ACTIVE TRANSEPITHELIAL ELECTROLYTE TRANSPORT BY TWO-DIMENSIONAL HUMAN SALIVARY GLAND MODELS

Short PhD Thesis

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INTRODUCTION

Saliva is essential to the functioning of the oral cavity (such as food bolus formation, speech, mastication, etc.) and to the maintenance of oral health. The majority of saliva is produced by the major salivary glands: the parotid, submandibular and sublingual glands. The primary fluid, electrolyte and protein secretory units of the salivary glands are the acinar cells. The ductal cells, on the other hand, are absorptive and relatively water-impermeable. Autoimmune diseases such as Sjögren's Syndrome and the radiotherapy of head and neck cancer can lead to irreversible loss of acinar cells. Consequently, patients lack the ability to produce adequate volumes of saliva. The resulting speech impairment, swallowing difficulty and increased susceptibility to oral infections, caries and periodontitis significantly impact the quality of life. Currently there is no adequate, permanent treatment for xerostomia (dry mouth). However, various promising therapeutic approaches are emerging for the re-establishment of damaged acinar function. These include salivary gland regeneration from stem cells, gene transfer-based treatments, development of new pharmacological tools, and development of a tissue-engineered artificial salivary gland. An *in vitro* salivary secretion model could be of great benefit to these studies. Human salivary acinar cells are not available for building *in vitro* functional salivary secretory units. The HSG human submandibular adenocarcinoma cell line is not able to form tight junctions, which is essential for epithelial fluid and electrolyte secretion; in addition, the cells are also incapable of controlled fluid transport. The PTHSG and huSMG human primary salivary gland cell cultures are known to have ductal phenotype, however, the electrolyte transport of these cells has never been characterized.

OBJECTIVES

1. To prepare primary cultures and polarized secretory epithelial monolayers of human submandibular gland and to provide optimal conditions for isolation and cell culturing.

2. To investigate whether these cells can maintain expression of acinar markers and amylase secretion during cell culturing and monolayer formation on Transwell Clear polyester membranes.

3. To study the monolayer formation properties and transepithelial ion transport of SMIE, Par-C10 and HPAF cell lines. The purpose of these preliminary experiments on cell lines was to set experimental conditions for functional studies on primary cells.

4. To investigate the transepithelial ion transport of the primary salivary cell monolayers. The aim was to answer the following questions:
   a. Are these cells capable of performing transepithelial anion secretion?
   b. Can transepithelial ion movement be stimulated via intracellular Ca^{2+} or cAMP mediated stimulation pathways?
   c. Which ion transporters are involved in the basolateral anion uptake?

METHODS

Cell culturing All cell types were cultured in standard conditions (37°C, in an atmosphere of air containing 5% CO_2). Par-C10 (rat parotid acinar) cells were cultured in a DMEM-F-12 medium supplemented with retinoic acid, triiodothyronine and hydrocortisone. HPAF (human ductal pancreatic) cells were grown in a MEM medium supplemented with non-essential amino acids and Na^+-piruvate. SMIE (rat submandibular) cells were grown in a DMEM medium. The PTHSG and the huSMG primary cells were isolated from human submandibular salivary gland. The salivary gland tissue was obtained from the Department of Oro-Maxillofacial Surgery and Stomatology, Semmelweis University. The salivary gland samples were collected from patients undergoing neck dissection surgeries (Regional Human Research Ethics Committee permission number: 67/2005). Patients involved (49 patients ages between 40 and 86 years) received no prior irradiation treatment or chemotherapy and none of the patients had salivary gland tumors or other pathological salivary gland conditions. The isolation protocol was based on protocols of Szlavik et al. for PTHSG and Tran et al. for huSMG, with several minor modifications. The tissue samples were mechanically minced, then the cells were dissociated using a sequential digestion protocol that minimized enzymatic damage. The cell suspension was incubated overnight in a Hepato-STIM medium. On the following day the supernatant was transferred into a second dish. The non-attached, epithelial-like cells form the huSMG cell culture, the PTHSG cell culture contained both quickly attaching and non-attached cells. We examined two different media for cell growth and monolayer formation: Hepato-STIM and MEM. The culture medium was partially changed three times a week to retain secreted autocrin and paracrin factors. The cells were harvested when the plate reached

