Characterization of transport processes in amelogenesis and saliva secretion

PhD thesis booklet

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Introduction

Epithelial fluid and electrolyte secretion has a particular importance in the development and maintenance of oral health. However, the final products may show great differences – see e.g. the example of hypotonic saliva secreted by the salivary glands or the ameloblast produced dental enamel, the hardest tissue in our body – some mechanisms are shared in different epithelia. While saliva secretion and its effects on oral health are well-studied in the scientific literature and research is moving towards practical applications of the accumulated knowledge, the transport processes of ameloblasts are barely known. The subject of this thesis was to investigate some biological therapeutic possibilities of salivary hypofunction on primary salivary gland cell cultures and a rat parotid gland cell line, as well as to present for the first time an *in vitro* model that is suitable for studying the HCO_3^- transport of ameloblasts.

Saliva is a hypotonic fluid containing electrolytes and proteins. It has an important role in the mechanical, chemical and microbiological defense of the oral cavity. Its importance is evident as each year approximately 500 000 of new patients suffer from the consequences of salivary gland hypofunction worldwide. In addition to the sensation of dry mouth (xerostomia), these patients have difficulties in mastication, swallowing and sometimes even in speaking. The incidence of caries and oral infections as well as the risk of periodontitis increase and in case of severely affected patients, mouth and esophageal ulceration may also occur.

Salivary gland hypofunction may be caused by certain drugs or systemic conditions, however, the two most common causes are the autoimmune Sjögren's syndrome and the therapeutic irradiation of head and neck cancers. Despite the different underlying conditions, salivary hypofunction occurs due to the loss of function of the acini that are responsible for fluid and electrolyte secretion in the gland while the less sensitive reabsorptive ductal system remain intact. Currently there are no effective pharmaceutical tools, but biological therapies (ductal-acinar transdifferentiation, stem cells, gene therapy and artificial salivary gland) may offer new possibilities to treat xerostomia. Research in this area focuses on retaining/restoring

acinar characteristics (ductal-acinar transdifferentiation, stem cells and artificial salivary gland) or the re-engineering of the ductal function (gene therapy).

Similarly to salivary gland cells, ameloblasts also have epithelial origin; they differentiate from the inner enamel epithelium during the bell stage of tooth development. Amelogenesis – just as saliva secretion – is a two-step process. During the secretory phase, in addition to Ca^{2+} and PO_4^{3-} transport, ameloblasts secrete matrix proteins (amelogenin, ameloblastin, enamelin) for initiating crystal growth. Later, during the maturation phase, these matrix proteins are degraded and reabsorbed by the ameloblasts, mineralisation continues, and by the end of maturation the mineral content grows from 30% to 96-98%. Characteristic markers of the maturation phase are the matrix protease kallikrein 4 (KLK4) and the less known amelotin. It seems that in addition to the above mentioned functions ameloblasts also play an important role in buffering the mineralization space. First of all, during the growth of hydroxyapatite crystals, a large amount of protons is liberated because in physiological conditions the phosphate precursors of the hydroxyapatite are mostly protonated. Secondly, several important pH regulators can be found to be expressed by ameloblasts such as carbonic-anhydrases (CAR), anion exchangers (AE), sodium-bicarbonate cotransporter (NBC), sodium-proton exchanger (NHE), cystic fibrosis transmembrane conductance regulator (CFTR) and v-type proton-ATPase. Additionally, the loss of these molecules can lead to enamel defects. Active HCO_3^- transport by ameloblasts may be the most important during the maturation phase when crystal growth is the most intensive.

The main obstacle for investigating ameloblasts is their difficult accessibility. Their *in vivo* or *ex vivo* examination is severely hampered as the cells form one single layer on the surface of the developing dental enamel. Sampling of the mineralization space is not viable; only the changes of the enamel may be indicative of the underlying processes. In addition, in non-continuously growing teeth, ameloblasts are lost after tooth eruption which means that in humans the investigation of embryonic tooth germs would be required thereby raising ethical

concerns. Consequently, histological, protein- and gene expression methods are applied mainly on continuously growing rodent incisors in which the different forms of ameloblasts are present at the same time on the different sections of the developing tooth surface. For this reason, the establishment of *in vitro* models suitable for functional measurements is of great importance.

Aims

The main foci of research on biological therapies targeting salivary gland hypofunction are the sustention/restoration of acinar characteristics and the re-engineering of ductal function by gene therapy. In association with this we aimed

1. to establish primary human submandibular gland cell cultures by the application of fractionated enzymatic digestion and conditioned medium and to study the expression of key salivary ion transporters and channels in these cultures investigate whether acinar characteristics could be preserved *in vitro;*
2. to provide evidence that the rat parotid gland derived cell line, Par-C10 is a suitable model for salivary gland gene therapy.

