 mM NH_4Cl)

in *HCO_3^-/CO_2 free* HEPES buffered solution was partly due to ammonium transport by NKCC, as there was a significant decrease in the rate of compensation in the basolateral presence of the NKCC specific inhibitor, bumetanide (100μM) ($p<0.5$ vs control). The presence of NKCC in HAT-7 cells is in consistence with our previous RT-PCR results.

d) Anion-exchanger activity at the basolateral side

Anion-exchanger proteins (AE) exchange HCO_3^- for Cl^- in a reversible, electroneutral manner. In our polarized HAT-7 cells, removal of Cl^- basolaterally from the *HCO_3^-/CO_2 free* HEPES buffered solution elicited an increase in pH_i as a result of *HCO_3^-* influx due to the reversed ion-gradient. This pH_i increase was sensitive to DIDS, an AE inhibitor ($p<0.5$ vs control), suggesting that anion-exchangers might be functional on the basolateral side.

The effect of fluoride on bicarbonate secretion in HAT-7 cells

Besides the direct transport of bicarbonate by NBC (using sodium gradient as a driving force), cells can also accumulate HCO_3^- by the diffusion of CO_2 into the cells, and its conversion into HCO_3^- and H^+ (catalysed by carbonic-anhydrases) and subsequent H^+

extrusion by NHE. As it was demonstrated in our 2016 publication, when HCO_3^- uptake is blocked on the basolateral side (by using NBC and NHE inhibitors) in the polarized HAT-7 cells, the continuing efflux (apical secretion) leads to a slow intracellular acidification, that can be detected using pH microfluorometry, and this base secretion can be further enhanced by simultaneous Ca^{2+} and cAMP-mobilizing stimuli (50μM ATP, 10μM forskolin, and 500μM IBMX) (Bori et al, 2016). In the present work the initial acidification rate, a measure of HCO_3^- secretion was used to test, whether fluoride has a direct or indirect effect on vectorial HCO_3^- transport. We have found that acute 0.03–1 mM fluoride exposure did not significantly affect stimulated bicarbonate secretion in our HAT-7 cellular model.

The effects of fluoride on cell viability

The cytotoxic effects of fluoride were evaluated by the alamarBlue viability assay. We observed that metabolic activity of the cells was not affected up to 0.6 mM fluoride concentration, while 1 mM fluoride slightly reduced their metabolic activity, but was still not toxic, as cells have grown to the full surface of membranes confluently. In contrast, 3 mM fluoride and higher doses were totally toxic, and killed cells after just 48 hours.

The effects of fluoride on transepithelial resistance (TER) development, and on the expression of tight junction proteins

Tight junction proteins are essential components of ameloblast polarization and differentiation. We monitored tight junction formation and polarization by measuring daily the transepithelial resistance (TER) of the cells cultured on Transwell membranes in the absence and presence 0.3-1 mM sodium-fluoride. The cells became confluent in all of the samples. We detected that 0.3-0.6 mM fluoride did not affect, while 1 mM fluoride significantly delayed TER development ($p<0.5$ vs control).

Examining the expression of tight junction proteins by quantitative PCR, to our surprise, fluoride exposure did not reduce the gene expression of the most important members of the TJ complex (Tjp1, Cldn1, Cldn4, Cldn8, Cldn16 és Cldn19), rather a small, but significant increase was observed.

CONCLUSIONS

1) Functionally active basolateral transporters that affect intracellular pH regulation can be identified in ameloblast-like HAT-7 cells grown as a polarized monolayer on Transwell membrane.
    a) Na^+-dependent sodium/proton exchange activity can be detected on the basolateral side of the cells. By removal of protons from the cell on the basolateral side, the transporter can provide intracellular accumulation of bicarbonate in ameloblast cells. This activity is also sensitive to the isotype-specific, more selective cariporide inhibitor, suggesting the functional presence of the NHE-1 isotype.
    b) In the presence of external bicarbonate, basolateral activity of the electrogenic sodium-bicarbonate cotransporter can also be detected, a mechanism that may contribute to the direct transport of bicarbonate ions into ameloblast cells.
    c) On the basolateral side of the cells, the activity of sodium-potassium-chloride cotransporter can also be

detected, which may provide replenishment of chloride ions in ameloblasts.

d) There is a significant basolateral anion exchange activity in the cells, a mechanism that may be an important component in the pH regulation of ameloblast cells.

2) Acute exposure to fluoride (over a wide concentration range) does not significantly affect the stimulated vectorial, basolateral to apical HCO_3^- secretion in HAT-7 cells grown on Transwell membrane, so fluoride is unlikely to have a direct effect on secretion.

3) Fluoride in the micromolar range (up to 1 mM) has no substantial effect on HAT-7 cell viability, but has been shown to be toxic at 3 mM concentration.

4) The non-toxic 1 mM fluoride concentration significantly delays the development of transepithelial resistance of HAT-7 cells differentiated on Transwell membrane. This indicates inhibition of the formation of tight junctions by fluoride. In contrast, 1 mM fluoride does not reduce (rather slightly increase) the gene expression of Tjp1, Cldn1, Cldn4, Cldn8, Cldn16 and Cldn19 tight junction proteins in HAT-7 cells. These observations suggest that instead of protein expression,

fluoride may inhibit the formation of tight junctions in their assembly and/or their membrane translocation, thus our work opens further interesting research directions in enamel fluorosis research.

Our data do not diminish the importance of other mechanisms hypothesized so far in the development of dental fluorosis. To some extent several of these mechanisms may contribute to hypomineralization depending on the actual local concentrations of fluoride. However, the putative role of fluoride in delaying the development of tight junctions may also be of great importance when considering the cyclic structural and functional changes in ameloblasts during enamel maturation (the approximately 8h cycle of ameloblast cell forms, TJ rearrangement, and pH modulation in rats).

LIST OF PUBLICATIONS BY THE AUTHOR

Original publications published in the topic of the dissertation:

1. **Racz R**, Nagy A, Rakonczay Z, Dunavari EK, Gerber G, Varga, G. *Defense mechanisms against acid exposure by dental enamel formation, saliva and pancreatic juice production.* CURRENT PHARMACEUTICAL DESIGN 24 : 18 pp. 2012-2022. (2018)

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3. **Racz R**, Foldes A, Bori E, Zsembery A, Harada H, Steward MC, DenBesten P, Bronckers ALJJ, Gerber G, Varga, G. *No Change in Bicarbonate Transport but Tight-Junction Formation Is Delayed by Fluoride in a Novel Ameloblast Model.* FRONTIERS IN PHYSIOLOGY 8 : 940. pp 1-12. (2017)

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Other publications:

1. Farkasdi S, Pammer D, **Rácz R,** Hriczó-Koperdák G, Szabó BT, Dobó-Nagy Cs, Kerémi B, Blazsek J, Cuisinier F, Wu G, Varga G. *Development of a quantitative preclinical screening model for implant osseointegration in rat tail vertebra.* CLINICAL ORAL INVESTIGATIONS 23 : 7 pp. 2959-2973. (2019)