80% confluence (5-6 days after seeding). Functional studies were performed on cells from passage 1.-3. The cells were seeded onto Transwell Clear polyester membranes in order to perform polarized monolayers. Transepithelial electrical resistance (TER) was measured daily with an EVOM voltohmmeter.

Expression of marker proteins The mRNA expression of Na^+-K^+-$2Cl^-$ cotransporter NKCC1 *(SLC12A2)*, amylase (*AMY1A*) and aquaporin 5 (*AQP5)* as acinar markers, epithelial sodium channel ENaC *(SLC12A2)* and claudin-1 *(CLDN1)* as ductal markers was examined using RT-PCR. Total RNA was isolated from the salivary gland tissue samples, as well as from cultured PTHSG and huSMG cells grown both on plastic Petri dishes and on Transwell membranes. The salivary gland samples were homogenized using a Potter homogenizer, and the RNA was isolated using a TRI Reagent. Total RNA from cell cultures was extracted by the spin column method, using a NucleoSpin RNA II kit, according to the manufacturer's protocol. RNA concentration was measured with a NanoDrop spectrophotometer, and RNA quality was checked by gel electrophoresis. For RT-qPCR measurements, one microgram of each high-quality RNA sample was reverse transcribed using the High-Capacity RNA-to-cDNAMasterMix. RT-qPCR amplification was performed using predesigned primers, ABI StepOne System with TaqMan Universal Master Mix. RPLP0 was used as a housekeeping control; to quantify gene expression we used the ΔΔCT method.

Amylase release measurements To measure secreted amylase activity of the PTHSG and huSMG cell cultures, samples of media were collected. Cells were incubated in Hepato-STIM for 72 h after seeding on either a plastic surface or on Transwell filters. In the case of Transwell membranes, samples were taken from medium bathing both the

apical and basal surfaces of the cell cultures. Amylase activity was measured by the Phadebas Amylase Test. Data were normalized to 1 m^2 of cultured monolayer and are given in U/L in 1 h.

Short circuit current measurements Transepithelial ion transport was estimated by short circuit current (I_{sc}) measurements. Confluent monolayers grown on Transwell membranes were mounted in a modified Ussing chamber. The apical and basolateral side of the monolayers were each perfused continuously with the buffer solution at a flow rate of 2mL/min. The transepithelial potential difference (V_m) was measured with agarose-KCl bridges and Ag-AgCl electrodes. The short-circuit current (I_{sc}) was measured by voltage clamping to 0 mV with Ag/AgCl wire current electrodes mounted directly in the bath fluid. A positive I_{sc} can represent either a negative charge transfer from the basolateral to the apical side or a positive charge transfer from the apical to the basolateral side. The effect of the agonists on the I_{sc} was presented as ΔI_{sc}: the basal I_{sc} was divided from the maximum value of I_{sc} after agonist application.

Statistical analyses Averaged values are presented as the mean ± SEM. The normality of the data was verified using the Kolmogorov–Smirnov test. Where data were not normally distributed nonparametric tests were used. Statistical comparisons of normally distributed data were performed using an analysis of variance followed by a Tukey post hoc test or a t-test where only two groups were to be compared. Significance levels: *p<0,05; ** p<0,01; *** p<0,005.