Our knowledge regarding the HCO_3^- transport of ameloblasts is mainly based on the results of histology and gene expression studies. So far, there was no chance to perform functional cell physiological measurements in the absence of appropriate models. For this reason we aimed to establish an *in vitro* model that would be suitable for the functional analysis of the HCO_3^- transport of ameloblasts. We investigated whether

3. rat dental epithelium derived from HAT-7 cells express protein markers characteristic to ameloblasts when seeded onto Transwell membranes and cultured in DMEM-F12-based Control and Differentiation or in HepatoSTIM media;
4. HAT-7 cells under circumstances described in the 3rd point are able to form tight junctions which is a prerequisite for vectorial transport;
5. HAT-7 cells under the circumstances described in the 3rd point are able to express those pH regulators (CAR2, NHE1, AE2, NBC1, CFTR) that were found (also) in ameloblasts *in vivo* according to the literature;
6. HAT-7 cells under any of the circumstances described in the 3rd point are able to transport HCO_3^- thereby constituting a suitable model of the HCO_3^- secretion of ameloblasts.

Methods

Primary human submandibular gland cells were isolated as described by Tran et al. and Szlavik et al. with modifications in order to enhance the viability of the cells and to prevent the damage of the more sensitive acinar cells: fractionated enzymatic digestion was applied during the isolation and conditioned medium was used during the culturing of the cells. We established two types of cell cultures: quickly attaching, mixed epithelial-mesenchymal PT-HSG and from one-day-old floating cell aggregates, huSMG culture that is enriched in less quickly attaching epithelial cells. Cells were seeded onto Transwell membranes and cultures in HepatoSTIM. Their gene expression was investigated by RT-PCR.

Par-C10 cells were maintained in DMEM-F12 medium supplemented with 10% FCS, 0.1 μM retinoic acid, 2 nM triiodothyronine and 0.4 μg/ml hydrocortisone. Adenoviral vectors encoding fluorescent protein (AdEYFP) and water channel (AdAQP1) were prepared and amplified as it was described previously by He et al. and Delporte et al. Expression of the transgene was detected at RNA and protein level by RT-PCR (AQP1) and fluorescent microscopy (EYFP), respectively. Fluid movement across Par-C10 monolayers seeded onto Transwell membranes were measured by gravimetry following transduction with 3 viral particles/cells.

HAT-7 cells were cultured on Transwell membranes in three different media. DMEM-F12 supplemented with 10% fetal calf serum (HyClone) was applied as Control medium. To enhance differentiation, two additional media were used: the so-called Differentiation medium which was a variant of the Control medium supplemented with $CaCl_2$ to 2.1 mM final concentration and 10^{-5} mM dexamethasone, while cells were also cultured in HepatoSTIM that was successfully applied previously for the maintenance of primary human salivary gland cell cultures. Gene- and protein expression of the cells was investigated by RT-PCR and immunocytochemistry. Transepithelial resistance that is an indicator of the formation of tight junctions was measured by epithelial volt-ohm meter (EVOM). HCO_3^- transport of the cells

was examined by microfluorometry. Briefly, the cells were loaded with pH sensitive fluorescent dye, BCECF. By the controlling of the extracellular environment (ionic composition, transporter inhibitors) the particular elements of HCO_3^- transport could be identified, transport processes active in the given conditions could be deduced from the changes of the intracellular pH.

Results

As expected according to the literature, in native salivary gland samples both the transporters involved in the Cl^- uptake of acini ($Na^+/K^+/2Cl^-$ cotransporter 1 (*NKCC1*), Na^+/H^+ exchanger 1 (*NHE1*) and anion exchanger 2 (*AE2*)) and the characteristic sodium channel of the ducts (epithelial sodium channel (*ENaC*)) were detected at RNA level. In addition to the transporters and channels above Na^+/HCO_3^- cotransporter 1 (*NBC1*) was also expressed. There was no significant change in the RNA level of *ENaC, NHE1* and *AE2* when primary cell cultures were compared to the native tissue. In contrast, the expression of *NKCC1* and *NBCe1* decreased both on plastic and on membrane surfaces. Practically no difference was found in the expression patterns of PT-HSG and huSMG cultures. Although the expression of the key acinar electrolyte transporter, *NKCC1,* significantly declined in PT-HSG and huSMG cultures indicating the dedifferentiation of the cells, the acinar characteristics could be retained partially.