RESULTS

Characterization of cell lines and the primary cells

When grown in Hepato-STIM medium, PTHSG and huSMG cells formed epithelium-like patches separated by thin lines of fibroblast-like cells. The epithelial areas were microscopically similar to Par-C10 cells. The huSMG contained more floating and semi-attached cells and cell aggregates then the PTHSG. With increasing passage number the number of semi-attached cell aggregates diminished. In MEM medium the cell cultures contained higher numbers of fibroblast-like cells and these cells soon became dominant in the culture. After passage No. 4 (3-4 weeks after isolation) the fibroblast cells grew over the epithelial cells even in Hepato-STIM medium. The formation of functional tight junctions leads to the formation of a polarized and confluent monolayer, which is essential for epithelial fluid and electrolyte secretion. The paracellular ion movement is restricted by functional tight junctions. Therefore not only the confluency of the monolayers but also paracellular permeability can be judged by measuring the transepithelial resistance level. The cells cultivated in Hepato-STIM medium reached maximum TER at 6-7 days after seeding (PTHSG: 595±93 Ωcm^2, n=55; huSMG: 622±117 Ωcm^2, n=45). In MEM medium, on the other hand, TER remained under 200 Ωcm^2. The use of conditioned Hepato-STIM medium resulted in faster cell growth and higher TER levels.

The Par-C10 cells (TER=2500-3000 Ωcm^2) and the HPAF cells (TER=500-700 Ωcm2) were capable of forming confluent polarized monolayers while the SMIE cells (TER=150-180 Ωcm2) formed a ’leaky’ epithelium on Transwell membranes. Therefore SMIE was not suitable for short circuit current measurements.

The mRNA expression of the acinar and ductal markers was measured by qRT-PCR. The acinar markers: *AMY1A, AQP5, NKCC1* were detected in the native salivary gland tissue and the isolated cell cultures as well. However, a decrease by three orders of magnitude was detected in the acinar markers *AMY1A* and *AQP5*, and a similar, but much less pronounced decrease was found in *NKCC1* expression in the cultured cells compared to the whole human salivary gland sample. On the other hand, culturing of the salivary cells induced a significant increase in *CLDN1* expression and a much less pronounced increase in *ENaC* expression. These results indicate that there is some de-differentiation in the cell culture. *AMY1A* expression was significantly higher, while *CLDN1* expression was significantly lower in cells cultured on Transwells than in cells cultivated on a plastic surface. We were able to measure amylase release in both PTHSG and huSMG cultures. Amylase discharge was significantly higher in cells cultured on Transwells than in cells cultivated on a plastic surface. Salivary amylase was discharged exclusively at the apical surface, providing further evidence of the polarization of the cells under these conditions on Transwell membranes. Amylase activity in huSMG was 742±214 U/L on plastic and 5022±1682 U/L on Transwells (apical surface medium); while in PTHSG we detected 568±193 U/L on plastic and 3043±882 U/L on Transwells (apical surface medium); (n=9-11). Taken together, these data suggest that monolayer formation significantly shifted PTHSG and huSMG cell cultures toward an acinar phenotype.

Short circuit current measurements

In the presence of HCO_3^- and Cl^-, without stimulation only a small I_{sc} was detected in Par-C10 and HPAF cells. (Par-C10 basal I_{sc}=1.01±0.27 μA/cm^2, n=12; HPAF basal I_{sc}=0.84±0.28 μA/cm^2, n=6). Application of 50 μM ATP to the apical

surface resulted in a biphasic increase in I_{sc}. ΔI_{sc}=2.31±0.31 μA/cm^2, n=12 (Par-C10) and 3.35±0.85 μA/cm^2, n=6 (HPAF).

In both PTHSG and huSMG cells in the HCO_3^--buffered solution a significant I_{sc} of approximately 6 μA/cm^2 was detectable in the absence of stimulation. Substitution of Cl^- and HCO_3^- on both sides of the epithelium results in a significant decrease in the basal I_{sc}. The remaining current in the absence of Cl^- and HCO_3^- was most likely due to Na^+ absorption mediated by the epithelial sodium channel (ENaC) on the apical surface of the ductal cells. ENaC can be selectively blocked by low concentrations (10 μM) of amiloride. In the presence of Cl^- and HCO_3^- in the bathing solutions, application of 10 μM amiloride to the apical surface reduced the basal I_{sc} by 40-60%. In the absence of Cl^- and HCO_3^-, I_{sc} was almost completely abolished by apical amiloride. These data suggest that the basal I_{sc} may be attributed partly to Cl^- and/or HCO_3^- secretion and partly to Na^+ absorption.