The Par-C10 cells were able to express transgenes following adenoviral transduction. The *AQP1* mRNA could be detected following the application of 1, 10 and 100 viral particles/cells virus dose. In contrast, those cultures that were not treated with the recombinant viral vectors did not show *AQP1* expression. Water permeability of the Par-C10 cells could be increased by AdAQP1 gene delivery. Water movement through the non-transduced monolayers was 0.4 ± 4.5 µl in isosmotic medium following 2 hours of incubation that increased to 14.0 ± 5.7 µl when hyperosmotic medium was applied apically even in the absence of viral treatment. Fluid movement through AdAQP1 treated cells in isosomotic conditions was 1.6 ± 2,3 µl and 31.2 ± 3.2 µl when hyperosmotic driving force was provided. This latter is considered significantly higher compared to non-transduced cells, respectively EYFP protein was also expressed by a great portion of the cells following transduction with 3 viral particles/cells dose.

HAT-7 cells reached confluence within 2-3 days on Transwell membrane surface independently of the culture media. They showed typical cobblestone morphology under

phase contrast microscope. Expression of the typical maturation phase markers, the matrix protease KLK4 and the recently discovered amelotin were found by immunocytochemistry.

There were striking differences between the transepithelial resistance values when HAT-7 cells where cultured in different media. Resistance curves typically reached a peak value on the fourth-fifth day and declined to a lower plateau phase by the seventh day. TER was the lowest in cells grown in Control medium and highest in HepatoSTIM medium, typical resistance values measured on the fifth day were 187±23 Ωcm^2, 567±170 Ωcm^2 and 1606±277 Ωcm^2 in Control, Differentiation and HepatoSTIM media respectively. The molecular investigation of tight junctions revealed gene expression of zonula occludens 1 (*Zo1*) and 2 (*Zo2*), occludin (*Occl*) as well as claudin 1, 4, 7 and 8 (*Cldn1, 4, 7 and 8*). The greatest difference was found in Cldn8 expression by semiquantitative analysis; it was the lowest in cells cultured in Control medium while the highest (approximately three times higher compared to Control) in HepatoSTIM in accordance with the results of the TER measurements. Claudins expressed in HAT-7 cells predominantly decrease paracellular permeability, and according to literature, all of these were found in the tight junctions of ameloblasts.

Expression of electrolyte transporters and channels involved in HCO_3^- secretion that are published to be found in ameloblasts such as *Nhe1, Ae2, Nbc1, Pendrin* and *Cftr* were also detected in HAT-7 cells independently of culture conditions, although at variable levels. The cytosolic carbonic anhydrase *Car2* was also expressed. Investigating cells cultured on Transwell membranes in Differentiation or HepatoSTIM medium by immunocytochemistry NBC1, AE2, Pendrin, PAT1, CFTR and CAR2 were found also at protein level. There was no difference in the quantity or the expression profile.

By microfluorometry, first of all, the HCO_3^- and CO_2 permeability of HAT-7 cell membranes were examined. After perfusing both the apical and basolateral sides of the cells with the HCO_3^-/CO_2-free HEPES-buffered solution, apical or basolateral perfusion was switched to the

HCO_3^-/CO_2 buffered solution. When measurement solution was switched apically, a rapid acidification of intracellular pH occurred as a result of CO_2 diffusion into the cells. Following this, pH reached a new equilibrium and remained at this more acidic level until the HCO_3^-/CO_2-free HEPES solution was restored at the apical side, indicating that there is no HCO_3^- uptake through the apical membrane. When the same change, from HEPES to HCO_3^-/CO_2 buffered solution was performed on the basolateral side, only a tiny and transient pH fall occurred before pH increased rapidly showing significant uptake of HCO_3^- ions. Basolateral HCO_3^- uptake was sensitive to the inhibitor of NBC, 500 μM H_2DIDS. The application of the membrane permeable carbonic anhydrase inhibitor, 100 μM acetazolamide, also decreased the pH elevation indicating the functional activity of these enzymes in HAT-7 cells.

When cells are exposed to acid load in HCO_3^-/CO_2 buffered solution, pH compensation may occur due to H^+ extrusion by proton pumps and/or Na^+/H^+ exchangers and/or due to HCO_3^- uptake by Na^+-HCO_3^- cotransporters. To check the presence of the above transporters Na^+ was withdrawn from the measurement solution right after the acid load. As a result, pH compensation was completely abolished showing that proton pumps cannot be found in HAT-7 cells, Na^+-dependent processes are responsible for the pH recovery alone. After the restoration of Na^+, intracellular pH recovered, however, it could be restrained by 85% by the joint application of 300 μM amiloride and 500 μM H_2DIDS, the inhibitors of NHE1 and NBC1, respectively.

The ability to block HCO_3^- uptake pathways across the basolateral membrane allows us to estimate the rate of HCO_3^- secretion across the apical membrane. Following basolateral blockade, HCO_3^- secretion continues at the apical membrane and pH_i decreases as a result. HCO_3^- secretion of HAT-7 cells was measured in HCO_3^-/CO_2 buffered solution by the application of basolateral amiloride and H_2DIDS inhibition. Different agonists were used to stimulate the secretion such as 50 μM ATP to mobilize the intracellular Ca^{2+} and/or the combination of membrane permeable 10 μM forskolin and 500 μM IBMX. Unstimulated

HAT-7 cells show minimal basal secretion however the intracellular acidification is measurable following 50 μM ATP basolaterally. Forskolin and IBMX greatly potentiate the effect of basolateral ATP indicating synergism between the Ca^{2+} and cAMP mediated signaling pathways that have been demonstrated earlier in several epithelia including salivary glands.

Conclusions

After reviewing the completed experiments and literature data we concluded that

1. Primary human submandibular gland cell cultures are successfully established/maintained by applying fractionated digestion and conditioned medium. In these cultures the cells partially retain acinar characteristics, for instance, the key acinar transporter *NKCC1* is expressed.
2. Par-C10 cell line is suitable for performing gene transfer experiments. The transgene is expressed by a great proportion of the cells and water permeability significantly increases following AdAQP1 transduction.
3. HAT-7 cells express maturation phase ameloblast markers (KLK4 and amelotin) when seeded onto Transwell membranes in DMEM-F12 based and HepatoSTIM media.
4. HAT-7 cells are able to form tight junctions indicated by the transepithelial resistance. The permeability of these tight junctions differs depending on culture conditions. In cells cultured in HepatoSTIM, paracellular permeability decreases significantly which is predominantly caused by the elevated expression of *Cldn8*.
5. HAT-7 cells express all important enzymes, transporters and channels (CAR2, NHE1, AE2, NBC1, Pendrin, PAT-1, CFTR) that have been found previously in ameloblasts and may play a role in the HCO_3^- transport process of ameloblasts.
6. HAT-7 cells are suitable to perform ion transport measurements when cultured in DMEM-F12-based medium supplemented with $CaCl_2$ and dexamethasone
    a) according to microfluorometric measurements, the cells are functionally polarized, their apical membrane is greatly permeable to CO_2, while HCO_3^- is taken up through the basolateral membrane;
    b) their HCO_3^- secretion is measurable and can be stimulated.

To sum up, we established an *in vitro* model for the first time that is suitable for the investigation of HCO_3^- transport related to ameloblasts.

List of publications

The thesis is based on the following articles:

2016

1. Bori E , Guo J , Rácz R , Burghardt B , Földes A , Kerémi B , Harada H , Steward M C , Den Besten P , Bronckers A L J J , Varga G
   Evidence for Bicarbonate Secretion by Ameloblasts in a Novel Cellular Model
   JOURNAL OF DENTAL RESEARCH 95: (5) pp. 588-596 (2016)

2015

2. Hegyesi O , Foldes A , Bori E , Nemeth Z , Barabas J , Steward MC , Varga G
   Evidence for Active Electrolyte Transport by Two-Dimensional Monolayers of Human Salivary Epithelial Cells.
   TISSUE ENGINEERING PART C METHODS 21:(12) pp. 1226-1236. (2015)

3. Varga G , Kerémi B , Bori E , Földes A
   Function and repair of dental enamel – Potential role of epithelial transport processes of ameloblasts
   PANCREATOLOGY 15:(4. Suppl) pp. S55-S60. (2015).

2014

4. Bori E , Rácz G , Burghardt B , Demeter I , Hegyesi O , Varga G , Földes A
   Par-C10 sejtek a parotis szöveti szerveződésének modellezésére
   FOGORVOSI SZEMLE 107:(3) pp. 99-105. (2014)

2013

5. Varga G , Bori E , Kallo K , Nagy K , Tarjan I , Racz GZ
   Novel Possible Pharmaceutical Research Tools: Stem Cells, Gene Delivery and their Combination.
   CURRENT PHARMACEUTICAL DESIGN 19:(1) pp. 133-141. (2013)