To stimulate ion secretion we studied the effect of carbachol, ATP and forskolin. Carbachol (CCh) activates basolateral muscarinic receptors and acts via the IP_3/Ca^{2+} signalling pathway. ATP also elevates intracellular Ca^{2+} levels, but it stimulates purinergic receptors. Forskolin elevates intracellular cAMP by stimulating adenylyl cyclase. Application of either 100 μM carbachol to the basolateral surface or 50 μM ATP to the apical surface increased I_{sc} significantly. Apical administration of 10 μM forskolin had no significant effect on I_{sc}. ATP stimulation produced the largest increase in short-circuit current: $\Delta I_{sc}\approx 5$ μA/cm^2 in both PTHSG and huSMG monolayers. However, the response was transient; the I_{sc} increased to a peak, and then fell slowly to base level, despite sustained stimulation. Substitution of HCO_3^- did not change the effect of ATP significantly. On the other hand, in the absence of both HCO_3^- and Cl^- the ΔI_{sc} evoked by ATP decreased significantly (PTHSG: ΔI_{sc} =2.8±0.8 μA/cm^2,

n=5; huSMG: ΔI_{sc} =1.6±0.7 μA/cm^2, p<0.05, n=8). Blocking the ENaC channels with 10 μM amiloride also significantly reduced the I_{sc} changes evoked by ATP (PTHSG: ΔI_{sc}=3.4±0.6 μA/cm^2, n=9; huSMG: ΔI_{sc}=2.4±0.3 μA/cm^2, p<0.05, n=5). These results suggest that both anion secretion and Na^+ absorption increase following purinergic stimulation. We found only a very weak association between the TER values (measured immediately before short circuit experiments) and the I_{sc} responses to ATP stimulation in individual Transwell preparations. These data indicate that I_{sc} activity is not strongly related to TER values. Transepithelial electrical resistance is primarily determined by tight junction formation, which does not directly affect active transcellular electrolyte transport.

The function of the basolateral membrane transporters of the cells were studied on PTHSG cell culture. The semi-quantitative RT-PCR studies of my colleague Erzsébet Bori identified the presence of the epithelial sodium channel ENaC, the Na^+-K^+-2Cl^--cotransporter NKCC1, the Cl^-/HCO_3^- exchanger AE2, the Na^+/H^+ exchanger NHE1 and the Na^+/HCO_3^- cotransporter NBC1 in both native human submandibular gland tissue homogenates and in PTHSG cells grown either on plastic or on Transwell membranes. (Results are discussed in Erzsébet Bori's Ph.D. thesis titled: 'Characterization of epithelial transport processes in amelogenesis and saliva secretion'). To evaluate the contribution of the basolateral NKCC1, the major pathway of Cl^- uptake on the basolateral side to the I_{sc}, we applied bumetanide, a selective inhibitor of NKCC1. There was no significant change in neither the basal I_{sc}, nor in the ΔI_{sc} following stimulation with apical ATP after basolateral application of 100 μM bumetanide in HCO_3^--buffered solution. However, in the absence of HCO_3^-, bumetanide pretreatment decreased the response to ATP in ~60%. To eliminate the contribution of ENaC-mediated Na^+ absorption, the experiment was repeated in the presence of apical

amiloride (10 μM). Once again, bumetanide pretreatment had significant decreasing effect on the I_{sc} changes evoked by ATP in the absence of HCO_3^- and with amiloride present in the solution. These data suggest that the bumetanide insensitive, HCO_3^--dependent component of ΔI_{sc} derives from anion secretion rather than Na^+ absorption.

In salivary acinar cells the coordinated activity of basolateral AE2, NHE1 and transporters provides an alternative Cl^- uptake pathway, while NBC1 mediates HCO_3^- uptake. These transporters contribute to the driving force of secretion across the apical membrane. To evaluate the contribution of HCO_3^- secretion to I_{sc}, the following experiments were performed in the absence of Cl^-. Simultaneous application of 30 μM EIPA and 500 μM H_2DIDS, selective inhibitors of NHE1 and NBC1 respectively, resulted in a decrease of approximately 20% in the basal I_{sc}. Addition of 10 μM amiloride at the apical membrane reduced the current further, but not completely. The absence of Cl^- had no significant effect in the I_{sc} changes evoked by ATP. On the other hand, in the absence of Cl^-, EIPA and H_2DIDS reduced ΔI_{sc} by approximately 50%. The remaining ΔI_{sc} current was abolished after inhibiting ENaC with amiloride. These data indicate that in PTHSG cells a significant component of the ATP-evoked current can be due to transepithelial HCO_3^- secretion via this pathway.

CONCLUSIONS

1. PTHSG and huSMG primary salivary cells are capable of forming confluent polarized monolayers on Transwell Clear membranes, but only when cultured in Hepato-STIM medium. MEM medium is not suitable for either maintaining the epithelial phenotype of the cell culture, or supporting monolayer formation. The epithelial character of PTHSG and huSMG cell cultures is similar in Hepato-STIM medium even in the presence of the quickly attached cells in the PTHSG cell culture. This suggests that the Hepato-STIM acts as an epithelial selection factor during cell culturing. The use of sequential digestion protocol and conditioned medium results in faster cell growth and more viable cells in cell cultures.

2. PTHSG and huSMG cell cultures are mixed, acinar/ductal phenotype. The expression of acinar markers (*AQP5, AMY1A, NKCC1*) can be detected in both PTHSG and huSMG cell cultures, however, the level of expression is significantly lower in the primary cells then in the native tissue. Both PTHSG and huSMG cells and monolayers are capable of amylase secretion, providing further evidence for the presence of acinar-like cells in the cell culture. Cells grown on Transwell membranes performed significantly higher amylase gene expression and amylase secretion then the cells on plastic surfaces. The monolayers released amylase only to the apical fluid compartment, which demonstrates the polarization of the cells.

3. Par-C10 and HPAF cells are capable of forming confluent, polarized monolayers and achieve transepithelial electrolyte secretion. The secretion can be stimulated

by purinergic stimulation. These cell lines are suitable for functional preliminary experiments that set experimental conditions for primary cell studies.

4. PTHSG and huSMG cells cultured in Hepato-STIM medium are capable of vectorial transepithelial electrolyte transport.

    a. The transepithelial ion movement in these monolayers has two major components: basolateral to apical anion (HCO_3^- and Cl^-) transport and apical to basolateral Na^+-current.

    b. The transepithelial ion movement increases significantly after application of purinergic (ATP) or cholinergic (CCh) agonists. The elevation of intracellular cAMP level by forskolin did not significantly change the $I_{sc.}$

    c. In PTHSG cells the ATP-stimulated anion transport decreased significantly after the basolateral application of either bumetanide in the absence of HCO_3^-, or EIPA and H_2DIDS in the absence of Cl^-. These results indicate the presence of functional NKCC1, NHE1 and NBC1 transporters in the basolateral membrane of PTHSG cells.

Taken together, monolayers of PTHSG and huSMG primary human submandibular salivary cells are capable of achieving vectorial transepithelial ion secretion. Thus, we successfully created a human salivary secretion model, although in some parameters it shows a mixed acinar/ductal phenotype.

The thesis is based on the following articles:

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.

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FOGORVOSI SZEMLE **107:**(3) pp. 99-105. (2014)

Demeter I, Szucs A, Hegyesi O, Foldes A, Racz GZ, Burghardt B, Steward MC, Varga G

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